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THE NONPHOTOSYNTHETIC FIXATION OF CARBON DIOXIDE BY THREE MICRO-ORGANISMS[1]

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Introduction

Studies by Lynch and Calvin (1952,1953) have established the nature of the compounds incorporating C^{14} nonphotosynthetically from $C^{14}O_2$ in thirteen micro-organisms: a yeast, a protozoan, two water moulds, one slime mould, three algae, three bacteria, and the green flagellate Euglena gracilis. With the exception of E. gracilis, and of Lactobacillus casei which fixed no detectable amounts of carbon dioxide, all these organisms fixed carbon dioxide into amino and organic acids derived from the tricarboxylic acid cycle, and into a few other compounds in individual cases (tyrosine, phenylalanine, polysaccharides [probably glucose polymers], acetic acid and butyric acid). The authors concluded that the presence of C^{14} in almost all these compounds could be accounted for by the carboxylation of pyruvate to yield oxalacetic or malic acids, followed by transaminase reactions.

In E. gracilis, however, considerable quantities of activity also appeared in phosphorylated compounds in the dark, especially in the sugar monophosphates, phosphoglyceric acid, and phosphoenolpyruvic acid. Only with this organism was a kinetic study performed to determine the identity and degree of labeling of the compounds containing C^{14} after varying periods of time. It was not stated definitely by which route carbon dioxide entered the photosynthetic intermediates, but it was implied (Fig.4, Lynch and Calvin, 1953) that it was incorporated directly into phosphoglyceric acid, and that the energy for this process, which in photosynthesis is derived from sunlight, was provided by respiration or fermentation. Certain

aspects of these presumptions however, do not adequately account for all the experimental data.

Stoppani, Fuller and Calvin (1955) have also investigated the dark fixation of carbon dioxide by the nonsulfur purple bacterium Rhodopseudomonas capsulatus. As in E. gracilis, this organism incorporated a considerable proportion of the total fixed C^{14} into phosphorylated compounds, as well as into the amino acids and organic acids.

A further investigation has now been carried out with three micro-organisms to elucidate in more detail the metabolic pathways by which carbon dioxide is assimilated nonphotosynthetically. New light has been shed on the route by which it may enter the phosphorylated intermediates common to respiratory and photosynthetic mechanisms. The three organisms studied were the algae Chlorella pyrenoidosa (also used by Lynch and Calvin (1952)) and Nostoc muscorum, and the fungus Zygorrhynchus moelleri, a member of the Phycomycetes.

METHODS

The culturing techniques for C. pyrenoidosa (Holm-Hansen, Hayes and Smith,1956), N. muscorum (Linko, Holm-Hansen, Bassham and Calvin,1957), and Z. moelleri (Moses, 1954, 1959) have been described elsewhere. After growth, the cells in each case were harvested, washed, and resuspended in distilled water at a concentration of 0.033 ml of wet-packed cells/ml of suspension for the algae, and 0.05 ml of wet-packed cells/ml of suspension for the fungus. Aliquots of the cell suspension (1 ml for the algae, and 5 ml for the fungus) were pipetted into a series of small Erlenmeyer flasks which were shaken at room temperature for 30 minutes on a Warburg

respirometer. The preliminary shaking and subsequent incubation of the algae with labeled substrate were performed in complete darkness; no such precautions to exclude light were taken with the mould. At the beginning of the incubation period, each 1 ml sample of algal cell suspension received 0.1 ml of 0.01887 M-$NaHC^{14}O_3$ (40 μc) and the cells were killed at the end of the incubation period by the addition of 4 ml of ethanol. To each 5 ml sample of fungal cell suspension was added 0.25 ml of the $NaHC^{14}O_3$ (100 μc), and 20 ml of ethanol was added at the end of the incubation period to kill the cells.

After extraction with 80% ethanol, the suspensions were centrifuged, and the cell residues extracted with 20% (v/v) ethanol, and then with water. The residues were discarded and the extracts concentrated *in vacuo* below 40° to a small volume. With the algal extracts, the whole of each sample was chromatographed on oxalic acid-washed Whatman No. 4 filter paper; the first solvent was phenol-water (10 hr), and the second solvent *n*-butanol-propionic acid-water (8 hr) (Benson, *et al*, 1950). Radioactive substances were located by exposure of the chromatograms to Dupont blue-sensitive single-coated X-ray film 507E. The concentrated fungal extracts were adjusted to a volume of 1.5 ml, of which 0.3 ml was chromatographed as described above. A further 0.8 ml from each extract was chromatographed for longer periods (24 hr in phenol-water, and 20 hr in *n*-butanol-propionic acid-water) in order to attain a better separation of phosphorylated compounds (Moses and Calvin, 1958). The remaining 0.6 ml of each extract was held in reserve. The chromatograms were exposed to X-ray film as above.

The radioactivity in each substance on the chromatograms was counted with a Scott-type Geiger-Müller tube having a thin (1 mg/cm^2) "Mylar" window. The counting tube was flushed with a mixture of 99.05% (v/v) He and 0.95% (v/v) isobutane.

Radioactive substances were identified in the first instance by their chromatographic positions, and identity was confirmed by cochromatography with known marker substances. Phosphates were first treated with human seminal acid phosphatase in 0.02 M-acetate buffer, pH 5, before being cochromatographed with appropriate sugars, acids, etc.

RESULTS

Chlorella pyrenoidosa.

