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(^{14}C)Glucose Metabolism in Fungal Cells

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(^{14}C)Glucose Metabolism in Fungal Cells*

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SUMMARY: The incorporation pattern of ^{14}C from (^{14}C)glucose by the fungus Zygorrhynchus moelleri has been investigated. The following substances became labelled in incubation periods of 6 seconds to 30 minutes: the monophosphates of glucose, fructose, sedoheptulose, ribose, maltose, glycerol, gluconic acid, glyceric acid, enolpyruvic acid and guanosine; the diphosphates of fructose, glucose, glyceric acid, adenosine, uridine, inosine and guanosine; the triphosphates of adenosine and uridine; uridinediphosphoglucose and uridinediphosphoribose; free maltose and fructose; aspartic acid, glutamic acid, alanine, valine, tyrosine, proline, histidine, threonine, citrulline and glutamine; malic acid, citric acid, succinic acid, fumaric acid and glyceric acid. A small percentage of the ^{14}C was present in unidentified substances. The kinetics of the incorporation of ^{14}C first into uridinediphosphoglucose, then into maltose phosphate, and finally into free maltose, suggests this sequence of compounds for the biosynthetic pathway of maltose formation.

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The main effects of starvation of the cells by shaking them in phosphate buffer for 24 hours was an increased incorporation of ^{14}C into maltose and the sugar phosphates, and a decreased incorporation into amino acids, organic acids and nucleotides. The addition of a small amount of ammonia to starved cells increased the activity in the nucleotides and decreased that in the sugar phosphates. In the presence of azide, the incorporation of ^{14}C by starved cells into maltose and the nucleotides was severely inhibited and the incorporation into amino acids was somewhat increased. Thus, one of the most important results of starvation was to prevent incorporation of ^{14}C into the nucleotides, while the addition of ammonia overcame the effects of starvation and promoted nucleotide synthesis. The effects of ammonia were abolished by azide.

A kinetic study of the sequence in which ^{14}C was incorporated into these compounds indicated that glucose was metabolized by both the glycolysis and the oxidative pathways, and later by the tricarboxylic acid cycle.

Investigation into the respiratory activities of fungi with labelled substrates has been used mainly to study the distribution of label within various molecules produced enzymically from substrates labelled in known positions. From the results it has been possible to arrive at certain conclusions as to the importance of particular pathways for the metabolism of the relevant substrates. Thus, Goldschmidt, Yall & Koffler (1956), and Moses (1957), have demonstrated that the tricarboxylic acid cycle plays a prominent role in the oxidation of acetate in two moulds, and Heath & Koffler (1956), and Bartlett & Moses (1957), have used variously labelled glucoses to investigate the importance of the oxidative and fermentative pathways in the utilization of glucose by Penicillium chrysogenum and Zygorrhynchus moelleri, respectively.

Relatively little work has been done, however, along the lines of Calvin (1956) and his co-workers, in which an organism is fed a labelled substrate for various short periods of time and then killed and extracted to determine the nature of all the substances incorporating label from the original substrate. Claridge & Werkman (1954) have studied the incorporation of label from (^{14}C)2-ketogluconate by Pseudomonas aeruginosa, and Milhaud & Aubert (1955) have supplied labelled glucose and ethanol to baker's yeast. In both cases a considerable number of products were detected on a subsequent analysis of the cells, and an attempt was made to obtain a picture of the kinetic relations between the compounds observed. A preliminary study by Bartlett & Moses (1957), using Z. moelleri, showed that this fungus converts (^{14}C)glucose into a large number of compounds in a very short time (15 seconds was the shortest incubation time used). At that time it was not possible to identify many of the products of glucose metabolism, and no very definite conclusions could be drawn.

The present communication reports a further attempt to elucidate this problem using cells of Z. moelleri in various experimental conditions, and analyzing the cells after several short periods of exposure to very high specific activity (^{14}C)glucose. Following the killing and extraction of the cells with hot ethanol, the extracts were analyzed by chromatography and radioautography as described by Benson, Bassham, Calvin, Goodale, Haas & Stepka (1950). The results are consistent with the operation of both the hexose monophosphate shunt and the glycolysis mechanisms for glucose breakdown, and with the subsequent involvement of the citric acid cycle. Considerable differences were found in the pattern of incorporation of ^{14}C between starved and fresh cells, and a study has been made of the effects

of sodium azide and of ammonium chloride on glucose metabolism in the starved cells.

METHODS

Cultures of Zygorrhynchus moelleri (Commonwealth Mycological Institute No. 21994) were cultured in the same medium as that used in previous studies (Moses, 1954), save that yeast or liver extracts were omitted. Spores obtained from several agar slants of the fungus were inoculated into 200 ml. of the growth medium in a 2 l. bottle which was shaken vigorously on a reciprocal shaker for about 16 to 18 hours at room temperature (ca. 25°C). After growth the cells were harvested by centrifugation, washed with distilled water, and subsequently resuspended in distilled water at a cellular concentration of about 4 ml. wet-packed cells/100 ml. suspension. The volume of the wet-packed cells was determined by centrifuging the suspension in a graduated 12 ml. conical tube for 5 minutes at 500 g.

If starved cells were required, the hyphae, after harvesting and washing, were resuspended in 200 ml. of 0.067 M-phosphate buffer, pH 6.8, and were shaken for a further 24 hours. They were then washed and resuspended in distilled water.

Each of several 50 ml. Erlenmeyer flasks received 5 ml. of cell suspension. At the beginning of the incubation period 40 µl. of (^{14}C)glucose solution (specific activity 240 µc./mg.; 33.6 µc./flask; final glucose conc. 1.6×10^{-4} M) were rapidly injected into one flask at a time. After the desired incubation period at room temperature the cell suspension was quickly poured through a sintered glass funnel; the latter had previously been inserted through a rubber stopper into the top of a Buchner flask, and a layer of Celite filter

aid deposited on the sintered glass. The suspension was sucked through the filter by a water pump, the funnel and the rubber stopper removed from the Buchner flask, inserted into a centrifuge tube of suitable size, and 5 ml. of boiling 80% (v/v) ethanol poured onto the cells on the sintered glass disc. The operations of filtering and killing the cells were performed as quickly as possible, and, with practice, only about 2 seconds elapsed between the pouring of the cell suspension onto the filter pad and the addition of the ethanol.

The cells were allowed to stand for 2 hours at room temperature, the funnel loosened in the tube, and the suspension filtered through the pad into the tube by pressure from a N_2 cylinder. The funnel was again inserted into the tube and 2 ml. of boiling 20% (v/v) ethanol poured onto the cells. After 1 hour the second extract was filtered and the process repeated once more with a further 2 ml. of boiling 20% (v/v) ethanol.

