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KINETICS OF THE ALKALINE DENATURATION OF HEMOGLOBIN IN THE SINGLE ERYTHROCYTE

By

B. Thorell* and G. Matioli**

INTRODUCTION

The question of whether two different types of hemoglobin (Hb) may coexist within a single erythrocyte is of interest as well from the cytogenetic as from the biochemical point of view. Attempts to determine whether a single red cell from fetal blood contains both the Hb types A and F have been made by analyzing the solubility in citrate-buffers of erythrocytic pigment in fixed blood smears.[1] The limitation of the above procedure essentially depends on the inability of the buffer to extract differentially the two Hb types with absolute specificity.[2]

Among the procedures capable of detecting the Hb F, the alkali denaturation method is one of the most specific. This method has been widely applied to the analysis of Hb solutions in the macroscale but not, for obvious reasons, to the analysis of the Hb in a single cell. The present investigation shows the possibility of using the alkali denaturation procedure with the help of microspectrophotometric recording of the denaturation kinetics, for the analysis of the type of Hb in a single erythrocyte.

METHOD

For the analysis of the alkali resistant Hb F within a single erythrocyte, the Brinkman and Jonxis procedure was modified to the ultramicroscale.[3] This method is based on the spectrophotometric recording of the absorption differences at 576mμ of the HbO_2 and its denaturation product, respectively, during the alkali treatment.

The preparation of the erythrocyte specimen for alkali denaturation was done in the following way. A small drop of a suspension of washed erythrocytes in glycerol was gently spread out on a coverslip. The erythrocytes were covered and imbedded in a 30 to 40μ-thick layer of 50% polyethylenimine in water (Polymin P, B.A.S.F.), the gel prepared beforehand on a circular, semipermeable cellophane membrane 7.5 mm in diameter and 30μ thick. This procedure imbedded the cells without causing hemolysis or morphological changes. By strips of coverslip glass a groove 12 mm in length, 100-110μ thick and 1 mm in width, was constructed on a microscope slide. The cellophane-Polymin-erythrocyte-smear on the coverslip was put onto the groove with cellophane layer down to make the lid of a chamber. The layer of

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Polymin P. with the erythrocytes (compartment A) was thus separated from the chamber (compartment B) by the cellophane.

The microspectrophotometer used has been described previously.[4] The optics used were the Zeiss Ultrafluar objective, 100 X, N.A. 0.85, and condenser N.A.= 0.60. The noise level of the recording, expressed in extinction units, was 10^{-4}. The measurements were made at 576mμ with 20% of sensitivity full-scale deflection on a Speedomax recorder and with a time constant of 2.5 seconds.

After having chosen the erythrocyte to be analyzed in the microspectrophotometer at the wave length of 576mμ, the compartment B was filled with a drop of a solution of 3.00 ml Polymin P in 4.70 ml 2N NaOH. The diffusion of the alkali from compartment B into compartment A across the cellophane membrane and the start of the alkali denaturation in the erythrocyte took about 40-50 seconds under the prevailing conditions, as evaluated by the start of the decrease in absorption of the erythrocytic pigment at 576mμ. Because the adjustment of the microspectrophotometer required about 10 seconds (focusing, recorder adjustment, etc.) the remaining half-minute was sufficient to record the 500-600mμ absorption spectrum of the erythrocyte pigment before the start of the alkali denaturation. The recording of the alkali denaturation at 576mμ was then continued until a constant value was attained that represents the end of the alkali denaturation reaction of the Hb in the cell (Fig. 1). Then another complete absorption spectrum was run and by comparing the absorption values read at zero time and at the end time at 522mμ, which is an isobestic wave length for the HbO_2 and its alkali denaturation product, a control was obtained for loss of pigment (hemolysis), changed cell position, etc. The percentage of undenatured Hb at the time t, was calculated according to the equation:

$$Hb\ t = \frac{E_t - E_\infty}{E_0 - E_\infty} \times 100$$

Where:

E_0: absorbancy at the start of the reaction.

E_∞: absorbancy at the end of the reaction.

E_t: absorbancy at the time t during the reaction.

DISCUSSION OF THE METHOD

Products of the Alkali Denaturation of HbO_2 in a Single Erythrocyte. The absorption spectra of the Hb in the erythrocytes of normal adult and fetal blood shows that 10 minutes after they had been imbedded in the Polymin P layer, their hemoglobin is still in the HbO_2 state (Fig. 2). The alkali denaturation reaction and its recording is completed within less than 5 minutes and also when erythrocytes with high alkali resistant Hb are analyzed. Thus, the pigment within the single erythrocyte at the zero time is represented by HbO_2. At the end time of the alkali denaturation reaction the spectra of the same cell show a leveling off in the visible region, decreasing towards the red end of the spectrum in good agreement

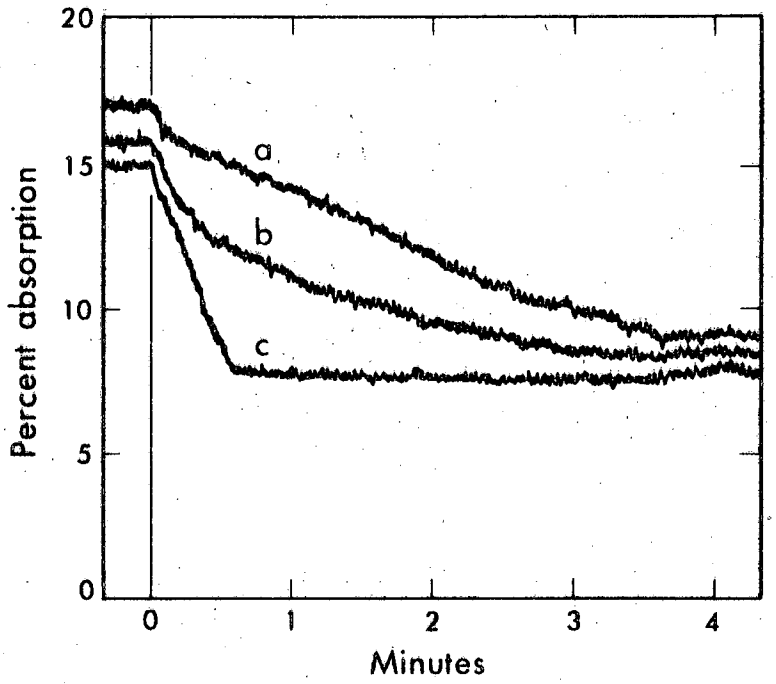

Figure 1. Microspectrophotometer tracings of the absorption changes at 576mμ during the alkali denaturation of the hemoglobin in single erythrocytes. Curves a and b represent the reactions in two different cells from fetal-cord blood. Curve c represents the denaturation reaction in a single, adult erythrocyte.

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with the spectrum of the alkaline globin-hemochrome. If the alkali reagent in compartment B, after the denaturation reaction has been completed, is replaced with a reducing agent (3.00 ml Polymin P + 2.00 ml of a water solution of $Na_2S_2O_4$), the absorption spectrum of the erythrocyte corresponds to that of the alkali globin hemochrome (Fig. 2). Moreover, the magnitude of the specific absorption peak shows that virtually no loss of pigment in the cell has occurred and that the physico-optical conditions, such as light scatter, are unchanged.

The Concentrations of Alkali and Hemoglobin During Denaturation. By experiments carried out in macroscale, it is known that the alkali denaturation of Hb follows a first order reaction only for certain concentrations of alkali in the reacting mixture.[5] The optimal pH for the alkali denaturation lies at 12.6. The pH in the single cell during the alkali denaturation cannot be directly estimated, however. The pH of the solution of Polymin P in NaOH is about 13.5, as measured with the Radiometer type pHM 23 C glass electrode. This pH roughly checks with the pH in compartment A, evaluated by the use of paper indicator strips, after the

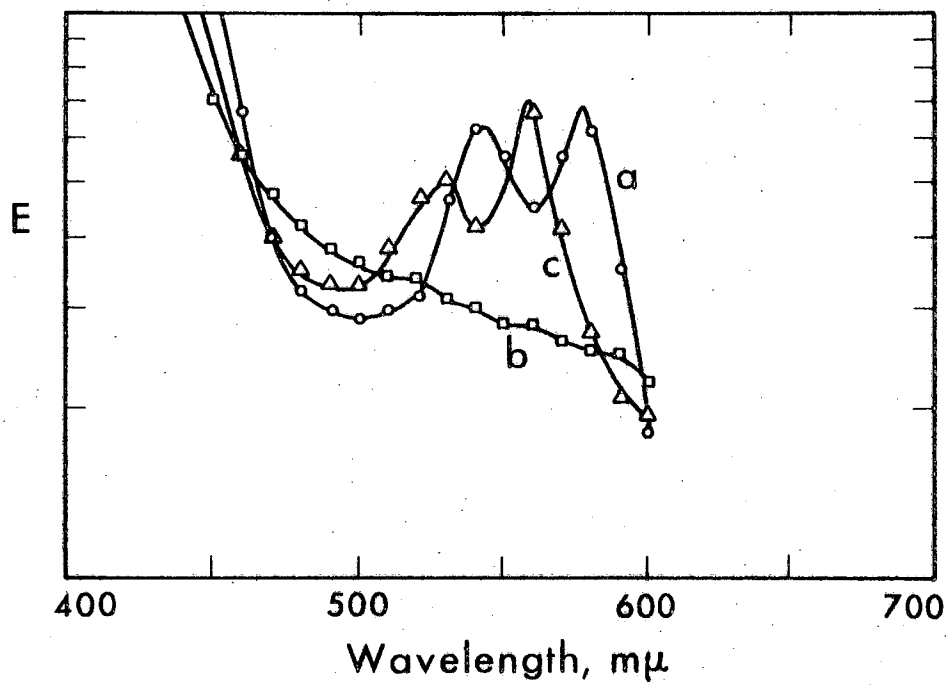

Figure 2. Spectral absorption of a single erythrocyte:
a: embedded in Polymin P
b: at the end of alkali treatment
c: after treatment with $Na_2S_2O_4$
The curves are plotted from the original transmission record as absorbancy (log $\frac{Jo}{J1}$) on a logarithmic ordinate.

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diffusion of the alkali from compartment B.

The alkali denaturation reaction in the macroscale uses a pigment concentration of about 0.4 - 0.6%. In this case, the buffering action of the protein in the reaction mixture is negligible. In the case of the alkali denaturation of Hb within a single erythrocyte, the buffering action of the protein should be considered higher than that in the macroscale because of the higher protein concentration (about 30%). A direct control of the pH within a single erythrocyte is not possible, however, and that is why the reaction must be rigidly standardized and evaluated from the empirical results. As shown by the results, the constant alkali denaturation kinetics recording in the normal adult erythrocytes is in agreement with a satisfactory standardization of the above-mentioned factors.

MATERIAL

The described ultramicroprocedure was applied to the analysis of the alkali denaturation kinetics of the Hb within single erythrocytes of normal human adult, human fetal and chicken blood. The relative concentrations of the alkali-resistant

Hb, as estimated according to the macroscale method, was 72% in the fetal blood samples and in the range of 30% in the chicken blood samples. In the normal human adult blood samples no Hb F was detectable.

RESULTS

Figures 3, 4 and 5 show the alkali denaturation kinetics of the Hb within single erythrocytes. The percentages of undenatured Hb at various times t of the reaction are plotted for each cell as a logarithmic function against time. Figure 3 shows the alkali denaturation kinetics of the Hb in 10 erythrocytes from normal adults. In each erythrocyte the alkali denaturation reaction was completed in 40-50 seconds. The rate of reaction is rather constant in all cells, which reflects the degree of reproducibility and standardization obtained. Figure 4 shows the alkali denaturation kinetics of the Hb in 15 fetal erythrocytes. In the cells number 1-14, the reaction reaches the end (not represented in the graph) at about 200 seconds, i.e., 5 times longer than in normal adult erythrocytes. Although the curve of the alkali denaturation of the cell number 15 is similar to the curves of the normal adult erythrocytes, the curves of the cells 1-14 differ significantly. The reactions in the erythrocytes N 1-2-3-5 are represented by straight lines; the reactions in the cells N 4-6-7-8-9, and especially those of the cells N 10-11-12-13-14, clearly show two components: the first (rapid) between zero time and 20 seconds; the second (slow) between 20 seconds and 100 seconds. Figure 5 shows the alkali denaturation kinetics of the hemoglobin in 7 chicken erythrocytes. In the cells, N 1-5, the reaction (not represented in the graph) is completed at about 160 seconds. Although the reactions in the cells N 6-7 are quite similar to those of normal adult erythrocytes, the reactions in the cells N 1-5 show two different rates: the rapid reaction displayed between zero time and 20 seconds and the slow reaction between 20 seconds and 100 seconds.

DISCUSSION

The normal, adult erythrocytes contain exclusively Hb A; i.e., the amount of Hb F is nil or in trace amounts only, as checked by the analysis in the macroscale. Thus, the Hb in the adult erythrocytes has a relatively low alkali resistance. The reaction time recorded for the single cell in the microspectrophotometer is significantly shorter than that for the fetal or chicken erythrocytes in which the Hb F ranges between 0 and 100%.[2] The denaturation process in the single adult erythrocytes seems not to follow strictly a first order reaction, however. About 20 seconds after the zero time the reaction appears to slow down but is not brought to a complete standstill. This anomaly in the reaction kinetics might be explained by the fact that the concentrations of Hb and alkali in the single cell scale are far from the optimal condition on which a first-order reaction depends. Nevertheless, the recorded kinetics of the alkali denaturation of the Hb in single adult erythrocytes

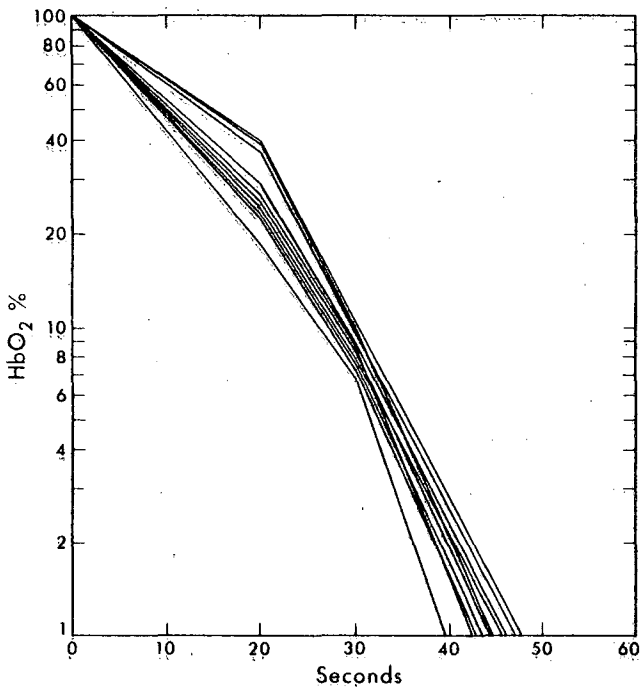

Figure 3. Alkali denaturation kinetics of the hemoglobin within ten single erythrocytes from normal, adult human. The curves are calculated from the microspectrophotometric records obtained at 576mμ (see Fig. 1).

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represent the behaviour of an Hb compound (Hb A) that is homogeneous in respect to the denaturating action of the alkali, although the rate of the reaction is modified by the micro-scale conditions.

From the classification of the Hb types according to their alkali resistance and the comparison of alkali denaturation rates obtained in normal adult erythrocytes with those recorded in the fetal and chicken blood red cells, it is evident that the bulk of the Hb in the cell N 15 (Fig. 4) (fetal blood) and N 6-7 (Fig. 5) (chicken blood) is represented by Hb A or I, and that the bulk of the Hb in the cells N 1-2-3-5 (Fig. 4) (fetal blood) is represented by a much slower reacting hemoglobin (Hb F or II). The other analyzed cells in the fetal and chicken blood samples obviously contain a mixture of the two types, represented by the Hb types I and II in the chicken cells, and by Hb A and F in the fetal erythrocytes.

It is not possible, however, to compute accurately the relative concentrations of the Hb A and F that are present in the same cell from the recorded reaction kinetics. Although the results clearly indicate that in those blood samples where the alkali denaturation procedure in macroscale detects a mixture of hemoglobins A and F, there are single cells that contain a mixture of the different types of hemoglobins. This, in turn, might be regarded as a result of the synthesis of different hemoglobins in one erythroblast. The present results also indicate that the relative concentrations of the Hb A and F in a single cell can differ over a rather large range

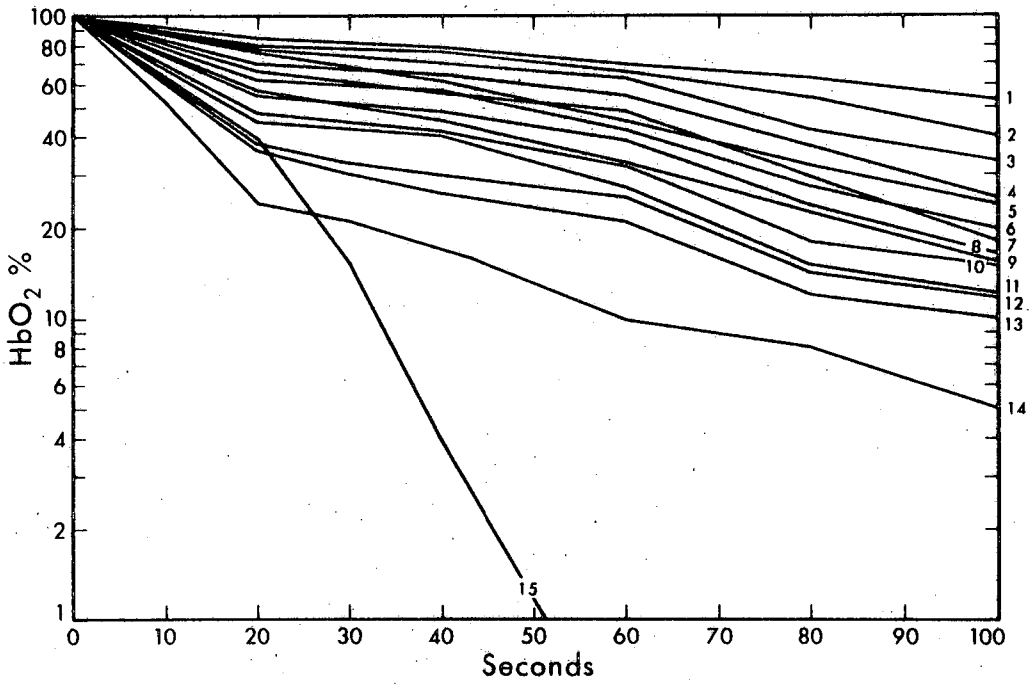

Figure 4. The alkali denaturation kinetics of the hemoglobin within 15 erythrocytes from human fetal blood. The overall relative concentration of Hb F in the sample was 72%.

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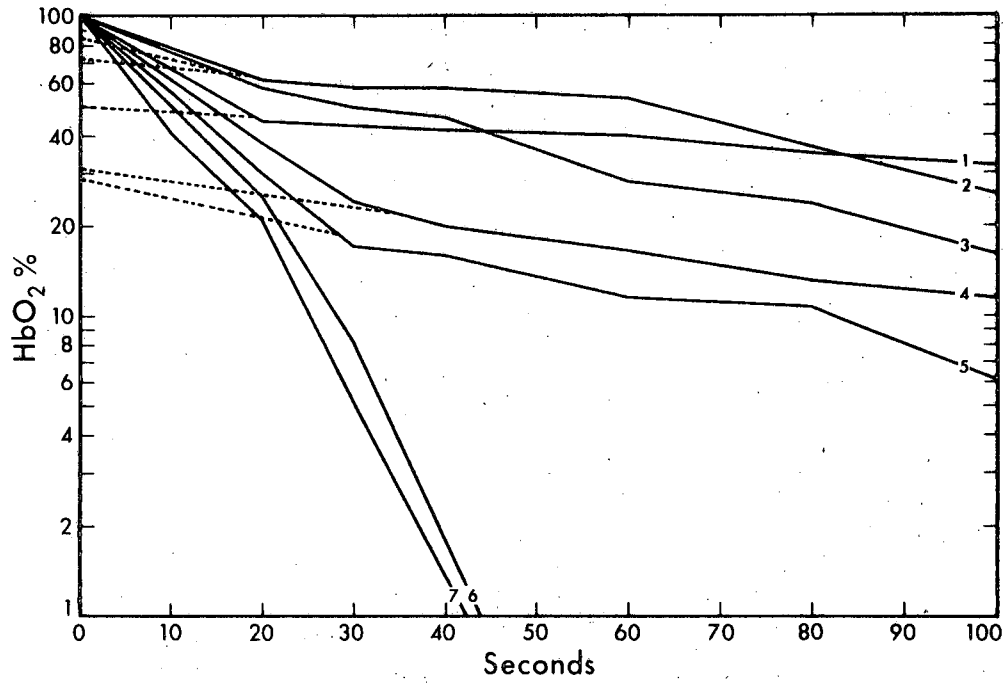

Figure 5. The alkali denaturation kinetics of the hemoglobin within 7 chicken erythrocytes. The relative concentration of Hb II in the chicken blood was 30%.

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that is in agreement with the microspectrophotometric results obtained with the differential extraction method.[2]

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INFECTIOUS MACROMOLECULES*

By

John H. Northrop**

The nature of viruses has been a subject of controversy ever since their discovery 60 years ago. They have been described as parasites, proteins, enzymes, genes, nucleo-proteins and finally, nucleic acids. The results of the last few years show that many, if not all, viruses are infectious nucleic acids, capable of transmitting genetic information from one cell to another. They are, therefore, closely related to the transforming-principle nucleic acids and not at all to cellular parasites.

The virus nucleic acids differ from the transforming-principle nucleic acids in that they transfer harmful genetic properties as well as beneficial ones, they are of higher molecular weight and are associated more or less closely with protective proteins.

In retrospect, it may be seen that several workers had guessed the correct solution of the problem, or nearly so, and also that if the discoveries had been made in logical instead of chronological order, the problem would have been solved much more readily.

Duggan and Armstrong, Muller, Bordet, Wollman, Darlington, Bawden and Euler all suggested at various times that viruses are related to genes, and Gratia pointed out that they have properties similar to the transforming principle. At the time these suggestions were made, no one knew what a gene or a transforming principle was, but it now appears that a gene, a virus and a transforming principle are all macromolecular nucleic acids. These macromolecules, therefore, are the master molecules of the living cell; within them is coded the information which controls their own reproduction and also the genetic constitution of the cell itself.

At the time when the viruses were discovered the only things known that had similar properties were micro-organisms, and it is not surprising that the viruses were automatically considered to be small parasites.

If the transforming principle had been discovered and isolated before the discovery of the viruses, the latter would have been classified with the transforming principle, and it is doubtful if the parasite theory would have been accepted at any time.

The logical sequence of events would have been somewhat as follows:

1. Avery, MacLeod and McCarty isolated the transforming principle and

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identified it as an infectious nucleic acid, capable of transmitting genetic information. This furnished the key to the puzzle.

2. Freeman transmitted by a virus a genetic property of a bacterium, virulence, and Lederberg found this process to be quite general. He named it transduction and pointed out that it was obviously related to transformation.

3. Hershey and Chase found, as the writer had suggested, that a nucleic acid was the essential part of a bacterial virus.

4. Tobacco mosaic virus and many other viruses were then isolated by Fraenkel-Conrat, Gierer and Schramm, and others and found to be nucleic acids. Bacterial viruses were also isolated as nucleic acids.

5. Further work showed that the virus nucleic acid was more or less closely associated with a protein which acted as a protective agent. The protein-virus complex of many viruses was isolated and crystallized. The bacterial virus nucleic acid is enclosed in a protein coat which assists the virus in penetrating the cell membrane. The virus alone can attack only a cell from which the membrane has been removed.

In view of these facts, the long list of complicated assumptions, that have been made from time to time in order to maintain the parasite theory, are unnecessary and may be discarded and filed away with other famous unnecessary assumptions; for example, "Enzymes are neither proteins, carbohydrates nor fats but are an entirely new and unknown class of compounds."

Because viruses have the same chemical and biological properties as the transforming principle, it is logical to assume that they originate in the same way, i.e., as a result of a mutation of the host cell. This assumption covers the facts and also satisfies the demand of the principle of conservation of hypotheses, otherwise known as "Occam's razor."[1]

Now it is known from the results of Drosophila genetics that mutagenic agents increase the number of all mutants in approximately the same proportion, although there are some exceptions. If the appearance both of virus nucleic acid and of transforming-principle nucleic acid is by mutation, mutagenic agents should increase the cells producing transforming-principle nucleic acid and the cells producing viral nucleic acid in about the same proportion.

Lwoff and his collaborators found that mutagenic agents do in fact, increase the proportion of virus-producing cells, and under certain conditions may cause nearly all the cells in a culture to produce virus.*

*This fact has been cited as an objection to the mutational origin. This result is due not to a peculiarity of the virus-producing cells but to the unusually large proportion of these cells in the population, often 1% or more. Hence, an increase of 10 to 100 times in the mutation rate will cause almost all the cells to mutate, whereas most mutants occur in the proportion of 1×10^{-6} or less, so that an increase of 100 times would still result in a proportion of only 1×10^{-4}.

It is also known, largely from the work of Hotchkiss and his collaborators and Herriott and his co-workers, that antibiotic-resistant mutants produce a specific transforming principle nucleic acid, and this nucleic acid may be isolated and used to infect other cells exactly as the viral nucleic acid does.

If the mechanism of the appearance of the viral nucleic acid is actually related to that of the appearance of the transforming-principle nucleic acid, the effect of various mutagens on the appearance of the two nucleic acids should be similar. In order to test this assumption the writer has studied the effect of a number of mutagens on the proportion of virus-producing cells and of antibiotic-resistant mutants in several B. megatherium cultures.

The results of these experiments are summarized in Table 1, showing that, in every experiment in which the proportion of antibiotic-resistant mutants was increased by the mutagen, the proportion of virus-producing cells was also increased. If the antibiotic-resistant mutants did not increase, the virus-producing cells did not increase either. In general, the relative increase in the proportion of the various mutants was about the same for all the mutants, but, in the case of ultraviolet light, hydrogen peroxide and butadiene dioxide, the increase in the virus-producing mutants was higher than that of the terramycin-resistant ones.* On the other hand, $MnCl_2$ increases the proportion of streptomycin-resistant mutants more than the virus.

Temperature coefficient of mutation rate of virus production and terramycin-resistant cells.[5] A further test of the similarity of the appearance of antibiotic-resistant cells and of virus-producing cells is a comparison of the temperature coefficients of the two processes. The mutation frequency rate constant for the terramycin-resistant cells in B. megatherium 899 cultures has a temperature coefficient of about 2 from 20° to 35° . ($\mu = 9 \times 10^3$ cal.) There is a maximum at 40°. That of the virus-producing cells is about 3 from 20° to 45° ($\mu = 20 \times 10^3$ cal.). The reactions that result in the production of the virus, therefore, have a slightly higher temperature coefficient than those that produce the terramycin-resistant mutants. It is possible that this difference is due to the fact that virus production occurs without cell division, but terramycin-resistant mutants may appear after cell division.

*The fact that the proportion of mutants in a culture increases does not prove that the mutation rate increases; the increase may be due to selection or, in the case of a virus, to an increase in the number of virus molecules formed per cell. The mechanism of the increase is of secondary importance for the purposes of comparison of the increase in virus with the increase in transforming principle. It may be shown, however, by direct determination of the mutation rate by means of the null fraction method, that the increase in some of the mutants shown in the Table is actually due to an increase in mutation rate and not to preferential selection of mutants nor to an increase in the number of virus molecules produced per cell.[2]

Table 1

THE EFFECT OF VARIOUS MUTAGENS ON THE PROPORTION OF MUTANTS AND OF VIRUS IN B. MEGATHERIUM CULTURES

B. Megatherium	Culture medium	Mutagen	Mutant: Virus	Mutant: Virus-resistant	Mutant: Terramycin-resistant	Mutant: Streptomycin-resistant	Reference
			Mutants per bacterium in presence of mutagen / Mutants per bacterium in control				
899 Y	Yeast extract peptone (YEP)	H_2O_2	3.5		3.3		(2)
899 Y	Ammonium sulfate (ASCM)	H_2O_2	3.3		2		"
899	YEP	H_2O_2	2.7		1.6		"
899	ASCM	H_2O_2	4.1		4.3		"
KM	YEP	H_2O_2	-		6		"
899	YEP	UV	6		1.3		"
899 SP	YEP	UV	25		2		"
899 Y	YEP	UV	1		1		"
899	YEP	$MnCl_2$	8	16	4.4	2	(3)
899	YEP	$NaNO_2$	6	3.5	6	1.3	(4)
899	YEP	X ray	4	5	2.5	1.3	(4)
20	YEP	Urethane	2			3.6	Unpublished
20	YEP	H_2O_2	3			2.1	"
20	YEP	Versene	5.1			2.2	"
20	YEP	Sodium desoxycholate	2.1			2.1	"
20	YEP	Maleic hydrazide	2.4			3.7	"
20	YEP	Butadiene dioxide	30			3	"
20	YEP	Ethyl urethane	2.5			2	"

The ratios are the means of 6 to 12 separate experiments. The probable errors of the means are 10 to 20%.

Calculation of rate of appearance of mutants.[6,7] The proportion of terramycin-resistant mutants in B. megatherium cultures may be satisfactorily calculated by means of the following equation:

$$\frac{M}{W} = \frac{C(e^{(B+C-A)t}-1)}{B+C-A} + \frac{M_o}{W_o}e^{(B+C-A)t} \qquad \text{(a)}$$

in which M/W is the number of mutants for normal (wild) cells, C is the mutation time rate constant, B is the growth rate of the mutant and A the growth rate of the wild cells.

In the case of a virus the equation becomes[2]

$$\frac{P}{W} = \frac{lC(e^{(C-A)t}-1)}{C-A} + \frac{P_o}{W_o}e^{(C-A)t} \qquad \text{(b)}$$

in which l is the number of virus molecules formed per cell.*

The appearance rate of several mutants in the presence of $NaNO_2$ is shown in Figure 1. The curves have been calculated by means of equations (a) or (b).

The effect of various concentrations of H_2O_2 on the production of virus is shown in Figure 2.

The curve was calculated by means of equation (b) with the additional assumption that C, the rate of virus production, is directly proportional to the H_2O_2 concentration. The ratio of virus to cells increases in proportion to the H_2O_2 concentration at first, but then much more rapidly. This is correctly predicted by the equation.

This experiment is of some interest, because, if only a few measurements were made, it would appear that there was a threshold value for the effect, whereas the mutation rate is directly proportional to the H_2O_2 concentration, and the sharp increase in virus is due to the form of the relation between virus production and growth rate.

The experiments, as a whole, show that mutagens that cause an increase in the number of antibiotic-resistant cells also cause a similar increase in the

*This equation assumes that the mutants appear without cell division. This condition is fulfilled by the virus, because under some conditions virus production is more rapid than cell division and hence must occur without it. The appearance of bacterial mutants has been found by Novick and Szilard, to occur without cell division, and Fox has shown that antibiotic-resistant mutants are produced without cell division after infection with transforming-principle nucleic acid. In order to conserve hypotheses, therefore, it will be assumed that all mutants appear without cell division. Practically, there is no difference if the time rate constant for the appearance of mutants C is small compared to the growth rate A. In this case, $C = 2\lambda A$, where λ is the mutation frequency rate constant. The equations derived on the basis of mutation with or without cell division are, therefore, identical except for the value of the constants, provided the mutation time rate constant is small compared to the growth rate constant.

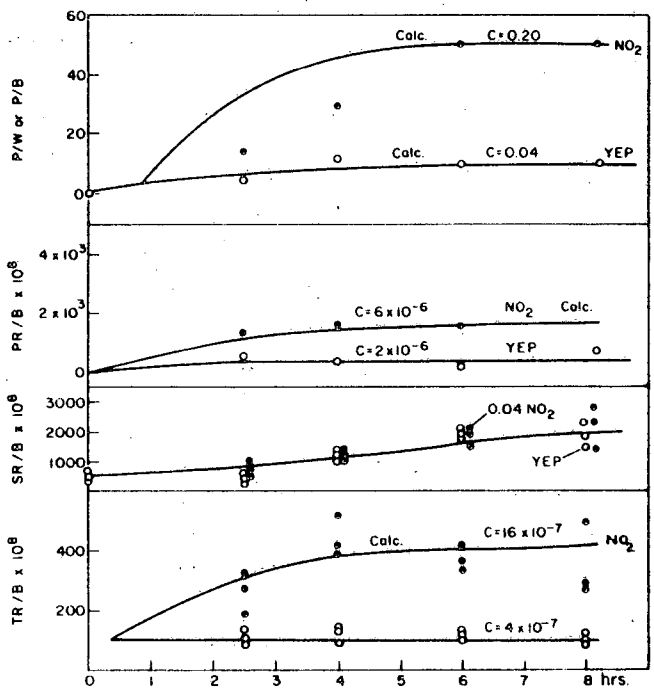

Figure 1. Increase in the proportion of virus (P/W), virus-resistant cells (PR/B), streptomycin-resistant cells (SR/B), and terramycin-resistant cells (TR/B) in the presence of $NaNO_2$. Curves calculated.[4]

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number of virus-producing cells. Both types of cells contain a new and different infectious nucleic acid. There appears at present to be no reason to assume that the cells that produce the transforming-principle nucleic acid do so as the result of a mutation, and the cells that produce the virus nucleic acid do so because they were infected some time in the past.

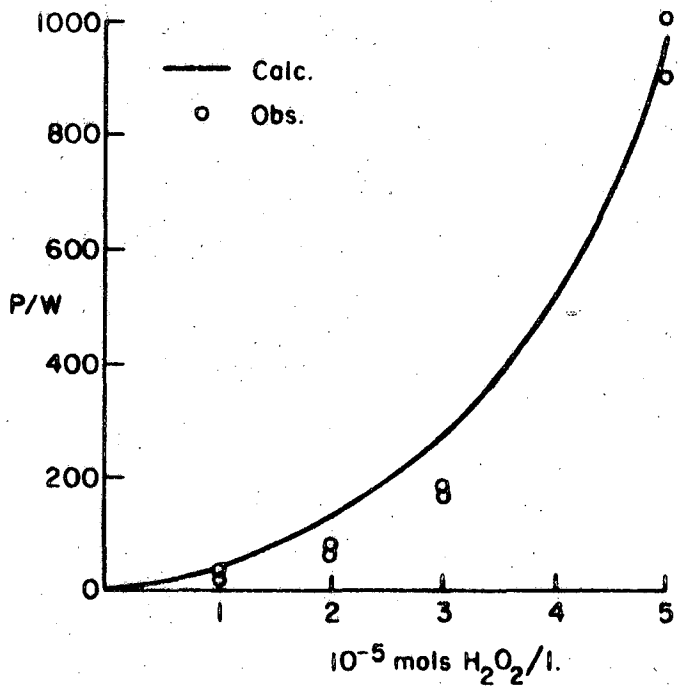

Figure 2. Increase in the proportion of virus to cells in the presence of various concentrations of H_2O_2. Curves calculated.

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THE PHYSICS OF SPACE RADIATION

By

Roger Wallace

INTRODUCTION

The several sources of radiation which one expects to encounter in the course of space exploration fall into five categories.

A. The most energetic radiations are the normal cosmic ray background, often referred to as galactic cosmic rays. These have been known for more than 50 years and their characteristics are fairly well understood.[1] Fortunately, in spite of their very high energies, their intensity is sufficiently low so that the dose one would expect to receive from them, even in an unshielded space ship, is quite small. These primary or galactic cosmic rays are divided naturally into two types, the lighter component consisting of protons with energies extending in some rare cases up to well over 10^{18} electron volts, and heavier ions such as He, Li, Be, B, C, N, O, F, and several other ions up to iron have been identified. Lithium, beryllium, and boron, although quite low in intensity, definitely are present in the primary cosmic rays.

B. The second natural category can be referred to loosely as solar cosmic rays These are more recently discovered than the galactic cosmic rays, their existence having been carefully investigated only since 1956. They are quite variable in their intensity and are produced in conjunction with visible outbursts from the sun. They probably constitute the most serious natural radiation problem in space. The majority of this report deals with them.

C. The third natural category is the Van Allen radiation, which is fairly intense but quite localized in space. This limited region consists of one or two belts circling the earth and symmetrical with respect to the equatorial plane of the magnetic field; it extends from 400 to 40,000 miles above the surface. For a space ship orbiting inside the Van Allen Belt or passing through the belt twice in every orbit the radiation encountered would be fairly intense. Because the belt may be rather easily avoided it is not a major problem for lunar or planetary travel, as was once feared.

D. The fourth possible source of radiation provides strictly an engineering problem. It is the radiation produced by nuclear reactors that might be carried in a space ship. The shielding criteria for such reactors have been voluminously documented in the literature in connection with the nuclear airplane project. There is in space no air scattering around the shield to complicate the direct shielding problem; therefore, no particularly new aspect is introduced by space travel, with the possible exception that in space it might be practical to separate the crew from the reactor by a greater distance than in the airplane.

E. There may be other sources of radiation as, for example, the surface of the moon or planets. There is no evidence at present that the surface of the moon contains unusual levels of radioactivity. At this time all the planets are beyond our ability to make direct measurements of surface activity.

In general it seems that the radiation danger is quite small for lunar trips lasting 1 to 3 weeks. The radiation problem is somewhat more severe for extended trips such as to Mars or Venus, on which the space ship will inevitably be exposed for periods of many months. Unfortunately it is not possible with the information now at hand to state categorically whether this is a serious problem or only an irritating one. Quantitatively small changes in dose measurements might push the conclusion either way. It is the object of this paper to bring together in a brief form as many presently available facts as possible to allow evaluation of this situation as the new data become available.

PRIMARY COSMIC RAYS

Primary cosmic rays have been investigated for many years and there is a vast body of literature on the subject. The synthesis of these many different reports has led to a fairly consistent picture, which indicates that the primary or galactic cosmic rays with which the earth is constantly bombarded are of extrasolar origin. There is a factor-of-2 change in their intensity during the 11-year solar cycle. They are twice as intense at the minimum of the solar cycle--that is, when the solar sun spot count is at its minimum value. This factor-of-2 change, which has been observed for many years, is interpreted as caused by a change in the sun's magnetic field, the solar field being stronger at times of solar sunspot maximum. The increased field protects the earth from the galactic cosmic rays. The magnetic field dwindles during the minimum of the solar cycle and consequently the intensity of cosmic rays reaching the earth increases.

With the exception of this solar-induced variation of the galactic cosmic rays we find that they are remarkably constant and have been so during the last 50 years. There is some experimental evidence that they have been constant for several hundred thousand years. They are thought to be of galactic origin--possibly from supernovae in the galaxy, or possibly from some synchrotron type of acceleration mechanism--or to be the result of an approach to an equilibrium energy distribution between charged particles and magnetic clouds. These several theories of their origin have been suggested principally by Enrico Fermi.

There is no general agreement about the origin of cosmic rays; however, several outstanding facts about the primary cosmic rays must be taken into consideration in any theory of their origin, and in the evaluation of their possible biological effect. Their energy spectrum is given roughly by

$$P(E>E_t) = 0.4\ E_k^{-1.15}$$

The similarity of the spectra of the various major components of the primary cosmic rays suggests that the protons, deuterons, alpha particles, carbon, nitrogen, oxygen and heavier ions have all been accelerated by the same mechanism in such a way that, although their spectral intensities vary with the type of ion, the energy dependence of their spectra is quite similar. This similarity of spectral shape rules out some of the theories of the origin of cosmic rays and indicates that some of the acceleration mechanisms proposed for their origin are not realistic. Although the most likely source of cosmic rays is not yet agreed on, it is very tempting, in view of the similarity of the energy spectra, to feel that a gross accelerating mechanism carrying along all atomic species in a shock wave or plasma bundle, as in an exploding supernova, may offer a logical explanation. Fortunately for our

purposes the understanding of their origin is not particularly critical.

The spectra given in Figure 1 are extrapolated to locations in space more than 10 earth radii away where the effect of the earth's magnetic field is negligible. The dotted lines in the figure, labeled with latitudes of the earth, refer to the fact that the net result of the earth's magnetic field acts on primary cosmic rays to prevent cosmic rays below a certain cutoff energy (a function of latitude) from reaching the earth's surface. At the earth's magnetic poles in Northern Canada and Antarctica there is no so-called energy cutoff, and cosmic rays of arbitrarily low energy can penetrate to altitudes of roughly 100,000 feet or lower, barring their encounter with atoms of the air. The energy spectrum at the magnetic poles is thought to be characteristic of that in free space, but spatial orientation of the cosmic rays is directed vertically rather than in the 4-π free-space orientation. With regard to their free-space orientation, no preferred direction for cosmic rays has ever been discovered, although the earth's magnetic field somewhat distorts the apparent direction of approach of cosmic rays. When corrections are made to eliminate the effect of the earth's magnetic fields it seems that the primary cosmic rays are probably isotropic in the solar system.

As a result of the energy cutoff and the resulting inability of cosmic rays with less than a certain energy to reach the surface of the earth, there is a shift with latitude of the energy spectrum observed at the top of the atmosphere or at the surface of the earth, because the earth's magnetic field extends to about 10 earth radii or about 40,000 miles, but the earth's atmosphere extends only to about 20 miles. The spectral situation at the top of the atmosphere is essentially that which one would have at the surface, if the earth's atmosphere did not exist but only the magnetic field were present.

Particles arriving at the geomagnetic equator, which roughly parallels the geographic equator, must have momentum of about 14 Bev/c in order to penetrate to the top of the atmosphere. At 30° geomagnetic latitude this value has declined to about 7 Bev/c; at 60° latitude it is only 1 Bev/c, and it goes to essentially zero at the geomagnetic pole. This phenomenon is most convenient for investigating the primary cosmic rays, because merely by making measurements at the top of the atmosphere (or, under special circumstances, at sea level) at different latitudes one can investigate the spectrum of the primary cosmic rays. This technique also applies to the particles associated with solar flares. If sea-level measurements are made, the atmosphere unfortunately introduces secondary particles that tend to mask the effect and to make the interpretation more difficult.

This magnetic effect on cosmic rays, which directly influences space travel only inside the Van Allen belts, is a basis for much of our understanding of the cosmic rays. It can be explained by noting that the earth's magnetic field is shaped about like the field of a bar magnet or dipole magnet located approximately at its center. The center of this equivalent magnet is located about 342 kilometers from the center of the earth, and its magnetic poles at latitudes of 80 degrees north and 76 degrees south, rather than at 90 degrees in each case. All magnetic effects are tilted in such a way that the magnetic latitudes over the entire North American continent are somewhat higher than its corresponding geographic latitudes.

Another result of the magnetic field is that positive particles of low momentum

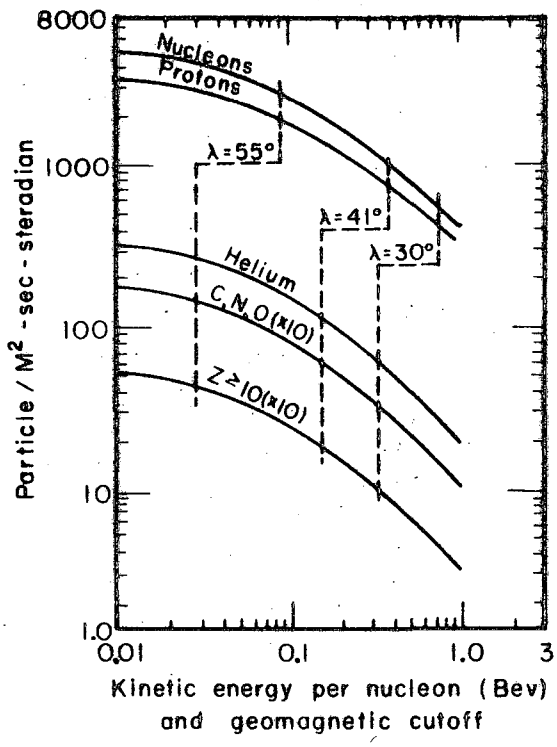

Figure 1. Integral energy spectrum of primary cosmic rays, separated into four constituents: nucleons as a whole; protons; helium; carbon, nitrogen and oxygen and $Z \geq 10$. The magnetic cutoffs for 30, 41 and 55 degrees geomagnetic latitude are shown. The cutoff at the equator for protons is 15 Bev. After B. Peters (ref. 1).

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at any particular longitude reach the earth more easily from the west than from the east. These directions are interchanged for negative particles, the so-called "east-west" effect that is the basis for our belief that essentially all primary cosmic rays are positive in charge. This does not mean that the earth has gradually accumulated an enormous net positive charge. There is a simultaneous flow of negative electrons to the earth which exactly balances the flow of positive charge to the earth. These electrons, however, have quite low energies compared with the energies of positive cosmic rays, and the major energy flow to the earth is carried by the positive, heavy cosmic rays. The spectral shape for charged particles is unaffected if the particles have sufficient energy to penetrate the earth's magnetic field. The spectra are "cut off" at their low-energy ends by the excluding effect of the magnetic field.

Figure 2 shows the relation between primary cosmic-ray intensity and distance from the earth at four different latitudes. This intensity rises to an asymptotic value of about 26 millirep per day at distances greater than 2 to 3 earth radii, and that at such distances the effect of the earth's magnetic field, in causing intensity variation with latitude, becomes very small. The dose rate of 26 mrep per day is that which would, in the absence of solar flares or other special situations, be received by an astronaut at large distances from the earth, for example, in the main cockpit of a space vehicle or on the surface of the moon or some other planet such as Mars. There is some question as to the proper RBE factor to be applied here to give this irreducible radiation dose in units of rem per day. It seems probable, however, that a reasonably small RBE factor, between 1 and 5, would be appropriate. This would mean that there would not be a serious radiation problem

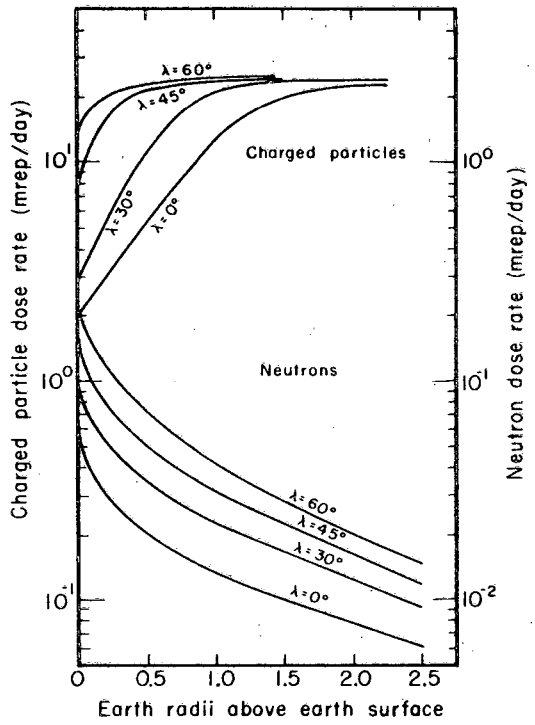

Figure 2. Dose rates in mrep/day for charged cosmic rays and for secondary neutrons escaping from the atmosphere. Note that the neutron curves have been shifted up by one cycle for compactness. From Wallace (ref. 6).

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involved in extended residence outside of a special solar flare shield at times when flares are not occurring.

The earth acts as a perfect cosmic ray absorber: no cosmic ray (except for neutrinos, which are not important to our considerations) is sufficiently energetic to pass through the earth. As a result of this shielding effect, the cosmic ray intensity at any point relatively near the earth is cut in half. This presumably is also the case near all other astronomical objects. The constant counting rate measured by the Russian rocket as it approached the moon in 1959 indicates that the radiation near the lunar surface is about one-half due to cosmic rays and one-half due to radioactive content of the lunar surface.

A great deal of experimental and theoretical work has been done on the secondary cosmic radiation particles knocked out of the air by the passage of the primary cosmic rays. Because the incident primary cosmic rays are largely absorbed at altitudes above 70,000 or 80,000 feet, these secondary cosmic rays are the principal ones available to surface investigators. The large amount of information available on these secondary radiations, which consist of protons, mesons, electrons, photons and neutrons, indicates that they do not constitute a serious dose hazard on the earth and would not under any other planetary atmosphere. Therefore we should concentrate our present attention on other types of radiation, which are observed largely in the vacuum outside our atmosphere, and neglect any further discussion of the production of secondaries in a planetary atmosphere.

SOLAR FLARES

Solar flares, although a relatively recent discovery, have been described by a number of authors, and summarized by several, as in the excellent review article by D. H. Robey.[2] In discussing the phenomena of solar flares a few words should be said about the sun itself and about the much more fully observed phenomena of solar sunspots. A few simple facts about the sun will be of value in our discussion. The sun rotates on its own axis in the same manner as a planet but much more slowly, the period of rotation being 27 days. The sun has its own magnetic field, which in an overall average at the solar surface is somewhat weaker than that of the earth; however, the local magnetic field strengths are often in the kilogauss region. The visible surface of the sun, called the photosphere, is a highly absorbant layer of incandescent gas and is the source of most of the visible radiation. It is the photosphere that emits the radiation received by the earth, which is similar to that which would come from a radiating black-body at 6000° Kelvin.

The blackbody concept cannot be extended to the short-wave-length end of the spectrum, because a negligible amount of radiation is emitted below 1500 Å. The 6000° blackbody analogy is good only near the visible region. Of interest in the prediction of solar flares are two extremely intense emission lines in the short-wave-length end of the solar spectrum, the hydrogen and alpha line at 1216 Å and the doubly ionized helium line at 304 Å. Neither can be seen at the surface of the earth because essentially all wave lengths shorter than 2850 Å are absorbed by the earth's atmosphere. Actually the intensity of the hydrogen alpha line at the distance of the earth from the sun amounts to a few tenths of a microwatt per cm^2. It is the most intense line in the entire solar spectrum. The solar corona seen during solar eclipses as a bluish-white halo with a greenish cast extending several million miles out from the sun, should not be confused in one's thinking with solar flares. The solar corona is an extremely low-pressure gas which has a temperature of approximately $1{,}000{,}000^{\circ}$ K, which is required to account for the expansion of the corona against the pull of the sun's gravitational field. The greenish tinge is apparently caused by iron atoms each of which has lost 13 electrons. The light from the corona does not have strong lines in it because the very high velocities of the atoms produce Doppler broadening of the spectral lines.

<u>The Solar Wind</u>. There is an important phenomenon known as the solar wind that has received attention only in recent years. It consists of ionized hydrogen atoms which continuously fly away from the sun in all directions. These form an electrically neutral plasma which is completely ionized and has as a result its own "frozen" magnetic field. It is apparently a consequence of this plasma streaming that the sun's otherwise dipole magnetic field is considerably flattened into a disc shape. The velocity of the solar wind is a few hundred to a few thousand kilometers per sec. The particle density ranges from 10^{-2} to 10^{2} ions/cm^3 at the earth's orbit. On at least one occasion there was a sixfold increase in the velocity of the solar wind as measured by Explorer X at a great distance from the earth several hours prior to a solar flare. There was an accompanying disturbance of the sun's magnetic field which ordinarily is between 20 and 32 gammas (1 gamma = 10^{-5} gauss), so it is possible that the solar wind and the accompanying magnetic field bear some relation to the impending onset of a solar flare.

The solar wind seems to carry the sun's distorted dipole field radially outward to a distance somewhere between the orbit of Mars and the orbit of Jupiter. In this region the magnetic field is sufficiently weak to equal roughly the galactic magnetic field. At this radius there is a disturbed field configuration. Several measurements have indicated that solar flare particles released from the sun are fairly well contained within a roughly spherical region whose perimeter lies somewhere between the orbits of Mars and Jupiter, because this barrier is known to reflect very energetic particles emitted by the sun. It is conceivable that the Martian-Jovian boundary may screen the earth from some external galactic cosmic radiation. However, no direct measurements of this possibility are at present available.

The average magnetic field on the surface of the sun seems to be 1 gauss and this value falls off roughly as the inverse square of the radius for considerable distances from the sun; only outside the earth's orbit does it seem to be of conventional dipole inverse cube behavior.

The sun is known to be a source of soft X rays which are emitted both by the corona and by the photosphere. The X-ray emission changes rather rapidly in both intensity and wave length. In some cases, for example, a hot spot in the corona may last for an hour and during this time increase the radiation of 8-to-20 Å wave length quite markedly. It has been observed that when a solar flare occurs, the total X-ray energy output may be as high as 10^{31} ergs, which represents 7000 ergs/cm^2 at the earth's distance from the sun. This X-ray emission may last for 30 minutes. It is relatively easy to shield against these soft X rays which apparently do not constitute a serious hazard.

Sun Spots. Sun spots are characterized by the sunspot number, R, which is a measure of sunspot frequency. It was first defined by R. Wolf of Zurich in 1849 as the number of sunspots, n, on the solar disc which appear in g groups, and the sunspot number is

$$R = K\ (n + 10\ g)$$

where K is an estimate of the local observing conditions (Figure 2). K is about 0.6 for Zurich. R would be 1 for a telescope in free space. The relative sunspot numbers for a period of more than 200 years are shown in Figure 3. Interestingly, the recent year 1958 had the highest number on record, when 910 sunspot groups were observed from Mt. Wilson in California. The sun was visible for 339 days during this year. The average time between peaks in the sunspot intensity curve is 11.1 years; however, variations as much as 3 or 4 years from this value have been known. There is also 90-year periodicity, which is superimposed on the 11-year period. We were at the peaks of both cycles in 1958.

Sunspots themselves occasionally can be seen with the naked eye through a dense absorbing filter. When studied in detail with a telescope they usually appear in pairs. Because their temperature is 1000° to 2000° lower than the 6000° average temperature of the surface, they have a somewhat darker appearance than the surrounding photosphere. Many of their characteristics are known. For example, there is an area surrounding the spot itself which appears to be covered by radial filaments. These filaments change with a lifetime of about 30 minutes. The spots themselves are shallow bowl-shaped depressed areas ranging in diameter from 150 to 50,000 miles. Several spots may overlap, forming patches as large as 150,000 miles across. Ordinarily a spot lasts only a few days, although some individual spots

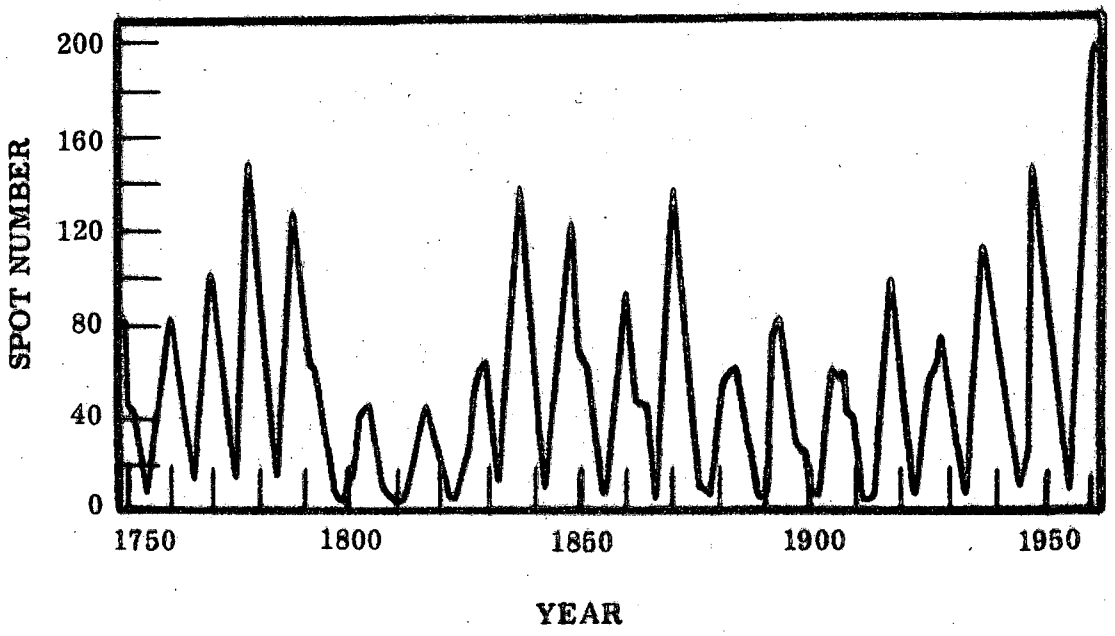

Figure 3. Relative sunspot number versus time, showing the short-period (11-year) and long-period (~90-year) solar cycles. From D. H. Robey (ref. 2).

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have lasted for months.