The distribution of activity in compounds from this organism after incubation periods in the dark with labeled carbon dioxide of 19 sec, 32 sec, 46 sec, 60 sec, 121 sec, and 183 sec, and, in a separate experiment, 1800 sec, is shown in Table 1. The plot of the percent activity with time showed negative slopes in the cases of malic acid, citric acid, and alanine, suggesting that all three compounds are early products of $C^{14}O_2$ fixation in the dark. The first phosphorylated compound to appear (phosphoglyceric acid) was not seen until 2 min with labeled substrate had elapsed. While most of the fixed activity remained in amino acids (65%) and organic acids (22%) after 30 min, some activity (about 3.5%) also appeared in phosphorylated substances. The fact that phosphoglyceric acid was the first of these to incorporate C^{14}, followed later by triose and hexose phosphates, suggests that C^{14} entered the sugars by a reversal of the glycolytic reactions.

The presence of label at the earliest incubation periods in citric and malic acids, and in alanine, indicates that three carboxylation reactions may proceed simultaneously. One of these is the carboxylation of pyruvate to produce oxalacetate; the pool size of the latter is presumably very small, and label first appeared in citrate. A second carboxylation of pyruvate by the malic enzyme

would form malic acid directly. However, the incorporation of label so rapidly into alanine suggests that another carboxylation also took place, that of a two-carbon substance (possibly acetylthioctic acid) to pyruvate, which was rapidly transaminated to alanine. Whether or not two different carboxylations of pyruvate took place, or whether both malic and citric acids arose from one carboxylation only, cannot definitely be decided from the data. One of the radioautograms of the Chlorella extracts is shown in Fig. 1.

Nostoc muscorum.

A kinetic study of the incorporation of $C^{14}O_2$ in the dark by the blue-green alga, N. muscorum, after several exposure periods to the isotope is reported in Table 2, and a radioautogram of the cell extract after 30min exposure shown in Fig. 2. In contrast to C. pyrenoidosa, only one compound, aspartic acid, showed a negative slope of percentage incorporation of activity with time. Activity appeared in citric and glutamic acids before malic acid, indicating that uptake of $C^{14}O_2$ by the malic enzyme was not significant in this alga. The main carboxylation reaction appeared to be that forming oxalacetic acid from pyruvic acid (with rapid transamination to aspartic acid). C^{14} was also incorporated into citrulline (Table 2). Citrulline has earlier been reported in this organism by Linko et al (1950), who found that the early label was in the carbamyl group,

Zygorrhynchus moelleri.

The suspension of fungal hyphae was exposed to labeled bicarbonate for fourteen different incubation periods. The distribution of activity in seven of these extracts (4 sec, 11 sec, 30 sec, 60 sec, 105 sec, 180 sec, and 1800 sec) is given in Table 3. Radioautograms of the extract after 30 min incubation, showing all the compounds present, and of a chromatogram of the extract after 165 sec dev-

eloped for long periods to spread the phosphate esters, are shown in Figs. 3 and 4, respectively.

With this organism, as with the two algae described above, there is evidence for carboxylation of pyruvate. As in the case of C. pyrenoidosa (Table 1), the percentage of activity in malic and citric acids showed a decrease with increasing time. Amino acid products (particularly aspartic acid) became prominent with increasing time. Citrulline appeared after 30 min. In a number of ways, however, the incorporation of C^{14} showed differences from the algae. Most prominent of these was the very early appearance of tracer carbon in phosphogluconic acid, which reached a maximum percentage activity in 11-15 sec, and thereafter showed a decline in the percentage activity incorporated. Phosphoenolpyruvate was the only other phosphorylated respiratory intermediate to contain C^{14} until sugar monophosphates and phosphoglyceric acid appeared after 30 min. Considerable amounts of radioactivity also appeared in various nucleotides, and in fact after 30 min more C^{14} was found in these compounds than in any other group of substances except the amino acids (Table 3).

The presence of unlabeled glucose in the medium (0.022 M), added 30 min before the labeled bicarbonate, increased the uptake of C^{14} at all the incubation periods studied (e.g., to 221%, 160%, and 131% of the C^{14} fixed in the absence of glucose after 45 sec, 180 sec, and 1800 sec, respectively). With glucose present, the percent C^{14} taken up into sugar phosphates, nucleotides, nucleotide-sugar complexes, and amino acids fell, after 30 min, by 45-75%, while that incorporated into organic acids of the Krebs cycle increased some 5.5 times (Table 3 and Fig. 5). This can probably be explained by the relatively large pools of unlabeled sugar phosphates remaining from the unlabeled glucose, while the lowered incorporation of C^{14} into the nucleotides and amino acids was probably due to the absence of available nitrogen for the

synthesis of such substances owing to the prior metabolism of external glucose in the absence of a source of nitrogen. Other studies (Moses, 1959) have shown that in hyphae starved of nirtogen the synthesis of nucleotides is greatly depressed unless an external source of nitrogen is supplied. However, the unlabeled glucose probably enabled more $C^{14}O_2$ to be fixed by the carboxylation of pyruvate derived from the sugar, with the formation of organic acids of the tricarboxylic acid cycle.

