Utilization of ^{14}C -Labelled Glucose by Human Cells (Strain HLM) in Tissue Culture

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The cultivation of cells *in vitro* in a well-defined medium provides a convenient system for studying pathways of glucose utilization. By using established cell lines it is possible to avoid the radical short-term changes in metabolism and in enzyme activity associated with freshly explanted tissues (Paul & Pearson, 1957*a, b*; Warburg, Gawehn & Geissler, 1958; Burlington, 1959; Leslie & Yarnell, 1960). Other factors that influence the glucose metabolism of cell cultures are cell type (Suschny, Kellner, Broda, Figdor & Rücker, 1958), the pH of the medium throughout the test (Broda, Suschny, Rücker & Kellner, 1959; Paul, 1959*a*) and the composition of the medium in which the cells are grown (Eagle, Barban, Levy & Schulze, 1958).

Previous investigations have shown that embryonic and malignant cells in tissue culture respond to the presence of insulin. In cultures of freshly explanted embryonic-chick-heart cells insulin stimulated cell proliferation, as measured by increments in DNA phosphorus, RNA phosphorus, lipid phosphorus and protein nitrogen (Leslie & Davidson, 1951; Leslie & Paul, 1954); it also stimulated glucose uptake, but decreased total α -oxo acid production by these cells (Leslie & Paul, 1954). The hormone elicited a different response, however, in some experiments on established strains. In cultures of HeLa, HEP 1 and HEP 2 cells, proliferation as measured by increments in DNA phosphorus was unaltered but there were significant increases in RNA phosphorus, and especially in glucose consumption and lactic acid production in the presence of insulin (Leslie, Fulton & Sinclair, 1957). Similar findings have been reported also for other established cell strains (Paul & Pearson, 1960; Etingof & Krichevskaya, 1960).

In the present study the application of a ^{14}C -tracer technique has permitted a fuller assessment of insulin effects in proliferating cells of the HLM strain. The incorporation of ^{14}C -labelled glucose carbon into nucleic acids, lipids, proteins and acid-soluble compounds has been measured, together with its conversion into lactic acid and carbon dioxide. By comparing patterns of disposal of

radioactivity from glucose labelled specifically in different carbon atoms, it has been possible to estimate the relative contributions of glycolysis and the pentose-shunt pathway to glucose breakdown, and to see whether these are subject to hormonal control as suggested by Glock & McLean (1955) and Glock, McLean & Whitehead (1956).

EXPERIMENTAL

Cell strain. Human-foetal-liver cells (HLM) were established as a strain in this Laboratory in 1956 (Leslie, Fulton & Sinclair, 1956). HLM cells are not contaminated with pleuropneumonia-like organisms, as confirmed by periodic tests kindly carried out at intervals since 1960 by Dr D. Dane, Department of Microbiology, Queen's University, Belfast.

Growth media. Throughout this work an attempt was made to adapt the cells to growth in chemically defined media as improved formulae became available. The experiments were begun with a medium composed of 20% of embryo extract, 20% of human serum and 60% of modified Connaught synthetic medium (Healy, Fisher & Parker, 1955). Later the chemically defined medium employed was based on the formula 'MB 752/1' of Waymouth (1959). This was modified by the inclusion of *p*-aminobenzoic acid (0.0125 mg./100 ml.) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.092 mg./100 ml.). As insulin stimulates culture growth (Leslie & Paul, 1954; Leslie *et al.* 1957), Boots Pure Drug Co. Ltd. crystalline insulin (1 unit/ml.) was included in the stock medium. Better growth was obtained when this latter medium was supplemented with 4% of human serum (type AB). Bacterial infection was avoided by including penicillin [Glaxo Laboratories Ltd. benzylpenicillin (50 $\mu\text{g.}/\text{ml.}$)].

^{14}C -labelled glucose. Replicate cultures were fed on a medium containing [^{14}C]glucose, obtained from The Radiochemical Centre, Amersham, Bucks.; the [^{14}C]glucose was labelled either uniformly in all positions in the molecule ([U- ^{14}C]glucose) or specifically in a single carbon atom ([1- ^{14}C] and [6- ^{14}C]glucose). Glucose was present in the media at concentrations of 2 or 5 mg./ml. and had a specific activity of 0.2 $\mu\text{C}/\text{mg.}$

Cultivation procedure. For routine maintenance the cells were grown in large roller tubes of an average capacity of 140 ml. in 10 or 15 ml. of medium. Because the pH of the stock medium stored at 0–4° tended to rise, it was brought to an initial value of between 7.2 and 7.4 by passing in a gas mixture of nitrogen containing 3–9% of carbon dioxide and 20% of oxygen. In making subcultures, cell suspensions were prepared by treating the cultures with 5 ml. of 0.25% trypsin (Difco) in salt solution (Waymouth, 1959) for 5–

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10 min. For the preparation of replicate cultures the same volume of inoculum was dispensed into each of a series of roller tubes in the manner described by Paul (1959*b*). Such replicate cultures were allowed to become established over a period of a few days (usually with one change of medium) before they were used for the tests on growth and on the distribution of ^{14}C .