Sunspots normally appear in groups that are elongated along latitudes of the sun, and they appear in zones both north and south of the solar equator. Most spots confine themselves to the region between 8° and 30° north and south latitude. They rarely come within less than 8° of the equator. Each 11-year cycle begins with the groups of spots located 30° north and 30° south of the equator. The succeeding groups gradually appear closer to the equator, fading out at about 8°.

The spots are magnetically polarized and the polarity reverses itself on succeeding solar cycles, so that there is a polarity cycle of 22 years. The large magnetic field of sunspots is apparently important in maintaining the temperature differential between them and the surrounding area. Many other phenomena of solar sunspots have been observed which indicate that in general they are strongly magnetic regions. They are related to each other in groups in turn related to the rotation of the sun, the phase of the solar cycle and the internal magnetic structure of the sun. Some knowledge of the qualitative behavior of the sunspots is perhaps of value in our subsequent discussion of solar flares. A small sunspot is likely to have a magnetic field of the order of 1000 gauss, which is greater by three orders of magnitude than the average solar magnetic field. The large groups may have magnetic field strengths extending up to 4000 gauss. The magnetic field of a spot tends to extend vertically along the solar radius.

Solar flares. Although magnetic storms observed on the earth have been correlated with large sunspots, it is more likely that they are caused by solar flares. When a flare is seen in profile on the limb of the sun it usually resembles a bright little mound directly above the photosphere. Loop prominencies are often seen in

conjunction with flares, and it is possible that they may represent the ejected flare plasma, which can be seen only near the limb of the sun. Most flares are red because they emit large quantities of hydrogen alpha light at 6562 Å. Occasionally a flare is sufficiently intense to be seen as white light. Usually they are best observed through a band-pass filter about a 2 Å wide that includes the hydrogen alpha line.

A typical flare is thought to consist of two phenomena, an expulsion of low-velocity particles contained in a magnetic capsule that take about a day to arrive at the earth, and prompt particles that travel along curved paths from the sun at about 60% of the velocity of light and are observed earlier than the low-velocity particles, but which do not themselves produce magnetic storms. However, these high-velocity particles are responsible for polar-cap blackouts, which are described later.

The bowl-shaped solar prominence associated with the flare may be as high as 20,000 kilometers above the photosphere surface; 10,000 kilometers is usually an upper limit, however. A flare can cover as much as 0.1% of the sun's surface. They are rarely seen more than 10,000 kilometers from the center of a sunspot group. Shortly after or at the same time as a flare starts, some of the surface region of the sun around the flare brightens considerably. At the same time the tenuous hydrogen clouds that frequently float above the photosphere and are occasionally seen in profile, may temporarily brighten or vanish. This phenomenon occasionally occurs with no accompanying effect on the filaments, which are also above the solar surface but at a lower altitude than the hydrogen cloud, and this is interpreted to mean that the disappearance of the hydrogen cloud is not the result of direct radiation from the flare bowl to the clouds but rather of the movement of ions along curved magnetic flux lines, thus avoiding the intermediate filaments.

It seems reasonable to assume that the energy contained in a flare comes from the magnetic field associated with the sunspots that surround it. The exact mechanism by which this is accomplished is not clear. The sunspots associated with flares are usually rather complex and disordered and their magnetic field somewhat nonsymmetrical. The flare usually arises in a null field position where the gradient of the field is quite steep but its absolute value perhaps near zero. In a typical large flare, an energy release amounting to 10^{32} to 10^{33} ergs may result from a rapid change in the magnetic field in this region.

The fraction of total flare energy that goes into X radiation, hydrogen alpha light, radio noise, auroral displays and, most importantly, cosmic rays is now known. For the largest flare on record, that of February 23, 1956, it has been estimated that about 1% of the total energy of 3×10^{32} ergs went into protons with energies above 2 Bev, and approximately 0.6×10^{32} ergs of hydrogen alpha light was produced.

The particles ejected in conjunction with flares are probably between 85 and 100% protons. Other particles have been detected, including heavy ions, but these are not particularly numerous. If neutrons were released by flares, then in view of their nonmagnetic behavior they would be predominantly observed at the solar noontime position on the earth. Such is not the case, and it is consequently presumed that the mechanism for solar flare production is not a nuclear process. The neutron half life of 13 minutes certainly considerably reduces the flux at the earth of any neutrons that might be emitted by the sun, because flight times from the sun

to the earth at least as long as the half-life are involved for high-energy neutrons. Neutrons have not been detected in solar flares, which indicates that neutron doses would not be a problem in conjunction with solar flares.

Actually, Severnyi has identified 25 different elements in the optical spectrum of solar flares.[3] How many of these are accelerated is not known, but some heavy ions have been detected in satellite experiments.

Bright shock waves have been seen moving out from flares at supersonic velocities. When these supersonic shock waves approach a sunspot they are somewhat retarded, their luminosity increases and they appear to follow curved magnetic channels. Sometimes these phenomena are accompanied by accelerations of the solar atmosphere which appear to be operating against the gravitational effect. In spite of the failure to observe neutrons, deuterium lines are often observed as intensified, showing that neutrons actually may be produced as a by-product of the flare. In evaluating the possible mechanisms for solar flares, it should be remembered that the ionization of the gas in which they occur is extremely high and that as a result the magnetic field is frozen into this solar plasma. Under these conditions it is not possible for large electrical currents to flow, because such currents would require a magnetic field reorientation. As a result, when a blob of solar plasma is ejected from the sun, it carries along trapped within it the magnetic field that was originally present in this particular mass of gas.

The initial explosion of a flare produces X rays which are apparently simultaneous with the visible hydrogen alpha light flash and have energies as high as 100 kilovolts. These produce sudden ionization of the D region of the upper atmosphere and also force the D layer to a lower altitude, especially in the polar cap region. A flux of high-energy protons may accompany this initial photon flux and lasts for an hour or more. The proton flux then decays in a manner that suggests that the protons may have been trapped in a large but leaky magnetic bottle partially closed by magnetic mirrors at each end.

At the earth the flux density of these protons has been known to reach two or three orders of magnitude above that of the average galactic cosmic ray flux. As mentioned previously, a low-energy magnetized plasma arrives at the earth about one day later. The average transfer time is about 33 hours. This low-velocity plasma that follows a day later behaves in a manner suggestive of the behavior of a shaped-charge jet produced by a chemical high explosive, where there is an initial outburst of material followed later by a large mass of material. The protons in this late-arrival plasma cloud are of relatively low energy; however, the magnetic field that it contains strongly influences the magnetic field of the earth, producing magnetic storms and intense auroral displays. The low-energy proton-containing plasmas which can induce geomagnetic storms do not seem to accompany flares of class less than 2.

Flares are classified according to the limits set forth in Table 1. We are principally concerned with flares of classes 3, 3+, and 4. Actually only flares of class 4 seem to be particularly dangerous to an astronaut with a minimum of shielding. Dangerous solar flares are generally accompanied by radio-wave reception. There are several different types of radio emission associated with solar outburst phenomena; they are outlined in Table 2.

Type II and type III solar radio noise outbursts, although not always present, often signal the initiation of a solar flare. Type II radio noise, that tends to

Table 1

FLARE CHARACTERISTICS*

Class	Number of flares (1959)**	Duration in min.	Relative intensity (H-alpha)	Mean area 10^{-6} visual hemispheres	Average H-alpha line width, Å	Maximum particle energy
1-	2209	5-20	1.5	≈25	1.5	
1 123 1+ 9	132	4-43	1.5	100-250	3.0	
2 21 2+ 6	27	10-90	2.0	250-600	4.5	
3	6	20-155	2.5	600-1200	8	several hundred Mev
3+	1	50-430	3.0	1200	15	1 Bev
4	0.02***	44-120	4	1200	23 or greater	2 to 40 Bev

*After Robey (ref. 2).
**The data in the second column are based on the measurements by G. E. Moreton of the Lockheed Solar Observatory (2375 flares during 1869 hr of observation).
***Based on eight class-4 flares in a 20-year period.

Table 2

NONTHERMAL SOLAR RADIO NOISE*

Type	Duration	Source	Speed through corona (km/sec)	Spectral character	Band width
I Noise storm burst	20 sec	"R" center	----	nearly fixed, narrow	a few Mc
II Outbursts excited by gas motions through the corona	minutes, 10-100 per hour	flare, limb ejections	1000 km/sec	slow-frequency drift to lower values, sharp cutoff	broad with narrow peaks, tens of Mc
III	seconds	initiation of flare	10^5 km/sec or more	fast-frequency drift to lower values	5-1000 Mc
IV Synchrotron radiation	several hours	occurs after flares	stationary after type II motion	spiraling electrons 100% polarized	continuous distributions 15-1000 Mc
V	minutes	often flare	3000 km/sec after type III motion	100 Mc region	tens of megacycles

*After Robey (ref. 2).

be associated with magnetic storms, is also associated with class 2 or class 3 flares. More than 90% of these slow-drift bursts were associated with flares of importance greater than 1. Conversely it can be said that all flares that have radio noise associated with them have either a group of type III bursts, or a type II burst followed by a type III burst.

The type II burst may be delayed as long as one minute after the optical onset of the flare, but it still provides ample warning of imminent arrival of the charged particles. The type IV noise is highly correlated with the solar flare particles, but comes too late to provide advance warning of the flare. The flare particles themselves provide several minutes of advance warning, and although the rate of rise of the flare radiation is quite steep, a man would be quite safe to rely on his radiation detectors to tell him when to take cover.

It is interesting to note that the number of sunspots occurring in the northern and southern solar hemispheres is approximately equal, but that sunspots in the northern solar hemisphere are about 6 times more likely to be associated with intense flares than are those in the southern solar hemisphere. This ratio is based on a 23-year study of 580 major solar flares. About 60% of these flares originated from spot groups located within 20° longitude of the central meridian as viewed from the earth. A plot of solar flare locations as a function of their magnetic type is shown in Figure 4,[2] and these data are summarized in Table 3.[2] Spots of the type $\beta\gamma$, that have a complex magnetic field and that are located in the northern hemisphere, are far more likely to produce flares (Table 3[2]). Actually none of these complex spots was identified in the southern hemisphere. Once a flare has occurred a "magnetic channel" appears to have been formed from the sun to the earth down which the flare particles pass. Some hours later this same channel from sun to earth conducts the low-energy plasma responsible for geomagnetic storms and auroral displays.

The character of the type III radio noise is somewhat altered by the superposition of harmonics thought to be caused by oscillations of the solar corona as the charged flare particles pass outward through it. This is a well-known effect in plasmas. The harmonic alteration of type III radio noise signals is thought to be a clear indication of the ejection of protons from the sun.

Table 4,[2] that lists the great solar flares, their points of origin on the sun and their subsequent geomagnetic activity, shows that there is an overwhelming preponderance of major flares located in the northwest quadrant of the sun, an effect thought due to the shape of the sun's magnetic field. The solar magnetic field is flattened in the plane of its equator by the conductivity of the solar winds into an inverse-square-law dependence. This solar-wind interaction with the sun's solar magnetic field produces also a spiraling of the radial field lines toward the west. The sun rotates in the same sense as the earth proceeds around the sun, so that on our side of the solar disc the apparent motion is from left to right (from east to west), and the solar magnetic flux lines approach us from the west of the sun. Therefore, the apparent source of particles approaching the earth and spiraling along solar flux lines is 40° west of the sun. This effect also strongly increases the probability that a flare on the west side of the sun will deliver particles to the earth. We view the sun from the west magnetically, or put another way, the solid angle for a solar-flare particle to reach the earth is much larger for events on the west side of the sun than on the east side. Statistical analyses have been made

Table 3

GEOMAGNETIC STORM FLARE DATA*

Magnetic sunspot type	Number of GSPF per 580 flares** N(north)	S(south)	Ratio N/S	Probability of GSPF/spot north	south
Unipolar - α	1	7	0.14	0.00172	0.0121
Bipolar - β	11	1	11	0.0190	0.00172
Semicomplex - β	31	0	∞	0.0534	0
Complex -	18	2	9	0.0310	0.0034

*After Robey (ref. 2).
**GSPF = great-storm-producing flares.

Table 4

SOLAR COSMIC RAY FLARES

Date	Magnetic type of associated spot	Disk position of flare	Subsequent geomagnetic activity
1942 Feb. 28		4° E 7°N	great storm
1942 Mar. 7		90° W 7°N	small storm
1946 July 25		15° E 22°N	great storm
1949 Nov. 19		70° W 2°S	no storm
1956 Feb. 23		80° W 23°N	great storm
1960 May 4		90° W 12°N	moderate storm
1960 Nov. 12		10° W 12°N	very great storm
1960 Nov. 15		50° W 25°N	great storm

*After Robey (ref. 2).

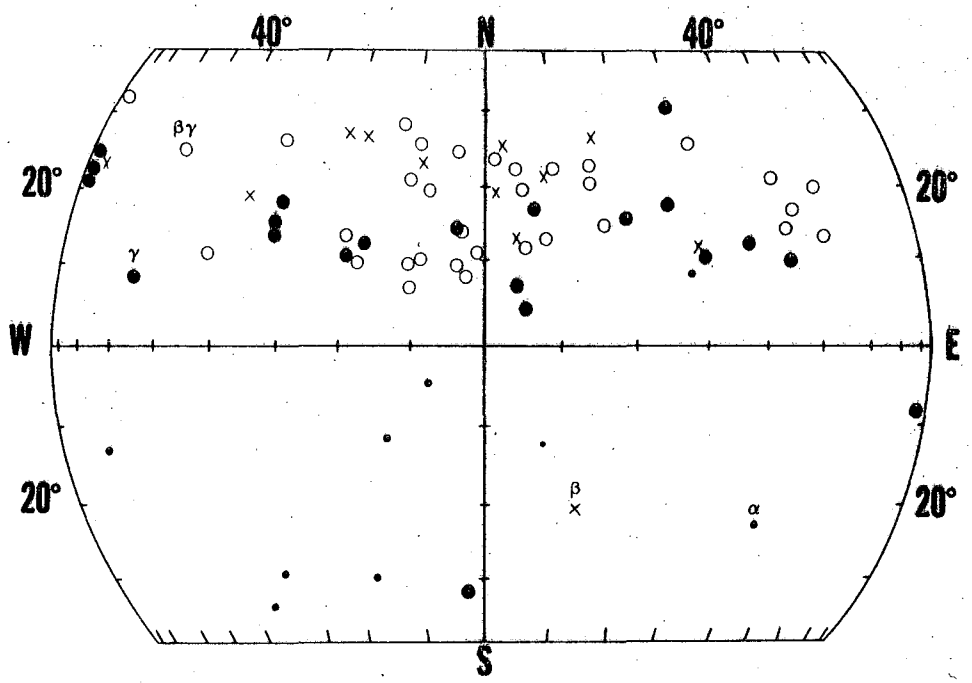

Figure 4. Positions of intense geomagnetic-storm-producing flares on the solar disk. The associated sunspot magnetic field type is also indicated. After Robey (ref. 2).

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of the solar sunspot frequencies and solar flare frequencies, that are closely correlated. However, predictions from one solar cycle to the next are very poor. For example, in 1959, two years after the peak of the cycle in 1957, the sun was still more active than in 1947, the peak of the preceding cycle. Although solar radio noise outbursts are so far not useful for predicting flares in advance, a noise outburst accompanying a flare is a good indication that particles have been ejected. Many flares, with associated radio noise, are observed although the earth does not subsequently receive particles.

A few hours prior to a major flare the region surrounding an associated sunspot group takes on a chaotic appearance, the so-called "ripe look", which an experienced solar observer can identify as having a high probability for emitting a solar flare. Unfortunately these criteria are difficult to describe objectively, the prediction being purely an art on the part of the solar observer. A sunspot or sunspot group that has already produced a flare has a much higher probability of producing subsequent flares, and it should be continuously watched even after it returns from having been on the far side of the sun for the 14-day half-rotation time.

Some general conclusions can be made about solar flare occurrence: flare probability is in phase with the solar cycle, although some large flares have been observed during times of solar minima; during a year there is a higher probability that flares will occur near the equinoxes than that they will occur during June or December. The critical fact in accounting for this phenomenon seems to be that the

earth passes through the plane of the solar equator on June 5 and December 5. (This transit is of course not the same as the equinoxes, when the sun crosses the plane of the earth's equator.) Apparently the probability of solar flares is enhanced when we view the sun from as high or as low a solar magnetic latitude as possible. The monthly distribution of particle-producing flares is closely correlated with the geomagnetic and the auroral events of that particular month. These qualitative conclusions suggest that December or June is a better choice for lunar trips of a few weeks than the spring or fall.

If at any time the solar disc contains a small number of sunspots, or if the sunspots are of the magnetic type, age or size having a low correlation with known flare production, it would seem that for the next week or two the probability of encountering a serious flare is relatively small. Apparently advance solar-flare predictions for short lunar trips are entirely within the realm of possibility. Purely on a statistical basis one can say that favorable launching dates can be recognized provided the trip is of sufficiently short duration that the solar conditions are unlikely to change during that period.

It should be noted that in all our discussions we are considering only the near side of the sun. Apparently there is a very small chance that flare particles ejected from the back side of the sun can reach the earth. Actually, out of all the flare events observed there is only one rather doubtful case in which particles came to the vicinity of the earth with no visible flare on our side of the disc. Thus as soon as a spot of known activity swings past the solar limb to the far side we can be reasonably safe from it.

Chupp, Dye, Marr, Oncley and Williams have made an expert analysis of the seriousness of the solar-flare proton radiation hazard.[4] This analysis is based on a statistical approach by which the flares from 1956 to 1960 were taken into consideration and estimates made of their spectral distribution. These spectral distributions were then converted into doses, assuming that the pertinent dose to be considered is at 4 cm depth in tissue. They made their calculations for unshielded tissue and for tissue shielded by 5 g/cm^2 of aluminum. The 5 g/cm^2 of aluminum is chosen to represent a practical minimum of essential hardware for a space capsule. Two or three times as much shielding consisting of hardware may be provided by a reasonable capsule design.

For the 43 events considered during this period, only six actually gave significant doses. Of these the largest was 150 rad. If these data are to be believed, it seems that for a relatively short flight of about 2 weeks there is a very small probability that a flare would be a serious problem even to an unshielded astronaut. Chupp concludes that the probability of encountering a dangerous dose level in the course of a week's space flight is about equal to the probability of a fatal accident in a week of flying in an operational military aircraft. He further concludes that if advance solar flare prediction is used, this hazard can be further reduced by a factor of 3 on short missions. If, in addition a small amount of extra shielding is included, the dose can be further reduced by a factor of 3 to 10.

Thus it would seem that in comparison with the other hazards of space flight the solar flare problem, although well worth serious consideration, is by no means a limiting factor. It is probably preferable to devote payload to components giving system reliability than to shielding. Figure 5 shows the intensity of flare particle

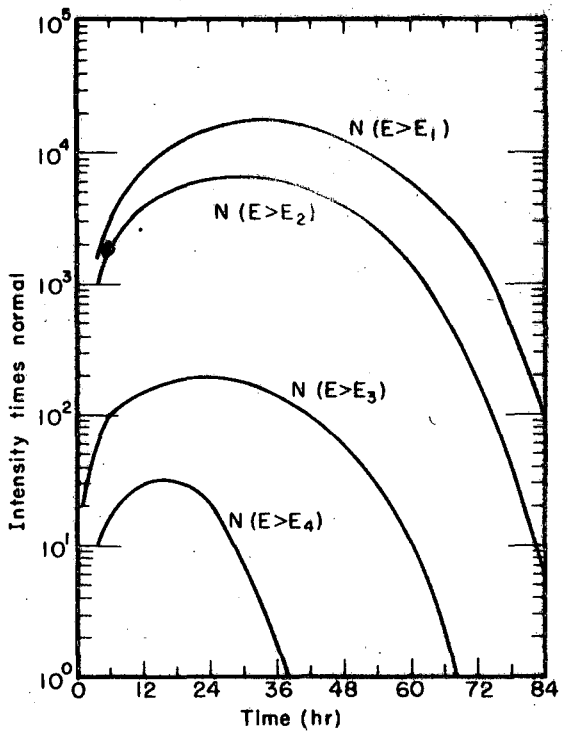

Figure 5. An idealized complete set of curves of integral intensity vs time for Method I. After Chupp et al. (ref. 4).

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radiation in the vicinity of the earth but outside the atmosphere, as a function of the time since the onset of the flare.[4] It is seen that particles of energy $E > E_4$, for example, arrive early and stop early; the low-energy particles--for example, $E > E_1$--arrive somewhat later and persist much longer. Thus there is a shift in intensity with time for particles of a given energy and also for the overall spectral shape. The net result of such a curve tends to be complicated. In terms of dose the dangerous part of a flare certainly occurs during its early phase, because the energies after a few hours usually drop below a few tens of Mev. Protons of these energies are quite easily shielded out by ordinary pressure-vessel walls.

The shift in spectral shape is generally attributed to the different flight times of particles of different energies. The direct flight time from the sun varies by several hours for particles of different energies, and as a result there is considerable dispersion of the originally coherent bundle of particles that were ejected together (within a short time) from the surface of the sun. Unfortunately, each flare differs somewhat from all other flares in its spectral shape as a function of time. Chupp has made some simplifying assumptions in the analysis of the problem in order to secure a numerical answer. For some flares he has assumed that there is a linear rise to the maximum, and that after the maximum is reached the intensity falls off as T^{-n}, where n is fitted to the available time history of the event. The maximum-intensity point is taken to be that one at which riometer maximum is achieved.

"Riometer" stands for relative ionospheric opacity meter. This instrument measures the galactic radio noise received at the earth's surface, a measurement usually made in the polar regions. When the ionosphere becomes more opague to radio waves owing to the formation of extra ions by bombarding particles in the upper atmosphere ionic layers, the galactic radio background noise is reduced. This is a

very simple and effective method for measuring the bombardment of the ion layers of the high atmosphere by particles from the sun. The riometer is especially useful in making measurements of quite low-energy protons, as low as 10 Mev. Such a low energy is quite harmless, because even the thinnest-walled Geiger counters shield out protons with energies less than 30 Mev. The riometer gives a good measurement of the spectrum of protons in the region of a few tens of Mev, which are incident at latitudes greater than 70°. Unfortunately our data on the spectrum of the incident flare protons, especially at energies above 100 Mev, are not as complete as we desire. There are extensive measurements with balloon-borne emulsions, satellites, sounding rockets and even some types of sea-level detectors. The most consistent picture measurements are now supplied by the riometer. The data gathered in other ways are reasonably consistent but show rather wide variations.

The most critical question in all flare measurements arises when we try to predict their spectral shape in a few-tens-of-Mev region in those cases in which the only measurements were at higher energies. This is the case when data for a flare depend largely on sea-level measurements which, because of the magnetic cutoff of the earth's field, are automatically limited to high-energy particles. In these cases the extrapolation from the measured high-energy spectrum to the low-energy spectrum is subject to considerable error, so that rather wide variations are possible in estimates of the seriousness of the dose problem. This has been especially true of the data obtained from some of the large flares of the past. Some methods of extrapolation to lower energies, such as the use of the same energy power law, have predicted colossal doses in the 10-to-100-Mev region. It seems, now that more data are becoming available on the spectrum of the protons in solar flares, that there is less of a tendency to make such linear extrapolations and more of a realization that a nonlinearity of the spectrum tends to de-emphasize the low energies. This places considerably lower estimates on the doses from flares, which it was thought a few years ago would be fatal even under thick shield.

HEAVY IONS

The second most serious and less known radiation problem to be encountered in space travel is that of heavy ions. Heavy ions, as was shown in Figure 1, are present in the primary cosmic rays with spectra whose energy dependence is similar to that of the primary protons.

Heavy ions associated with solar flares were detected in emulsions under about 0.1 g/cm^2 of shielding. This is a very thin shield. The elemental distribution of the heavy ions associated with flares is the same as that for solar material, indicating that they do indeed come from the sun. Their ranges were from 2 to 3 mm in emulsion, which is such a short range that there is certainly no significant radiation problem associated with flare-produced heavy ions. As a result of the apparently very modest energy of the occasionally produced solar-flare heavy ions it seems unlikely that they present a radiation problem that needs to be considered seriously for interplanetary travel.

Heavy ions present a radiation problem in quite another direction, the importance of which is not yet completely evaluated. The heavy ions associated with the primary cosmic rays, although very low in intensity, certainly do have ample energy to penetrate many grams of material. Simons has pointed out that in view of the

potential lethality of a few of the largest solar flares, either measures must be taken to protect against them or they must be avoided by some form of prediction.[5] In any event, once these measures have been taken, the radiation dose received by a crew of a spaceship during a flare, although perhaps still undesirably large, does not differ greatly in its biological effect from doses received on earth from natural and man-made sources. Although some biological and dosimetry experiments are needed to evaluate flare radiation hazards, the control of the dose received during a flare can perhaps be relegated to the status of an engineering problem or of a statistical prediction problem. Hence, one sees that in planning ahead for space flight it will perhaps be necessary to provide a small heavily shielded region in the space craft. One would hope that occupancy of this cramped shielded space might be for only a very small part of the time. When the space traveler is not huddled inside this flare shield he presumably will be in a thin pressure tank whose walls will be lined with 5 to 20 g/cm^2 of hardware, which, although it offers good protection from solar flares, offers little protection against penetrating primary cosmic radiation.

An important problem is the determination of the relative biological effectiveness of the heavy fragments present in cosmic rays. Much physical and biological work has been done on heavy ions by many investigators. The high charges present on the heavy ions produce intensely ionizing tracks in materials that they strike. There is a question whether a single such dense particle track can cause a gross biological effect, as for example in the brain. It is not the purpose of this article to discuss this latter point in any detail from the biologic point of view, but rather to gather together some of the large amount of published material now available on the subject of heavy ions. Heavy ions are still to some extent an unknown in the astronaut radiation problem, and adequate earth or satellite experimental facilities are not now available to assess their seriousness.

The first indication that ions heavier than protons are present in the primary cosmic radiation was secured from observations of densely ionizing tracks in emulsions flown just below the top of the atmosphere. Since the Z of a particle can often be found by an examination of its track of developed silver grains in nuclear emulsions, it was soon realized that carbon, nitrogen and oxygen, as well as ions as heavy as iron, were present in decreasing numbers with increasing Z. The number of lithium, beryllium, and boron ions is rather small, as indicated in Tables 5, 6, and 7, but nevertheless these ions are present. The changes in the intensity of the heavy ions with the latitude at which they are measured, caused by the earth's magnetic field, allows their energy spectrum to be ascertained in a manner analogous to that used to secure the spectrum of the primary protons. The use of the earth's magnetic field as a large spectrometer yields the results shown in Figure 1. The equations for these spectra are given in Table 8.

In evaluating the behavior of heavy ions in a stopping medium it is important to know their charge state as a function of position along their path. The charge state usually is not single-valued, nor is it equal to Z, but it has some intermediate value between zero and Z. The charge state changes with velocity for a particular ion, but the change in charge state with different stopping media for a given velocity and density is quite small. The equilibrium charge state should be thought of as the result of a competition between the loss of electrons to and the capture of electrons from the medium. The charge of any one ion fluctuates along

Table 5

COMPOSITION OF GALACTIC COSMIC RAYS RELATIVE TO THE COMPOSITION OF THE UNIVERSE NORMALIZED TO HYDROGEN

Element	Cosmic rays	Universe
H	100	100
He	16	8
Li, Be, B	0.24	$\approx 10^{-6}$ (light component)
C, N, O, F	1.2	0.2 (medium component)
$Z \geqslant 10$	0.4	0.3 (heavy component)
Fe group	0.1	0.003
$Z > 26$	$\sim 10^{-4}$	10^{-6} (very heavy component)

Table 6

ABUNDANCE OF CONSTITUENTS OF THE HEAVY-ION COMPONENT OF GALACTIC COSMIC RAYS

Element	Abundance (%)
Li	3.9
Be	1.7
B	11.6
C	26.0
N	12.4
O	17.9
F	2.6
$Z > 10$	23.9
	100.0

Table 7

ABSOLUTE FLUXES OF GALACTIC COSMIC RAY PARTICLES

Element	No./m.^{2}sec.ster
H	610 ± 30
He	88 ± 2
Li, Be, B	1.6 ± 0.4 (light component)
C, N, O, F	5.7 ± 0.28 (medium component)
Z ⩾ 10	1.94 ± 0.25 (heavy component)

Table 8

ENERGY SPECTRA OF PRIMARY COSMIC RAYS*

Z	Range of validity E_t = Gev/nucleon	Integral spectrum in particles/cm^2 sec steradian	Limits of exponent
1	$2 < E_t < 20$	$0.4\ E_t^{-1.15}$	1.05 - 1.25
2	$1.5 < E_t < 8$	$0.046\ E_t^{-1.6}$	1.3 - 1.7
3,4,5		~50% of CNOF flux	
6,7,8,9	$3 < E_t < 8$	$0.0024\ E_t^{-1.6}$	1.45 - 1.75
< 10	$3 < E_t < 8$	$0.0016\ E_t^{-2.0}$	1.85 - 2.25

*After Peters, ref. 1.

its path and eventually declines to zero at the end of the path.

There is at present no rigorous theory relating the velocity and charge state for a given ion in a given medium. An excellent review of the theoretical situation has been given by Neufeld.[6] Bohr suggested that an ion of large Z will lose all its electrons that have orbital velocities less than its translational velocity through the stopping medium. This is certainly qualitatively true. Bohr argued that the slower and less firmly bound electrons are the most easily removed, and that in the inverse process those electrons are most likely to be captured in an orbit whose characteristic velocity is close to that of the ion moving through the medium. Lamb, on the other hand, stated the stripping conditions in another way, that the moving ion is stripped of its outermost electrons until the ionization potential of the next stage of ionization is greater than the kinetic energy of an electron having the velocity of translation of the ions through the medium. At best, the experimentally measured values for the ionic charges as a function of the velocity agree only roughly with those predicted from either of these theories. Furthermore, the theory of the stopping medium and its density has not been considered at all. There are experimental data to show that fission fragments have a higher ionization in argon than in helium, an effect that is not found with either oxygen or neon ions passing through hydrogen, helium, nitrogen, or argon. It is possible that the effect is too small with oxygen or neon but is large enough to observe with fission fragments.

The average charge of an ion increases with the pressure of a stopping gas up to a plateau value. This effect depends on the relative length of the average time between successive charge-exchange collisions compared with the average time for radiation de-excitation of the ion from its excited state. If the collisions are infrequent, electrons that have been excited but not separated completely from their ions can radiate energy and drop back to their original state. If the collisions are closer together in time than the de-excitation processes, the orbital electrons are successively excited to orbits of higher and higher energy and are eventually removed from the ion. In an ion that is in equilibrium with its stripping medium, having been several times ionized and yet retaining several electrons, these remaining electrons are not in their ground state but are in a continual state of excitation. One of the difficulties encountered in heavy-ion theory is that the ions are actually in an excited state whenever moving inside a medium. So far all the theories have been for ground-state ions.

It would be desirable, in order to use the Bohr or Lamb theories in a solid or liquid, to know the distribution of electron orbital velocities or energies for an ion that is continually perturbed by collisions with a relatively high frequency. Neufeld does consider the effect of the moving ion on the medium and the back-reaction of the rearrangement of the electrons in the medium on the electrons of the ion.[6] This refinement has the advantage of being applicable to any ion, but the Bohr theory is applicable only to ions near the upper end of the periodic chart.

Many experiments have been conducted to measure the ranges of heavy ions of different energies in a variety of materials. There are data for range vs energy, as well as stopping power as a function of range. This material has been brought together in one reference.[7]

At present, experimental work to evaluate the biological and physical problems that arise in connection with heavy ions is being carried out at the three heavy-ion

accelerators, at Yale and at Berkeley in the United States, and at Kharkov in the USSR. In addition, work is being carried on under much more difficult conditions in balloons, rockets, and projected satellites. Unfortunately the heavy-ion accelerators available at present are capable of imparting only about 10 Mev/nucleon to their heavy ions. This is a very modest energy when compared with the energy of heavy cosmic ray primary ions. Only a very limited range of biological samples can be irradiated with these low-energy ions, because the ranges of all HLAC ions in tissue are less than 1 mm. Enzymes, bacteriophage, bacteria and yeast cells have been studied and described by several authors.[8-16]

The very low intensity of primary cosmic ray heavy ions makes it unusually difficult to assess their possible danger in space travel. It is conceivable that a few heavy ions producing rather large ionization tracks in sensitive regions of the brain might seriously impair the performance of an astronaut. This is a debatable point unsubstantiated by any neurological experiments available at present. In view of the serious implications it raises, this possibility should be further investigated.

It is known experimentally that there is a sensitive reaction of hair color, at least in certain strains of black mice, whereby a single heavy ion may bring about the cessation of color-pigment production in several adjacent hair follicles leaving a small streak and producing an effect which has been referred to as a "mouse cloud chamber." This extensive work by Chase is described by Simons.[5] It seems that this particular reaction is an unusually sensitive one, and it is not likely to be repeated with equal sensitivity in other heavy-ion interactions. Experiments closer to the problem of space flights have been reviewed by Simons,[5] who limited his considerations to those experiments which bear on the immediate medical problem of heavy-ion damage. Figure 6, based on Simons' data, shows the number of ion pairs formed per micron of path in water, an approximately tissue-equivalent material, plotted against the range of these particles in water. Carbon, neon and iron are represented in separate curves. The iso-energy curves are also shown in this figure, which contains a condensation of most of the important information available on the interaction of heavy ions with tissue, except for the radial variation of track ionization. The variation of the energy of the particles in the region of the earth during a sunspot cycle is indicated. Except for a few earth radii the earth's magnetic field becomes quite small and the magnetic cutoff is absent, allowing penetration by particles down to energies that can pass through the much weaker solar magnetic field. The regions in which tracks terminate by thin-down rather than by nuclear collisions is indicated; this distinction probably has considerable biological significance. The region of cosmic ray hits as defined by Schaefer is shown.[17] The three regions differing in the qualitative appearance of their radial distribution of ionization are roughly shown. Finally, the region presently covered by accelerators and the region that could possibly be covered by projected accelerators are indicated. Figure 7 shows particle energies plotted as a function of their velocities.

Unfortunately no experiments performed thus far in balloons or rockets have been arranged to resolve the three types of radiation pattern indicated in Figure 6. It is clear that this question can be resolved only by an accelerator with energies up to about 500 Mev/nucleon. Such an accelerator would make it possible for all three of the different δ-ray pattern regions to be investigated. It seems that

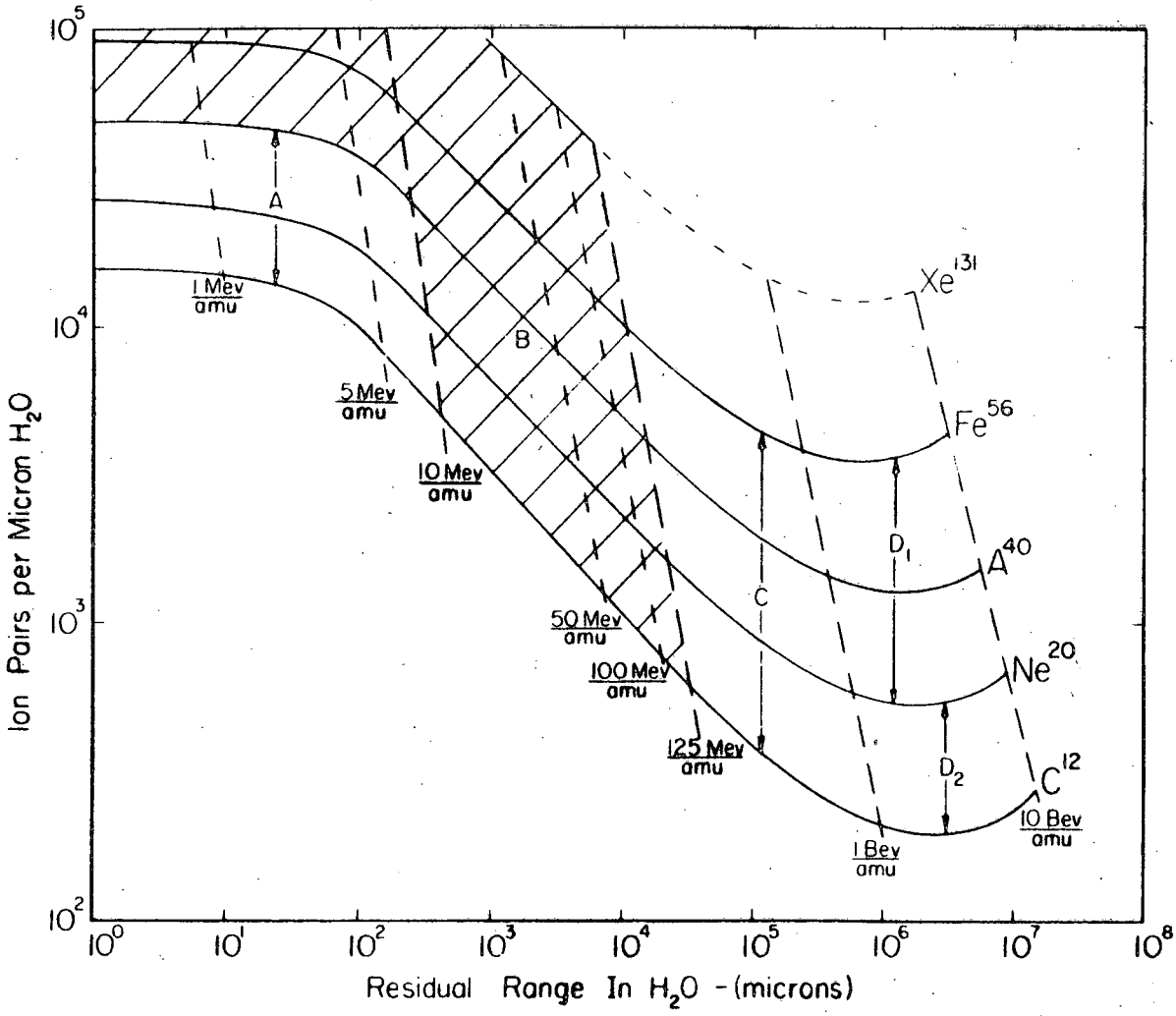

Figure 6. Specific ionization of heavy ions as a function of residual range in water. Adapted from Simons (1960). Regions:

A. HLAC operating conditions (1 to 40 amu; up to 10 Mev/amu). Tracks have densely ionizing cores and limited radial δ rays, ending by thin-down tracks.

B. Additional region included by projected future accelerators (1 to 131 amu; to about 125 Mev/amu).

C. Minimum energy of particles that can penetrate to the top of the atmosphere at middle latitudes varies within this region, from 125 Mev/amu at sunspot minima to 1 Bev/amu at sunspot maxima. Tracks have densely ionizing cores and extensive radial δ-ray patterns, and may have either thin-down or collision endings. The smaller particles in this region have somewhat less densely ionized track cores than the heavier particles.

D_1. 1 to 10 Bev/amu; 20 to 56 amu. Tracks of particles in this region have densely ionizing cores and extensive radial δ-ray patterns, and end by collisions.

D_2. 1 to 10 Bev/amu; 12 to 20 amu. Tracks of particles in this region have extensive radial δ rays and less densely ionizing cores, and end by collisions. From Wallace (ref. 7).

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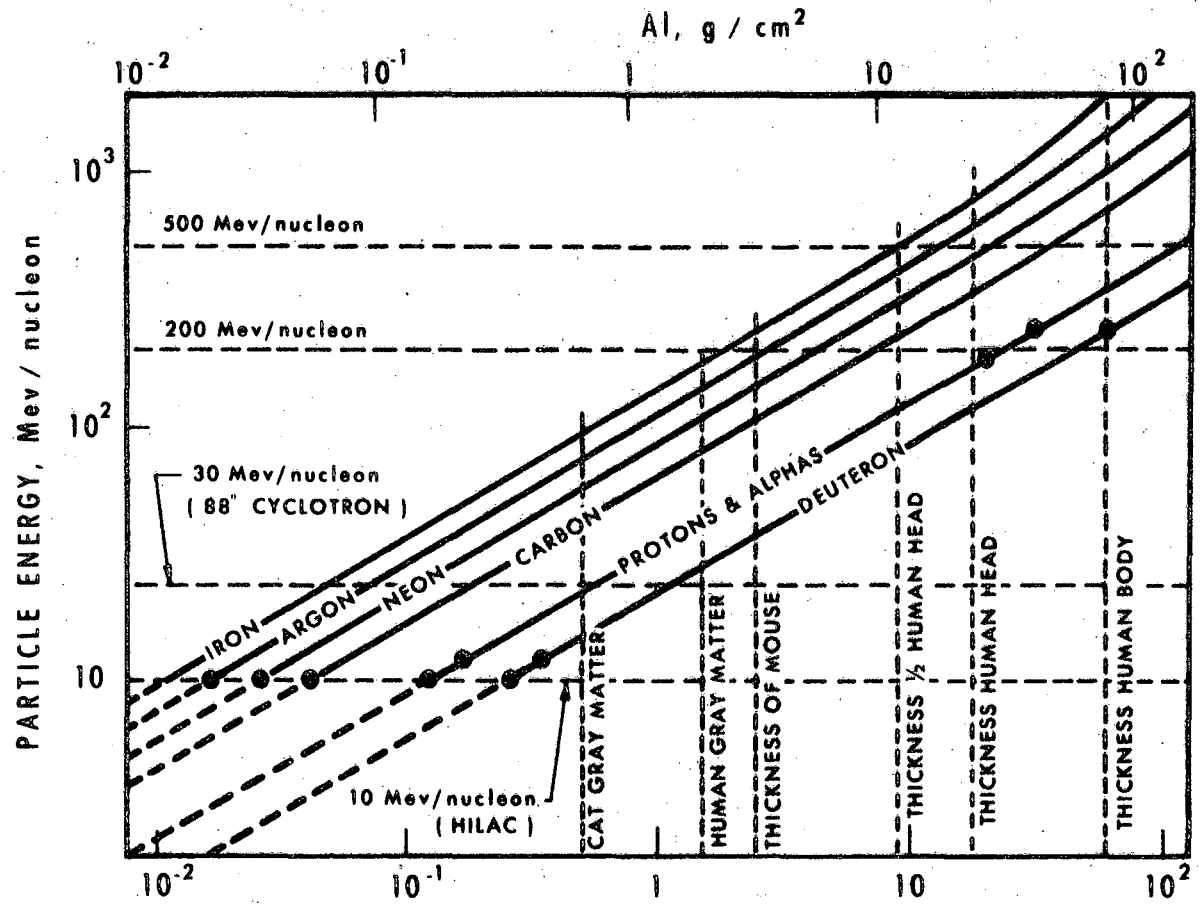

Figure 7. Particle energy vs. range in aluminum for several ions from deuterium to iron. Important biological thicknesses are shown as well as the energy presently available in two accelerators. The 200 and 500 Mev/nucleon levels are for proposed medical accelerators.

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going to energies higher than 500 Mev/nucleon would be of less interest, because the heavy-ion track patterns give way to collisions producing stars which may not be very different from those provided by proton accelerators presently available. Stars made in tissue by ion-nucleus collisions would have more prongs than those made by protons, but this may have only modest biologic implications.

The incident intensity of the heavy ion is such that at altitudes above 100,000 feet there is about 1 dense thin-down track per cm^3 of tissue per 24 hours. This rate is just sufficient so that the experiments that are quite sensitive to heavy ions, such as the "mouse cloud chamber" work of Chase, are barely possible. During a total of 4525 mouse hours above 90,000 feet, five gray streaks were observed, giving one identifiable gray streak per 38 mouse days. The streaks are interpreted as being caused by heavy-ion damage to the mouse hair follicles by a particle traveling roughly parallel to the skin. Mouse hair follicles are separated by about 100 microns. If the radial extent of a heavy primary track is of the order of only 10 microns it would seem that more experimental work is called for to resolve this apparently greater effective radial range than would be expected. It seems inescapable that the spots and streaks of gray hair are due to single ionization events. There is some evidence that the effect of these single ionization particles is transmitted to the affected follicles by their supporting cells.

It can be concluded at present that the physical understanding of heavy-ion behavior in matter is on a fair basis and that the technology for producing and accelerating heavy-ions to several hundred Mev is well developed. Although the maximum energy now available from accelerators is disappointingly only 10 Mev/nucleon, the biological experiments that have been performed with them indicate that heavy ions produce quite different effects from those of X rays: for example, there is evidence for single-hit large effects in addition to the mouse hair-graying reactions. The possibility that variables not now available such as a variable radial charge distribution may become available in bioradiology experiments with heavy ions is of some interest.

VAN ALLEN RADIATION BELT

In order to make this discussion reasonably complete, something must be said about the Van Allen radiation belt. Such a large number of measurements have been made and an even larger number of papers written on this subject, that it does not seem to be worth-while to go into it at length. Briefly, the Van Allen radiation belts are localized with regard to space travel on a lunar or planetary scale and would be traversed very quickly. There seems to be no advantage to orbiting satellites with human occupants at the particular altitude covered by these radiation belts. If a satellite did orbit for some time in one of the Van Allen radiation belts, the accumulated dose would of course be fairly high.

For a belt traversal of a few hours at the velocities appropriate to a lunar mission, it seems reasonable to expect a dose of about 5 r to be received during one pass. This is such a small amount of radiation that it seems relatively unimportant to make shielding preparations specifically for it. The only serious radiation problem in connection with the Van Allen belt might arise if the vehicle on its return from a lunar or planetary mission were to establish itself in geocentric orbit that through error passed through the Van Allen belt many times before finally landing.

Under these circumstances the Van Allen radiation might prove serious; however, the 5 to 20 g/cm^2 that seems to represent the average inherent shielding value of a functioning space capsule would certainly go a long way toward eliminating this radiation problem.

ADDITIONAL RADIATION SOURCES

There are some other possible sources of radiation encountered in space travel. Probably the principal one is the reactor that might be carried in a vehicle either for propulsion or as an electrical power supply. It seems unnecessary to discuss the radiation shielding and reactors in this report, because a vast amount of data has been accumulated on this subject in connection with the aircraft nuclear propulsion program. It is not easy to carry the required shielding and to have a reactor of adequate power. Fortunately it is fairly easy when in space to take advantage of the inverse-square law. Serious reactor radiation problems still remain during landing or take off of such a vehicle.

The last possible source of radiation which we have not discussed would be the surface of the moon or of a planet such as Mars or Venus if it had an inherently highly radioactive surface. This certainly seems unlikely. At present there is some evidence that the radiation emanating from the surface of the moon is either quite small or negligible. It is reported that the Soviet rocket that struck the moon in September 1959 did not record either a rise or fall in its radiation counting rate as it approached the lunar surface. If the moon were completely devoid of radioactivity one should have expected a drop by a factor of 2 as the surface was approached. Such a drop was not observed, perhaps indicating that there is a modest amount of radioactivity roughly equal to half the cosmic ray rate. Certainly this is not a serious amount.

It would seem unlikely that the surface of the moon or of a planet would contain large quantities of radioactive material that would produce important biological effects. It seems much more likely that the distribution of natural radioactive materials is more locally concentrated than on the earth. There are a few specialized regions on the earth, for example in uranium mines, where dose rates are rather high compared with the permissible occupational level, but these regions are so restricted that they certainly do not in any way limit our activity; the same situation probably prevails on other planets because there is no particularly obvious reason why radioactive materials should be concentrated by any known geologic process. The tendency seems rather to be toward dispersion, and it is our experience that radium is one of the most widely distributed elements on earth. This observation indicates that surface radiation is not likely to limit space operation.

CONCLUSION

In summary it can be said that solar-flare protons are the only radiation problem that seems to offer potential hazards for space travel in any way commensurate with those inherent in the travel itself due to propulsion and guidance problems. These protons have been discussed in fair detail, and it is seen that their incidence and intensities are such that the hazard they probably present is not nearly as great as other hazards inevitable in such travel; however, they do offer a hazard of sufficient potential to warrent extensive study of their properties, and an effort should be made to provide predictions of their incidence. It would seem that

the occurrence of serious flares is sufficiently rare so that large, comfortable, shielded regions in space vehicles are not called for, but it might be desirable for missions lasting several months or years to provide cramped and perhaps completely inoperative locations (for example, inside a fuel tank) where the crew could hide for a few hours once a year. The radiation problem in space, although well worthy of further study, should certainly not be a limitation to the overall possibility of space travel.

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RELATIVE BIOLOGIC EFFECTIVENESS OF 730 MEV PROTON PARTICLES FOR ACUTE LETHALITY OF MICE

By

C. C. Wang, J. Lyman and C. A. Tobias

Man's travel in space has become a reality. During the coming decade, attention will focus on the solution of many technical and radiobiologic problems. Of these, adequate understanding of the biologic effects of highly penetrating radiations is essential. High-energy protons, ranging from a few million volts to several billion volts are the most abundant component of the various space radiations.[1,2,3] Because unnecessary shielding may be too heavy for space exploration, a basic radiobiologic inquiry into the relative biologic effect of high-energy proton radiations is of academic interest and of practical importance.

The importance of ionization density to the biologic effect of various radiations on mammalian tissues was first realized in studies on whole mice and on transplantable mouse tumors carried out in 1935,[4,5] when it was found that the RBE of fast neutrons (average LET around 20 kev/μ) as compared with 200 kv X rays (average LET about 3 kev/μ) was of the order of 2.1.[4] Since then, studies on mammalian cells have shown increasing RBE with increasing LET up to about 30 kev/μ then a falling off.[6]

In recent years accelerators have produced heavier and heavier particles with greater and greater energies. This study concerns the RBE of 730 Mev protons from the Berkeley 184-inch cyclotron as compared with 200 kv X rays on mice following whole-body irradiation.

In any study of radiation lethality effects it is imperative that the end-points in each experiment be closely reproducible. This is particularly true of $LD_{50(30)}$ after the whole-body irradiation. It has been shown in this laboratory,[7] and in others,[8] that the reproducibility of lethality in mice after whole-body irradiation is possible if certain physical and biologic experimental procedures are rigidly adhered to and proper caution is exercised in the care of the animals.

METHOD

Namru strain, random-bred Swiss white mice from this laboratory were used. All were males 5 to 7 weeks old, weighing 24 ±5 gm. The mice were individually caged in labeled 1-pint, wide-mouth glass Mason jars that were supplied with sterile wood shavings and cleaned weekly (Figure 1). The mice were fed with Simonsen Laboratory white diet and water ad lib and were observed for 30 days after irradiation.

RADIATION AND DOSIMETRY

The X radiation was from a Phillips unit, and the technical factors were

Figure 1. Mason-jar individual mouse cages.

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200 kv, 15 MA with added filters of 0.25 mm Cu and 1 Al; the half-value layer was 1 mm Cu.

For the past five years a rotating positioning wheel with lucite individual-mouse canisters has been used to hold the animals for total-body irradiation (Figure 2). The wheel rotated at the rate of 2.5 rpm, and the dose rate delivered to each mouse was approximately 30 rad/min.

For proton radiation a 730 Mev beam from the 184-inch cyclotron was used. The proton beam was deflected from the cyclotron, magnetically sorted and collimated by the quadrupole-focusing method. Following its emergence from the vacuum collimating system into air, it consisted of nearly parallel streams of particles in a homogeneous beam nearly two inches in diameter. Dosimetry was performed by inserting in the beam a parallel-plate ionization chamber made of thin aluminum foils.[9,10] (Figure 3) It is known that any absorber through which the protons pass will give rise to secondary particles, including electrons, mesons and nuclear recoils that increase the dose to the animal and alter the distribution of LET. As much as possible the present experiments avoid creation of such secondaries, and the ionization in the body of mice, as shown by phantom experiments, is probably uniform within 5%. The dose rate ranged from 500 to 1000 rad per minute. Each mouse was irradiated in an individual cylinder on a rotating wheel. Usually 20 mice were exposed to the beam for each dose level, as in X irradiation. The

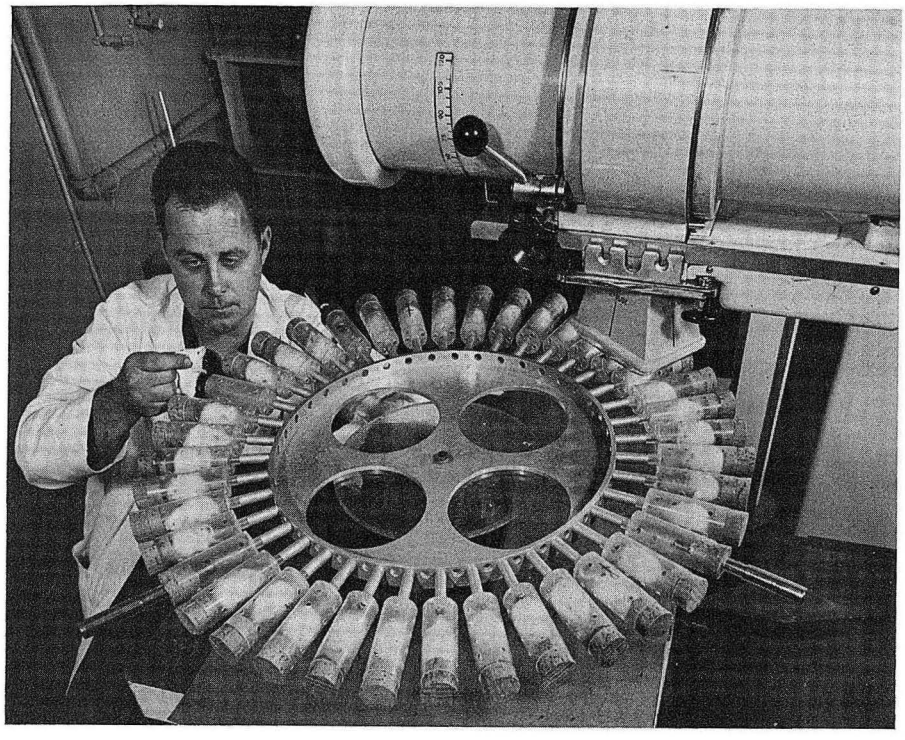

Figure 2. Rotating positioning wheel to hold mice for total-body irradiation.

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pre- and post-irradiation care of the animals was identical in both groups. The mice were followed for 30 days, and the dead ones were autopsied. Five of each group of survivors at the end of the experiment were sacrificed and the organs studied.

RESULTS

Figure 4 shows the dose-survival curves of the mice after either X-ray or proton irradiation. The $LD_{50(30)}$ after 200 kv X radiation was 580 rad* and that after 730 Mev proton radiation was 775 rad. The relative biologic effect of protons is therefore approximately 0.75 as compared to that of 200 kv X-rays. In view of the fact that the RBE of Co^{60} gamma rays for acute lethality of mice, $LD_{50(30)}$ is about 0.75,[12] it may be that the RBE of 730 Mev proton radiation is qualitatively similar to that of Co^{60} gamma rays. The findings are in harmony with those that the LET distribution of Co^{60} gamma rays and 730 Mev protons are not very different.

At autopsy the spleens from those mice dead of radiation were weighed. Figure 5 shows more than 50% reduction of the spleen weight following similar doses of X-ray or proton radiation. Therefore the RBE of proton radiation for

*Considering 1 roentgen measured in a phantom equivalent to 1 rad.[11]

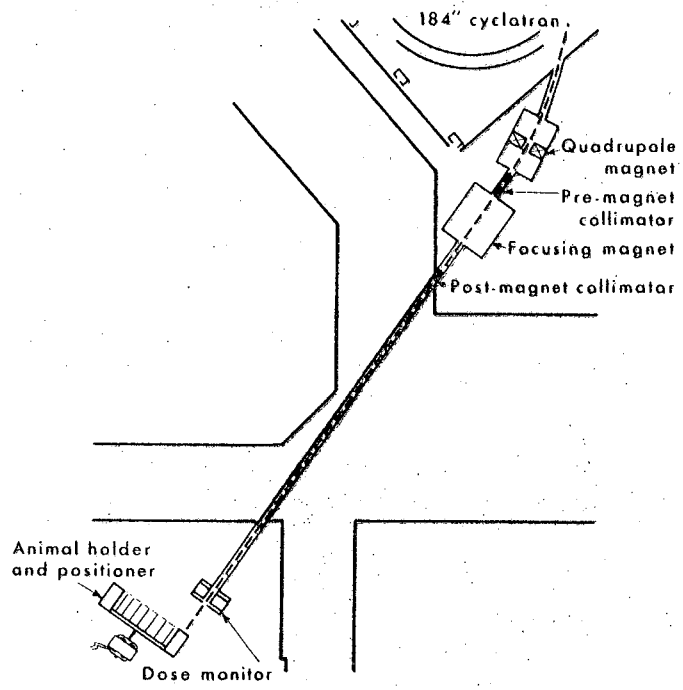

Figure 3. Diagram of set-up for mouse whole-body irradiation.
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splenic atrophy is about 1 as compared with 200 kv X rays. Those mice surviving irradiation more than 30 days showed nearly-normal or normal sized spleens in both the X-ray and the proton groups.

