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L. R. B. Mann and V. Moses

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Properties of *Escherichia coli* Grown in Deuterated Media

BY L.R.B. MANN* AND V. MOSES

Laboratory of Chemical Biodynamics and Lawrence Radiation Laboratory
University of California, Berkeley, Calif., U.S.A. 94720

SUMMARY

Acetate-grown cells of *Escherichia coli* would not grow in highly deuterated media containing deutero-acetate without a training sequence in which the level of enrichment with deuterium was gradually increased. No training was required for growth in deuterated medium containing protio-glucose. After adaptation to D_2O-acetate, the growth rate was about half that in H_2O-acetate. The effects on a number of parameters of transferring cells between protonated and deuterated media were studied; these included the synthesis of DNA, RNA, total protein, β-galactosidase, growth and oxygen consumption. Similar measurements were made on cells fully adapted to growth on deuterated media. The amino acid compositions of deuterated and protonated cellular protein were similar, but in deuterated cells the ratio protein:DNA was doubled. Deutero- and protio-β-galactosidase had similar K_M values and turnover numbers in D_2O and H_2O. The kinetics of β-galactosidase synthesis were not changed by deuteration; it was found, however, that lower concentrations of inducer were required

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*Present address: Department of Biochemistry, University of Auckland, Auckland, New Zealand. Please address requests for reprints to Berkeley.

to achieve particular levels of induction. Brief exposure to inducer in one medium, followed by removal of inducer and expression of enzyme forming potential in either D_2O or H_2O, showed that mRNA synthesized by deuterated cells was translated equally well in both media. mRNA synthesized by protonated cells was translated about twice as efficiently in H_2O. We do not know whether this was a direct consequence of deuteration on the translation mechanism, or a secondary response involving catabolite repression. Inducible strains (but not a regulator constitutive) lost the capacity to synthesize enzymically active β-galactosidase after more than 100 generations in D_2O-acetate. The defect persisted when such cells were grown in H_2O-acetate, but enzyme activity was restored by growth in H_2O-glycerol. The failure to produce active enzyme was not due to a failure of the induction mechanism; gel electrophoresis revealed the presence of an inactive protein species. The nature of adaptation to deuteration is discussed. It is concluded that an important factor is a reduction of catalytic efficiency for a wide variety of enzymes, which is compensated by increased derepression for these proteins (Orgel, 1964). There is evidence for a number of other differences between protonated and deuterated cells.

INTRODUCTION

Deuteration is a particularly interesting biological problem. Because of the universal importance to living systems of hydrogen bonding in determining the conformations and activities of macromolecules, and in biochemical recognition phenomena, it is to be

expected that widespread biological repercussions would follow from deuteration. Slight changes have been detected in hydrogen bond length when deuterium is substituted for protium (Gallagher, 1958). In some reactions the rates for deuterated substrates are different from those of their protiated analogues (Pereira-Farjaz & Jacobsohn, 1936; Thomson, 1960). It has been suggested that deuteration would result in a marked decrease in the rate of spontaneous genetic mutation (Löwdin, 1963); experimental evidence on this point is currently contradictory (Pollard & Lemke, 1965; Jung, 1968).

The potential hazards that deuteration holds for a living cell are varied, and many of them must be biological novelties. One cannot regard it as likely that at any time in its evolutionary history an organism found itself in a nutritional environment highly enriched for deuterium. Thus, exposing a contemporary cell to such an environment presents it with a threat to its fundademtal molecular organization, which will not previously have been encountered.

Media highly enriched in D_2O have long been known to be inhibitory to living organisms (Lewis, 1933). Microbial studies have appeared on the behaviour in deuterated media of algae (Weinberger & Porter, 1954; Moses, Holm-Hansen & Calvin, 1958; Walker & Syrett, 1959; Chorney, Scully, Crespi & Katz, 1960; Crespi, Archer & Katz, 1960; Katz, 1960) and bacteria, including E. coli (Rittenberg & Borek, 1961; Henderson, 1962; Henderson, Dacus, Crespi & Katz, 1967). A summary of many biological effects is given by Thomson

(1963). Many questions remained unanswered, and it seemed to us of interest to investigate the effects of deuteration in such a well-understood and versatile organism E. coli. We recognized at the outset that we were unlikely to elucidate the whole story, and indeed we have not done so. Nevertheless, a number of interesting observations have been made, and we have no doubt that further investigations into these phenomena will increase our understanding of fundamental molecular biological realities.

MATERIALS AND METHODS

Bacterial strains. The following strains of E. coli were used; their sources and genotypes are noted: 300U (lac $i^+o^+z^+y^-$) and 230U (lac $i^-o^+z^+y^-$) from Dr. J. Monod; 2000-o^c (lac $i^+o^cz^+y^+$) from Dr. C. Willson; 3000-o^c_{67} (lac $i^-o^cz^+y^+thr^-$; see Davies & Jacob, 1968) from Dr. E. Steers; Cavalli (lac $i^+o^+z^+y^+met^-thy^-$) from Dr. A. Simmons; E203 (carries two or three wild-type lac operons) from Dr. A. Novick. All genotypes were checked before use.

Growth media. Media were based on minimal medium 63 (Pardee & Prestidge, 1961). When required, amino acids were added to 50 mg./l., and thymine to 2 mg./l. Carbon sources were either acetic acid (33 mM) or glucose (11 mM).

Fully deuterated media were usually made by dissolving all the solid components, and fully-deuterated acetic acid, in a minimal vol. of D_2O (99.8 atom % D). The pD was adjusted with solid NaOH to 7 (the pH of protonated medium 63) using electrodes soaked for some hr. in D_2O. (It is known (Glasoe & Long, 1960) that a pH meter

coupled to electrodes soaked in D_2O gives a reading of pH - 0.4). After some hr. at room temp. to permit complete exchange of H linked to N, O and S, the solution was freeze-dried. The residue was dissolved in a minimum vol. of 99.8% D_2O and lyophilized again. The residue was finally dissolved in the required vol. of 99.8% D_2O and the D/H ratio determined by nuclear magnetic resonance against standards of known composition. The D/H ratio was always at least 200/1 even after the medium had been used to grow cells. The ^3H content, determined by scintillation counting, was never more than 90 nCi./ml., and usually less than 0.9 nCi./ml. Fully-deuterated acetic acid was used as the carbon source for most of this work, as it is by far the least expensive fully-deuterated carbon source acceptable to E. coli. Carbon sources other than acetate, and supplements for auxotrophes, were not enriched in D at the non-exchangeable positions.

The only medium we have used in which the carbon source was fully deuterated was the one just described. This will be referred to as "all-D acetate medium", and cells grown in it will be described as "all-D cells". The corresponding media based on H_2O and protio-acetate, and the cells grown in it, will be called "all-H acetate medium" and "all-H cells", respectively. Media and cells other than these will be identified separately.

A close approximation to all-D acetate medium was achieved without the freeze-drying procedure. Dissolution of all the medium components (as anhydrous salts) in 99.8% D_2O, and adjustment of the pD with solid NaOH, reduced the enrichment of D from 99.8% to 99.43%.

The addition of an all-H carbohydrate at a concn. of 0.2% reduced the D enrichment to about 99.37% by equilibration of the exchangeable H. Non-exchangeable H in the carbohydrate might contribute more directly than H_2O in the medium to cellular H. Media were sterilized by filtration through dry sterile membrane filters (0.45 μ pore size).

Growth conditions. Cells were grown aerobically in stoppered vessels at 37° with agitation. Growth was measured by E_{650} in a 1 cm. cuvette using a Beckman DK-2 double-beam spectrophotometer. The validity of measuring growth in this way is considered in the Results section. Stock cultures were transferred every 3 days for all-H cultures, and every 7-14 days for all-D cultures

In transferring cells from one medium to another during experiments, they were harvested on a membrane filter, washed extensively in the new medium, and resuspended in the latter. The temp. was maintained at 37° during these manipulations.

Induction and assay of β-galactosidase. The lactose enzymes were induced with 0.5 - 1 mM-isopropylthiogalactoside (IPTG) which, while not enriched in D, was dissolved in D_2O. Samples for assay of enzyme activity were taken as described by Palmer & Moses (1968). Differential rates of β-galactosidase synthesis are expressed as P values: increase in nmoles ONPG hydrolysed/min./ml. culture/increase in E_{650} (Herzenberg, 1959).

Labelling with radioactive precursors. For cumulative labelling, cells were grown for the desired period in the presence of the labelled precursor. Samples were taken at intervals into an equal

vol. of 10% (w/v) trichloroacetic acid at 0°, and held on ice for 1-3 hr. They were then filtered and the precipitated radioactivity measured in a scintillation counter (Moses & Prevost, 1966). For pulse-labelling experiments, samples (0.5 ml.) of the growing culture were mixed with 0.01 ml. of the precursor solution. The cells were shaken at 37° for an appropriate period, and incorporation terminated by the addition of an equal vol. of cold 10% (w/v) trichloroacetic acid containing unlabelled precursors. They were then treated as above.

When a labelling experiment was being performed with an all-D culture, the labelled compounds were dissolved in D_2O.

Cell size measurements. Cell sizes were determined with a modified Coulter-type particle counter connected to a pulse-height analyser, read-out unit and plotter, similar to that described by Harvey & Marr (1966).

Amino acid analysis. Cells in exponential growth were harvested by centrifugation and extracted for 30 min. at 45° with 75% (v/v) ethanol. This treatment extracts free amino acids but very little protein (Roberts, Abelson, Cowie, Bolton & Britten, 1955). The solids were then sedimented by centrifugation and hydrolysed with 6 N-HCl at 110° for 8 hr. in sealed tubes *in vacuo*. After cooling, HCl was removed by evaporation under reduced pressure, the residues dissolved in water and again evaporated to dryness. They were finally dissolved in citrate buffer, and the amino acids separated with an Amino Acid Analyzer Model 120C (Beckman Instrument Co., Fullerton, Calif., U.S.A.).

Soluble protein profiles. After harvesting, the cells were disrupted by sonication and the soluble proteins separated by electrophoresis in polyacrylamide gel (Moses & Wild, 1969).

Preparations of β-galactosidase. Crude extracts were prepared from late exponential cultures. The cells were washed and suspended in fresh all-H or all-D medium as required. They were disrupted with a French pressure cell, and cell debris removed by centrifugation.

Purified preparations were made with the hyper-producing strain E203. β-Galactosidase produced by this strain shows a normal K_M value and thermal inactivation profile (Horiuchi, Tomizawa & Novick, 1962). All-D or all-H cells from a late exponential culture were concentrated several fold in the appropriate medium supplemented with 0.01 M-2-mercaptoethanol (Craven, Steers & Anfinsen, 1965). The cells were disrupted by sonication at 0°. Proteins in the cell extract were separated on cylindrical polyacrylamide gels. Staining with Amido black of gels from induced and uninduced cells showed the presence in induced cells of an additional prominent band corresponding to the position of β-galactosidase (Fairbanks, Levinthal & Reeder, 1965; Craven et al., 1965; Moses & Wild, 1969). To detect the enzyme on unstained gels, they were rolled on filter paper moistened with a solution of o-nitrophenylgalactoside (ONPG). A fairly sharp yellow band appeared on the gel corresponding to the position presumed from the stained gels to be that of β-galactosidase. The enzyme was extracted from the ONPG-stained part of the gel by homogenization in the desired buffer. Analysis of these solutions

by gel electrophoresis showed the presence of a major sharp band of β-galactosidase; minor bands of β-galactosidase were sometimes detected (Alpers, Steers, Shifrin & Tomkins, 1968). The enzymic activities of the preparations were determined as above, and the protein content measured by the method of Lowry, Rosebrough, Farr & Randall (1951), using standards of bovine serum albumin.

Respiration. The rates of O_2 uptake were measured in standard Warburg manometers. All solutions were enriched with D to the appropriate degree. Thus, the solutions of KOH used to absorb respiratory CO_2 had the same D/H ratio as the medium in which the cells were suspended. The filter paper concertinas used to aid CO_2 absorption were dehydrated at 110° and equilibrated after cooling over D_2O/H_2O mixtures of the appropriate ratios.

Chemicals and radiochemicals. All labelled precursors were from New England Nuclear Corp., Boston, Mass., U.S.A. IPTG and ONPG were obtained from Calbiochem, Los Angeles, Calif., U.S.A. D_2O (99.8% atom % D) and deuteroacetic acid (99.5 atom % D) were from International Chemical and Nuclear Corp., City of Industry, Calif., U.S.A.

RESULTS

Growth in deuterated media

Adaptation to deuterated media

Considerable variability was observed in the rapidity with which cells adapted to growth in deuterated media. No strain was consistently more adaptable, and the same strain on different occasions demonstrated widely differing capacities to adapt.