Test procedures. A minimum of seven replicate roller-tube cultures was used in a standard test. The amount of cell material per tube may be estimated for each sample by using the appropriate conversion factor, namely that 1 mg. of protein nitrogen is equivalent to 13.5 mg. dry wt. of HLM cells (Tables 1 and 3). Since the medium was necessarily buffered with bicarbonate (Swim & Parker, 1958) it was essential to arrange that carbon dioxide should not be collected until the end of the growth period. Three tubes with cultures in radioactive medium were equipped with traps designed for this purpose (see Fig. 1). Of the remaining four cultures, in non-radioactive medium, two were removed at the beginning and two on completion of growth, for the respective measurement of initial and final amounts of cell components. After growth, 6 ml. samples of medium were collected from each of these four tubes, together with a 6 ml. sample of the initial medium. Protein in the samples was precipitated by the addition of 10 ml. of tungstic acid reagent [3 vol. of 10% (w/v) sodium tungstate plus 2 vol. of *n*-sulphuric acid plus 10 vol. of water]. Cells and media were stored at -15° until analysed. A control tube, containing radioactive medium only, was included in each test to measure the small amount of $^{14}\text{CO}_2$ produced by the serum enzymes in the absence of tissue (Suschny *et al.* 1958).

To achieve a satisfactory growth rate in the stock cultures, insulin was normally incorporated in the medium at a concentration of 1 unit/ml. Accordingly, on inoculation of replicate cultures for tests with insulin, two series were set up, the test series being prepared in insulin-containing medium and the control series in insulin-free medium, to ensure that the cells in the latter were thoroughly depleted of endogenous insulin at the start of the test period. The hormone was then added to batches of test medium as required from a stock solution containing 44 units/ml., prepared by dissolving the calculated weight of pure crystalline insulin (Boots Pure Drug Co. Ltd.) in water to which a few drops of *n*-hydrochloric acid were added.

Collection of carbon dioxide from ^{14}C -labelled cultures. The carbon dioxide in the air space and in the medium was collected at the end of the growth period in the following manner. Each trap consisted of a perforated glass cylinder, which was built into the ground-glass stopper of the roller tube and contained a roll of filter paper (Fig. 1). The interior end of the trap was closed with a silicone-rubber cover and the outer end was sealed with a thin rubber cap. At the end of the test the filter paper in each trap was saturated with 0.5 ml. of 30% (w/v) potassium hydroxide injected through the rubber cap. The tubes were then allowed to stand for 1 hr. at 4° for complete absorption of the gas. The traps were removed and the filter papers transferred rapidly to stoppered centrifuge tubes containing carbon dioxide-free water. The glass cylinders were washed with water and these washings added to the centrifuge tubes. Potassium carbonate was eluted from the papers by soaking overnight.

To recover 'bound' carbon dioxide the medium that remained in the four roller tubes was transferred quickly to clean dry roller tubes, together with the residual medium

washed from the cells by adding 2 ml. of ice-cold 0.9% sodium chloride solution to the tubes and rotating them rapidly. Each tube containing medium and sodium chloride wash was fitted with a clean carbon dioxide trap, and alkali was again injected on to the paper, as described above. To liberate the 'bound' carbon dioxide 1 ml. of 5*N*-hydrochloric acid was injected through the topmost hole of the glass cylinder. The tubes with the acidified medium were kept at -15° until the liquid solidified; after thawing, the papers were removed and eluted as described above. The eluates from the two filter papers corresponding to each roller tube were combined, and sodium carbonate (about 70 mg.) was added as carrier to bring the final activity within the desired counting range. The carbonate was precipitated as barium carbonate by the addition of 1 ml. of saturated barium chloride to each tube. The precipitates were centrifuged and washed twice with water. The barium carbonate was collected on sintered-glass filters, washed with small volumes of ethanol and ether and dried to constant weight (sample range 217–305 mg.) under an infrared lamp. Samples (at least 50 mg. in wt.) were plated at 'infinite thickness' on 2 cm.² planchets, and assayed for radioactivity as described below.

The radioactive media from which carbon dioxide had been removed were neutralized with dilute sodium hydroxide and made up to a known volume. Samples (6 ml.) were deproteinized with tungstic acid as described above.

By adopting the above procedure of acidifying the medium after it was transferred from the original tubes, it was possible to harvest the cells and to use them for the analysis of ^{14}C activity in nucleic acids, proteins, lipids and acid-soluble compounds.

Chemical analysis of cells. The cells were scraped off the glass surface into 3 ml. of ice-cold *n*-perchloric acid, and the suspension was transferred to a pointed-end roller tube (Leslie & Paul, 1954). After thorough mixing to ensure extraction of acid-soluble material, the cells were centrifuged down at 1800 *g* for 5 min. at 0° . The supernatant was removed and the pellet was washed twice with 2 ml. of 0.5*N*-perchloric acid. All operations at this stage were carried out as quickly as possible at $0-4^\circ$. These combined acid extracts formed the acid-soluble fraction; before analysis for total phosphorus content or ^{14}C activity, the excess of perchloric acid was precipitated by the addition of the required amount of 30% (w/v) potassium hydroxide solution.

The lipids were extracted with 2 ml. volumes of each of the following solvents in turn (Paul, 1958): (a) 80% (v/v)

Fig. 1. Roller tube with trap for collection of carbon dioxide at the end of the test period. Details are given in the Experimental section.

ethanol, (b) ethanol, (c) ethanol-ether (3:1, v/v), (d) ether-carbon tetrachloride (3:1, v/v) and (e) ether. At stages (c) and (d) the tissue was kept in contact with the solvent for 15 min. at room temperature. The five extracts from each tube were combined and analysed for lipid phosphorus after evaporation of the solvents at 70°. The dry tissue residue was separated into protein and nucleic acid fractions by a modification of the method of Schmidt & Thannhauser (1945). Nucleic acids were determined by measuring the phosphorus content (Allen, 1940; Fontaine, 1942), and proteins by the Folin reaction (Lowry, Rosebrough, Farr & Randall 1951).