The total body weights of mice were recorded before and after irradiation. Those mice that died of radiation invariably showed loss of weight, in contrast to those surviving radiation. Figure 6 shows that the rapidity of weight loss is directly proportional to the dose delivered. Both X-ray and proton irradiated mice succumbing to irradiation showed a 25% weight loss during 13-16 days post-irradiation at the 650-700 rad level. It appears that the rapidity and degree of weight loss may be used as an index to determine the RBE of the two kinds of radiation, approximated though it is.

DISCUSSION

These findings are in harmony with previous work[13] on the relative biologic effects of heavy-particle radiation having LET like that of 730 Mev protons. Our previous studies with high energy deuterons[14] are in harmony with the present studies. In the present studies the RBE being less than one suggests that, although the LET of 200 kv X rays and 730 Mev protons is similar, the greater absorption of 200 kv X rays in bone may account for the lower RBE of the protons.

Because radiation from space has heavy particles above the Z of protons,

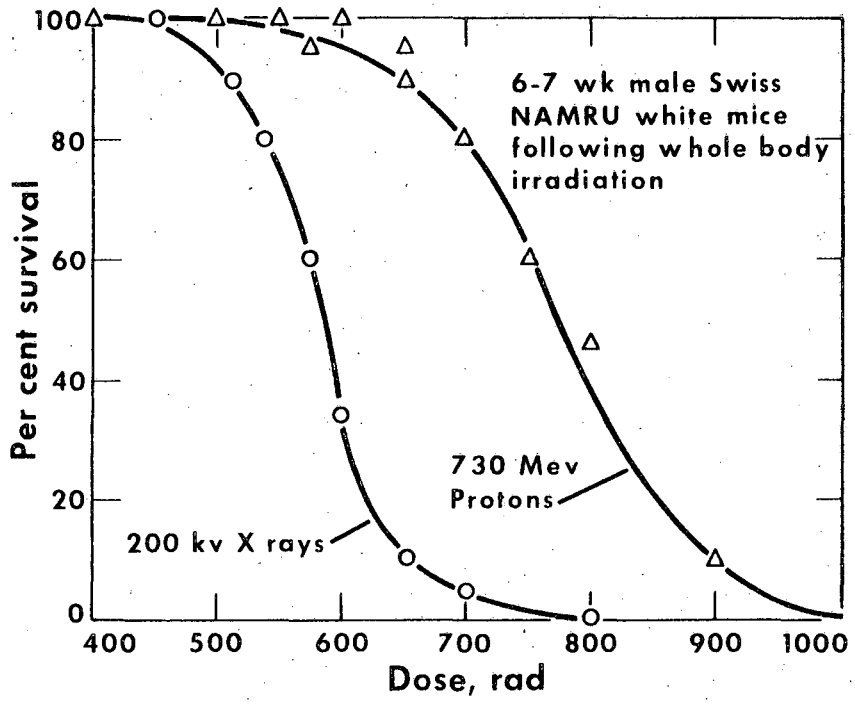

Figure 4. Dose survival curves of mice after either X-ray or proton irradiation.

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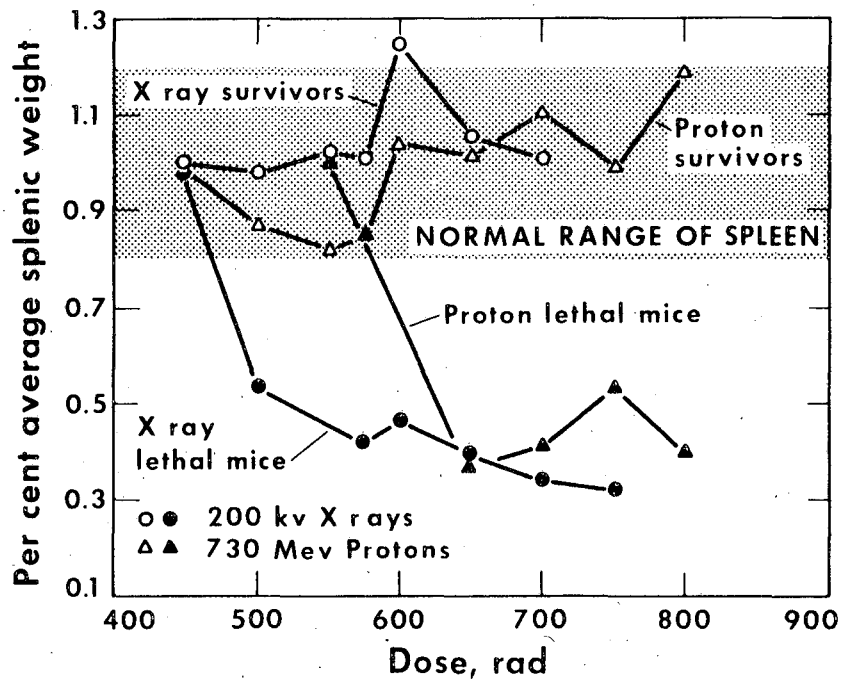

Figure 5. Changes in spleen weight of mice following whole-body X-ray or proton irradiation.

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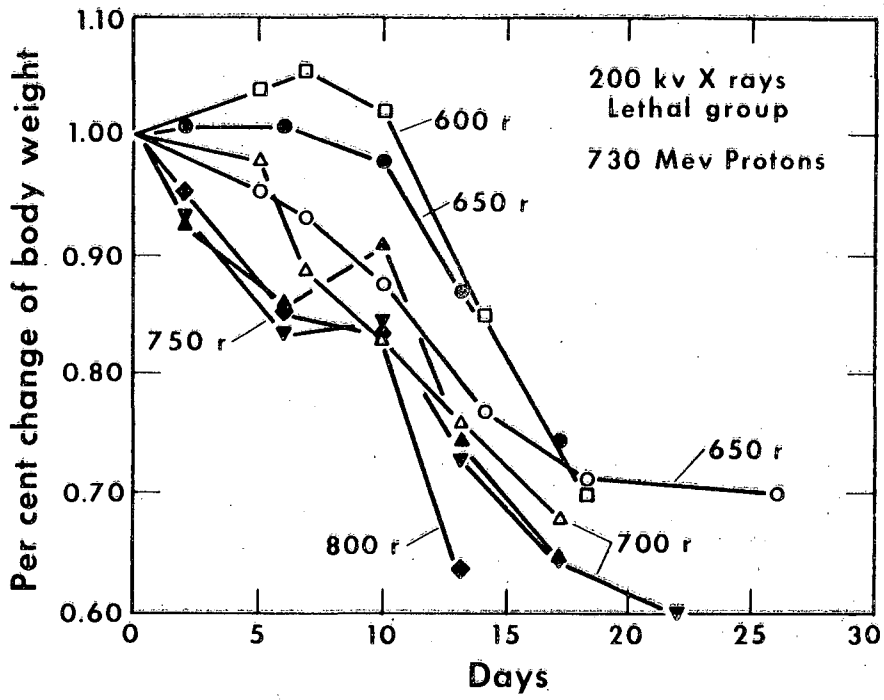

Figure 6. Change in body weight of mice following whole-body irradiation.

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we have also been studying the RBE of heavier particles that give greater LET. This work will appear in a future report.

SUMMARY

The RBE of 730 Mev proton particles as compared to 200 kv X ray: a) for acute lethality of mice $LD_{50(30)}$ is approximately 0.75; b) for splenic atrophy it is approximately 1; and c) for rapidity and degree of weight loss following lethal radiation it is approximately 1.

ACKNOWLEDGEMENTS

We wish to express our thanks to Dr. John H. Lawrence for his interest and support and to Mr. Warren Clark and Miss Alice Beckman for their technical assistance.

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RADIATION CHEMISTRY OF COMPOUNDS CONTAINING THE PEPTIDE BOUND*

By

Warren M. Garrison and Boyd M. Weeks

A few years ago we proposed that major aspects of the radiation chemistry of a wide diversity of organic nitrogeneous compounds, both in the solid state and in aqueous solution, could be correlated and generalized in terms of a common locus of reaction.[1-4] These earlier considerations of mechanism are consistent with the viewpoint that the formation of free-radical intermediates and molecular products from compounds of the type $R_2N\text{-}CH(R)_2$ can be characterized in terms of chemical changes involving the aliphatic carbon atom adjacent to the nitrogen function. Subsequent applications of this concept to the radiation chemistry of primary amines, secondary amines, N-alkyl amides and peptides have been made, and in a more recent report we have given detailed chemical evidence of the validity of this approach to the study of the indirect action of ionizing radiation on the variously substituted N-C bonds of protein in oxygenated solution.[5] In the present paper we are concerned more specifically with the radiation-induced reactions of the peptide bond in simpler chemical compounds both in solution and in the solid state. The N-acylamino acids provide a convenient model for such studies. The chemical and physical properties of the acetylated amino acids are fairly well defined and, in addition, there is considerable literature on analytical methods for the separation and determination of this particular class of peptide. In fact, the techniques of partition chromatography were originally developed by Martin and Synge[6] for the express purpose of analyzing protein hydrolyzates following acetylation.

REACTIONS IN AQUEOUS SOLUTION

The radiation-induced oxidation of peptides in oxygenated solution can be represented[5] in terms of the reactions

$$RCONH\text{-}CHR_2 + OH \longrightarrow RCONH\text{-}CR_2 + H_2O \qquad (1)$$

$$RCONH\text{-}\dot{C}R_2 + O_2 + H_2O \longrightarrow RCONH_2 + RCOR + HO_2. \qquad (2)$$

The relative importance of reactions 1 and 2 in the over-all radiation chemistry of a particular peptide system will depend on the nature of the substitutions, R. However, even for aqueous protein solutions, reaction of type 1 and 2 represents the single most important path for removal of hydroxyl radicals. In the γ-radiolysis of oxygenated gelatin solutions, for example, both amide and carbonyl products are formed with $G \simeq 1$.[5] It is noted that step 2 represents an over-all reaction and does not specify the nature of the intermediate involved in the reaction of $RCONH\text{-}CR_2$

* This paper appears as UCRL-10192 in a limited number of copies. This work was performed under the auspices of the Atomic Energy Commission.

with O_2 to yield the acid amide and the corresponding carbonyl product. As discussed elsewhere,[5] the simplest representation of the intermediate processes of reaction 2 involved the formation of the dehydropeptide

$$RCONH\text{-}CR_2 + O_2 \longrightarrow RCON{=}CR_2 + HO_2 \qquad (3)$$

followed by

$$RCON{=}CR_2 + H_2O \longrightarrow RCONH_2 + RCOR. \qquad (4)$$

On the other hand, the peroxy radical $RCONH\text{-}C(O_2)R_2$ may be formed and undergo reactions of the type

$$RCONH\text{-}C(\dot{O}_2)R_2 + H_2O \longrightarrow RCONH\text{-}C(OH)R_2 + HO_2. \qquad (5)$$

The product of 5 represents a hydrated dehydropeptide which decomposes according to

$$RCONH\text{-}C(OH)R_2 \longrightarrow RCONH_2 + RCOR. \qquad (6)$$

Recent studies of oxygenated acetylalanine and acetylglycine systems indicate that reaction 2 occurs largely through the formation of the peroxy radical. It is found that the rate of formation of pyruvic acid hydrazone, on addition of 2,4-dinitrophenylhydrazine (2,4-DNPH) to an irradiated acetylalanine solution, approximates that given by a control solution of authentic pyruvic acid. Dehydroacetylalanine, $CH_3CON{=}C(CH_3)COOH$, is relatively stable towards carbonyl reagents such as 2,4-DNPH under standard analytical conditions.[7] However, we cannot positively identify the initial product as <u>free</u> pyruvic acid, because the hydrated dehydropeptides $RCONH\text{-}C(OH)R_2$ also react rapidly with 2,4-DNPH.[8] Current studies should resolve this ambiguity. The reaction sequence 1-2 is identical with that proposed earlier for the radiation-induced deamination of the free α-amino acids.[2] The nature of the intermediate formed on reaction of $NH_2\dot{C}(R)COOH$ with O_2 via step 2 is, however, not readily subjected to experimental inquiry, because for primary amines both the dehydro intermediate (i.e., $NH{=}C(R)COOH$) and the intermediates derived from the peroxy radical represent extremely labile forms.

In the radiolysis of oxygen-free amino acid solutions, removal of $NH_2\dot{C}(R)COOH$ occurs almost exclusively through disproportionation to give $NH{=}CH(R)COOH$ that immediately hydrolyzes to ammonia and α-keto acid; dimerization of $NH_2\dot{C}(R)COOH$ also occurs but in quite low yield.[2] The role of corresponding reactions in the radiation chemistry of peptides in oxygen-free solution are now under study.[9] Typical data for the acetylated amino acids are described below.

Oxygen-free 0.1 <u>M</u> solutions of acetylglycine containing added $CH_3CONHC^{14}H_2COOH$ were irradiated in evacuated pyrex ampules with Co^{60} γ-rays under conditions which have been previously described.[2] Aliquots of the irradiated solution were analyzed directly by the method of partition chromatography adapted in this laboratory from the original method of Martin and Synge[6] and the later modifications of Marvel and Rands.[10] Water adsorbed on silicic acid acts as the immobile phase; chloroform

containing increasing amounts of butyl alcohol was used as the developing liquid. A second series of aliquots was hydrolyzed in 2 N hydrochloric acid for four hours at 100°C. The spectrum of nitrogen products in the hydrolyzate was then examined chromatographically on Dowex 50 (hydrogen form) after Moore and Stein.[11] Hydrochloric acid in continuously increasing concentration (0 to 4N) was used as the eluting agent. Analytical details including the radiometric techniques have been given.[2]

Figure 1 shows a typical elution curve for a hydrolyzed fraction. Peak I contains the organic acid products which pass directly through the acid form of Dowex 50 with little or no retention. Peak II co-chromatographs with authentic aspartic acid. The major peak III is comprised of glycine liberated in the post-irradiation hydrolysis of unreacted solute. Peak IV and the smaller adjacent peak correspond to the meso and racemic forms of diaminosuccinic acid. Exact correspondence between authentic diaminosuccinic acid and activity from both peaks was obtained chromatographically. Although the relative yields of these two peaks vary somewhat from one irradiation to the next, the total yield of diaminosuccinic acid is quite reproducible.

Figure 2 shows a product spectrum for unhydrolyzed acetylglycine solution as revealed by partition chromatography. Peaks III and IV have been identified as the acetylated isomeric forms of diaminosuccinic acid. The activity of peak I corresponds to free glyoxylic acid, and it is noted that the magnitude of this peak varies with the time interval between irradiation and chromatographic separation. Presumably the labile precursor of glyoxylic acid co-chromatographs with acetylglycine or with the product peaks III and IV. Analysis for total glyoxylic acid production was made following acid hydrolysis of the irradiated solutions. In effect, this amounted to re-chromatographing peak I of Figure 1 on silicic acid. As shown in Table 1, glyoxylic acid is the major organic acid product, although formic acid is also found in low yield.

The Conway diffusion method[12] was used to determine free ammonia in the unhydrolyzed solutions. The diffusates were assayed by means of the Nessler reaction. The measured free ammonia yields were uniformly low and did not increase appreciably above the limiting value with prolonged diffusion times. "Amide-like" ammonia was liberated by hydrolyzing the irradiated solutions *in vacuo* at 100°C in the presence of 2 N hydrochloric or acetic acid. Aliquots were taken at intervals, chilled and assayed for free ammonia. Essentially, identical amide yields were obtained under the two conditions. Hydrolysis times required to reach the limiting values were approximately 3 hrs. and 24 hrs. for hydrochloric and acetic acids respectively.

Yields of major products formed in the γ-radiolysis of oxygen-free solutions of glycine and acetylglycine are compared in Table 1. Although these data were not taken under identical conditions, they serve to illustrate the nature of the differences involved in the radiolytic response of the two systems. It is clear

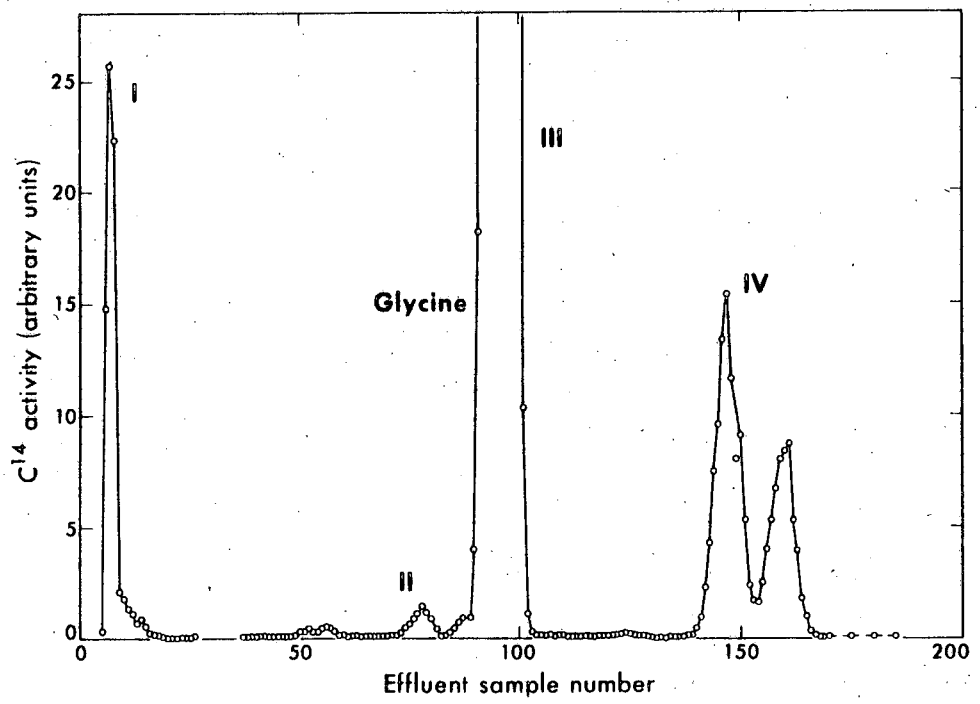

Figure 1. Typical elution curve (Dowex 50) of irradiated acetylglycine solution, hydrolyzed (Weeks, Cole, and Garrison, ref.9).

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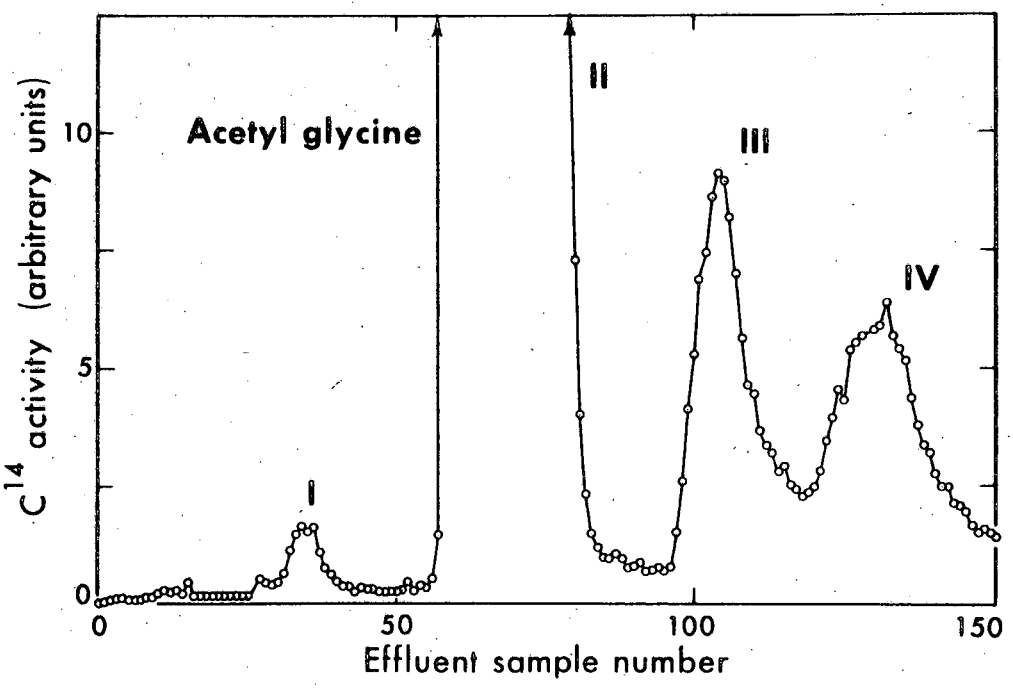

Figure 2. Typical elution curve (silicic acid) of irradiated acetylglycine solution, unhydrolyzed (Weeks, Cole, and Garrison, ref. 9).

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TABLE 1.

PRODUCT YIELDS IN THE γ-RADIOLYSIS OF OXYGEN-FREE SOLUTIONS OF GLYCINE AND ACETYLGLYCINE

Product	Yield[a]	
	NH_2CH_2COOH[b]	$CH_3CONH-CH_2COOH$[c]
Ammonia	4.3	0.90
Glyoxylic acid	2.4	0.50
Acetic acid	1.3	$\sim$.02
Diaminosuccinic acid	$\sim$.08	1.6
Aspartic acid	0.25	0.13

[a] Molecules per 100 ev absorbed in water

[b] Compiled from data reported in reference 2 and 13

[c] Measured after hydrolysis

that a principal effect of acetylation is manifested in a pronounced increase in the yield of the diamino derivative and in a concomitant decrease in the over-all yield of the degradation reactions. Qualitatively similar results have been obtained with acetylalanine and a number of other acetylated amino acids and related compounds. By analogy with the mechanism proposed for radiolysis of oxygen-free amino acid solutions (2), we may write for the acetylated derivatives

$$2\ \mathrm{RCONH\text{-}\dot{C}(R)COOH} \longrightarrow \begin{matrix}\mathrm{RCONH\text{-}C(R)COOH}\\ \mathrm{RCONH\text{-}C(R)COOH}\end{matrix} \qquad (7)$$

$$\longrightarrow \mathrm{RCON{=}C(R)COOH} + \mathrm{RCONH\text{-}CH(R)COOH} \qquad (8)$$

$$\mathrm{RCONH\text{-}\dot{C}(R)COOH} + H_2O_2 \longrightarrow \mathrm{RCONH\text{-}C(OH)(R)COOH} + \mathrm{OH.} \qquad (9)$$

On this basis, formation of "amide-like" ammonia on postirradiation hydrolysis of the peptide solutions may be identified with a hydrolytic decomposition of the dehydropeptide and/or its hydrated derivative through reaction of type 4,6. Current studies of the rate of hydrolytic release of ammonia and carbonyl products indicate that both types of intermediates could be contributing. Reductive cleavage of the N-C bond via

$$\mathrm{H} + \mathrm{RCONH\text{-}CH(R)COOH} \longrightarrow \mathrm{RCONH_2} + \mathrm{.CH(R)COOH} \qquad (10)$$

$$\longrightarrow \mathrm{R\dot{C}O} + \mathrm{NH_2CH(R)COOH}, \qquad (11)$$

does not appear to be involved to any appreciable extent in the aqueous acetylglycine system. This conclusion is based on the finding that degradation products containing the CH_2COOH radical viz glycine, acetic acid, succinic acid and aspartic acid are found only in low yield (Table 1).

The possibility that chemical change akin to the Strecker reaction[14] might be involved in the postirradiation hydrolysis of these systems has been considered

in some detail.* Reactions of monocarbonyls with glycine, alanine, etc., are known to lead to transamination of the type $RCHO + R'CH_2NH_2 \rightarrow R'CH_2NH_2 + RCHO$ in neutral or alkaline solution.[15] However, under the conditions of acid hydrolysis such reactions are suppressed, although on prolonged heating some evidence for transamination is obtained.[16] Similar conclusions were reached in the earlier study of carbonyl products formed in the radiolysis of oxygenated peptide solutions.[5] However, the diamino acids formed in oxygen-free solutions have been found to be somewhat more reactive towards monocarbonyl compounds. Hence, although the G value for carbonyl production remains constant on prolonged hydrolysis, the identity of the carbonyl product fraction is not retained. Furthermore, at the longer hydrolysis times some additional ammonia appears -- presumably through hydrolysis of aminoaldehydes formed via transamination. Similar reactions have been observed between 1,2-dicarbonyls and monoamino acids under the same condition. The problem here is that H reduction of acetylglycine at the acetyl group followed by dimerization of the reduced radical species would yield a 1,2-dicarbonyl derivative which may not be detected as such by our standard methods for carbonyl analysis.[5] These aspects of the problem are now being investigated.

REACTION IN THE SOLID STATE

From the nature of the reactions observed in our initial studies on the radiation-induced cleavage of the N-C bond in aqueous peptide systems, we concluded that the observed reactivity of the α-carbon locus might also be a factor in the radiation chemistry of the solid state. And, as part of these earlier studies, we found through application of the analytical methods developed for aqueous protein systems that irradiated solid pepsin and gelatin (evacuated) on dissolution in water do indeed yield high-molecular-weight compounds containing the carbonyl function.[3] Subsequent hydrolysis of the products showed the constituent carbonyl compounds to include α-keto acids. Hence, we tentatively proposed that dehydrogenation followed by the hydrolysis step 4 could be involved. Meanwhile, Caputo and Dose[16] in a comparative study of solid proteins and their corresponding model hydrolyzates found that proteins, unlike the hydrolyzates, exhibited amide-like function as a major radiation chemical change. Caputo and Dose also established that the product fragments (separated electrophoretically) contained carbonyl groups, and they concluded that dehydropeptides were involved.

* As pointed out in reference 5, one cannot assume a priori that product groups formed on radiolysis of protein and other peptides are unreactive during post-irradiation treatment. It is quite likely that part of the reported "radiation sensitivity" of the more labile amino acid residues may be attributed to "dark" reactions between these residues and the initial products. Reactions of carbonyl groups (and other unsaturated linkages) with substances such as tryptophane, histidine and sulfur-containing moieties are well-known, and these could contribute to the observed chemical and biological properties of irradiated proteins.

The more recent studies of Alexander and Hamilton[17] and Bowes and Moss[18] appear to confirm generally the idea that a major chemical effect of ionizing radiation on protein in the solid state leads to degradation of the peptide chain with formation of amide ammonia and carbonyl products, both in the presence and in the absence of oxygen. However, concepts of the nature of the elementary process involved remain largely speculative.

The initial proposal that the aliphatic carbon atom α to the NH group represents a major locus of the direct action of ionizing radiation on compounds containing the peptide bond was derived strictly from chemical evidence based on a comparison of products formed in aqueous solutions and in solid systems. Although characteristic ESR spectra of irradiated peptides had been observed, it was not until several years later that these spectra were interpreted.[19, 20] The ESR studies of a variety of irradiated proteins, simple peptides, N-alkylamides, and N-acylamino acids indicate that the species $RCONH\text{-}C(R)_2$ represents the principal free radical product at room temperature.[19-23].

The ESR spectra of acetylglycine and acetylalanine have been attributed to $CH_3CONH\text{-}\dot{C}HCOOH$ (doublet) and $CH_3CONH\text{-}\dot{C}(CH_3)COOH$ (tetraplet) respectively. The fact that these radical species are stable in the absence of oxygen for prolonged periods of time makes it possible to distinguish, at least in part, their separate contribution to the over-all chemistry of the irradiated solid. Three different experimental treatments are involved: (I) irradiation <u>in vacuo</u>, dissolution in oxygen-free water; (II) irradiation <u>in vacuo</u>, dissolution in oxygenated water; (III) irradiation and dissolution in the presence of oxygen. It has been shown in the case of the acetylated amino acids that radicals of the type $RCONH\text{-}C(R)COOH$, as formed in aqueous solution, react almost exclusively in the absence of oxygen to give (acetyl) diaminosuccinic acid via step 7, whereas, in the presence of dissolved oxygen, cleavage of the N-C bond occurs through reaction akin to step 2. Yields of diaminosuccinic acid and ammonia from solid acetylglycine under the three prescribed experimental conditions are summarized in Table 2. These data, obtained by direct application of the analytical methods described earlier in the section on aqueous systems, substantiate the ESR assignment of $CH_3CONH\text{-}CHCOOH$ as the long-lived radical species, and also are in accord with the mechanisms outlined here. Comparison of the diaminosuccinic acid yields under condition I and II gives $G \simeq 1.2$ as a minimum value for the yield of $CH_3CONH\text{-}CHCOOH$ in the solid state.

$$CH_3CONH\text{-}CH_2COOH \rightsquigarrow CH_3CONH\text{-}\dot{C}HCOOH + H. \qquad (12)$$

It is also seen from Table 2 that the loss in diaminosuccinic acid is balanced by an equivalent increase in amide ammonia on changing the experimental condition from case I to case II. This finding is in quantitative agreement with the concept that reaction of $CH_3CONH\text{-}CHCOOH$ with O_2 leads to cleavage of the N-C bond. The fact that the amide yield is increased even more if oxygen is present during irradiation (condition III) may be attributed to an interference of O_2 with radical

Table 2

YIELDS OF NITROGEN-CONTAINING PRODUCTS IN THE γ-RADIOLYSIS OF ACETYLGLYCINE, SOLID[a]

Experimental conditions	Yield[b,c] Ammonia	Diaminosuccinic acid
I	2.0	0.60
II	3.2	.12
III	4.4	.12

[a] Dose=1 x 10^{21} ev/gm

[b] Molecules per 100 ev absorbed in solid.

[c] Measured after hydrolysis

or change recombination and/or to reaction of O_2 with excited states which are otherwise removed *in vacuo*. A more complete material balance is required to distinguish the various possibilities.

The formation of amide ammonia with G≃2.0 under the conditions in which both irradiation and dissolution are made in the absence of oxygen cannot be represented simply in terms of the dehydrogenation reaction

$$CH_3CONH\text{-}CH_2COOH \rightsquigarrow CH_3CON{=}CHCOOH + H_2. \qquad (13)$$

As shown in Table 2, the combined yield of glyoxylic acid plus formaldehyde corresponds to a G value of approximately 0.65. This accounts for less than one-half the amide yield. Furthermore, some glyoxylic acid and ammonia (G 0.2) are detected prior to hydrolysis, and this suggests the possibility that part of the carbonyl yield arises from the molecular rearrangement

$$CH_3CONH\text{-}CH_2COOH \rightsquigarrow CH_3CHO + NH{=}CHCOOH. \qquad (14)$$

Studies of the C^{14}-labeled degradation products formed in the radiolysis of $CH_3CONHCH_2C^{14}OOH$ indicate that a major fraction of the "amide" ammonia produced in excess of the carbonyl yield arises from processes involving direct cleavage of the N-C bond, for example

$$CH_3CONHCH_2COOH \rightsquigarrow CH_3CONH + CH_2COOH, \qquad (15)$$

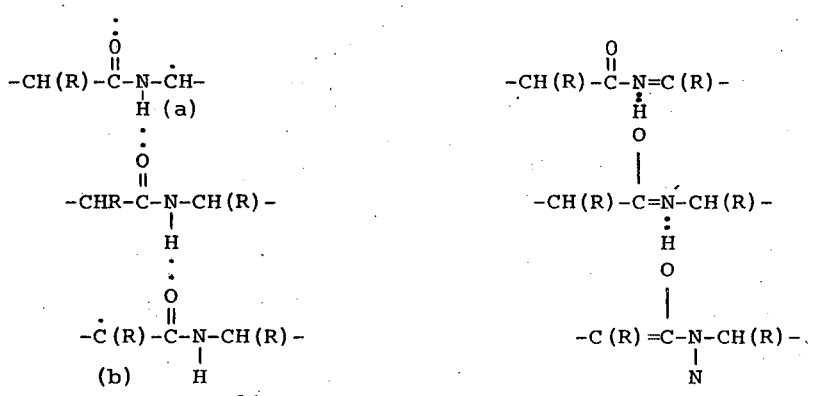
followed by immediate hydrogen abstraction reactions of the products of 15 with neighboring acetylglycine molecules. Evidence has also been found for degradation of the N-C bond through intramolecular rearrangement. However, the major rearrangement process in the acetylated amino acids is the decarboxylation reaction

$$CH_3CONHCH_2COOH \rightsquigarrow CH_3CONHCH_3 + CO_2, \qquad (16)$$

which is characteristic of organic acids in general, both in radiolysis and photolysis.[24]

Finally, we should like to mention briefly some observations that indicate that

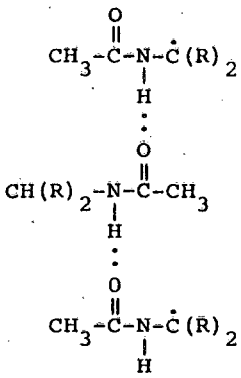
the hydrogen-bond system of solid peptides are involved in the co-valent chemistry of radiolysis. The G values for production of long-lived radicals of the type -NH-CR- in the γ-radiolysis of simple peptides, N-acylamino acids, N-alkylamides, etc., are in the order of unity;[20,21,23] in some instances, even higher values have been reported. On the other hand, G values for radical production in native protein are uniformly quite low; in most cases the production of a resonance center requires some 10^3 ev or more.[25] Of course, part of this effect may be attributed to differences in the radiation chemical contributions of the various side chains which have not, in all cases, been separately studied in simple peptide systems. However, Blyumenfel'd and Kalmanson[25] have shown that thermal denaturation of protein prior to irradiation results in a striking increase in the yield of resonant centers per unit of absorbed energy. They find that thermal disruption of the hydrogen-bond network increases the free-radical yield by several hundred times and have interpreted this effect as evidence for electron or hole migration. We propose an alternate explanation. Consider the schematic representation of the α-helix configuration in native protein containing the radical sites (a), (b) as shown in I



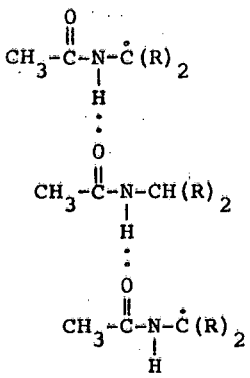
On the basis of the spur model[26] the radical sites in the solid are taken to be formed in distributions that are confined. Interaction of (a) and (b) can occur without appreciable change in the position of atomic nuclei. Chemically, the net effect is a disproportionation reaction

$$2\sim\mathrm{CONH\text{-}\dot{C}(R)}\sim \longrightarrow \sim\mathrm{CONH\text{-}CH(R)}\sim + \sim\mathrm{CON{=}C(R)}\sim ,$$

accompanied by enolization of the intervening hydrogen-bond bridge. The latter chemical change provides a co-valent mechanism for the localized disruption of the hydrogen-bond configuration of irradiated protein. Consider now a similar pair of radicals in a simple acylamine--either as III or IV



III



IV

The point here is that in these simple model compounds, radical re-combination cannot occur simply through electron re-arrangement as is possible in the polypeptide structure, although both systems have the peptide bond in the trans configuration. On the other hand, it is readily shown that the amino acid anhydrides as simple peptides containing the cis configuration can undergo radical disproportionation and enolization. A similar formulation can be written for the nucleic acids. Chemical evidence for radiation-induced enolization of the hydrogen-bond has recently been observed in studies of the radiation synthesis of pyrazine-like derivatives from amino acid anhydrides in the solid state.[27] These preliminary concepts will be treated in detail in a forthcoming publication on the role of hydrogen-bonds in the co-valent radiation chemistry of peptide systems.

SUMMARY

Radiation-induced reactions of peptides in solution and in the solid state are described. It is shown that the original proposal regarding the reactivity of the ~NH-CH(R)~ locus is in accord with the more recent chemical and physical evidence. A specific radiation-chemical role is assigned to the hydrogen-bond structure.

ACKNOWLEDGMENTS

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EFFECTS OF X-IRRADIATION UPON CELL POPULATION AND MORPHOGENESIS IN THE DEVELOPING BEETLE WING *

By

James S. Beck** and Thomas L. Hayes

Recent studies have disclosed the dose-incidence relation, the time course of radiosensitivity and the location of the sensitive site for the wing deformity in Tribolium confusum. The membranous wing consists of a flat, closed envelope of cuticle around a double layer of cells of a single type that form the cuticle with its microscopic projections (spines). The construction of this cuticle occurs within a short time after the end of the radiosensitive period of development. The total mass of the deformed and normal wings are equal within limits of error. To determine the relative total carbon content of the wings, groups of experimental and control wings were exposed to 900 MeV α-particles, producing the $C^{12}(\alpha,\alpha n)C^{11}$ reaction. The positron-emitting C^{11} was then counted. No significant difference was found. The total phosphorous content was explored by the thermal neutron reaction, $P^{31}(n,\gamma)P^{32}$. Individual wings were thus compared, as little as 0.03μgm P being measured. It was found that 2.3±0.7 times as much P is in the deformed wing. By quantitatively removing the imaginal wing from the pupal cuticle, the phosphorous content at various stages of development has been determined. The smallness of the cells and spines makes optical microscopy of little use in determining the basic nature of the deformity. But the remarkable thinness of the adult wing -- 0.5μ-- has made possible rapid sampling with the electron microscope. A general increase in density to electrons, ultramicroscopic osmophilic foci, and a 50% decrease in number of spines have been found in the maldeveloped wing. Studies are now under way to prove the hypothesis that there is a one-to-one correspondence between cells and spines, thus furnishing a permanent record of cell population and morphogenetic activity.

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** Postdoctoral research fellow of the National Cancer Institute.

CHEMICAL ELEMENTS OF THE BLOOD OF MAN II*

A) THE LEVELS OF SIXTY-SIX CHEMICAL ELEMENTS IN THE SERUM OF OSTENSIBLY HEALTHY ADULT MALES

By

John W. Gofman, O. F. deLalla, G. Johnson, E. L. Kovich, O. Lowe, D. L. Piluso, R. K. Tandy, and F. Upham

A program directed toward the development of a systematic set of procedures for the analysis of most of the chemical elements in blood and tissue was described in our previous semi-annual report.[1] The current phase of the program is devoted to the evaluation of X-ray spectrochemical analysis as a method to encompass a large fraction of all the elements ultimately to be studied. In the previous report the principles of X-ray spectrochemical analysis, its advantages and its limitations, were described, and observations were reported for the serum level of 40 elements in a group of 22 subjects (male and female) in ostensible health. In the recent period the method has been extended to encompass 66 chemical elements, from phosphorus (Z=15) through uranium (Z=92), including observations of the levels observed in ostensibly healthy individuals for these 66 elements, and some progress has been made in each of these major directions.

The broad long-term objectives of this program include:

a) An effort to learn more concerning the factors limiting analytical sensitivity so as to attempt study of elements present at extremely low levels.
b) Evaluation of the relation of prime physiological parameters such as age, sex or familial factors to the serum levels of the various chemical elements.
c) An exploration of possible derangements in metabolism of the chemical elements in various pathophysiological states.

METHODOLOGY

Effect of holder thickness. One source of error in the earliest group of analyses was the variation in thickness of the lucite holders in which the pressed serum disks are mounted. Some of the primary radiation was scattered from the lucite holders. Differences in scatterer thickness will have a larger effect upon relatively hard X rays than upon soft X rays. Probably the variation in levels among the early group of subjects was to some extent increased by this spurious source of variability. All the recent analyses, here reported, have been made since the narrowing of the primary beam by apertures and by introduction of rigid tolerances for the lucite holders. It is impossible to prevent primary radiation from impinging upon the holders to some extent, but any possible effects are minimized by constancy of holder dimensions within narrow limits.

Pulse height discrimination stability. A second source of significant difficulty in the earlier work was drift in the pulse-height discrimination systems. In part this has been remedied by replacement of obsolete equipment. Temperature fluctuation continues to be a serious problem with the gas flow counters used to measure the soft X rays. The magnitude of such drift has proved negligible for the scintillation crystals utilized in the measurement of the relatively hard X rays. It is hoped that air-conditioning will become available to reduce this general source of error.

*This work supported by the United States Atomic Energy Commission.

Time required for analysis. In the earlier studies each blood sample was analyzed for all sixty-six elements studied. Because 20-30 minutes of time in the spectrometer is required per analysis (at current levels of sensitivity), a total time of 30 hours is required for a complete analysis of a single blood specimen, that imposes a serious limitation upon the potential breadth of the studies contemplated. Although multiplication of the number of spectrometers increases capacity, it is achieved at high cost of funds and maintenance. We have, therefore, explored alternative approaches to increase analytical capacity. One highly attractive possibility is in the utilization of pooled-blood samples instead of individual blood samples as a first step in any study of a physiologic subgroup or in a disease category. If for a group of 50 subjects in a particular category a single pooled sample can be studied, there will be an obviously enormous contraction of the analytical load. If pooling is carried out for such a group and for any reference control group, a reasonable determination of the difference in mean level for any particular element should be correctly determined. It is a prerequisite that pooling provide a true representation of all the individual samples, adequately mixed. In many types of studies such pooling proves unfeasible, because the collection of individual samples for the pool extends over too long a time compared with the tolerable storage period for the material. In our program this limitation is nonexistent. Blood samples are converted into lyophilized powdered serum shortly after collection. Such lyophilized powder has been stored without apparent deterioration for years, eliminating difficulty with case-collection time. A rigorous study of the technical feasibility of pooling lyophilized sera has been performed using a series of samples containing varying proportions of three large original batches of lyophilized serum. Aliquots were weighed, the powders triturated together and pellets prepared from the mixed powders. The X-ray spectrochemical analyses of the pooled mixtures were within analytical error, as was expected from the proportions of the individual powders represented in the pool. Furthermore, our experience demonstrates that the pooled samples give means essentially identical with the mean of the individual samples for such elements as phosphorus emitting soft X rays, and for such elements as bromine emitting relatively hard X rays.

What is lost by the procedure of studying pools rather than individual bloods? First, it is evident that biological variation within a population sample is not observable for a pooled sample. It is possible that a particular state of disordered physiology may be characterized by the same mean value as the healthy state but show a significantly greater or lesser variability than the reference state. Such altered variability with unaltered mean level could represent an important physiologic aberration worth discovering. It would be completely speculative to estimate how frequently this situation might arise.

At present we feel it worth-while to sacrifice the possibility of observing states of altered variability without altered mean levels in order to achieve the ability to extend the breadth of the search for states characterized by altered mean levels. This latter endeavor is clearly possible through the pooling procedure.

A second major consideration must be made concerning pooling. When mean levels of any two groups of subjects are compared, the biological variation within each group enters prominently into the determination of the statistical significance of any observed difference. The study of pools does not provide such a required estimate

of biological variation, so that this information must be provided otherwise. From the initial studies on 22 subjects we have a reasonable estimate of the variation for 66 elements within a sample of ostensibly healthy subjects. As a first approximation one can estimate that any special group under study will show the same variability as the reference population, which allows for a calculation of the significance of observed differences. Should the biological variation in the special group prove to be less than in the reference population sample, the first approximation above is conservative, in that one claims less than the data truly would allow. "Claiming less" is unfortunate in a sense because certain small, but important, differences may be missed. Should the biological variation in the special group prove to be greater than in the reference population sample, the same approximation above will lead to claiming more than the data truly allow, because a difference in mean levels may be falsely judged significant.

"Claiming more" would be serious were one to stop at this point. We feel this is the least worrisome of all points concerning the pooling procedure, because in every case where a difference based on pools is adjudged significant we are interested in knowing the distribution of levels in the special group under study. Whenever the pooled blood study indicates an apparently significant difference, the individual samples in the pool will be studied for that particular element. Abnormally high biological variability is thereby uncovered, and the possibility of claiming more than the data justify is obviated.

For this reason in our present and immediately future studies we are adopting the study of pooled bloods as a "screening" procedure coupled with selective study of individual bloods for elements where the pools indicate differences of interest.

EVALUATION OF PHYSIOLOGIC PARAMETERS IN RELATION TO SERUM ELEMENTAL LEVELS

Ultimately we hope to ascertain the influence of age, sex and other parameters upon the levels of all the chemical elements. The earliest study of 22 subjects, including males and females, provided only an estimate of the magnitude of levels of the various elements. As a beginning of the study of the influence of physiologic variables, a series of 39 ostensibly healthy adult males has been analyzed using the pooling procedure. This will be followed by the study of pooled groups of females subdivided by age and other parameters.

The adult males range in age from 21 to 53 years, with a mean of 35.3 years. All subjects in the group were free of major disease discoverable by a complete physical examination. All were residents of the Northern California Bay Area, and included urban and rural dwellers. Common allergies and minor dermatoses did not constitute bases for exclusion of cases. Acute febrile illnesses did constitute a basis for exclusion, because certain elements, as copper, are altered during infection.

The details of analytical procedure have previously been presented.[1] The point may be stressed again here that absolute values of elemental levels, while highly desirable, are not as important as is the difference in levels between samples. The difficulties that may arise in the determination of absolute levels were discussed in detail in our earlier report. The net result of such difficulties is that for a particular element, e.g. copper, all values may be low or all may be high by a constant amount. Although this fact demands caution in comparing absolute levels

determined through X-ray spectrochemical analysis with values determined by alternative procedure, it is not of moment in comparing one sample with another by a single method of analysis. This comparison depends upon knowing the magnitude of the error of the analysis rather than whether the absolute baseline is slightly low or high.

The mean values for 66 chemical elements in the series of 39 ostensibly healthy males are presented in Table 1. Presented also is the estimated standard error of the mean obtained by combining the biological variation estimated in our earlier studies with the technical error of measurement measured on the pooled bloods by use of 10 separate counting intervals. From these data it has been possible to estimate the 99% confidence limits within which the true mean value for such subjects may be expected to lie. Although we believe that these limits possess absolute validity for nearly all elements, it is true that some systematic absolute error may be present in some cases. However, the confidence limits reported provide a ready estimate of the kind of differences that would have to exist between values to render significance highly probable.

RESULTS

Now that data are available for all 66 elements of potential interest with Z values of 15 or higher, some general comments concerning "trace" and "non-trace" elements in human blood serum are possible. The absolute levels of the chemical elements from Z=15 through Z=20 (including phosphorus, sulfur, chlorine, potassium and calcium, but excluding argon) may be regarded as enormously high in comparison with the very low values observed for all elements of Z greater than 20. At least this is true for this group of male adult subjects in ostensible health. Although between Z=15 and Z=20 (excluding argon) mean levels of 100 p.p.m. and much higher are encountered, there is not a single element of $Z > 20$ for which a mean level as high as 5 p.p.m. exists in this series of healthy subjects. The only relatively prominent element beyond Z=20 is bromine (Z=35) for which a mean value of 4.12 p.p.m. has been determined in this group. Additionally, only three elements of $Z > 20$ (serum iron, copper and zinc) are in the one part per million class. These latter data are in good agreement with numerous chemical and optical spectroscopic measurements of the same elements. Many elements which have aroused intense biological interest, such as manganese, nickel, cobalt, chromium, cadmium, selenium, strontium and molybdenum, are apparently controlled at very low levels (order of 0.1 p.p.m. and lower). It is important to avoid equating low absolute levels with lack of importance to the physiologic economy. Thus, for serum zinc, with a mean level in these males of 0.98 p.p.m. and a standard deviation of 0.07 p.p.m., it may well result that a deviation of 0.3 p.p.m. is of much greater biological importance than a difference of 30 p.p.m. for such elements as potassium or sulfur. For such elements as chromium, manganese and cobalt, with mean levels measured in the neighborhood of 0.00 - 0.05 p.p.m., it is plausible that alterations in level of the order of a few parts per hundred million may reflect significant pathophysiology.

The studies reported here describe a population sample of adult males in ostensible health residing in the Northern California Bay Area. It is impossible to assure that this represents a valid sample of what is statistically considered "The population." This is by no means a belabored minor issue. This area of investigation of elemental levels, more than many others, will ultimately demand rigorous

Table 1

MEAN LEVELS FOR SIXTY-SIX CHEMICAL ELEMENTS IN THE SERUM OF OSTENSIBLY HEALTHY ADULT MALE SUBJECTS
(Mean Age = 35.3 years)
(39 Cases)

Element	Atomic number (Z)	Mean level (p.p.m.)	Standard error of mean (p.p.m.)	99% confidence limits on mean value (p.p.m.)
Phosphorus	15	163	3.1	155-171
Sulfur	16	1201	14.7	1161-1241
Chlorine	17	3798	20.0	3744-3852
Potassium	19	177	3.1	169-185
Calcium	20	115	1.3	111-119
Scandium	21	0.0	0.1	0.0-0.3
Titanium	22	0.0	0.07	0.00-0.18
Vanadium	23	0.01	0.04	0.00-0.11
Chromium	24	0.01	0.01	0.00-0.04
Manganese	25	0.00	0.02	0.00-0.06
Iron	26	0.65	0.08	0.44-0.86
Cobalt	27	0.00	0.02	0.00-0.05
Nickel	28	0.00	0.07	0.00-0.18
Copper	29	1.29	0.05	1.16-1.42
Zinc	30	0.98	0.02	0.93-1.03
Gallium	31	0.04	0.02	0.00-0.09
Germanium	32	0.00	0.04	0.00-0.10
Arsenic	33	0.02	0.01	0.00-0.05
Selenium	34	0.1	0.02	0.05-0.15
Bromine	35	4.12	0.16	3.70-4.54
Rubidium	37	0.15	0.05	0.02-0.28
Strontium	38	0.00	0.02	0.00-0.05
Yttrium	39	0.02	0.01	0.00-0.05
Zirconium	40	0.1	0.04	0.00-0.20
Niobium	41	0.0	0.06	0.00-0.16
Molybdenum	42	0.2	0.04	0.10-0.30
Ruthenium	44	0.0	0.09	0.00-0.23
Rhodium	45	0.0	0.06	0.00-0.16
Palladium	46	0.0	0.04	0.00-0.10
Silver	47	0.2	0.07	0.02-0.38
Cadmium	48	0.0	0.09	0.00-0.23
Indium	49	0.0	0.08	0.00-0.21
Tin	50	0.0	0.18	0.00-0.47
Antimony	51	0.0	0.12	0.00-0.31
Tellurium	52	0.0	0.19	0.00-0.49
Iodine	53	0.0	0.22	0.00-0.57
Cesium	55	0.2	0.2	0.00-0.52
Barium	56	0.18	0.1	0.00-0.44
Lanthanum	57	0.0	0.08	0.00-0.21
Cerium	58	0.1	0.17	0.00-0.55
Praseodymium	59	0.0	0.05	0.00-0.13
Neodymium	60	0.0	0.03	0.00-0.08
Samarium	62	0.0	0.07	0.00-0.18
Europium	63	0.0	0.2	0.00-0.52
Gadolinium	64	0.0	0.1	0.00-0.26
Terbium	65	0.0	0.09	0.00-0.23
Dysprosium	66	0.0	0.1	0.00-0.26
Holmium	67	0.0	0.16	0.00-0.41
Erbium	68	0.00	0.03	0.00-0.08
Thulium	69	0.0	0.14	0.00-0.36
Ytterbium	70	0.1	0.08	0.00-0.31
Lutetium	71	0.0	0.05	0.00-0.13
Hafnium	72	0.0	0.16	0.00-0.41
Tantalum	73	0.2	0.08	0.00-0.41
Tungsten	74	0.0	0.07	0.00-0.18
Rhenium	75	0.1	0.06	0.00-0.26

Table 1 (Continued)

Osmium	76	0.1	0.18	0.00-0.57
Iridium	77	0.0	0.05	0.00-0.13
Platinum	78	0.1	0.14	0.00-0.46
Gold	79	0.0	0.09	0.00-0.23
Mercury	80	0.1	0.08	0.00-0.31
Thallium	81	0.0	0.01	0.00-0.03
Lead	82	0.3	0.09	0.07-0.53
Bismuth	83	0.1	0.04	0.00-0.20
Thorium	90	0.00	0.04	0.00-0.10
Uranium	92	0.02	0.03	0.00-0.10

definition of the character of population samples under study. There is good reason to suspect that such environmental factors as soils, water, air pollution and occupational environment may prominently influence the steady-state level of certain elements. To study aberrant levels in disease states it is of the greatest importance to choose appropriate reference population samples in health. Such features as age or sex matching of appropriate reference populations are self-evident; others may be much more subtle, such as water and food supply or institutionalization versus non-institutionalization. Provision of data for elemental levels in appropriate reference populations represents a very large task--one not readily accomplished in its entirety in the early phases of investigation. At best, matching is never perfectly achieved, but if the issue itself is recognized clearly, spurious association of an apparently altered elemental level with a particular disorder of physiology may be avoided. It is possible that appropriate matching of population samples will ultimately represent a greater task than the analytical measurements themselves.

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CHEMICAL ELEMENTS OF THE BLOOD OF MAN II*

B) THE LEVELS OF SIXTY-SIX CHEMICAL ELEMENTS IN DOWN'S DISEASE (MONGOLISM)

By

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In 1959 LeJeune, Gauthier and Turpin[1,2] discovered that mongolism is characterized in general by a chromosomal aberration of 47 chromosomes in somatic cells instead of the 46 usually present in the cells of normal humans. The extra chromosome is usually one of the small acrocentric chromosomes assigned to chromosome 21 (Denver nomenclature). This predominant abnormality in mongolism (21 trisomy) is believed to result from non-disjunction in maternal gametogenesis.[3,4] Additional types of chromosomal aberration have less commonly been reported in some cases of mongolism, including translocations of what is apparently the largest part of chromosome 21 to some other chromosome, as to one in the 13, 14, 15 group.[3] In any event, the various translocations lead to the presence of additional chromosomal material (of probably the same type) even though the total number of chromosomes is 46 rather than 47 as is the case for trisomy of chromosome 21.

Subjects with mongolism demonstrate clearly that the presence of the additional chromosomal material (either as an extra No. 21 chromosome or a translocation equivalent) produces massive biological abnormalities, including mental retardation, physical abnormalities encompassing essentially all somatic tissues, and a striking increase in the risk of development of leukemia.[3] Because translocation mongolism appears somatically similar to trisomy mongolism, it is suspect that extra chromosomal material rather than position accounts for the biologic abnormalities. In accord with Stern's estimate of 10,000 genes for man,[4] one extra chromosome represents the order of 200 extra genes. Because a particular type of chromosome is involved, a particular set of genes may be estimated to be in excess. At this time no biochemical explanation has appeared for the widespread tissue maldevelopment in either embryonic or post-embryonic life. Biochemical deviations in mongolism have been observed, but none has yet been implicated as crucial for any particular feature of the abnormal development.[3,4]

The elemental distribution in the blood, especially in electrolyte form, describes one important aspect of the internal environment in which numerous biochemical processes occur. For further study of mongolism and for its intrinsic interest, it is worth-while to know the effect of a particular complement of extra genes upon the elemental distribution in blood. Any alterations deserve investigation in order to relate them to the mental and/or somatic abnormalities of mongolism. The paucity of distinctive biochemical aberrations is striking in view of the prominent tissue abnormalities in structure and function.[6,7] Because of all these considerations we have initiated a study of the chemical elements of the serum of subjects with mongolism, the preliminary results of which are reported here.

*This work supported by the United States Atomic Energy Commission.

MATERIALS AND METHODS

Subjects. All the subjects with mongolism are residents of the Sonoma State Hospital at Sonoma, California. Although ultimately it will be pertinent to study cases of mongolism in several age groups, these early studies are limited to males in the age range 19 to 28 years and to females in the age range 16 to 41 years, an age range close to that for the ostensibly healthy subjects already studied. The fact that the subjects with mongolism are institutionalized at Sonoma State Hospital argues in favor of an institutionalized group of controls from the same hospital, but without mongolism. On the other hand, such "controls" are not really ostensible normals, so that they too may not represent valid controls. We have elected the following procedure of study. At present the subjects with mongolism will be contrasted with non-institutionalized ostensibly healthy subjects, and we are fully aware that inadequacy of the reference control sample may be responsible in part or wholly for any differences in elemental levels. In several on-going studies other disease entities from the Sonoma State Hospital are under investigation, so that it will be possible at a later date to contrast mongolism with diverse other diseases from the same institution. Should geography, diet, or institutionalization represent overriding influences, it is anticipated that most or all groups of institutionalized subjects will show elemental level distributions like that of mongolism. Should the other categories of disease not show the pattern observed in mongolism, the suggestion would be strengthened that an observed deviation in mongolism is truly related to the mongolism itself.

Analytical procedures for the X-ray spectrochemical analysis of all elements of interest between Z=15 and Z=92 were identical with those previously described.[8] The reference samples of individuals in ostensible health are re-analyzed along with the analysis of every disease category. In this manner nearly all factors of methodologic drift are self-compensating, especially because total analytical time is divided into sub-intervals in which the reference group and the disease group are alternately measured.