Generally, cells would not grow when transferred from all-H acetate medium directly into all-D acetate, though they did so occasionally. A training sequence was required in which cells were grown consecutively in media containing 50, 75, 87, 94, 98 and 99.8 atom % D. Occasionally, growth would fail to continue when the enrichment with D exceeded 90 atom % D. The ability to grow in all-D acetate without training was not affected by inoculum size.

The effects were tested of adding a number of amino acids, singly or in combination, on the ability of cells to grow on first exposure to all-D acetate. Casein hydrolysate (1 mg./ml.) clearly permitted growth at about 50% of the rate of cells from the same batch inoculated into all-H acetate containing this mixture. Investigation of the effects of individual amino acids, and of some pairs, yielded erratic results only.

The specific effects of deuteration of the acetate on the growth of cells in deuterated and protonated media were also studied. Cells previously grown in all-H acetate medium were inoculated into deuterated or protonated media containing CH_3COOD, CD_3COOD, CH_3COOD or CH_3COOH. Table 1 shows that there was a marked inhibition by $[CD_3]$acetate in deuterated medium, with a slight inhibition in protonated medium.

[INSERT TABLE 1 NEAR HERE]

After adaptation to all-D acetate was complete, the growth rate was typically 0.17 - 0.25 doublings/hr., compared with 0.4 - 0.5 doublings/hr. in all-H medium. On being returned from all-D acetate to all-H acetate, no growth lag was observed and, indeed, an abnormally high rate of growth was usually obtained (0.56 - 0.77

doublings/hr.). The rate of loss of adaptation to deuterated medium was measured by transferring cells grown in all-D acetate to all-H acetate. At various times thereafter, their ability to grow without lag in all-D acetate was examined. Table 2 shows that 1.5 - 2 generations of growth in the absence of D_2O were sufficient to abolish completely adaptation to all-D acetate.

[INSERT TABLE 2 NEAR HERE]

With glucose instead of acetate as the carbon source, growth in highly deuterated media was much more readily achieved, though it must be noted that we did not use fully-deuterated glucose. In one experiment, cells of 300U grown in all-H acetate were filtered, washed, and inoculated into protonated and deuterated media containing glucose. The rates of growth were 0.94 and 0.26 doublings/hr., respectively; there was no significant lag period. Cells grown in glucose media did not lose their ability to grow in a highly deuterated medium after a period of growth in a protonated environment. A culture of strain 300U, grown for many generations in D_2O-glucose medium, was resuspended in H_2O-glucose medium. At intervals, portions of the culture were filtered, washed and resuspended in D_2O-glucose medium. Table 3 shows that the growth rates in deuterated medium actually increased after a period of growth in protonated medium.

[INSERT TABLE 3 NEAR HERE]

Respiration in deuterated media

The rates of O_2 uptake were measured in cells grown in all-H acetate. The cells were harvested, washed and suspended in all-D

acetate. Samples (0.2 ml.) were placed in the side-arms of pairs of Warburg flasks, the main compartments of which contained 1.8 ml. media of appropriate D/H ratios to give, on mixing, enrichments of 10, 60, 80, 90, 95 and 99.8 atom % D. One flask from each pair contained chloramphenicol (100 μg./ml.); the antibiotic was omitted from the other flask of the pair. KOH/KOD in H_2O/D_2O mixtures of the appropriate D enrichments were added to the centre-wells to absorb CO_2. The cells were mixed with the medium, and O_2 uptake followed for 135 min. at 37°. Fig. 1 shows the total vol. of O_2 absorbed during the experiment, in the presence and absence

[INSERT FIG. 1 NEAR HERE]

of chloramphenicol, as a function of enrichment with D. In the presence of chloramphenicol, growth was blocked and O_2 was linear with time. In the absence of the inhibitor, the cells were able to grow, and the rates of O_2 consumption gradually increased. Thus the ratio, O_2 uptake without chloramphenicol: O_2 uptake with chloramphenicol, provides an indication of the extent of growth during the experiment. Fig. 1 shows that the inhibition of growth by deuteration closely paralleled the inhibition of respiration. We conclude that the inability of cells to oxidize acetate on initial exposure to all-D acetate medium constitutes at least part of the reason for their failure to grow.

In a similar experiment, the ability of cells to oxidize $[CD_3]$acetate and $[CH_3]$acetate was compared. Table 4 shows a slight consistent inhibition by $[CD_3]$acetate.

[INSERT TABLE 4 NEAR HERE]

Protein/DNA ratios in protonated and deuterated cells

Orgel (1964) suggested that adaptation to deuteration involved the derepression of many enzymes whose catalytic activity would be impaired by deuteration. He predicted that a consequence would be an increase in the protein/DNA ratio.

Deuterated and protonated lines of Cavalli strain were grown in media containing L-[$^{14}CH_3$]methionine and [C^3H]thymine. This auxotrophic strain requires both these precursors so that all of these components incorporated into macromolecules would have the same specific radioactivity as the precursors. It has been shown (Moses & Calvin, 1965) that essentially all the ^{14}C from L-[$^{14}CH_3$] methionine is incorporated into protein methionine. Fig. 2 shows that cells fully adapted to all-D acetate incorporated 2.1 times more methionine/mole of thymine than did those grown in all-H acetate.

[INSERT FIG. 2 NEAR HERE]

Amino acid composition of protonated and deuterated cells

The molar proportions of amino acids in hydrolysates of cells grown in all-H acetate and all-D acetate are shown in Table 5. The values for all-H acetate agree closely with those given by Roberts

INSERT TABLE 5 NEAR HERE]

et al. (1955). Few differences were observed between protonated and deuterated cells, except for methionine. The lower proportion of methionine in the protein of the deuterated cells indicates that the increased protein/DNA ratio shown in Fig. 2 is a minimum value.

It can be calculated from the data of Table 5 and Fig. 2 that the increased protein/DNA ratio in deuterated cells is largely the result of more protein rather than less DNA. As would be expected from such a result, deuterated cells were larger than those grown in protonated medium. Fig. 3 shows size distributions, corresponding to cell vol., using a simultaneous pulse-height

[INSERT FIG. 3 NEAR HERE]

analyser with a Coulter counter. At stationary state, the peak vol. of deuterated cells was about 1.5 times that of protonated cells.

Catalytic properties of protonated and deuterated β-galactosidase

The Michaelis constants (K_M) of β-galactosidase from protonated and deuterated cells of strain 3000-o^c_{67} were measured in D_2O and H_2O. Fig. 4 illustrates a slight solvent effect on the K_M of the protonated enzyme, while the effect on the deuterated enzyme was smaller and probably not significant.

[INSERT FIG. 4 NEAR HERE]

Table 6 shows that the turnover numbers for the catalytic activities of protonated and deuterated enzymes, measured in H_2O, were essentially identical. The value for the protonated enzyme

[INSERT TABLE 6 NEAR HERE]

was some 40% higher than that reported by Cohn (1957), and may reflect a greater purity of our preparation.

Synthesis of DNA, RNA and protein

The exposure without prior adaptation of all-H cells to all-D medium rapidly terminated the synthesis of RNA and protein, the induced synthesis of β-galactosidase, and the increase of turbidity

(Fig. 5). The transfer of D-adapted cells to all-H acetate produced a rapid increase in RNA and protein typical of a growth

[INSERT FIG. 5 NEAR HERE]

upshift (Fig. 6). The specific rate of RNA synthesis declined after about 0.5 generations in protonated medium. DNA synthesis was delayed, and showed no marked increase until nearly 1.3 generations

[INSERT FIG. 6 NEAR HERE]

in protonated medium. When deuterated cells which had been grown for 1.7 generations in all-H acetate were returned to all-D medium, the synthesis of all macromolecules was reduced to a low rate (Fig. 6). Both growth and the incorporation of precursors into macromolecules were lower in deuterated medium after exposure to protonated medium (cf. Table 2).

The synthesis of β-galactosidase in deuterated media

Experiments to study the synthesis of β-galactosidase have conventionally relied on measurements of enzyme activity as an indication of amounts of enzyme protein (Monod & Cohn, 1952). Having shown that the specific catalytic activity of deuterated β-galactosidase is essentially identical to that of the protonated enzyme (Table 6), we have used the same approach for investigations of β-galactosidase synthesis in deuterated systems.

Rates of β-galactosidase synthesis

The differential rates of β-galactosidase synthesis with and without IPTG (0.5 - 1 mM) were compared in several strains before and after 30-50 generations of growth in all-D acetate. No significant changes were observed in 300U, 230U, 2000-$^{c}_{67}$ and Cavalli, although the growth rates were much lower in deuterated medium.

Thus, β-galactosidase synthesis was reduced in cells adapted to all-D acetate to the same degree as that of other proteins, with no additional discrimination against this enzyme. After more prolonged periods of adaptation to all-D acetate a discrimination was observed, and this is discussed below.

β-Galactosidase synthesis in shifts to and from deuterated media

Cells from a fully deuterated culture of strain 300U were transferred to all-H acetate medium and grown for more than 20 generations. IPTG was added during exponential growth to establish the induced differential rate of enzyme synthesis. The cells were then transferred to all-D acetate medium containing IPTG. The growth rate fell from 0.68 doublings/hr. to 0.10 doublings/hr., and the P values for β-galactosidase synthesis from 3000 to about 450. After 2.5 hr. in deuterated medium the cells were replaced in all-H acetate containing IPTG; growth immediately accelerated to 0.80 doublings/hr., with a P value of 3800 (Fig. 7).

[INSERT FIG. 7 NEAR HERE]

When induced cells of a strain fully adapted to deuterated medium were transferred to protonated medium containing inducer, the increase in growth rate (0.11 to 0.43 generations/hr.) was not matched by a corresponding increase in the P value. The latter fell from 6000 to 1650 (Fig. 8).

[INSERT FIG. 8 NEAR HERE]

Kinetics of β-galactosidase synthesis

The induction lag in deuterated cells was about 3 min. (Fig. 9), corresponding closely to the well-established value for protonated

[INSERT FIG. 9 NEAR HERE]

cells (Pardee & Prestidge, 1961; Boezi & Cowie, 1961; Branscomb & Stuart, 1968).

The kinetics of β-galactosidase synthesis were investigated following removal of inducer (Kepes, 1963; Nakada & Magasanik, 1964). Cells from a deuterated culture of strain Cavalli were returned to all-H medium and maintained for several subcultures. Exponentially growing cells in all-D acetate and all-H acetate were induced with 0.5 mM-IPTG. After incubation at 37° for 75 sec., portions of each culture were filtered through Millipore filters (1.2 μ pore size), and rapidly washed four times with IPTG-free medium of the same isotopic content as that in which they had been grown. The filters were bisected, and the cells from one half resuspended with shaking in all-D acetate, and from the other half in all-H acetate, both at 37° without inducer. The filtering procedure was carried out at 37° and took 30-45 sec. Samples were taken from each suspension into chloramphenicol for later measurements of β-galactosidase activities. Measurements were made of E_{650} for each suspension immediately after resuspension; enzyme activities were later normalized to constant E_{650}. (It was shown separately that a sample of cells diluted into all-H or all-D media gave identical E_{650} values.) Fig. 10 shows that cells induced in all-D medium produced equal quantities of enzyme when resuspended,

[INSERT FIG. 10 NEAR HERE]

after removal of inducer, in either protonated or deuterated medium. However, cells induced in all-H medium consistently yielded more enzyme in protonated than in deuterated medium. For cells induced

in all-H acetate, decay plots of the fall in the rates of enzyme synthesis with time, after removal of inducer, showed half-times at 37° of 2.0 and 2.2 min. for cells in protonated and deuterated media respectively (Kepes, 1963).

Differential rate of β-galactosidase synthesis versus inducer concentration

Herzenberg (1959), using permeaseless strains, found that the rate of enzyme synthesis was max. at about 0.4 mM-IPTG, and half max. at about 0.16 mM-IPTG. Using the permeaseless strain 300U, we have measured the induced rates of enzyme synthesis in deuterated and protonated media at inducer concn. below 0.1 mM. The protonated culture of 300U used for this experiment was one which had earlier been fully adapted to deuterated medium, and then grown for several generations in protonated medium. Fig. 11 shows that IPTG at low concn. was significantly more effective as an inducer in deuterated cells than it was in protonated cells.