DISCUSSION

The experiments with the dark fixation of $C^{14}O_2$ by the two algae have confirmed and extended many of the findings of Lynch and Calvin (1952). The kinetic curves for the entry of C^{14} into the compounds of these cells have indicated that the only carboxylation reaction of importance was that of pyruvic acid to oxalacetic or malic acids, except for the synthesis of citrulline in N. muscorum, and of alanine in C. pyrenoidosa. The appearance of label in the sugar phosphates of various types is consistent with a reversal of glycolytic reactions being the route by which this was accomplished. Phosphoglyceric acid was the first of the phosphorylated compounds in which C^{14} appeared, and only much later was tracer found in the hexose phosphates (the pool of phosphoenolpyruvate was probably too small for the detection of label in this substance in the early stages).

Evidence is also presented for $C^{14}O_2$ uptake by carboxylation of pyruvate in Z. moelleri. In this organism, however, label did not appear to enter the hexose monophosphates by a reversal of glycolycis, but rather by a reversal of the well-known decarboxylation of 6-phosphogluconic acid to pentose phosphate (Gunsalus, Horecker and Wood, 1955). Labeled carbon appeared in phosphogluconate

in significant quantities at the shortest incubation times, and was found in the hexose monophosphates without first appearing in the hexose diphosphates; the activity in phosphoglyceric acid was also very low compared with that in the hexose monophosphates, particularly when unlabeled glucose was present. Nevertheless, minute quanitites of C^{14} did appear in phosphoenolpyruvate after about 60 sec, and much later showed up in phosphoglycerate, so that while hexose synthesis by a reversal of glycolysis does not seem to be impossible in this mould, $C^{14}O_2$ entered the hexose much more readily by carboxylation of pentose.

Other work (Moses, 1959) on the metabolism of labeled glucose by Z. moelleri has shown that glucose is degraded by both the pentose phosphate cycle and via glycolysis. The first three substances in which C^{14} appeared from uniformly labeled glucose were the hexose monophosphates and diphosphates, and phosphoglyceric acid. Yet when the hyphae were supplied with $C^{14}O_2$ while simultaneously oxidizing glucose, partly via fructose diphosphate, activity appeares in the sugar monophosphates but not in the diphosphates. These results appear to be particularly significant with regard to the physical separation within the cells of different metabolic pools of the same substances, and they suggest that there must be two pools, at least of phosphogluconate and of hexose monophosphate, to account for the absence of label in hexose diphosphate. Other work with C. pyrenoidosa (Moses, Holm-Hansen, Bassham and Calvin, 1959) has also suggested that pools of respiratory and photosynthetic intermediates are not in a state of rapid equilibrium, even though many of the substances in both systems are chemically identical.

These conclusions appear to be relevant in the findings of Lynch and Calvin (1953) with E. gracilis. There, too, C^{14} from $C^{14}O_2$ entered the hexose monophosphates very rapidly, and little variation in the percentage of the total fixed C^{14} which was present in the hexose monophosphates was apparent in the period from 5

to 40 min. Furthermore, no activity was present in the sugar diphosphate area on the radioautogram of dark fixation of carbon dioxide in E. gracilis (Lynch and Calvin, 1953). It seems reasonable, therefore, that the initial carboxylation into hexoses in the dark in E. gracilis, like Z. moelleri, may be via phosphogluconic acid, and not by carboxylation of ribulose diphosphate to yield phosphoglyceric acid.

SUMMARY

An investigation has been made of the nonphotosynthetic uptake of $C^{14}O_2$ by the algae Chlorella pyrenoidosa and Nostoc muscorum, and the mould Zygorrhynchus moelleri. In both the algae the entry of carbon dioxide in the dark was primarily by carboxylation of pyruvate to organic acids in, or close to, the citric acid cycle. Tracer appeared after about half an hour in phosphorylated sugars, probably by a reversal of glycolysis reactions.

While carboxylation reactions leading to amino and organic acids also were prominent in the fungal hyphae, the first labeled phosphate observed was phosphogluconic acid, which made its appearance as early as 4 seconds after the addition of labeled carbon dioxide in the cells: this was the shortest period studied. C^{14} appeared in the hexose monophosphates without first being present in the hexose diphosphates or phosphoglyceric acid. Very little C^{14} was seen in phosphoglyceric acid after 30 min, and none at all in the diphosphates. It is concluded that $C^{14}O_2$ was incorporated into hexoses by carboxylation of pentose phosphate to 6-phospho-3-keto-gluconate, which was later reduced to glucose-6-phosphate through 6-phosphogluconate. The significance of these findings with regard to separate pools of metabolic intermediates is discussed.

Another significant difference between the fungus and the algae was the large amount of C^{14} which, in the fungus, appeared in several nucleotides.

Earlier investigations of the dark fixation of $C^{14}O_2$ by *Euglena gracilis* (Lynch and Calvin, 1953) have been considered in view of the present studies, and alternative conclusions from those originally proposed have been suggested.

TABLE 1

Fixation of labeled carbon dioxide in the dark by Chlorella pyrenoidosa

1 ml aliquots of cell suspension, containing 0.033 ml of wet-packed cells, incubated with 0.1 ml of 0.01687 M-$NaHC^{14}O_3$ (40 μc) for the periods indicated. The values given represent the percentages of the total fixed C^{14} in soluble material which were present in each compound.

