[¹⁴C]Glucose Metabolism in Fungal Cells

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SUMMARY: The incorporation pattern of ¹⁴C from [¹⁴C]glucose by the fungus *Zygorhynchus moelleri* has been investigated. The following substances became labelled in incubation periods of 6 sec. to 30 min.: 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 ¹⁴C was present in unidentified substances. The kinetics of the incorporation of ¹⁴C, first into uridinediphosphoglucose, then into maltose phosphate, and finally into free maltose, suggests this sequence of compounds for the biosynthetic pathway of maltose formation.

The main effects of starvation of the cells by shaking them in phosphate buffer for 24 hr. were an increased % incorporation of ¹⁴C 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 absolute rate of incorporation of ¹⁴C from labelled glucose into soluble cellular constituents; however, while the % of the total soluble ¹⁴C found in the nucleotides was markedly increased when ammonia was added, the % in the sugar phosphates decreased. In the presence of azide, the incorporation of ¹⁴C 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 ¹⁴C into the nucleotides, organic acids and amino acids, while the addition of ammonia overcame the effects of starvation and promoted nucleotide synthesis. Ammonia had very little effect on the cells in the presence of azide.

A kinetic study of the sequence in which ¹⁴C was detected in 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 about the importance of particular pathways for the metabolism of the relevant substrates. Thus, Goldschmidt, Yall & Koffler (1956) and Moses (1957) 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) used variously labelled glucoses to investigate the importance of the oxidative and fermentative pathways in the utilization of glucose by *Penicillium chrysogenum* and *Zygorhynchus moelleri*, respectively.

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Relatively little work has been done, however, along the lines of Calvin (1956) and co-workers, in which an organism is fed a labelled substrate for short periods of time and then killed and extracted to determine the nature of all the substances incorporating the label from the original substrate. Claridge & Werkman (1954) studied the incorporation of label from [¹⁴C]2-ketogluconate by *Pseudomonas aeruginosa*, and Milhaud & Aubert (1955) 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 *Zygorhynchus moelleri*, showed that this fungus converts [¹⁴C] glucose to a large number of compounds in a very short time (15 sec. was the shortest incubation time used), but they did not identify many of the products of glucose metabolism, and no very definite conclusions were drawn.

The present communication reports a further attempt to elucidate this problem by using cells of *Zygorhynchus moelleri* in various experimental conditions, and analysing the cells after several short periods of exposure to very high specific activity [¹⁴C]glucose. Following the killing and extraction of the cells with hot ethanol, the extracts were analysed 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 ¹⁴C as 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 *Zygorhynchus moelleri* (Commonwealth Mycological Institute No. 21994) were grown in a medium similar to that used in previous studies (Moses, 1954), but with yeast or liver extracts omitted. Spores obtained from several agar slope cultures 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 16–18 hr at room temperature (*c.* 25°). After growth the organism was harvested by centrifugation, washed with distilled water, and subsequently resuspended in distilled water at a concentration of about 4 ml. wet packed organisms/100 ml. suspension. The volume of the wet packed organisms was determined by centrifuging the suspension in a graduated 12 ml. conical tube for 5 min. at 500 g.

When 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 hr. They were then washed and resuspended in distilled water.

Each of several 50 ml. Erlenmeyer flasks received 5 ml. cell suspension. At the beginning of the incubation period 40 µl. of [¹⁴C]glucose solution (specific

activity 240 $\mu\text{c.}/\text{mg.}$; 33.6 $\mu\text{c.}/\text{flask}$; final glucose conc. $1.6 \times 10^{-4}\text{M}$) was rapidly injected into one flask at a time. After the desired incubation period at room temperature the suspension was quickly poured on to a sintered glass funnel bearing a layer of Celite filter aid and inserted through a rubber stopper into the top of a Buchner flask. The liquid of 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 in water poured on to the organism on the sintered glass disk. The operations of filtering and killing the cells were performed as quickly as possible, and, with practice, only about 2 sec. elapsed between pouring the suspension on to the filter-pad and the addition of the hot aqueous ethanol.

The cells were allowed to stand for 2 hr. at room temperature, the funnel loosened in the centrifuge tube, and the liquid of the suspension forced through the filter-disk and Celite 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 in water poured on to the organism. After 1 hr. the second extract was filtered and the process repeated once more with a further 2 ml. of boiling 20 % (v/v) ethanol in water.