[INSERT FIG. 11 NEAR HERE]

Fig. 11 also shows an unusually high level of induction at very low IPTG concn., reminiscent of the behaviour of y^+ cells (Herzenberg, 1959). We therefore investigated the allelic state of the y gene in our strain. Herzenberg (1959) found that in the y^+ strain ML30 the induced rate at 2×10^{-5} M-IPTG was 70% of that at 4×10^{-4} M-IPTG. Branscomb & Stuart (1968), using the cryptic strain 300U obtained from us before any history of growth in deuterated medium, observed that at 2.5×10^{-5} M-IPTG the rate was 41% of that at 2×10^{-4} M-IPTG. From Fig. 11 one can calculate that in our

system the inducer concn. giving 41% of the rate at 10^{-4} M-IPTG was 2.3×10^{-5} M. Thus, our results correspond to the $\underline{y}^-$ state, rather than the $\underline{y}^+$. Further, Herzenberg (1959) showed that, in $\underline{y}^+$ cells induced at low inducer concn. (2×10^{-5} M and below), there was a period (about 1 generation long) of increase in the differential rate of enzyme synthesis before a constant rate was attained. Herzenberg (1959), Pardee & Prestidge (1961) and Branscomb & Stuart (1968), using $\underline{y}^-$ cells, all showed that a constant rate was attained with 3 min. of adding inducer. Our cultures behaved in the $\underline{y}^-$ manner when induced at low concn. of inducer (Fig. 12). We also grew our deuterated strain of 300U on eosin-methylene blue-lactose

[INSERT FIG. 12 NEAR HERE]

agar plates. Each of 10^4 colonies examined was light pink in colour; a $\underline{y}^+$ strain spread on plates of the same batch of medium produced only dark colonies. Finally, R. N. Stuart (personal comminication) has found max. induction with a $\underline{y}^+$ strain at 10^{-5} M-IPTG. All these tests therefore suggest that our strain was still $\underline{y}^-$.

However, 300U, both from our deuterated stocks and in other studies, has been found to grow slowly on lactose-minimal medium without the selection of $\underline{y}^+$ revertants as determined by the colour of colonies on eosin-methylene blue-lactose agar. One criterion for the existence of permease function in induced cells is the ratio of β-galactosidase activity with and without prior treatment with toluene or detergent. For a number of $\underline{y}^+$ strains we have found at 37° that measured enzyme activity without detergent was 15% of that with detergent; with $\underline{y}^-$ strains the value was about 2%.

In strain 300U with no history of exposure to deuterated media, the value was 3.1%, while our deuterated stocks showed a value of 20%.

It appears possible from these data that two consequences result from deuteration which are not readily reversed on returning the cells to protonated medium. One is a greater affinity of the lactose induction mechanism for inducer. The other is an alteration of cell membrane properties permitting a more rapid entry of galactosides but without the intervention of permease. Further work will be necessary to substantiate these conclusions and elucidate the mechanisms involved.

Effects of prolonged growth in deuterated medium

As noted above, maximal P values for all strains grown for 30-50 generations in all-D acetate were about 3000, a typical value found for protonated strains never exposed to deuteration. After prolonged growth (more than about 100 generations) in all-D acetate medium, the induced P values for the inducible strains 300U and Cavalli, and the partial (9%) operator-constitutive mutant 2000-o^c_{67}, fell to very low levels of 2-5 even in the presence of 1 mM-IPTG. The basal levels remained unchanged at P = 1. Such a decline did not occur with strain 230U, the only regulator-constitutive strain tested. With the latter, the P value remained at 2000 after more than 200 doublings in all-D acetate medium.

This phenomenon was examined in some detail in strain 300U. The loss of inducibility was a function of growth in deuterated medium, not growth on acetate. None of the strains showing loss of

inducibility in all-D acetate demonstrated a loss after hundreds of doublings in all-H acetate. After some 120 doublings in all-D acetate, the induced P value was about 2. Transfer of such cells into all-H acetate for 20 doublings yielded induced P = 1-2. When those cells were then grown in all-H glycerol medium, the induced P value reached 2000 after a few doublings. If the deuterated cells in all-D acetate with low P values were transferred directly to all-H glycerol, the induced P value reached 2500 after 10 doublings, and 3000 after 20 doublings. Subculture of the latter in all-H acetate then again reduced the P value to about 10 in a few generations. The uninduced P values in all these cultures were about 1. These results are summarized in Fig. 13. Each arrow indicates one subculture providing for 10-20 doublings.

[INSERT FIG. 13 NEAR HERE]

We sought to ascertain whether the low P values in acetate medium resulted from a failure to synthesize enzyme protein or from the synthesis of a protein devoid of catalytic activity. The soluble protein profiles of cells from the different cultures were separated by electrophoresis in polyacrylamide gels. Densitometer scan tracings are shown in Fig. 14. Figs. 14 A and B show the induction of active enzyme in cells grown in all-H glycerol.

[INSERT FIG. 14 NEAR HERE]

The prominent peak at 1 cm. had a similar mobility to that reported in gel separations with purified β-galactosidase (Craven et al., 1965) and with enzyme in cell extracts (Fairbanks et al., 1965; Moses & Wild, 1969); it travelled with the same mobility as the

β-galactosidase detected directly on gels by ONPG hydrolysis (see Methods), and it constituted a considerable fraction of the soluble protein. We are thus confident that this peak represented β-galactosidase. Figs. 14 C and D, and Figs. 14 E and F show that a prominent component, with a similar mobility, was induced by IPTG in those cultures (both in D_2O and in H_2O) which showed no β-galactosidase activity. The loss of inducibility was, therefore, due not to a failure to synthesize β-galactosidase protein, but to inactivity of the protein so produced.

Comparison of Figs. 14 C and E suggests that a number of differences exist between the soluble protein profiles of protonated and deuterated cells. Similar indications have been obtained from other experiments, but these differences have not yet been rigorously examined.

DISCUSSION

The general nature of adaptation to deuteration

Many studies have been made of the effects of D_2O on individual enzyme reactions (Thomson, 1963). Theoretical max. values of kinetic isotope effects (k_H/k_D) at 30° are 7, 8 and 10 for reactions in which the rate-limiting steps break C-H, N-H and O-H bonds, respectively (Wiberg, 1955). Typical experimental values for enzyme reactions *in vitro* have been in the range 1-3 (Thomson, 1963). In nearly all these studies the system employed protonated enzyme and substrate in deuterated solvent, so that the k_H/k_D values were, in a wide sense, only solvent effects.

Rittenberg & Borek (1961) prepared cell-free extracts from cells grown on a medium similar to our all-D acetate. The preparation catalysed oxidation of succinate by ferricyanide, and the rate of succinate oxidation was the same with tetradeuterosuccinate and all-protonated succinate. A similar system from protonated cells oxidised protonated succinate twice as fast as deuterosuccinate. Thus, the fully-deuterated enzyme was better fitted to catalyse reaction with the deuterated succinate, which, of course, was its substrate *in vivo*.

One can envisage many possible ways in which deuteration might affect cellular activities. Among these are hydrogen-bonding between nucleic acid bases, affecting the rate and fidelity of the replication and transcription of DNA, and of other reactions involving nucleic acids (Holland & Antoni, 1968); hydrogen-bonding changes altering the conformation of protein molecules (Hattori, Crespi & Katz, 1965; Tomita, Rich, de Loze & Blout, 1962); alterations to the quaternary structures of macrostructures; and changes in the network of metabolic activities resulting from changes in the activities and quantities of enzymes.

Adaptation to deuteration is rather a rapid process, and is unlikely to involve selection of D-resistant mutants. However, prolonged growth in deuterated media might do so, although it is difficult to imagine their nature. It seems unlikely that any evolutionary ancestor of *E. coli* became adapted to media containing 99.8 atom % D, and the existence of dormant genes for life in D_2O thus seems improbable. Nor is there evidence of mutants arising during growth in D_2O. Once adaptation was complete in a few

generations, no increase in growth rate was observed during hundreds of doublings in D_2O. Ability to grow in D_2O resulted in no loss of ability to grow in H_2O. And, most conclusively, adaptation to D_2O was lost, for cells in acetate medium, after only 2 doublings in protonated medium.

Orgel (1964) has proposed a general qualitative theory of adaptation to widespread metabolic disturbances, such as those resulting from growth in D_2O. He postulated that no single reaction is critically inhibited, but that many are slowed to some degree. Short-term adaptation cannot consist of genetic selection because there is a negligible probability that pre-existing mutants are present for all the affected enzymes. Stepwise mutation and selection are conceivable but would take many generations, and, as has just been shown, are to be neglected here. Orgel suggests that widespread derepression of enzymes is a likely mechanism for physiological adaptation. Thus, the lowered turnover rates of affected enzymes might be partly or fully compensated by an increase in their concentrations. An expected consequence is a higher ratio of protein to DNA. This has been reported for cells adapted to trifluoroleucine (Rennert & Anker, 1963), in which the ratio increased threefold; our results for deuterated cells have shown a twofold increase.

Extensions of Orgel's proposal may be used to account for some of our findings. We have often observed that cells grow particularly rapidly in H_2O when they have been adapted to D_2O, a phenomenon which may result from cells possessing increased quantities of certain enzymes. The loss of adaptation to D_2O after only

2 doublings in H_2O may also be considered in the light of this theory. One might envisage 3 reasons for this loss: (1) Adaptation to deuteration depends only upon deuteration of metabolic intermediates. Were this the case, one would expect a rapid loss of adaptation on transfer to protonated medium, since intermediary metabolic reactions are rapid. The replacement of deuterated metabolites by their protonated counterparts would be expected to occur in minutes, not in hours. (2) Adaptation depends on altered conformations and other structural characteristics of deuterated macromolecules. If this were true, one would not expect adaptation to be lost so rapidly. After 1, 1.5 and 2 generations of growth in H_2O, 50%, 33% and 25% of the macromolecules would still be deuterated at the non-exchangeable positions. Yet the growth lag in D_2O was already 5 hr. after 1 generation in H_2O, and became very long indeed after 2 generations (Table 2). (3) If adaptation depended on the relative amounts of the various cellular proteins its loss could be more readily understood. Although adaptation might result in some enzymes becoming more efficient in D_2O, it is improbable that many would do so, and it is likely that most would remain inefficient. Orgel's theory suggests that these variously inefficient enzymes are produced in variously greater yield, which need not be in proportion to their inefficiency. Almost certainly they would still be able to function better in H_2O than in D_2O. Thus, on returning the cell to H_2O, it would be enzymically unbalanced, and would so adjust its protein synthesizing activities as to bring itself back to that balanced content of macromolecules

which is the result of a long evolutionary history in H_2O. It follows that while a cell may yet contain a considerable fraction of its macromolecules in the deuterated form, the relative distribution of macromolecules may no longer be appropriate for growth in D_2O, and adaptation is lost.

Orgel's theory would not predict equal derepression for all enzymes. Some of them may not be significantly inactivated by D_2O, and there would be no metabolic need for increased derepression. On the other hand, some enzymes might be derepressed willy-nilly*, by virtue of being part of a regulon that is derepressed to provide sufficient amounts of one of its other enzymes. An adapted deuterated cell might therefore be very different from a protonated one, and possible evidence for this has been found in the soluble protein profiles (Fig. 14). Some residue of a differently balanced macromolecular content and range of activities might persist for many generations after changing from a deuterated to a protonated medium. Again, we have obtained evidence of this in the loss, after prolonged growth in D_2O, of β-galactosidase inducibility, which persisted after the cells were transferred to protonated acetate medium (Fig. 13). It has also been found, with strain 300U in protonated medium, that some features of the soluble protein profile characteristic of growth in one medium survive for at least 12 generations of growth in another (Moses & Sharp, 197).

Effect of deuteration on translation

Our evidence suggests that deuteration has little effect on the rate of transcription. Recent data by Manor, Goodman & Stent

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*nolens volens

(1969) indicate a period of 1-3 min. to assemble lac mRNA. Since induced enzyme first appears 3 min. after the addition of inducer (Branscomb & Stuart, 1968), most of the delay may be ascribed to the synthesis of message. We found that deuteration had no effect on the induction lag, and conclude that the rate of transcription was probably unchanged.

In contrast, there is some evidence that mRNA synthesized in a protonated environment is translated more slowly in deuterated than in protonated medium; mRNA made in a deuterated cell appears to be translated equally well in both. While this result indicates that deuteration can affect polypeptide synthesis, we do not know whether the effect is general for proteins, or restricted to a few, β-galactosidase among them. It was shown by Yudkin & Moses (1969) that catabolite repression can reduce the rate of translation in the lactose system.

The loss of ability to synthesize active β-galactosidase after prolonged growth in deuterated acetate might result from a persistent mis-reading during transcription or translation. Clearly no genetic mutation was involved, since activity could be restored in a few generations of growth in a suitable medium exerting no direct selection. Yet an altered protein molecule was synthesized which possessed similar electrophoretic mobility to the native protein but was devoid of catalytic activity. It is tempting to surmise that deuteration can produce "phenotypic mutations" (based on consistent mis-reading) without the advent of genotypic changes. Such "mutations" might be common, and would doubtless revert to

wild-type at different rates when the cells were replaced in protonated medium.