The three ethanol extracts from each sample were pooled and evaporated *in vacuo* below 40° to a small volume. Two-dimensional descending chromatograms were run of each extract on 18" x 22" sheets of Whatman No. 4 filter paper previously washed with 2% (w/v) oxalic acid. Phenol-water was used as the solvent in the first dimension and *n*-butanol-propionic acid-water as that in the second dimension (Benson *et al.*, 1950). Generally, two chromatograms were run for each sample. For the first, 1/5 of each extract was chromatographed so that each solvent just reached the edge of the paper (8 to 10 hours for phenol-water, and 7 to 8 hours for *n*-butanol-propionic acid-water) to obtain a picture of all the soluble substances in the extract. On such chromatograms sugar phosphates and nucleotides, important compounds in this type of investigation, move but a small distance from the origin, and the separation of

many of these compounds is very poor. To overcome this difficulty, a further 2/5 of each extract was chromatographed for 24 hours with phenol-water and for 20 hours with n-butanol-propionic-water acid (Moses & Calvin, 1958). This spread out the area containing the sugar phosphates and nucleotides much further, and good separation was usually achieved. The final 2/5 of each extract was kept in reserve in case one of the chromatograms was unsatisfactory. The locations of the radioactive materials were determined by exposing the chromatograms to 14" x 17" sheets of single-coated blue-sensitive Dupont X-ray film 507-E for a suitable period. Radioactive ink markers were stamped on the corners of the chromatograms to facilitate the subsequent alignment of the film with the paper.

The ^{14}C present in each compound on the chromatogram was determined with a Scott-type large window Geiger-Muller tube connected to a scaler. The window was of Du Pont "Mylar" Polyester film and the counting tube was flushed continuously with "Q gas" (99.05% (v/v) He and 0.95% (v/v) isobutane). When this counter was used to count ^{14}C from Whatman No. 4 filter paper, about 6 to 7% of the total disintegrations were counted. The spots were usually counted on both sides of the paper and the values obtained were averaged.

The identity of labelled compounds on the chromatograms was established partly by their position and their susceptibility to hydrolysis by acid phosphatase, and was confirmed by co-chromatography with authentic marker substances, if necessary, after removal of the phosphate groups from the unknown substances. Co-chromatography was performed in the same solvent systems as those mentioned above. Exact coincidence between the labelled substance and the authentic marker was taken as the criterion for identity.

The following techniques were used for the location of the authentic markers: Amino-acids were sprayed with 0.1% (w/v) ninhydrin in 95% (v/v) ethanol and the chromatograms heated at 100° for 5 minutes. Organic acids were sprayed with an 0.04% (w/v) ethanolic solution of bromcresol green, made just alkaline with a little NH_3. Sugars and other reducing substances (i.e., glyceric acid) were detected by dipping the chromatograms into an acetone solution of $AgNO_3$, allowing them to dry in air, and then spraying with ethanolic NaOH. After development of the spots, the excess $AgNO_3$ was washed off in NH_3 solution and the papers dried (Trevelyan, Procter & Harrison, 1950). In addition, ketoses were detected with the orcinol-trichloracetic acid reagent (modified from Klevstrand and Nordal, 1950). Nucleosides were located by their quenching of fluorescence under u.v. illumination.

Spots suspected of being phosphates were eluted from the paper with water, treated either with purified Polidase S (Cohen, 1953) plus acetate buffer, pH 5, or with human seminal acid phosphatase (a gift from Professor H. A. Barker) and acetate buffer pH 5, and allowed to stand overnight at 37°. Authentic markers were added, and the substances chromatographed.

Labelled glucose was prepared together with K. K. Lonberg-Holm from Canna leaves allowed to carry out photosynthesis in the presence of $^{14}CO_2$ (Putman & Hassid, 1952). Owing to the radiation decomposition of the very high specific activity glucose, samples of the glucose were chromatographed immediately before use, and then eluted from the chromatograms. This achieved a satisfactory purification of the substrate and, except for one experiment in which this was not done (see Results), no interference from contaminants in the (^{14}C)glucose was apparent on the chromatograms.

RESULTS

Identification of substances formed from glucose

A considerable number of substances were produced from (^{14}C)glucose by the hyphae, some in very short periods. Aspartic acid, glutamic acid, alanine, valine, tyrosine, glutamine, proline, histidine, threonine, citrulline, malic acid, citric acid, succinic acid, fumaric acid, glyceric acid, maltose and fructose were identified by elution and co-chromatography with the appropriate authentic markers. The maltose spot contained only glucose after acid hydrolysis (N-HCl at 60° for 12 hours).

Analysis of the area on the chromatograms known to contain the sugar monophosphates (Benson et al., 1950; Bassham & Calvin, 1957) was performed by treatment with phosphatase followed by co-chromatography. Phosphoglyceric acid, phosphoenolpyruvic acid, ribose monophosphate, glycerol phosphate, maltose phosphate and phosphogluconic acid were identified, after hydrolysis, by co-chromatography with glyceric acid, pyruvic acid, ribose, glycerol, maltose and gluconic acid, respectively. The area suspected of being mixed hexose and heptose monophosphates was shown to contain glucose, fructose and sedoheptulose monophosphates. Sedoheptulose lies chromatographically between glucose and fructose, and is not easy to detect in the presence of large amounts of the hexoses. To confirm its presence, the phosphatased monophosphate area was heated with 0.1 N-HCl at 100° for 5 minutes. Sedoheptulose was partly converted into sedoheptulosan, which chromatographs quite separately from the hexoses, and was detected by the orcinol-trichloracetic acid spray (Klevstrand & Nordal, 1950). The area containing the sugar diphosphates (Benson et al., 1950; Bassham & Calvin, 1957) was

shown, after phosphatasing, to contain overwhelmingly fructose, with traces of glucose, gluconic acid, glyceric acid and possibly sedoheptulose.

The spot labelled "UDPG" in Figs. 1-4 was shown, after treatment with phosphatase, to contain uridine and glucose, and was probably uridinediphosphoglucose; the spot designated "UDPR" similarly contained uridine and ribose, and may, by analogy with uridinediphosphoglucose, be uridinediphosphoribose. In neither case, nor with any of the other nucleoside derivatives identified, was any attempt made to determine whether the ^{14}C was present in the sugar, or the base of the nucleoside, or in both.