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 in. $\times$ 22 in. 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, one-fifth of each extract was chromatographed so that each solvent just reached the edge of the paper (8–10 hr. for phenol + water, and 7–8 hr. 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 two-fifths of each extract was chromatographed for 24 hr. with phenol + water and for 20 hr. with *n*-butanol + propionic acid + water (Moses & Calvin, 1958). This spread out the area which contained the sugar phosphates and nucleotides much further, and good separation was usually achieved. The final two-fifths of each extract was kept in reserve in case one of the chromatograms was unsatisfactory. The locations of the radio-active materials were determined by exposing the chromatograms to 14 in. $\times$ 17 in. sheets of single-coated blue-sensitive Du Pont X-ray film 507-E for a suitable period. Radio-active 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-Müller 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 ¹⁴C from Whatman No. 4 filter-paper, about 6–7 % 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, when 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 of 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 in water and the chromatograms heated at 100° for 5 min. Organic acids were sprayed with a 0.04 % (w/v) ethanolic solution of bromocresol green, made just alkaline with a little ammonia. Sugars and other reducing substances (e.g. glyceric acid) were detected by dipping the chromatograms into an acetone solution of AgNO₃, allowing them to dry in air, and then spraying with ethanolic NaOH. After development of the spots, the excess AgNO₃ was washed off in ammonia solution and the papers dried (Trevelyan, Procter & Harrison, 1950). Ketoses were also detected with the orcinol + trichloroacetic acid reagent (modified from Klevstrand & Nordal, 1950). Nucleosides were located by their quenching of fluorescence under u.v. irradiation.

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

Labelled glucose was prepared from *Canna* leaves allowed to carry out photosynthesis in the presence of ¹⁴CO₂ (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 [¹⁴C]glucose was apparent on the chromatograms.

RESULTS

Identification of substances formed from glucose

A considerable number of substances were labelled from [¹⁴C]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 appropriate authentic markers. The maltose spot contained only glucose after acid hydrolysis (N-HCl at 60° for 12 hr.).

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 monophosphate area after treatment with phosphatase was heated with 0.1 N-HCl at 100° for 5 min. Sedoheptulose was partly converted into sedoheptulosan, which chromatographs quite separately from the hexoses, and was detected by the orcinol + trichloroacetic acid spray (Klevstrand & Nordal, 1950). The area containing the sugar diphosphates (Benson *et al.* 1950; Bassham & Calvin, 1957) was shown, after treatment with phosphatase, to contain overwhelmingly fructose, with traces of glucose, gluconic acid, glyceric acid and possibly sedoheptulose.

The spot labelled 'UDPG' in Pl. 1, figs. 1-2 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 radio-autograms showed the presence of up to ten weakly active substances which were not identified: in most cases not more than 2-5 % of the total activity incorporated from [^{14}C]glucose appeared in these unidentified compounds.

Kinetic studies with unstarved cells

Freshly harvested organisms, suspended in distilled water, was incubated with [^{14}C]glucose for fifteen different incubation periods between 6 sec. and 3 min., and in another experiment for 30 min. The distributions of the activities in a

Table 1. *Incorporation of ^{14}C from $[^{14}\text{C}]\text{glucose}$ by unstarved Zygorhynchus moelleri*

Samples (5 ml.) of unstarved cell suspension (containing 0.2 ml. wet packed cells) were incubated with $[^{14}\text{C}]\text{glucose}$ (33.6 μC . and 0.8 $\mu\text{moles/sample}$) for the periods stated. The cells were killed and extracted with ethanol, and the extracts chromatographed. The ^{14}C in each compound on the chromatogram was counted. The values given are expressed as the % of the total ^{14}C incorporated into all soluble substances from labelled glucose, which is present in each individual substance. The ^{14}C remaining in the residual glucose was excluded from all calculations.