Incubation period	19 sec	32 sec	46 sec	60 sec	121 sec	183 sec	1800 se
Sugar monophosphates							1.2
Triose phosphate**							0.59
Phosphoglyceric acid					0.60	0.40	0.47
Phosphoenolpyruvic acid**							0.14
Uridine-phospho-sugar**							0.95
Malic acid	27.5	22.3	19.4	16.3	13.3	14.3	15.7
Citric acid	10.1	6.5	7.0	3.8	4.6	3.9	1.8
Fumaric acid		1.8	6.9	3.3	3.0	3.6	3.0
Succinic acid							1.7
Glucose**							1.0
Aspartic acid	40.4	50.7	45.5	56.2	58.2	55.6	26.9
Glutamic acid			6.8	5.9	9.3	11.9	35.2
Alanine	21.9	18.6	14.5	14.6	11.0	10.4	2.0
Serine plus glycine***							1.1
Unidentified subatances							8.1
Total C^{14} fixed in soluble materials (dis/min/ml cells x 10^{-5})	0.089	0.245	0.377	0.597	0.822	1.88	12.1

* Separate experiment
** Identity not confirmed by cochromatography
*** Not separated chromatographically

TABLE 2

Fixation of labeled carbon dioxide in the dark by Nostoc muscorum

1 ml aliquots of cell suspension, containing 0.033 ml of wet-packed cells, incubated with 0.1 ml of 0.01687 M-$NaHC^{14}O_3$ (40 μc) for the periods indicated. The values given represent the percentages of the total fixed C^{14} which were present in each compound.

Incubation period	3 sec	10 sec	30 sec	60 sec	180 sec	1800 sec
Sugar monophosphates					1.5	5.0
Pentose phosphates*					1.6	1.2
Phosphoglyceric acid					1.8	1.1
Phosphoenolpyruvic acid*						0.21
Sucrose						2.2
Malic acid					0.91	0.30
Citric acid				4.8	1.8	2.5
Aspartic acid	100.0	100.0	100.0	95.2	80.2	40.5
Glutamic acid					4.6	24.8
Alanine					1.4	2.3
Glutamine					1.3	1.7
Threonine						2.6
Citrulline					4.1	7.0
Unidentified substances					0.79	8.6
Total C^{14} fixed in soluble materials (dis/min/ml cells x 10^{-5})	0.0235	0.0085	0.034	0.165	1.59	4.50

* Identity not confirmed by cochromatography

TABLE 3

Fixation of labeled carbon dioxide by Zygorrhynchus moelleri

5 ml aliquots of cell suspension, containing 0.25 ml of wet-packed cells, incubated with 0.25 ml of 0.01687 M-$NaHC^{14}O_3$ (180 μc) for the periods indicated. The values given represent the percentages of the total fixed C^{14} which were present in each compound. Glucose concentration, 0.022 M.

Incubation period (sec)	4	11	30	60	105	180	1800*	1800* (plus glucose)
Sugar monophosphates**							1.2	0.59
Phosphoglyceric acid							0.8	0.05
Phosphoenolpyruvic acid				0.6	0.2	0.02	0.3	0.07
Phosphogluconic acid	10.9	11.1	6.5	1.1	0.4	0.2	0.1	0.23
Uridine triphosphate					0.4	1.1	1.0	0.01
Uridine diphosphoribose (plus inosine triphosphate)			0.7	2.0	5.3	4.0	1.3	0.23
Uridine diphosphoglucose				0.1	1.2	1.8	1.3	0.31
Adenosine triphosphate (plus uridine diphosphate)			0.3	2.7	4.4	2.8	1.0	2.3
Adenosine diphosphate								0.39
Nucleotide diphosphates***			0.5	0.7	3.1	4.9	21.5	6.0
Unidentified nucleoside monophosphate				0.8	1.0	0.8		
Maltose				0.8	0.5	0.3	0.2	1.1
Fructose					1.5	3.0		
Sucrose****								0.40
Malic acid	23.6	21.2	11.3	14.8	9.0	8.0	6.6	46.1
Citric acid	65.5	39.0	31.0	11.2	7.6	5.2	2.4	6.1
Fumaric acid		6.0	1.0	2.3	1.4	1.5	0.9	4.0
Succinic acid							1.1	
Aspartic acid		22.6	40.9	51.5	48.9	49.5	19.9	4.1
Glutamic acid			2.2	3.3	4.4	5.1	20.5	13.8
Serine plus glycine				1.0	2.0	3.3	3.9	0.85
Threonine			2.2	0.7	1.3	2.3	2.3	0.36
Alanine			3.5	5.9	6.0	4.2	2.3	0.52
Proline					0.4	0.5	0.4	1.1
Valine				0.6	0.4	0.3	3.4	0.30
Glutamine					0.6	0.7	3.8	4.4
Citrulline							0.2	1.2
Tyrosine								0.05
Histidine								1.8
Unidentified substances						0.5	3.9	3.5
Total C^{14} fixed in soluble materials (dis/min/ml cells x 10^{-5})	0.183	0.965	3.38	14.8	44.3	81.2	103	111