In all, 30 male subjects and 29 female subjects with mongolism have been studied. At this time no reference control population of female subjects has been studied, so that the study of female mongolism can only be regarded as indicative of possible consistent alterations in mongolism. Later the female subjects with mongolism will be contrasted with appropriate female reference groups.

RESULTS

The experimental findings for the analysis of 66 chemical elements are presented in Table 1 (male mongolism) and Table 2 (female mongolism) tentatively contrasted in both cases with the reference population sample of ostensibly healthy males reported in part A of this report. In these tables the following items are available:

a) The absolute mean values in the reference group and the difference in mean values (mongolism--"controls"). The consistent procedure will be used of subtracting the mean value for the reference population from that for the mongolism group. Thus, a positive difference means that the mongolism group is higher in level than the reference group; a negative value, that the mongolism group is lower in level than the reference group.

b) Significance test upon differences. For both the mongolism series and the

Table 1

COMPARISON OF THE SERUM LEVELS OF SIXTY-SIX CHEMICAL ELEMENTS IN MALES WITH MONGOLISM AND THE REFERENCE GROUP (MALES) (in p.p.m.)

Element	Z	Mean value in reference group "controls"	Mongolism minus "controls"	Standard error of difference of means	Significance at 1% level	Mongolism "controls" 99% confidence limits Limit A	Limit B
Phosphorus	15	163	-10.7	3.8	Yes	-0.7	-20.7
Sulfur	16	1201	-90	15	Yes	-50	-140
Chlorine	17	3798	-233	53	Yes	-93	-373
Potassium	19	177	+9.2	2.2	Yes	+3.4	+15.2
Calcium	20	115	-9.3	0.9	Yes	-6.9	-11.7
Scandium	21	0.0	-0.15	0.26	No	-0.84	+0.54
Titanium	22	0.0	+0.06	0.07	No	+0.25	-0.13
Vanadium	23	0.01	0.00	0.02	No	+0.05	-0.05
Chromium	24	0.01	0.00	0.02	No	+0.05	-0.05
Manganese	25	0.00	-0.01	0.02	No	-0.06	+0.04
Iron	26	0.65	-0.19	0.07	Yes	0.00	-0.38
Cobalt	27	0.00	+0.01	0.01	No	+0.04	-0.02
Nickel	28	0.00	+0.01	0.01	No	+0.05	-0.03
Copper	29	1.29	+0.15	0.06	No	+0.31	-0.01
Zinc	30	0.98	-0.11	0.02	Yes	-0.03	-0.13
Gallium	31	0.04	0.00	0.02	No	+0.05	-0.05
Germanium	32	0.00	-0.01	0.02	No	-0.06	+0.04
Arsenic	33	0.02	-0.02	0.02	No	-0.07	+0.03
Selenium	34	0.1	0.00	0.01	No	+0.03	-0.03
Bromine	35	4.12	-1.18	0.17	Yes	-0.73	-1.63
Rubidium	37	0.15	+0.04	0.05	No	+0.17	-0.09
Strontium	38	0.00	+0.03	0.02	No	+0.08	-0.02
Yttrium	39	0.02	0.00	0.04	No	+0.11	-0.11
Zirconium	40	0.1	-0.02	0.05	No	-0.15	+0.11
Niobium	41	0.0	-0.03	0.08	No	-0.24	+0.18
Molybdenum	42	0.2	+0.05	0.07	No	+0.24	-0.14
Ruthenium	44	0.0	+0.06	0.05	No	+0.19	-0.07
Rhodium	45	0.0	+0.05	0.07	No	+0.24	-0.14
Palladium	46	0.0	+0.05	0.08	No	+0.25	-0.17
Silver	47	0.2	-0.03	0.10	No	-0.30	+0.24
Cadmium	48	0.0	-0.01	0.16	No	-0.43	+0.41
Indium	49	0.0	+0.10	0.22	No	+0.68	-0.48
Tin	50	0.0	-0.09	0.27	No	-0.81	+0.63
Antimony	51	0.0	-0.14	0.22	No	-0.72	+0.44
Tellurium	52	0.0	+0.33	0.24	No	+0.97	-0.31
Iodine	53	0.0	+0.83	0.33	No	+1.70	-0.04
Cesium	55	0.2	-0.21	0.19	No	-0.71	+0.29
Barium	56	0.18	-0.16	0.09	No	-0.40	+0.08
Lanthanum	57	0.0	+0.04	0.08	No	+0.25	-0.17
Cerium	58	0.1	-0.01	0.17	No	-0.46	+0.45
Praseodymium	59	0.0	+0.05	0.05	No	+0.18	-0.08
Neodymium	60	0.0	0.00	0.03	No	+0.08	-0.08
Samarium	62	0.0	+0.06	0.07	No	+0.25	-0.13
Europium	63	0.0	-0.11	0.19	No	-0.61	+0.39
Gadolinium	64	0.0	+0.03	0.11	No	+0.32	-0.26
Terbium	65	0.0	0.00	0.09	No	+0.24	-0.24
Dysprosium	66	0.0	+0.03	0.11	No	+0.32	-0.26
Holmium	67	0.0	0.00	0.16	No	+0.42	-0.42
Erbium	68	0.00	0.00	0.03	No	+0.06	-0.06
Thulium	69	0.0	-0.04	0.14	No	-0.41	+0.33
Ytterbium	70	0.1	-0.07	0.10	No	-0.34	+0.20
Lutetium	71	0.0	-0.07	0.16	No	-0.49	+0.35
Hafnium	72	0.0	-0.01	0.09	No	-0.25	+0.23
Tantalum	73	0.2	-0.01	0.07	No	-0.20	+0.18

Table 1 (Continued)

Tungsten	74	0.0	−0.08	0.07	No	−0.27	+0.11
Rhenium	75	0.1	+0.05	0.14	No	+0.42	−0.32
Osmium	76	0.1	−0.03	0.13	No	−0.37	+0.31
Iridium	77	0.0	+0.08	0.05	No	+0.21	−0.05
Platinum	78	0.1	−0.10	0.07	No	−0.29	+0.09
Gold	79	0.0	−0.05	0.07	No	−0.24	+0.14
Mercury	80	0.1	+0.08	0.06	No	+0.24	−0.08
Thallium	81	0.0	+0.02	0.03	No	+0.10	−0.06
Lead	82	0.3	−0.03	0.08	No	−0.24	+0.18
Bismuth	83	0.1	+0.02	0.04	No	+0.13	−0.09
Thorium	90	0.00	+0.51	0.34	No	+1.41	−0.39
Uranium	92	0.02	+0.00	0.65	No	+1.72	−1.72

Table 2

COMPARISON OF THE SERUM LEVELS OF SIXTY-SIX CHEMICAL ELEMENTS IN FEMALES WITH MONGOLISM AND THE REFERENCE GROUP (MALES) (in p.p.m.)

Element	Z	Mean value in reference group "controls"	Mongolism minus "controls"	Standard error of difference of means	Significance at 1% level	Mongolism "controls" 99% confidence limits Limit A	Limit B
Phosphorus	15	163	-11.9	3.8	Yes	-1.9	-21.9
Sulfur	16	1201	-109	18	Yes	-61	-157
Chlorine	17	3798	-157	53	Yes	-17	-297
Potassium	19	177	+3.8	2.2	No	+9.6	-2.0
Calcium	20	115	-9.7	0.9	Yes	-7.3	-12.1
Scandium	21	0.0	-0.02	0.26	No	-0.96	+0.67
Titanium	22	0.0	+0.04	0.06	No	+0.20	-0.12
Vanadium	23	0.01	+0.03	0.02	No	+0.08	-0.02
Chromium	24	0.01	-0.02	0.02	No	-0.07	+0.03
Manganese	25	0.00	+0.01	0.02	No	+0.06	-0.04
Iron	26	0.65	+0.02	0.07	No	+0.21	-0.17
Cobalt	27	0.00	+0.01	0.01	No	+0.04	-0.02
Nickel	28	0.00	0.00	0.01	No	+0.03	-0.03
Copper	29	1.29	+0.19	0.06	Yes	+0.03	+0.35
Zinc	30	0.98	-0.09	0.02	Yes	-0.04	-0.14
Gallium	31	0.04	+0.02	0.02	No	+0.07	-0.03
Germanium	32	0.00	0.00	0.02	No	+0.05	-0.05
Arsenic	33	0.02	-0.01	0.02	No	-0.06	+0.04
Selenium	34	0.1	-0.02	0.01	No	-0.05	+0.01
Bromine	35	4.12	-0.86	0.17	Yes	-0.41	-1.31
Rubidium	37	0.15	+0.06	0.05	No	+0.19	-0.07
Strontium	38	0.00	-0.01	0.03	No	-0.09	+0.07
Yttrium	39	0.02	-0.03	0.04	No	-0.14	+0.08
Zirconium	40	0.1	-0.10	0.07	No	-0.29	+0.09
Niobium	41	0.0	-0.04	0.06	No	-0.20	+0.12
Molybdenum	42	0.2	+0.03	0.07	No	+0.22	-0.16
Ruthenium	44	0.0	+0.01	0.05	No	+0.14	-0.12
Rhodium	45	0.0	+0.14	0.07	No	+0.33	-0.05
Palladium	46	0.0	+0.04	0.08	No	+0.25	-0.17
Silver	47	0.2	+0.11	0.10	No	+0.38	-0.16
Cadmium	48	0.0	+0.08	0.13	No	+0.42	-0.26
Indium	49	0.0	-0.08	0.22	No	-0.66	+0.50
Tin	50	0.0	-0.38	0.27	No	-1.10	+0.34
Antimony	51	0.0	-0.10	0.14	No	-0.47	+0.27
Tellurium	52	0.0	-0.21	0.26	No	-0.91	+0.48
Iodine	53	0.0	+0.06	0.25	No	+0.73	-0.61
Cesium	55	0.2	-0.11	0.19	No	-0.61	+0.39
Barium	56	0.18	+0.03	0.12	No	+0.35	-0.29
Lanthanum	57	0.0	+0.09	0.10	No	+0.36	-0.18
Cerium	58	0.1	+0.04	0.17	No	+0.49	-0.44
Praseodymium	59	0.0	+0.01	0.06	No	+0.17	-0.15
Neodymium	60	0.0	+0.11	0.07	No	+0.30	-0.08
Samarium	62	0.0	+0.04	0.07	No	+0.23	-0.15
Europium	63	0.0	+0.12	0.23	No	+0.72	-0.48
Gadolinium	64	0.0	+0.17	0.13	No	+0.51	-0.17
Terbium	65	0.0	-0.06	0.10	No	-0.33	+0.21
Dysprosium	66	0.0	-0.06	0.09	No	-0.30	+0.18
Holmium	67	0.0	+0.07	0.15	No	+0.46	-0.32
Erbium	68	0.00	-0.05	0.03	No	-0.13	+0.03
Thulium	69	0.0	-0.01	0.13	No	-0.35	+0.33
Ytterbium	70	0.1	+0.18	0.11	No	+0.47	-0.11
Lutetium	71	0.0	-0.18	0.12	No	-0.50	+0.14
Hafnium	72	0.0	+0.05	0.10	No	+0.32	-0.22
Tantalum	73	0.2	+0.07	0.11	No	+0.36	-0.22

Table 2 (Continued)

Tungsten	74	0.0	-0.05	0.08	No	-0.26	+0.16
Rhenium	75	0.1	-0.06	0.14	No	-0.43	+0.31
Osmium	76	0.1	+0.19	0.13	No	+0.53	-0.15
Iridium	77	0.0	+0.08	0.05	No	+0.21	-0.05
Platinum	78	0.1	-0.06	0.06	No	-0.22	+0.10
Gold	79	0.0	-0.2	0.09	No	-0.4	+0.2
Mercury	80	0.1	-0.06	0.05	No	-0.20	+0.08
Thallium	81	0.0	+0.02	0.02	No	+0.07	-0.03
Lead	82	0.3	-0.03	0.07	No	-0.22	+0.16
Bismuth	83	0.1	-0.01	0.05	No	-0.15	+0.13
Thorium	90	0.00	+0.02	0.29	No	+0.80	-0.76
Uranium	92	0.02	-0.27	0.52	No	-1.7	+1.1

reference series the standard errors of the means were estimated (see part A, this report) and the standard error of the difference in means calculated by standard procedures. For the numbers of cases involved, a t value of 2.65 corresponds approximately to the probability value of 0.01. For conservatism we have chosen the p=0.01 level as a requirement for considering a difference as "significant." Thus, a rating for each difference as "yes" or "no" is based upon whether or not the p=0.01 level is reached or exceeded. The arbitrariness of this criterion hardly requires further comment here.

c) Confidence limits upon the differences in mean values. Perhaps of greater interest than the "yes" versus "no" answer of the significance test is an estimate of the extent to which differences in means at a reasonable level of probability, might differ, even though the significance test is "no." By adding 2.65 times the standard error of the difference to the difference in means and by subtracting this same quantity from the difference in means, one obtains the 99% confidence limits upon the difference in means. The use of these confidence limits may be illustrated by using the data for cobalt in Table 1, showing that the male mongolism group is 0.01 p.p.m. higher than the reference group, but the difference is not significant. The standard error of the difference in means is 0.01, and rounding off, we have (2.65)(Standard Error) $\cong$ 0.03. The 99% confidence limits are 0.01 + 0.03 = 0.04 and 0.01-0.03 = -0.02. From these limits, exclusive of any bias in choice of populations, there is only one chance in 100 that further studies of this problem will find male mongolism more than 0.02 p.p.m. lower than the reference group or more than 0.04 p.p.m. higher than the reference population. Even though no significant difference has been demonstrated for cobalt, we are in a position to say much more than this. We can predict with high probability how large a difference may ever be discovered. It was stated above that all this is true provided bias in choice of populations is excluded. Because reservations exist concerning what constitutes a reference population for the mongolism group, reservations must be kept in mind concerning bias. However, if future studies show the reference population to be adequate, confidence limits described can be useful in determining the probability that certain hypotheses concerning trace elements in mongolism may be reasonable. It is evident that the confidence limits represent a very narrow range for some elements and an appreciably larger range for others. In the main this is the direct result of the difference in the sensitivity (or error) of the X-ray spectrochemical analysis for some elements compared with others.

DISCUSSION OF RESULTS

Several chemical elements are shown by this study to be significantly different in the male mongolism group from the reference population. Although the differences are significant, they are not large. Listed in Table 3 are the significant differences expressed as a percentage of the mean values in the reference population sample.

Phosphorus, as measured here, includes all inorganic and organic sources of serum phosphorus and among the organic compounds includes protein bound phosphorus and phospholipid phosphorus. Therefore it is not possible to determine, within these data, which phosphorus compound accounts for the difference observed. Previous workers have observed that inorganic phosphate is essentially unchanged in mongolism.[6,9] Such observations are not at variance with the present results, for

the present results include so many forms of phosphorus. Indeed, if inorganic phosphate is unchanged in mongolism, it will be of interest to know what form of phosphorus is truly lowered in mongolism.

Table 3

SIGNIFICANT DIFFERENCES EXPRESSED AS A PERCENTAGE OF THE MEAN VALUES IN THE REFERENCE POPULATION SAMPLE

Element	Z	Mean value in reference population	% alteration in males with mongolism
Phosphorus	15	163	6.6% lower
Sulfur	16	1201	7.5% lower
Chlorine	17	3798	6.1% lower
Potassium	19	177	5.2% higher
Calcium	20	115	8.1% lower
Iron	26	0.65	29.2% lower
Zinc	30	0.98	11.2% lower
Bromine	35	4.12	28.6% lower

For the phosphorus results, as for all others discussed below, the preliminary character of these findings cannot be overstressed. It must be borne in mind that some feature of the matching of the reference population with the mongolism group (such as age, geography, diet, institutionalization) may account for the observed difference rather than mongolism per se. Only the evolution of future studies of such features as diet, etc., can settle the issue.

Sulfur, as measured by X-ray spectrochemistry, also includes a variety of compounds, sulfates, organic and inorganic, and protein-bound sulfur. Undoubtedly the bulk of the sulfur measured here is protein-bound sulfur. Whether this is the form differing in mongolism will require separate studies to establish.

Chlorine is probably essentially in the chloride form in serum. The fact that several elemental levels in mongolism are lower than in the reference population might give a preliminary impression that hydremia is important. However, this fact fails to explain why the potassium levels are elevated in the mongolism group.

Although the mongolism group is older than that reported by Stern and Lewis, the observed magnitude of lowering of calcium in mongolism is in excellent agreement with their prior findings.[9]

Bromine is especially interesting because the mongolism group shows a much lower standard deviation, meaning a much tighter distribution, than does the reference group. The standard deviation for the reference group is 0.99 p.p.m. versus 0.34 for the mongolism group. Further, not a single case of mongolism showed a bromine level as high as 4.0 p.p.m., whereas one-half of the cases in the reference group showed values above 4.0 p.p.m.

The group of female mongol subjects showed deviations similar in direction to those observed in the male mongol subjects. Except for potassium, the magnitude of the difference was closely analogous in male and female mongol subjects. For potassium the elevation in female mongolism does not reach the 1% significance level. The elevation in copper level in females with mongolism must be viewed with reserve in view of Cartwright's data indicating that females in health show slightly higher

serum copper levels than do males.[10]

SUMMARY

Apparently significant differences are observed for eight elements in males with mongolism. Whether any spurious basis is responsible for the observed differences will become apparent from further study of the problem of reference controls, as discussed above. Should the observed differences truly be specifically associated with the mongolism per se, it will be of interest to speculate upon what possible bearing these findings may have upon the numerous anatomic and functional peculiarities in mongolism. Perhaps as surprising as the eight elements apparently different in mongolism is the fact that the overall serum elemental pattern is so similar in mongolism to that in reference controls.

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CHEMICAL ELEMENTS OF THE BLOOD OF MAN II*

C) THE SERUM LEVELS OF SIXTY-SIX CHEMICAL ELEMENTS IN SCHIZOPHRENIA AND ALCOHOLISM

By

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Various known toxic substances, several of them chemical elements, are capable of inducing seriously deranged central nervous system function including frank psychosis. Lead, mercury and bromine are well-known in this connection. Many workers suspect copper deposition to be the toxic process underlying the central nervous system manifestations of hepato-lenticular degeneration (Wilson's Disease). It is reasonable to consider that derangement in metabolism of some common or uncommon chemical element may underlie other central nervous system dysfunctions. Because so many organic compounds occurring in biological systems form highly stable complexes or chelates with metals, it is further possible that primary disturbances in metabolism of such organic compounds may secondarily provoke elemental derangements of a potentially toxic nature. Evaluation of this concept may ultimately require the study of elemental levels in blood and in such tissues as brain, liver and kidney, or in excreta. As a first step in this general area we have undertaken the study of the chemical elements of the blood serum in two disorders, schizophrenia and alcoholism.

MATERIALS AND METHODS

Subjects. Two groups of humans with schizophrenia are under investigation. The first group is represented by 21 subjects with schizophrenia of relatively short duration, who are regarded as having a high potential for early return to society. The second group is represented by 25 patients regarded as "chronic" schizophrenics with long-term hospitalization having, at least at this time, a low potential for discharge from institutionalization. The first of these two groups is reported here.

Two groups of alcoholic subjects are under investigation. The first is represented by 23 patients with alcoholism involuntarily committed to the Napa State Hospital for a period of 30 days. The second group of 29 patients with chronic alcoholism show the central nervous system derangement characterized as the Korsakoff Syndrome. The first of these two groups of alcoholics is reported here.

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As discussed in section B of this report, the question of who constitute appropriate reference control subjects is a thorny one in such studies as these. The additional problem that a variety of therapeutic agents are administered to the hospitalized patients is superimposed. The concomitant study of alcoholism and schizophrenia in patients from wards of the same institution (the Napa State Hospital at Imola, California) provides a degree of compensating internal control. While we are well aware of the pitfalls associated with proper "controls." we feel that a first-order approach can be made to the study even without a perfect solution of this particular aspect of the overall problem. Any relatively blatant derangements in the control of blood elemental level will probably be apparent from the studies. Second and higher order refinements will always be possible.

Analytical procedures for the X-ray spectrochemical analysis of all elements considered to be of potential interest between Z = 15 (phosphorus) and Z = 92 (uranium) were identical with those described previously.[1,2] As has become standard practice in this work, the reference control samples for the group of ostensibly healthy males are re-analyzed along with those for the study groups (in this case alcoholism and schizophrenia) in order to compensate for known or unknown methodological drifts.

Results. The findings in the alcoholic group and the short-duration schizophrenia group are presented in Tables 1 and 2 respectively. In both series the elemental levels are contrasted with the previously described reference group of ostensibly healthy males. The format of presentation of the tabular material is identical with that used for the report on mongolism (section B of this report).

DISCUSSION OF RESULTS

A. Alcoholism

Significant differences are noted for seven chemical elements in the males with alcoholism when contrasted with the ostensibly healthy reference group. The elements demonstrating differences are not identical with those shown (in part B, this report) to be apparently different in mongolism, and it would, therefore, appear that there is no systematic difference necessarily the result of institutionalization per se.

Sulfur. The serum sulfur shows a significant depression in the alcoholism group. It will be recalled that protein must be the major source of the sulfur being analyzed under these conditions. This does not necessarily imply that protein sulfur is the form here found to be low. Inspection of these data plus those concerning mongolism and schizophrenia indicates a lowering of sulfur in all groups. It will be of considerable interest to determine (a) what biochemical form of sulfur is low, and (b) whether the lowering truly relates to the diseases under study. Because only one reference population sample is available at this time, the possibility that this reference group is fortuitously high in sulfur

Table 1

COMPARISON OF THE SERUM LEVELS OF SIXTY-SIX CHEMICAL ELEMENTS IN MALES WITH ALCOHOLISM AND IN THE REFERENCE GROUP (OSTENSIBLY HEALTHY MALES)

Element	Z	Mean value in reference group "controls"	Alcoholism minus "controls"	Standard error of difference of means (in p.p.m.)	Significance at 1% level	Alcoholism "controls" 99% confidence limits Limit A	Limit B
Phosphorus	15	163	+9.2	3.8	No	-0.9	+19.3
Sulfur	16	1201	-114	21	Yes	-59	-169
Chlorine	17	3798	-17.6	30	No	-97.6	+62.4
Potassium	19	177	+10.4	3.7	Yes	+0.6	+20.2
Calcium	20	115	-4.3	1.2	Yes	-1.1	-7.5
Scandium	21	0.0	-0.27	0.24	No	-0.91	+0.37
Titanium	22	0.0	+0.05	0.06	No	+0.21	-0.11
Vanadium	23	0.01	+0.01	0.03	No	+0.09	-0.07
Chromium	24	0.01	-0.03	0.02	No	-0.08	+0.02
Manganese	25	0.00	-0.02	0.02	No	-0.07	+0.03
Iron	26	0.65	-0.40	0.07	Yes	-0.59	-0.21
Cobalt	27	0.00	-0.01	0.01	No	-0.04	+0.02
Nickel	28	0.00	+0.01	0.02	No	+0.06	-0.04
Copper	29	1.29	+0.12	0.06	No	+0.28	-0.04
Zinc	30	0.98	-0.14	0.02	Yes	-0.19	-0.09
Gallium	31	0.04	+0.01	0.02_5	No	+0.08	-0.06
Germanium	32	0.00	-0.02	0.02	No	-0.07	+0.03
Arsenic	33	0.02	0.00	0.02	No	+0.05	-0.05
Selenium	34	0.1	-0.01	0.01	No	-0.04	+0.02
Bromine	35	4.12	+4.90	3.3	No	+13.6	-3.8
Rubidium	37	0.15	+0.02	0.05	No	+0.15	-0.11
Strontium	38	0.00	-0.03	0.02	No	-0.08	+0.02
Yttrium	39	0.02	-0.12	0.05	No	-0.26	+0.02
Zirconium	40	0.1	0.00	0.06	No	+0.16	-0.16
Niobium	41	0.0	-0.05	0.06	No	-0.21	+0.11
Molybdenum	42	0.2	+0.01	0.08	No	+0.22	-0.20
Ruthenium	44	0.0	+0.02	0.06	No	+0.18	-0.14
Rhodium	45	0.0	+0.14	0.27	No	+0.41	-0.13
Palladium	46	0.0	+0.09	0.21	No	+0.30	-0.12
Silver	47	0.2	-0.06	0.14	No	-0.43	+0.31
Cadmium	48	0.0	+0.11	0.16	No	+0.54	-0.32
Indium	49	0.0	+0.12	0.26	No	+0.81	-0.57
Tin	50	0.0	-0.27	0.28	No	-1.02	+0.48
Antimony	51	0.0	+0.20	0.17	No	+0.73	-0.33
Tellurium	52	0.0	+0.19	0.31	No	+1.01	-0.63
Iodine	53	0.0	+0.40	0.28	No	+1.15	-0.35
Cesium	55	0.2	-0.06	0.22	No	-0.65	+0.53
Barium	56	0.18	-0.29	0.11	Borderline	0.00	-0.58
Lanthanum	57	0.0	+0.11	0.07	No	+0.30	-0.08
Cerium	58	0.1	+0.14	0.14	No	+0.51	-0.23
Praseodymium	59	0.0	+0.03	0.06	No	+0.19	-0.13
Neodymium	60	0.0	+0.03	0.04	No	+0.14	-0.08
Samarium	62	0.0	-0.04	0.09	No	-0.28	+0.20
Europium	63	0.0	-0.07	0.20	No	-0.60	+0.47
Gadolinium	64	0.0	+0.11	0.11	No	+0.40	-0.18
Terbium	65	0.0	+0.03	0.09	No	+0.27	-0.21

Table 1 (Continued)

Dysprosium	66	0.0	-0.09	0.11	No	-0.38	+0.20
Holmium	67	0.0	+0.15	0.16	No	+0.58	-0.28
Erbium	68	0.00	+0.01	0.03	No	+0.09	-0.07
Thulium	69	0.0	+0.04	0.14	No	+0.41	-0.33
Ytterbium	70	0.1	-0.17	0.14	No	-0.54	+0.20
Lutetium	71	0.0	-0.09	0.16	No	-0.52	+0.34
Hafnium	72	0.0	+0.04	0.09	No	+0.28	-0.20
Tantalum	73	0.2	+0.08	0.08	No	+0.29	-0.13
Tungsten	74	0.0	0.00	0.08	No	+0.21	-0.21
Rhenium	75	0.1	+0.04	0.14	No	+0.41	-0.33
Osmium	76	0.1	+0.09	0.13	No	+0.44	-0.26
Iridium	77	0.0	+0.07	0.05	No	+0.20	-0.06
Platinum	78	0.1	-0.11	0.08	No	-0.32	+0.10
Gold	79	0.0	-0.07	0.09	No	-0.31	+0.17
Mercury	80	0.1	-0.04	0.05	No	-0.17	+0.09
Thallium	81	0.0	-0.01	0.02_4	No	-0.07	+0.05
Lead	82	0.3	-0.07	0.07	No	-0.26	+0.12
Bismuth	83	0.1	0.00	0.06	No	+0.16	-0.16
Thorium	90	0.00	+0.19	0.29	No	+0.96	-0.58
Uranium	92	0.02	-0.26	0.52	No	-1.64	+1.12

Table 2

COMPARISON OF THE SERUM LEVELS OF SIXTY-SIX CHEMICAL ELEMENTS IN MALES WITH SCHIZOPHRENIA AND IN THE REFERENCE GROUP (OSTENSIBLY HEALTHY MALES)

Element	Z	Mean value in reference group "controls"	Schizophrenia minus "controls"	Standard error of difference of means (in p.p.m.)	Significance at 1% level	Schizophrenia "controls" 99% confidence limits Limit A	Limit B
Phosphorus	15	163	-0.2	3.8	No	-10.3	+9.9
Sulfur	16	1201	-60.0	21	Yes	-115	-5.0
Chlorine	17	3798	-177	27	Yes	-249	-105
Potassium	19	177	-10.2	3.7	Yes	-19.1	-1.3
Calcium	20	115	-5.2	1.2	Yes	-8.4	-2.0
Scandium	21	0.0	+0.46	0.32	No	+1.31	-0.39
Titanium	22	0.0	+0.06	0.06	No	+0.22	-0.10
Vanadium	23	0.01	+0.04	0.02	No	+0.09	-0.01
Chromium	24	0.01	+0.02	0.02	No	+0.07	-0.03
Manganese	25	0.00	0.00	0.02	No	+0.05	-0.05
Iron	26	0.65	-0.12	0.08	No	-0.32	+0.09
Cobalt	27	0.00	-0.01	0.01	No	-0.04	+0.02
Nickel	28	0.00	-0.01	0.02	No	-0.06	+0.04
Copper	29	1.29	+0.11	0.06	No	+0.27	-0.05
Zinc	30	0.98	-0.04	0.02	No	-0.03	+0.07
Gallium	31	0.04	0.00	0.02	No	+0.05	-0.05
Germanium	32	0.00	0.00	0.02	No	+0.05	-0.05
Arsenic	33	0.02	+0.01	0.02	No	+0.06	-0.04
Selenium	34	0.1	0.00	0.01	No	+0.03	-0.03
Bromine	35	4.12	+0.36	0.27	No	+1.08	-0.36
Rubidium	37	0.15	+0.01	0.05	No	+0.14	-0.12
Strontium	38	0.00	0.00	0.03	No	+0.08	-0.08
Yttrium	39	0.02	-0.10	0.04	No	-0.21	+0.01
Zirconium	40	0.1	-0.10	0.05	No	-0.23	+0.03
Niobium	41	0.0	-0.04	0.08	No	-0.25	+0.17
Molybdenum	42	0.2	+0.01	0.08	No	+0.22	-0.20
Ruthenium	44	0.0	+0.08	0.05	No	+0.21	-0.05
Rhodium	45	0.0	+0.11	0.07	No	+0.30	-0.08
Palladium	46	0.0	+0.14	0.08	No	+0.35	-0.07
Silver	47	0.2	+0.15	0.10	No	+0.42	-0.12
Cadmium	48	0.0	+0.18	0.13	No	+0.53	-0.17
Indium	49	0.0	-0.19	0.24	No	-0.83	+0.45
Tin	50	0.0	-0.40	0.22	No	-0.99	+0.19
Antimony	51	0.0	+0.12	0.18	No	+0.60	-0.36
Tellurium	52	0.0	+0.46	0.22	No	+1.05	-0.13
Iodine	53	0.0	-0.04	0.24	No	-0.68	+0.60
Cesium	55	0.2	-0.19	0.19	No	-0.69	+0.31
Barium	56	0.18	-0.17	0.11	No	-0.46	+0.12
Lanthanum	57	0.0	+0.12	0.08	No	+0.33	-0.09
Cerium	58	0.1	-0.10	0.18	No	-0.58	+0.38
Praseodymium	59	0.0	+0.08	0.06	No	+0.24	-0.08
Neodymium	60	0.0	+0.01	0.06	No	+0.17	-0.15
Samarium	62	0.0	+0.09	0.09	No	+0.33	-0.15
Europium	63	0.0	-0.04	0.22	No	-0.63	+0.55
Gadolinium	64	0.0	+0.04	0.14	No	+0.41	-0.33
Terbium	65	0.0	+0.01	0.08	No	+0.22	-0.20

Table 2 (Continued)

Dysprosium	66	0.0	-0.04	0.09	No	-0.28	+0.20
Holmium	67	0.0	-0.09	0.15	No	-0.50	+0.32
Erbium	68	0.00	+0.01	0.03	No	+0.09	-0.07
Thulium	69	0.0	+0.02	0.10	No	+0.29	-0.25
Ytterbium	70	0.1	-0.10	0.14	No	-0.47	+0.27
Lutetium	71	0.0	-0.18	0.18	No	-0.64	+0.30
Hafnium	72	0.0	+0.09	0.10	No	+0.36	-0.18
Tantalum	73	0.2	+0.01	0.10	No	+0.28	-0.26
Tungsten	74	0.0	-0.08	0.07	No	-0.27	+0.11
Rhenium	75	0.1	+0.01	0.14	No	+0.38	-0.36
Osmium	76	0.1	+0.19	0.13	No	+0.54	-0.16
Iridium	77	0.0	+0.05	0.05	No	+0.18	-0.08
Platinum	78	0.1	+0.06	0.07	No	+0.25	-0.13
Gold	79	0.0	-0.01	0.09	No	-0.25	+0.23
Mercury	80	0.1	+0.04	0.06	No	+0.20	-0.12
Thallium	81	0.0	+0.04	0.03	No	+0.12	-0.04
Lead	82	0.3	-0.05	0.07	No	-0.24	+0.14
Bismuth	83	0.1	-0.02	0.05	No	-0.15	+0.11
Thorium	90	0.00	+0.15	0.34	No	+1.06	-0.76
Uranium	92	0.02	+0.00	0.52	No	+1.38	-1.38

must be considered. This could account for mongolism, alcoholism and schizophrenia being low in sulfur. Or, because all the disease groups are institutionalized (either at Sonoma State Hospital or Napa State Hospital), the sulfur lowering may relate to some aspect of institutionalization. It is highly doubtful that lack of perfect age matching accounts for the observations, because the mongolism group is slightly younger than the reference group, and the alcoholism group is somewhat older than the reference group, yet the sulfur alterations are in the same direction for both. Only further studies of additional reference group samples can resolve these issues.

Potassium. The serum potassium is significantly elevated in the alcoholism group in contrast with the reference group. In this case we have the interesting observation that the schizophrenia group from the same institution is significantly lower than the reference group to essentially the same extent. Institutionalization per se does not seem, therefore, to be a major factor for this element.

Calcium. The serum calcium is significantly low in the alcoholism group. Considerations discussed above for sulfur are possible pertinent here too, because all disease groups (mongolism, alcoholism and schizophrenia) studied so far seem to show this lowering.

Iron. The finding of a marked lowering in serum iron in the alcoholism group is the most striking single finding observed in all the elemental studies to date. The serum iron in this series of alcoholism cases is depressed to less than 50% of its value in the reference group.

Possible spurious bases for this marked alteration deserve consideration. The question of hemolysis needs consideration. Because the X-ray spectrochemical method does not distinguish iron in various bonding forms, it is evident that hemoglobin iron will be measured along with other iron, should any hemolysis have occurred. It is our standard practice to inspect sera visually. If any visible hemolysis is present, the serum is excluded from this analysis both in the alcoholism and in the control group, but the visible inspection does not rule out hemolysis completely. We know of no reason to suspect more hemolysis in the reference group than in the alcoholism group from the procedures used to obtain the bloods. Furthermore, it is unlikely that appreciable hemolysis can have occurred at the subvisible level in the reference group, because the mean value in the reference group is in good agreement with literature values for serum iron (the X-ray method gives a slightly low value compared with chemical methods that are reported to be insensitive to hemoglobin iron). Hemolysis in the alcoholism group cannot account for the difference, for any excess of hemolysis in this group would mean that the serum iron is even lower truly than the marked lowering already apparent. All these considerations make it unlikely that invoking hemolysis can explain this striking alteration of serum iron in alcoholism.

It is of interest to inspect the individual values of serum iron observed in

the alcoholism and the reference groups. These data are presented in Table 3. At least five of the nineteen cases in the alcoholism group show serum iron values hardly different from zero values. Fully 59% of the ostensibly healthy subjects show serum iron levels higher than the highest value observed among the alcoholic subjects. Clearly the difference in serum iron levels between the groups is a marked overall trend rather than an effect resulting from one or a few aberrant values.

Hepatic disease must, of course, be considered in the alcoholism group. In this preliminary study no studies of specific hepatic status were made. Acute hepatitis has been studied by several investigators,[3,4] and the result is a marked elevation in the serum iron, the opposite of the effect observed in this alcoholism group. Cirrhosis of the liver has also been investigated by several workers.[5,6] In cirrhosis the serum iron values have been reported to be normal or slightly low. It is conceivable that our alcoholic group may show a high incidence of fatty livers and that this state of liver disease is associated with the markedly lowered serum iron values observed.

The prominent role of iron as an integral part of the molecule of several enzymes involved in oxidation is known. Whether the use of alcohol as a major fuel by the alcoholic alters iron metabolism in relation to such enzymes presents an intriguing question for future study.

Zinc. The serum zinc level is significantly depressed in the alcoholism group as compared with the reference group in ostensible health. The schizophrenic subjects from the same institution did not show a comparable lowering. Vallee has reported lowered zinc levels in alcoholism before.[7] Our findings represent a corroboration of his observations, although the absolute levels by the different methodologies are not in agreement.

Bromine. The absolute mean level of serum bromine is more than double the value for the reference group, yet this elevation cannot be proved significant. This apparently anomalous result finds ready explanation in a consideration of the individual bromine results for the alcoholism group. One subject showed a serum bromine of 42 p.p.m.; another showed a serum bromine of 56.8 p.p.m. These two cases, some ten to fifteen times the mean for the reference group, account for the high mean value in the alcoholism group. No bromine-containing medications were being administered to these subjects while they were at the Napa State Hospital. However, it is entirely possible that these two subjects with extremely high serum bromine levels had been taking bromide-containing medications themselves before admission. The relatively long decay period for blood bromide would allow for appreciable levels still to have been present at the time the subjects were sampled (less than 30 days after hospital admission). While the prior use of bromides is a highly plausible explanation of the observations, we cannot yet rule

Table 3

SERUM IRON VALUES (p.p.m.) IN INDIVIDUAL CASES OF ALCOHOLISM AND OSTENSIBLY HEALTHY CONTROL SERIES*
(Arranged from lowest to highest values in each series)

Alcoholism	Reference Group (Ostensibly Healthy)
-0.11**	0.28
-0.05**	0.29
0.00	0.30
0.03	0.39
0.07	0.39
0.12	0.39
0.12	0.39
0.18	0.40
0.22	0.42
0.27	0.44
0.33	0.47
0.33	0.50
0.34	0.51
0.35	0.51
0.40	0.55
0.43	0.57
0.44	0.57
0.46	0.58
0.55	0.58
	0.58
	0.61
	0.62
	0.63
	0.68
	0.68
	0.72
	0.74
	0.76
	0.85
	0.85
	0.92
	0.93
	0.95
	0.97
	1.08
	1.23
	1.23

*Cases with visible hemolysis are excluded from both groups.

**As explained in detail in our previous report (1), when the true value is close to zero, it is possible by X-ray spectrochemistry to record a negative value. Obviously the true value cannot be negative.

out some metabolic basis. The similarity of serum bromine levels in the remaining 17 alcoholic subjects to those in the controls militates against a metabolic factor.

B. Schizophrenia

The finding of significant lowering of sulfur, potassium and calcium has been discussed above in connection with the consideration of similar findings in the alcoholism group. It deserves reiteration that even though both the schizophrenia and the alcoholism series are in the same institution, the serum potassium alterations are in opposite directions when contrasted with those of the ostensibly healthy group.

It is of interest to note that chlorine is significantly lower in the schizophrenic subjects but not in the alcoholism group in the same institution.

Aside from the small, but significant, lowering in sulfur, chlorine, potassium and calcium levels observed in this group of schizophrenic subjects, no other single element can be proved significantly different in level for the schizophrenia group in contrast with the reference group.

We are inclined to minimize attachment of special importance to these results in the schizophrenic subjects, since the differences are all in the same direction; all represent approximately the same percentage lowering and hence could possibly be an effect of comparative differences in hydremia. The group of schizophrenic subjects here reported are biased in a purposeful sense. They were chosen as a group of schizophrenic subjects who appeared to have a high chance of being discharged from the hospital. We are currently studying a group of schizophrenics with 5 years or more of hospital residence. Whether this increased "severity" will demonstrate elemental deviations not shown by the "milder" group will be of interest. At this time these preliminary studies do not offer any promising biochemical clues to this major disease entity.

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A PORTABLE DEVICE FOR CONSTANT INFUSION OF THE AMBULATORY PATIENT*

By

Reed Johnson, Edward Dowling and Donald Van Dyke**

When constant infusion with a drug is necessary in medical treatment but the patient would benefit from being ambulatory, it is advantageous to have the infusion equipment portable. Certain improvements in routinely available equipment have made it possible to incorporate a lightweight constant-infusion device into a hinged cast that the patient can wear with very little inconvenience and that allows him to perform most routine activities.

The application of such a device is simplified by the ready availability of indwelling polyethylene venous catheters that are free of tissue reaction.*** The fabrication is facilitated by the use of a disposable plastic syringe, the plunger of which can be driven at any rate without leakage and without jamming. Improvements in routinely available power units (mercury batteries and lightweight motors) have made it possible to keep the weight within comfortable limits.

MATERIAL AND METHODS

The portable infusion pump is assembled on a lightweight plastic cast that is hinged so that it is easily put on and off. High-impact styrene plastic is moulded in a vacuum mould over a plaster-of-paris cast of the human arm. A range of sizes can easily be made by making the first cast to the plaster mould and forming several others on top. In this way each new cast (layer) is larger by the thickness of the plastic used (2.5 mm). High-impact styrene is sufficiently strong to bear the hinges and yet be lightweight. A flange on which the syringe and motor drive are mounted is left on the lower half of the cast (Figs. 1 and 2).

The infusion device consists of a disposable plastic syringe (Tomac) and a spiral-drive cam mounted to the top of the flange on the cast with the batteries (two Mallory type RM 12R) and motor (Cramer, Model 800, shaft speed 1 revolution per 12 hours, 3 volts, direct current) mounted underneath. A block of Teflon anchors the syringe and guides the shaft of the plunger. The drive cam is mounted with a wing nut for easy adjustment of position. For use with solutions that might deteriorate at room temperature, hollow shells of thin butyrate plastic to fit over the syringe are vacuum-formed to the contours of the syringe barrel and filled with water (Fig. 1). The "ice cubes" are held in place by a spring and can be easily and frequently changed by the patient, several spares being kept in the deep-freeze. A handle is attached for use with a sling if desired.

* This paper appears as UCRL-9958 in a limited number of copies.

** Supported in part by the U. S. Atomic Energy Commission.

*** Adams intramedic polyethylene tubing, PE 10, was used.

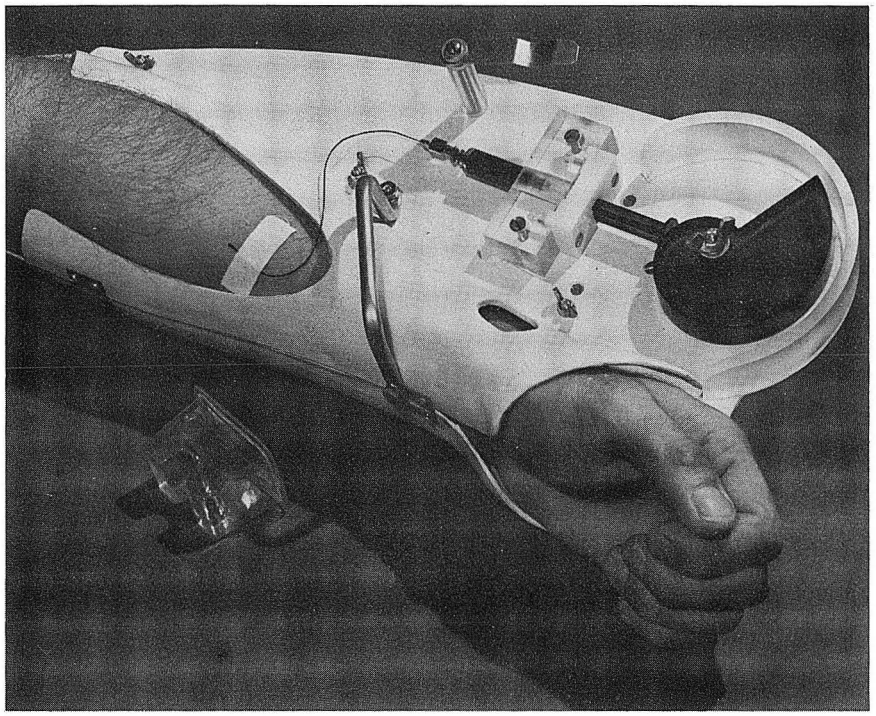

Fig. 1. The cast as it would be worn, with the drive cam and plastic syringe mounted to the top of the flange. The plastic "ice cube," that can be held over the syringe barrel by the metal spring, is shown detached.
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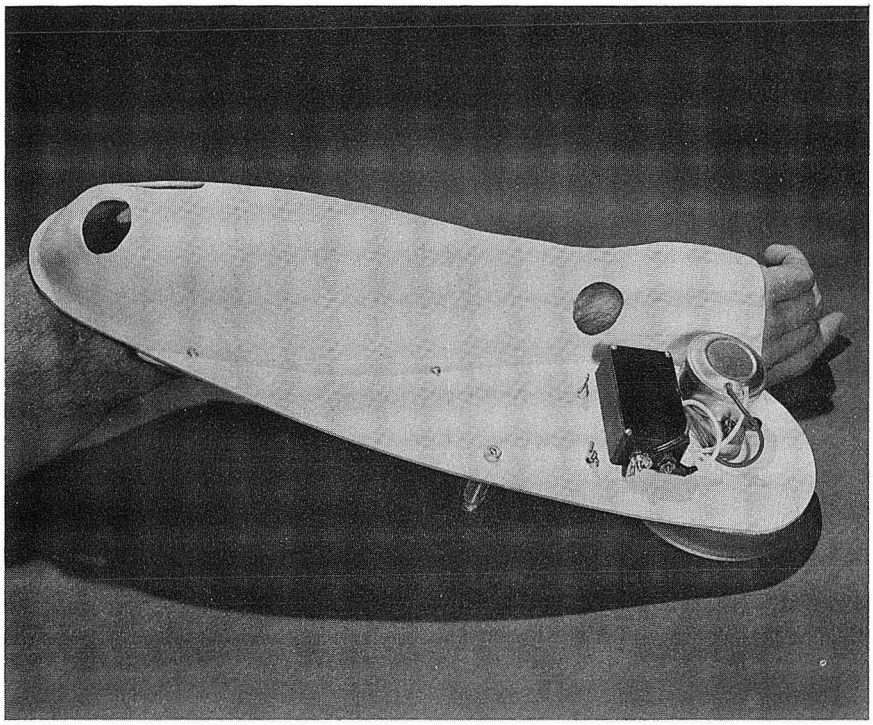

Fig. 2. The cast from the bottom, showing the battery case and motor.
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RESULTS AND DISCUSSION

The entire infusion device and cast weighs 1 lb. 14 oz., and as designed pushes the syringe barrel 1-1/2 inches (0.7 ml. with a 1-ml. syringe and 11 ml. with a 20-ml. syringe) in 12 hours. The patient is able to carry on ordinary activities without great inconvenience. An office worker could perform most of his usual tasks. However, typewriting and driving a car are not possible.

The infusion can be continued for 3 or 4 days on a single pair of batteries. An indwelling venous catheter can be left *in situ* for several days. When the infusion is discontinuous, the catheter can be detached from the syringe, the needle socket sealed with a Teflon plug, the cast removed and the catheter protected by a bandage. The catheter must always be firmly secured by tape to avoid any possibility of its being lost into the vein.

A LOW-SPEED SWINGING-BUCKET ULTRACENTRIFUGAL ROTOR*

By

Frank T. Lindgren

INTRODUCTION

Although swinging-bucket-type rotors have been designed[1] or are available commercially, such as the Spinco (SW39L, SW 25.1, and K6) and Serval (HS) rotors, none provides for use of both full-size 6-ml and 9-ml 1/2-inch preparative tubes. Use of these convenient full-length tubes, in particular the 9-ml tubes, would present a long radial path (8 cm) in which to achieve nearly ideal sedimentation or flotation. Because of the widespread use of Spinco-type equipment it seemed desirable to design a swinging-bucket type of rotor that would operate in either the standard Model L or Model E ultracentrifuge. Because Spinco-type equipment provides for high-vacuum operation, a requirement for the swinging-bucket assembly would be for an effective vacuum seal to prevent possible escape of fluid from the centrifuge preparative tube into the vacuum chamber during operation. Another potentially desirable feature includes sealing these preparative tubes with standard preparative cap assemblies to achieve an additional safety vacuum seal as well as for ease of removal of the preparative tube from the centrifuge bucket. These features have been incorporated in a low-speed swinging-bucket rotor described below.

CONSTRUCTION AND TESTING

Because of the convenience of either two- or four- place operation it seemed advantageous to design a four-place rotor employing 90 deg symmetry. The rotor assembly shown in Figures 1 and 2 consists of three parts: a rotor base, the cylindrical rotor, and a locking cylinder that also serves as a suspension coupling for use on the Model E ultracentrifuge. The rotor base allowing operation in the Spinco Model L ultracentrifuge was turned down from an old Spinco preparative rotor that had been retired after prolonged use at its rated speed. The cylindrical rotor was made from a forged billet of 7075 aluminum. After the cylindrical rotor had been machined slightly oversize it was subjected to a T-6 heat treatment. This process consisted of maintaining the rotor at 880°F for 1 hour followed by a 125°F water quench. The rotor was then reheated to 250°F for 24 hours and air quenched. A final machining of the tempered rotor was made to the specified tolerance (±.002 in. for all dimensions).

Perhaps the most critical parts of this centrifuge rotor assembly are the trunnions, which should not be excessively massive yet must support their own weight and that of the preparative tube assemblies in the centrifugal field. For

* This work was done under the auspices of the U. S. Atomic Energy Commission.

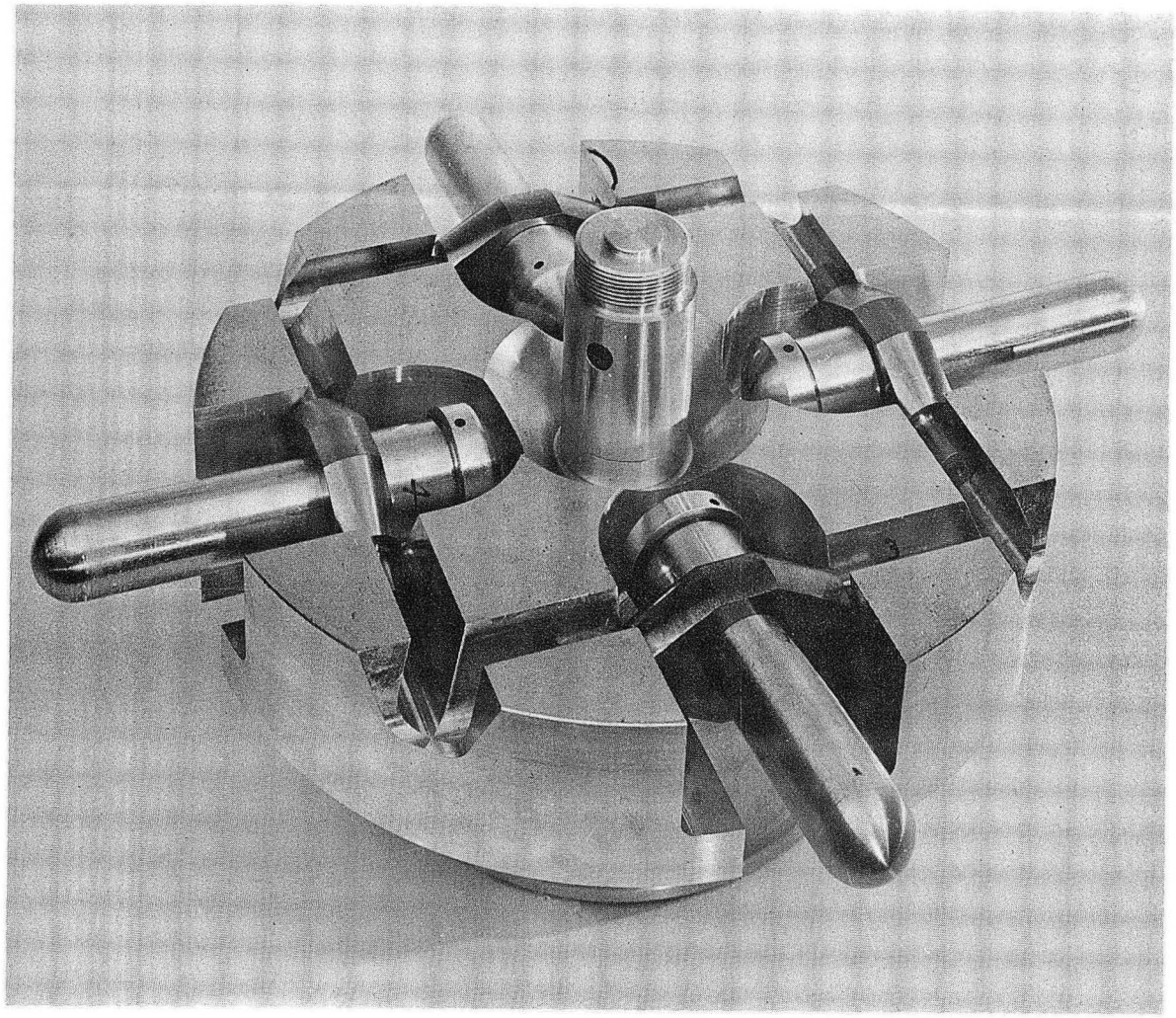

Figure 1. The complete rotor equipped with 9-ml swinging buckets in operational position.

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this reason a special silico-manganese type of steel (Allegheny Ludlam 609) was selected for constructing these trunnions, because of its high elastic limit and its ability to withstand severe stress before bending or breaking. The trunnions shown in Figure 1 were machined first to 0.010-in. oversize before the heat treatment. A hardening temperature of 1550° to 1650°F was maintained for 1/2 hour followed by an oil quench at room temperature. Thereafter a drawing temperature of 800°F was maintained for 12 to 18 hours with the trunnions contained within a "neutral pack" to prevent scaling. After this heat treatment the trunnions were machined to full tolerance, which included balancing such that no trunnion differed from another by more than ±0.020 gram.

Each trunnion was tested in a special fixture shown in Figure 3. A hydraulic

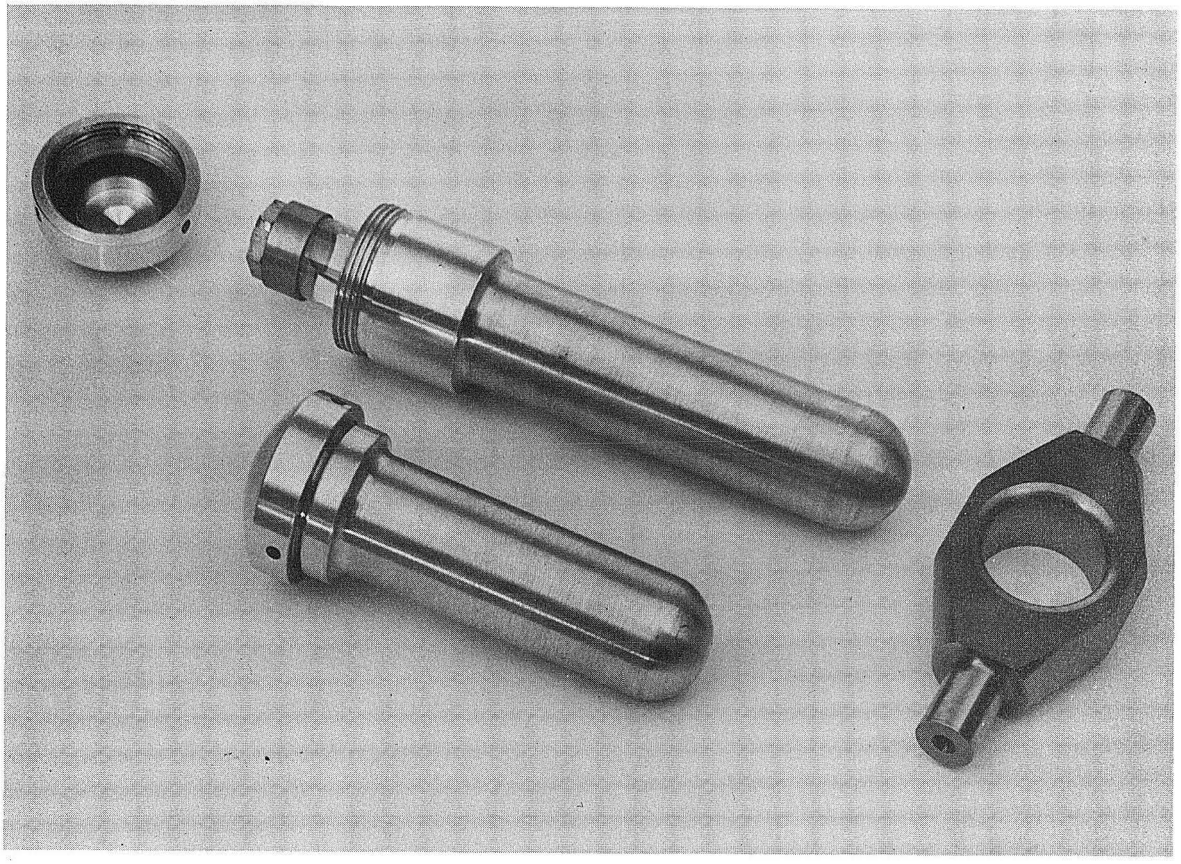

Figure 2. Trunnion, swinging buckets (9 and 6 ml capacity) and cap. Swinging buckets and cap allow use of full size standard 1/2" diameter Spinco preparative tubes (of 6 ml and 9 ml capacity) and 1/2" Spinco cap assemblies.