In later experiments an alternative method (Schneider, 1945) was employed for fractionation of nucleic acids and protein. The defatted tissue from each roller tube was treated twice with 2 ml. of *N*-perchloric acid at 70° for 20 min. and centrifuged after each extraction. The residue was washed once with 1 ml. of cold *N*-perchloric acid and the three supernatants containing the extracted DNA and RNA were combined. The total supernatant was diluted to 10 ml. with water, making a final concentration of 0.5 *N*-perchloric acid. One portion was diluted tenfold with water, and the extinction read at 260 m μ . The amount of DNA in the 10 ml. extract was determined by the diphenylamine procedure of Burton (1956). By taking $E_{260} = 0.275$ as the extinction of a DNA solution containing 1 μ g. of DNA phosphorus/ml., the contribution due to DNA can be subtracted from the observed extinction, and the RNA content can then be calculated by using the value $E_{260} = 0.322$ for 1 μ g. of RNA phosphorus/ml. The precipitate after perchloric acid extraction contained the protein, which was dissolved in dilute sodium hydroxide and determined by the method of Lowry *et al.* (1951) as described above.

Chemical analysis of the medium. The deproteinized medium was analysed for the uptake or production of the following components: glucose by the anthrone method of Trevelyan & Harrison (1952); lactic acid as described by Hullin & Noble (1953); and α -oxo acids (mainly pyruvic acid) by the method of Friedemann & Haugen (1943) as modified by Leslie & Paul (1954).

Distribution of ^{14}C activity in tissue components. In some experiments the acid-soluble material from three cultures (extracted with perchloric acid as described above) was pooled and fractionated further by the method of Smith & Mills (1954). A glycogen fraction was first precipitated in 48% (v/v) ethanol. Nucleotides in the supernatant were then isolated by precipitation with barium acetate in 80% (v/v) ethanol at -15° overnight; the remaining carbon was obtained as a dry residue by evaporation at 50°.

Lipids were extracted as described above and the combined extracts were evaporated at 50°. The defatted cell material was then incubated overnight at 37° in 1 ml. of 0.3 *N*-potassium hydroxide. DNA and protein were precipitated by the addition of ice-cold perchloric acid to give a final concentration of 0.5 *N*. The RNA nucleotides were removed completely by washing the precipitate twice with 0.5 *N*-perchloric acid at 0-4°. Excess of acid in the combined supernatant plus washings was neutralized with 30% (w/v) potassium hydroxide solution, and the insoluble potassium perchlorate removed by centrifuging. DNA in the tissue precipitate was separated from protein by extracting twice with 2 ml. of *N*-perchloric acid at 70° for 20 min. and washing once with 1 ml. of cold *N*-perchloric acid.

Excess of acid in the combined extracts was removed as described above. The protein residue was dissolved in dilute sodium hydroxide and transferred to combustion tubes.

The freeze-dried samples containing radioactive carbon were oxidized by a wet combustion method (Van Slyke & Folch, 1940; Van Slyke, Plazin & Weisiger, 1951), by using a simple U-tube apparatus described by Greenberg & Rothstein (1957). In each combustion (with the exception of the acid-soluble residues) 20 mg. of dried glucose was added to provide carrier carbon and the carbon dioxide was trapped in 25 ml. of saturated barium hydroxide containing 8.5% (w/v) of barium chloride, filtered immediately before use and cooled in ice. The carbonate precipitate was washed free of excess of alkali and treated as described above for the collection of carbon dioxide from the roller tubes. The barium carbonate samples (range 132-232 mg.) were assayed at 'infinite thickness' with a thin end-window Geiger-Müller tube connected to a fast scaling unit (type D 4100; Labgear, Cambridge).

In each assay at least 10 000 counts were recorded, thus decreasing the random error to 1% or less (Faires & Parks, 1958). Only two corrections therefore had to be applied to each count rate: (a) the background value of 7-10 counts/min. was subtracted; (b) the count was multiplied by a factor $x/100$ where x is the known weight of barium carbonate obtained by the combustion procedure or by the collection of carbon dioxide, as described above. The latter adjustment converted the specific activities, as measured at 'infinite thickness', into relative total activities. It was established by combustion of glucose samples of known ^{14}C activity that the count rate corrected in this way was proportional to the activity over the range used.

RESULTS

Metabolism of $[\text{U-}^{14}\text{C}]$ glucose by HLM cells. The results of the growth measurements in two representative tests are given in Table 1. After 48 hr., the values shown in columns 3 to 7 had all increased by 25-75% of the amounts initially present. This was the extent of growth generally obtained under the experimental conditions described. The ratios of cell components to DNA were not significantly changed at the end of the experiments. The changes in the composition of the medium are also shown in Table 1. The total acid production (i.e. the sum of the lactic acid and α -oxo acids formed) did not account for all of the glucose utilized, the molar ratio of total acid produced to glucose utilized being 1.64 in Expt. 1 and 0.61 in Expt. 2.