* Separate experiments

** Contains monophosphates of glucose, fructose, and possibly sedoheptulose

*** Contains diphosphates of inosine, uridine, and guanosine **** Identity not confirmed by

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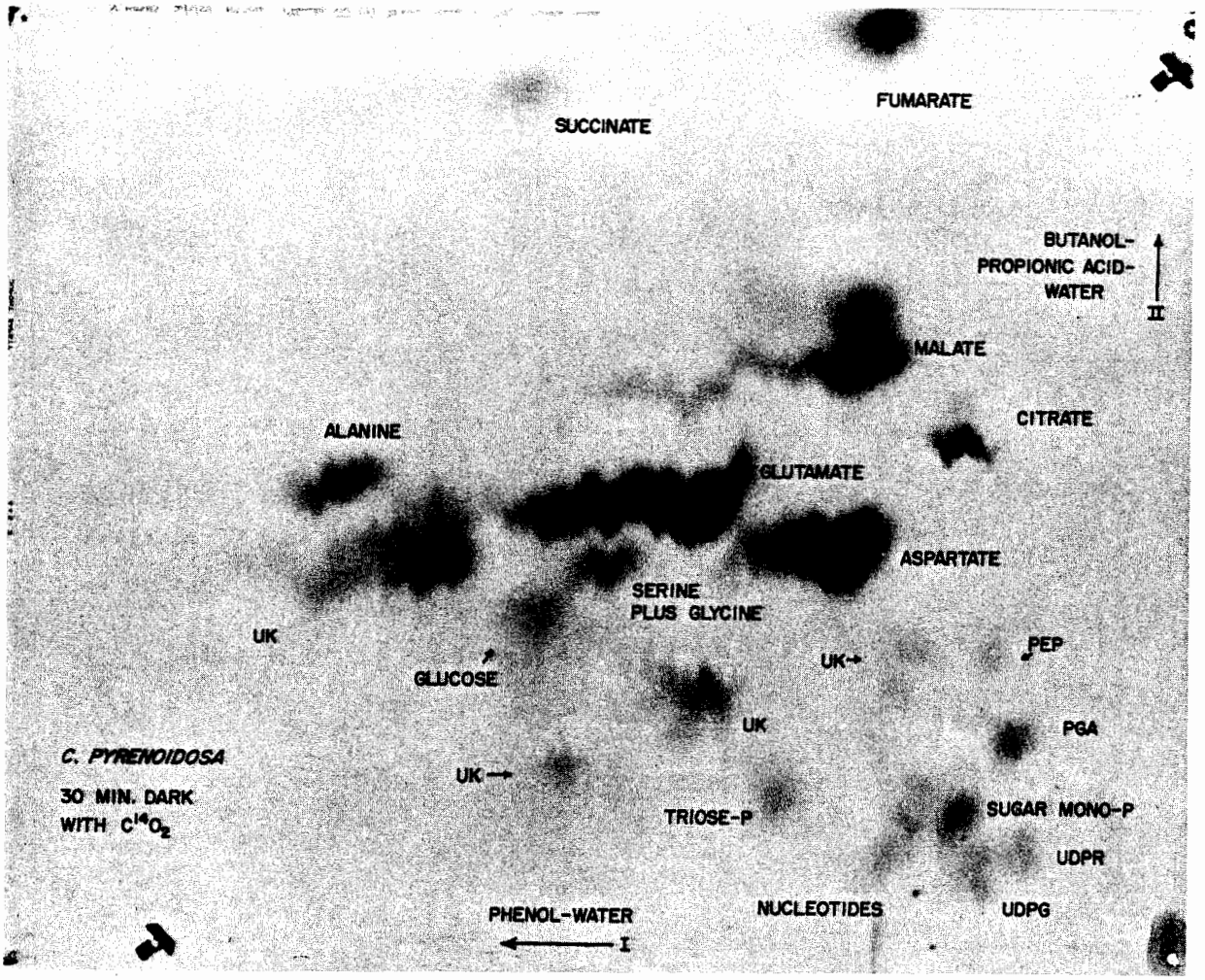
FOOTNOTES

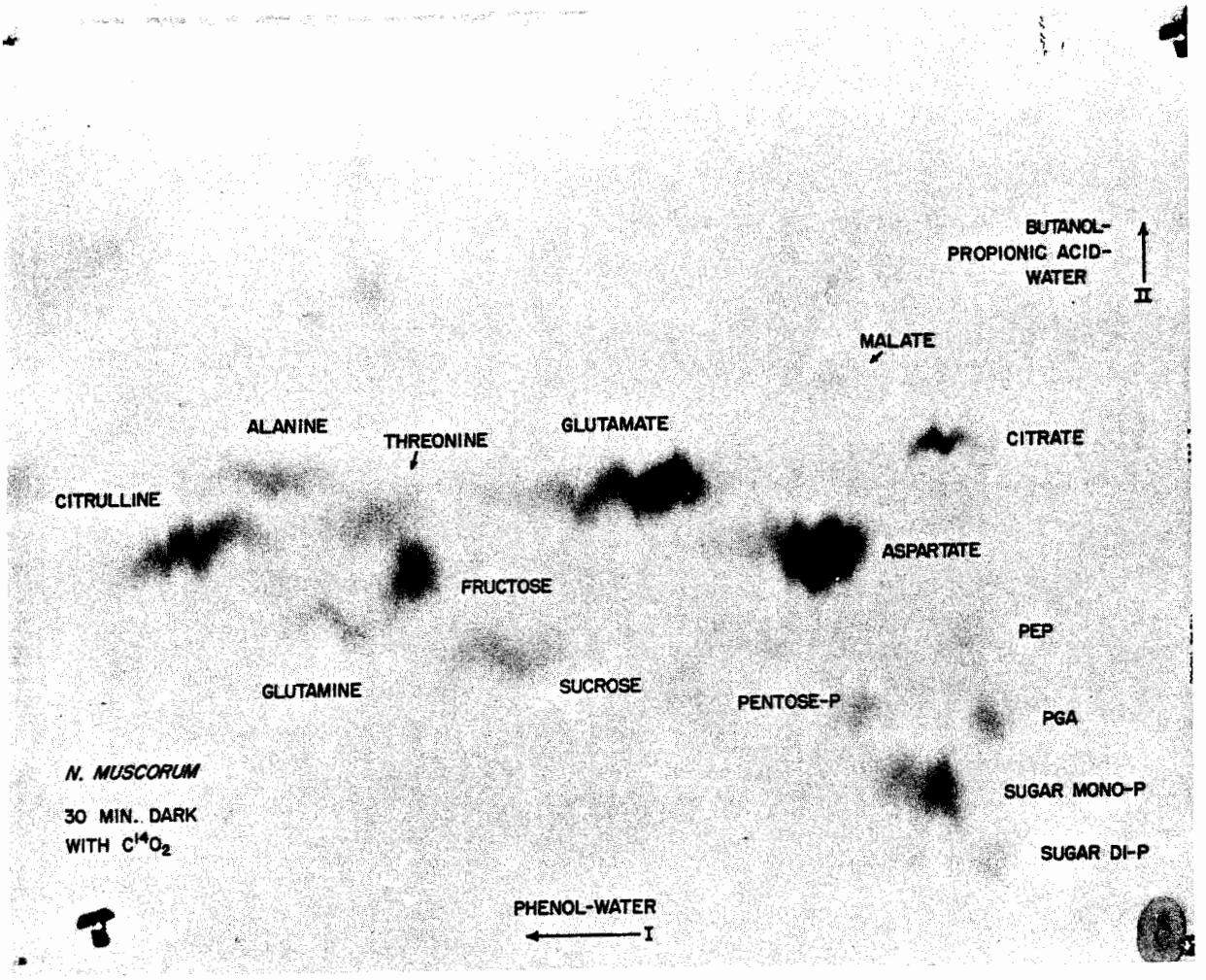
1. The work described in this paper was sponsored in part by the U.S. Atomic Energy Commission, and in part by the Department of Chemistry, University of California, Berkeley, California.