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press carried each trunnion to a full 20,000 lb stress. The stress-strain relation is determined by the downward deflection of the center of the trunnion. Also shown along the stress axis are the comparable operational rotor speeds using the fully loaded 9 ml preparative tube - trunnion assemblies. One trunnion was carried to failure, which occurred at a loading of 33,200 lb. Although a radial cross section through the center of the preparative tube hole of the trunnion was approximately 90% of the combined area of the two trunnion support points, failure occurred simultaneously across these latter two support points. Close examination of the trunnion subjected to failure (Fig. 4) suggests that the near pivot failed in shear, and the far pivot failed as a result of the severe bending movement developed in the region of failure. It would appear from this failure (at approximately 150,000 lb/in.^{2}) that enlarging the radius in the region joining the support pivot and the body of trunnion would potentially increase trunnion strength. The expected ultimate strength of Allegheny Ludlam 609 with our heat treatment is in the neighborhood of

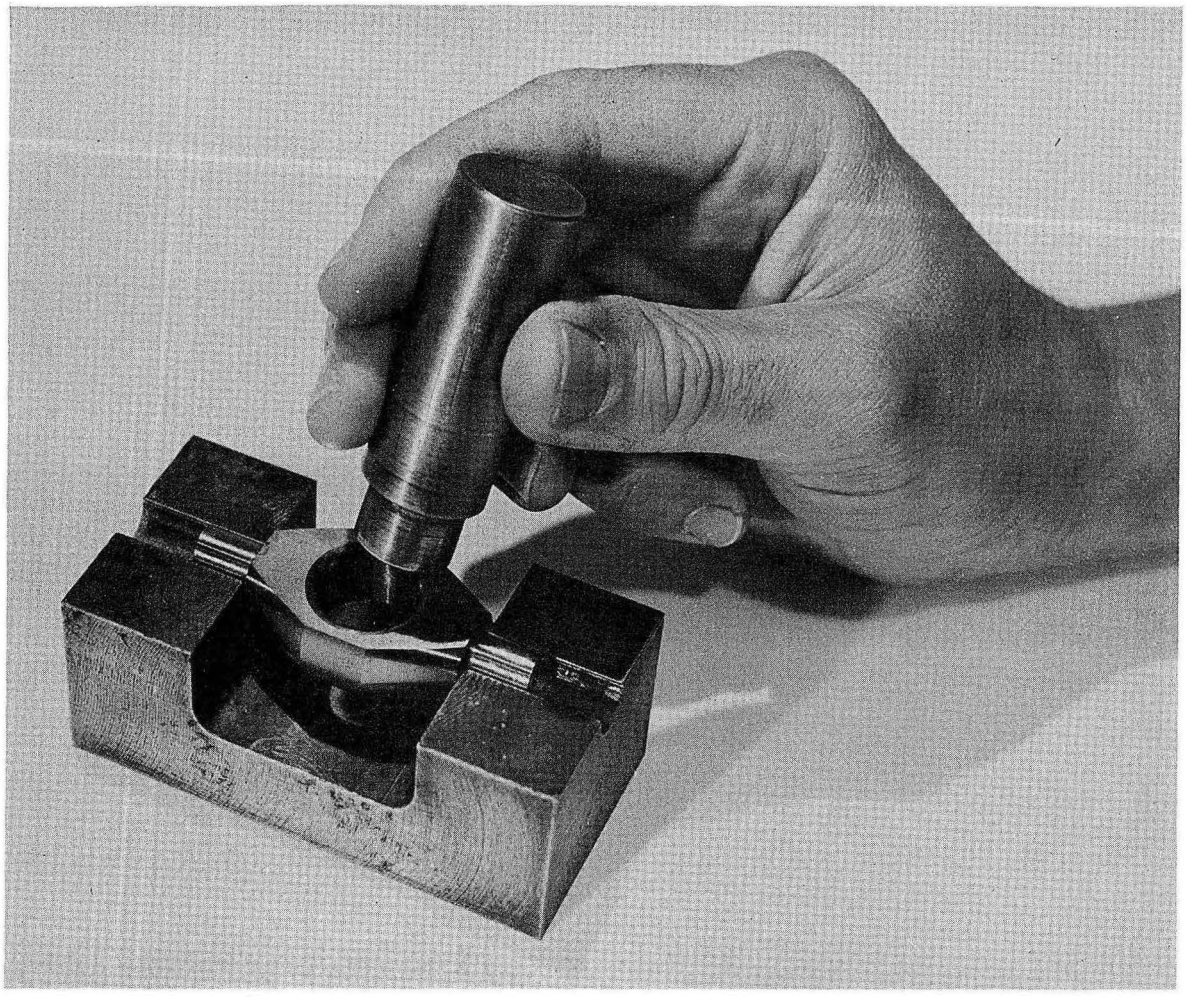

Figure 3. Support fixture and plunger for testing trunnion.
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300,000 lb/in.^{2}

The 6- and 9-ml swinging buckets and caps (shown in Fig. 2) were machined out of ST24 duraluminum and balanced to within ±0.020 g. A thrust shoulder of 3/32-in. radius was machined on each of the buckets, matching a complementary supporting rim on the trunnions to provide for accurate and reproducible centering of the trunnion assembly during operation. Failure to provide such centering could lead to potentially detrimental rotating couples during operation. Each bucket cap is fitted with an O-ring type of seal.

APPLICATION

Practical operation of this rotor thus far has been confined to rotor speeds of from 4000 to 10,000 rpm (1,200 to 6,800 x g). However, useful operation at from 15,000 to 20,000 rpm (corresponding to a mean force of 15,000 to 27,000 x g)

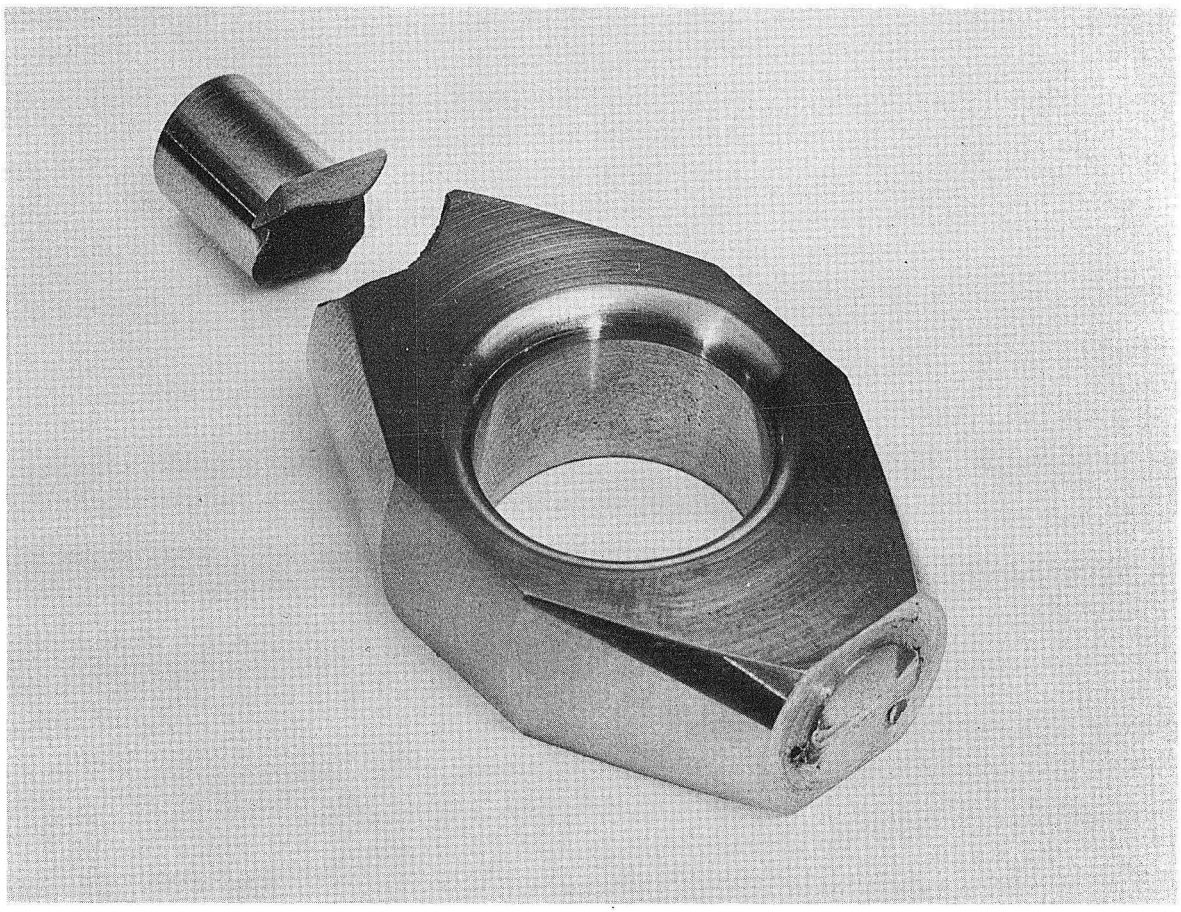

Figure 4. Trunnion carried to failure illustrating shear failure (right support) and failure resulting from bending-movement stress (left support).

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can be expected with safety factors of approximately 6.4 and 3.5, respectively. Unfortunately, because of the reduced chamber diameter of the Model E ultracentrifuge (relative to the model L), only the 6-ml swinging buckets can be used in this machine. Thus far this rotor has been used to fractionate,[2] by means of density-gradient techniques, certain of the human serum lipoproteins of the chylomicron class (above $S_f 400$). Potential applications include ultracentrifugal fractionation of subcellular components, including microsomes, mitochondria and such granules or substructural granules as are found in mass cells and eosinophils.

ACKNOWLEDGMENT

I thank Mr. Leslie O. Seaborn of the Inorganic Materials Research Laboratories (Lawrence Radiation Laboratory, Berkeley) for carrying out the stress and failure tests on the trunnions, as well as Mr. George E. Jolis and Mr. Jack L. Hill (Lawrence Radiation Laboratory, Livermore) for radiographs and ultrasonic inspection of the

rotor assembly. I particularly wish to thank Mr. Pete Dowling and Mr. Jack Kroll (Lawrence Radiation Laboratory, Berkeley) for their helpful suggestions and craftsmanship during the construction of the complete rotor assembly and test fixtures. Also, I much appreciate the helpful analysis made by Dr. Karl S. Pister (University of California Engineering Department, Berkeley) of the bending movement developed during stress of the trunnion.

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CELL DIVISION AND PHAGOCYTIC ACTIVITY IN LIVER RETICULO-ENDOTHELIAL CELLS

By

Lola S. Kelly, Barbara A. Brown and Ernest L. Dobson

INTRODUCTION

Evidence has been presented that both stimulation to hyperactivity of the reticulo-endothelial (R. E.) system by estradiol or zymosan and recovery of the R. E. system from "blockade" by colloids are accompanied by liver littoral* cell proliferation.[1] A question has frequently been raised as to which littoral cells undergo division under these conditions. Are the active phagocytes in the liver the cells that divide, or is there a group of less differentiated stem cells that supplies the highly active phagocytic population? The experiments reported here were designed to elucidate these points under conditions which previously have been shown to stimulate liver littoral cell proliferation: estradiol administration and recovery from "blockade" by saccharated iron oxide.

METHODS

Swiss male mice approximately 8 weeks old were used in all experiments. In experiment 1, liver littoral cell division was stimulated by the subcutaneous administration of 1 mg of estradiol as previously described.[1] Three days later tritiated thymidine (25 μc per mouse, 360 μc/μ mole, Schwartz Laboratories) was injected intraperitoneally to label the cells synthesizing DNA in preparation for division. In order to determine whether cells preparing for division were phagocytic, saccharated iron oxide (Proferrin, Sharp and Dohme) containing 1 mg iron was injected intravenously one hour after the thymidine, and the mice were sacrificed two hours later (experiment 1a). In another group (experiment 1b) the saccharated iron oxide was injected one day after the thymidine, and the mice were sacrificed two hours later in order to determine whether newly divided cells were phagocytic.

In experiment 2, the same dose of saccharated iron oxide was employed for a different purpose, i.e. as a "blockading" agent. In order to determine which cells were preparing for division in the recovery from "blockade", tritiated thymidine was given two days after the intravenous injection of the "blockading" dose of colloid, and the mice were sacrificed 1 hour later.

Following paraffin embedding, 6μ liver sections were covered with stripping film (Kodak AR10), exposed for 3 weeks and developed and stained with hematoxylin and eosin. In each liver approximately 1000 cells in both the perilobular regions

*The term littoral cell rather than reticulo-endothelial cell has been used here after Abercrombie and Harkness,[2] because some uncertainty exists as to whether all the sinusoidal lining cells are capable of phagocytosis and thus whether they belong to the reticulo-endothelial system.

and in the areas closely surrounding central veins were examined at a magnification of 900x. The cells were scored as to whether or not they contained saccharated iron oxide and whether they had incorporated labeled thymidine into their DNA.

In order to demonstrate more clearly the phagocytized colloidal iron, a duplicate set of sections was stained with Perls' ferrocyanide method and counter stained with hematoxylin to make the nuclei clearly visible. In each liver approximately 400 cells in the perilobular regions and an equal number in areas closely surrounding central veins were scored at a magnification of 900x.

RESULTS AND DISCUSSION

TABLE 1

EXPERIMENT	PERCENT LIVER LITTORAL CELLS LABELED WITH H^3-THYMIDINE				PERCENT PARENCHYMAL CELLS LABELED WITH H^3-THYMIDINE		PERCENT LITTORAL CELLS WITH COLLOID	
	With colloid		Without colloid					
	Centri-lobular	Peri-lobular	Centri-lobular	Peri-lobular	Centri-lobular	Peri-lobular	Centri-lobular	Peri-lobular
(1a) Phagocytosis by stimulated cells during DNA synthetic period (3 day estradiol; 3 hour H^3-thymidine; 2 hour colloid)	11.6	11.8	0.0	0.0	3.3	0.0	64	59
	9.3	9.3	0.0	0.0	0.9	0.6	56	64
	6.7	11.4	0.0	0.0	0.5	1.3	56	65
Avg	9.2	10.8	0.0	0.0	1.6	0.6	59	63
(1b) Phagocytosis by newly divided stimulated cells (4 day estradiol; 1 day H^3-thymidine; 2 hour colloid)	10.9	16.0	0.0	0.4	1.4	0.0	58	64
	11.1	13.4	0.0	0.0	2.7	0.0	57	61
	10.4	12.8	0.0	0.0	1.0	1.2	42	52
Avg	10.8	14.1	0.0	0.1	1.7	0.4	52	59
(2) Stimulation of proliferation by colloid loading (2 day colloid; 1 hour H^3-thymidine)	8.7	5.3	1.5	0.6	2.3	5.4	49	44
	5.1	2.6	0.0	0.2	1.9	3.3	49	49
	9.1	5.4	1.4	1.2	2.8	1.2	46	45
	10.3	5.0	2.1	1.1	0.6	5.2	42	54
Avg	8.3	4.6	1.2	0.8	1.9	3.8	46	48
Unstimulated controls (n=8) (H^3-thymidine only) Avg ± S.E.	1.5 ± 0.4				0.02 ± 0.13		---	

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The data are presented in Table 1. Each entry represents the scoring on one liver. From the data of experiment 1 it is clear that DNA synthesis is markedly increased in both littoral and parenchymal cells following the administration of estradiol. The littoral cells which have been stimulated are capable of phagocytizing colloid during the DNA synthetic period (experiment 1a) and shortly after they presumably have divided (experiment 1b).

From 10 to 15% of the liver littoral cells were labeled with tritiated thymidine in the estradiol-treated mice, and all of these labeled cells contained colloid. However, phagocytized colloid was observable in only 50-60% of the total littoral cell population in these livers. Had labeled and non-labeled cells possessed equal phagocytic potential, we should have expected only half of the labeled cells to contain colloid. These observations suggest that the cells that have been stimulated to divide by estradiol treatment are particularly avid phagocytes.

Experiment 2 was designed to test whether colloid loaded cells are capable of division or whether the recovery from "blockade" is a result of proliferation of unloaded cells or "stem cells." Because most of the thymidine-labeled littoral cells contained colloid, we must conclude that the active phagocytes are capable of DNA synthesis after ingesting colloid, and that to a large extent the littoral cells preparing for division come from the colloid-containing population.

In this experiment we noted that some of the cells that do not appear to contain colloid are also preparing for division. This observation, though suggestive, cannot establish with certainty that the labeled cells without observable colloid were stimulated by some intercellular mechanism. There are three alternative explanations for this observation: these cells may have contained colloid that was not recognized; the labeled cells without colloid could have resulted from a colloid shedding division during the two-day period before the tritiated thymidine was injected; they may have been in the normal pool of dividing cells and did not happen to phagocytize at the time the colloid was available.

Although cell division normally follows DNA synthesis, it seems possible that those cells containing colloid might fail to enter the mitotic division for which they had prepared. Therefore, sections were scanned for mitotic figures. About 0.5 to 1% of the littoral cells were found to be in mitosis. Colloid was visible in approximately 80% of these cells, so that the fraction of dividing cells containing colloid was similar to the fraction of DNA-synthesizing cells containing colloid. This indicates that the ingested colloid did not interfere with the division process to any major extent and confirms the observation made many years ago by Beard and Rous[3] that, under <u>in vivo</u> conditions, Kupffer cells that had ingested colloidal material were capable of division in culture.

We have previously reported[1] a very high liver DNA specific activity 2 days after the intravenous injection of saccharated iron oxide. In the present experiment the autoradiographic data (Expt. 2, Table 1) indicate that this DNA synthesis is not confined to the littoral cells in the colloid-loaded livers. The large increase that occurred in the percentage of parenchymal cells synthesizing DNA and undergoing mitosis, together with their relative abundance and polyploidy, make their contribution a sizeable fraction of the total liver DNA synthesis. The explanation for the parenchymal cell proliferation is unknown but the proliferation may be a response to mild injury.

TABLE II

IRON DISTRIBUTION (PERLS' STAIN) AND CELL POPULATIONS IN LIVERS	PERCENT LITTORAL CELLS WITH COLLOID		LITTORAL CELL NUCLEI PER 100 PARENCHYMAL CELL NUCLEI	
	Centri-lobular	Peri-lobular	Centri-lobular	Peri-lobular
(1a) Mice given estradiol 3 days, and colloid 2 hours before sacrifice	65	74	110	160
	68	64	76	103
	80	88	131	175
Avg	71	75	106	146
(1b) Mice given estradiol 4 days, and colloid 2 hours before sacrifice	62	68	96	141
	70	74	116	170
	67	76	104	184
Avg	66	73	105	165
(2) Mice given colloid 2 days before sacrifice	52	61	118	115
	49	45	90	106
	38	46	87	132
	42	49	95	116
Avg	45	50	98	117
(Controls) Mice given colloid 2 hours before sacrifice	74	79	76	101
	57	60	66	95
	50	61	66	106
Avg	60	67	69	101

MU 26416

The fraction of littoral cells containing colloid was found to be the same in perilobular and centrilobular regions in sections which had been prepared primarily for autoradiography. Howard et al.[4] had, however, reported marked perilobular distribution of colloidal iron. Duplicate sections were therefore re-examined after staining with Perls' stain for ferric iron in order to make the colloid more clearly visible. When these sections were examined under low magnification, it was obvious that most of the iron was located in perilobular regions confirming the observation of Howard et al. However, scoring under high power again showed no essential difference in the percentage of littoral cells containing colloid in the two regions (Table 2). Two factors appear to account for the observation that the perilobular areas contain a large proportion of the total iron. The individual phagocytes in this region generally contain a larger amount of colloid per cell than do the centrilobular phagocytes, and the concentration of littoral cells is higher in the perilobular region, as indicated by the ratio of littoral cell nuclei to parenchymal cell nuclei (Table 2).

The overall percentage of littoral cells containing recognizable colloid was slightly higher in sections stained with Perls' stain (Table 2) than in the sections used for autoradiographs (Table 1). This is probably due to the fact that the unstained colloid was more difficult to see and some cells containing small amounts of colloid were therefore scored as negative.

The livers of mice, stimulated with estradiol had a higher ratio of littoral cell to parenchymal cell nuclei than the controls (Table 2), confirming this point in our previous report.[1] Both the relative increase in littoral cell number (Table 2) and the percent labeling (Table 1) were similar in the perilobular and centrilobular regions, indicating that cells in both regions proliferated equally. In contrast, the thymidine labeling in the animals recovering from "blockade" was always higher in the central areas than in the perilobular areas (Table 1, experiment 2). It has been pointed out by Bensch et al.[5] that fibroblasts that have ingested small amounts of particulate material divide normally but that cells that have ingested very large amounts do not divide. It may be that the perilobular phagocytes, that have ingested a great deal more colloid than the centrilobular phagocytes, are to some extent prevented from undergoing DNA synthesis and division. This may also be the explanation for the fact that the relative increase over control values in cell number (littoral cell nuclei/100 parenchymal cell nuclei) is greater in the central than in the perilobular areas in the colloid-loaded livers (Table 2).

It is important to note that the experiments reported here relate only to the proliferation of the littoral cells of the liver and that this is but one of the possible mechanisms of R. E. system activation.[6] Howard[7] has described conversion of "F" type to "G" type littoral cells following endotoxin injection,[8] and an increase in acid phosphatase content of existing liver littoral cells.

SUMMARY

Autoradiographs of liver sections from mice given tritiated thymidine were used to identify the cells that were preparing for division, and colloidal saccharated iron oxide was used to identify the active phagocytic cells. It was established that, in livers of mice whose reticulo-endothelial system was stimulated by estradiol, the cells preparing for division and those that had recently divided were actively phagocytic. In livers of mice whose reticulo-endothelial system had been "blockaded" with saccharated iron oxide, the cells that had phagocytized colloid were able to divide in the process of recovery from "blockade." No evidence was found for a stem cell that proliferates and differentiates to provide the active phagocytic population.

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LIPOPROTEIN FATTY ACIDS AND OIL INGESTION

By

Alex V. Nichols, Carl S. Rehnborg, Frank T. Lindgren and Robert D. Wills

INTRODUCTION

Various facets of the effects of acute fat ingestion on human serum lipids and lipoproteins have been investigated and reported.[1-12] While these studies have helped to increase our understanding of certain specific aspects of the influence of fat ingestion on serum lipids, detailed investigations directed towards establishing an integrated picture of these phenomena have been lacking. To describe adequately the physical and chemical changes in serum lipids following fat ingestion we feel it is necessary to evaluate the alterations occurring in (a) the concentrations of the major serum lipoprotein classes, in (b) the concentrations of the lipid constituents (cholesteryl esters, glycerides, phospholipids and cholesterol) of these major lipoprotein classes, in (c) the fatty acid compositions of these various esterified lipid constituents, and in (d) the fatty acid composition of the albumin-bound free fatty acids (FFA). This report presents the results of preliminary studies in which most of the above variables were determined in humans following ingestion of vegetable oils (safflower and olive oil).

METHODS AND MATERIALS

Two fasting males (I.W. age 33; B.W. age 41) were given 85 grams of oil (safflower to I.W. and olive oil to B.W.) dispersed by a Waring Blendor in 300 ml of skim milk containing a small amount of fruit flavoring. The oil preparation was well tolerated by both subjects. Two-hundred ml of blood was drawn immediately prior to oil ingestion and 5 hours afterwards. Directly after drawing the blood, the serum was separated and subjected to preparative ultracentrifugal procedures[13] for the isolation of the following three major lipoprotein classes, the two low-density classes, S_f 20-10^5 and S_f 0-20, and the total class of high density lipoproteins (HDL) d <1.20 and >1.063. All salt solutions used in the preparative ultracentrifugal procedures contained 0.05 g/liter of the disodium salt of ethylenediaminetetraacetic acid. Very low-density lipoprotein species S_f 1300-10^5 were separated by low-speed ultracentrifugal techniques[14] using a swinging bucket rotor[15] from the serum of subject I.W. after oil ingestion. In addition, the serum of subject I.W. before and after oil ingestion was subjected to preparative and analytic ultracentrifugation and analysed according to procedures previously described.[16] A 3 ml aliquot of each of the sera was also directly adjusted to d = 1.21 by addition of 3 ml of a D_2O-NO_3 solution of d = 1.393, and subjected to preparative ultracentrifugation for separation of the lipoprotein-free protein residue containing primarily the bulk of the albumin-bound FFA. Errors in recovery in each of the above ultracentrifugal operations are

estimated to be of the order of 5-10%.

Lipids were extracted from lipoprotein solutions by a modified method of Sperry et al[17] and each total lipid extract was separated by silicic acid chromatography.[18] The chromatographed lipids were quantitated by infra-red spectrophotometric techniques previously reported.[19] The various lipid fractions were then transmethylated by the method of Stoffel et al.[20] FFA's were extracted from protein solutions by the method of Dole et al[21] and methylated by the procedure of Metcalfe et al.[22] The gas-liquid chromatographic system and chromatogram analysis techniques used for the fatty acid determinations have been described in detail elsewhere.[23] The major fatty acids are tabulated according to the nomenclature proposed by Dole et al.[9] Minor and unidentified constituents are designated by: class A, methyl esters eluting before 16:0 (methyl palmitate); class B, methyl esters eluting between 16:0 and 18:0 (methyl stearate); class C, methyl esters eluting between 18:2 (methyl linoleate) and 20:4 (methyl arachidonate); and class D, methyl esters eluting after 20:4. These class designations refer to elution time interval positions of the minor and unidentified components relative to the more abundant identified esters using the diethylene glycol polysuccinate liquid phase. The error estimated for the overall procedure of component fatty acid analysis is approximately 5% for the major components, which are in the range of 10-90% of the reported fatty acid composition.

RESULTS

Although non-turbid before the experiment, the sera of both subjects were markedly lipemic following oil ingestion. The results of the analytic ultracentrifugation of the serum lipoproteins of subject I.W. before and 5 hours after ingestion of oil are tabulated in Table 1. These results show significant elevations in the concentrations of the S_f 20-400, S_f 8-20, and the $S_{f(1.20)}$4-8 lipoprotein classes following oil ingestion. Elevations in the levels of lipoprotein species of $S_f > 400$ were also noted but these could not be quantitated by the analytic ultracentrifugal techniques used.

Table 1

EFFECT OF SAFFLOWER OIL INGESTION ON SERUM LIPOPROTEIN CONCENTRATION

Sample	Lipoprotein concentration (mg/100ml)				
	S_f20-400	S_f8-20	S_f0-8	$S_{f(1.20)}$4-8	$S_{f(1.20)}$0-4
Control	32	63	349	65	255
5 hours after oil	113	94	342	84	261

Lipid analyses of the major lipoprotein classes before and 5 hours after oil ingestion are presented in Tables 2 and 3. In each subject the following observations are made: after oil ingestion (a) the $S_f 20-10^5$ lipid concentrations increased significantly, (b) the percentage lipid composition (in terms of cholesteryl esters, glycerides, phospholipids and cholesterol) of the $S_f 20-10^5$ remained relatively constant, (c) except for the glyceride concentration increase following safflower oil ingestion and the cholesterol concentration decrease following olive oil, the S_f 0-20 lipid concentrations showed no appreciable change, and (d) consistent increases occurred in HDL glyceride concentrations.

Table 2

EFFECT OF SAFFLOWER OIL INGESTION ON LIPOPROTEIN LIPID CONCENTRATIONS

Lipoprotein class	Sample	Lipid concentration (mg/100ml)			
		Cholesteryl esters	Glycerides	Cholesterol	Phospholipids
$S_f 20-10^5$	control	13.8	61.4	8.1	19.3
	5 hours after oil	28.8	134.6	14.6	38.4
S_f 0-20	control	170.4	19.8	36.3	86.9
	5 hours after oil	170.4	25.0	35.6	89.1
HDL	control	60.6	8.3	7.0	65.8
	5 hours after oil	63.1	15.6	7.9	79.8

Table 3

EFFECT OF OLIVE OIL INGESTION ON LIPOPROTEIN LIPID CONCENTRATIONS

Lipoprotein class	Sample	Lipid concentration (mg/100ml)			
		Cholesteryl esters	Glycerides	Cholesterol	Phospholipids
$S_f 20-10^5$	control	7.0	32.9	7.7	11.9
	5 hours after oil	20.7	87.4	15.8	28.7
S_f 0-20	control	160.6	17.4	50.4	88.6
	5 hours after oil	159.3	17.9	40.1	87.9
HDL	control	76.1	8.6	13.3	101.6
	5 hours after oil	69.4	13.5	13.0	107.1

The fatty acid data for the esterified lipids of the S_f 1300-10^5 class following safflower oil ingestion are shown in Table 4. Control S_f 1300-10^5 fatty acid compositions were not obtained due to the extremely low concentration of this lipoprotein class in the subject's serum prior to oil ingestion. Following safflower oil, however, this lipoprotein class increased markedly, and Table 4 compares primarily the fatty acid composition of its glyceride moiety with that of the ingested oil. It is apparent that the fatty acid composition of the S_f 1300-10^5 glycerides following safflower oil ingestion is very similar to that of the oil fed. The fatty acid composition of the cholesteryl esters is similar to that found in cholesteryl esters of the other lipoprotein classes (see Table 5) following safflower oil ingestion. No arachidonic acid was detected in this ester of the S_f1300-10^5 class, although in the remaining lipoprotein classes it ranged from 5 to 7% of the fatty acid composition.

Table 4

FATTY ACID COMPOSITION OF S_f 1300-10^5 LIPIDS (after Safflower Ingestion) and FED SAFFLOWER OIL

Fatty acids	Lipid fatty acid composition (percent of total methyl esters)			
	Cholesteryl esters	Phospholipids	Glycerides	Fed safflower oil
Class A	4.0	2.6	0.4	0.1
16:0	11.2	23.6	8.5	5.5
16:1	3.3	3.6	0.3	0.1
Class B	1.3	1.3	-	0.1
18:0	3.2	17.9	2.9	2.3
18:1	20.8	12.7	13.2	11.4
18:2	53.2	27.7	72.7	79.9
Class C	3.2	3.6	1.7	0.7
20:4	-*	7.0	0.3	-
Class D	-	-	-	-

* none detected.

The influence of safflower oil ingestion on the fatty acid compositions of the esterified lipids of the three major lipoprotein classes is shown in Tables 5, 6, and 7. Since in these tables the fatty acid data are reported as percentage composition, it is clear that by themselves these values will indicate only whether shifts in fatty acid composition do or do not occur. To evaluate changes in the serum concentration of specific fatty acids it is necessary to combine these percentage

Table 5

EFFECT OF SAFFLOWER OIL INGESTION ON FATTY ACIDS IN LIPOPROTEIN CHOLESTERYL ESTERS

Fatty acids	Lipoprotein class (percent of total methyl esters)					
	$S_f 20-10^5$		$S_f 0-20$		HDL	
	control	5 hours after oil	control	5 hours after oil	control	5 hours after oil
Class A	2.1	1.3	1.0	0.8	0.8	1.1
16:0	11.4	9.5	10.4	10.3	9.5	9.1
16:1	2.6	2.2	2.3	2.3	2.2	2.3
Class B	1.1	0.8	0.9	0.7	0.5	0.8
18:0	3.2	2.0	1.5	1.5	1.1	1.2
18:1	22.5	20.4	19.2	19.4	18.3	18.3
18:2	47.6	55.0	54.4	55.3	57.0	56.4
Class C	3.7	2.9	3.1	2.8	3.2	2.7
20:4	5.3	5.9	6.4	6.5	7.4	7.2
Class D	0.5	-	0.8	0.6	-	1.0

Table 6

EFFECT OF SAFFLOWER OIL INGESTION ON FATTY ACIDS IN LIPOPROTEIN GLYCERIDES

Fatty acids	Lipoprotein class (percent of total methyl esters)					
	$S_f 20-10^5$		$S_f 0-20$		HDL	
	control	5 hours after oil	control	5 hours after oil	control	5 hours after oil
Class A	2.4	1.4	1.8	1.9	1.9	1.7
16:0	22.7	16.1	22.0	18.6	22.3	18.2
16:1	3.5	2.2	2.7	2.7	3.1	2.6
Class B	1.0	0.9	0.9	1.1	1.1	1.1
18:0	4.2	3.5	5.3	4.6	6.2	4.8
18:1	39.8	24.4	40.4	30.3	37.7	26.9
18:2	21.0	47.1	20.6	37.2	21.2	40.1
Class C	4.3	3.2	3.4	2.2	2.9	2.3
20:4	1.0	1.2	1.8	1.4	1.6	1.3
Class D	0.2	-	1.2	-	2.0	1.1

Table 7

EFFECT OF SAFFLOWER OIL INGESTION ON FATTY ACIDS IN LIPOPROTEIN PHOSPHOLIPIDS

Fatty acids	Lipoprotein class (percent of total methyl esters)					
	S_f 20-10^5		S_f 0-20		HDL	
	control	5 hours after oil	control	5 hours after oil	control	5 hours after oil
Class A	1.9	1.3	1.4	1.3	1.7	1.5
16:0	22.6	21.6	23.1	23.3	23.0	21.7
16:1	1.1	0.9	0.9	0.8	1.0	0.9
Class B	1.1	0.8	1.0	0.8	0.9	1.0
18:0	15.6	15.7	14.8	14.9	14.8	14.9
18:1	12.9	12.1	12.0	11.2	12.0	12.0
18:2	25.9	28.2	25.9	28.4	24.6	27.1
Class C	5.3	5.6	5.7	5.3	5.8	5.4
20:4	8.9	9.8	9.2	8.8	10.1	10.4
Class D	4.8	7.6	6.0	5.3	6.2	5.2

data with the lipid concentration data given in Table 2. In this section we will consider only whether changes in lipoprotein lipids do or do not occur following oil ingestion. Composition changes appear in the following lipoprotein lipids 5 hours after safflower oil ingestion: (a) the cholesteryl esters of the S_f20-10^5 class, and (b) the glyceryl esters of all of the broad lipoprotein classes investigated. In addition consistent small changes are also observed in the composition of the lipoprotein phospholipids after safflower oil ingestion. The major change in the above lipids is an increase in the percentage of linoleic acid in their fatty acid composition.

The fatty acid composition data following olive oil ingestion for the cholesteryl esters and glycerides (phospholipids were lost) of the three lipoprotein classes are presented in Table 8 and 9. Fatty acid composition changes were observed in the S_f20-10^5 cholesteryl esters and the glyceryl esters of the S_f20-10^5, S_f 0-20, and HDL. The major change in these lipids was an increase in the percentage of oleic acid in their fatty acid composition. The cholesterol ester changes are much smaller following olive oil than after safflower oil but are larger than the estimated error in the fatty acid analysis. The possible error introduced by biological variation was not determined, but judging from the constancy of the fatty acid compositions of the S_f 0-20 and HDL cholesteryl esters in both experiments, it is reasonable to assume that it is quite small during the experimental period. This assumption is also made for the fatty acid composition data obtained from the other lipid components. The possibility of glyceride contamination of the

Table 8

EFFECT OF OLIVE OIL INGESTION ON FATTY ACIDS IN LIPOPROTEIN CHOLESTERYL ESTERS

Fatty acids	Lipoprotein class (percent of total methyl esters)					
	S_f 20-10^5		S_f 0-20		HDL	
	control	5 hours after oil	control	5 hours after oil	control	5 hours after oil
Class A	1.8	1.3	0.7	0.6	0.7	0.7
16:0	9.6	9.6	9.2	9.8	9.0	9.0
16:1	2.8	2.5	2.0	2.1	2.1	2.1
Class B	0.3	0.4	0.2	0.2	0.3	0.3
18:0	1.5	1.5	0.8	0.8	0.8	0.9
18:1	17.6	21.1	15.9	15.9	15.4	15.9
18:2	54.6	52.2	59.4	59.1	58.8	58.1
Class C	3.8	2.5	2.3	2.4	2.4	2.3
20:4	8.1	8.4	8.8	8.5	9.8	9.9
Class D	-	0.6	0.8	0.7	0.9	0.8

Table 9

EFFECT OF OLIVE OIL INGESTION ON FATTY ACIDS IN LIPOPROTEIN GLYCERIDES

Fatty acids	Lipoprotein class (percent of total methyl esters)					
	S_f20-10^5		S_f0-20		HDL	
	control	5 hours after oil	control	5 hours after oil	control	5 hours after oil
Class A	2.4	1.6	0.9	0.6	1.8	1.6
16:0	25.5	22.3	22.3	20.2	22.9	22.5
16:1	4.5	3.4	3.6	2.8	3.7	3.6
Class B	0.5	0.3	1.2	1.0	1.5	0.9
18:0	3.1	2.8	5.9	4.1	6.3	4.1
18:1	36.8	46.5	38.2	42.8	35.6	41.6
18:2	22.1	19.9	20.7	20.3	20.0	19.8
Class C	3.7	2.1	3.3	3.1	4.2	2.9
20:4	1.2	1.4	3.1	4.2	2.2	1.7
Class D	0.3	-	0.9	0.8	2.0	1.4

cholesteryl esters during silicic acid chromatography has also been carefully avoided in this investigation.

The composition data for the ultracentrifugal protein residue containing the FFA are found in Table 10. Significant alterations in fatty acid composition were detected after oil ingestion. In the safflower oil experiment the percentage of linoleic acid increased, and in the olive oil experiment the percentage of oleic acid rose. Possible limitations on these FFA composition data will be treated in the Discussion section.

Table 10

FATTY ACID COMPOSITION OF THE ULTRACENTRIFUGAL PROTEIN RESIDUE FRACTION FOLLOWING OIL INGESTION

Fatty acids	Safflower oil ingestion		Olive oil ingestion	
	control	5 hours after oil	control	5 hours after oil
Class A	2.1	1.1	0.9	0.8
16:0	18.7	14.3	21.7	19.4
16:1	2.1	1.4	2.3	2.1
Class B	1.8	1.0	1.1	0.6
18:0	14.4	7.8	9.8	5.8
18:1	29.5	21.4	35.8	46.2
18:2	21.1	47.1	21.2	19.9
Class C	5.3	2.4	3.2	2.3
20:4	2.4	1.9	3.1	1.9
Class D	2.6	1.7	1.0	1.1

DISCUSSION

Ultracentrifugal lipoprotein concentration data show that following safflower oil ingestion there occurs an increase in $S_{f(1.20)}$4-8 lipoprotein species in addition to the expected elevation in S_f20-400 lipoproteins. This concentration increase in $S_{f(1.20)}$4-8 species is associated with a significant elevation in the glyceride content of the total HDL lipid composition. In humans, elevations of HDL phospholipid concentrations have been reported by Havel et al,[6] and in dogs, Hillyard et al[24] observed increases of all lipid constituents of d=1.063-1.107 lipoproteins after a fat mean. Some elevation in HDL phospholipids after oil ingestion was also noted in the present investigation but represented a much smaller percentage change than the glyceride elevation. The fact that cholesteryl ester and phospholipid change so little compared with the glyceride increase argues

against an increase in level of the usual type HDL molecules. Either new types of HDL molecules high in glyceride content must be appearing, or glyceride is attaching to the usually-occuring HDL species. Thus, the observed increase in HDL species may arise in part from the metabolism of very low density lipoprotein (S_f 1300-10^5) which may yield some HDL-type molecules relatively high in glyceride content. Indirect evidence for this possibility may be drawn from the study of Scanu et al[25] in which they observed the appearance of radioactivity in the HDL fraction following the administration of labelled (with protein I^{131}) chylomicrons to dogs. Under the conditions of an acute ingestion of oil there may occur a detectable "pile-up" of some HDL species which may be involved in the "clearance" of the very low density lipoprotein species. Further evidence for some metabolic association between serum glycerides and HDL may be drawn from existing reports[26,27] of similarities in the protein moieties of chylomicrons and HDL. Additional chemical information on the $S_{f(1.20)}$4-8 species both under fasting and under post-prandial conditions is now being obtained for evaluation of their possible origin.

Some direct attachment of additional glyceride to normal HDL may also play a part in the observed HDL changes, and studies are now in progress to evaluate the <u>in vitro</u> interaction of glycerides with HDL species. Evidence[28] is available for the attachment of HDL to artificial glyceride emulsions of very large particle size, but no data are available on the binding of small numbers of glyceride molecules by intact HDL molecules.

The glyceride increase in HDL species following oil ingestion suggests a possible metabolic relation between this lipoprotein class and serum glycerides. Thus, certain species of HDL may actively participate in serum glyceride transport and metabolism, and, as a result, may play a regulatory role on serum levels of S_f20-10^5 lipoproteins. This is suggested from the lipoprotein data on human populations by deLalla et al,[29] in which significant inverse relations are found between serum concentrations of S_f20-400 species and lipoproteins of $d < 1.107$ and > 1.063 (HDL_2). On the average, lower levels of S_f20-400 lipoproteins would be expected in individuals having elevated serum HDL_2 levels. In particular, the data of deLalla show that females who have, at all ages, mean serum HDL_2 levels approximately double those in males, exhibit significantly lower mean S_f20-400 levels than males at all ages. Further, the recent studies by Fredrickson et al[30] on Tangier disease, where there is apparently an almost complete absence of serum HDL, also support the above hypothesis that HDL species may play a regulatory role in S_f20-10^5 concentrations. In this unique disease a significant elevation of $d < 1.019$ lipoproteins and glycerides has been reported. Other disease states manifesting elevated S_f20-10^5 levels, such as idiopathic hyperlipemia, xanthoma tuberosum and glycogen storage disease, have also been reported to exhibit lower levels of HDL species.[31,32]

Bragdon et al[9] studied the effects of oil ingestion on the fatty acids of the total mixture of lipids in the chylomicron class, and they reported an almost identical composition for this mixture and the ingested oil. Farquhar et al[33] in similar studies separated the total lipid mixture extracted from the chylomicron class into two fractions: (A), the non-phospholipid fraction and (B), the phospholipid fraction. Their fatty acid composition analyses indicated that the fraction (A) resembled the fed fat, and the fraction (B) reflected the fed fat but to a lesser extent than (A). In the present study the $S_f 1300-10^5$ glyceride moiety is very similar in fatty acid composition to the ingested oil, while the fatty acid compositions of its cholesteryl esters and the phospholipids resemble those observed in the $S_f 20-10^5$ following oil ingestion.

The present data show that oil ingestion influences the fatty acid composition of the $S_f 20-10^5$, $S_f 0$ 20, HDL and the ultracentrifugal protein residue fraction. Bragdon et al[11] studied the effects of corn oil ingestion on the fatty acid compositions of total lipid extracts from various lipoprotein classes and have reported changes in composition based on the ratios of linoleic to oleic acids before and after oil. These authors found shifts in this ratio primarily in the chylomicrons, lipoproteins of $d < 1.019$ and the fraction of $d > 1.21$. Since total lipid extracts were used for the evaluation of fatty acid composition changes, only gross changes in lipoprotein fatty acid composition could be detected.

An approximate calculation has been made for the fatty acid composition of the glyceride increment to the $S_f 20-10^5$ and the HDL classes for both oils studied. Considering only the major fatty acids we find for the safflower oil study the following fatty acid composition for the glyceride increment: for the $S_f 20-10^5$, 16:0, 11%; 18:1, 12%; and 18:2, 69% and for the HDL, 16:0, 14%; 18:1, 15% and 18:2, 61%. In the olive oil study we find: for the $S_f 20-10^5$, 16:0, 20%; 18:1,51% and 18:2, 18%; and in the HDL, 16:0, 21%; 18:1, 53%; and 18:2, 21%. Although the values of these increments approach the fatty acid distributions of the fed oils (safflower: 16:0, 5%; 18:1, 11%; 18:2, 80%; olive: 16:0, 15%, 18:1,64%; 18;2, 16%) they are somewhat different. The fatty acid composition of the total $S_f 20-10^5$ glyceride increment however is very similar to that of the $S_f 1300-10^5$ species. Because the fatty acid compositions of the glyceride increments in the major lipoprotein classes are not identical with the ingested oil, some dilution is probably taking place. This dilution may be due in part to exchange of the newly ingested glyceride with endogenous glycerides. Bragdon et al[11] in their ingestion experiments found no detectable in vitro exchange between chylomicrons and other lipoprotein fractions from their measurements of linoleic to oleic acid ratios in the total lipid extracts from the various lipoprotein fractions. In the present report an indirect estimate of the extent of the exchange that may be occuring can be made by inspection of the fatty acid composition changes in the $S_f 0-20$ following olive oil ingestion. In this particular fraction there was little, if any, increase in

glyceride content, and changes in fatty acid composition would thus be expected to be due primarily to exchange.

In conflict with the observations of Dole et al,[8] Bragdon et al[9] reported significant shifts in the linoleic to oleic acid ratio of the d > 1.21 fraction following corn oil ingestion. In studies on the effects of long-term ingestion of corn oil Hirsch et al[34] also observed FFA composition changes reflecting the fed oil. In the present report major alterations are found in the composition of this fraction following safflower and olive oil ingestion. The changes are significant and are in the direction of the fatty acid composition of the fed oil. These values, although indicative of composition changes in the albumin-bound FFA, may not represent the exact *in vivo* composition, possibly because of two factors involved in such an investigation. The first factor may be a minimal but unavoidable hydrolysis of some serum lipids during the ultracentrifugal procedures,[21] and the second may be some differential displacement of fatty acids from albumin to lipoproteins under the influence of the relatively high ionic strengths used during the ultracentrifugal procedures.[35] To minimize the possible influence of the first of these factors, the serum was separated without delay and immediately adjusted to d = 1.21 and centrifuged for 24 hours at 40,000 rpm at a temperature of 17°C. To minimize the second factor, a D_2O-$NaNO_3$ solution was used for the density adjustment instead of an aqueous solution of higher salt content such as H_2O-NaBr. The D_2O-$NaNO_3$ solution, however, may have produced some differential displacement of FFA from the albumin in spite of its lower salt content. Quantitative contributions of these two factors were not evaluated in the present study. Bragdon et at[11] found no significant change in the linoleic to oleic acid ratio of the d > 1.21 fraction upon *in vitro* incubation of chylomicrons and fasting serum.

It is interesting to note that following safflower oil ingestion the fatty acid composition of the ultracentrifugal protein residue fraction is similar to that of the S_f20-10^5 glycerides. This suggests that the composition changes in the residue fraction may be due either to the binding of FFA released during *in vivo* hydrolysis of ingested oil or to the appearance of some of the ingested glyceride in this fraction following oil ingestion.

SUMMARY

This report describes preliminary investigations on the influence of safflower and olive oil ingestion on (a) the lipid composition of the major serum lipoprotein classes, (b) the fatty acid composition of the lipoprotein lipids, and (c) the fatty acid composition of the ultracentrifugal protein residue fraction. Significant glyceride increases occur in the S_f20-10^5 and the high density lipoproteins (HDL) following oil ingestion. Fatty acid composition changes occur in the glyceride moieties of the S_f20-10^5, S_f0-20, and HDL after oil ingestion. Marked alterations in the composition of the fatty acids associated with the ultracentrifugal protein

residue fraction occur following oil ingestion. The origin of the HDL lipid following oil ingestion is discussed in relation to the metabolism of the $S_f 20-10^5$ lipoproteins.

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ERYTHROPOIETIC STIMULATION IN THE HYPERTRANSFUSED GUINEA PIG FOLLOWING INJECTIONS OF HUMAN URINARY ERYTHROPOIETIN*

By

Joseph F. Garcia and John C. Schooley

Considerable evidence has accumulated in recent years that supports the concept of a humoral component as part of the mechanism for control of erythropoiesis. The humoral agent has been termed erythropoietin or erythropoietic stimulating factor and has been obtained from plasma and urine of various anemic animals and from urine of patients with various hematologic disorders. Erythropoietin does not appear to be species specific; human, rabbit and sheep erythropoietin stimulate erythropoiesis in the mouse, rat and rabbit. Reports of the effectiveness of erythropoietin obtained from various sources in stimulating erythropoiesis in the guinea pig have been variable. Gordon[1] reports that injections of urine from a child with thallassaemia major did stimulate erythropoiesis in the guinea pig, but that other urine samples that were quite erythropoietically active in rats failed to stimulate guinea pigs. Osmond[2] failed to observe erythropoietic stimulation in guinea pigs following the injection of plasma extracts from anemic ponies. Osmond et al[3,4] on the basis of quantitation of marrow cellularity found that injections of human urinary erythropoietin into normal guinea pigs not only were not erythropoietic but actually were granulopoietic. Van Dyke,[5] using the Fe^{59} assay, found an absence of response in the guinea pig to human urinary erythropoietin. Keighley[6] is reported to have found that a filtrate of anemic rabbit plasma, erythropoietically active in other species, was inactive in guinea pigs. In general, these workers conclude that failure of guinea pigs to respond to injections of extracts which are erythropoietically active in other rodents may be due to the presence in the extracts of antigens to which the guinea pig is more responsive than the other animals studied. Possibly these antigens indirectly influence the erythropoietic response by stimulating the cellular proliferation of other cell lines such as granulocytes, lymphocytes and plasma cells. This increased proliferation may compete with erythropoiesis for stem cells or essential nutrients. This competition, if it does occur, should be less in assay animals not undergoing active erythropoiesis prior to the administration of exogenous erythropoietin. Thus, in the present experiments the responsiveness of guinea pigs to human erythropoietin has been reinvestigated using hypertransfused guinea pigs.

Male guinea pigs of the Hartley strain, initially weighing about 400 gm, were used. On each of the first two days of the experiment the animals were given

* This work was supported by the United States Atomic Energy Commission.

intraperitoneally 10 ml of a 60% suspension of washed red cells obtained from donor guinea pigs. This was followed by 5 days of treatment with 2 mg daily of erythropoietin. The erythropoietin contained 16 cobalt units per mg dry weight. The daily dose was given in 0.5 ml of saline, half of the dose being given intraperitoneally and the other half subcutaneously. The control animals were injected in the same manner with saline. The erythropoietin was obtained from the urine of a patient with aplastic anemia and was extracted by ultrafiltration as described elsewhere.[7] The day following the last injection of erythropoietin the guinea pigs were injected intraperitoneally with 0.5 microcurie of Fe^{59} in 0.5 ml of saline. The animals were anesthetized with ether 24 hours later and approximately 10 ml of blood withdrawn from the aorta into a heparinized syringe. An aliquot of this blood was counted for radioactivity and based on a value to 5% of the body weight for the blood volume, the radioactivity in the total blood volume was calculated. This figure is given as a percent of the Fe^{59} injected. Microhematocrits and reticulocyte counts were done on the same sample of blood. A total of 1,000 cells were counted in smears made of blood previously incubated 20 minutes with 1% brilliant cresyl blue in saline for the reticulocyte determinations. The spleen was fixed in formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin. Smears of the bone marrow were also examined.

The 24 hour Fe^{59} incorporation and the reticulocyte response in the erythropoietin and saline-injected hypertransfused guinea pigs is shown in Table 1. We observed, as did Osmond et al.,[3] that a weight loss occurred in the guinea pigs receiving erythropoietin. The estimated blood volume is obviously low because of the hypertransfusion and weight loss in the erythropoietin injected animals, so that the estimated Fe^{59} uptake in the calculated blood volume indicates only qualitatively the nature of the erythropoietic response. These results and a comparison of the cytological changes in the marrow and the histological changes in the spleen (Fig. 1) indicate that injection of human urinary erythropoietin can specifically stimulate erythropoiesis in the hypertransfused guinea pig, i.e., the marrow or spleen devoid of erythropoietic foci shows marked erythropoietic activity after erythropoietin injections.

Table 1

RESPONSE OF HYPERTRANSFUSED GUINEA PIGS TO HUMAN URINARY ERYTHROPOIETIN

Guinea Pigs Transfused With A Total Of 11.54 ml Of Packed Red Cells During The Two Days Prior To Treatment.

	Number of animals	Weight (final)	Hematocrit	24-hr Fe^{59} red cell incorporation		Reticulocytes
		gm	%	%	p*	%
Control (saline)	10	472	60	2.1		0.01
Erythropoietin (2 mg/day for 5 days)	8	380	62	11.0	<.001	1.01

*Fisher's "t" test

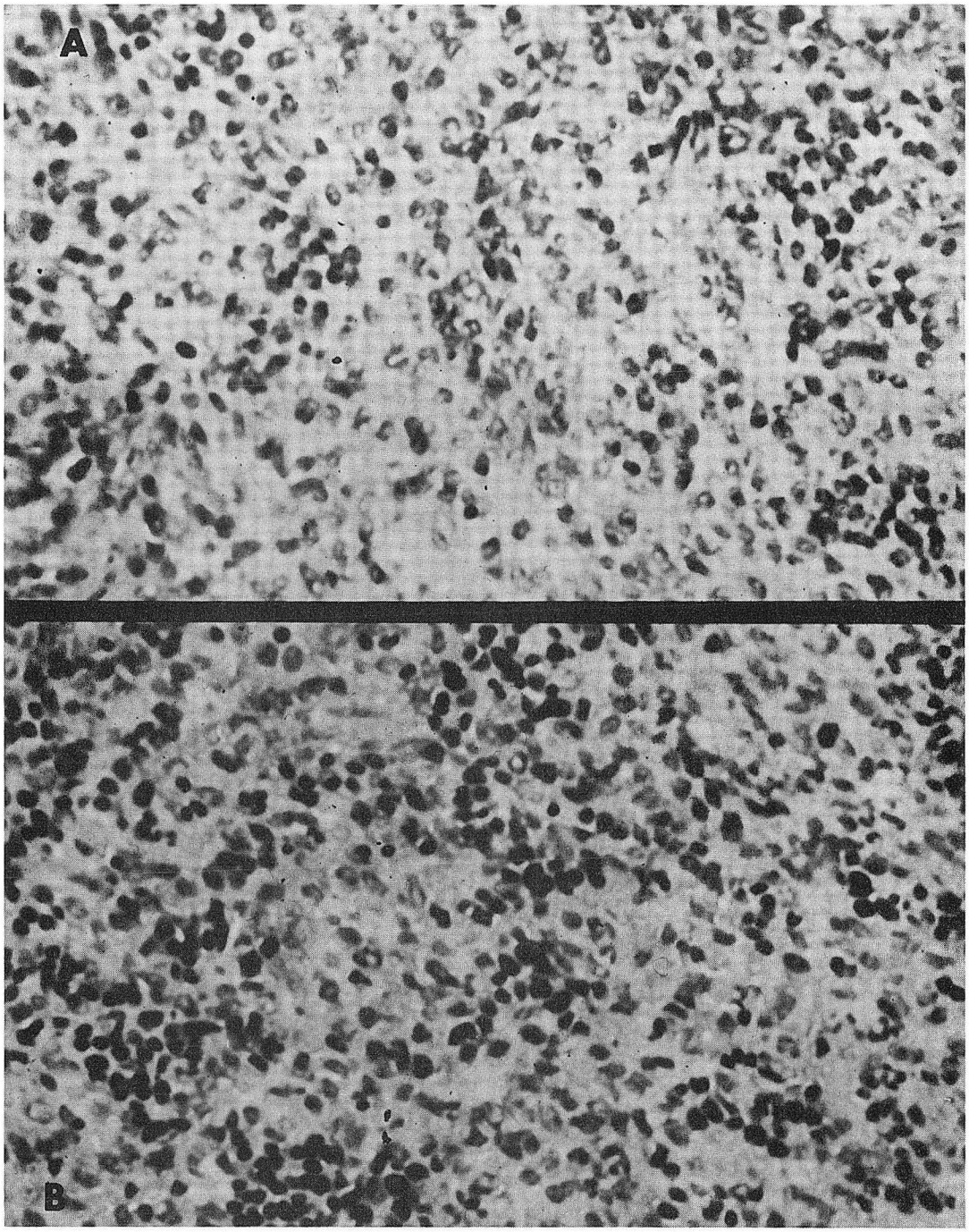

Figure 1. A. Spleen of saline injected hypertransfused guinea pig. Non-erythroid.