A number of spots were observed, which, from their chromatographic positions and u.v. absorption were suspected of being nucleotides. Those labelled "ATP" and "ADP" gave adenosine, uridine and ribose on treatment with phosphatase, and were possibly ATP and ADP with a uridine-sugar contaminant. The "Nucleotide diphosphate" spot contained uridine, inosine and probably guanosine, and chromatographically corresponded to the diphosphates of these compounds. Uridine triphosphate (UTP) contained only uridine and corresponded with the position of the triphosphate ester; another spot was identified in a similar way as being guanosine monophosphate. A further spot (called "unknown nucleotide monophosphate") may have been xanthosine monophosphate but its identity was not confirmed. In addition to all these compounds which have been identified with a greater or lesser degree of certainty, the radioautograms showed the presence of up to about 10 weakly active substances which were not identified: in most cases not more than 2 to 5% of the total activity incorporated from (^{14}C)glucose appeared in these unidentified compounds.

Kinetic studies with unstarved cells

Freshly-harvested cells, suspended in distilled water, were incubated with (^{14}C)glucose for fifteen different incubation periods between 6 seconds and 3 minutes, and in another experiment for 30 minutes. The distributions of the activities in a number of different compounds is presented in Table 1.

(Insert Table 1 near here).

These values are expressed for each compound as a percentage of the total activity in the soluble fraction of the cells which was incorporated from the labelled glucose into that compound; in addition, the total activities in terms of disintegrations per minute for a standard aliquot of the total soluble fraction are given.

The time sequence of the incorporation of ^{14}C from (^{14}C)glucose into the various substances detected is consistent with the operation of both the glycolytic and oxidative pathways. Thus, in the first few seconds ^{14}C appeared in the sugar monophosphates and diphosphates, in phosphogluconate, ribose monophosphate, phosphoglycerate, phosphoenolpyruvate, and in malate (Table 1). A small amount of UDPG was present. Aspartic acid was apparently very active, but this was probably an artifact due to chromatographic coincidence of aspartic acid with a radioactive contaminant in the (^{14}C)glucose, as in this experiment the glucose was not first purified chromatographically (see Methods). In other experiments it has been found that the first three radioactive substances to appear were the sugar monophosphates, diphosphates and phosphoglyceric acid. With the exception of aspartic acid, three substances showed a fall in the percentage of the total ^{14}C from the shortest incubation times (sugar monophosphates, phosphogluconate and ribose

Table 1. INCORPORATION OF ^{14}C FROM (^{14}C)GLUCOSE BY UNSTARVED CELLS

The values given are expressed as the % of the total ^{14}C incorporated which is present in each compound. The cells were killed with ethanol after the incubation periods stated, extracted, and the extracts chromatographed.

Incubation period (sec.)	6	8	10.5	17.5	31	45	61	75	90	106	120	135.5	151	167	180	1800*
Sugar diphosphates**	2.1	4.9	4.9	4.2	3.4	3.9	3.6	2.7	2.5	2.9	2.4	2.1	2.4	3.2	2.3	0.18
Sugar monophosphates***	52.2	35.8	34.8	31.8	38.2	33.8	29.5	26.2	25.4	24.7	22.5	19.3	17.7	17.2	16.5	1.4
Phosphoglycerate	5.0	7.7	9.6	8.3	10.8	8.5	8.4	8.0	6.8	5.5	5.5	5.7	4.9	4.6	4.6	0.48
Phosphoenolpyruvate	6.1	6.6	3.9	2.7	2.7	2.4	2.1	1.7	1.5	1.3	1.5	1.7	1.1	1.3	1.1	0.10
Phosphogluconate	4.6	4.7	3.5	4.1	3.2	2.0	1.9	1.5	1.1	1.1	1.1	1.1	0.88	0.87	0.85	0.09
Ribose monophosphate	5.6	4.0	4.1	1.9	2.6	1.5	1.9	1.2	0.74	1.6	1.0	1.2	0.89	0.96	1.1	0.55
Glycerol phosphate		3.3	2.4	2.4	2.2	2.1	2.0	1.5	1.1	1.1	1.4	1.2	0.86	1.3	0.73	0.55
Maltose phosphate							0.26	0.43	0.62	1.2	1.6	1.8	1.9	1.9	1.8	
Uridinediphosphoglucose	0.89	1.6	1.1	2.2	2.5	3.1	3.3	3.5	6.0	3.9	5.2	4.6	4.4	4.2	4.4	1.1
Uridinediphosphoribose				0.41	0.62	0.66	0.94	1.2	1.1	1.1	1.5	1.3	1.4	1.0	1.1	0.44
Nucleotide diphosphates****		0.85	1.1	3.8	4.6	7.0	7.8	9.3	9.9	9.2	10.3	9.0	8.2	9.5	9.2	26.4
Guanosine monophosphate		3.1	2.7	1.9	2.6	3.6	2.4	2.4	1.9	1.8	1.7	2.1	1.6	1.5	1.0	0.67
Uridine triphosphate					0.10	0.44	0.33	0.37	0.52	0.64	0.87	0.92	0.86	0.96	1.2	1.1
Adenosine diphosphate						0.32	0.40	1.1	0.56	0.45	0.43	0.31	0.25	0.32	0.32	
Adenosine triphosphate						0.32	1.1	0.42	1.7	3.5	1.4	1.0	1.0	0.93	1.1	
Maltose									1.3	1.7	1.9	2.4	2.7	2.5	2.5	1.2
Succinate																0.07
Fumarate									0.02		0.03	0.16	0.11	0.14	0.16	0.84
Citrate			2.0	1.3	1.1	1.3	1.2	1.2	1.0	1.0	1.1	1.4	1.4	1.4	1.4	6.7
Malate	3.5	3.4	3.9	5.1	1.8	1.8	2.0	1.7	1.1	0.88	1.0	1.4	1.2	1.1	1.3	6.0
Aspartate*****	20.1	17.5	14.3	14.4	11.5	8.5	9.6	10.0	9.4	10.2	10.0	10.3	10.7	10.8	11.0	20.3
Glutamate			4.5	3.5	2.1	2.9	3.9	4.5	5.1	5.1	6.0	6.8	8.0	8.1	8.2	22.1
Alanine		7.5	7.3	12.0	10.1	14.5	16.3	18.6	17.5	16.9	17.6	19.0	20.7	20.2	20.9	4.1
Glutamine								1.6	1.4	2.1	2.1	2.0	3.1	2.3	2.5	1.6
Valine						0.38	1.0	0.55	0.63	0.82	0.83	1.1	1.7	1.6	1.8	0.62
Proline																0.35
Histidine																0.37
Tyrosine								0.40	0.25	0.41	0.36	0.41	0.48	0.49	0.42	0.30
Unknown substances									0.83	0.99	0.86	1.6	1.7	1.4	2.5	2.4
Total in sugar phosphates	75.6	67.0	63.2	55.4	63.1	54.2	49.7	43.2	42.8	39.4	37.0	34.1	30.6	31.3	29.0	3.3
Total in uridine-sugars	0.89	1.6	1.1	2.6	3.1	3.8	4.2	4.7	7.1	5.0	6.7	5.9	5.8	5.2	5.5	1.5
Total in nucleotides		4.0	3.8	5.7	7.3	11.7	12.0	13.6	14.6	15.6	14.7	13.3	11.9	13.2	12.8	28.2
Total in free sugars									1.3	1.7	1.9	2.4	2.7	2.5	2.5	1.2
Total in organic acids	3.5	3.4	5.9	6.4	2.9	3.1	3.2	2.9	2.1	1.9	2.1	2.9	2.7	2.6	2.9	13.6
Total in amino acids	20.1	25.0	26.1	29.9	23.7	26.3	30.8	35.7	34.3	35.5	36.9	39.6	44.7	43.5	44.8	49.7
Total activity/100 ul. cells (dis./min. x 10^{-5})	0.58	0.63	1.01	1.87	2.87	4.36	4.98	5.59	6.20	8.36	8.46	9.91	14.7	16.1	16.9	28.5