2. Present address:



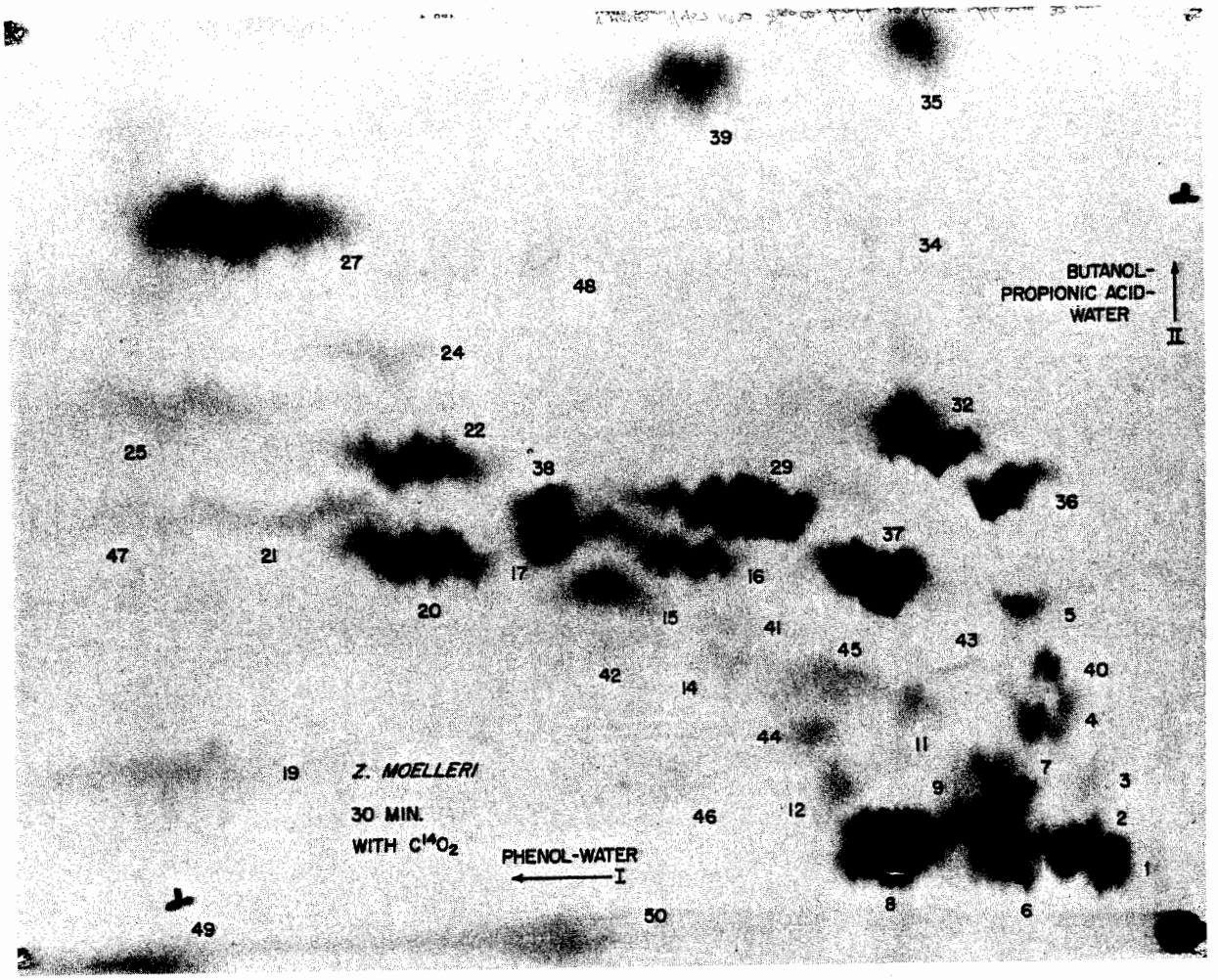
ZN-1982

Figure 1. Dark fixation of labeled carbon dioxide by C. pyrenoidosa. Radioautogram of extract of Chlorella cells incubated in the dark for 30 min with $NaHC^{14}O_3$. Key to abbreviations: PEP, phosphoenolpyruvate; PGA, phosphoglyceric acid; Sugar mono-P, sugar monophosphates; Triose-P, triose phosphate; UDPG, uridine diphosphoglucose; UDPR, uridine diphosphoribose; UK, unidentified.