	Incubation period (sec.)															180	1800*
	6	8	10.5	17.5	31	45	61	75	90	106	120	135.5	151	167	180		
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	
Phosphoglucuronate	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	0.0	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	0.0	0.0	0.0	0.0	0.0	0.0	0.26	0.43	0.62	1.2	1.6	1.8	1.9	1.9	1.8	0.0	
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	0.0	0.0	0.0	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§	0.0	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	0.0	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	0.0	0.0	0.0	0.0	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	0.0	0.0	0.0	0.0	0.0	0.32	0.40	1.1	0.56	0.45	0.43	0.31	0.25	0.32	0.32	0.0	
Adenosine triphosphate	0.0	0.0	0.0	0.0	0.0	0.32	1.1	0.42	1.7	3.5	1.4	1.0	1.0	0.93	1.1	0.0	
Maltose	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	1.7	1.9	2.4	2.7	2.5	2.5	1.2	
Succinate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.07	
Fumarate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.02	0.0	0.03	0.16	0.11	0.14	0.16	0.84	
Citrate	0.0	0.0	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	0.0	0.0	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	0.0	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	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	1.4	2.1	2.1	2.0	3.1	2.3	2.5	1.6	
Valine	0.0	0.0	0.0	0.0	0.0	0.38	1.0	0.55	0.63	0.82	0.83	1.1	1.7	1.6	1.8	0.62	
Proline	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.35	
Histidine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.37	
Tyrosine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.40	0.25	0.41	0.36	0.41	0.48	0.49	0.42	0.30	
Unknown substances	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	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	0.0	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	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	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 μl . cells (dis./min. $\times 10^{-5}$)	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 radio-active glucose used as substrate.

number of different compounds are presented in Table 1. These values are expressed for each compound as % total activity present in compounds other than glucose in the soluble fraction of the cells, omitting radio-activity present in the residual substrate glucose; in addition, the total activities in terms of disintegrations/min. for a standard sample 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 radio-active, but this was probably an artefact due to chromatographic coincidence of aspartic acid with a radio-active contaminant in the [^{14}C]glucose, as in this particular experiment the glucose had not first been purified chromatographically (see Methods). In other experiments it was found that the first three radio-active substances to appear were the sugar monophosphates, diphosphates and phosphoglyceric acid. With the exception of aspartic acid, four substances showed a fall in the percentage of the total ^{14}C from the shortest incubation times (sugar monophosphates, phosphogluconate, phosphoenolpyruvate, and ribose monophosphate) indicating that these substances play an early role in glucose metabolism. As time progressed a peak of the percentage of the radio-activity incorporated passed through the sugar diphosphates, phosphoglyceric acid, and phosphoenolpyruvic acid, with activity later accumulating in citrate, glutamate and malate (Table 1). Later, synthetic products took on an increasingly greater significance as activity appeared in ever greater % amounts in the amino acids and nucleotides, and a corresponding fall occurred in the early intermediates (i.e. sugar phosphates). UDPG contained ^{14}C even after 6 sec.; after 61 sec. label appeared in maltose phosphate, and in free maltose after 90 sec., 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 sec.) 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 min.) 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 2–3 hr. was apparent before the rate of oxidation became maximal (Moses, 1954), and that the lag applied not only to the rate of oxygen uptake but also to the rate of glucose utilization (Moses, 1955*a*). Bartlett & 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 as 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 ¹⁴C from [¹⁴C]glucose was made by using incubation times between 8 sec. and 30 min.; the patterns at seven of these times are given in Table 2. Compared with unstarved cells (Table 1) several differences are apparent. Activity remained at a much higher degree in the sugar phosphates, still being 23 % of the total after 30 min., as compared with 3.5 % in unstarved cells. The processes of metabolism appeared to have been slowed down: in starved cells radioactivity did not appear in phosphogluconate until 31 sec. (6 sec. 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 sec. as compared with 8–10 sec. 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.

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

Although with the present techniques it was not possible to compare the absolute rates of [¹⁴C]glucose utilization in fresh and starved cells, an earlier study showed that when the same batch of cells was used as the source of both fresh and starved material, the latter were less active than the former (Bartlett & Moses, 1957).

Effects of ammonia and azide on glucose metabolism

It was reported by Moses (1954) that glucose oxidation in starved cells was greatly stimulated by 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 ¹⁴C from [¹⁴C]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 min. before the [¹⁴C]glucose. The % incorporation into maltose, the amino acids, and the organic acids was virtually unaffected, but there

Table 2. *Incorporation of ^{14}C from ^{14}C glucose by starved Zygorhynchus moelleri*

Starved cells were supplied with labelled glucose; experimental conditions as in Table 1. The values given are expressed as the % of the total ^{14}C incorporated into all soluble compounds from labelled glucose which is present in each individual substance. The ^{14}C remaining in the residual glucose was excluded from all calculations.