* Separate experiment. **Contains diphosphates of fructose, glucose and glyceric acid. ***Contains monophosphates of glucose, fructose and sedoheptulose. ****Contains diphosphates of uridine, inosine, and guanosine. *****Contains a contaminant from the radioactive glucose used as substrate.

monophosphate) indicating that these substances play an early role in glucose metabolism. As time progressed a peak of the percentage of the radioactivity incorporated passed through the sugar diphosphates, phosphoglyceric acid, phosphoenolpyruvic acid, citrate, glutamate and malate (Table 1). Later, synthetic products took on an increasingly greater significance as activity appeared in ever greater percentage amounts in the amino acids and nucleotides, and a corresponding fall occurred in the early intermediates. UDPG contained ^{14}C even after 6 seconds; after 61 seconds label appeared in maltose phosphate, and in free maltose after 90 seconds, suggesting a biosynthetic route for maltose via UDPG and maltose phosphate. Thirty minutes after the addition of the (^{14}C)glucose almost all the activity had disappeared from the sugar phosphates (which contained over 75% of the total activity after 6 seconds) and appeared in nucleotides, amino acids and organic acids, with a small amount (1.2%) in maltose. The rapidity with which ^{14}C entered the nucleotides (28% of the total activity after 30 minutes) is significant and it will be shown later that nucleotide synthesis is one of the metabolic activities most sensitive to starvation and to metabolic inhibition.

Kinetic studies with starved cells

Earlier work with starved cells had shown that when these cells were supplied with glucose a lag period of two to three hours was apparent before the rate of oxidation became maximal (Moses, 1954), and that the lag applied not only to the rate of oxygen consumption but also to the rate of glucose utilization (Moses, 1955 a). Bartlett and Moses (1957) were able to confirm that (^{14}C)glucose was incorporated into various cellular components, insoluble

as well as soluble, more slowly by starved cells than by freshly-harvested hyphae. They also found considerable differences in metabolic patterns between starved and unstarved cells, but were unable to identify many of the compounds which showed most of the differences.

In the present investigation a kinetic study of the incorporation of ^{14}C from (^{14}C)glucose was made at various incubation times between 8 seconds and 30 minutes; the patterns of seven of these times are given in Table 2. Compared with unstarved cells (Table 1) several differences are apparent.

(Insert Table 2 near here)

Activity remained at a much higher level in the sugar phosphates, still being 23% of the total after 30 minutes, compared with 3.5% in unstarved cells. The processes of metabolism appeared to be slowed down: in starved cells activity did not appear in phosphogluconate until 31 seconds (6 seconds in fresh cells), ribose monophosphate never incorporated as much activity as it did in unstarved cells, and activity did not reach a peak in the sugar diphosphates until 31 seconds compared with 8 to 10 seconds for unstarved cells. This agrees with the earlier findings (Bartlett & Moses, 1957) that the oxidative pathway played a less prominent part in glucose metabolism following starvation of the cells.

The most important differences, however, appeared in the quantitative distribution of activity in substances formed after a longer incubation period (30 minutes). Considerable activity still remained in the sugar phosphates, lesser amounts of amino-acids, organic acids and nucleotides were formed (25 to 50% of the amounts in fresh cells), and enormous amounts of ^{14}C were diverted into maltose, so much so that some 30% of the total ^{14}C was present in this sugar. At an earlier stage, free fructose also appeared.

Table 2. INCORPORATION OF ^{14}C FROM (^{14}C)GLUCOSE BY STARVED CELLS

The values given are expressed as the % of the total ^{14}C incorporated which is present in each compound. The cells were killed with ethanol after the incubation periods stated, extracted, and the extracts chromatographed.

Incubation period (sec.)	9	31	60.5	90.5	122	149	1800*
Sugar diphosphates**	4.2	5.8	4.2	2.8	2.0	3.3	1.1
Sugar monophosphates***	85.2	61.8	76.5	74.9	63.3	61.3	19.9
Phosphoglycerate	1.2	1.2	0.87	0.90	1.1	1.7	0.9
Phosphoenolpyruvate	0.31	0.31	0.65	0.43	0.52	0.63	0.2
Phosphogluconate		0.54	0.55	0.60	0.56	0.50	
Ribose monophosphate	2.0	1.4	1.5	1.3	0.88	1.2	0.5
Glycerol phosphate	1.3	3.0	0.74	0.93	0.52	1.4	0.6
Maltose phosphate			0.41	1.1	5.5	3.9	
Uridinediphosphoglucose	1.5	5.8	4.5	4.8	7.6	6.0	2.0
Uridinediphosphoribose			0.43	0.62	0.51	0.08	2.2
Nucleotide diphosphates****		0.34	0.65	0.72	1.3	1.3	11.4
Guanosine monophosphate							1.1
Uridine triphosphate						0.08	0.8
Adenosine diphosphate							0.9
Adenosine triphosphate							0.1
Unidentified nucleotide monophosphate		1.4	1.8	1.9	1.7	1.5	
Maltose			0.74	0.57	4.3	2.9	30.6
Fructose	4.3	12.2	4.2	5.2	6.5	7.8	
Succinate							0.12
Fumarate							0.27
Citrate			0.05	0.10	0.05	0.13	1.4
Malate				0.03	0.01	0.12	1.9
Glycerate			0.09	0.10	0.20	0.22	
Aspartate			0.47	0.70	0.75	0.96	3.7
Glutamate				0.20	0.10	0.21	10.8
Alanine		4.2	1.1	1.4	1.9	3.7	5.7
Glutamine							2.1
Valine		2.0	0.33	0.43	0.62	0.85	0.3
Histidine							0.3
Tyrosine			0.05	0.10	0.10	0.13	0.1
Citrulline							0.3
Unknown substances			0.12	0.20		0.12	1.4
Total in sugar phosphates	94.2	74.1	85.4	83.0	74.4	73.9	23.4
Total in uridine-sugars	1.5	5.8	4.9	5.4	8.1	6.1	4.2
Total in nucleotides		1.7	2.5	2.6	3.0	2.9	14.5
Total in free sugars	4.3	12.2	4.9	5.8	10.8	10.7	30.6
Total in organic acids			0.1	0.2	0.3	0.5	3.7
Total in amino acids		5.2	2.0	2.8	3.5	6.7	23.4
Total activity/100 ul. cells (dis./min. x 10^{-5})	2.52	8.15	16.94	23.98	31.88	39.24	24.2