ZN-1985

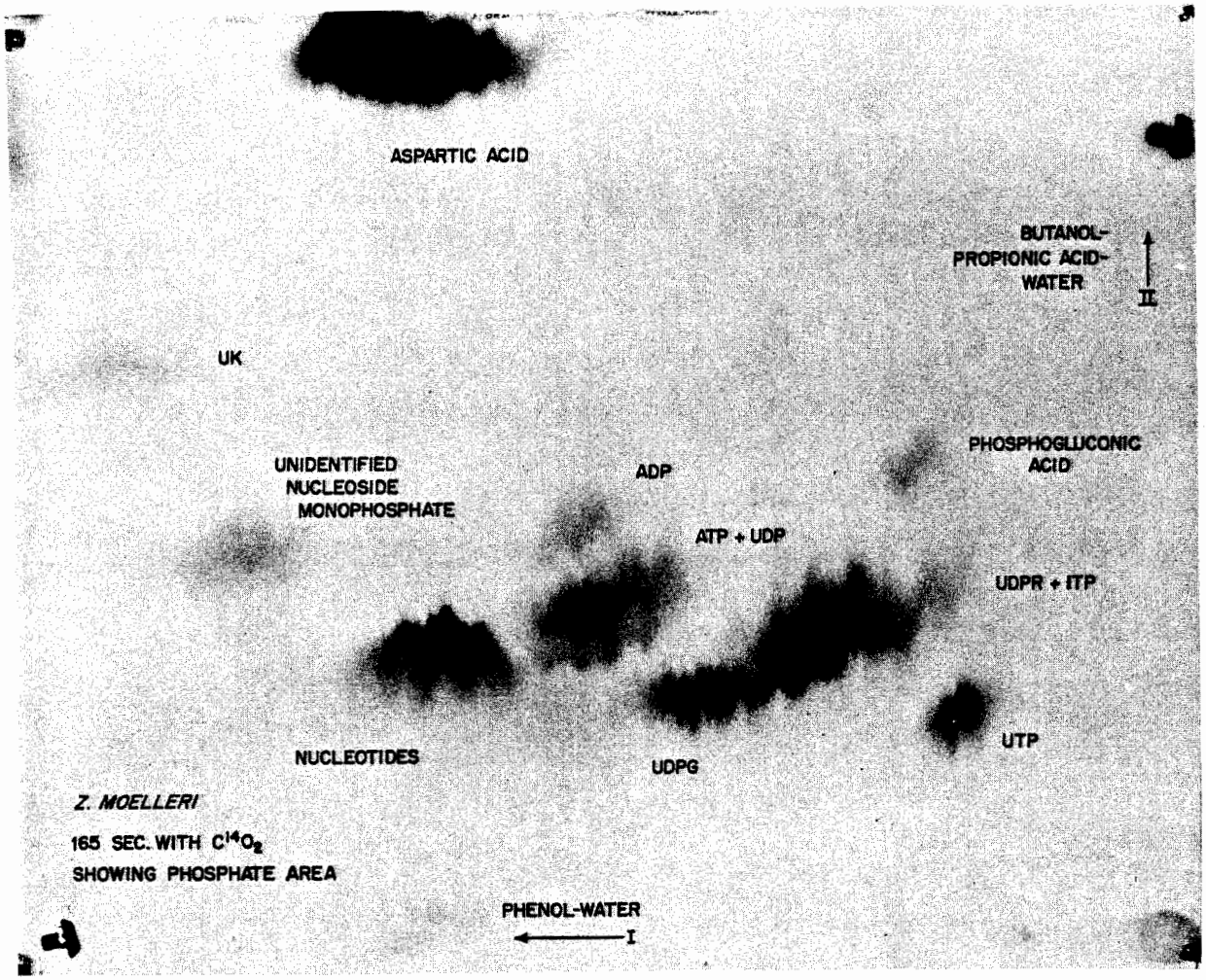
Figure 2. Dark fixation of labeled carbon dioxide by N. muscorum. Radioautogram of extract of Nostoc incubated in the dark for 30 min with $NaHC^{14}O_3$. Key to abbreviations: Pentose-P, pentose phosphates; Sugar di-P, sugar diphosphates; others as in Figure 1.



ZN-1981

Figure 3. Fixation of labeled carbon dioxide by Z. moelleri.