The distribution of ^{14}C activity from $[\text{U-}^{14}\text{C}]$ -glucose metabolised by HLM cells is shown in Table 2. Radioactivity measurements on barium carbonate samples prepared from initial medium incubated in the absence of cells permitted calculation of the total ^{14}C activity introduced into each tube. This initial activity remained satisfactorily consistent from one test to another. It had a value of $160\,078 \pm 2095$ counts/min. (mean from nine experiments $\pm$ s.e.) in medium with a glucose con-

centration of 5.0 mg./ml. From the value for each batch of medium and from glucose determinations on the initial and spent media, the following equation gives the values denoted (*A*) in Table 2:

Radioactivity (counts/min.) of [U-¹⁴C]glucose metabolized

$$= \frac{\mu\text{moles of glucose utilized}}{\mu\text{moles of initial glucose}}$$

$$\times \text{initial radioactivity (counts/min./tube) of [U-}^{14}\text{C]glucose}$$

In Expt. 1 the highest activity, namely 9.37 % of glucose counts metabolized, was found in the carbon dioxide fraction [row (*C*) of Table 2]. In later experiments carried out to confirm the pattern of distribution of radioactivity in these cells, the observations were extended to include separate estimations on RNA, on DNA and on three sub-fractions of the material extracted with cold perchloric acid, i.e. glycogen, nucleotides and the residue. The distribution of activity from [U-¹⁴C]-glucose in such a test is shown in Table 2 (Expt. 2).

Table 1. *Growth and metabolic activity of HLM cells*

In Expt. 1 the cells were grown in Connaught 858 medium supplemented with 20 % of human serum and 20 % of embryo extract. In Expt. 2 they were grown in Waymouth medium MB 752/1 supplemented with 4 % of human serum and 0.25 % of yeast extract. Determinations of RNA phosphorus and DNA phosphorus were carried out by the Schmidt & Thannhauser (1945) procedure. Each value is the mean of duplicate determinations (triplicate for glucose) on the contents of each of two tubes.

Expt. no.	Time (hr.)	Acid-soluble phosphorus (μg.)	Lipid phosphorus (μg.)	RNA phosphorus (μg.)	DNA phosphorus (μg.)	Protein nitrogen (μg.)	Glucose (μmoles)	Total acid* (μmoles)	Total acid produced Glucose utilized (molar ratio)
1	0	73.5	39.5	46.9	25.5	728	202	31.2	—
	48	102.0	68.0	77.0	40.5	1170	31.9	309.2	1.64
2	0	159.5	74.2	115.5	54.0	1508	382	20.84	—
	48	200.0	114.2	177.6	89.0	2375	267	91.28	0.61

* Lactic acid plus α-oxo acids.

Table 2. *Distribution of ¹⁴C activity from [U-¹⁴C]glucose metabolized by HLM cells*

The results shown refer to Expts. 1 and 2 of Table 1. Cells were grown for 48 hr. in the presence of labelled glucose at concentrations of 2 mg./ml. (Expt. 1) and 5 mg./ml. (Expt. 2) and at a specific activity of 0.2 μC/mg. The initial radioactivity of the medium was in Expt. 1 49 066 counts/min./tube and in Expt. 2 161 333 counts/min./tube. The values given are means of determinations on material from four replicate cultures in Expt. 1 and from two replicate cultures in Expt. 2.

Expt. no.	Component	¹⁴ C activity	
		(counts/min./tube)	(% of [¹⁴ C]glucose radioactivity metabolized)
1	(A) [¹⁴ C]Glucose metabolized	41 200	100.0
	(B) Nucleic acid	340	0.83
	Protein	494	1.20
	Lipid	436	1.06
	Acid-soluble compounds	599	1.45
	(C) CO ₂	3 862	9.37
	(B+C) Total cell components + CO ₂	5 731	13.91
	(D) Total acid	35 469	86.10
2	(A) [¹⁴ C]Glucose metabolized	48 500	100.0
	(B) DNA	552	1.1
	RNA	1 528	3.2
	Protein	2 037	4.2
	Lipid	2 875	5.9
	Acid-soluble glycogen	415	0.85
	Acid-soluble nucleotides	924	1.90
	Acid-soluble residue	816	1.68
	(C) CO ₂	11 449	23.6
	(B+C) Total cell components + CO ₂	20 596	42.4
	(D) Total acid	27 904	57.6

Table 3. *Effect of insulin on growth and metabolism of HLM cells*

The cells were grown in Waymouth's medium MB 752/1 supplemented with 4 % of human serum. Values are means from determinations on the numbers of replicate cultures indicated.

Test no.	Tracer compound	Time (hr.)	No. of tubes	Cell analysis				Medium analysis			
				Insulin (unit/ml.)	Acid-soluble phosphorus ($\mu\text{g./tube}$)†	Lipid phosphorus ($\mu\text{g./tube}$)†	Nucleic acid phosphorus ($\mu\text{g./tube}$)†	Protein nitrogen ($\mu\text{g./tube}$)†	Glucose ($\mu\text{moles/tube}$)†	Total acid* ($\mu\text{moles/tube}$)†	Total acid produced Glucose utilized (molar ratio)
Is 18	[U- ¹⁴ C]Glucose	0	3	0	79.1	30.6	45.9	610	387.0	10.09	—
		50	2	0	114.6	50.3	71.1	1024	308.2	112.1	1.30
		0	3	1	94.0	36.2	65.1	822	391.0	11.29	—
Is 21	[6- ¹⁴ C]Glucose	50	2	1	132.0	57.6	78.4	1269	230.2	217.8	1.29
		0	2	0	72.1	22.4	55.2	453	218.0	9.40	—
		48	2	0	97.5	34.2	87.1	703	168.0	69.5	1.20
Is 22	[1- ¹⁴ C]Glucose	0	2	1	112.0	37.6	87.1	760	227.0	9.60	—
		48	2	1	150.0	62.1	133.8	1206	71.8	221.6	1.37
		0	2	0	54.2	21.0	40.0	330	211.0	10.3	—
		48	2	0	75.6	36.1	58.9	469	177.7	44.9	1.04
		0	2	1	74.8	28.5	48.4	428	221.0	10.6	—
		48	2	1	118.4	51.0	86.8	866	130.3	122.0	1.23

* Lactic acid plus α -oxo acids.