B. Spleen of hypertransfused guinea pig injected with human urinary erythropoietin. Active erythropoiesis.

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CELL DIFFERENTIATION AND RADIOPATHOLOGY IN THE WING OF TRIBOLIUM CONFUSUM*

By

James S. Beck

INTRODUCTION

Neither biologic development nor radiation damage to living systems is close to being understood; both are fascinating and elusive problems. The radiobiology of development is itself of great practical importance. The techniques of radiobiology--both experimental and analytic--may yet contribute to a meaningful knowledge of development. The actions of ionizing radiations upon embryogeny were studied very soon after Roentgen's disclosure of his observations of X rays in 1895, and Becquerel's discovery of natural radioactivity in 1896. In 1903, a French journal published two articles reporting observations of embryos of the toad[1] and the sea urchin[2] after exposure to radiation; in both organisms the author noted radiosensitivity variation with the state of developing tissues, successive acceleration and retardation of certain processes, and the delayed appearance of monsters. He remarked that only tissues in the process of differentiation were affected; that those already determined were not affected. In the same year in the United States, Gilman and Baetjer reported acceleration and subsequent retardation and morphologic abnormalities following exposure of both amphibian and avian eggs to X rays.[3] In 1909 and 1911, Bardeen wrote of his observations on comparative sensitivity to X rays of amphibian sperm, ovum, zygote and blastula;[4,5] he concluded that "the period of greatest susceptibility is the period during which there is the most rapid production of nuclear material".[4] W. M. Baldwin, making early observations of X ray effects,[6] used partial irradiation to produce malformations of specific structures in frog ova and zygotes,[7] and found specific malformations of late stages following X irradiation of one- and two-cell stages in the bullfrog.[8]

Since these early investigations, widely varying animal forms have been subjected to ionizing radiation in various stages of development and observed in different ways. Two papers, dealing with frog embryos irradiated very early in development, emphasize the delay of appearance of damage and suggest that this delay and the apparent variation in sensitivity were effects of damage that prevented subsequent metabolic work.[9,10] Butler studied histologically the effects in the first minutes and hours as well as later after exposure of 48-hour chick embryos and reported a series of cellular changes.[11] (This same author wrote a review in a 1936 book on biological effects of radiation.[12] A later review by

*Thesis, May 1962, Division of Medical Physics. This work was done under the auspices of the United States, Atomic Energy Commission.

Packard is of historical as well as scientific value.[13]) Other work done on the avian system, conveniently provided by the hen's egg, is presented by Strangeways and Fell[14] and by later authors.[15,16,17] The amphibians that were part of the earliest studies reappear in the later work of Rugh,[18] Allen[19] and Allen and Ewell.[20] The sea urchin, also an early starter, was not forgotten by later investigators.[21,22] The humble worm has made its contribution. Cook[23] and Vinogradov[24] used species of Ascaris. Trichinella and Rhabditis have been studied after Co^{60}- and X irradiation.[25,26] Henley and Costello employed an annelid, Chaetopterus.[27] Some very detailed and careful descriptive work has been done with fishes by Welander and co-workers,[28,29] and by others.[30] Mammals have been studied more extensively in this respect than most other groups. Such investigations on a large scale and with such thoroughness are difficult and expensive. Significant work has been done on the general problem with rats[31,32,33] and with mice.[34,35,36] Hicks has concerned himself with the development of the central nervous system after early exposure[37] and considers the general problem[38] in both rats and mice. Local exposure of the heads of young mice and subsequent observations of them are reported by Brunst and Figge.[39] Detailed observations of vibrissae in mice irradiated in utero have revealed loss or gain in number compared with those of controls, depending upon time of exposure.[40] Mouse hair follicles were observed after irradiation as a synchronized population of differentiating cells.[41]

This idea of using a regenerating part as a developing system had been exploited earlier to great advantage in amphibians and is certainly pertinent to the problem. Rather than cite the many papers[42,43,44,45,46,47,48,49,50,51,52,53,54,55] devoted to the subject of irradiation with ultraviolet and X rays of regenerating salamander limbs and developing organs, I shall merely point out the results most important to the subject of this paper. By combinations of amputation and local irradiation, histologic examinations and transplantations, experimenters have shown that the effects of irradiation are, for the most part, direct (in the anatomical sense), mitosis can be arrested without loss of ability of cells to differentiate, and radiation damage can cause redundancy in differentiation, i.e., supernumerary complex structures, as well as stunting. Other conclusions have been drawn by the several investigators active in using this system, but these points are especially apropos. Again, we must recognize the humble worm. The study of radiation effects upon regenerating tissue has also been pursued in transected planarians[56,57] and in Lumbriculus.[58]

The insects have been reserved for special mention. The characteristics of many insects and their stages of development have made them effective subjects for study of the variation of radiosensitivity with development. Probably the earliest such study reported was by Runner in 1916; he reported only lethality.[59] Then in 1923 and again in 1927, Mavor reported on the variation of lethality in Drosophila and stated that development either is interrupted by death or proceeds

normally to completion.[60,61] But in 1929, Patterson reported production of somatic mutations in Drosophila.[62] In the following year, Packard saw a relation between mitotic rate and susceptibility of Drosophila larvae to X irradiation. One year later, effects of X irradiation on larval forms of the wasp Habrobracon were reported to include the production of structural abnormalities in later stages as well as the usual lethality.[63] The morphologic effects were thought to be physiologic, not somatic mutation and mosaicism. Another paper in the same issue of the journal reported an increasing proportion of somatic abnormalities with increasing doses of X rays in the same species of Habrobracon.[64] A paper lacking in data but with interesting microscopic findings appeared two years later in 1933, reporting X irradiation of larvae of a blow-fly, Calliphora. The pupae that developed looked grossly normal but the hypodermis consisted of larval rather than imaginal cells; other structures were larval rather than imaginal, and bristles and some muscles were absent.[65] In four successive papers Evans, having used the grasshopper Melanoplus differentialis and X rays, described large-scale experiments.[66,67,68,69] He found clear variations in types and proportions of deformity with respect to time of exposure. Evans, in the fourth of these papers,[69] suggests that there are two classes of abnormality, local aberrations resulting in duplications of structures and secondary outgrowths appearing to be efforts at formation of new embryos. He speculates that X ray "destruction of certain cells probably results in 1) splitting of anlagen and, 2) removal of inhibitory control...of cells... which apparently have retained the potentiality of forming whole embryos."

In 1939, Lamy and Muller offered the opinion that embryonic damage from X irradiation was "physiologic" or cytoplasmic rather than genetic or chromosomal. They were brought to this conclusion by their failure to observe the expected differences in Drosophila of different ploidy. In 1940, Henshaw and Golomb supplemented Mavor's earlier work on Drosophila, reported effects of X rays upon emergence and O_2 uptake.[70] Though lacking a quantitative approach, two papers by Waddington in 1942 are very interesting and present worthwhile ideas.[71,72] He irradiated Drosophila at stages from larval to over 24 hr. after puparium formation. He noted lack of bristles and cessation of the morphogenesis of wings after late exposure, reduplication of wings and other structures after intermediate exposure, normal adults after earliest exposure. Waddington points out that these abnormalities are not of single gene type and that somatic mutations affect far fewer cells. He emphasizes tissue interaction and an amplifying sequence of cause-and-effect events following the initial injury. Other insect forms were studied with more emphasis upon variation in dose of X rays.[73] In 1946, Villee's paper[74] added data to Waddington's work and supported his interpretations. Publications concerning exposure of developing Drosophila[75] and Habrobracon[76] to P^{32} radiation were seen in 1949 and 1950. The physiologic vs. genetic damage issue was rejoined by Clark and Kelley in 1950,[77] and Clark and Mitchell in 1951.[78] These authors

used the appropriate system Habrobracon and found X-ray-induced developmental malformations to be more frequent in haploid than in diploid groups. They interpreted this to indicate that somatic damage is due to injury of genetic material. In a later paper, Clark and Mitchell found an exception in the greater sensitivity of diploids during the cleavage stages--syncytial in Habrobracon.[79] This they felt was due to a different mechanism of damage, damage to duplicating chromosomal apparatus in which more chromosomes meant more vulnerability. Meanwhile, Drosophila larvae exposed to X rays were reported to show sex-related difference in adult eye facet reduction that was proportional to dose given in the sensitive period.[80] Fritz-Niggli found the pupal death of X rayed Drosophila to be due mostly to a defect in differentiation and development of the body wall.[81] In 1951 a cytological investigation of X irradiated embryos of the grasshopper Chortophaga demonstrated a swelling of nuclei without loss of desoxyribonucleic acid (DNA), which the authors suggested could reflect a depolymerization of DNA.[82] In 1955 following an earlier abstract[83] Amy reported, in addition to the typical findings in Habrobracon, nuclear enlargement without arrest of mitosis.[84] Another approach revealing the same general pattern of effect was made by growing the parasitic Habrobracon on host Ephestia, made radioactive with P^{32}.[85] In this study wing abnormalities were most common. Tahmisian and his co-workers have shared Evans' earlier interest in the grasshopper Melanoplus differentialis as a developing system. They used chemical and cytological techniques and found continued cell division without the normal chromosomal configuration[86] and other nuclear changes.[87] Later, in 1961 Tahmisian and Devine applied light and electron microscopy to this system after X irradiation and described a number of such changes as supernumerary centrioles in organelles and nuclear disorganization.[88] Takeda added his observation of wing abnormalities following X irradiation of eggs,[89] as did Pohley for Ephestia.[90] Pohley gives an account of wing development and states that wing anlagen are capable of regenerating parts during larval growth and that this capability can be destroyed by X irradiation. Baldwin and Salthouse cleverly used a good system in studying X irradiation burn of a small area on Rhodnius prolixus.[91,92] They found that the burn remained latent until a certain stage of cellular activity and that this was accompanied by decreased oxidase activity, decreased number of cells, and absence of epicuticle. A later third paper by Baldwin alone describes developmental delay in this system in terms of mitotic stages.[93] Another clever approach to the problem was presented in 1960 by two Japanese investigators who X irradiated Drosophila larvae and then tissue-cultured imaginal discs.[94] They found the wing disc more sensitive than some others.

The information represented by the above review is difficult to bring to a meaningful condensation without scientific levity. This should not be surprizing for we lack knowledge of the principles by which biologic development operates,

but some generalizations can be made and are really necessary at some point. Viewing all the animal forms tested, we can say that development includes relatively sensitive processes and amplifies initial damage, that radiation damage is, in part, direct (in the spatial sense) and probably is manifest through an interaction of cells or tissues, and that radiosensitivity varies in time in a particular site and varies from site to site. I have omitted considerations such as O_2 effect, and recovery in the realm of radiobiology and much in the realms of embryology and cell differentiation.

The experiments presented in what follows were started as a search for a system in which to study effects of ionizing radiation upon development. Such a system was found in the developing wing of the confused flour beetle. This beetle had been used in radiation studies before. The first instance was reported by Davey in two similar papers in 1917.[95,96] Davey looked only for effects upon adult longevity, being primarily concerned with X ray technology and mode of biologic damage. In 1958 Park, DeBruyn and Bond published observations of effects of X irradiation upon fecundity and fertility,[97] and in 1960 Ducoff and Walburg stated in an abstract their findings regarding survival and developmental delay after X irradiation of larvae.[98] Without involving radiation at all, abnormalities, including some of wing development, were reported in 1926 by Chapman, who exposed larvae to the gas produced by adults,[99] and in 1934 by Nagel, who used temperature shocks.[100] In 1961 X rays and wing abnormality were joined in an abstract reported by me after the discovery described below.[101] Because the system consists of normal and perturbed wing development, it should be mentioned that the normal process has been studied in beetles[102,103] and a brief presentation appears on pages 195-197 in Comstock's textbook.[104]

MATERIALS AND METHODS

A. The Experimental Animal

1. Characteristics. The animals used in the experiments described in this paper are insects of the order Coleoptera, known as *Tribolium confusum*. Their common name is confused flour beetle. They are brown, measure 4 mm in length, and weigh 2 mgm as adults. They reach adulthood about 30 days after oviposition and live 1-2 years. The egg, larval, pupal and adult (imaginal) stages are shown in Fig. 1. Much information about life cycle, habits, effects of various culture conditions, and handling procedures is available.[105,106,107,108,109,110]

2. Culturing. Beetles used in the experiments described below were bred and grown in culture medium composed of whole wheat flour (without additives) sifted through a 20 mesh/inch sieve (.833 mm openings) and mixed with 3-4% nutritional yeast. One-quart glass jars were half to two-thirds filled with this medium and covered with perforated lids. Stock cultures contained perhaps 1500 adults and were kept in an unlighted incubator at 30°C and ambient humidity. By

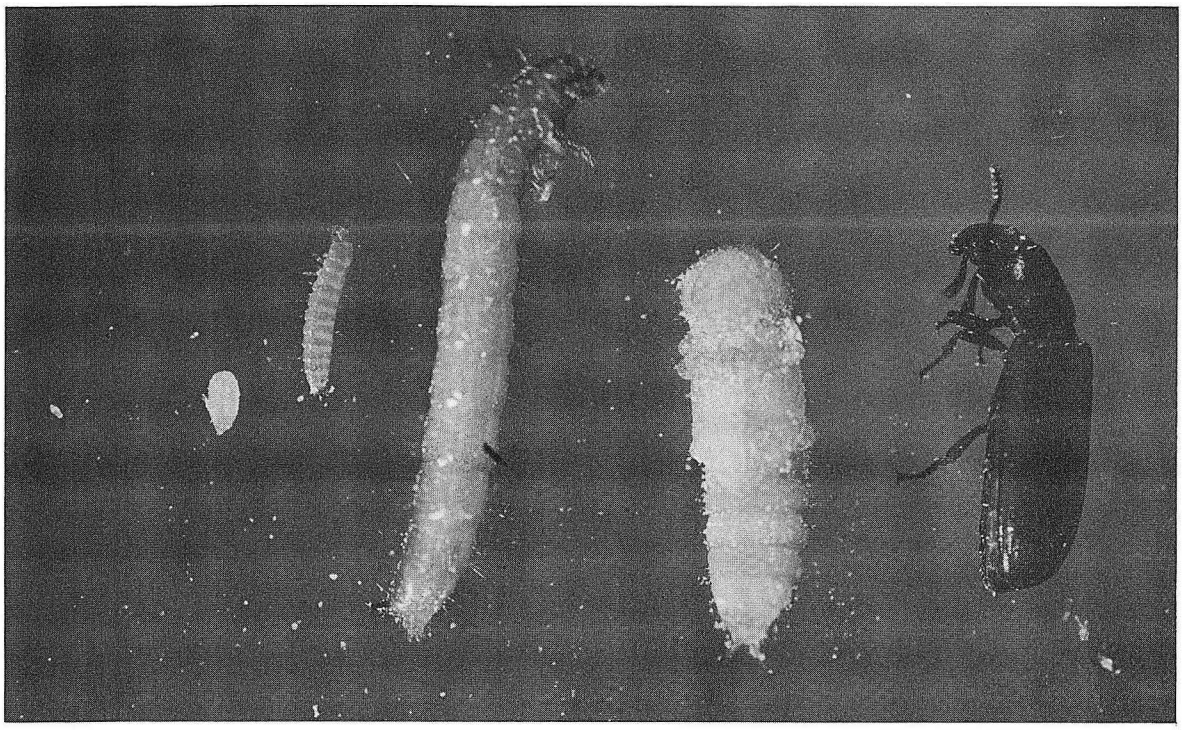

Figure 1. Stages of the life cycle. Left to right; egg, larvae, pupa, adult.

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monthly replacement, which consisted of sifting out and apportioning adults to fresh medium, cultures were maintained in good condition without use of any sterilizing procedures.

3. Handling. The stages of the life cycle were separated by sifting the cultures in steps with appropriate sieves, the final separation being by camel-hair brush into white enamel trays. Individuals were conveniently lifted by brushing onto a histologic section lifter or onto stiff paper. The mobility and photophobia of the adults may be exploited to separate them from other stages of the same size. A 20 mesh/inch sieve will retain adults and pupae; eggs and smaller larvae quickly pass through it; most of the larger larvae crawl through more slowly. To obtain pupae of a certain age, the following procedure was used:

i) Larger larvae were separated from stock cultures, put onto fresh medium and incubated for one or two days at 30°C.

ii) These larval cultures were cleared of pupae by sieve and brush and returned to the incubator; the time was noted.

iii) Then after the appropriate interval these same cultures were harvested for pupae.

Thus, when an experiment is designated as done with pupae less than 24 hr. old, it is to be understood that the pupae were harvested and the experiment done

24 hr after the cultures had been cleared of all pupae. Similarly, "pupae irradiated at 8±2 hr" means that the exposure occurred 10 hr after clearing and 6 hr after harvesting. The moult of the last larval instar marks the end of the feeding larval stage and the beginning of the 6-day inactive pupal period.

After separation and experimental procedures, pupae were kept in the 30°C incubator in glass Petri plates covered by lids of copper wire screen. Observation of experimental results was aided, when necessary, by a magnifier viewing lamp and a binocular dissecting microscope. In cases where larvae or adults were kept for periods longer than several days, standard culture medium was spread over the bottom of the Petri plates.

4. Dissection. Some of the determinations presented here depend for usefulness upon the definition of "wing" and the reproducible isolation of wings. The membranous wing is a flat surface, narrowed very sharply at its end to a "point" where it hinges to the mesothorax. (Fig. 2). As used herein, "membranous wing" means the translucent white posterior or inside wing structure from this hinge point distally to its natural boundaries. The dissection of a wing from an adult was done under a dissecting microscope with two pairs of fine forceps as follows: the beetle was grasped between head and thorax and decapitated; the elytra were removed by pulling; the forceps were wiped clean; the membranous wings were pulled free by grasping very near the hinge point and gently pulling away from the body. Carefully done, this procedure yields a complete wing which has broken away from the body at a naturally weak point. The dissection of the imaginal membranous wing from the pupa requires much more care and patience. The pupa may be brushed to remove loose flour particles, if the sample is for a chemical determination. The animal was killed by decapitation and the elytra removed under the dissecting microscope. The pupal membranous wing is closely applied to the pupal elytra but is somewhat shorter, leaving a free place to grasp for removing the elytra. Then the pupal membranous wing was removed in a way similar to that described for the adult. The structure is smaller, more delicate and less well delimited at the proximal end. After removal, the pupal wing containing the imaginal wing was put onto the surface of distilled water in a small cuvette filled so as to form a flat surface without a meniscus. Using either reflected or transmitted light, one can see the convoluted imaginal wing within the clear pupal cuticle. Usually the end of the imaginal wing is short of the closed end of the pupal cuticle. Grasping the case at this point, one can pull out the imaginal wing from the open proximal end of the pupal cuticular sac. This should be done with clean forceps in the surface of the water, not beneath it. Then the surface tension will neatly spread the rather limp imaginal wing that may be picked up from below with a metal spatula. With due attention, surface properties of wing, water and metal can be of great help. For sampling of the cell populations

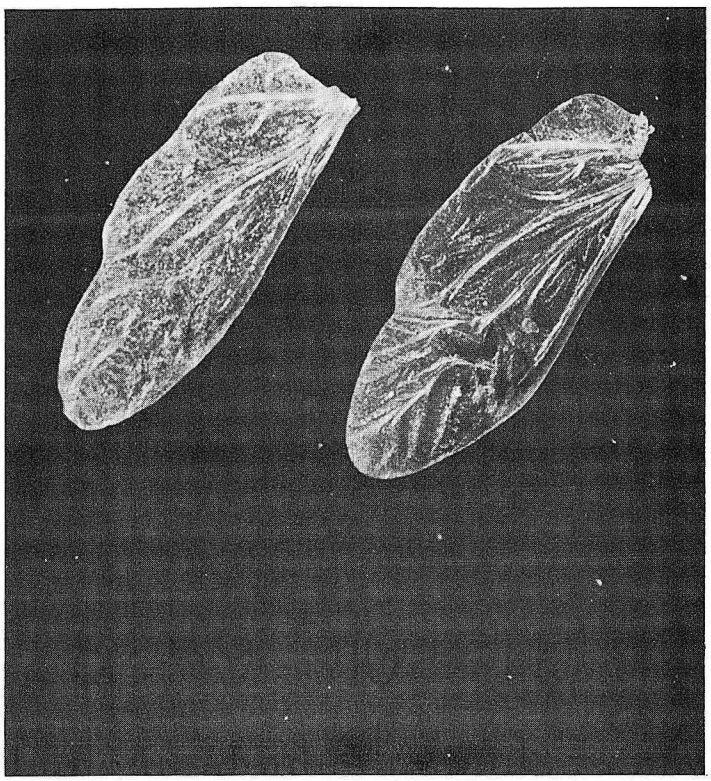

Figure 2. Membranous wings. Left, deformed; right, normal. Scale: smallest division = 0.1 mm.

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before an imaginal cuticle is formed and separated from pupal cuticle, the pupal cuticle was used as a transparent carrier. No chemical determinations were done at these stages.

B. X Irradiation

X irradiation was done with a Picker X-ray Corporation NR12 control and an OEG 60 tube, at 50 KvP, 25 ma., with an external filter of 0.1 mm. Cu, 0.4 mm. Al, at 86 r/min. The dose was measured at the position of the pupae irradiated with a 100 r Victoreen chamber on a Plexiglas shelf. For the experiment testing the effect of a higher energy X ray spectrum, a Phillips X ray unit, type 11645, with a Phillips 250 Kv, 15 ma. tube, type 23405, was operated at 150 KvP, 15 ma., with external filter of 0.25 mm. Cu and 1.00 mm. Al; dose rate was 86-92 r/min. The dose was measured by a Radocon integrating dosimeter, made by the Victoreen Instrument Company, with input from a Victoreen chamber placed beside the sample being irradiated. The dose rates above and those given in what follows do not account for backscatter from the glass of the Petri plates which held the pupae; this has been reported to alter dose by factors around 1.4, for 250 KvP X rays.[111,112,113]

C. Activation Analysis

The activation of carbon was achieved in the medical cave at the Berkeley 184-inch synchrocyclotron. The samples were exposed in polystyrene vials near the

center of a beam of α-particles. Particle energy was 900 Mev; beam intensity was approximately 1.7×10^{-7} μa.; length of exposure was 20.5 min. The activation of phosphorous occurred in an S tube of the 2 megawatt pool-type uranium reactor at the Livermore site of the University of California Lawrence Radiation Laboratory.[114] The thermal neutron flux was 5×10^{12} cm.$^{-2}$ sec.$^{-1}$ and the exposure time 8 hr. The samples were packaged in cleaned polyethylene and sealed inside an aluminum container for exposure. The P^{32} was counted with a Tracer-Lab end-window Geiger-Mueller tube as input to a Systron automatic scaler-timer. Samples were changed by a Nuclear Chicago planchet sample changer. Pulse height analyses to check the identity of the radioactive emissions from the reactor-activated wings were done with a Radiation Counter Laboratories 256-channel pulse-height analyzer at the Livermore Laboratory.

D. Electron Microscopy

Most of the electron microscopy was done with the RCA EMU-2E 50 Kv electron microscope at the Donner Laboratory. Some observations were made with an RCA EMU-3 100 Kv electron microscope in the Department of Zoology, also at the University of California, Berkeley.

E. Preparations for Optical Microscopy

1. Transverse sections. For transverse sections of wings, processing was done with wings *in situ*. Lillie's alcohol-acetate-formaldehyde and Helly's fixatives were used, the latter being more satisfactory. The heads and last 1 or 2 abdominal segments were cut off and the remainder fixed for about 2 days. These specimens were then dehydrated by ethanol and "cleared" in methyl benzoate. Then, after 10 min. in benzene, they were vacuum-embedded with 63°C melting-point paraffin. The resulting blocks were cut on an ordinary microtome and the sections mounted on glass slides. Sections of tissue fixed with Helly's fixative containing $HgCl_2$ were treated with Lugol's solution and thiosulfate before staining. Two staining procedures were used: Groat's hematoxylin[115] and Mallory's 1936 method.[116,117] In general, these histological techniques were unrewarding. The wings were often lost in the process sometime after blocking. The Mallory's stain does, however, show more structures and with differential staining of cuticle, nuclei, etc.

2. Whole preparations. The preparation of whole wings has been more satisfactory than sectioning. During the first 3 to 4 days of pupal life samples are taken by dissecting pupal wings off the pupae, making 3 or 4 cuts from the posterior edge to within a few tenths of a millimeter of the anterior border and then putting them into 10% formaldehyde. Later in pupal life, the imaginal wings are removed from the pupal cuticle, similarly cut with a scalpel and similarly fixed. After 24 hr fixation, the wings are mounted on albumin-coated glass slides, dried at 50-60°C and stained with hematoxylin (very close to Ehrlich acid hematoxylin[116]

with the substitution of potassium alum for ammonium alum, spontaneously ripened) until the nuclei show well, usually for 2 to 3 days. These preparations are made permanent under cover slips and examined with the light microscope. Additional information about techniques and materials is given in the descriptions of the experiments.

EXPERIMENTS AND INTERPRETATIONS

A. The Normal Development of the Membranous Wing

The information presented here is a result of correlation of observations of microscopic transverse sections and whole wing preparations. The membranous wing originates as an invagination of the hypodermal cell layer of the body wall. This new structure develops during larval life into a double layer of hypodermal cells that everts at the last larval moult and thereby appears externally as the pupal wing. From this time the wing remains a population of probably over 1/2 million apparently identical cells that are accessible for isolation and observation. (Hemolymph, leukocytes and tracheae are also present in the wing.) During most of the first day of pupal life these cells and the pupal cuticle are contiguous, forming a flat structure symmetric about a middle "membrane". The cells are cuboidal or columnar with round nuclei and tapering processes that appear as strands extending to the middle membrane. Near the end of the first day the cells flatten against the cuticle and by this time, exuvial fluid may appear spottily between the cell layer and the pupal cuticle. The flattening of the cells continues during the second day near the end of which the cell layer begins to undulate with respect to the flat pupal cuticle. This extension and convolution of the cell layer continues during the succeeding 3 days, and the opposite cell layers get progressively closer to one another. The imaginal cuticle is being formed on the outside surface of the cell layer during this time. Early in the fifth day the spines form suddenly. The spines are cuticular outward projections formed on both surfaces. Individually they are essentially conical with rounded tips, continuous with the imaginal cuticle and a micron or two in length. In one series of samples, a wing taken at 102±1 hr (pupal age) had no spines, whereas a wing taken at 113±1 hr had its full complement. The spines form a beautifully precise pattern such that each one is equidistant from its 6 nearest neighbors. The relation between the cells and the cuticle and spines is being studied. When cells and spines are both observable, there is a one-to-one correspondence. (The number of cells in a wing, given above, was estimated by determination of the number of spines per unit area.) By the end of the fifth day, the imaginal wing consists of a double layer of hypodermal cells with a visible cuticle highly convoluted within the pupal cuticular sac. Upon eclosion this imaginal wing spreads to an area perhaps twice that of the pupal wing, folds in a precise manner within 30-40 min. after eclosion and remains thereafter lying

unused beneath the elytra. A day or so after eclosion the cells of the wing seem sparser and in another day or two are not found at all. No debris or degenerating cells were seen at this stage. By this time the wing is a strong rigid structure of variable thickness. It has been found to be as thin as 1/2 μ, though 1 μ may be closer to the average thickness. This thinness permits examination of whole mounts with the electron microscope, a technique discussed below. This qualitative description is presented primarily as a basis for evaluation and interpretation of the quantitative experiments with this system.

B. Description and Scoring of the Deformity

The only externally apparent deformities consistently elicited in this laboratory by X irradiation of T. confusum in larval, pupal or adult life are deformities of the membranous (posterior) wings and the elytra (external or anterior wings). The deformity of the elytra appears as a slight, irregular warping, a displacement dorsally and laterally from the normal position over the membranous wings and the dorsolateral aspect of the abdomen, and as lessened pigmentation. The deformity of the membranous wings is externally manifest by warping, failure to fold after eclosion, and lateral displacement from the normal position over the dorsal aspect of the abdomen. Though the deformity appears to be specific, it is not an all-or-none phenomenon; with doses producing lower incidences there is a variation in degree of deformity. The experiments are scored by classifying adult beetles as 1) normal, 2) mildly deformed, 3) moderately deformed, 4) severely deformed, and 5) moulting failure. Each category is defined according to clearly observable features. The data below is presented as if only two classes were defined, normal and deformed, the 3 subclasses of wing deformity being consolidated. Pupae that failed to eclose are recorded as pupal deaths or moulting failures and excluded from the analyses, although they are included in "number of pupae" in the tables. The data seem to justify such a convention that is motivated by a desire to make full use of the specificity of the deformity and the advantages afforded by its temporal isolation from other developmental radiosensitivities. Figure 3 demonstrates the visible characteristics of the radiation-induced aberration in wing development. The tables and graphs show that the pupal stage is about 1 day longer for the irradiated pupae. I have studied this effect and found it to be reproducible and proportional to dosage. However, it is not the primary objective of these investigations and will receive only brief mention where indicated.

C. Dose-incidence Relation

The first series of experiments determines the incidence of the deformity as a function of dose. The pupae irradiated were within the first day of the pupal stage and were irradiated with the beam incident from the ventral aspect. Subsequent experiments varying the direction of the beam relative to the pupae

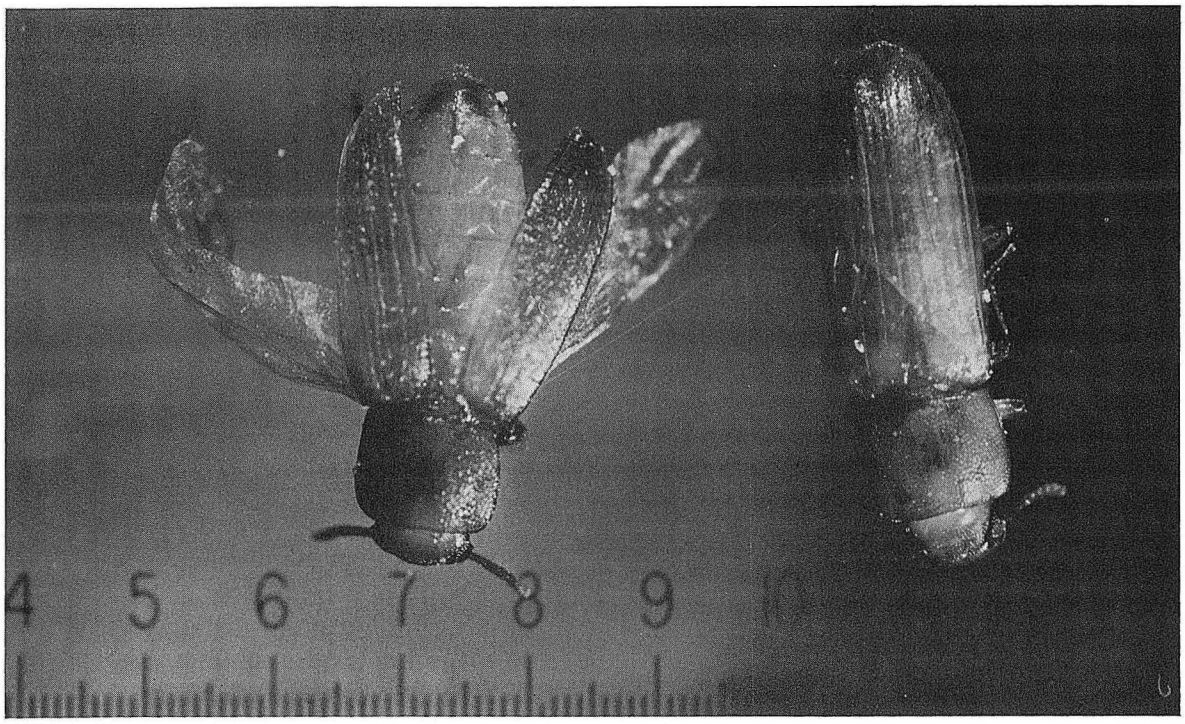

Figure 3. Adults. Left, severe wing deformity; right, normal. Scale: Smallest division = 0.1 mm.

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showed no differences. Therefore, later experiments were done without specific orientation. Results of one series of exposures are shown in Table 1 for 50 KvP X rays. Table 2 presents data for 150 KvP X rays, obtained in a single experiment. The dose is given in r for comparison, but caution in interpretation is in order, because 1) the dosimetry may be inaccurate, 2) dosimetry was done with different instruments for the different energy spectra, and 3) the backscatter from the glass Petri plates may differ for the different energy spectra. However, if an increase in dose due to backscatter is due primarily to photoelectrons, these are probably absorbed superficially in the pupae and may not appreciably widen the differences in the effective doses. Figure 4 presents the results graphically as a survival curve. The ordinate is the fraction having normal wings, plotted logarithmically. Points plotted are computed on pooled data where more than one experiment was done for a given dose. Standard deviations in the tables are computed from the equation:

$$\sigma = \sqrt{\frac{f(1-f)}{n}}$$

where f is the fraction deformed and n is the sample number (number normal + number deformed). The uncertainty of the last point for 50 KvP does not permit

Table 1

FRACTION AFFECTED BY GIVEN DOSES
OF 50 KVP X RAYS

Dose (r)	Number of pupae	Number deformed, D	Number normal, N	$\frac{D}{N+D}$	σ
0	451	10	428	.024	.007
560	300	13	283	.044	.004
1120	303	32	267	.107	.018
1400	294	131	156	.701	.027
1680	297	265	20	.930	.015
1960	299	285	3	.990	.006
2240	299	266	2	.993	.005

Table 2

FRACTION AFFECTED BY GIVEN DOSES
OF 150 KVP X RAYS

Dose (r)	Number of pupae	Number deformed, D	Number normal, N	$\frac{D}{N+D}$	σ
0	371	6	355	.017	.007
500	300	13	275	.045	.012
1000	295	26	259	.091	.017
1250	298	85	206	.292	.027
1500	299	216	74	.745	.026
2000	299	283	3	.990	.006

the conclusion that the curve is tailing off. Mr. Nabil Amer has done more extensive dose-incidence determinations with X rays at this laboratory. His sample variances are smaller and his work (unpublished as yet) confirms the relation presented here.

The wide shoulder of the semilogarithmic survival curve and the steep slope beyond suggest that the effect depends upon disturbing many functional units of high sensitivity. Beyond this, conclusions are probably not justified for a multicellular system as incompletely defined as this.

D. Determination of the Critical Period

The experiments presented in this section establish the critical period of pupal life for this radiosensitivity. Problems in handling make precise grouping of larvae according to age difficult. A single crude experiment was done with larvae: a group of larvae over 6 mm in length (probably of the last one or two instars) and a group of larvae 1.3-3.0 mm in length were irradiated in the usual way with 50 KvP X rays for 26 min. These two groups were incubated at 30°C with flour in Petri plates. Pupae were removed and allowed to eclose. Of

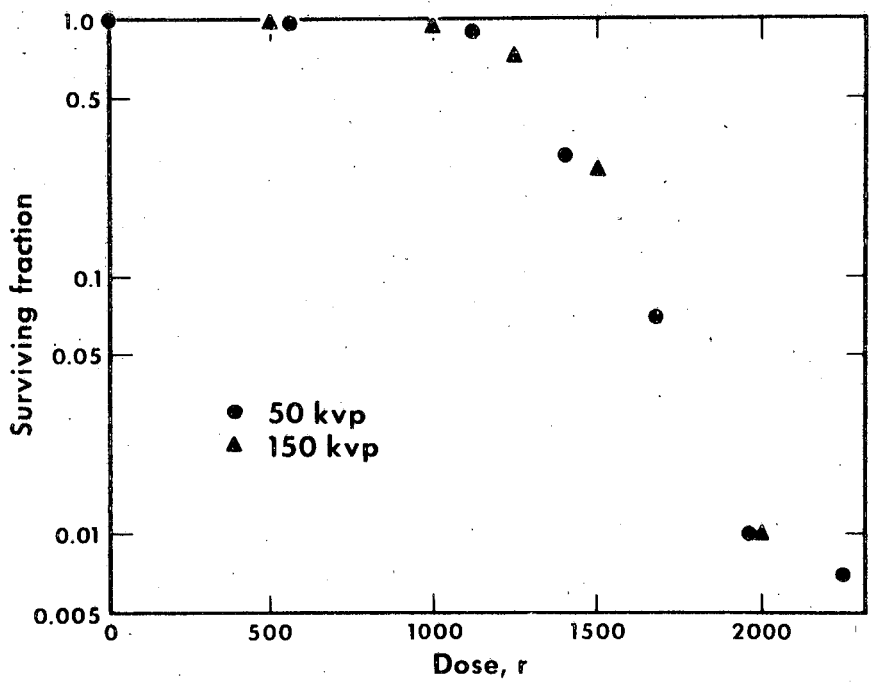

Figure 4. Fraction affected by given doses.

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the adults developed from the group of initially larger larvae, there were 31 deformed and 85 normal, i.e. 27% deformed. Of those developed from the group of initially smaller larvae, there were 13 deformed (11 of them only mildly deformed), 62 normal, and 5 excluded, i.e. 17% deformed. Abnormalities were not apparent in structures other than wings.

The results of irradiation of pupae of various ages are presented in Tables 3 and 4. Table 3 constitutes the result of irradiation with 2240 r, a dose sufficient to cause very nearly 100% incidence of the wing deformity, yet not over the right-hand shoulder of the dose-incidence curve. Table 4 is constructed from experiments done with 1540 r, a dose that lies within the steep rise range of the dose-incidence curve. Figure 5 is a plot of these results. For both doses the incidence falls rather sharply around 35 hr after the beginning of pupal life. In view of the near equality--5 and 6 hr--of the age ranges of all the groups and the active differentiation of the cells of the membranous wings at this time, these experiments suggest that the cells are probably well synchronized and the end of the critical period is quite sharp.

E. Chemical Determinations

1. Mass. Determination of the masses of the normal and deformed wing is a first step in characterizing the nature of the deformity. However, the small size and high surface-to-volume ratio have made weighing problematical. Groups

Table 3

FRACTION AFFECTED BY 2240 R
AT GIVEN PUPAL AGES

Age range (hr)	Number of pupae	Number deformed, D	Number normal, N	$\frac{D}{N+D}$
0-6	30	27	0	1.00
17-1/2-23-1/2	29	29	0	1.00
18-23	74	73	0	1.00
23-28	74	70	2	.97
28-33	76	72	0	1.00
33-38	75	55	19	.74
38-43	78	23	54	.30
42-48	29	4	24	.14
66-72	27	2	21	.09

Table 4

FRACTION AFFECTED BY 1540 R
AT GIVEN PUPAL AGES

Age range (hr)	Number of pupae	Number deformed, D	Number normal, N	$\frac{D}{N+D}$
0-5	230	73	154	.32
31-36	84	24	58	.29
32-37	199	53	144	.27
38-43	200	16	177	.08
42-47	113	9	94	.09
48-53	121	5	110	.04
66-71	120	3	109	.03

of 13 each of normal and deformed wings were weighed on an analytical balance which reads to hundredths of a milligram. The control group had a mass of 0.24 mgm.; the experimental group, 0.20 mgm. This would indicate 18 μgm. and 15 μgm. individual wing masses, respectively. But the weighing is probably not good enough to justify the assumption of a difference in mass. These wings were weighed after neutron activation and before counting for phosphorous content. The results of the phosphorous determination (discussed below) showed 1.7 times as much phosphorous in deformed wings as in control wings. It can be concluded that the difference in phosphorous content is not due to a proportional increase in all components of the deformed wing.

2. Carbon content. As another step toward analyzing the difference between normal and deformed membranous wings the total C content was determined. The technique used was activation analysis by the reaction $C^{12}(\alpha,\alpha n)C^{11}$. C^{11} produced by this reaction has been studied in humans and in a rat.[118] C^{11} emits

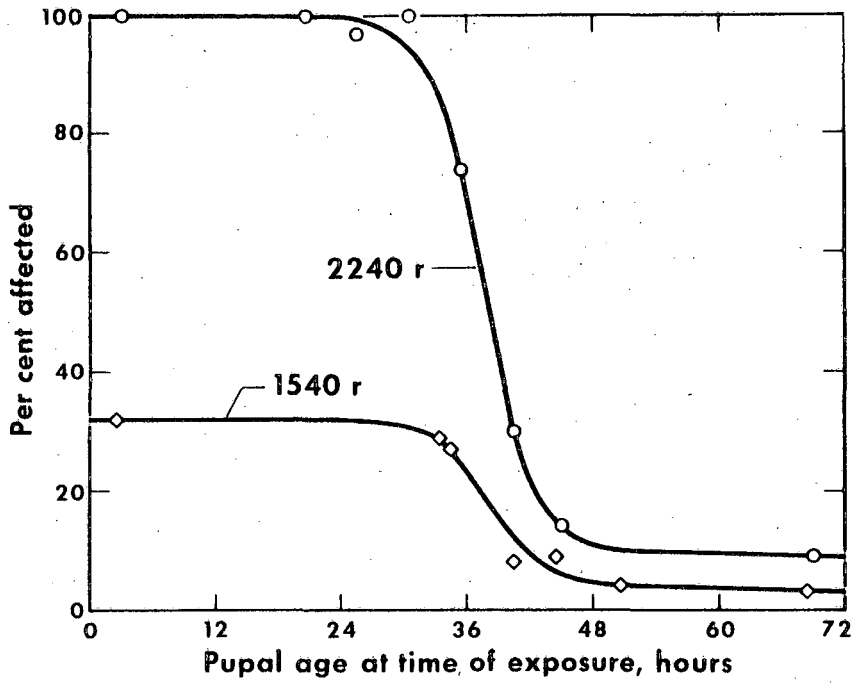

Figure 5. Fraction affected at given pupal ages.

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a 0.98 Mev positron with half-life of 20.5 min.[119] The α-source and counting system are described in Materials and Methods. The derivation of the activation equation and the basis for comparative measurement by counting radioactivities is given in the Appendix of this paper. The sensitivity of the measurement with the counting facilities available is such that individual wings could not be analyzed. The wings were activated and counted in a control group of 17 wings and an experimental group of 22 deformed wings. The wings were dissected from adults in the standard manner within 24 hr after eclosion and stored until activation. The adults developed from pupae less than 24 hr old at the time of harvest. The experimental group was exposed to 2240 r at less than 24 hr pupal age. Results of the counting appear in Table 5. The times given are the intervals in minutes between the beginning of the first count (about 10 min. after the end of the activating exposure) to the middle of the counting interval. The standard deviations are computed by the following relation:

$$\sigma = \sqrt{\sigma_i^2 + \sigma_b^2}$$

where

$$\sigma = \sqrt{\frac{R_i}{t_i}} ,$$

Table 5

RADIOACTIVE DECAY COUNTS FOR C CONTENT

Control group (17 wings)		Experimental group (22 wings)	
Time (min.)	Count*	Time (min.)	Count*
1.0	130±10	4.3	169±11
8.5	89±6	14.2	116±6
19.7	51±5	25.2	79±5
33.3	35±4	45.0	41±4
53.3	21±3	64.3	18±3

*Counts represent counts per min. above background (56±2 CPM) $\pm\sigma$.

and R_i is the count for the ith sample, t_i is the counting interval in minutes, and σ_b^2 is the variance of the background count, similarly computed. This is based upon the variance of counting and is in no way a reflection of the variance in the populations of wings. Figure 6 is a semilogarithmic plot of these counts and times. Lines drawn for 20.5 min. half-life were fitted to the points. The zero-time intercepts are 185 for the deformed group, 113 for the control group; correcting the controls for the numbers of wings: 22/17 x 113 = 146; and the relative C content of experimental wings compared to control wings is 185/146 = 1.3.

Before any conclusion is drawn, it should be noted that 1) the fitting of a proper C^{11} decay line to the observed points is a procedure of doubtful accuracy, and 2) the lack of comparisons among individual wings prevents any conclusion as to the reliability of the sampling.

3. Phosphorous content. The search for quantitative differences between normal wings and those deformed by irradiation during development produced the first striking find with the measurement of the total P. In addition to the significance for the phenomenon being studied, it has added to the technological knowledge of microanalysis of biological systems. Activation analysis has been applied to P detection before: in a general analysis of ashed samples,[120] on chromatograms,[121,122] in a survey of elements in normal and leukemic blood,[123] and in measuring elements in teeth.[124] But the following represents the only measurement of P in untreated tissue in so small an amount.

Three successful experiments were done. The first was an analysis of normal wings from adults developed from control pupae and deformed wings from adults developed from pupae irradiated with 2240 r 50 KvP X rays within the first 24 hr of pupal life. Both membranous wings and elytra were analyzed in this experiment. Over a period of several hours, more than 24 hr after eclosion, a number of wings were dissected from the adults and stored in marked vials. Several days later these

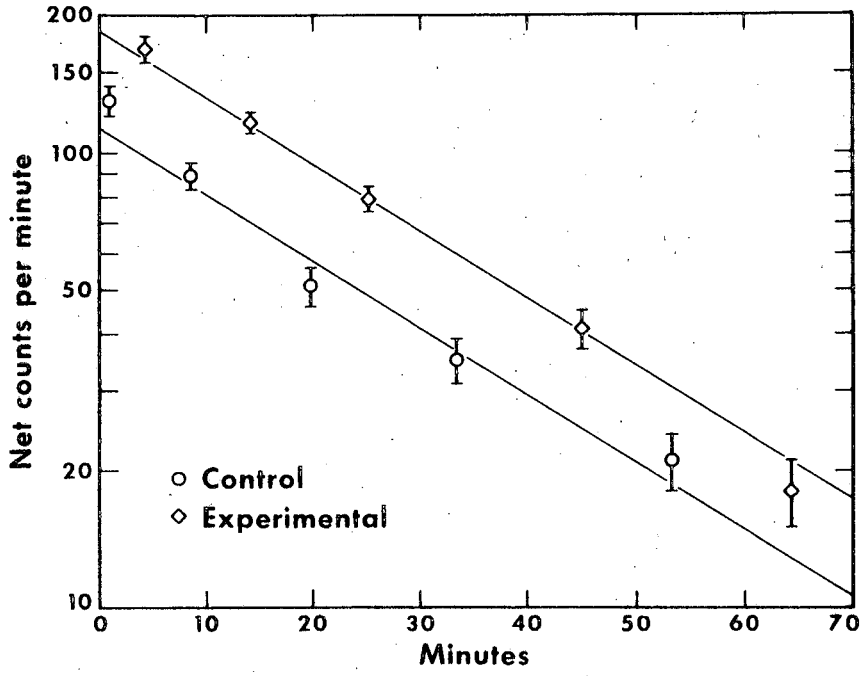

Figure 6. Radioactive decay counts for C content.

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wings were taken to the Livermore Laboratory reactor building. Ten of each of the normal membranous wings and elytra were put into a single polyethylene vial and ten each of the deformed membranous wings and elytra were put into another vial. A single lump of KH_2PO_4 weighing 6.20 mgm. (by an analytical balance reading to hundredths of a milligram) was put into another polyethylene vial. These containers were then exposed on the following day for 8 hr as described in Materials and Methods. The critical reaction is P^{31} $(n, \gamma)P^{32}$. Interfering reactions were no problem. (The proof of this lies in the data that show a long period of decay with the 14.3 day half-life of P^{32}. In addition, pulse-height analysis showed no detectable amounts of products of possible interfering reactions.) The pulse-voltage plateau for the Geiger-Mueller tube was found and the voltage set appropriately. The sensitivity of the counting system--the amplifier and scaler--was for 0.25 v. tube output. The time delays for sample changes and recycling of samples were determined so that the times of counts were known for the given counting periods. The target material had been retracted to a shielded position in the reactor and left until preparation for counting. It was retrieved and the wings placed individually on sticky tape with adhesive on both sides, on plastic counting dishes. These numbered disks were put into metal planchets and stacked in the sample changer. Handling of the dried wings with fine forceps was, on the whole, successful; two of the ten control membranous wings crumbled and were

discarded. Two of the experimental elytra were combined on one holder. The KH_2PO_4 sample was quantitatively transferred to a 10 ml volumetric flask, diluted to volume and shaken on a mechanical shaker. 2 λ ($\lambda = 10^{-6}$l.) of this solution was prepared in duplicate for counting by evaporating on one of the plastic counting dishes. Empty plastic holders were included for background counts and a Sr^{90} standard was included as a check on the performance of the tube and counting equipment. The KH_2PO_4 standards consisted of 2.82×10^{-4} mgm. P. The counting interval was 10 min. for the earlier counts and 30 min. for the later; the first count began a few hours less than 1 day after the end of the exposure to neutrons; the last series of counts was done 28 days later. The counts were printed out on tape. The time of the midpoint of each counting interval and the counts per minute over background were determined. Rather than extrapolating by graphic means, comparisons were made by correcting the counts for a zero-time arbitrarily chosen as the beginning of the first count. Because

$$A = A_o e^{-\lambda t},$$

then

$$A_o = A/e^{-\lambda t},$$

where A is the activity at time t and A_o is the activity at t = 0. A table of values of $e^{-\lambda t}$ for P^{32} for 3-hour intervals is available.[125] These values were found by linear interpolation, and the results of the divisions were found. Table 6 is a summary of the data accumulated from 15 series of counts (the first 2 days' series are not included). Each of these activities represents a single wing or standard. The counts entered in this table are corrected for time and background. Background was quite consistent; typically it was determined to be 28±1 counts per minute. The computed zero-time activities are entered into the table from left to right in the order of the counts from which they were computed. The high early values reflect the rapidly decaying non-P nuclides. Averages for each of the 4 classes of wings are given in Table 7, top to bottom in the order of the observations. The standard deviations given for the groups are computed from the counts in Table 6 by $\sigma = \sqrt{\frac{1}{n} \Sigma (x-\bar{x})^2}$. The accuracy of the counting and the precision of the sampling of the wings are shown graphically by a plot of 10 observed counts for membranous wings and standards in Figure 7. The ratio of P content of deformed to that of normal is obtained from any of the averages from the period where effectively only P^{32} is being counted (region of stable zero-time activity). The standard error of this ratio, Q, may be computed by

$$\frac{\sigma_Q}{Q} = \sqrt{\left(\frac{\sigma_1}{s_1}\right)^2 + \left(\frac{\sigma_2}{s_2}\right)^2}$$

The result is 2.4±0.8 for membranous wings. Elytra were not different. Comparison

Table 6

SUCCESSIVE TIME-CORRECTED COUNTS

Sample Nos. 1-10, Control Elytra; 11-18, Control Membranous; 19-26, Experimental Elytra; 27, Two Experimental Elytra; 28-37, Experimental Membranous; 38-39, Standards.

1.	1969	749	684	693	662	695	---	699	694	688	669	741	720	729	736
2.	2252	761	728	698	697	671	730	705	723	726	716	725	730	721	769
3.	1946	734	696	694	693	663	735	709	719	696	672	696	733	700	742
4.	2009	714	694	681	694	658	694	708	667	702	680	718	726	705	743
5.	1963	737	670	669	690	686	658	674	706	702	710	709	723	711	755
6.	1918	710	643	664	662	628	664	663	645	660	644	673	670	648	694
7.	1935	755	707	694	674	686	728	717	680	717	712	737	736	737	756
8.	2198	854	780	640	794	763	789	802	793	782	783	814	807	815	844
9.	1762	714	699	692	685	674	687	689	708	720	705	722	726	721	758
10.	1650	640	608	591	618	606	621	619	618	641	623	633	637	633	662
11.	283	128	136	137	131	138	143	141	126	135	147	149	148	141	153
12.	239	117	109	111	119	111	125	120	105	115	108	122	128	122	---
13.	231	95	105	105	90	88	97	101	100	105	92	102	114	107	118
14.	154	76	77	72	72	74	79	75	65	83	71	80	88	68	86
15.	178	93	91	84	93	95	88	96	98	80	92	97	99	95	102
16.	222	108	104	98	100	103	105	109	107	95	126	114	108	105	122
17.	231	122	112	121	127	114	124	118	140	126	113	130	138	123	134
18.	276	119	118	119	116	114	120	122	124	126	124	119	135	123	130
19.	1499	643	640	614	632	617	634	649	646	650	631	629	654	641	655
20.	1332	669	664	651	659	658	675	664	673	660	644	671	694	667	692
21.	1364	687	684	678	675	670	690	673	680	693	655	711	612	630	704
22.	1464	726	732	729	719	706	752	752	717	767	718	778	760	779	777
23.	1682	752	716	723	718	717	738	733	708	722	722	744	740	752	757
24.	1590	703	680	672	669	670	673	686	678	666	675	694	712	707	707
25.	1472	744	739	736	750	717	734	735	728	752	762	739	768	756	794
26.	1464	695	665	668	659	687	691	684	677	682	709	711	707	705	720
27.	3027	1522	1459	1452	1453	1427	1488	1442	1454	1484	1463	1489	1512	1497	1568
28.	656	353	338	339	334	322	325	340	326	343	351	345	365	345	359
29.	503	272	287	277	263	275	275	280	276	280	267	289	282	289	279
30.	770	375	388	369	366	371	369	368	355	381	362	379	386	396	264
31.	408	265	255	244	264	238	259	251	243	247	243	256	261	256	272
32.	343	216	201	199	199	190	209	206	204	199	195	203	211	203	192
33.	501	336	326	331	323	331	324	334	320	328	333	337	330	344	328
34.	382	228	221	225	222	228	228	227	215	227	238	234	233	238	241
35.	400	253	249	243	248	240	252	240	239	255	251	268	256	279	265
36.	382	222	215	226	234	222	214	231	226	205	206	220	233	226	221
37.	245	152	143	137	150	147	142	151	143	131	138	156	143	141	153
38.	1131	900	872	894	883	889	889	872	869	874	860	868	886	887	870
39.	1145	883	923	883	878	898	890	906	884	869	876	888	904	887	890

Table 7

SUCCESSIVE AVERAGE TIME-CORRECTED COUNTS
FOR P CONTENT BY GROUPS

Control elytra (10)	Experimental elytra (10)	Control membranous wings (8)	Experimental membranous wings (10)	KH_2PO_4 standards (2)
1960	1489	227	459	1138
737	714	107	267	892
691	698	107	262	897
672	692	106	259	889
687	693	106	260	881
673	687	105	256	893
701	708	110	260	889
699	702	110	263	889
695	696	108	255	877
703	704	108	260	871
691	698	109	258	868
717	717	114	269	878
721	726	120	270	895
712	713	111	272	887
746	737	121	257	880

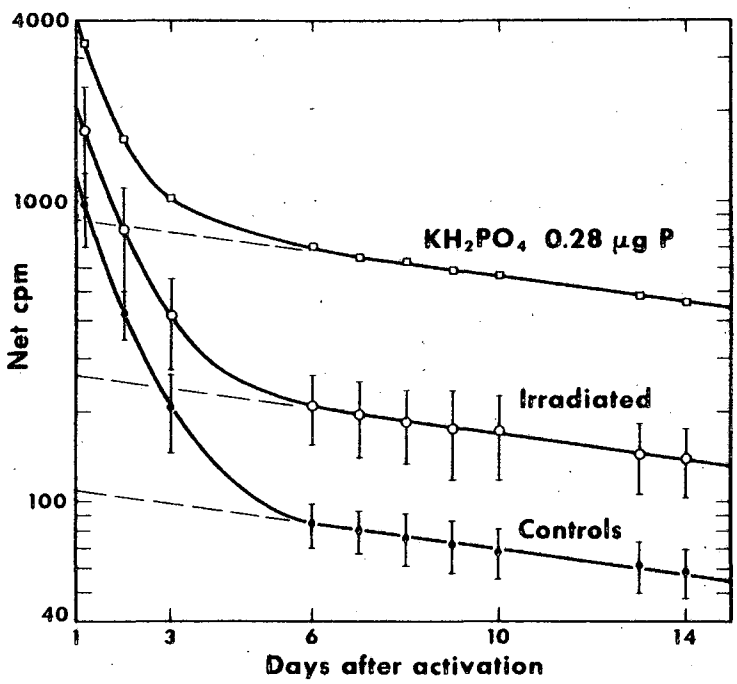

Figure 7. First 10 counts of neutron-activated membranous wings and standard.

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with the standard gives 0.03 μgm. P in control, 0.08 μgm. in deformed membranous wings.

The second determination of P content was done with the same facilities. The technique differed in that sampling was done again one day after eclosion but with a considerable variation in timing. Otherwise the only differences were that exposure in the reactor was for 16 hr, and the counting was begun 7 days after the end of exposure. Only membranous wings were analyzed. There were 13 control wings, counted individually, and 13 experimental wings, counted individually. Four complete series of counts were taken with 50-min. counting periods. The data were handled as in the previous experiment. The ratios of activity in deformed wings to that in normal for the 4 series were 1.7±0.9, 1.8±1.3, 1.7±1.2, 1.7±1.2. The large standard errors will be explained in the interpretation of the next experiment.