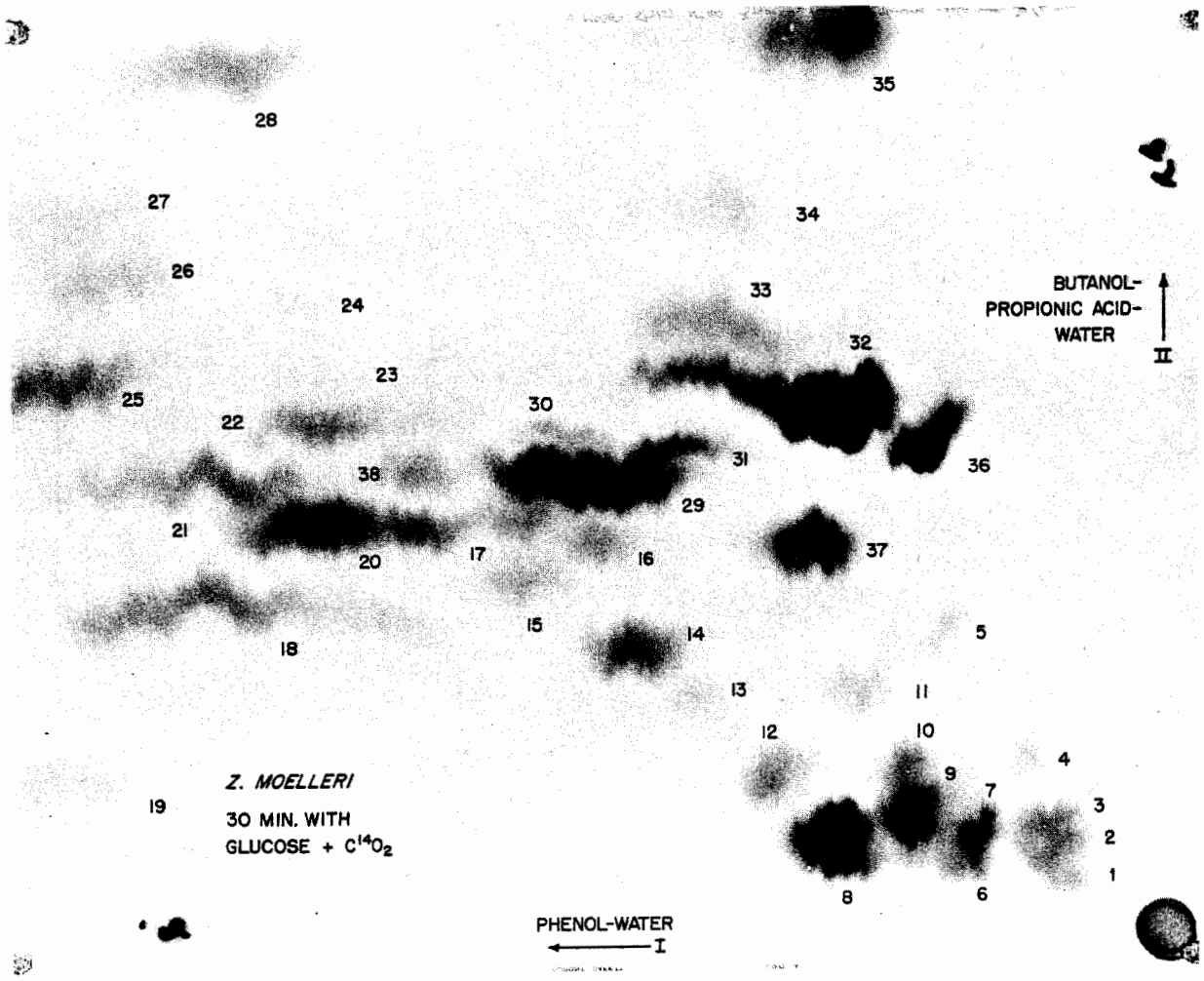
Radioautogram of extract of Zygorrhynchus cells incubated for 30 min, with $NaHC^{14}O_3$. Key to numbers: 1, uridine triphosphate; 2, uridine diphosphoribose plus inosine triphosphate; 3, phosphogluconate; 4, phosphoglycerate; 5, phosphoenolpyruvate; 6, uridine diphosphoglucose; 7, sugar monophosphates; 8, nucleoside diphosphates; 9, adenosine triphosphate plus uridine diphosphate; 11, unidentified; 12, unidentified; 14, maltose; 15, sucrose; 16, serine; 17, glycine; 19, unidentified; 20, glutamine; 21, citrulline; 22, alanine; 24, tyrosine; 25, proline; 27, valine; 29, glutamic acid; 32, malic acid; 34, unidentified; 35, fumaric acid; 36, citric acid; 37, aspartic acid; 38, threonine; 39, succinic acid; 40, phosphoglycollic acid; 41-50, unidentified.



ZN-1984

Figure 4. Fixation of labeled carbon dioxide by Z. moelleri.

Radioautogram of extract of Zygorrhynchus cells incubated for 165 sec with $NaHC^{14}O_3$. Chromatogram developed for 24 hr with phenol-water, and for 20 hr with n-butanol-propionic acid-water in order to spread the phosphate area. Key to abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; ITP, inosine triphosphate; UTP, uridine triphosphate; others as in Figure 1.



ZN-1983

Figure 5. Fixation of labeled carbon dioxide in the presence of glucose by Z. moelleri. Radioautogram of extract of Zygorrhynchus cells incubated for 30 min with $NaHC^{14}O_3$ in the presence of unlabeled glucose (0.022 M). Key to numbers: 10, adenosine diphosphate; 13, unidentified; 18, histidine; 23, unidentified; 26, unidentified; 28, unidentified; 30, unidentified; 31, unidentified; 33, unidentified; rest as in Figure 3.