† Each culture analysed in duplicate.

‡ Each medium sample analysed in triplicate.

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† Each culture analysed in duplicate.

‡ Each medium sample analysed in triplicate.

As before the greatest measured activity (namely 23.6 % of glucose counts metabolized) appeared in carbon dioxide, with the remainder, in order of decreasing activity, in lipid, protein, nucleic acid and acid-soluble compounds. In the glycogen fraction which contained the lowest activity (only 0.85 %) the count rate was still in the measurable range.

Extent of fixation of carbon dioxide in HLM cells.

It was observed in a series of similar experiments that 5–25 % of the radioactivity of [U-¹⁴C]glucose metabolized by human-cell strains growing in roller-tube culture could be recovered in carbon dioxide. It was decided to investigate whether any fixation of carbon dioxide occurred in the cells under these circumstances.

Sodium [¹⁴C]bicarbonate was used as tracer. A specific activity of 0.2 $\mu\text{C/mg.}$ was employed, giving a final activity of about 0.41 $\mu\text{C/ml.}$ in the Waymouth medium containing 224 mg. of sodium bicarbonate/ml. During the experiment the cells proliferated well; the DNA phosphorus content increased by about 83 % after 48 hr. and amounts of other cell constituents increased proportionately. Although the cell population almost doubled, the incorporation of ¹⁴C into the tissue fractions was very low. The labelling of the various fractions was never greater than 0.11 % and total uptake corresponded to only 0.32 % of the [¹⁴C]bicarbonate activity present in the initial medium.

Effects of insulin on the glucose metabolism of HLM cells. Table 3 shows the results of the growth analysis in three representative experiments in which the cells were grown in Waymouth medium MB 752/1 containing 5 mg. of glucose/ml. ¹⁴C-labelled glucose was incorporated at a specific activity of 0.2 $\mu\text{C/mg.}$ With HLM cells it was generally found that there was some stimulation of growth in the presence of insulin, as shown for example by the increments in the tissue components in tests Is 21 and Is 22. Owing to the preliminary insulin-depletion process, the cell populations were thus larger in the hormone-treated cultures throughout the test periods. In each test, two or three times as much glucose was utilized in the hormone-treated cultures as in the controls, but as the quantity of total acid (i.e. lactic acid plus α -oxo acid) produced also increased, the molar ratio of total acid produced to glucose utilized tended to rise (Table 3). Moreover, when the glucose utilization is expressed as a quotient (i.e. uptake/unit of nucleic acid phosphorus/unit of time), which eliminates weighting due to the differences in cell number between the control and hormone-treated cultures, the values of the quotients are increased approximately twofold by the presence of insulin.

The effect of insulin on the distribution of radioactivity from [U-¹⁴C]glucose in HLM cell cultures is shown in Table 4. A somewhat higher uptake of ¹⁴C (as counts/min./tube; columns 2 and 4 of Table 4) was found with insulin in every fraction except DNA. However, when the results were expressed as percentages of the total [¹⁴C]glucose activity metabolized, then lower values were obtained in the hormone-treated cultures in the cell components (with the exception of the acid-soluble glycogen) and in carbon dioxide, as a consequence of the relatively greater increase in glucose converted into lactic acid and α -oxo acids (see columns 3 and 5 of Table 4).

In comparable experiments the cells were given glucose labelled at either C-6 or C-1, and having the same specific activity as before (0.2 μ C/mg.). The results, together with those obtained with [U-¹⁴C]glucose, are presented in Table 5. The activities in cell components, total acids of the medium, and carbon dioxide are given relative to each 1000 counts/min. of the [¹⁴C]glucose metabolized. Though this mode of expression conceals the fact that insulin stimulated the glucose utilization per cell, it indicates how the distribution of ¹⁴C activity varied with altered labelling of the atoms within the glucose molecule.

It is evident from Table 5 that [1-¹⁴C]glucose gave rise to a considerably higher labelling of carbon dioxide than did [6-¹⁴C]glucose, both in the presence and absence of insulin (cf. column 4 with 6, and 5 with 7, in Table 5). This suggests that an

active pentose pathway is operating in these cells. Consistent with this interpretation is the fact that labelling of the lipid fraction was higher with [6-¹⁴C]glucose than with [1-¹⁴C]glucose. It is also a feature of the ¹⁴C distribution expressed in this way that the relative activities found in the total acid produced from [6-¹⁴C]glucose, [U-¹⁴C]glucose and [1-¹⁴C]glucose diminish in that order. (The last row of Table 5 relates the values to those for [6-¹⁴C]glucose.)

It was desirable to have a form of expression of ¹⁴C distribution which was directly related to cell proliferation, and which would reflect the increased growth of cells in the presence of insulin. Because of the stability of DNA (Thomson, Paul & Davidson, 1956, 1958; Graham & Siminovitch, 1957) and the constancy of DNA per cell nucleus (Davidson & Leslie, 1950; Thomson, Heagy, Hutchison & Davidson, 1953), it was considered reasonable to relate the radioactivity in each cell fraction to the ¹⁴C activity in DNA. Distribution patterns for the tests Is18, Is21 and Is22 with the ¹⁴C activities expressed in this way are presented in Table 6. In each experiment the chief effect of insulin was again an acceleration of glucose metabolism, in which most of the extra glucose utilized was channelled towards lactic acid production. Some increases in the ¹⁴C activities of the tissue fractions were noted, but these were, for the most part, small and insignificant.