*Separate experiment. **Contains diphosphates of fructose, glucose, and glyceric acid.
***Contains monophosphates of glucose, fructose, and sedoheptulose.
****Contains diphosphates of uridine, adenosine, and guanosine.

It will be noted from Tables 1 and 2 that the starved cells appeared to utilize labelled glucose more rapidly than did the unstarved hyphae. However, the experiments on which these two Tables are based were performed on separate occasions and with different batches of cells; the apparently greater incorporation of radioactivity by the starved cells is probably to be explained by uncontrolled variation in the biological material and in the experimental conditions. When the same batch of cells was used as the source of both fresh and starved material, it was found that the latter were indeed less active than the former (Bartlett & Moses, 1957).

Effects of ammonia and azide on glucose metabolism

It has been reported earlier (Moses, 1954) that glucose oxidation in starved cells is greatly stimulated by the presence of small amounts of ammonia, a stimulation which could be totally inhibited by sodium azide. Azide prevented the incorporation of ammonia nitrogen by the cells and also increased both the endogenous respiration and that percentage of the glucose utilized which was completely oxidized to carbon dioxide and water. It therefore appeared to be of interest to study the effects of ammonia and azide, alone and together, on the patterns of glucose metabolism.

Fresh, unstarved cells were, as might be expected, not short of nitrogen and showed little or no response to the presence of 2×10^{-3} M-ammonium chloride; the incorporation pattern of ^{14}C from (^{14}C)glucose showed little or no difference when ammonia was added to fresh cells.

With starved cells there was a marked effect due to 2×10^{-3} M-ammonium chloride added 2 minutes before the (^{14}C)glucose. The percentage incorporation into maltose, the amino acids, and the organic acids was virtually unaffected,

but there was a noticeable drop in the activity in the sugar phosphates and a corresponding rise in the nucleotides (Table 3). The addition 10 to 15 minutes before the (^{14}C)glucose of azide (10^{-3} M) resulted in the almost

(Insert Table 3 near here)

total inhibition of both nucleotide and maltose production, a fall in the total activity incorporated, and of the percent activity in the sugar phosphates, and a rise in the ^{14}C in the amino-acids and organic acids (Table 3). The presence of both azide and ammonia resulted in a picture very similar to that with azide alone. There was a slight rise in the sugar phosphates and a corresponding fall in the organic acids, and some redistribution of activity between aspartate, glutamate and alanine, but otherwise the pictures presented when azide was present, with and without ammonia, were almost identical (Table 3).

The effects of azide thus appeared to be directed mainly towards energy-requiring processes: synthesis of disaccharides (maltose) and nucleotides. Protein synthesis was apparently also impaired; Moses (1954) demonstrated that the incorporation of ammonia nitrogen into the insoluble nitrogen of the cells (i.e., protein) was blocked by azide. The high incorporation of ^{14}C into amino acids and organic acids might then result from a blocking of protein synthesis with a subsequent accumulation of the raw materials for the manufacture of proteins, the amino acids. This resulted, in turn, in a backing-up of the organic acid precursors of the amino acids.

The well-known uncoupling of respiration and oxidative phosphorylation by azide (Simon, 1953), and the consequent increased and probably uncontrolled endogenous respiration of the cells, would also account for the lowered activity in the sugar phosphates when azide was added to the cell suspensions.

Table 3. EFFECT OF AMMONIUM CHLORIDE AND SODIUM AZIDE ON THE INCORPORATION OF ^{14}C FROM (^{14}C)GLUCOSE BY STARVED CELLS

The values given are expressed as the % of the total ^{14}C incorporated which is present in each compound. The cells were killed with ethanol after the incubation times stated, extract and the extracts chromatographed. Concn. of added substances: NH_4Cl, 2 x 10^{-3} M; NaN_3, 10^{-3}