Comparison with other studies on deuterated bacterial systems

Henderson (1962) used E. coli 112 (genotype not specified) for studies of β-galactosidase synthesis shortly after introducing the cells to deuterated media. The carbon sources (glucose and lactose) were not enriched in D. Some of Henderson's conclusions clearly differ from ours, but we regard them as not warranted by his data. His measurements were made in the first 4 hr. after introducing the cells to deuterated medium, and it does not seem likely that a steady state of adaptation was reached. During those 4 hr. "..... the formation of enzyme [i.e. β-galactosidase] appeared to be even more sensitive to environmental deuterium than was the increase in turbidity". Replotting Henderson's data in the form of a differential plot of enzyme activity/ml. of culture versus E_{600} of the culture gave a graph with too much uncertainty to permit drawing firm conclusions. Most of his studies appeared to concern enzyme induction of cultures in non-exponential states of growth, which make his results impossible to compare with ours.

Studies by Henderson, Dacus, Crespi & Katz (1967) are closer to our own, and there is good agreement on results with exponentially-growing cultures. Their induction values with IPTG were considerably higher in H_2O, in contradistinction to our measurements. However, the effects of varying the IPTG concn. were studied in glucose cultures undergoing linear growth as a result of admitting glucose solution to the culture drop by drop in an effort to minimize catabolite repression; this makes direct comparison difficult.

Conclusions

The mechanisms responsible for reduced growth rates in deuterated media are clearly complex. The data we have presented indicate a number of specific changes which take place when unadapted cells are inoculated into deuterated medium. As Orgel (1964) suggested, there is probably a reduction of catalytic activity for an unknown variety of essential enzymes, and time is required for this to be compensated by derepression. There appears to be an impairment of the translation mechanism, at least for β-galactosidase, and this may also apply to other proteins. Cells dependent on acetate as a carbon and energy source are impaired in their ability to oxidize the substrate in a deuterated medium, particularly when the acetate itself is deuterated. Finally, there is reason to suspect changes in cell membrane properties as evidenced by the more ready access of external ONPG to β-galactosidase in cells not treated with toluene or detergent. We think it probable that further investigation will disclose many more differences between protonated and deuterated cells.

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Running title: Escherichia coli in deuterium oxide

Correspondence and proofs to:

Dr. V. Moses
Laboratory of Chemical Biodynamics
Lawrence Radiation Laboratory
University of California
Berkeley, California 94720

CAPTIONS FOR FIGURES

Fig. 1. Effect of enrichment with D_2O on the respiration of cells grown in protonated medium. Cells of strain 300U, in all-H acetate, were resuspended in acetate media of varying degrees of enrichment with D. O_2 uptake was measured at 37° with and without chloramphenicol (100 μg./ml.). Δ, O_2 uptake with chloramphenicol; o, O_2 uptake without chloramphenicol; ●, O_2 uptake without chloramphenicol/O_2 uptake with chloramphenicol.

Fig. 2. Incorporation of [C^3H_3]thymine and L-[$^{14}CH_3$]methionine into trichloroacetic acid-precipitable material. Strain Cavalli was fully-adapted to all-H acetate or all-D acetate. o, all-H; ●, all-D. The ratio of the slopes is 2.1.

Fig. 3. Measurements of relative cell sizes of strain Cavalli fully adapted to all-H acetate or all-D acetate. 1, all-H cells, stationary phase; 2, all-D cells, stationary phase; 3, all-D cells, exponential phase.

Fig. 4. Woolf plots to determine the Michaelis constant of β-galactosidase. ●, enzyme from all-D cells in D_2O buffer [K_M = 0.16 mM]; ▲, enzyme from all-D cells in H_2O buffer [K_M = 0.17 mM]; o, enzyme from all-H cells in H_2O buffer [K_M = 0.13 mM]; Δ, enzyme from all-H cells in D_2O buffer [K_M = 0.18 mM].

Fig. 5. Effect on macromolecule synthesis of transferring all-H cells to all-D medium. Cells of strain 300U, growing exponentially in all-H acetate medium, were supplied with [^3H]uracil,

CAPTIONS FOR FIGURES (continued)

Fig. 5 (continued)

L-[G-^{14}C]phenylalanine and 0.5 mM-IPTG. Samples were taken for measurements of incorporated radioactivity and for β-galactosidase activity. The cells were transferred to all-D acetate containing 0.5 mM-IPTG and labelled precursors at the same specific radioactivity as before. Sampling was continued for a further 140 min. at 37°. Δ, β-galactosidase; ▲, E_{650}; ●, ^{14}C; o, ^{3}H.

Fig. 6. Synthesis of RNA, DNA and protein in a double shift with strain Cavalli. Cells in all-D acetate at 37° were pulse-labelled for 16 min. periods with [^{3}H]uracil, [C^{3}H$_{3}$]thymine and L-[G-^{14}C] phenylalanine. They were transferred to all-H acetate, and again pulse-labelled at intervals for about 2 hr. Finally, they were returned to all-D acetate, and pulse-labelled at intervals for a further 11 hr. A, uracil incorporation; B, phenylalanine incorporation; C, thymine incorporation; D, E_{650}.

Fig. 7. Growth and β-galactosidase synthesis in a double shift with strain 300U. Cells in all-H acetate at 37° were induced with 0.5 mM-IPTG. They were later transferred to all-D acetate containing IPTG, and finally returned to all-H acetate plus IPTG. A, β-galactosidase activity; B, E_{650}; ●, in all-H acetate; Δ, after transfer to all-D acetate; o, after return to all-H acetate.

Fig. 8. Growth and β-galactosidase synthesis in 300U shifted from all-D acetate to all-H acetate. IPTG (0.5 mM) was used to induce enzyme synthesis. The arrow indicates the time of changing the

CAPTIONS FOR FIGURES (continued)

Fig. 8 (continued)

medium. A, β-galactosidase activity; B, E_{650}; ●, in all-D acetate; o, in all-H acetate.

Fig. 9. Early induction kinetics in strain 300U fully adapted to all-D acetate. Cells were grown exponentially, and 0.5 mM-IPTG added at 0 min.

Fig. 10. Translation in inducer-free medium. Cells of strain Cavalli were fully adapted to all-H acetate or all-D acetate. Each culture received 1 mM-IPTG for 75 sec. at 37°. The cultures were then washed free from inducer and each was resuspended in all-D acetate and all-H acetate, without IPTG. Samples were taken during the subsequent 11 min. for measurement of β-galactosidase activity. ●, cells induced in H_2O and resuspended in H_2O; ▲, cells induced in H_2O and resuspended in D_2O; o, cells induced in D_2O and resuspended in D_2O; Δ, cells induced in D_2O and resuspended in H_2O.

Fig. 11. Differential rate of β-galactosidase synthesis as a function of inducer concn. Cells of 300U, fully adapted to all-H acetate or all-D acetate, were induced with varying concn. of IPTG. ●, all-D cells; o, all-H cells.

Fig. 12. Kinetics of induction of strain 300U by a sub-maximal concn. (2 μM) of IPTG. Cells were fully adapted to all-D acetate.

CAPTIONS FOR FIGURES (continued)

Fig. 13. Differential rates of β-galactosidase synthesis in strain 300U after prolonged culture in all-D acetate, and subsequent transfer to various media. Each arrow indicates one subculture permitting about 10 mass doublings. For explanation see text.

Fig. 14. Densitometer scan tracings of stained electrophoretograms of soluble proteins from sonically disrupted cells of strain 300U. In each pair of tracings, the upper is from an uninduced culture, and the lower from the same culture about 1 generation after receiving 1 mM-IPTG. A and B, cells in all-H glycerol (P = 3000); C and D, cells in all-D acetate (P = 1-2); E and F, cells in all-H acetate (P = 1-2). For explanation, see text. The peak at the extreme left end of each tracing is material remaining at the origin.

Table 1. Growth of strain 300U in protonated and deuterated media

Cells grown in all-H acetate were resuspended in protonated and deuterated media containing various carbon sources. Growth was measured turbidometrically for the following 12 hr.

Medium	Carbon source	Growth rates (doublings/hr.)	
		experiment 1	experiment 2
all-H	CH_3COOH	0.58	0.67
	CD_3COOH	0.52	0.62
	glucose	0.94	1.09
all-D	CH_3COOD	0.08	0.13
	CD_3COOD	0.04	0.00
	glucose	0.26	0.32

Table 2. Loss of ability to grow in all-D acetate medium after varying periods in all-H acetate medium

Cells of strain 300U, fully adapted to all-D acetate, were suspended in all-H acetate medium. At intervals, portions of the suspension were resuspended in all-D acetate medium and growth followed for more than 24 hr. The growth rate in all-D acetate is compared with the number of mass doublings in all-H acetate.

No. of mass doublings in all-H acetate	Growth rate after being returned to all-D acetate (doublings/hr.)
1.0	0.2
1.5	0.05
2.0	0.0*

*no growth in 24 hr.

Table 3. Effect of glucose on the growth of D-adapted cells in deuterated medium after varying periods in protonated medium

Strain 300U, growing exponentially in deuterated medium plus glucose, was transferred to protonated medium plus glucose. At intervals, samples were resuspended in deuterated medium plus glucose, and growth followed for several hr.

	Growth rates (doublings/hr.)
In D_2O before transfer to H_2O	0.26
In H_2O	0.81
Returned to D_2O after following periods in H_2O:	
80 min.	0.34
110 min.	0.36
160 min.	0.39
190 min.	0.40
1300 min.	0.49

Table 4. Oxygen consumption in deuterated media

Cells of strain 300U grown in all-H acetate medium were resuspended in fully deuterated medium without a carbon source. Samples were dispensed into Warburg flasks where they were diluted with medium containing the desired carbon source to give varying levels of enrichment with D_2O. CO_2 was absorbed by alkali. The uptake of O_2 was measured for 3 hr. at 37°.

Substrate	Enrichment with D (atom %)	µl. O_2 taken up in 180 min.
CH_3COOH	10	77
	80	29
	90	21
	99.8	18
CD_3COOD	10	75
	80	23
	90	17
	99.8	15

Table 5. Amino acid composition of protein hydrolysates from protonated and deuterated cultures of strain 300U

Amino acid	Concn. (moles %)*	
	Protonated cells	Deuterated cells
Alanine	10.5	10.9
Arginine	4.7	5.0
Aspartic acid + asparagine	10.3	10.3
Glutamic acid + glutamine	11.0	11.4
Glycine	9.2	9.0
Histidine	1.6	1.6
Isoleucine	4.9	5.0
allo-Isoleucine**	0.35	0.27
Leucine	8.8	8.6
Lysine	5.4	5.9
Methionine	1.0	0.55
Phenylalanine	3.9	3.5
Proline	3.7	3.9
Serine	4.8	4.7
Threonine	5.5	5.5
Valine	6.7	6.5
Unidentified 1	0.56	0.36
Unidentified 2	1.2	1.6

*Since tryptophan and cysteine are not determined by this method, their mole % values have been taken as 1.7 and 1.04, respectively (Roberts *et al.*, 1955). It is not asserted that these values hold for deuterated cells; the purpose of the assumption is only to bring those mole % values which were determined as nearly as possible to a total protein basis.