Estimations of the percentage of glucose molecules metabolized by the pentose shunt. From a knowledge

Table 4. *Effect of insulin on the distribution of radioactivity from [U-¹⁴C]glucose metabolized by HLM cells (test Is18)*

The cells were grown for a period of 50 hr. in the presence or absence of insulin. The medium (Waymouth's MB 752/1 supplemented with 4% of human serum) contained [U-¹⁴C]glucose (concn. 5 mg./ml.; specific activity, 0.2 μ C/mg.). For details of growth see Table 3. Values given are means of analyses on three replicate cultures.

Component (1)	¹⁴ C activity			
	Without insulin in medium		With insulin in medium	
	(counts/min./tube) (2)	(% of [¹⁴ C]glucose radioactivity metabolized) (3)	(counts/min./tube) (4)	(% of [¹⁴ C]glucose radioactivity metabolized) (5)
(A) [¹⁴ C]Glucose metabolized	38 250	100.0	78 740	100.0
(B) DNA	239	0.62	218	0.28
RNA	836	2.19	870	1.11
Protein	843	2.21	979	1.25
Lipid	2 086	5.71	2 315	2.94
Acid-soluble glycogen	22	0.06	140	0.18
Acid-soluble nucleotides	619	1.62	918	1.17
Acid-soluble residue	798	2.09	1 165	1.48
(C) CO ₂	9 939	25.95	11 007	14.0
(D) Total acid*	22 868	59.7	61 128	77.6

* Calculated by subtracting total counts in (B) and (C) from counts in (A). Total acid refers to lactic acid and α -oxo acids in the medium.

of ^{14}C activities in various metabolites formed from glucose labelled in different positions of the molecule, it is possible to estimate the percentage of glucose catabolized by the Embden-Meyerhof pathway and the pentose shunt, provided that the following assumptions are made: (1) The terminal carbons C-1 and C-6 of glucose are identically treated in the Embden-Meyerhof pathway at the triose and pyruvate stages. (2) When the total radioactivities (counts/min.) recovered in carbon dioxide are added to those of the cellular components and then subtracted from the total radio-

activity (counts/min.) of [^{14}C]glucose metabolized, the residual radioactivities (counts/min.) represent the ^{14}C activity in lactic acid and α -oxo acids in the medium. (For convenience, this activity is referred to as ^{14}C activity of lactate, since lactate is the major constituent.) (3) Extensive recycling of glucose carbon occurs in the pentose shunt by operation of transketolase-transaldolase reactions. (4) When glucose is metabolized via the pentose shunt, C-1 cannot be incorporated into pyruvate and lactate. (5) A negligible quantity of glucose is polymerized to glycogen.

Table 5. *Recovery of ^{14}C activity in HLM cells, per 1000 counts/min. of glucose metabolized, with variously labelled glucose*

The cells were grown in Waymouth's medium MB 752/1 supplemented with 4% of human serum, in the presence or absence of insulin (1 unit/ml.) for 48 hr. (tests Is21 and Is22) or 50 hr. (test Is18). The medium contained [^{14}C]glucose labelled either uniformly or specifically in a single carbon atom as indicated. Details of growth are given in Table 3. Each ^{14}C activity value is the mean result of determinations on three replicate cultures.

^{14}C activity (counts/min. per 1000 counts/min. of glucose metabolized)						
(1)	Is18 [U- ^{14}C]Glucose		Is21 [6- ^{14}C]Glucose		Is22 [1- ^{14}C]Glucose	
	Without insulin in medium	With insulin in medium	Without insulin in medium	With insulin in medium	Without insulin in medium	With insulin in medium
	(2)	(3)	(4)	(5)	(6)	(7)
[^{14}C]Glucose metabolized	1000	1000	1000	1000	1000	1000
DNA	6	3	13	7	13	8
RNA	22	11	45	27	40	23
Protein	22	12	36	27	38	29
Lipid	57	29	80	50	51	37
Acid-soluble fraction	38	29	55	31	51	28
Total cell components	145	84	229	142	193	125
CO_2	260	140	74	46	254	165
Total acid*	595	776	697	812	553	710
Total acid as % of value with [6- ^{14}C]glucose	86	95	100	100	79	87

* Calculated as described in footnote of Table 4.

Table 6. *Recovery of ^{14}C activity in HLM cells relative to radioactivity in DNA, with variously labelled glucose*

Experimental details are as given for Table 5.

^{14}C activity (counts/min./tube)						
	Is18 [U- ^{14}C]Glucose		Is21 [6- ^{14}C]Glucose		Is22 [1- ^{14}C]Glucose	
	Without insulin in medium	With insulin in medium	Without insulin in medium	With insulin in medium	Without insulin in medium	With insulin in medium
[^{14}C]Glucose metabolized	1670	3610	768	1430	768	1250
DNA	10	10	10	10	10	10
RNA	37	37	35	39	31	29
Protein	37	43	28	39	29	36
Lipid	95	97	62	72	39	46
Acid-soluble fraction	63	96	43	44	40	35
Total cell components	242	283	178	204	149	156
CO_2	431	466	56	66	195	207
Total acid*	997	2861	534	1160	424	887

* Calculated as described in footnote of Table 4.