	Plus NH_4Cl		Plus NaN_3		Plus NH_4Cl & NaN_3	
Incubation period (sec.)	180	1800	180	1800	180	1800
Sugar diphosphates*	1.2	0.22	0.82	0.36	0.99	0.35
Sugar monophosphates**	39.3	6.3	9.3	3.0	9.1	3.0
Phosphoglycerate	3.1	1.8	12.2	2.7	10.6	6.6
Phosphoenolpyruvate	0.76	0.42	2.9	0.62	2.9	1.7
Phosphogluconate	***	0.08	0.94	0.08	0.82	0.39
Ribose monophosphate	1.1	0.23	1.0	0.20	0.59	0.13
Glycerol phosphate	3.3	0.65				
Maltose phosphate	0.93	0.47		0.08		0.07
Uridinediphosphoglucose	11.0	3.0	1.2	1.5	1.0	1.8
Uridinediphosphoribose	4.5	1.5				
Nucleotide diphosphates****	3.6	20.2	0.23	1.1	0.19	1.2
Guanosine monophosphate	2.7	0.97				
Uridine triphosphate	1.5	1.4		0.06		
Unidentified nucleotide monophosphate	0.18	0.91		0.05		0.05
Maltose	7.6	34.4	3.1	2.3	3.0	1.0
Fructose	4.0	0.43		0.62	2.2	0.97
Succinate				1.4	0.45	0.81
Fumarate	0.1	0.19	0.42			0.48
Citrate	1.3	2.7	14.7	11.0	12.7	3.6
Malate	0.32	0.70	1.7	3.8	2.1	3.7
Glycerate	0.91	0.63	1.4		3.2	1.2
Aspartate	3.0	6.0	9.1	19.3	12.3	28.5
Glutamate	1.1	6.1	9.7	21.8	7.3	22.4
Alanine	5.7	5.2	26.1	24.3	25.8	15.2
Glutamine		1.9	0.56	0.82		1.0
Citrulline		0.29		0.31		0.40
Valine	1.1	0.73	0.75	2.2	1.7	1.4
Proline		0.14				
Histidine		0.26				
Tyrosine		0.35				
Unknown substances	0.80	1.6	3.8	2.4	2.9	3.8
Total in sugar phosphates	49.7	10.2	27.2	7.0	25.0	12.2
Total in uridine-sugars	15.5	4.5	1.2	1.5	1.0	1.8
Total in nucleotides	8.0	23.5	0.23	1.2	0.20	1.3
Total in free sugars	11.6	34.8	3.1	2.9	5.2	2.0
Total in organic acids	3.6	4.2	18.2	16.2	18.5	10.0
Total in amino acids	10.9	21.0	46.2	68.7	47.1	68.9
Total activity/100 ul. cells (dis./min. x 10^{-5}	68.10	64.07	10.24	47.76	14.51	51.79

*Contains diphosphates of fructose, glucose, and glyceric acid. **Contains monophosphates of glucose, fructose, and sedoheptulose. ***Did not separate on chromatogram.
****Contains diphosphates of uridine, inosine, and guanosine.

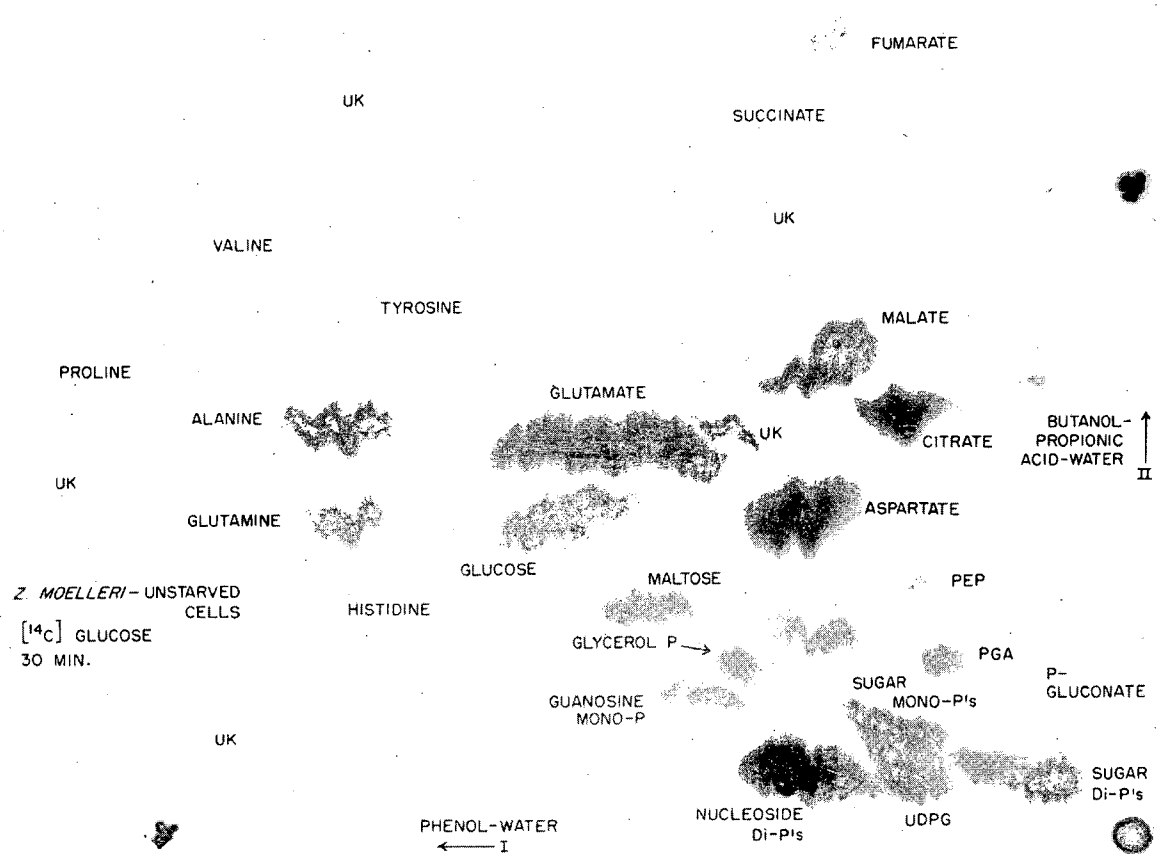
Typical radiosutograms, showing the effects of starvation and of azide, the chromatographic relations of the substances investigated and the advantages of long-time chromatography for separating the substances in the phosphate area, are shown in Figs. 1-4.

(Insert Figs. 1-4 near here)