The third experiment to explore P content was designed to follow the P content of the membranous wings through pupal development. This is made possible by the technique, outlined in Materials and Methods, for obtaining imaginal wings from pupae. It should be pointed out that such a dissection in early stages of development of the pupae, and in early stages of development of the technique, is not an ideal way of sampling for a measurement as sensitive as this P determination. The pupae harvested at the beginning of the experiment were within 0-9 hr of the beginning of pupal life. The experimental group received 2240 r 50 KvP X rays 1 hr after harvesting. The earliest samples were lost either by disruption at dissection or at transfer after activation. Sampling and handling was successful for wings from pupae 4-1/2 days or older. Samples were taken from adults after eclosion, so that the span of development observed is from the fifth day of pupal life to the fifth day of adult life. Of course, the wings can be seen to be deformed only after eclosion and perhaps after dissection from very late pupae. The dose used permits the assumption that the wings from irradiated pupae were indeed destined to be visibly deformed. The results support this assumption. The activation exposure was for 8 hr. The wings were packaged individually in custom-made polyethylene envelopes unsealed at one edge. These were stacked by wrapping in an Al strip and fitted snugly into the Al cannister for good thermal contact (prior experience had shown heat-induced changes in the polyethylene). The counting was done as before on individual wings; the counting period was 60 min. and counting was continuous until twice through the series, having begun 7 days after the activation ended. Included in this activation and counting were 14 each of normal and deformed wings from the carbon activation experiment. The corrected counts from the latter groups average to 4219±760 for controls and 5592±1480 for experimentals. The ratio, then, is 1.3±0.4 for P in deformed to P in normal. The results for temporal variation in P content for control and experimental are presented in Table 8. Comparisons can be made between control and experimental

Table 8

TIME-CORRECTED P^{32} RADIOACTIVITIES (ARBITRARY UNITS) OF CONTROL AND EXPERIMENTAL MEMBRANOUS WINGS AT GIVEN TIMES OF PUPAL LIFE

	Control		Experimental	
Age (days)	Count 1	Count 2	Count 1	Count 2
4-2/3	59±30	60±31	-----	-----
5-1/4	81±6	80±9	53±10	54±12
5-1/2	73±17	75±17	59±12	59±13
6	71±3	72±3	68±4	69±5
6-5/8	38±3	39±3	53±7	54±8
7-5/8	19±4	18±4	42±5	43±5
8-5/8	11±2	12±2	36±10	36±10
9-7/8	9±2	9±3	32±8	32±9

samples at a given developmental time as well as among developmental times, because the activation and counting were done at the same time with the same apparatus. The Sr^{90} standard uncovered some instability--in the Geiger-Mueller tube probably--but no consistent drift. Figure 8 displays the results graphically. The magnitude of the control P content is nearly matched by that of the experimental, though later in development, corresponding to the delay in eclosion. The deformed wings, however, do not subsequently lose P to the extent that the normal wings do. This temporal variation explains much of what has been presented here. It can be seen, for example, that an experimenter can find any ratio, (P in deformed)/(P in normal), from 0.6 to 3.6 by picking the appropriate sampling time. In addition, variability in sampling time with respect to age makes for large variance in a sample of wings. This was not realized at the time of the earlier experiments and this mistake is reflected in the results. Observations of the cell populations, presented below suggest a plausible explanation of these chemical observations.

F. Studies of the Cell Population and Morphogenesis

The investigation of the character of the hypodermal cells, their relation to one another and their relation to the cuticle they construct is currently being studied during normal development and following irradiation. Two methods are used: light microscopy and electron microscopy. A general outline of what is known about the normal development is given above in part on Experiments, The normal development of the membranous wing. The wing exposed to X rays in the first hour of pupal life differs morphologically from normal as soon as 16 hr after irradiation. These differences were observed by light microscopic examination of both sectioned and whole-mounted stained wings. The whole mounts were from a series irradiated with 2240 r 50 KvP X rays at 1±1 hr pupal age. The first observed evidence of radiation damage is a sharply defined, round, dark-staining

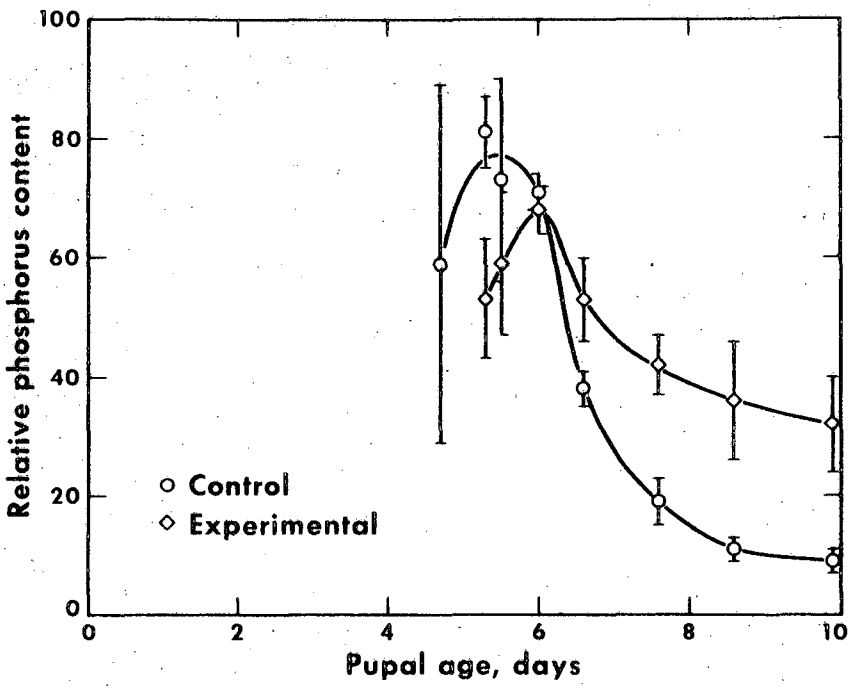

Figure 8. P^{32} radioactivities of wings at given times of pupal life.

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homogeneous body from 1/5 to 1/2 the size of a nucleus. These are first apparent in numbers great enough for certainty at 17 hr (16 hr after irradiation). These bodies appear in the same focal plane as the nuclei (Fig. 9). Because the cytoplasm is not stained adequately and the cell boundaries are not defined in whole mounts, the location and origin of these bodies is yet unsolved. Numerical data have not yet been obtained, but at 17 hr in one series the number of these bodies was approximately 1% of the number of nuclei, evenly distributed over the wing. At 24 hr these bodies number 25-50% of the number of nuclei. At 41 hr they are still present and can be seen also in the sectioned wings. These bodies exist in control preparations, but they are rare, many fewer than 1% of the number of nuclei. At 61 hr the cell layers are converging but the convolution within the pupal cuticle has not begun. At 71 hr fewer of the radiation-damage bodies can be seen. At 85 hr and beyond, exuvial fluid appears between the cell layer and the pupal cuticle. By 102 hr the pathological bodies number 1% of the number of nuclei or fewer; at 113 hr they are gone. At this time and at 127 hr there are no spines. At 136 hr imaginal cuticle can be seen in cross-sections, but the convolution of the imaginal wing is still not evident. Spines appear in the 145 hr preparations. At 164 hr the imaginal wing is convoluted. After the spines are formed their pattern is imperfect. There are occasional "holes" in the array and occasionally 2 or more spines where one is expected. The process of development is both delayed

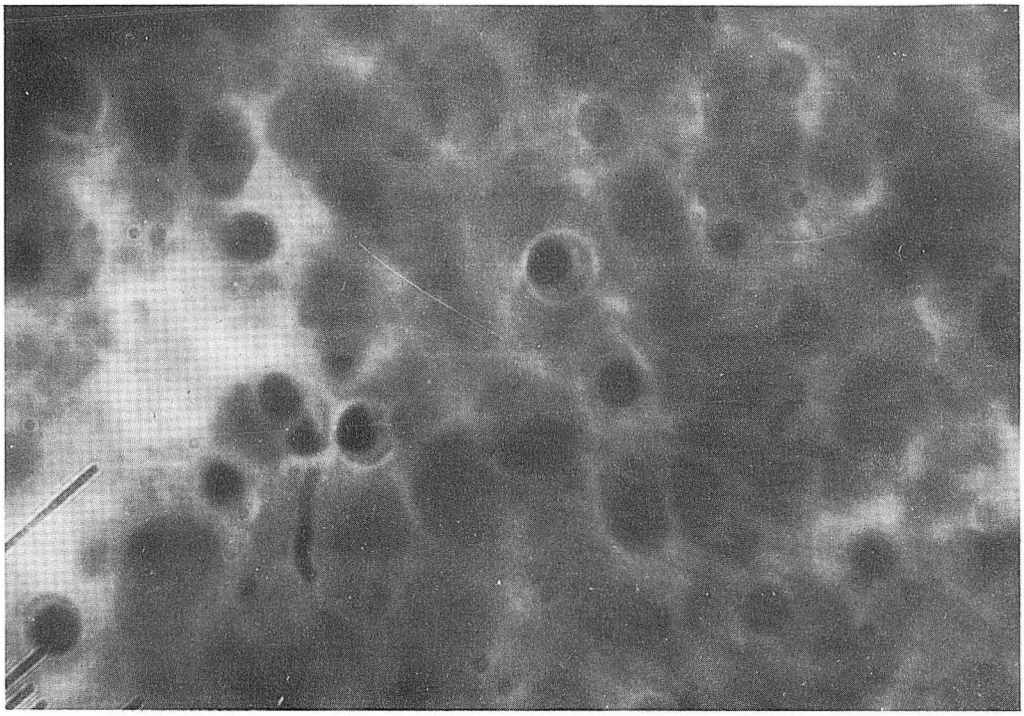

Figure 9. Membranous wing 24 hr after X irradiation. Hematoxylin stain. Magnification: 5700X.

and deranged. The disappearance of the cells after eclosion is slower than in controls and is incomplete as long as 3 days after eclosion. The wings have not been carefully observed beyond this time.

As pointed out above, the thinness of the membranous wing has made it possible to observe whole mounts by electron microscopy. This permits a rapid survey of ultramicroscopic detail over the entire wing. Because the two-dimensional expanse of the wing includes thousands of fields, it was necessary to devise a method of sampling certain regions reproducibly. Such a method was achieved in collaboration with Dr. Thomas Hayes of this laboratory. After dissection the wing was lifted from the surface of distilled water by bringing a formvar-coated 200 mesh/inch copper grid (Ernest F. Fullam, Inc.) for the electron microscope up under the wing so as to position it on the formvar surface, where it sticks. This preparation was then inverted over a 1% solution of OsO_4 for 30 min. for fixation and "staining." After drying, this was placed in position on the electron microscope specimen holder under the dissecting microscope with the wing up. Then an uncoated second grid was carefully superimposed on the grid supporting the wing so that the grid openings matched. This was done under a dissecting microscope (60X magnification) with fine forceps and drawn glass needles. The specimen-holder cap was put on, usually before the final grid adjustments, and tightened, sandwiching the wing

between the 2 grids. In this condition the wing remains in position, and heat is conducted away adequately while it is in the beam of electrons. After this preparation was made the wing was mapped. Mapping consisted of sketching outline and landmarks of the wing on two representations of the grid sandwich which held the wing. The first map represents the image of the wing in the light microscope; the second was a transformed likeness that characterized the image formed on the screen of the electron microscope. By reference to the maps the chosen areas could be viewed and the images photographed. Three of the 5 frames (exposures) of a photographic plate were taken in a selected area near the notch on the posterior edge of the wing and 2 in an area at the center of the wing determined by a geometric convention which compensated for any differences in dimensions among wings. Neither of these regions contains veins or tracheae. This technique permitted accurate localization and even made possible repeated re-examination of a given spine after indefinite storage of the prepared wing that was kept in a gelatin capsule taped to its map. Figure 10 displays electron micrograms of such preparations.

Using this method, two types of quantitative observation were made. A spotty increase in density to electrons had been noted in the OsO_4- fixed deformed wings as compared with the normal. This was measured by exposing a photometer through the individual frames of the exposed plate in an enlarger. The photometer reads higher for less light. Thus the higher readings represent less density to electrons. The results of such an experiment are presented in Table 9. These were 50 Kv electrons. It is quite certain that the deformed wing after OsO_4 fixation absorbs and scatters electrons more than the normal. The density to electrons is largely a reflection of reaction with OsO_4. In view of the results of the P determinations and the optical microscopy this difference in electron transmission is almost certainly the result of retained cellular material.

A second type of observation consisted of enumeration of the spines. Using the same plates, prints were made and the spines counted. The results are shown in Table 10. The densities described above may have obscured some of the spines, but it is thought that no more than 10% or so were obscured enough to be missed in counting. In addition, many of the spines are malformed in comparison with controls. These findings are in accord with what has been seen in following development by light microscopy. An attempt to correlate all of the experimental results and relate them with earlier work follows in the discussion.

DISCUSSION

The foregoing observations, in summary, have shown that the morphogenesis of the wing is quite radiosensitive at a certain critical time and that the units being damaged are probably many. We know also that the normal membranous wing is formed by a well synchronized changing group of cells. The radiation-damaged wing

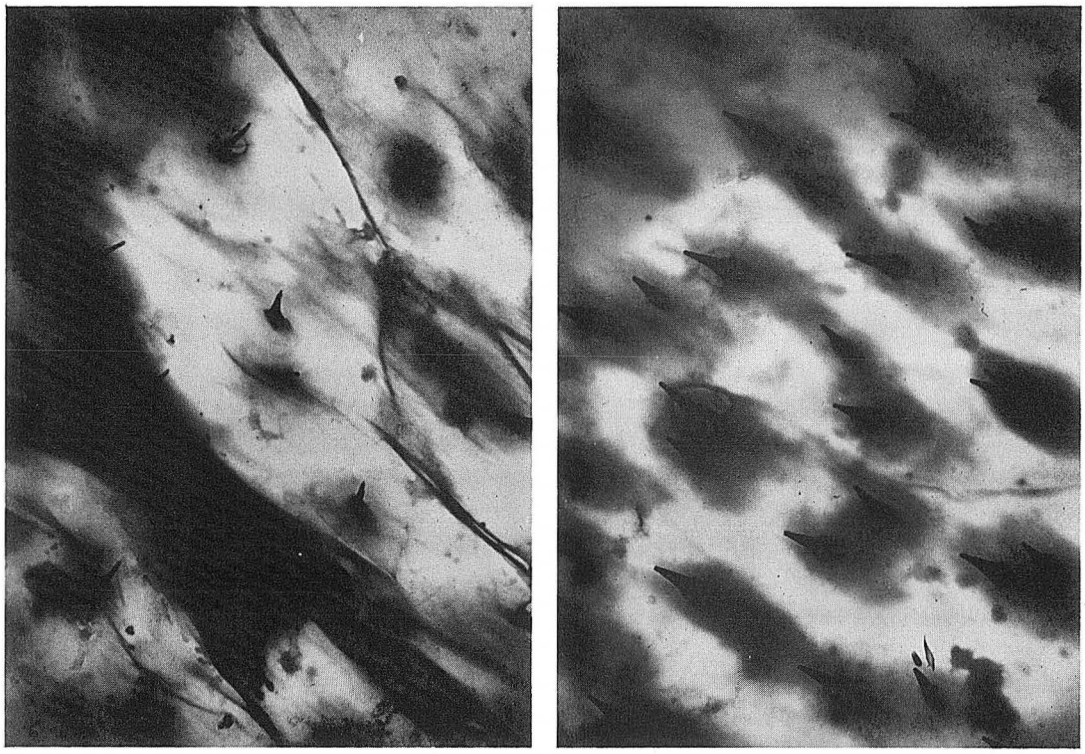

Figure 10. Electron micrograms of membranous wings: left, deformed; right, normal.

acquires and subsequently loses peculiar basophilic bodies that appear only rarely in the normal cell population. The cells of the damaged wing form the cuticle by a disordered sequence of events, and the outcome is abnormal. The spines of the affected wing are missing in some sites, duplicated in other sites, and often malformed. The normal and deformed wings differ little, if at all, in mass at about 1 day after eclosion. If there is such a difference, the normal is greater in mass. If different at all, the C content is greater in the deformed within one day after eclosion. There is no doubt that the P content is markedly different at all times. The difference seems to reflect primarily an aberration in timing until after eclosion, when the P content remains higher for the deformed wing. The loss of P and cells after eclosion and the failure to observe degeneration of cells or cellular debris suggests that the normal hypodermal cell either migrates out of the wing or rapidly degenerates and is removed by circulation. This process, whichever it may be, is less effectively carried out in the affected wing.

Though the critical period for this radiosensitivity has been defined only at the end, the one experiment with larvae suggests a distinct beginning. Many examples of critical periods for specific effects were mentioned in the Introduction.

Table 9

ELECTRON TRANSMISSION
(ARBITRARY UNITS)

Frame	Control	Deformed
1	107	27
2	97	46
3	107	35
4	102	21
5	91	43
Average	101	34

Table 10

NUMBERS OF SPINES ON
ELECTRON MICROGRAMS

Frame ($0.01\ mm^2$)	Control	Deformed
1	57	27
2	56	22
3	42	14
4	38	18
5	41	26
Total	234	107

Often exposure of very early embryos produces specific regional effects.[126] Where dividing cells are sensitive, non-dividing embryonic cells have been found resistant and capable of subsequent normal activity.[20] In general, damage to a tissue or anlage before it has lost the capacity of regulation, and exposure after the sensitive process has occurred, will both be ineffective, whereas the time between loss of regulatory capacity and completion of the process is the critical period. Regulatory capacity in Coleoptera exists up to a point,[127] but the wing sensitivity in Tribolium apparently is a nearly end-stage phenomenon. There may be other aberrations undetected, the wing being merely more obvious rather than uniquely sensitive. Still, the wing hypodermal cell has remained quite sensitive beyond the sensitive periods of other cell populations. This is a reflection of a relatively undifferentiated state at a late stage of development.

Radiation-induced aberrations in timing of developmental events are not uncommon. Mostly such phenomena have been reported for insects as prolongations of stages.[128,129,130,131] More specific cellular events have been observed to be displaced in time. For example, the appearance of actin has been retarded while

the appearance of myosin has been advanced by X irradiation in a developing embryo,[132] and it has been suggested that cells may be prodded into differentiating by irradiation.[133] Similarly specific cellular events have been differentially affected in the case of the Tribolium wing. If some single process were delayed at an early stage of differentiation, the mechanisms for the various capacities of the hypodermal cell may very well be responding in different ways to the altered intracellular environment. Thus the flattening of the cell may occur on schedule or with a delay proportional to the overall slowing of development, but the formation of cuticle and spines may be more sensitive to the first order effect and thus be more severely delayed.

Little can be said of the radiation-induced bodies which appear and disappear in the abnormally developing wing. Pyknotic chromatin and osmophilic material associated with chromosomes[87] and supernumerary organelles[88] and swollen nuclei[82] have been reported in irradiated grasshopper embryos. But the nature of the pathological bodies in the Tribolium wing is unknown. This problem is under study with the electron microscope. It is interesting to consider a possible relation between these bodies and the subsequent inadequacy of the cells to perform normally a task completed long after the bodies have disappeared. Such a visible damage presents the possibility of relating the early effect on a cell to the later defect in a structure such as a spine.

The spine pattern and structure are observable and precise results of the activity of a single cell type. It may be expected that one cell makes one spine, a condition that defines the system even better. A one-to-one correspondence of cells and spines was claimed by Dobzhansky for Drosophila.[134] Lees and Waddington make the statement again for Drosophila and suggest that every cell at the surface forms such a process.[135] (An extensive analysis of normal and mutant bristle formation appeared in 1945[136] and an interesting investigation of spine formation was published in 1951.[137]) The radiation-induced aberrations in spine pattern and structure are a promising aspect of this sytem. The many instances of redundancy of structures after irradiation are reviewed in the Introduction, but mostly they are redundancies of whole organs or tissue regions. In the case of the spine, presumably made by a single cell, the redundancy may be quite a different matter. Certainly it presents a better defined problem. Supernumerary organelles produced by X irradiation in grasshopper embryos, recently reported by Tahmisian and Devine,[88] may be related. Morphogenesis involves more than the production of an enzyme or stroma; it includes rather intricate temporal and spatial relation that are presumably controlled according to some information within the morphogenetically-acting cell. The supernumerary spines are probably the product of cells that have the capacity to make spines but have some defect in the control of the process. The absence of spines where there should be spines

is less surprizing. A cell could probably be damaged in many ways with the same effect of not producing a spine. However, cuticle still overlies such areas.

An absence of distal parts of the tracheal system has been observed in deformed wings, though tracheae are present during development. This particular aspect of the deformity has not been given much attention. Two interesting papers by Locke have to do with the physical processes of trachea formation[138] and with the control of tracheal growth.[139]

The radiation damage in this case is not somatic mutation. Others have excluded somatic mutation for Habrobracon[63] and for Drosophila.[71,72] Muller discusses the problem of genetic and non-genetic effects in Drosophila and other systems.[140] As a test for transmissable effects upon germ cells, I crossed mildly deformed (wing) Tribolium and found no more deformed among the offspring than among offspring of control pairings. The nature of the damage to differentiating cells is a complicated problem in any system. In insects a given group of cells responds repeatedly to hormonal stimuli, normally with different effects each time.[32,141,142,143] Growth in insects is humorally controlled in stop, go, rate fashions, while differentiation is humorally controlled in stop, go, direction fashions.[143] In the case of Calliphora, the pupae developed from irradiated larvae had retained some larval type cells and tissues.[65] The cuticle of the pupal wing of Tribolium has no spines. So the absence of a spine is in this sense a pupal characteristic in an adult.

It seems clear that more than one process suffer damage. Whatever the nature or location of the injury, it is probably an injury to some intracellular control mechanism rather to a synthetic process.

APPENDIX

The Nuclear Activation Equation and Comparative Measurement by Activation Analysis.

Consider the general nuclear reaction A(x,y)B, where A is the nuclide to be measured and B is a radioactive nuclide with decay constant λ sec.$^{-1}$. Let F (cm^{-2} sec.$^{-1}$) be the flux of x and σ (cm^{2}) be the cross section of A for the reaction A(x,y)B. Now, if N is the number of A atoms in the sample and N* is the number of B atoms in the sample, then, in dt sec.,

$$dN^* = (F\sigma N - \lambda N^*)dt$$

(The change in N due to the reaction is negligible.)
And,

$$dN^* = F\sigma N(1-\frac{\lambda}{F\sigma N}N^*)dt$$

$$\frac{dN^*}{1-\lambda N^*/F\sigma N} = F\sigma N dt$$

Integrating,

$$-\frac{F\sigma N}{\lambda}\ln\left(1-\frac{\lambda}{F\sigma N}N^*\right) = F\sigma Nt$$

$$1-\frac{\lambda}{F\sigma N}N^* = e^{-\lambda t}$$

$$N^* = \frac{F\sigma N}{\lambda}\left(1-e^{-\lambda t}\right)$$

Now, from the end of exposure the species B is decaying. Thus, in time τ sec. after the end of activation,

$$N^* = \frac{F\sigma N}{\lambda}\left(1-e^{-\lambda t}\right)e^{-\lambda\tau}$$

Now, the count of radioactivity, R, is proportional to the decay of N* nuclei, the proportionality factor, k, being dependent upon the counting apparatus. Then,

$$R = k\, dN^*/d\tau = -kF\sigma N\left(1-e^{-\lambda t}\right)e^{-\lambda\tau}$$

Finally, for the same reaction, the same flux and exposure time, and the same counting apparatus, the relative content of the activated nuclide in two samples is given by:

$$\frac{R_1}{R_2} = \frac{N_1}{N_2}\cdot\frac{e^{-\lambda\tau_1}}{e^{-\lambda\tau_2}}$$

And,

$$\frac{N_1}{N_2} = \frac{R_1}{e^{-\lambda\tau_2}}\,\frac{e^{-\lambda\tau_1}}{R_2}$$

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ISOLATION AND ESTIMATION OF SERUM ORGANICALLY-BOUND IODINE. I. EFFECTS OF THE REMOVAL OF IONS FROM SERUM PROTEINS

By

Gilles La Roche, Charles Oakes and Paul Blanquet

In the preparation of serum prior to protein-bound iodine (PBI) estimations or in the extraction of butanol extractable iodine (BEI), the primary concern is the removal of iodide. Several methods for the isolation of proteins or the extraction of thyroid metabolites have been described, reviewed and discussed by Chaney.[1] Most of the procedures pay little attention to the lability of serum proteins and involve their denaturation in the course of precipitation or extraction.

Several workers have used ion-exchange resins in estimating PBI^{131} and have shown how radioactive iodide can be effectively separated from organically-bound iodine. Slade[8] appears to be the first to use this method in the isolation of serum proteins and the evaluation of protein bound $iodine^{127}$ (PBI).

In the present study, care has been taken to prevent the denaturation of serum proteins, and evidence is furnished to indicate possible ill-effects of the procedures followed. The dual object of preventing denaturation was to insure that none of the organically-bound iodine was lost and that further concentration or fractionation of iodinated products could be performed without the production of artifacts. With this in mind, two methods appeared particularly suited: one by passing serum on an anion-exchange resin and the other by dialysis. The various serum preparations were tested for protein denaturation by paper electrophoresis and antigen-antibody reaction in gel, before treatment and after restoring serum halogen concentration. Results were compared with those obtained with fresh serum. The effectiveness of iodide removal was tested by microiodine determinations.

MATERIALS AND METHODS

To prevent contamination, all glassware that were to come in contact with serum were washed in a sulfochromic acid mixture, rinsed several times in distilled water followed by double-distilled water and dried prior to use. The double-distilled water used in this work was obtained from the apparatus described in Figure 1. This continuous distillation apparatus has been in operation for over two years and has demonstrated a remarkable degree of reliability.

The animals used were adult male Long-Evans rats, aged 10 - 16 months, which had been fed ad libitum a low iodine diet* from the time of weaning. The pooled

* Low iodine "Diablo Laboration" made by the Diablo Laboratory 1320 Third Street, Berkeley, California, was assayed by the Albert Chaney Chemical Laboratory, 1503 East Chevy Chase Drive, Glendale 6, California, on January 2, 1959, and found to contain 1.8 μgm iodine per gm.

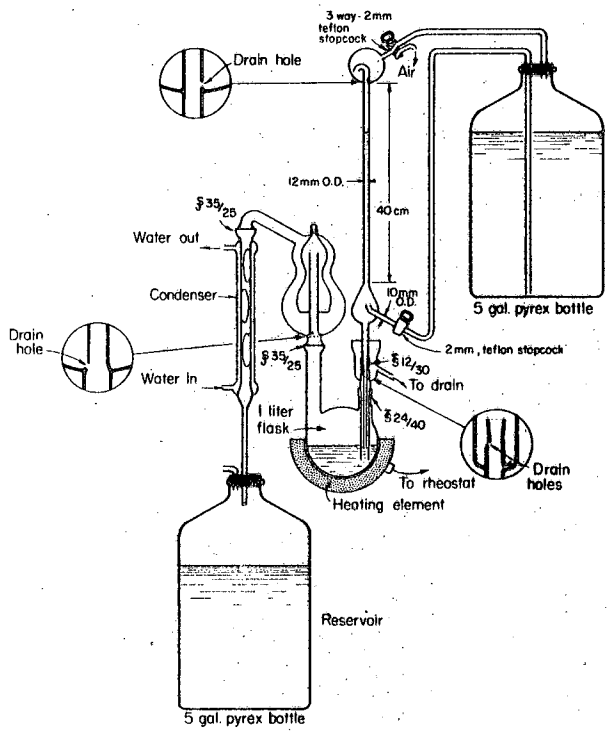

Figure 1. Continuous distillation apparatus used to obtain double-distilled water

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blood from 32 animals was used to establish the standard values for the rat. It was allowed to clot and the serum separated by contrifugation in the cold (5°C) for 30 minutes at 3500 rpm. The serum pools used experimentally were obtained from 6 to 10 rats, and aliquots of these pools were tested against controls in every instance.

The weak anion-exchange resin used in this work was Amberlite CG-45, type 2.* The exchange column was prepared as follows: a glass wool plug was first inserted in a cylinder 1.5 - 15 cm (Figure 2), at the top of which was a 250 ml conical reservoir fitted with a ground glass ball-and-socket joint (size 35/25) designed for use under pressure. Approximately 8 gm of resin (4 meg/gm) suspended in water were poured into the column and packed with 16 lbs pressure from compressed nitrogen. Any resin remaining in the conical reservoir was washed into the cylindrical part of the column, and a 0.5 cm layer of water was maintained above the resin in order to prevent drying or channeling. Amounts of serum ranging from 10 to 45 ml were

* This resin is the chromatographic grade of a synthetic weakly basic anion exchange resin (polyamine type) in its hydroxyl form. It is produced by Rohm and Haas Co., Washington Square, Philadelphia 5, Pa., and supplied by Fisher Scientific Co. (Cat. No. A-528.) Mallinckrodt Chem. Works also distribute an Amberlite OH (code No. 3343) resin which is known as CG 4B 100-200 mesh which is relatively pure.

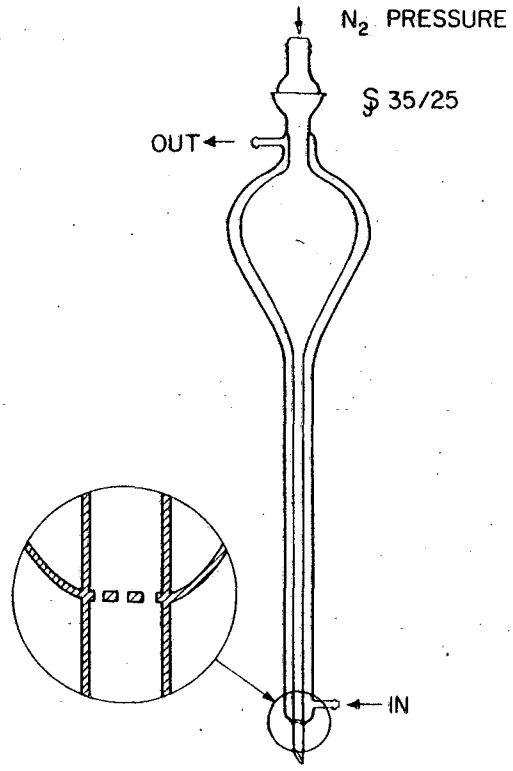

Figure 2. Jacketed chromatography column for constant temperature operation
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gently added to the column. The serum collection did not begin until the serum front had appeared by visual inspection and a few milliliters had been discarded as possibly diluted.

Dialysis of 25 ml volumes occurred through cellophane bags 0.5 cm in diameter that had previously been tested for leakage. Dialysis proceded with constant mechanical stirring against 1 liter volumes of double-distilled water at 1-3°C. The dialysis was considered complete after a total of 18 hours during which time the double-distilled water was changed once after 6 hours.

Free-moving boundary paper electrophoresis* employing a veronal buffer pH 8.6 at ionic strength 0.075 for a duration of 16 hours at 2.75 ma** was used to detect variation in the mobility of serum proteins. The dyed strips were scanned on a

* Electrophoresis was carried out in a Durrum Type Cell, Model R-Series C, made by Spinco Division of Beckman Instrument Co., Belmont, Calif. The veronal buffer (Spinco B-2, Cat. No. 300-824A) contained 2.76 mg diethyl barbituric acid and 15.4 gm sodium diethyl barbiturate per liter. Other Spinco parts were the B-4 dye (1.0 gm bromphenol blue prepared in 1 l. of methanol, Cat. No. 300-847), the electrophoresis wicks (Cat. No. 300-029), and the paper strips (Cat. No. 300-846).

** An Heathkit, Model PS-3, Voltage Regulator, made by Heath Co., Benton Harbor, Mich., was used as the power regulator.

Model RA Analytrol, made by Spinco Division of Beckman Instrument Co.*

If any delay occurred during the stepwise processing of the various fractions, care was taken to maintain these samples at 3°C.

The immunological test of serum proteins for detection of possible denaturation involved the use of a modification of the gel-diffusion technique described by Ouchterlony.[9-13]

The antiserum for this reaction was obtained from adult male New Zealand white rabbits which were injected I.V. with 1 ml rat plasma twice a week for three weeks. The rabbits were sacrificed at the end of the fourth week following beginning of immunization. The serum was separated, lyophilized and stored in a dessicator under vacuum. The antiserum was redissolved in water prior to use.

Each petri dish used to carry out the diffusion-precipitation technique was 9 cm in diameter and 2 cm deep, and contained 15 ml of gelified 1% agar (Difco) prepared in Sorenson phosphate buffer pH 6.7 - 7.2 and clarified by centrifugation. This agar preparation also contained 0.2% merthiolate (Lilly) as preservative and 0.01% trypan blue to offer greater contrast for observation of precipitation bands.[14]

Micro-iodine determinations were performed by a method similar to that of Bodansky et al.[15] which will be described and discussed in Part II.

RESULTS AND DISCUSSION

The paper electrophoresis of normal rat serum shows fairly well-defined bands for each of the five major protein complexes (Figure 3A). In the early studies on normal rat serum it was practically impossible to resolve the α-1 globulin from the albumin. At best, this globulin peak appeared as a slight inflection on the slow moving edge of the albumin band. Explanation for this is suggested from studies in which the effect of various amperages on protein mobility were studied. With a 2.5 milliamp current using a veronal buffer pH 8.6 for human serum, the resolution with rat serum was very poor. It was then determined after several trials that 2.75 milliamp offered the best resolution under the conditions specified above.

Normal rat serum electrophoretic patterns and immunological properties (Figure 3A control; Table 1 control) were established over a period of several months on different serum pools. Aliquots of the above sera were de-ionized by passing on CG-45 and examined, as were the controls. At the same time, other aliquots were passed on CG-45 and dialyzed in the usual manner against 0.85% NaCl in an attempt to re-establish normal serum halogen concentration. Typical paper

* Operation of the Model RA analytrol involved use of a No. 3 Empirical Balancing Cam. The No. 4 slit aperature of 1 mm proved satisfactory in providing sharp peaks. The instrument was calibrated to 8.2 cm on Analytrol charts (Cat. No. 300-542) with a Wratten Filter in "B" glass (Corning 5031) of 0.9 Neutral Density.

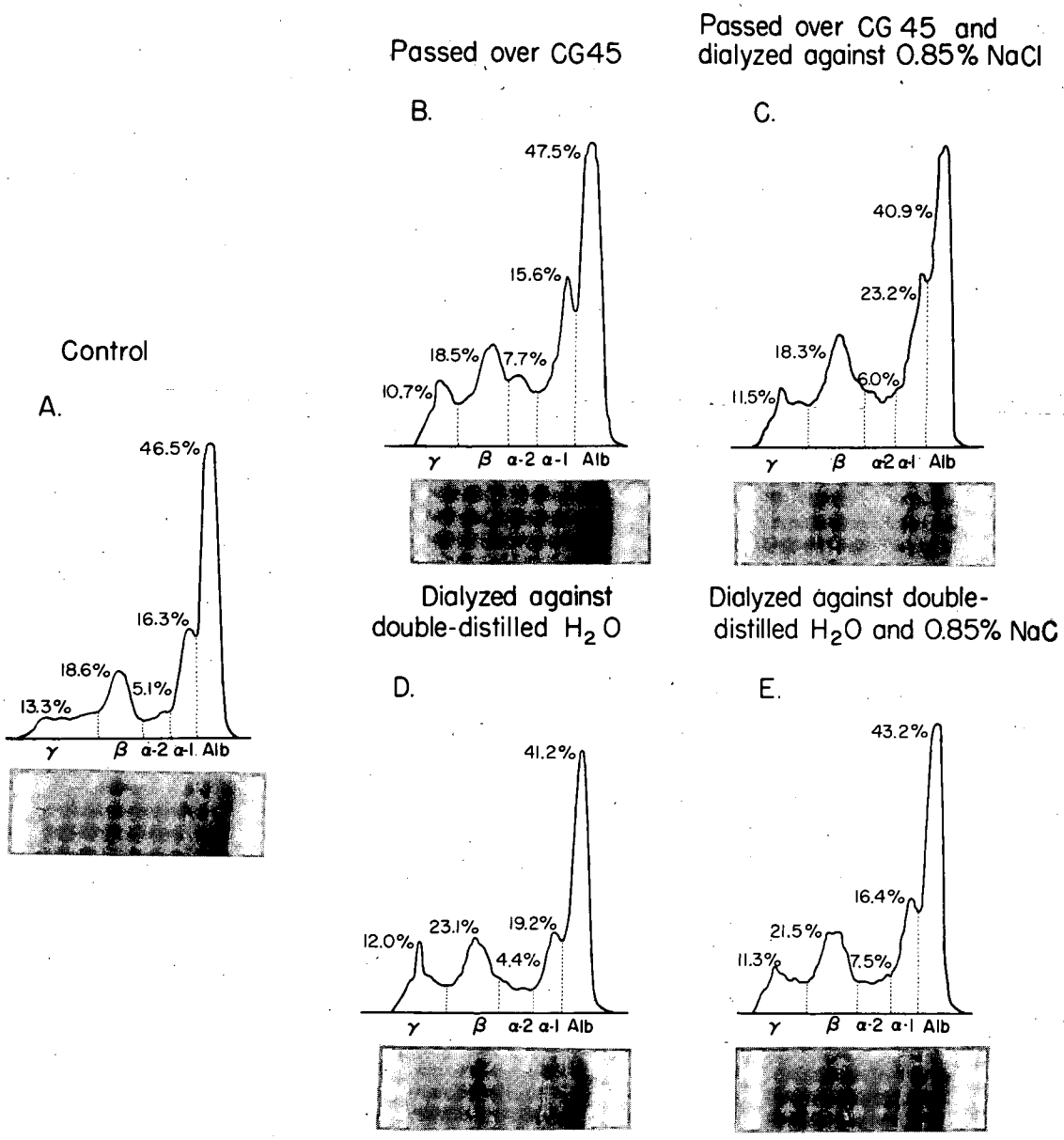

Figure 3. Rat Serum. Analytrol scanning of subjacent paper electrophoresis

electrophoretic patterns for these serum samples are shown on Figure 3A, B, C, and summarized in Table 1, along with the respective number of antigen-antibody precipitation bands.

From Figure 3A and B it seems as if passage on CG-45 sharpens the various protein peaks, and that in re-establishing normal halogen concentration (Fig. 3C) some of the acquired resolution is lost. The diagram then resembles that of control serum (Fig. 3A,C) except for a sharp little peak over the γ-globulin fraction.

Table 1

Serum samples	Electrophoretic patterns (% protein)[a]					Antigen-antibody bands[b]	μg Iodine/100 ml[c]
	γ Globulin	β Globulin	α_2 Globulin	α_1 Globulin	Albumin		
Control	13.3±2.1	18.6±3.1	5.1±1.5	16.3±2.5	46.5±2.0	6	8.4±0.3
Passed over CG-45	10.7±0.6	18.5±0.5	7.7±0.5	15.6±2.0	47.5±1.9	6	1.7±0.1
Passed over CG-45 and dialyzed against 0.85% NaCl	11.5±0.7	18.3±0.9	6.0±0.1	23.2±0.6	40.9±1.3	6	1.8±0.2
Dialyzed against double-distilled water	12.0±2.7	23.1±2.6	4.4±1.4	19.2±3.0	41.2±4.8	4	1.8±0.4
Dialyzed against double-distilled water and 0.85% NaCl	11.3±1.3	21.5±2.0	7.5±0.3	16.4±1.0	43.2±1.0	4	1.8±0.3

[a]These represent the closest values to the mean 6 separate electrophoretic patterns on the same serum fraction.

[b]This represents the number of precipitation bands developing on a single agar plate.

[c]These represent an average of three separate determinations on the same sample.

Similar amounts of rat serum were dialyzed against double-distilled (d.d.) water or both d.d. water and 0.85% NaCl. The results of these experiments can be compared to control serum on Figure 3A, D, E, Table 1.

Even though little, if anything, can be deducted from the percent protein variations (Table 1), it is possible to notice a sharp band (Fig. 3D) that remained at the origin (γ-globulin peak) and that is absent in control serum (Fig. 3A). A similar but much smaller peak can still be observed on water-dialyzed serum re-dialyzed against 0.85% NaCl (Fig. 1E). From this, it would appear that long standing under dialysis conditions induces denaturation of some proteins.

Passage of serum on CG-45 or dialysis against double-distilled water are both effective in removing iodide (Table 1) but only the former treatment will allow serum to retain its original antigenic properties. It is of interest to mention that the changes occuring in the water dialysis are permanent because the original immunological properties were not regained after re-dialysis against 0.85% NaCl. This phenomenon is further confirmed by the appearance of a precipitate in the cellophane bags at the end of dialysis in distilled water. The insoluable material does not diminish appreciably during re-dialysis in 0.85% NaCl.

In the immunization of rabbits to rat plasma, it appears that 6 groups of antibodies were formed (Table 1, control). It is probable that serum protein constituents not present in large enough concentration did not produce specific antibodies in the rabbit. For this reason, treatment of serum by CG-45 may have

produced denaturation of such proteins which would remain undetectable under the present methods of evaluation.

From these results, it would appear that paper electrophoresis alone is not sufficient to establish that proteins have retained their original configuration during processing. It is only in combination with the analysis of antigen-antibody reactions that it is possible to further confirm whether denaturation of major protein fractions has occurred or not.

Because the antigen-antibody precipitation in gel and paper electrophoresis give only qualitative estimates of protein modification it is probable that some denaturation occurred in CG-45 treated serum later dialyzed against 0.85% NaCl. This is somewhat substantiated by the sharp little knob on the γ-globulin fraction (Fig. 3C), although in this case denaturation would not have been as extensive as it was after water-dialysis (Fig. 3D), because enough proteins (antigens) remained to give the usual number of precipitation bands.

SUMMARY

Two methods for removal of free ions, including iodide, were used in an effort to study their effects on serum proteins: one was by passage on an anion-exchange resin and the other by dialysis.

Passage on CG-45 resin and dialysis seems to sharpen the electrophoretic pattern. Sixteen hour dialysis in the cold seems to induce a certain degree of protein denaturation, which is discussed.

Use of CG-45 to remove serum free ions including iodide, seems to represent an elective procedure because it is very rapid and does not appear to alter serum proteins significantly.

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A METHOD FOR THE PRODUCTION OF CONCAVE GRADIENTS FOR USE IN COLUMN CHROMATOGRAPHY AND ITS APPLICATION TO THE SEPARATION OF PHOSPHOLIPID AND NEUTRAL LIPID MIXTURES WITH SILICIC ACID COLUMNS*

By

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Gradient elution, first introduced by Alm and coworkers[1,2] in 1948, has only recently been applied to column chromatography of lipids.[3,4,5] Early studies with gradient elution systems considered only gradients in which the concentration of the polar or eluting solvent was increased very rapidly at the start of a run and then slowly approached 100% concentration asymptotically in the latter part of the run; this is often termed a convex*** gradient. This type of gradient is relatively simple to produce in the laboratory, but is necessarily a much poorer system than the corresponding linear or concave gradient in which the eluting solvent is introduced gradually at first and them more rapidly so that a continually increasing rate of change of the eluting solvent concentration is presented to the column.

In the study of serum lipids being carried out in this laboratory,[6,7] stepwise silicic acid column chromatography of phospholipids proved inadequate, and a system of concave gradient elution silicic acid column chromatography was developed to provide better separation of the various phospholipids. This paper will present the details of this system, certain aspects of column performance will be discussed, and the results of several chromatographic runs on standard mixtures and natural samples will be shown.

THEORETICAL CONSIDERATION

Martin and Synge[8] developed a theory for partition chromatography that while not rigorous for adsorption, can be adapted to adsorption processes. Lakshmanan and Lieberman[9] presented a discussion of gradient elution chromatography utilizing partition chromatography theory and concluded that concave gradient elution will provide the best possible elution characteristics if the substance has an adsorptivity described by a non-linear isotherm of the type proposed by Freundlich[10] for batch processes and mathematically represented by the equation:

$$C_s = K\ (C_m)^n \qquad (1)$$

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***Alm et al.[1] proposed the termed "gradient elution" to cover the method of chromatography discussed in their paper. The authors discussed a gradient having a large initial slope and that approached an abscissa asymptotically. Lakshmanan and Lieberman[9] later showed that a gradient of this type had definite disadvantages compared to a gradient whose slope was initially low and gradually increased as the chromatography was continued. Later investigators have tended to differentiate these two types of gradients by designating the first as a "convex gradient" and the second as a "concave gradient". A third type of gradient, called "linear", has a constant slope during the chromatography. Mathematically the second derivative of convex gradient is negative; of a concave gradient, positive; and of a linear gradient, zero.

where C_s is the amount of solute adsorbed by a given amount of absorbent, C_m is the concentration of the solute, and k and n are constants and n is less than 1.

A more rigorous treatment applying to adsorption chromatography per se has been proposed by deVault[11] and provides additional theoretical support for the belief that concave gradient elution will give optimum resolution in absorption column chromatography. If diffusion effects are neglected, a single solute with a Freundlich adsorption isotherm will elute off an adsorption column with a sharp front and a gradually tapering tailing edge. In practice, diffusion effects will tend to spread the boundary further. deVault has shown that a substance with a linear adsorption isotherm will elute off a column with the shape identical to that of the initial absorption band on the column, which will be rectangular in a well packed column if diffusion effects are neglected.

Concave gradient elution can be considered a way to increase the linearity of a non-linear isotherm. For a single substance a gradient can be constructed to keep the adsorptivity of that substance almost always in the linear region of the absorption isotherm. With complex mixtures this is not possible, but by varying the shape of the gradient in critical regions, much improved separations can be obtained over stepwise elution schemes, and "false peaks" caused by discontinuous solvent changes can be entirely avoided.

EXPERIMENTAL APPARATUS

Alm and coworkers[1] originally proposed a double reservoir system in which an eluting solvent* was added to a mixing chamber containing a carrier solvent. As the rates into and out of the mixing chamber were equal, the volume in it remained constant. The gradient produced was convex and given by the equation

$$C_t = C_o\,(1-e^{\frac{-RT}{V}}) \qquad (2)$$

where C_t is the composition of solvent leaving the mixing chamber at time t, in terms of the composition of solvent added, C_o. R is flow rate into (or out of) the mixing chamber and V is the volume of solvent in the mixing chamber.

Lakshmanan and Lieberman[9] added greatly to flexibility of the double reservoir arrangement by allowing the rate into, R_1, and out of, R_2, the mixing chamber to be independent of each other. In this manner they obtained concave, convex or linear gradients depending on whether R_1 was greater, equal to or less than R_2, and devised mathematical solutions for each case. More recently several other authors have described apparatus for the production and maintenance of complex gradients.[12,13,14] Perhaps the ultimate in flexibility for the production of gradients was achieved in the "Varigrad" designed by Peterson and Sober.[15]

During this study linear as well as convex gradients were found to be inferior to concave gradients, and will not be discussed further here.

Wren[3] proposed a system for the specific production of a concave gradient adapted

*The solvents used in gradient elution systems have a confused nomenclature. Some authors refer to different solvents by letters or numbers, others use "polar" or "non-polar" or "relatively more polar." In this paper a solvent that will not cause a noticeable migration of a compound on a column during the usual period of a chromatographic run is termed a "carrier solvent" referring to the fact that this solvent is a carrier or diluent for the solvent producing the migration of the compounds on the column. This latter solvent is referred to as the "eluting solvent".

from a proposal of Boman.[16] Although Wren's system gave a wide, uniformly varying gradient, it was not easily adaptable to close regulation of flow rates or to an exact mathematical description of the resulting gradient. It was possible to modify this system, however, to allow better regulation as well as greatly to increase the flexibility.

The siphons and pressure differentials were replaced by controlled-volume pumps. The shape of the gradient produced in this system is then given by the differential equation in terms of C_t the concentration of eluting solvent leaving the mixing chamber.

$$C_t = \frac{\int C_o R_1 \, dt - \int C_t R_2 \, dt}{V_o + \int R_1 \, dt - \int R_2 \, dt} \tag{3}$$

where R_1 and R_2 are as given previously, C_o is the concentration of eluting solvent added to the mixing chamber, and V_o is the volume of carrier solvent in the mixing chamber at time zero. This equation is similar to that given by Lakshmanan and Lieberman[9] except that R_1 is a variable of the form:

$$R_1 = at \tag{4}$$

where a is an arbitrary constant determined by a pump setting and has units of ml/min^2.

Substituting equation (4) into equation (3) and differentiating yields

$$\frac{dc}{(C_o - C)} = \frac{at \, dt}{(V_o + \frac{at^2}{2} - R_2 t)} \tag{5}$$

which can be solved immediately by integration. The forms of the solutions are:

If $2aV_0 = R_2^2$

$$C_t = C_0 \left[1 - \frac{R_2^2 e^{2\left(1 + \frac{R_2}{at - R_2}\right)}}{(at - R_2)^2} \right] \quad \text{(5)}$$

If $2aV_0 < R_2^2$

$$C_t = C_0 \left[1 - \frac{V_0 \left(\frac{R_2 + \sqrt{R_2^2 - 2aV_0}}{R_2 - \sqrt{R_2^2 - 2aV_0}} \right)^{\left(\frac{R_2}{\sqrt{R_2^2 - 2aV_0}} \right)}}{\left(V_0 + \frac{at^2}{2} - R_2 t \right) \left(\frac{at - R_2 - \sqrt{R_2^2 - 2aV_0}}{at - R_2 + \sqrt{R_2^2 - 2aV_0}} \right)^{\left(\frac{R_2}{\sqrt{R_2^2 - 2aV_0}} \right)}} \right] \quad \text{(6)}$$

If $2aV_0 > R_2^2$

$$C_t = C_0 \left[1 - \frac{V_0 e^{\frac{2R_2}{\sqrt{2aV_0 - R_2^2}} \tan^{-1} \left(\frac{-R_2}{\sqrt{2aV_0 - R_2^2}} \right)}}{\left(V_0 + \frac{a}{2} t^2 - R_2 t \right) e^{\frac{2R}{\sqrt{2aV_0 - R_2^2}} \tan^{-1} \left(\frac{at - R_2}{\sqrt{2aV_0 - R_2^2}} \right)}} \right] \quad \text{(7)}$$

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The three solutions are necessary because the radical $\sqrt{\pm(R_2^2 - 2aV_o)}$ appears and is indeterminate where $\pm (R_2^2 - 2aV_o)$ assumes a negative value or zero. Figure 1 gives examples of the curves obtained from each solution with various initial conditions. The curves are sigmoidal over the full range. The parameters a, R_2 and V_o can each be varied independently; thus, one solution is capable of yielding quite

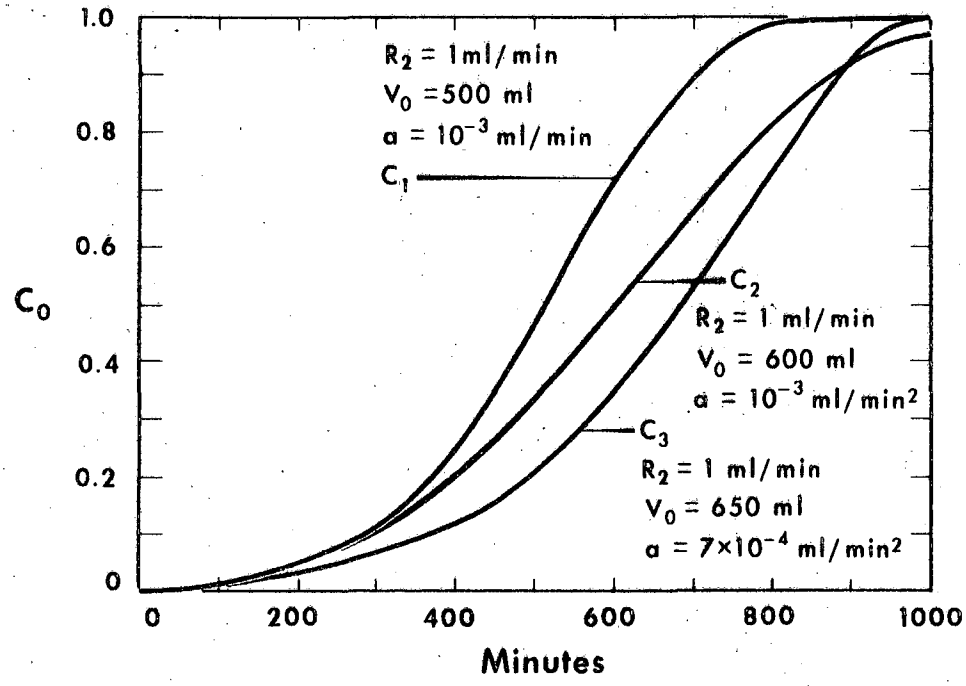

Figure 1. Graphical presentation of the gradients obtained under the conditions indicated on the figure for the three forms of the solution to equation 3 in the text. Curves C_1, C_2 and C_3 are solutions for equations 6, 7 and 8 respectively. The values of the parameters a, V_o, and R_2 are listed for each solution.

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varied gradients. Figure 2 illustrates this using equation 7.

Design of Apparatus. Figure 3 is a diagrammatic sketch of the apparatus capable of producing the gradients described above mathematically. The volume of solvent chambers shown in the figure are those found convenient for the 20 gm columns most commonly used in this laboratory. The critical parts of the assembly are the gradient and monitor pumps and the control for the gradient pump. Both pumps are "chromatographic miniPumps" No. CH-MM-29B designed by the Milton Roy Company, Philadelphia, Pa. These pumps have no organic soluble substances in the liquid head and are capable of pumping quite accurately liquids of low viscosity and high vapor pressure. The pumping action is accomplished by a reciprocating piston whose stroke can be varied from zero to 100% of capacity by a micrometer adjustment.

The motor on the gradient pump is a 1/15 h.p., D.C. motor made by Bodine Electrical Company, Chicago, Illinois, and is connected to an automatic control which increases the rpm of the motor linearly with time, starting at very low rpm. This control was designed by Frank Upham of this laboratory's Electronics Division. Figure 4 is the schematic circuit diagram of this control.

A linear increase in rpm of the motor produces a linear increase in pumping rate. The total volume, V, delivered by the pump at time, t, is

$$V = 1/2 \quad at^2 \qquad (9)$$

a can be calculated from this relation by collecting the volume delivered by the

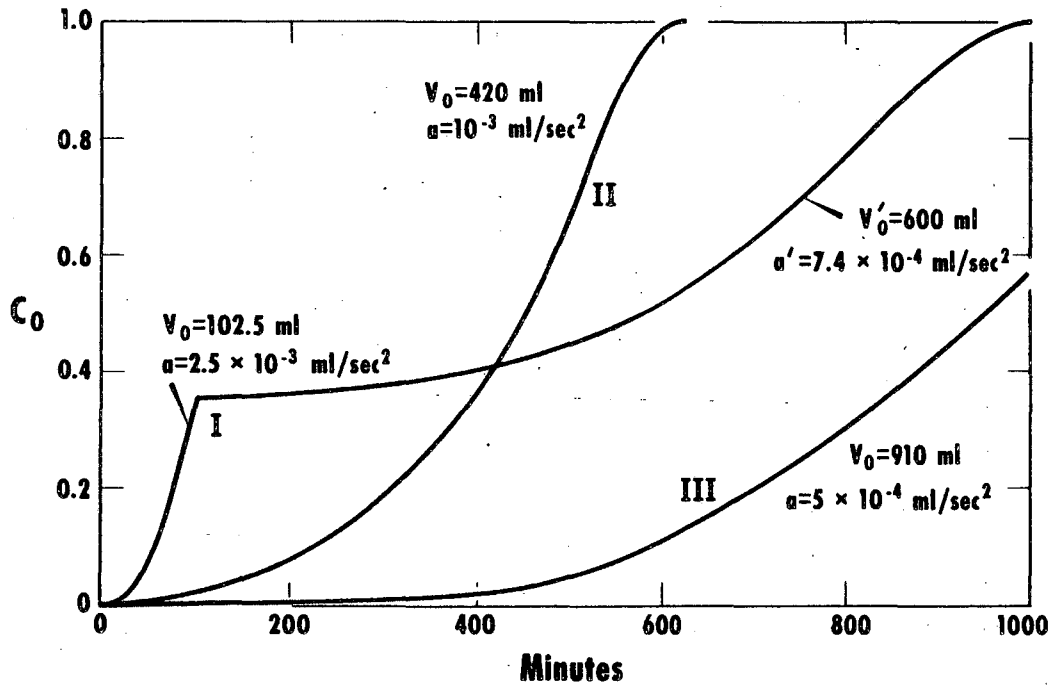

Figure 2. Some of the possible variations in the gradients produced by this system are shown. Solutions are obtained from Equation 7. R_2=1 ml/min. in all cases. Curve I presents a case in which additional solvent was added to the mixing chamber 100 minutes after the start of the run. The values for a and V_o are listed for each solution.