The deductions, based primarily on the results of experiments with [1-¹⁴C]glucose and [6-¹⁴C]glucose, may be followed by reference to Table 5, in which activities have been expressed relative to each 1000 counts/min. of the [¹⁴C]glucose metabolized. The last row of Table 5 gives the ¹⁴C activities in lactate as percentages of the values obtained with [6-¹⁴C]glucose, and indicates that lactate was labelled to a lesser degree when [1-¹⁴C]glucose was used. If glucose were metabolized solely by the Embden-Meyerhof pathway, equal ¹⁴C activities would be expected in lactate from [1-¹⁴C]glucose and [6-¹⁴C]glucose [see assumption (1)]. Hence the difference observed between lactate ¹⁴C activities with these two tracer compounds should reflect the amount of glucose metabolized via the pentose shunt. In the absence of insulin, the difference is about 20 % (Table 5). When a similar deduction is made from results obtained in the presence of insulin (Table 5), about 13 % of the glucose appears to pass through the pentose shunt.

The percentages of glucose metabolized by the two pathways may also be estimated on the basis of the ¹⁴C activities recovered in carbon dioxide. In this case it is assumed that, when [6-¹⁴C]glucose is the substrate, all the labelled carbon dioxide produced comes from glucose which is metabolized via the Embden-Meyerhof pathway but not released to the medium at the lactate stage. Table 5 shows that in the absence of insulin a ¹⁴C activity of 74 counts/min. was detected in carbon dioxide for every 1000 counts/min. of [6-¹⁴C]glucose metabolized. In the presence of insulin, the corresponding value was

46 counts/min. in carbon dioxide per 1000 counts/min. of [6-¹⁴C]glucose metabolized.

By using these values one may calculate the relative degrees of labelling expected in carbon dioxide with [1-¹⁴C]glucose and [6-¹⁴C]glucose respectively for particular percentage contributions of the pentose shunt to glucose metabolism (see Table 7). For example, if the shunt contribution is 20 % then for every 100 μmoles of [1-¹⁴C]glucose metabolized 20 will form 20 μmoles of labelled carbon dioxide. Of the remaining 80 μmoles of glucose metabolized via the Embden-Meyerhof pathway in the absence of insulin 7.4 % or 5.9 μmoles will be oxidized via the Krebs cycle to carbon dioxide. Thus, with [1-¹⁴C]glucose as substrate, 25.9 μmoles of ¹⁴CO₂ will be formed (see column 2 of Table 7), whereas with [6-¹⁴C]glucose only 5.9 μmoles of ¹⁴CO₂ will be produced (column 3 of Table 7). The ratio of these two values is 4.36 (column 4 of Table 7).

For HLM cells grown in the absence of insulin, the experimentally determined value of the ratio was 254/74 = 3.48 (Table 5). Comparison of this value with the theoretical values in columns 1 and 4 of Table 7 would seem to indicate that about 16 % of glucose utilized was degraded via the pentose shunt. The corresponding percentage for cells grown in the presence of insulin, derived from the measured ratio 165/46 = 3.58 (cf. Table 5) and the calculated values of columns 1 and 7 (Table 7), is about 11 %.

This method of calculation gives somewhat lower values for pentose-shunt activity both in the

Table 7. *Expected ratios of radioactivities in carbon dioxide formed from [1-¹⁴C]glucose and [6-¹⁴C]glucose respectively, by HLM cells for certain values of percentage pentose-shunt contribution*

The ratios are calculated on the assumption that all the $^{14}\text{CO}_2$ formed from $[\text{6-}^{14}\text{C}]\text{glucose}$ is produced by metabolism through the Embden-Meyerhof pathway, and by using the experimental findings that in the absence and presence of insulin 7.4 and 4.6 % respectively of the radioactivity of metabolized $[\text{6-}^{14}\text{C}]\text{glucose}$ is converted into $^{14}\text{CO}_2$. The method of calculation is explained in the text.

Percentage of glucose metabolized via the pentose shunt	Insulin absent: 7.4 % of glucose carbon metabolized via the Embden- Meyerhof pathway converted into CO_2 $^{14}\text{CO}_2$ produced			Insulin present: 4.6 % of glucose carbon metabolized via the Embden- Meyerhof pathway converted into CO_2 $^{14}\text{CO}_2$ produced		
	$(\mu\text{moles}/100 \mu\text{moles})$		a/b	$(\mu\text{moles}/100 \mu\text{moles})$		c/d
	of $[\text{1-}^{14}\text{C}]\text{glucose}$ metabolized)	of $[\text{6-}^{14}\text{C}]\text{glucose}$ metabolized)		of $[\text{1-}^{14}\text{C}]\text{glucose}$ metabolized)	of $[\text{6-}^{14}\text{C}]\text{glucose}$ metabolized)	
	(1)	(2)	(3)	(4)	(5)	(6)
0	7.4	7.4	1.00	4.6	4.6	1.00
10	16.7	6.66	2.51	14.14	4.14	3.40
12	18.52	6.52	2.84	16.05	4.05	3.97
15	21.3	6.29	3.38	18.9	3.91	4.84
18	24.1	6.06	3.96	21.8	3.78	5.76
20	25.9	5.92	4.36	23.7	3.68	6.47
30	35.2	5.18	6.80	33.2	3.22	10.30
40	44.4	4.44	10.00	42.8	2.76	15.60
50	53.7	3.70	14.50	52.3	2.30	22.80

presence and absence of insulin, as compared with the estimations based on the radioactivity in lactate. However, all values are clearly approximations in view of the nature of the assumptions on which the deductions are based. For example, the calculations based on carbon dioxide activities do not allow for the amount (presumed small) of labelled carbon dioxide which is formed from the labelled triose arising in the pentose shunt with [6- ^{14}C]glucose as tracer. Further, in calculating the theoretical ratios a/b and c/d for various pentose-shunt contributions, it has been assumed that either 7.4 % or 4.6 % of glucose carbon entering the Embden-Meyerhof pathway and Krebs cycle is completely oxidized to carbon dioxide. These values would be modified if C-6 of glucose were less readily oxidized than C-2-C-5.