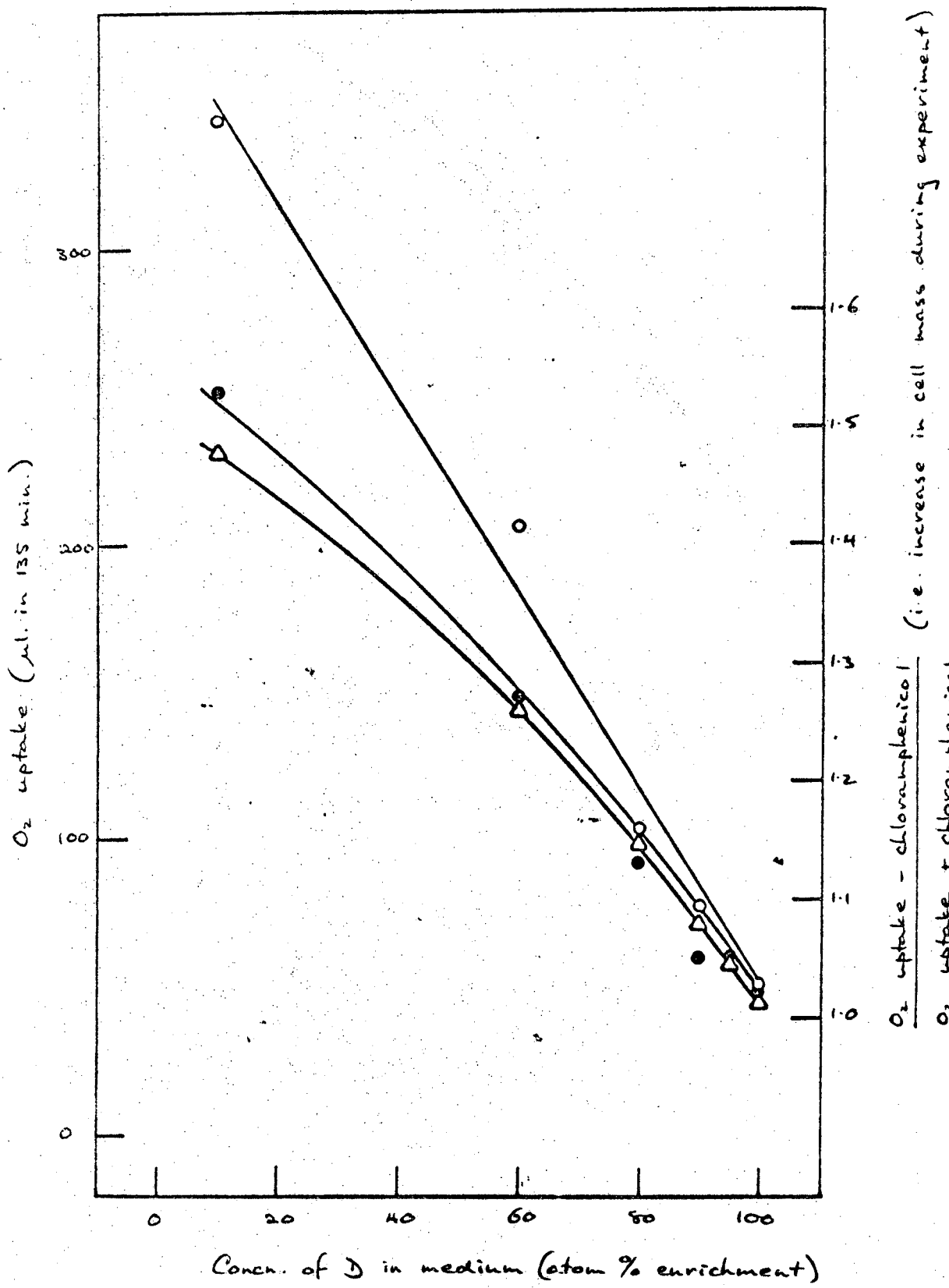
**Identification by retention time; colour constant of isoleucine used.

Table 6. Turnover numbers of protonated and deuterated β-galactosidase

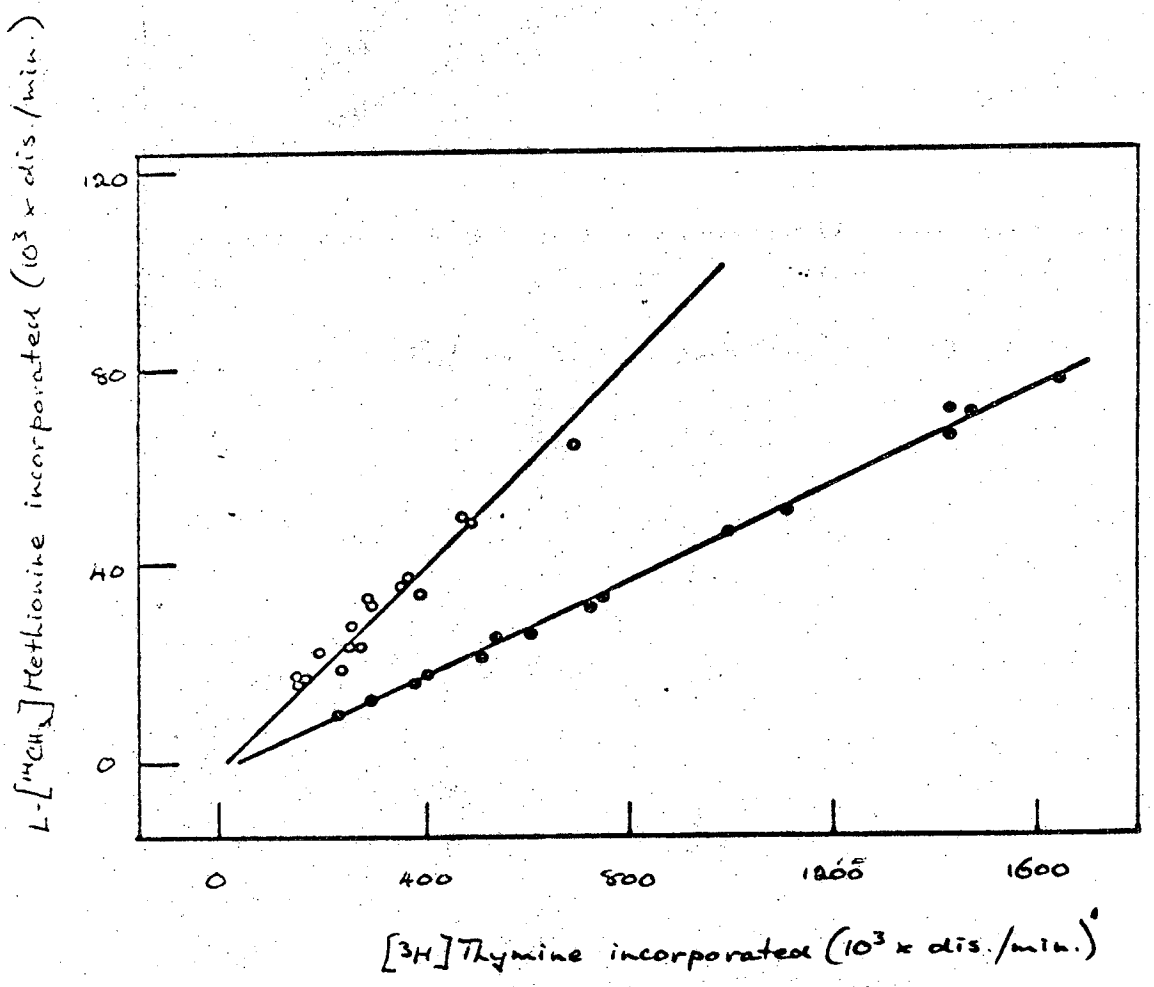
The enzyme was isolated from protonated or deuterated cultures of strain E203 as described in the Methods section. Measurements of catalytic activity were made at 21° in H_2O.

Enzyme	Turnover number (moles of ONPG hydrolysed/sec./μg. enzyme)
Protonated	5.0×10^{-9}
Deuterated	4.8×10^{-9}
Protonated (from Cohn, 1957)	3.9×10^{-9}

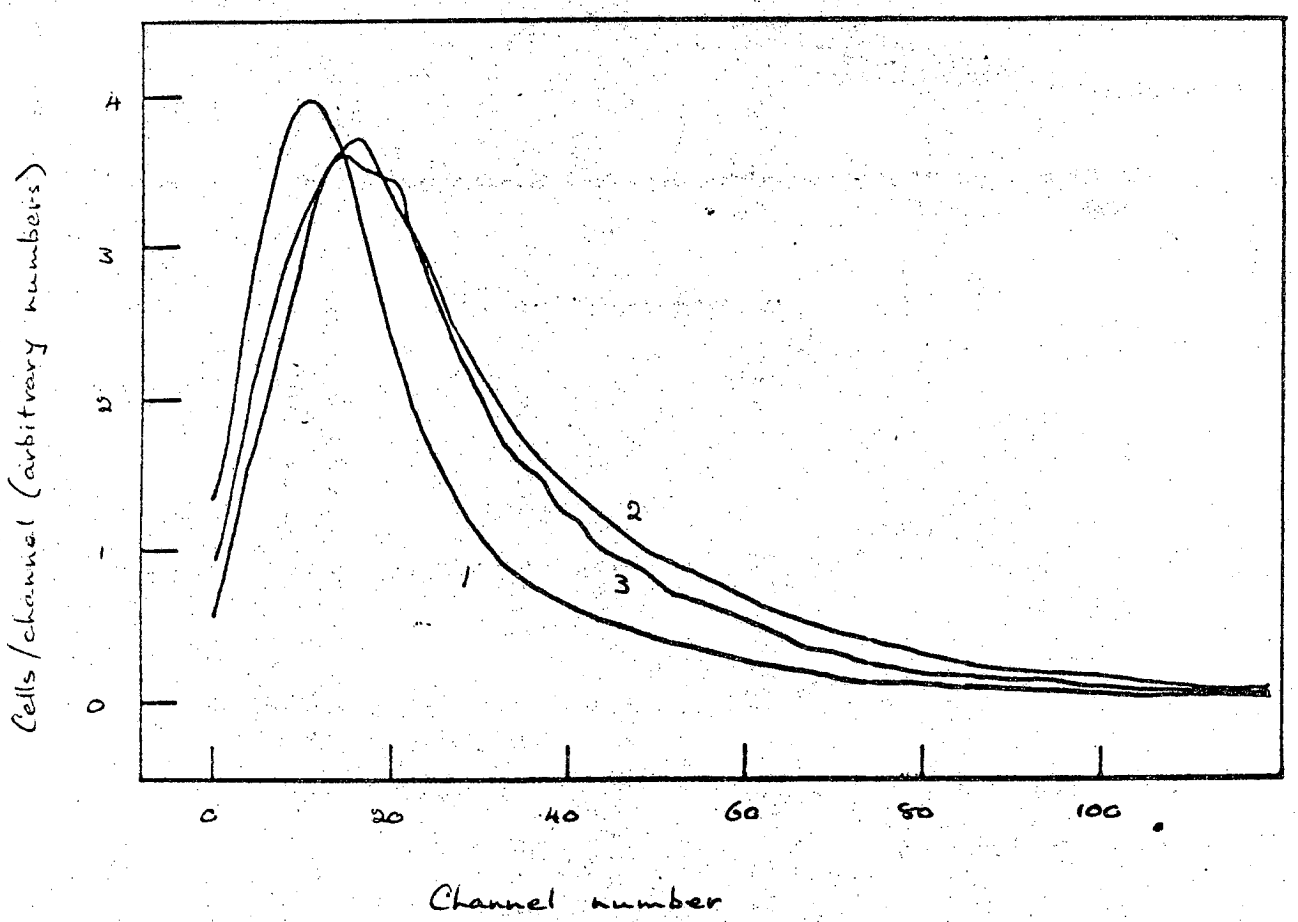
MANN + MOSES FIG. 1.



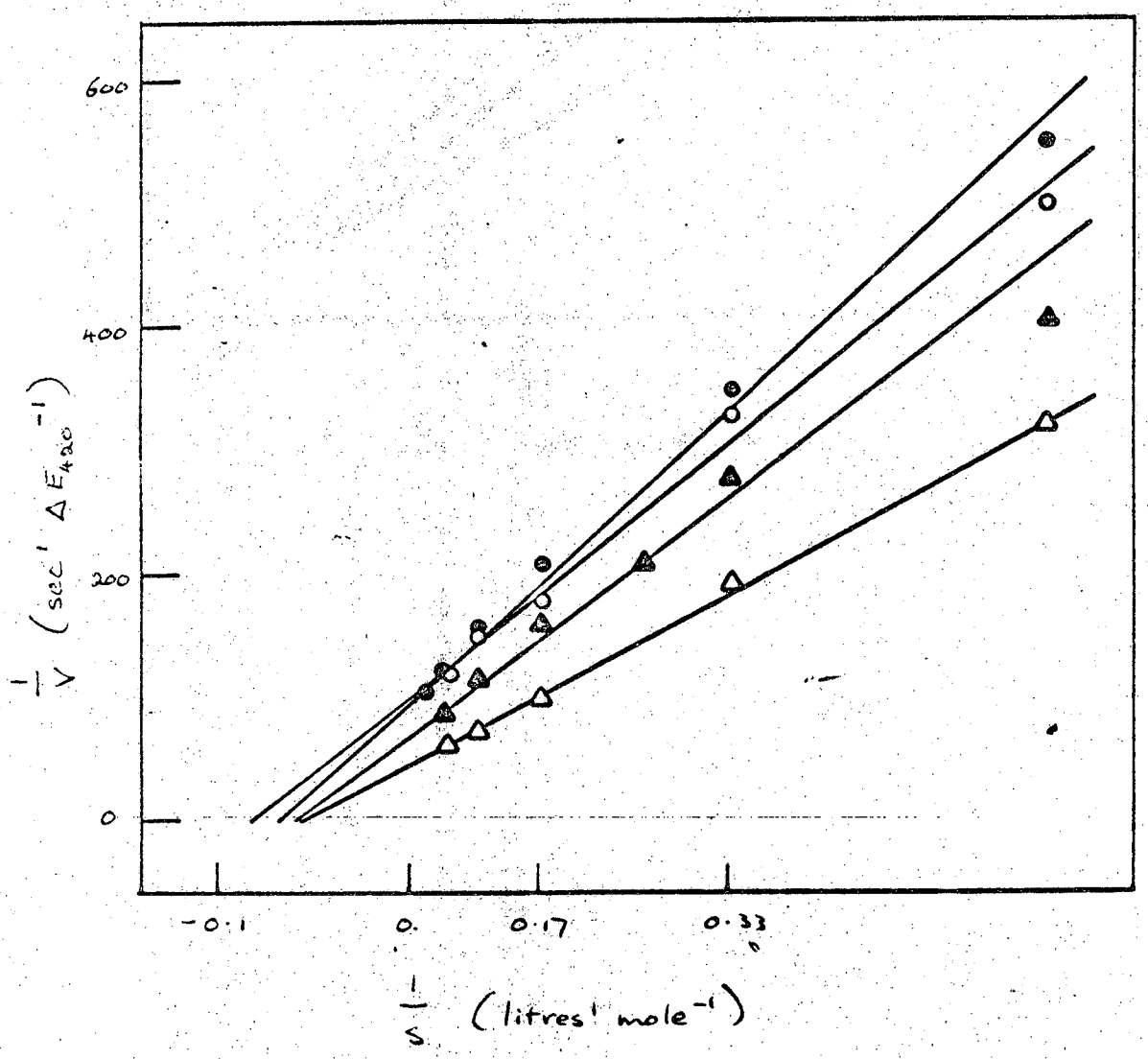
HANN + MOSES FIG. 2.