	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	0.91
Phosphoenolpyruvate	0.31	0.31	0.65	0.43	0.52	0.63	0.24
Phosphogluconate	0.0	0.54	0.55	0.60	0.56	0.50	0.0
Ribose monophosphate	2.0	1.4	1.5	1.3	0.88	1.2	0.58
Glycerol phosphate	1.3	3.0	0.74	0.93	0.52	1.4	0.69
Maltose phosphate	0.0	0.0	0.41	1.1	5.5	3.9	0.0
Uridinediphosphoglucose	1.5	5.8	4.5	4.8	7.6	6.0	2.0
Uridinediphosphoribose	0.0	0.0	0.43	0.62	0.51	0.08	2.2
Nucleotide diphosphates§	0.0	0.34	0.65	0.72	1.3	1.3	11.4
Guanosine monophosphate	0.0	0.0	0.0	0.0	0.0	0.0	1.1
Uridine triphosphate	0.0	0.0	0.0	0.0	0.0	0.08	0.85
Adenosine diphosphate	0.0	0.0	0.0	0.0	0.0	0.0	0.96
Adenosine triphosphate	0.0	0.0	0.0	0.0	0.0	0.0	0.16
Unidentified nucleotide monophosphate	0.0	1.4	1.8	1.9	1.7	1.5	0.0
Maltose	0.0	0.0	0.74	0.57	4.3	2.9	30.6
Fructose	4.3	12.2	4.2	5.2	6.5	7.8	0.0
Succinate	0.0	0.0	0.0	0.0	0.0	0.0	0.12
Fumarate	0.0	0.0	0.0	0.0	0.0	0.0	0.27
Citrate	0.0	0.0	0.05	0.10	0.05	0.13	1.4
Malate	0.0	0.0	0.0	0.03	0.01	0.12	1.9
Glycerate	0.0	0.0	0.09	0.10	0.20	0.22	0.0
Aspartate	0.0	0.0	0.47	0.70	0.75	0.96	3.7
Glutamate	0.0	0.0	0.0	0.20	0.10	0.21	10.8
Alanine	0.0	4.2	1.1	1.4	1.9	3.7	5.7
Glutamine	0.0	0.0	0.0	0.0	0.0	0.0	2.1
Valine	0.0	2.0	0.33	0.43	0.62	0.85	0.33
Histidine	0.0	0.0	0.0	0.0	0.0	0.0	0.33
Tyrosine	0.0	0.0	0.05	0.10	0.10	0.13	0.15
Citrulline	0.0	0.0	0.0	0.0	0.0	0.0	0.33
Unknown substances	0.0	0.0	0.12	0.20	0.0	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	0.0	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	0.0	0.0	0.1	0.2	0.3	0.5	3.7
Total in amino acids	0.0	6.2	2.0	2.8	3.5	6.7	23.4
Total activity/100 μl . cells (dis./min. $\times 10^{-5}$)	2.52	8.15	16.94	23.98	31.88	39.24	24.25

* Separate experiment. † Contains diphosphates of fructose, glucose, and glyceric acid.

‡ Contains monophosphates of glucose, fructose, and sedoheptulose. § Contains diphosphates of uridine, inosine, and guanosine.

was a noticeable decrease in the % activity in the sugar phosphates and a corresponding rise in the nucleotides (Table 3). The addition of azide (10^{-3}M) 10–15 min. before the [^{14}C]glucose resulted in the almost total inhibition of both nucleotide and maltose production, a decrease in the total activity incorporated, and of the % activity in the sugar phosphates, and a rise in the ^{14}C in the amino acids and organic acids (Table 3). The presence of azide + 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) showed 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 found in the sugar phosphates when azide was added to the cell suspensions.

Typical radio-autograms, showing 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 Pl. 1, figs. 1 and 2.