DISCUSSION

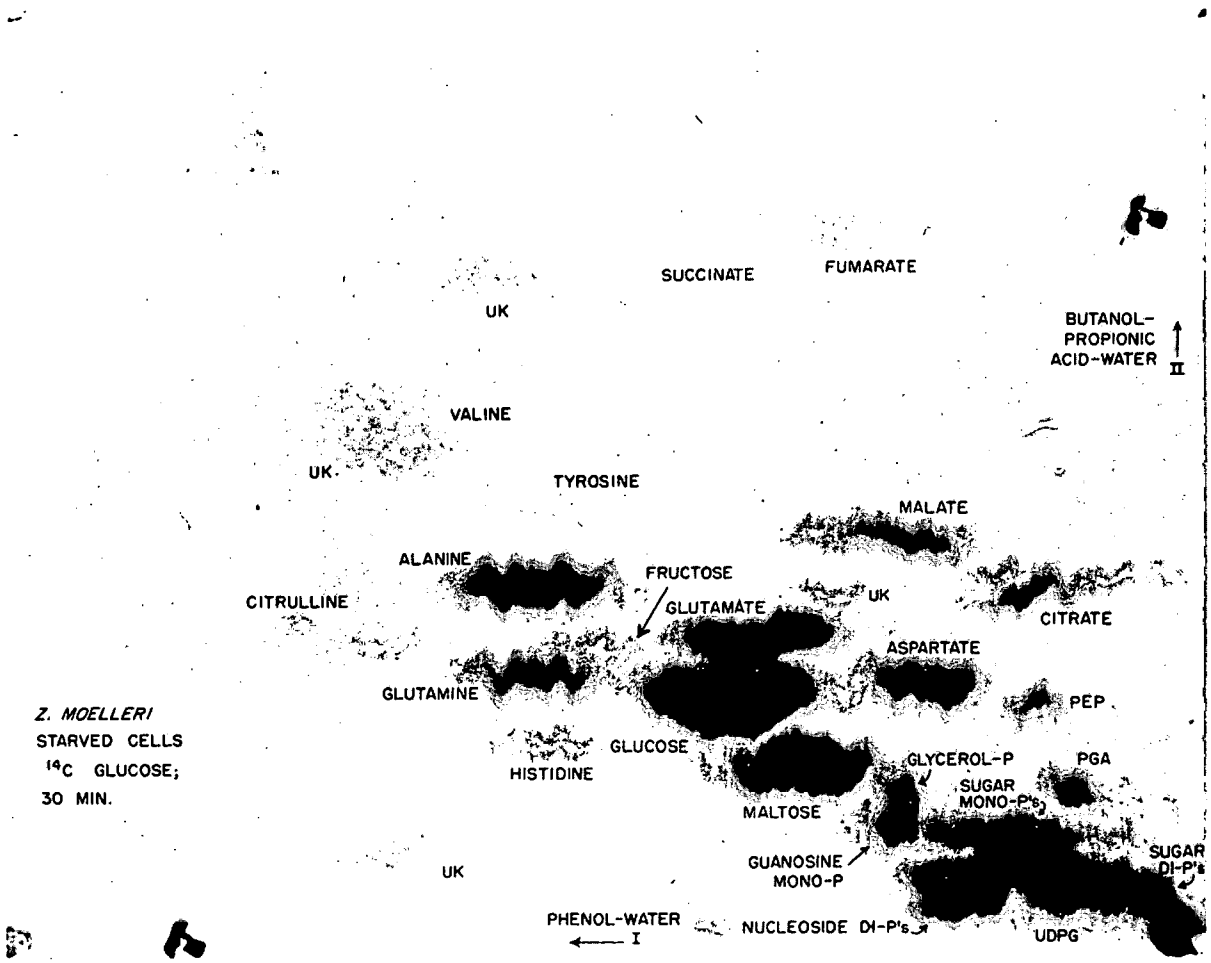
The path of glucose metabolism in this organism which emerges from the present investigations is entirely consistent with earlier studies to elucidate the routes of glucose breakdown in biological systems. The rapid formation from free glucose of glucose and fructose monophosphates, of fructose diphosphate and small quantities of glucose diphsophate, of phosphoglyceric acid, phosphoenolpyruvic acid, together with traces of glyceric acid in the diphosphate area (suggesting the presence of diphosphoglyceric acid), points to an involvement of the glycolysis pathway. This is supported by the appearance of ^{14}C in glycerol phosphate (presumably from triose phosphate) and in alanine and valine, probably from pyruvate. Similarly, a number of intermediates of the oxidative pathway have been detected: phosphogluconic acid, ribose monophosphate and sedoheptulose monophosphate. These compounds, too, appeared after only a few seconds of incubation with (^{14}C)glucose.

At a later stage substances associated with the tricarboxylic acid cycle appeared, and this cycle has earlier been shown to play a role in the respiratory metabolism of this mould (Moses, 1955 b; 1957). The uridine diphosphate derivatives of glucose and ribose incorporated activity very early, with the later appearance of maltose phosphate and free maltose.


FUMARATE
UK
SUCCINATE
UK
VALINE
TYROSINE
MALATE
PROLINE
GLUTAMATE
ALANINE
UK
CITRATE
BUTANOL-
PROPIONIC
ACID-WATER
II
UK
GLUTAMINE
ASPARTATE
GLUCOSE
MALTOSE
PEP
Z. MOELLERI – UNSTARVED
CELLS
HISTIDINE
[14C] GLUCOSE
30 MIN.
GLYCEROL P
PGA
SUGAR
MONO-P's
P-
GLUCONATE
GUANOSINE
MONO-P
UK
SUGAR
Di-P's
NUCLEOSIDE
Di-P's
UDPG
PHENOL-WATER
I

ZN-2012

Fig. 1


SUCCINATE
FUMARATE
UK
BUTANOL-
PROPIONIC
ACID-WATER
II
VALINE
UK
TYROSINE
MALATE
ALANINE
FRUCTOSE
CITRULLINE
GLUTAMATE
UK
CITRATE
ASPARTATE
GLUTAMINE
PEP
Z. MOELLERI
STARVED CELLS
14C GLUCOSE;
30 MIN.
GLUCOSE
HISTIDINE
GLYCEROL-P
PGA
SUGAR
MONO-P's
MALTOSE
GUANOSINE
MONO-P
SUGAR
DI-P's
UK
PHENOL-WATER
I
NUCLEOSIDE DI-P's
UDPG