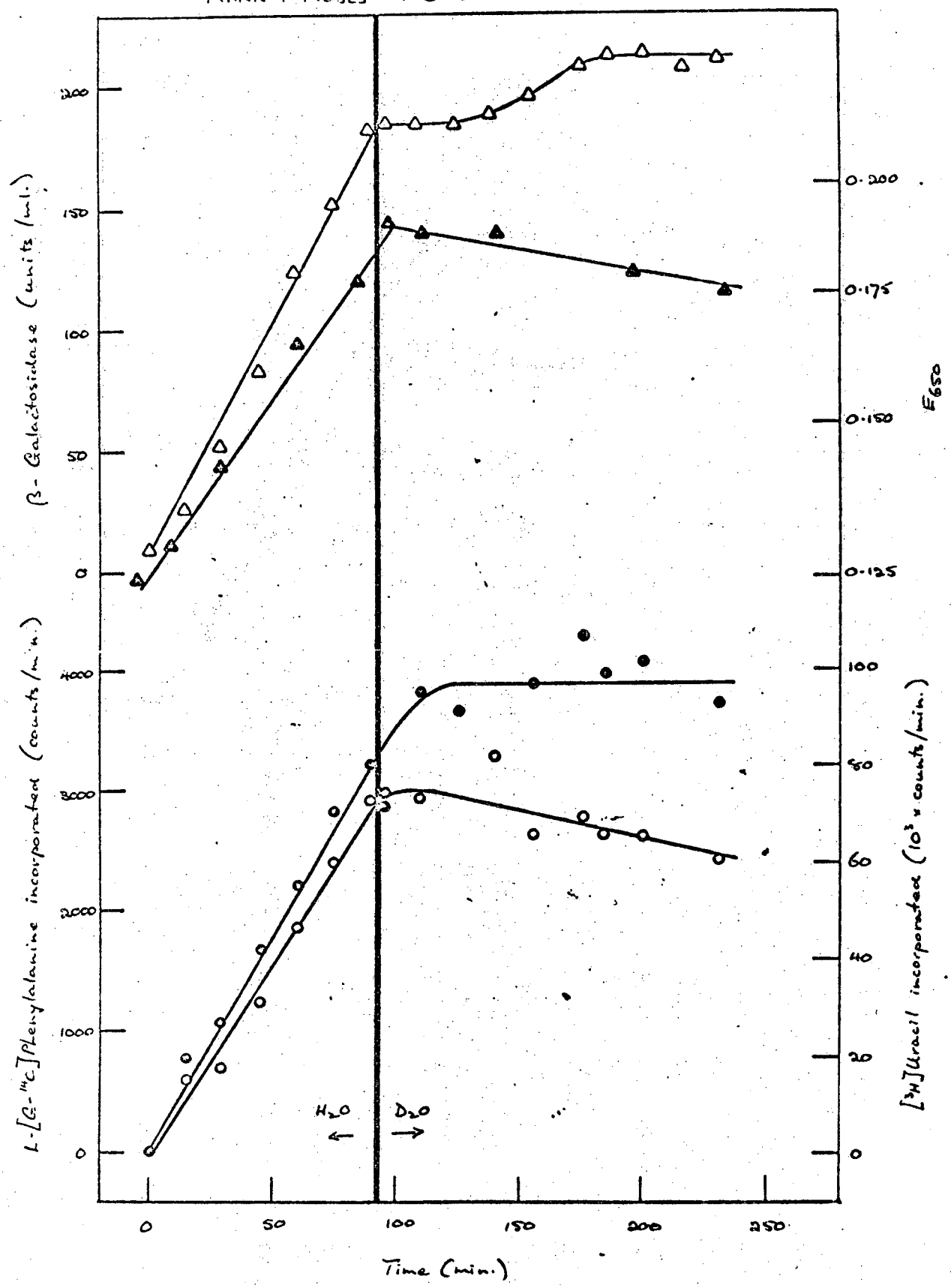
MANN & MOSES FIG. 3.



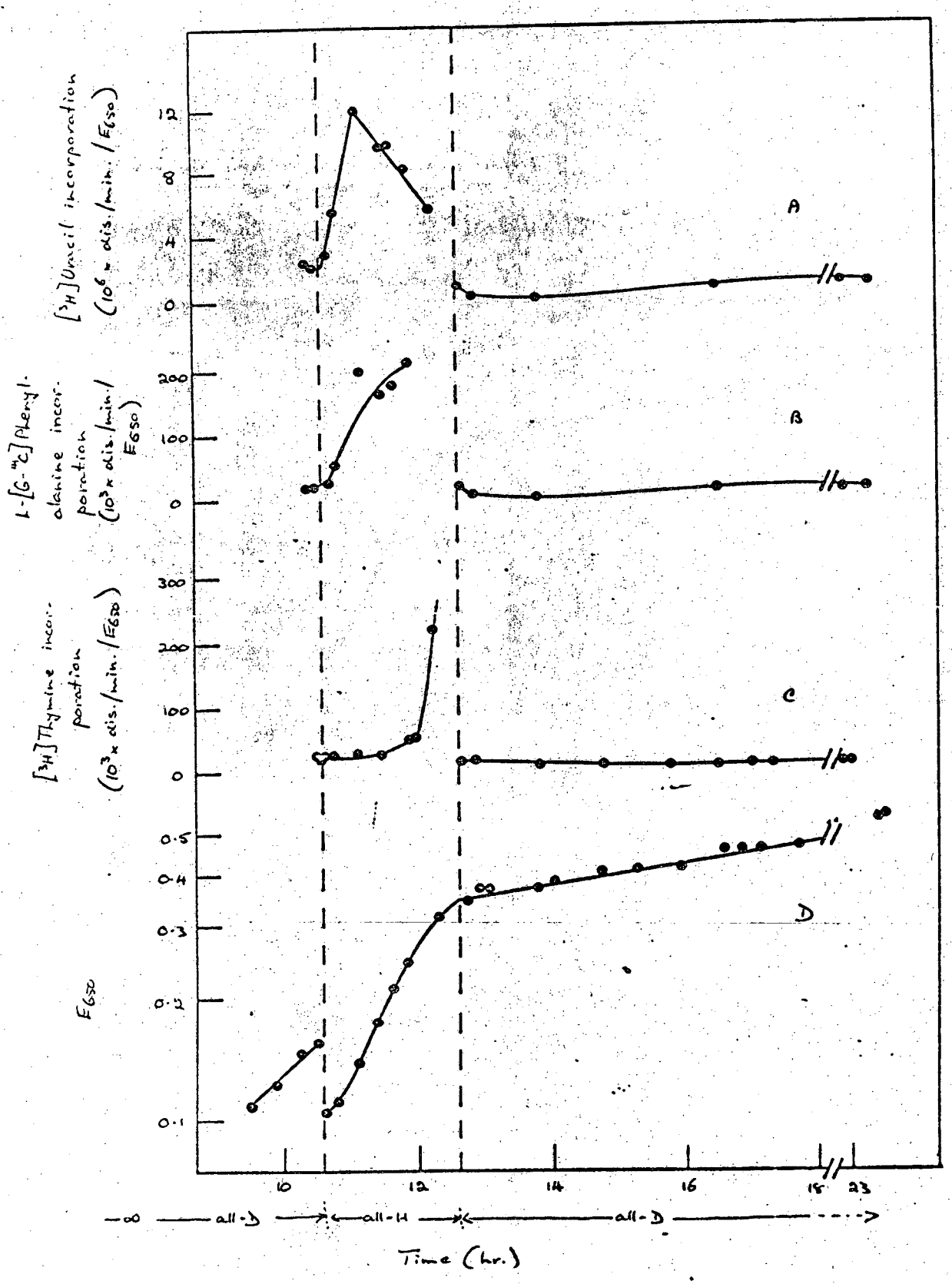
MANN + MOSES (FIG. 4).