DISCUSSION

The path of glucose metabolism in *Zygorhynchus moelleri* 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 diphosphate, 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

Table 3. *Effect of ammonium chloride and sodium azide on the incorporation of ^{14}C from $[^{14}\text{C}]\text{glucose}$ by starved Zygorhynchus moelleri*

Starved cells were supplied with labelled glucose; experimental conditions as in Table 1. The values given are expressed as the % of the total ^{14}C incorporated into all soluble substances, which is present in each individual compound. The ^{14}C remaining in the residual glucose was excluded from all calculations. Conc'n. of added substances: NH_4Cl , $2 \times 10^{-3}\text{M}$; NaN_3 , 10^{-3}M .

Incubation period (sec.)	...	Plus NH_4Cl		Plus NaN_3		Plus NH_4Cl and NaN_3	
		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	0.0	0.0	0.0	0.0
Maltose phosphate		0.93	0.47	0.0	0.08	0.0	0.07
Uridinediphosphoglucose		11.0	3.0	1.2	1.5	1.0	1.8
Uridinediphosphoribose		4.5	1.5	0.0	0.0	0.0	0.0
Nucleotide diphosphates§		3.6	20.2	0.23	1.1	0.19	1.2
Guanosine monophosphate		2.7	0.97	0.0	0.0	0.0	0.0
Uridine triphosphate		1.5	1.4	0.0	0.06	0.0	0.0
Unidentified nucleotide monophosphate		0.18	0.91	0.0	0.05	0.0	0.05
Maltose		7.6	34.4	3.1	2.3	3.0	1.0
Fructose		4.0	0.43	0.0	0.62	2.2	0.97
Succinate		0.0	0.0	0.0	1.4	0.45	0.81
Fumarate		0.1	0.19	0.42	0.0	0.0	0.48
Citrate		1.8	2.7	14.7	11.0	12.7	3.6
Malate		0.82	0.70	1.7	3.8	2.1	3.7
Glycerate		0.91	0.63	1.4	0.0	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		0.0	1.9	0.56	0.82	0.0	1.0
Citrulline		0.0	0.29	0.0	0.31	0.0	0.40
Valine		1.1	0.73	0.75	2.2	1.7	1.4
Proline		0.0	0.14	0.0	0.0	0.0	0.0
Histidine		0.0	0.26	0.0	0.0	0.0	0.0
Tyrosine		0.0	0.35	0.0	0.0	0.0	0.0
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 μl . cells (dis./min. $\times 10^{-6}$)		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.

metabolism of this mould (Moses, 1955*b*; 1957). The uridinediphosphate derivatives of glucose and ribose incorporated activity very early, with the later appearance of maltose phosphate and free maltose. Various nucleotides began to become prominent after about 1 min. of incubation, and increased to such an extent that after 30 min. with unstarved cells more activity was found in the nucleotides 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 decreased formation of amino acids and nucleotides by the starved cells. Furthermore, a smaller % of the total ^{14}C incorporated in starved cells appeared in intermediates of the oxidative pathway; 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, increased mainly the % of the total ^{14}C incorporated into nucleotides, with a corresponding fall in the % activity in the sugar phosphates. There was no increase in the % of ^{14}C found in the amino acids and the production of maltose remained at a high level. It was the synthesis of nucleotides, together with that of maltose, which was mainly affected by azide, which did not appear to inhibit the formation of amino acids. When azide was present the total amount of ^{14}C incorporated into the soluble fraction of starved cells was increased after 30 min. of incubation. Both the absolute amounts and the % of ^{14}C found in the organic and amino acids increased very greatly in the presence of azide, while the ^{14}C observed in the sugar phosphates, nucleotides and maltose showed a marked fall on both an absolute and a percentage basis.

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) was reflected by the lack of influence of this substance on the ^{14}C -incorporation pattern when azide was present.

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EXPLANATION OF PLATE

Fig. 1. Radio-autogram of chromatogram of extract of unstarved cells supplied with [¹⁴C] glucose for 30 min.

Fig. 2. Radio-autogram of chromatogram of extract of unstarved cells supplied with [¹⁴C]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, uridine-diphosphoglucose; UDPR, uridinediphosphoribose; UTP, uridine triphosphate; FMP, fructose monophosphate; SeMP, sedoheptulose monophosphate; GMP, glucose monophosphate.

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Fig. 1

Fig. 2

V. MOSES— $[^{14}\text{C}]$ GLUCOSE METABOLISM IN FUNGAL CELLS. PLATE 1

(Facing p. 196)