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pump during a specific interval. The time for the pump motor to reach its maximum rpm is taken as t_{max}; t_{max} is varied by a gear change on the motor control. a can be varied independently of t_{max} by changing the length of the piston stroke. Generally t_{max} is constant for columns of the same size.

The motor is started at an rpm somewhat greater than zero, approximately 1/20 of its maximum rpm. This produces a small deviation in the value of a predicted by equation (9) and consequently in the gradient for times close to zero. Figure 5 presents a calculated and observed gradient over the first 400 minutes of a run in which t_{max} had a value of 1000 minutes. For runs with t_{max} equal to or greater than 1000 minutes the effect of this small deviation from the theoretical gradient should be negligible. However, on short runs corrections must be applied.

The second or monitor pump, also a "chromatographic miniPump" identical to the gradient pump except that a constant speed A.C. motor is used in place of variable speed motor on the gradient pump, is used to transfer the eluting solvent from the mixing chamber to the column at a constant rate, R_2. Originally, gravity and pressure feeds were used in place of this pump but were inadequate to maintain a constant flow rate for any extended time period. A metering valve* attached to this pump at the outflow increases its accuracy. Experimentally, the pump delivered 1 ± .08 ml/min when pumping a gradient that varied from 100% chloroform to 100% methanol during

*Obtained from Nuclear Products Co., Cleveland 10, Ohio.

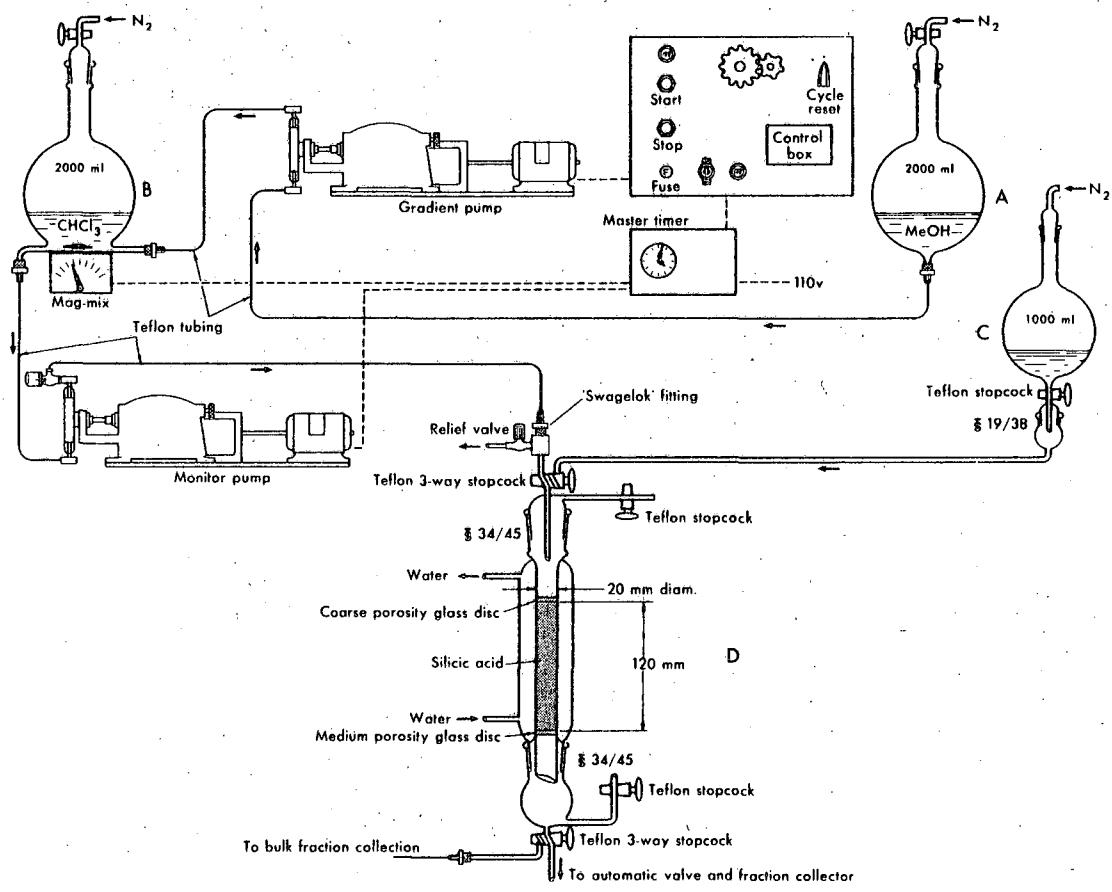

Figure 3. A diagrammatic sketch of the gradient elution column chromatographic system described in the text. A is the eluting solvent reservoir from which the solvent is transferred to the mixing chamber (B) by the gradient pump. A specific volume of carrier solvent is placed in the mixing chamber before the start of the run. The exact amount is determined by shape of the gradient and length of the run and calculated by equations 6, 7, or 8 given in the text. The outflow of the mixing chamber is pumped to the column (D) by the monitor pump. C is an additional solvent reservoir for non-gradient elution.

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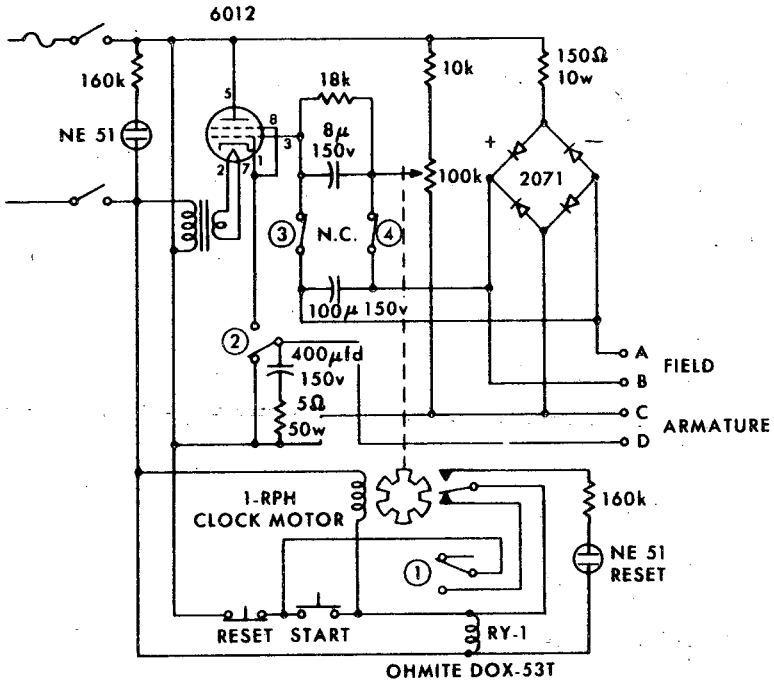

Figure 4. Schematic circuit diagram for the gradient pump motor control.

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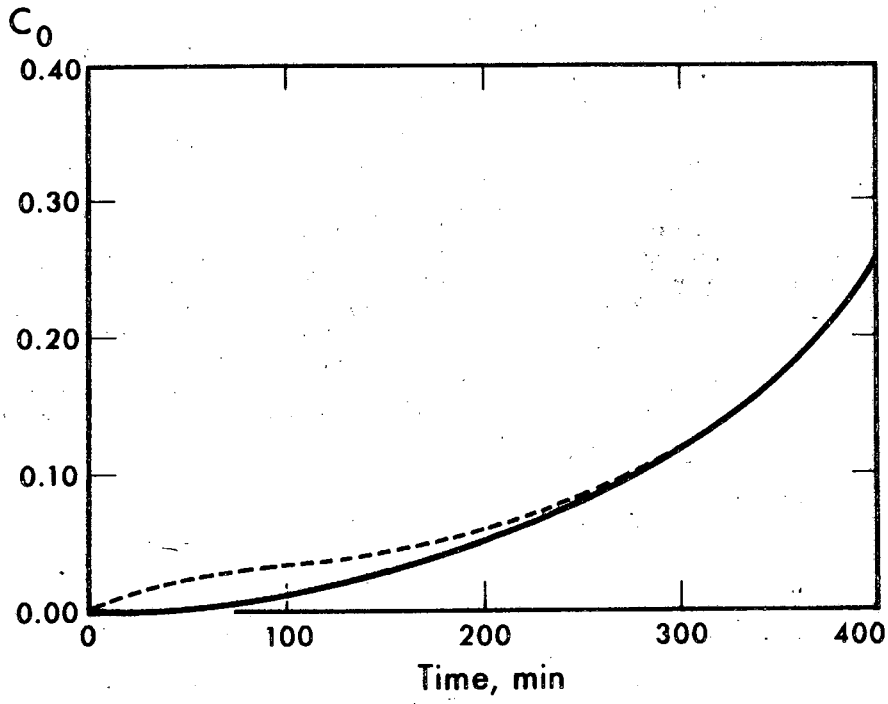

Figure 5. Curves showing experimentally observed deviation of gradient from ideality in the low concentration range caused by the gradient pump's initial rpm being greater than zero. Dotted curve is experimentally observed gradient. Solid curve is gradient calculated by equation 7 in the text.

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1000 minutes.

Column Housing. A water jacketed, glass-column housing similar to the design of Hirsch and Ahrens[17] was used. The laboratory water supply varied only 3°C, making thermostatic control unnecessary. A medium porosity-fritted glass disc* held in place by a carefully machined Teflon gasket that rested on a slight constriction at the bottom of the column supported the packing in the column. A coarse porosity disc* was used to protect the top of the column from disturbance after packing. Teflon stopcocks** were used throughout the apparatus to prevent leakage and to eliminate the need for stopcock grease.

Preparation of Column Packing. As the monitor pump is capable of maintaining a constant flow rate against high back pressures, no filter aid was added to the silicic acid packing. Columns were packed with pure silicic acid obtained from Mallinckrodt Chemical Co., 100 mesh, that was re-sieved to give a fraction passing a 100-mesh screen but retained by a 325-mesh screen. The sieved silicic was then washed with successive portions of distilled water, methanol*** and acetone and dried for 24 hours at 120°C, then stored in a sealed jar.

To pack a column, 20 gm of silicic acid was weighed and immediately before use, was heated in a vacuum oven between 110° - 130°C for 48 hours, then cooled, slurried with a solvent and poured as the slurry into the column housing. A minimum amount of additional solvent was used to wash the packing down the column, the coarse porosity glass disc was placed on top of the packing and pressed down until the column appeared closely packed. The extra solvent above the level of the disc was removed and the charge added to the column.

Chromatographic Procedure. The lipid extract was added to the column in the same solvent used to prepare the column packing. If neutral lipids were being analyzed, normal hexane was used; if only the phospholipids were of interest, chloroform was used. The charge was added to the column in 2 ml of solvent and washed into the column with an additional 2 ml. When neutral lipids were being fractionated, gradient elution with a gradient of ethyl ether in normal hexane is begun immediately after the charge is placed on the column. When the gradient reaches pure ethyl ether a methanol in chloroform gradient can be started to fractionate the phospholipids.

If, as was the case in most studies carried out here, neutral lipids were not to be separated in the same run, the column was prepared with chloroform. Before the gradient separation of the phospholipids was begun, 1000 ml of chloroform were run through the column to remove all neutral lipids in a single fraction.[8] The flow rate through a 20 gm column was 1 ml per minute. The charge added to the column yielded good resolution for most mixtures when it was between 3 and 4 mg of phospholipid per gm of silicic acid. A slightly higher value was found for the neutral lipids. Because resolution of the neutral lipid and phospholipids fractions was found to be independent, a 50-50 mixture of phospholipids and neutral lipid could be added to the column at 8 to 12 mg per g of silicic acid. No single

*Obtained from Fischer and Porter Co., Warminster, Pennsylvania.

**Kimax brand Teflon Stopcocks obtained from Kimble Glass Co. through various laboratory supply houses.

***All solvents used in all phases of this work were reagent grade commercial solvents. The chloroform contained 0.75% ethanol.

component should exceed 3 mg per gm of silicic acid in this system.

In most runs where multiple fractionation was carried out 10 ml aliquots were collected. As the flow rate through the column was constant at 1 ml per minute, time indexing was used to collect fractions.*

Lipid Extraction. Lipids from serum and liver were extracted as previously reported.[8,18] Red cell lipids were extracted in essentially the same manner except that the cells were first resuspended and washed twice with 0.9N saline solution. 20 ml of packed cells were then transferred to 180 ml. of 50-50 methanol-chloroform solution and homogenized for 2 min. in a Waring Blender. The subsequent extraction steps were carried out as described.[18]

Standard samples of 1-α dipalmitoyl phosphatidyl ethanolamine and 1- α dipalmitoyl phosphatidyl choline were obtained from California Corporation for Biochemical Research, Los Angeles, California. Sphingomyelin standards were prepared by the method of Rapport and Lerner[19] and rechromatographed on silicic acid. Lysolecithin was prepared by the method of Long and Penny.[20] Monophosphoinositol was prepared from mouse liver.[18] No pure phosphatidyl serine or cardiolipin standards were obtained.

Neutral lipid standards were obtained from the following sources: palmitic acid and tripalmitin from the Hormel Foundation, Austin, Minnesota; free cholesterol from Armour Laboratories, Chicago, Illinois. Cholesteryl laurate was synthesized from the free cholesterol and lauric acid by Dr. D. Kritchevsky. Saturated mono- and diglycerides were prepared from hydrogenated cottonseed oil by partial hydrolysis in 1N NaOH for 1 hour at 37° C and isolated by silicic acid chromatography.

Analytical Methods. Neutral lipids were characterized by infrared spectrophotometric techniques[21,22] and thin layer chromatographic methods.[23] Phospholipids also were analyzed by these methods, and fractions were monitored by chemical phosphorus determinations performed as described previously.[24] The solvent system used for separation of the phospholipid mixture on the thin layer chromatograms was diisobutylketone, acetic acid, water (70-20-10 V/V). Spots were developed by spraying the dried plates with concentrated H_2SO_4 and charring them.[18]

In addition, specific chemical determinations were performed on aliquots taken from various chromatographic fractions by the methods listed below: glycerol was determined by the method of Van Handel and Zilversmit[25] as modified by Van Handel;[26] ethanolamine and serine by the method of Dittmer and coworkers;[27] choline by the procedure of Wheeldon and Collins;[28] and sugars were analyzed by the anthrone reaction of Radin and coworkers.[29] Sphingosine was determined by its infrared spectra after isolation from the neutralized aqueous phase of the transmethylation procedure[7] with chloroform.

These tests were run only on a qualitative basis to indicate the absence or presence of the various compounds. No quantitative results were obtained, and no criterion of purity was established. Naturally, in any specific study more rigorous control procedures would be necessary, but exact procedures would vary with the purpose of the separation.

*The automatic fraction collector with time indexing units was obtained from Research Specialities Co., Richmond, California.

RESULTS

Figure 6 shows the elution pattern of a standard mixture of neutral lipids from a 20 gm silicic acid column. Table 1 gives the recoveries of the various components on a weight basis. The cholesteryl esters and triglycerides were fully resolved, but the free fatty acid, cholesterol and diglyceride peaks overlapped slightly as is reflected in the lower recovery of free fatty acids and diglycerides, and the cholesterol is greater than 100%. This column was not fully activated, which probably decreased its resolving power. Satisfactory methods of neutral lipid silicic chromatography have been developed by other workers,[17] so no further attempt was made to improve this system for neutral lipids.

Table 1
RECOVERIES OF NEUTRAL LIPID FROM CHROMATOGRAPHY OF STANDARD MIXTURE
Shown in Figure 6
(Gravimetric Determination)

Lipid	Collection tube numbers	Wt. added to column mg.	Wt. recovered mg.	Percent recovery
Cholesteryl laurate	2-10	58.3	58.1	99.6
Tripalmitin	11-20	59.1	60.7	102.8
Palmitic acid	21-30	21.2	19.8	93.4
Free cholesterol	31-46	37.9	40.3	105.9
Diglyceride, Monoglyceride	47-110	12.3	11.1	90.2
Total		188.8	190.0	100.7

Figure 7 is the elution pattern of a known mixture of phospholipids obtained from the sources listed above. All components except lecithin and sphingomyelin are well resolved, and even those hard-to-separate compounds are fairly well resolved. Infrared spectrophotometric measurements indicated that the collection tubes 80 through 95, the sphingomyelin peak, contained 5% of the lecithin added to the column. Tubes number 69 through 86, the lecithin peak, contained approximately 3% of the sphingomyelin. Most of the contamination is in tubes 77 through 85. Table 2 gives the recoveries of the various phospholipids in terms of moles percent phosphorus added to the column.

In figures 8, 9 and 10 the phospholipid elution patterns obtained from normal human serum, normal human red blood cells and mouse liver are shown. The shape of the gradient, the column size and other conditions were the same in each case. It can be seen from these runs that over a wide variation in the amount of individual components present, the major phospholipids are eluted at the same point in the system, plus or minus 2 collection tubes (20 ml of solution). This allows batch collection of the major phospholipids eluted by such a system after initially

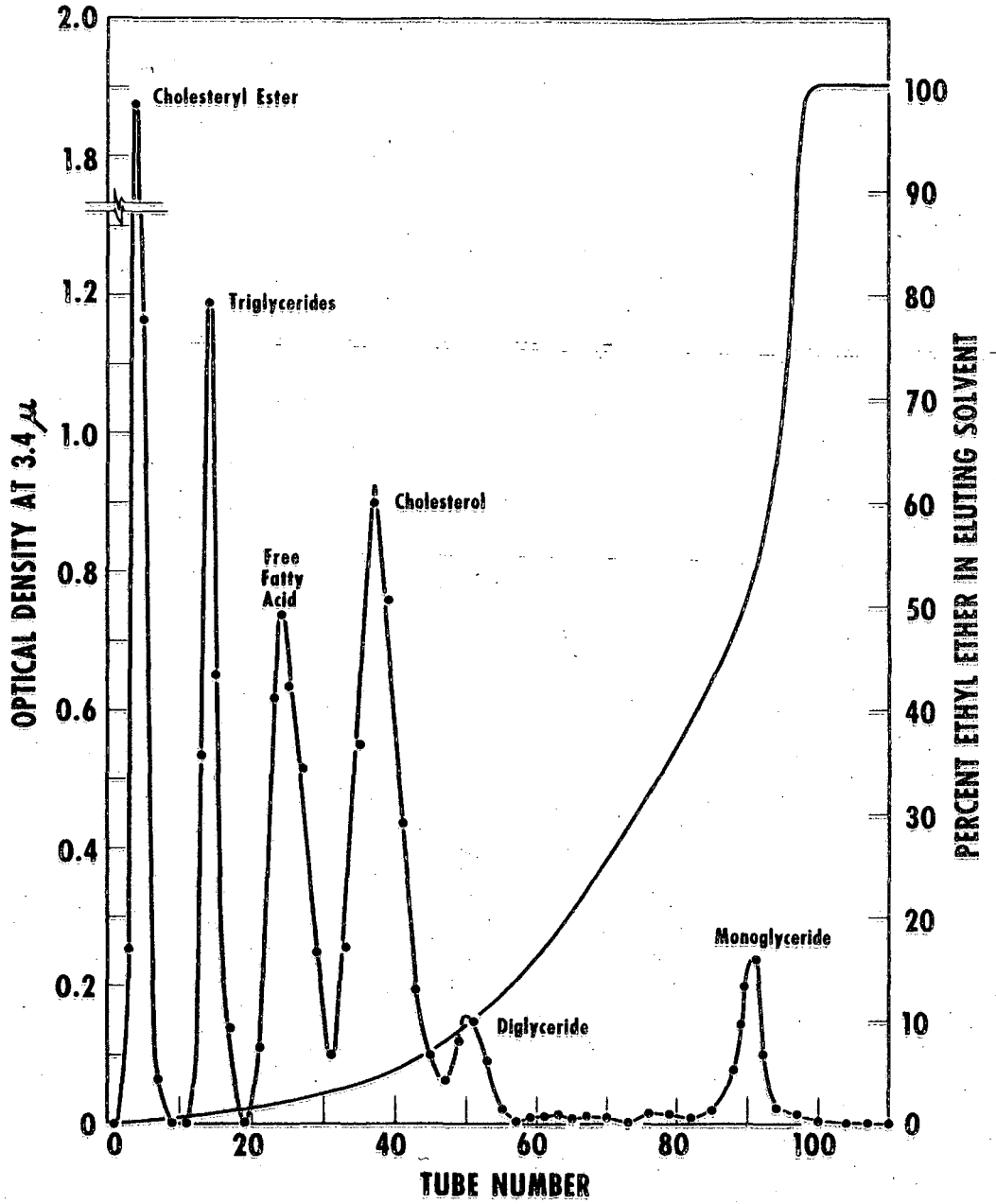

Figure 6. The experimentally determined elution pattern obtained by gradient elution chromatography of a known mixture of neutral lipids from a 20 gm silicic acid column. The gradient was diethyl ether in normal hexane. The shape of the gradient is shown on the figure. The left ordinate is the optical density of a 0.15 ml solution of the material, recovered from 1.0 ml of eluant solvent, in CCl_4 in a 3.0 mm path-length cell. The various peak heights are dependent on the specific absorption coefficient of each substance and not directly comparable to each other in the Figure. The right ordinate is the percent of diethyl ether in the gradient. See text for details of method and recovery data.

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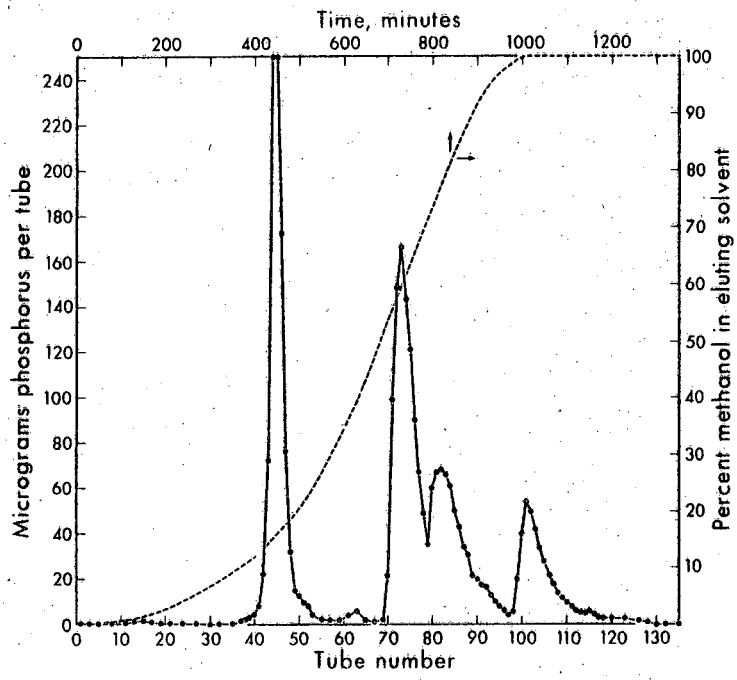

Figure 7. The experimentally determined elution pattern obtained by gradient elution chromatography of a known mixture of phospholipids obtained from sources listed in the text. 20 gm silicic acid column. The shape of gradient is indicated by the dotted line. The gradient was methanol in chloroform, and determined theoretically by equation 7 of the text. The values for the various parameters were: $a = .0007$ ml/min^2; $R_2 =$ 1 ml/min; $V_o = 650$ ml; and $t_{max} = 1000$ min. After 1000 min. elution was continued with pure methanol for an additional 400 min. Collection tubes 40 to 50, 69 to 85, 75 to 97, and 98 to 120 contained phosphatidyl ethanolamine, lecithin, sphingomyelin, and lysolecithin respectively. See text for detail of method and recovery data.

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Table 2

RECOVERIES OF PHOSPHOLIPIDS FROM CHROMATOGRAPHY OF STANDARD MIXTURE

Shown in Figure 7

(Mold % Phosphorus Added to the Column)

Phospholipid	Collection tube numbers	μgm P added to column	μgm P recovered	Percent recovery
L- α(dipalmitoyl) phosphatidyl ethanolamine	35-55	880	885	100.7
L -α(dipalmitoyl) phosphatidyl choline	69-85*	1976	1940	98.1
Sphingomyelin	75-97*	735	738	100.4
Lysolecithin	98-120	370	394	106.5
Total		3961	3957	99.9

*Overlapping tubes analyzed by infrared spectrophotometry

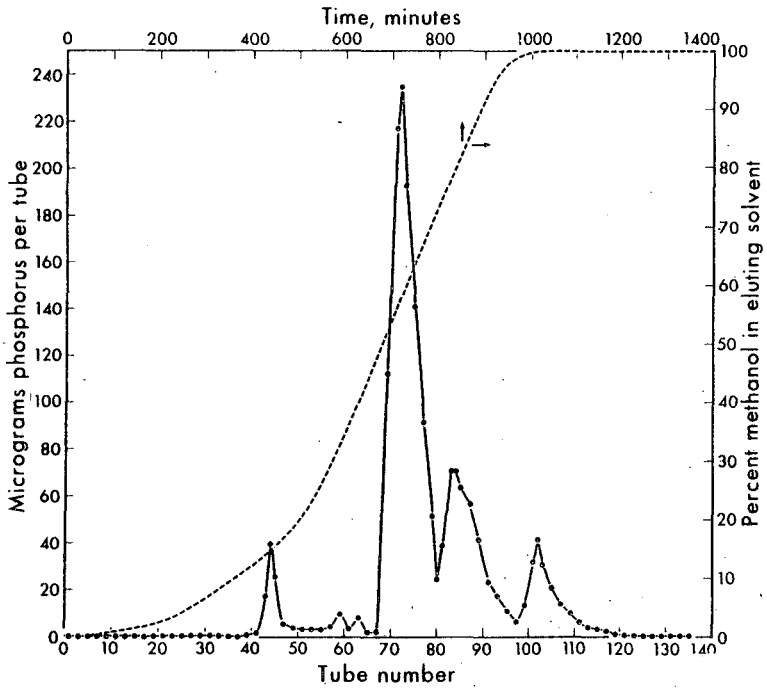

Figure 8. Experimentally determined elution pattern obtained by gradient elution chromatography of phospholipids extracted from normal human serum. Points on the curve represent phosphorus determinations on samples from every odd-number collection tube. The methanol in chloroform gradient was produced by conditions identical to those described in the caption to Fig. 7. Weight added to column was 2.89 mg of P, and 2.94 mg. were recovered as determined by chemical P analysis. Main components can be located by reference to Fig. 7. See text for details of method.

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determining the elution position of the various components that will depend on the shape of gradient used.

Regardless of the shape of the gradient the order of elution of different phospholipids from silicic acid column remains constant. Figure 11 summarized this sequence as determined by the analytical methods listed above for the gradient elution from 20 gm columns in this laboratory. Aside from phosphatidyl ethanolamine, phosphatidyl serine and the plasmalogen analogues of various phospholipids, most naturally occurring phospholipids can be separated by this scheme. Lecithin and sphingomyelin overlap slightly. Variations in the ratios of the phospholipids present in a mixture will affect the resolution to some extent although perhaps less than one would expect and certainly less than in non-gradient systems.

In the samples investigated here no material eluted off the column after lysolecithin, although the triphosphoinositol reported recently by Tomlison and Ballou[30] and by Dittmer and Dawson[31] would be expected to elute after lysolecithin if it can be eluted from silicic columns at all.

In serum and liver little or no cerebrosides or gangliosides are present and, hence, would not contaminate the various phospholipid fractions obtained by chromatography. In erythrocytes and other tissues various amounts of these compounds are present and have been observed to elute with the phospholipids. In chromatography of

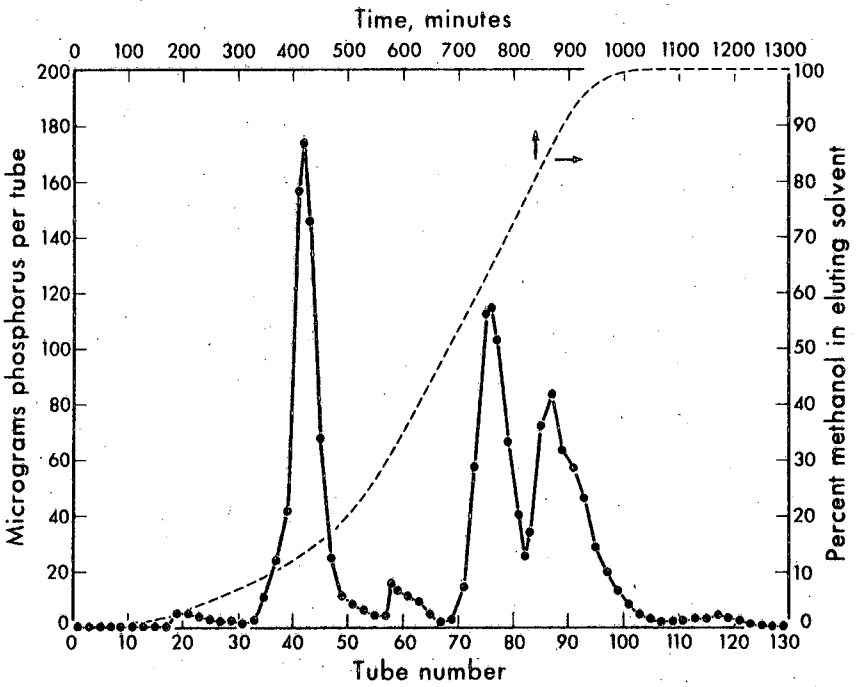

Figure 9. Experimentally determined elution pattern obtained by gradient elution chromatography of phospholipids extracted from normal human erythrocytes. Chromatographic conditions are described in the captions to Fig. 7 and 8. Weight added to column, 2.73 mg P; recovery, 2.77 mg P. Collection tubes 18 to 30 and 56 to 66 contained polyglyceryl phosphatides and monophosphoinositol respectively. Main components can be located by reference to Fig. 7. See text for details of method.

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the red blood cell phospholipids shown in Figure 9, cerebroside-like material was found in tubes 15 through 20 and 45 through 60, amounting to approximately 10% of the total lipids extracted from the erythrocytes by methanol-chloroform. Although no detailed studies of the elution characteristics of the cerebrosides were attempted, it is feasible that these sphingolipides also can be resolved by this procedure from phospholipids.

The phospholipids that eluted with low concentrations of methanol from the column exhibit much more symmetric elution peaks than those needing higher concentrations. The primary reason for the overlapping of the lecithin and sphingomyelin peaks is not the similarities of the migration rates, but the tailing of lecithin fraction into the sphingomyelin peak caused by this asymmetry. The synthetic lecithin, containing only palmitic acid, produced an asymmetric elution peak as did the natural lecithins. Hence, this tailing can not be attributed solely to differences in the fatty acid moieties of the molecule, but is more likely a result of the non-linear adsorption isotherm of lecithin on silicic acid.

Conversely the distortions of the phosphatidyl ethanolamine elution peak at its base, observed in natural samples, is most probably due to unidentified lipids, because the pure synthetic phosphatidyl ethanolamine gave a symmetrical elution pattern. Generally, the symmetry of the elution peak is inversely related to the

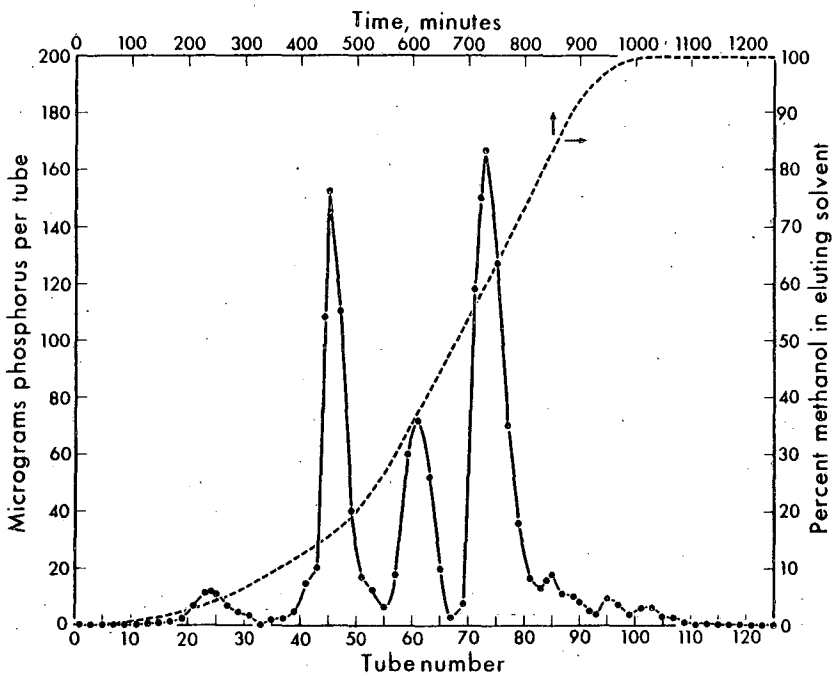

Figure 10. Experimentally determined elution pattern obtained by gradient elution chromatography of phospholipids extracted from normal mouse liver. Chromatographic conditions are described in the text and in the captions to Fig. 7 and 8. Weight added to column, 2.95 mg P; recovery, 2.90 mg P. Components can be located by reference to Figs. 7, 8, and 9.

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concentration of methanol needed to remove the substance from the column. The shape of the elution gradient can also influence the shape of the elution peak.

DISCUSSION

The results presented in this study indicate that concave gradient elution provides a technique capable of separating lipid mixtures with good resolution and without the necessity of multiple solvent changes during the run. Not only are so-called "false peaks" avoided but the method lends itself to complete automation. Once a run is started no attention need be payed to it until it is finished, thus allowing a run to continue overnight and for extended periods not feasible in manual operations.

Although Hirsch and Ahrens[17] as well as Hanahan and coworkers[32] had previously indicated that gradient elution column chromatography did not offer any advantages over stepwise schemes and, in fact, actually reduced the resolving power of the column only convex gradients were investigated by those workers. Conversely, the present study indicates that concave gradient elution of phospholipid mixtures yields better separations than any constant solvent or stepwise system operated under comparable conditions.

The activation of the column packing to the same state is absolutely necessary to obtain reproducible results from run to run, and a packing is good for only one

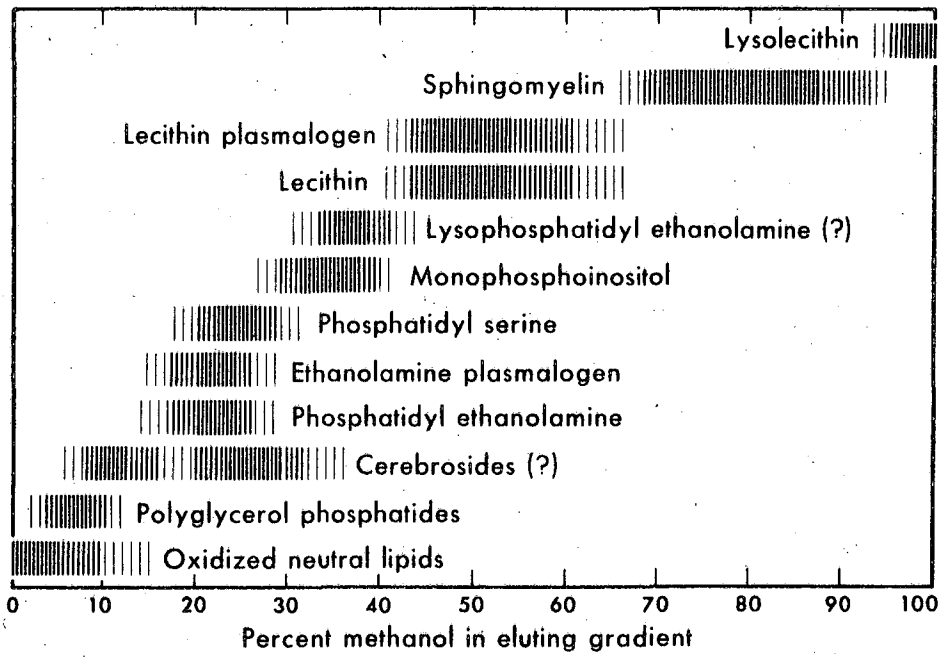

Figure 11. A schematic presentation of the relative elution characteristics of different phospholipids in the gradient elution system described in the text as determined by chromatography of reference compounds and specific chemical tests on the fractions obtained from chromatography of natural phospholipid mixtures extracted from serum, red cells and liver. Fully activated column eluted with methanol in chloroform gradient. The order of the sequence will be constant in any gradient elution although the particular concentration of methanol at which any individual phospholipid will elute from a column depends on the size of the column, the flow rate of the solvent and the shape of the gradient.

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run in this system. No solvent rejuvenation was able to return a packed column to its initial degree of activation. Furthermore, a column packing that was not fully activated showed poorer resolving power and shorter elution times than columns fully activated. The most prominent change observed with deactivated columns was a shortened retention time for the various lipids, either neutral lipids or phospholipids.

Several authors[33,34,35] have added water to their solvents in stepwise elution systems. In the recent scheme of Newman, Chiu and Zilversmit[35] the authors indicated that additions of 3% H_2O to their methanol greatly improved the resolution of the system. Addition of 3% H_2O to the methanol (the commercial reagent grade methanol used here had .05% H_2O) produced no noticeable improvement in resolution with the gradient elution system reported here.

The initial choice of a gradient is arbitrary. When some prior knowledge of the elution characteristics of the system is available, a gradient can be choosen to approximate a stepwise scheme. If two components are now well resolved, a reduction in the slope of the gradient will often proivde a better separation. Sometimes it is possible to separate compounds having identical elution times under one set of conditions by appropriate changes in the shape of the gradient.

The total cost of duplicating such equipment should be under 2,000 dollars which, although high, relative to most chromatographic systems, is not expensive compared to the cost of many commonly used analytical instruments found in modern laboratories. The superior performance of the system is believed to warrent the added cost.

In operation the system was found to be dependable. A reliable automatic fraction collector and indexing device is necessary for monitoring runs during developmental stages of an investigation. Batch collection can be used after the reproducibility of the system for a given mixture has been determined. It is advantageous to use a master timer to supply power to all components of the system. The timer then automatically shuts off all apparatus at the end of the run.

The use of this system for producing concave gradients is not restricted to silicic acid column chromatography of lipids. Any column chromatographic system would be applicable, as would many ion exchange separations. Piez and Morris[36] have already indicated that gradient elution of amino acid hydrolysates on the ion exchange column, using buffered solutions of different ionic strengths greatly enhances the operation.

Another modification of this system involves the addition of a second gradient pump leading to the mixing chamber, that would add a third solvent to the mixing chamber and lead to tertiary, rather than binary gradients. The mathematics of this type of system have already been investigated by the author and present no unusual difficulties. Whether such complex gradients can provide additional advantages to the chromatographic separation of lipid mixtures remains to be investigated.

SUMMARY

An automatic method utilizing concave gradient elution for the column chromatographic separation of lipid mixtures, particularly phospholipids, is described in detail. The gradient in this system is produced by an automatic pumping system using accurate, controlled-volume pumps and variable pumping rates. A discussion of the theoretical aspects of gradient elution in relation to non-linear absorption isotherms in included, and several chromatographic runs using the system are presented. The system is capable of excellent resolution of naturally-occurring phospholipid mixtures and is quite reproducible. Considerable flexibility is available in the shape of the gradient produced by the pumping system, allowing modification for particular tissue extracts containing phospholipids in varying ratios. The system is completely automatic and, once a run is started, operates untended until completion.

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IMMUNOLOGIC STUDIES ON THE MECHANISM OF ACTION OF ERYTHROPOIETIN*

By

John C. Schooley and Joseph F. Garcia

INTRODUCTION

The ability of serum obtained from rabbits previously immunized with human urinary erythropoietin to neutralize the erythropoietic activity of human urinary erythropoietin has been demonstrated.[1] Injection of such sera into normal mice markedly decreased their normal 24-hour incorporation of Fe^{59} into circulating red blood cells and their peripheral reticulocyte count. This neutralization was considered to be the result of an antigen-antibody reaction directed against either the erythropoietically active molecule or the general class of molecules to which erythropoietin belongs. The present experiments are concerned with the temporal changes in the relative numbers of different types of bone marrow cells and their proliferative capacities following the injection of such immune sera into normal mice. In addition, data are presented which further support the concept that the observed changes in the marrow are the result of neutralization of endogenous erythropoietin.

MATERIALS AND METHODS

Serum obtained from two rabbits previously immunized with alum precipitates of human urinary erythropoietin according to the schedule given by Kabat and Mayer[2] was pooled and termed anti-E. The human urinary erythropoietin was prepared by ultrafiltration on collodion as previously described.[3] The potency of this serum was such that four daily injections of 0.25 ml depressed the 24-hour Fe^{59} incorporation into circulating red blood cells of 10 normal mice to 1.5±0.2% compared to 26.9±2.1% following similar injections of normal rabbit serum.

Twenty male, Swiss mice, each weighing about 25 gm, were injected subcutaneously with 0.25 ml of anti-E on 4 consecutive days. A second group was injected similarly with normal rabbit serum. On the day following the first injection and for the next 9 days, two animals were sacrificed from each group and smears made of the bone marrow contained in one femur. The other femur, sternum, spleen, kidney and liver were fixed in buffered formalin. After decalcification of the femur and sternum, the tissues were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

One hour before sacrificing, the animals received an intravenous injection of 2 μc per gm body weight of tritiated thymidine (360 μc/μM, Schwarz Bioresearch, Inc.). Autoradiographs of the bone marrow smears were made using Kodak AR-10

*This work was supported by the United States Atomic Energy Commission.

stripping film. The autoradiographs, exposed for two weeks, were developed, fixed and stained with Giemsa. Bone marrow smears not used for autoradiographs were stained with Ralph's stain,[4] followed by Giemsa in order to identify more readily the erythroid cells. The percentage of erythroid cells, mature neutrophils, "small round cells" or lymphocyte-like cells, and disrupted or unidentified cells was determined for each marrow smear. A total of at least 1000 cells was counted. Bone-marrow smears of hypertransfused mice were also examined at various times after hypertransfusion.

Female Swiss mice, each weighing about 23 gm were hypertransfused by intraperitoneal injection of 1 ml of 80% donor red-blood cells on two consecutive days. The donor red-blood cells were washed three times in heparinized saline and the buffy coat removed before injection into the recipient animals. Five days after the last transfusion a wave of erythropoiesis was initiated by the intravenous injection of 50 μgm* of human urinary erythropoietin in 0.1 ml of saline. At various times before and after the injection of erythropoietin, groups of 10 mice were injected intravenously with 0.1 ml of anti-E. Control groups consisted of ten saline-injected mice and ten mice that received only erythropoietin. Two days after the erythropoietin injection, 0.5 μc of Fe^{59} in 0.2 ml saline was injected intraperitoneally. Three days later the percent of the injected Fe^{59} in the total circulating red blood cells was determined, assuming that the blood volume was 5% of the total body weight. The hematocrits of the assay animals at the time of sacrifice averaged 65%. These and other aspects of this assay have been presented by DeGowin et al.[5]

RESULTS AND DISCUSSION

The temporal changes in the percentage of erythroid cells in marrow of normal mice injected with anti-E is shown in Figure 1. Values shown for each day are the average for two animals. The percentage of erythroid cells present in the marrow of normal mice injected with normal rabbit serum was not significantly different during the 10 days following the first injection; therefore the mean value for all animals is indicated with the corresponding standard error. Following the injection of anti-E there was a progressive daily decrease in the percentage of marrow erythroid cells that became maximal on the 5th and 6th day after the first injection of anti-E or on the 2nd and 3rd day after the last injection. The few erythroid cells observed in the marrow at this time were primarily the more mature erythroid cells, although cells representing all stages of erythroid maturation could be found. The percentage of erythroid cells found in 5 mice on the 5th day after the initiation of transfusion-induced polycythemia is also indicated in Figure 1

*Direct comparison of the human urinary erythropoietin with Armour Lot No. K103-124 sheep erythropoietin in the starved-rat assay indicates that this dose contains approximately 1.2 cobalt units.

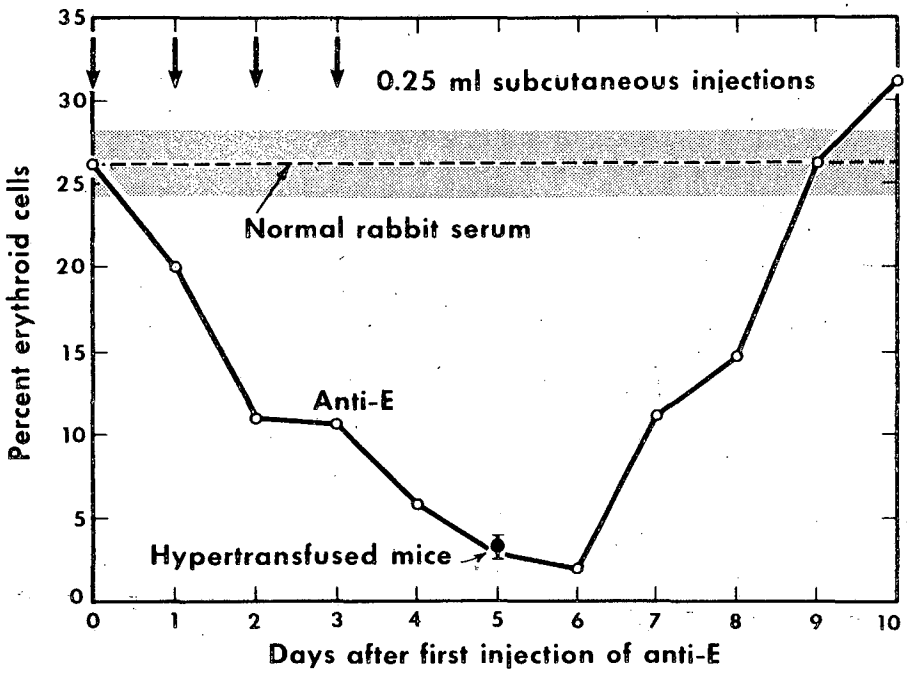

Figure 1. Temporal changes in the percentage of erythroid cells in marrow of normal mice injected with anti-E.

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(hypertransfused mice). The cytologic and histologic appearance of the bone marrow of anti-E injected normal mice and polycythemic mice are, at this time of maximal erythroid suppression, indistinguishable, except that more mature red blood cells are observed in the polycythemic marrow.

A progressive daily increase in the percentage of erythroid cells occurs after the 6th day, and by the 9th day the percentage of erythroid cells has returned to that found in mice injected with normal rabbit serum. Presumably this increase in erythroid cells is due to the presence of endogenous erythropoietin. This erythropoietin may result from an increased rate of production by the mouse and/or, more likely, a decreased level of neutralizing antibody as a result of the elimination of heterologous rabbit serum and the stoppage of anti-E injections.

An increased number of mature marrow neutrophils was observed following the injection of both anti-E and normal rabbit serum. There was some suggestion that increased percentages of "small round cells" resembling lymphocytes occurred in the anti-E injection animals prior to the reinstitution of erythropoiesis; however, too few mice were studied in these experiments to evaluate this suggestive difference. It is our impression that a similar increase in these cells occurs in polycythemic mice. Quantitative measurements of these relative changes in the marrow population are in progress. Injections of anti-E did not affect the relative numbers of

megakaryocytes in sections of the femur or sternum when compared to animals injected with normal rabbit serum, nor was there any obvious difference in the histological appearance of the kidneys, spleens and livers.

Autoradiographs made of bone-marrow smears one hour after the injection of tritiated thymidine were examined as an adjunct to the previous study. Injection of anti-E did not obviously affect the endogenous DNA synthetic capacity of individual marrow cells. Even at the time of maximal decrease in the percent erythroid cells following anti-E injections, the few immature erythroid cells found in the marrow were labeled and therefore presumably able to synthesize DNA. Similar observations have been made in polycythemic mice. Cells of the granulocytic series normally capable of DNA synthesis were labeled after anti-E injection or polycythemia. The subsequent ability of the labeled erythroid cells to divide could not be determined in the present experiments; however, granulocytic cells were apparently capable of division, because no particular decrease in their relative numbers was observed.

Erythropoietin is believed to control erythropoiesis by regulating the differentiation of stem cells into the erythroid series. It has been postulated that this regulation is the result of an enzyme induction in the stem cells.[6] The temporal changes seen in the erythroid population, the similarity of these changes to those observed after hypertransfusion, and the ability of immature erythroid cells to synthesize DNA after injection of anti-E support the concept that anti-E exerts its effects on erythropoiesis by neutralizing erythropoietin. The fact that anti-E injections into normal mice decrease the erythroid population also supports the concept that erythropoietin is involved in the normal control of erythropoiesis. Cytotoxic effects of anti-E on erythroid cells might also result in a decrease in erythroid cells. Several observations argue against extensive cytotoxic effects. The orderly gradual decrease observed in erythroid cells would not be expected if anti-E non-discriminately lysed the erythroid cells. Hemoglobinuria or hemoglobinemia, that might be expected if extensive cytolysis of hemoglobin-containing cells occurred, was not observed.

Support for the concept that anti-E neutralizes erythropoietin and evidence against a cytotoxic effect were obtained by determining the effect of anti-E injections on erythropoiesis in polycythemic mice. Filmanowicz and Gurney[7] have observed an orderly wave of erythropoiesis in the spleen of hypertransfused mice after the injection of purified sheep plasma erythropoietin. We have observed a similar wave in the bone marrow following injections of human urinary erythropoietin. During the first 12 to 18 hours, the marrow of polycythemic erythropoietin-stimulated mice appears cytologically little different from that of non-stimulated animals, although it is our impression that an increase in the relative numbers of reticulum cells or hemocytoblasts occurs. One day after erythropoietin stimulation, a peak percentage increase in proerythroblasts occurs followed on the second day by a peak in the percentage of basophilic erythroblasts. On the third day the peak percentage

of reticulocytes is found in the peripheral blood and a day or so later the marrow is again devoid of erythroid cells.

The effect of anti-E injections on the erythropoietic response of polycythemic mice to exogenous erythropoietin, as a function of the time of anti-E administration (hours) after erythropoietin stimulation, is shown in Figure 2. The amount of anti-E injected was calculated to be in excess of that required to neutralize completely all the exogenous erythropoietin injected. When this amount of anti-E was injected one hour before or at the same time as the erythropoietin, no stimulation of erythropoiesis occurred; i.e., the Fe^{59} incorporation in the calculated blood volume of 0.10 ± 0.01% was not different from the saline injected value of 0.13 ± 0.02%. The Fe^{59} incorporation observed when erythropoietin was injected alone is indicated in Figure 2.

Injection of anti-E one or two days after the initiation of erythropoiesis, when identifiable erythroid cells are present in the marrow, had no significant effect upon the subsequent ability of these erythroid cells to develop into mature red blood cells as measured by Fe^{59} incorporation. This indicates that anti-E did not exert a cytotoxic effect on these erythroid cells and that anti-E did not alter the ability of these erythroid cells to synthesize DNA, to divide, or to utilize Fe^{59} for hemoglobin synthesis. We have found that the iron levels and iron-combining capacities of plasma obtained from normal mice injected with anti-E are within normal limits, and that absorption of anti-E with transferrin (Cohn Fraction IV-4, human) does not alter its neutralizing ability, which suggests that anti-E does not disturb the transport of iron to the marrow. These observations support the concept that erythropoietin is not concerned with the maturation of erythroid cells but only with the differentiation of stem cells into the erythroid series.

Any interpretation of the effects of anti-E on the wave of erythropoiesis during the first 12 to 18 hours after injection of erythropoietin must be speculative. It seems reasonable to assume, although direct experimental evidence is lacking, that during these times a certain amount of the erythropoietin has acted in some fashion on receptive bone marrow stem cells so that some are destroyed in a non-selective manner without exerting any erythropoietic stimulation, and some free erythropoietin remains to stimulate stem cells or to be destroyed at a later time. Injection of anti-E apparently neutralizes the free erythropoietin that remains in the animal, and further erythropoietic stimulation by this erythropoietin cannot occur. That the erythropoietin-stimulated cells do continue to differentiate and mature in the presence of anti-E provides further evidence that anti-E does not exert a cytotoxic effect even at earliest stages of erythroid maturation. The absence of any effect on these early stimulated erythroid cells suggests that the combining sites on the erythropoietin molecule are no longer available for an antigen-antibody reaction once erythropoietin has stimulated the cell. Possibly erythropoietin exerts its stimulatory effect within the stem cell rather than on the cell surface.

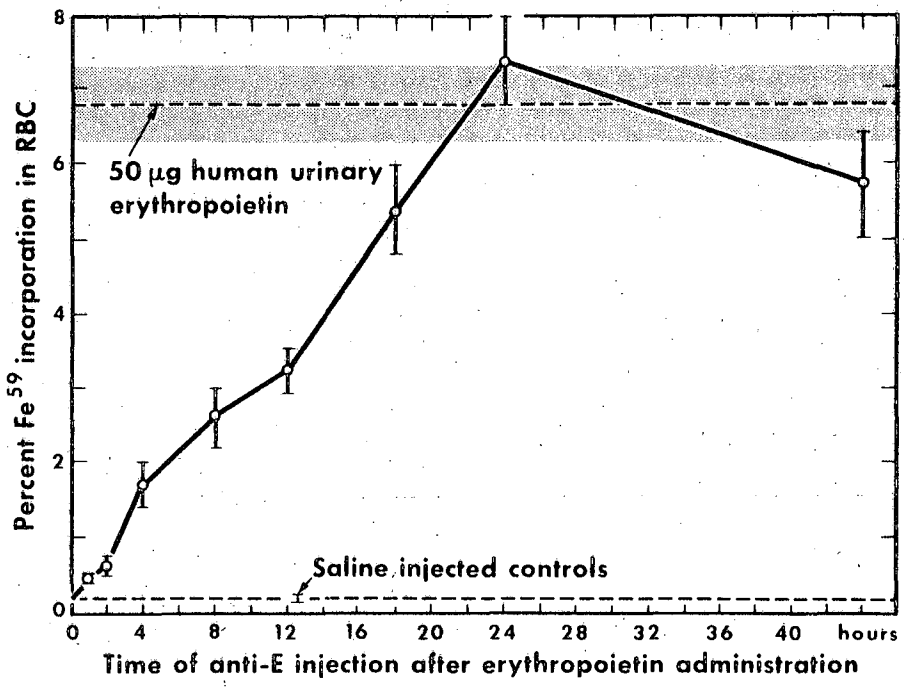

Figure 2. Hypertransfused mouse assay
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When erythropoietin is allowed to act for 12 hours before the injection of anti-E, a sufficient number of stem cells are stimulated so that their subsequent proliferation results in the formation of enough red cells to give an Fe^{59} incorporation approximately equal to half that obtained if the erythropoietin is allowed to act for 24 hours or if no anti-E is injected. Reference to a dose-response curve for human urinary erythropoietin obtained by using this same assay indicates that less than half of the injected erythropoietin stimulated erythropoiesis at this time. Thus, the rate of disappearance of erythropoietin in these hypertransfused mice is much slower than has been observed in rats.[8] Erythropoietic stimulation decreases as the time between erythropoietin and anti-E injections decreases, but even 0.5 hour exposure of the animal to erythropoietin is sufficient to stimulate some stem cells.

Why does erythropoietin require at least 24 hours to stimulate erythropoiesis maximally in these animals? The ability of erythropoietin to stimulate erythropoiesis and the magnitude of this stimulation during any time interval may depend upon a variety of factors, such as the size of the stem cell compartment, the availability of erythropoietin to cells within this compartment, the ability of a particular stem cell to respond when exposed to erythropoietin, and competition for stem cells between erythropoietin and factors stimulating the differentiation of stem cells into other types of marrow cells. It seems reasonable to assume that within an

hour or so after intravenous injection erythropoietin is available to all potential stem cells. The generation times of 8 to 12 hours, generally found for rapidly dividing cells, suggests that an increase in the stem cell compartment as the result of cell proliferation could not occur before 8 to 12 hours; however, after this time such a proliferation may be important. We are inclined, therefore, to attach more importance to the last two factors listed above in explaining the difference in the erythropoietic response observed when the same dose of erythropoietin is allowed to act for 2 and 8 hours.

SUMMARY

Injections into normal mice of serum obtained from rabbits previously immunized with human urinary erythropoietin results in a progressive daily decrease in the relative numbers of erythroid cells in the bone marrow. Injections of this serum into polycythemic mice 24 to 44 hours after the initiation of a wave of erythropoiesis with erythropoietin has no effect on the magnitude of the erythropoietic response, although injections of the serum one hour before or at the time that exogenous erythropoietin is administered completely abolished the erythropoietic response. These results support the concepts that erythropoietin is involved in the normal regulation of red cell production and that this regulation is the result of the effect of erythropoietin on the differentiation of stem cells. Some speculative interpretations of the effect of injections of the immune serum in polycythemic mice during the first 18 hours after the injection of exogenous erythropoietin are also presented.

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