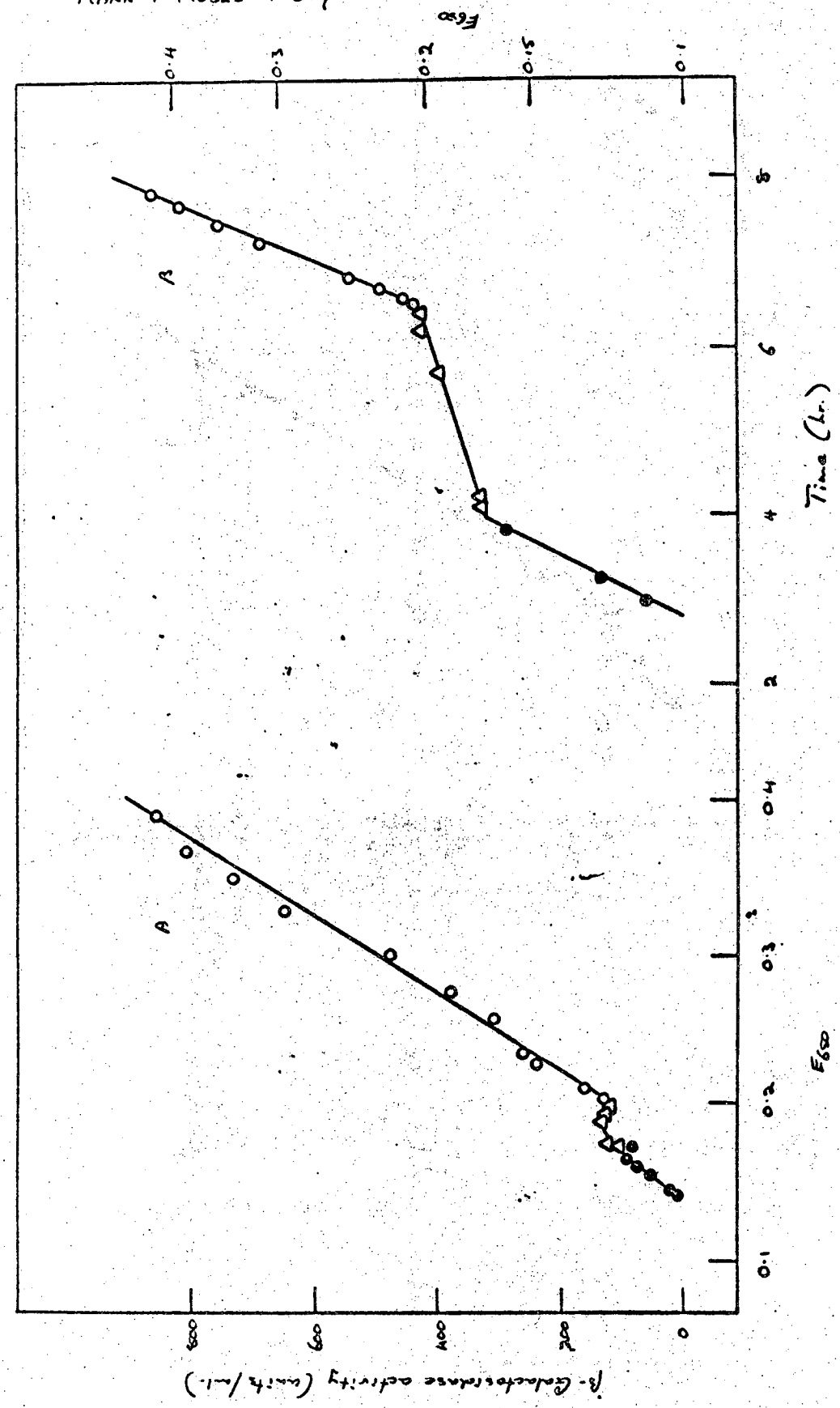
MANN + MOSES FIG. 5



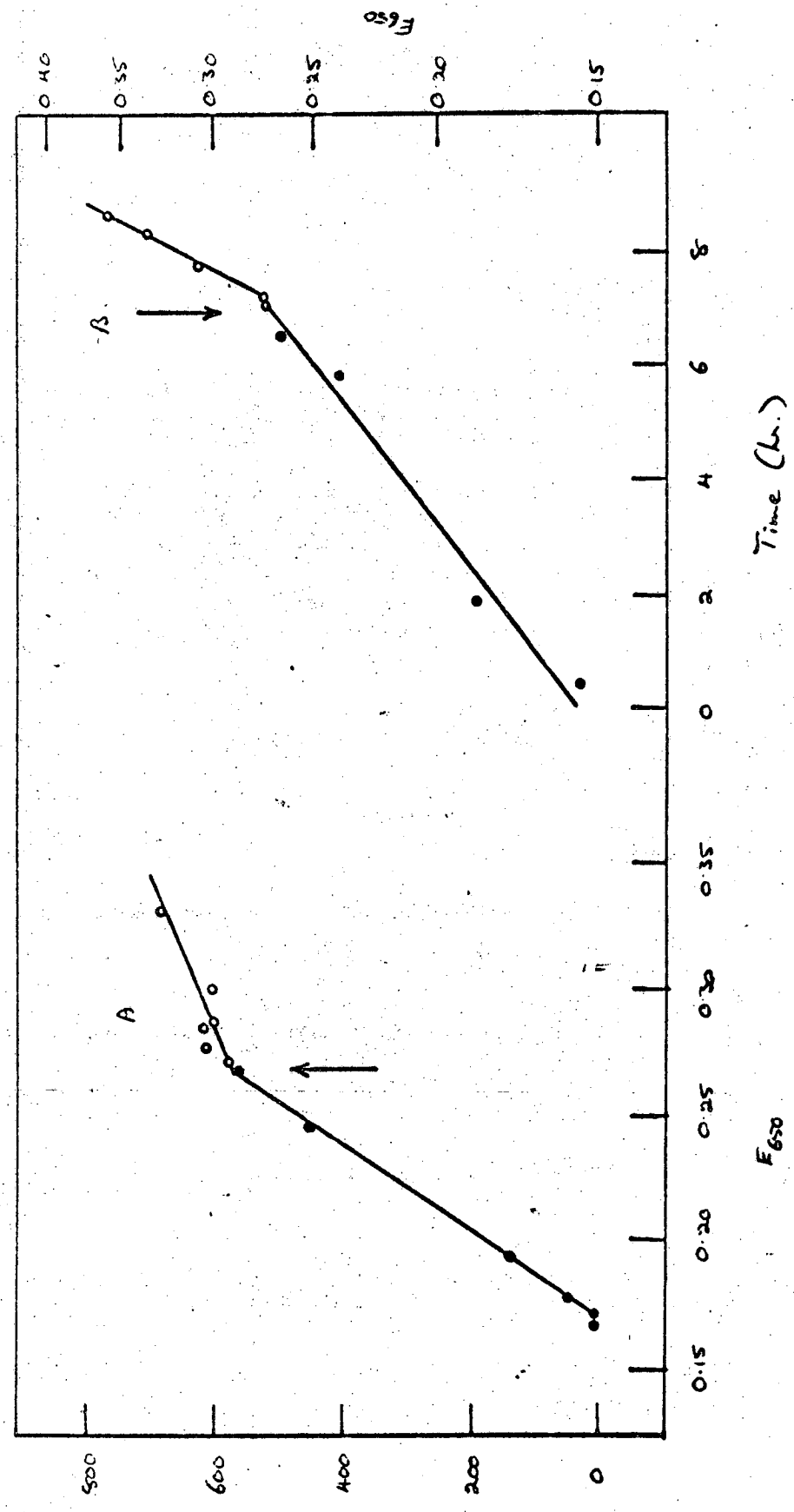
MANN & MOSES FIG. 6.



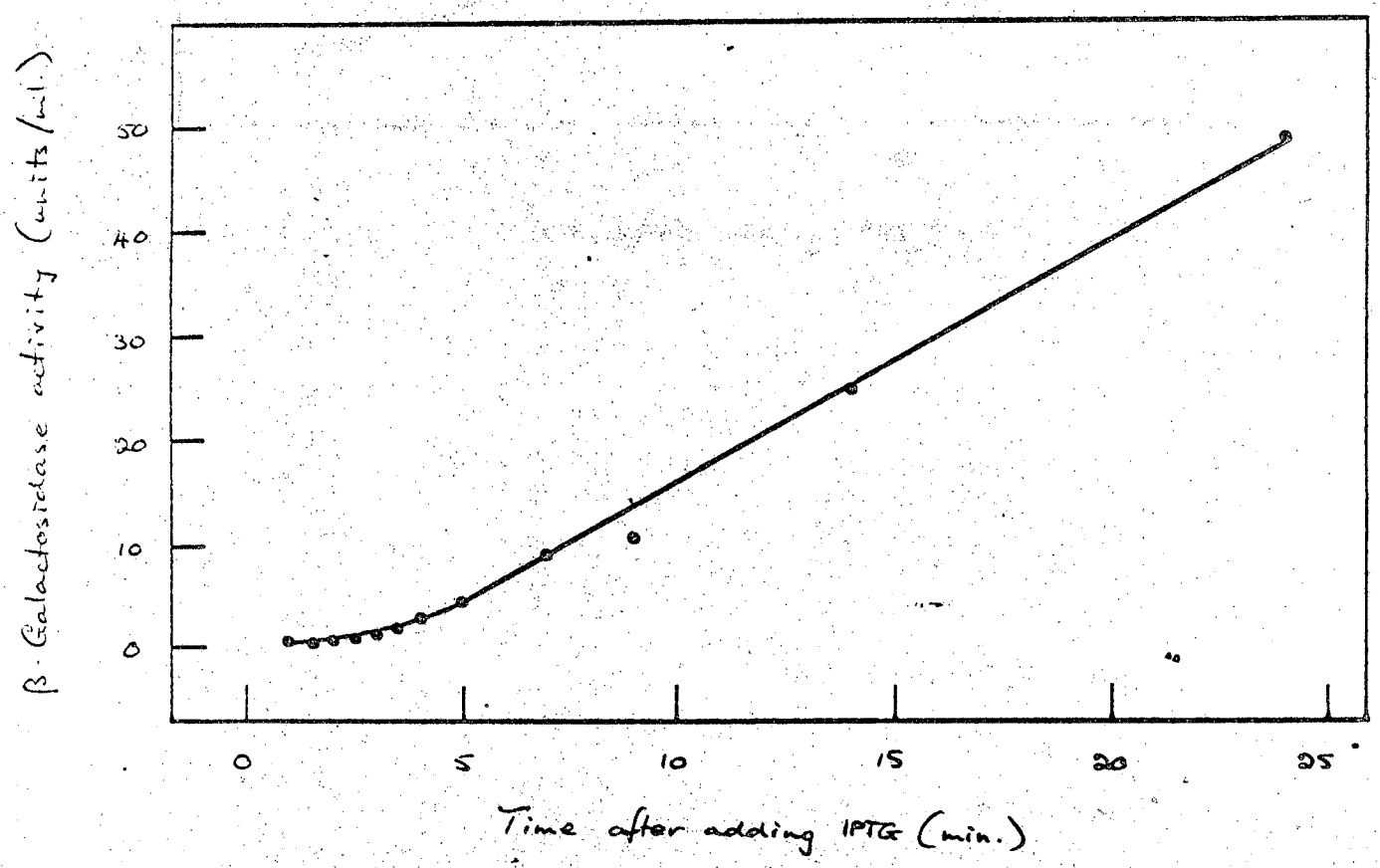
MANN + MOSES FIG. 7



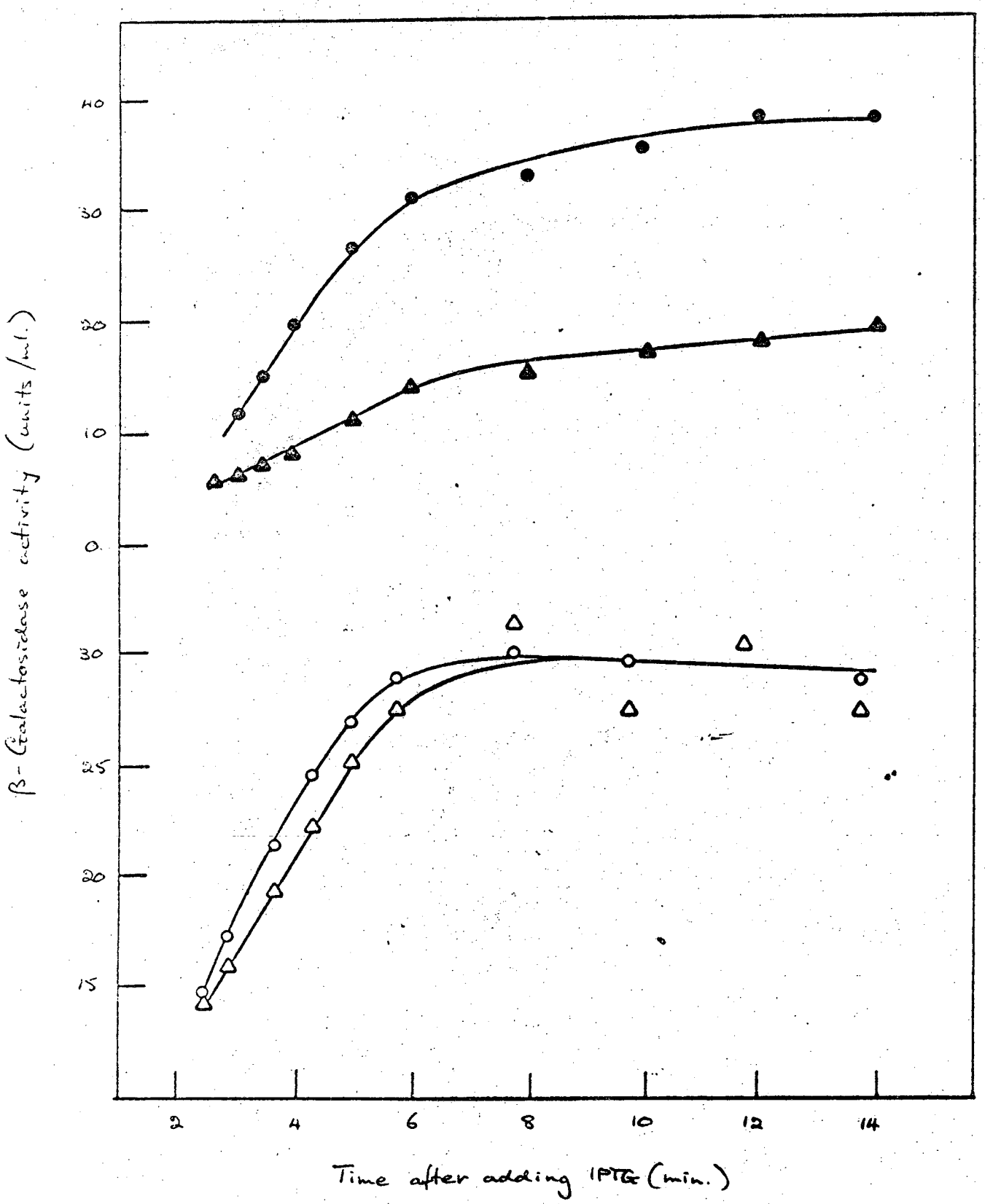
MANN + MOSES FIG. 8.