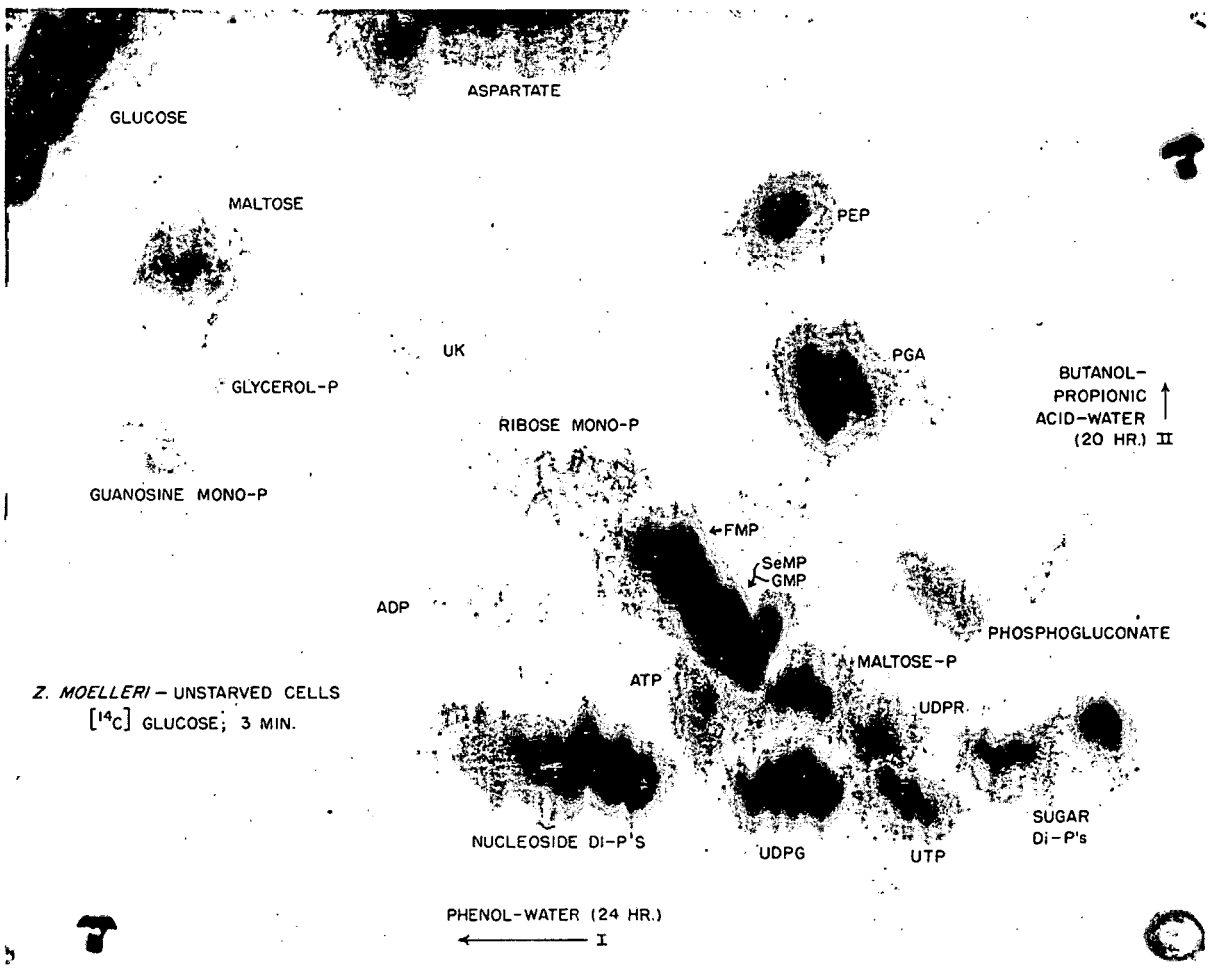
ZN-2010

Fig. 2

FUMARATE
SUCCINATE
UK
UK
UK
BUTANOL-
PROPIONIC
ACID-WATER
II
VALINE
GLYCERATE
MALATE
UK
ALANINE
CITRATE
CITRULLINE
GLUTAMATE
ASPARTATE
FRUCTOSE
PEP
Z. MOELLERI-STARVED CELLS
[14C] GLUCOSE + NH4Cl
+ NaN3
30 MIN.
GLUCOSE
GLUTAMINE
PGA
MALTOSE
SUGAR
MONO-P's →
PHENOL-WATER
← I
NUCLEOSIDE Di-P's
UDPG
SUGAR
Di-P's

ZN-2011

Fig. 3


ASPARTATE
GLUCOSE
MALTOSE
PEP
UK
PGA
GLYCEROL-P
BUTANOL-
PROPIONIC
ACID-WATER
(20 HR.) II
RIBOSE MONO-P
GUANOSINE MONO-P
FMP
SeMP
GMP
ADP
PHOSPHOGLUCONATE
MALTOSE-P
ATP
Z. MOELLERI – UNSTARVED CELLS
[14C] GLUCOSE; 3 MIN.
UDPR
SUGAR
Di-P's
NUCLEOSIDE DI-P'S
UDPG
UTP
PHENOL-WATER (24 HR.)
I

ZN-2009

Fig. 4

Various nucleotides began to become prominent after about 1 minute incubation, and increased to such an extent that after 30 minutes in unstarved cells, more activity was found in them than in any other group of compounds, except the amino acids.

The main differences to be seen between fresh and starved cells are those to be expected from nitrogen and carbohydrate starvation: a rapid production of maltose, probably later converted into insoluble polysaccharide reserve materials, and a reduced formation of amino acids and nucleotides by the starved cells. Furthermore, a smaller percentage of the total ^{14}C incorporated in starved cells appeared in intermediates of the oxidative pathway and this agrees with a previous estimate of the role of the oxidative versus the glycolytic pathways in these cells under various physiological conditions (Bartlett & Moses, 1957).

Ammonia had little effect on the incorporation patterns of unstarved cells, and in starved cells stimulated mainly the formation of nucleotides with a corresponding fall in the activity in the sugar phosphates. There was no increase in the amino acids and the production of maltose remained at a high level. It is the synthesis of nucleotides, together with that of maltose, which was mainly affected by azide: the latter did not appear to inhibit the formation of amino acids. Although the percentage of the total radioactivity in the amino acids increased when the cells were treated with azide, this was partly the result of a fall in the total incorporation of ^{14}C illustrated by the low activity in maltose and in the nucleotides; the increased absolute activity in the amino acids did not entirely account for the fall in ^{14}C normally incorporated into the nucleotides and maltose. Ammonia had only a slight effect in the presence of azide, and this concerned

mainly the relative distribution of activity in glutamate, aspartate and alanine; the inhibition of ammonia uptake by azide (Moses, 1954) is reflected by the lack of influence of this substance on the ^{14}C-incorporation pattern when azide was present.

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Running title: (^{14}C)Glucose metabolism in fungal cells

Captions for figures

Fig. 1. Radioautogram of chromatogram of extract of unstarved cells supplied with (^{14}C)glucose for 30 min.

Fig. 2. Radioautogram of chromatogram of extract of starved cells supplied with (^{14}C)glucose for 30 min.

Fig. 3. Radioautogram of chromatogram of extract of starved cells supplied with (^{14}C)glucose for 30 min. in the presence of ammonium chloride and sodium azide.

Fig. 4. Radioautogram of chromatogram of extract of unstarved cells supplied with (^{14}C)glucose for 3 min. Chromatogram developed with both solvents for a long period in order to spread the phosphate area.

Key: UK, unknown; PEP, phosphoenolpyruvate; PGA, phosphoglycerate; P-gluconate, phosphogluconate; Mono-P, monophosphate; Di-P, diphosphate; UDPG, uridinediphosphoglucose; UDPR, uridine-diphosphoribose; UTP, uridine triphosphate; FMP, fructose monophosphate; SeMP, sedoheptulose monophosphate; GMP, glucose monophosphate.