DISCUSSION

The experiments demonstrate that in HLM cultures larger proportions of [^{14}C]glucose carbon were converted into carbon dioxide than were incorporated into the cell materials. It is also evident that the more actively respiring cultures incorporated a higher proportion of glucose carbon into the cell components (Tables 2 and 5). Between 14 and 45 % of the activity of glucose metabolized (A) can be accounted for in the combined radioactivity ($B + C$) of the cell components and carbon dioxide, and it is assumed that the balance (D) of 55–86 % is largely associated with the lactic acid and α -oxo acids that accumulated in the medium during cell growth. The results of chemical determinations of glucose, lactic acid and α -oxo acids (Tables 1 and 3) show that, with one exception, 50–82 % of the glucose metabolized could be accounted for by the total acid that accumulated in the medium. The exceptionally low value of 30 % (Expt. 2 of Table 1) was associated with very large cultures, as indicated by the amounts of cell constituents in the roller tubes.

The major effect of insulin on cultures of proliferating HLM cells was the pronounced acceleration of glycolysis. There was, too, a clear growth-promoting effect, since cells that were cultivated in the absence of insulin for several days before the tests multiplied more slowly than cells kept throughout on medium containing 1 unit of insulin/ml. This second observation runs contrary to previous reports that insulin did not promote growth in cultures of HLM cells and other established strains (Leslie *et al.* 1957; Paul & Pearson, 1960), and it seems probable that the type of response to the hormone depends on the medium employed: in the present experiments the cells were grown in Waymouth's medium MB 752/1, supplemented with 4 % of human serum. Here, it is

unlikely that the insulin acted in a non-specific way, as its contribution to the total protein concentration of the medium was less than 2 %. No significant change in the distribution of glucose carbon amongst cell components and carbon dioxide was noted on the addition of insulin to the medium.

By the use of variously labelled forms of glucose, estimates were obtained for the relative contributions of the Krebs cycle and the pentose shunt to glucose metabolism in the HLM cells. It was found that 10–20 % of glucose utilized passed through the pentose shunt, while 4–8 % was oxidized to carbon dioxide via the Krebs cycle. Calculations showed that the maximum amount of pentose which could have been generated, if 20 % of the glucose utilized passed through the pentose shunt, was about eight times the quantity required for the nucleic acids synthesized during the test period. Presumably a considerable recycling of glucose carbon occurs in this pathway by the action of transketolase and transaldolase enzymes. Because of the assumptions that have to be made in deriving these percentages no significance could be attached to the slight lowering of the pentose-shunt contribution observed when insulin was added to the medium.

Estimations of proportions of glucose metabolized along different pathways, based solely on the levels of activity recovered in carbon dioxide, have been criticized by Wood (1955), on the grounds that they indicate only the ratio of the percentage of C-1 of glucose metabolized to carbon dioxide via the pentose shunt to the percentage of C-1 converted into carbon dioxide by the Embden-Meyerhof pathway and Krebs-cycle enzymes. Since in the pentose shunt carbon dioxide is released immediately in three consecutive reactions, whereas in the alternative route about 20 intermediate stages are involved, values for the ratio:

$$\frac{^{14}\text{C activity in CO}_2 \text{ from [1-}^{14}\text{C]glucose}}{^{14}\text{C activity in CO}_2 \text{ from [6-}^{14}\text{C]glucose}}$$

will be influenced by loss of ^{14}C activity through side reactions. In the present instance, there is reasonable agreement between the estimates based on ^{14}C activities in lactic acid and α -oxo acids, and those derived from the activities measured in carbon dioxide.

SUMMARY

1. A ^{14}C -tracer technique has been developed and used to investigate the utilization of carbon from ^{14}C -labelled glucose in proliferating cell cultures of the permanent strain HLM, derived from human foetal liver.

2. With uniformly labelled glucose ([U- ^{14}C]glucose), 9–26 % of the ^{14}C activity metabolized was found in carbon dioxide and 5–18 % was incorporated into the cell constituents (i.e. acid-

soluble compounds, lipids, nucleic acids and proteins), in cultures analysed at different times and in different media. The incorporation of carbon from sodium [^{14}C]bicarbonate was negligible in these cells.

3. The addition of insulin (1 unit/ml.) to the culture medium produced a marked stimulation of glycolysis. HLM cells cultivated for sufficient time in the absence of insulin grew more slowly than replicate cultures kept in medium containing insulin. The presence of insulin did not significantly alter the distribution of glucose carbon amongst cell constituents and carbon dioxide.

4. [$1\text{-}^{14}\text{C}$]Glucose produced higher activity in carbon dioxide than did [$6\text{-}^{14}\text{C}$]glucose, indicating some metabolism via the pentose-shunt pathway.

5. From the differences observed in the amounts of activity located in total acid produced or in carbon dioxide, by using [$1\text{-}^{14}\text{C}$]glucose and [$6\text{-}^{14}\text{C}$]glucose respectively, and on the basis of certain assumptions, it was estimated that 10–20 % of the glucose utilized was metabolized through the shunt.

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