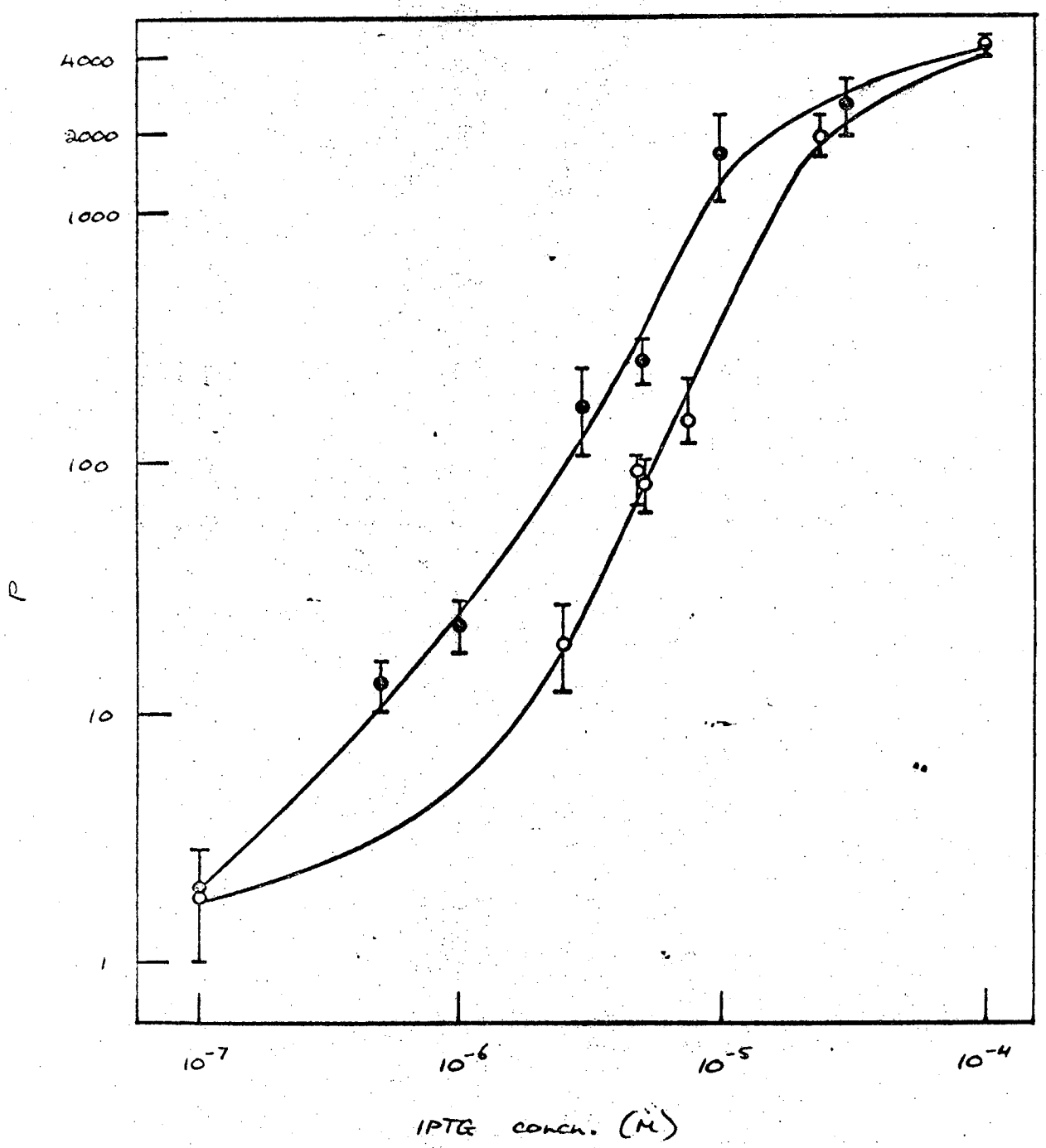
MANN + MOSES FIG. 9



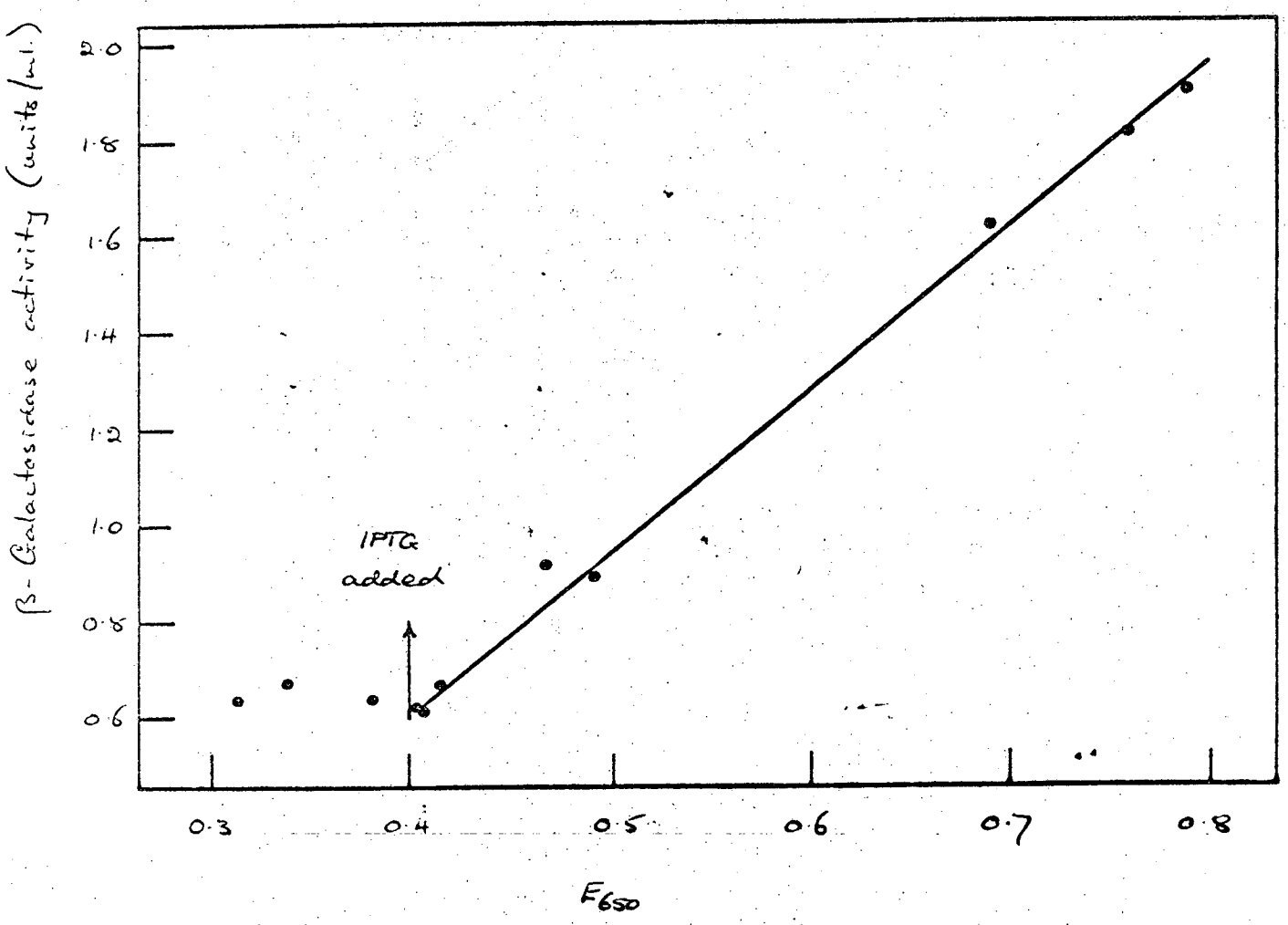
MANN + MOSES FIG. 10.



MANN + MOSES FIG. 11



MANN + MOSES FIG. 12.



MANN + MOSES FIG. 13

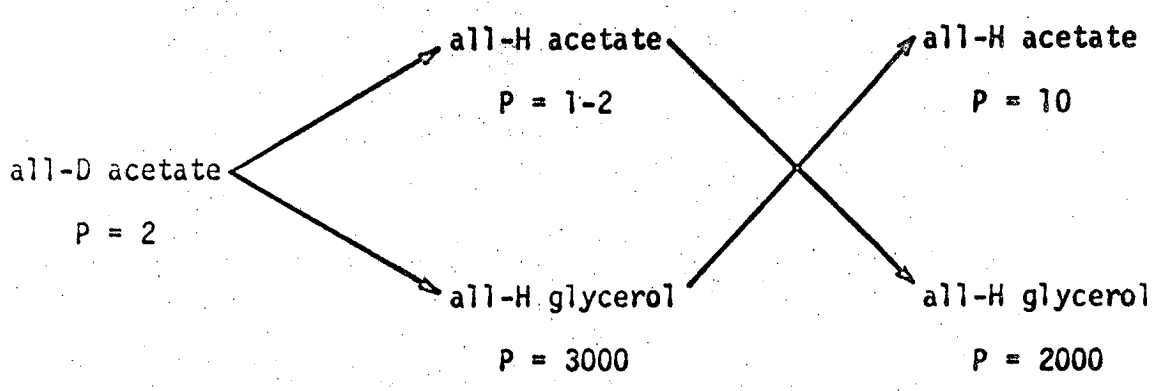
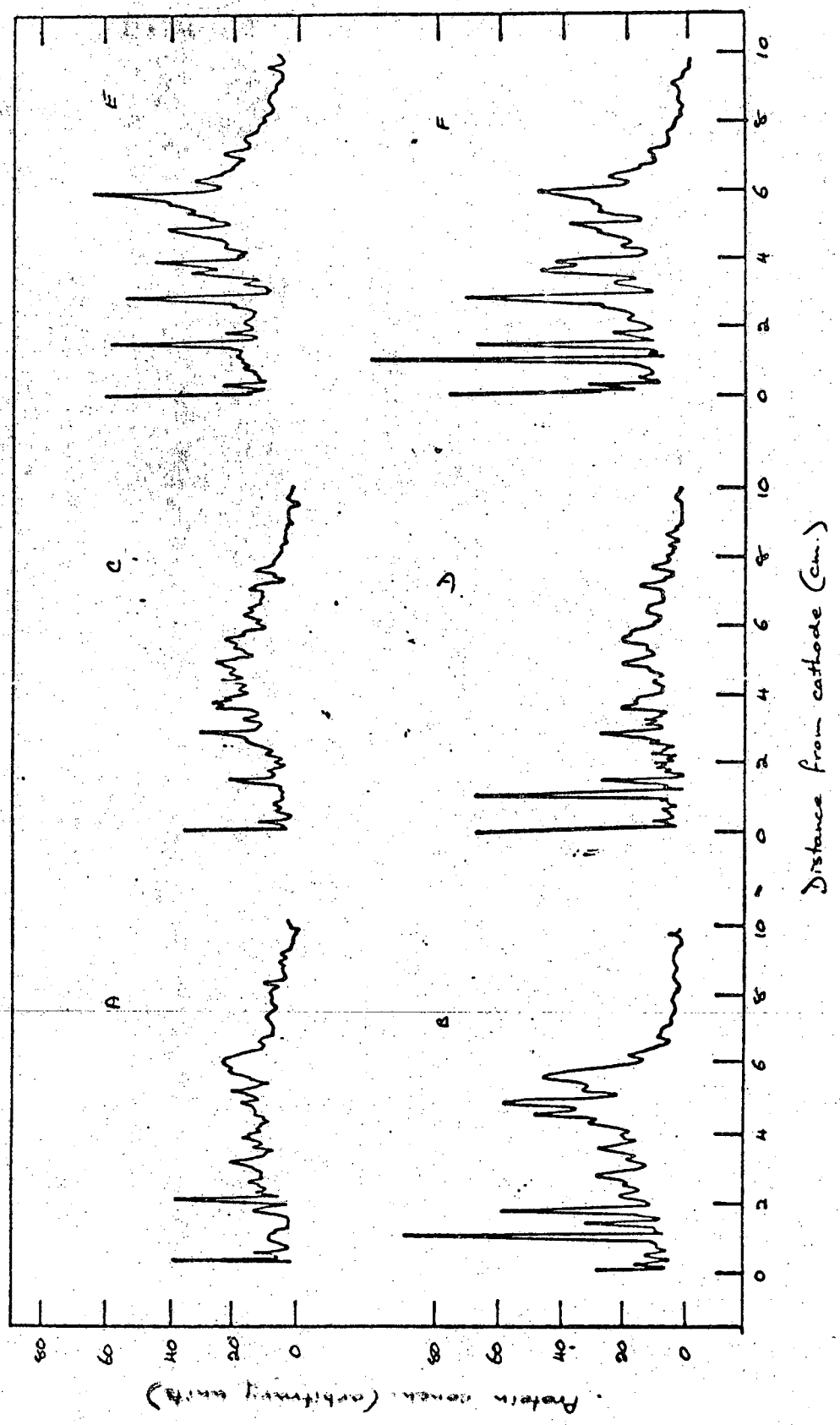


Fig. 13

MANN + MOSES FIG. 14



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TECHNICAL INFORMATION DIVISION
LAWRENCE RADIATION LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720