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Studies on the Metabolism of Hydroxypyruvate in Animal Tissues

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The preparation of pure hydroxypyruvate (Dickens & Williamson, 1958a) has made possible the use of this substance in metabolic experiments, e.g. with tissue slices, and in this paper we describe experiments on the utilization of hydroxypyruvate by the whole rat and by rat-liver slices. By the use of specifically labelled hydroxy[^{14}C]pyruvate an incorporation of radioactivity into the molecules of glycogen and glucose has been observed in both series of experiments. By stepwise degradation of the glucose molecule the pattern of radioactivity has been determined and evidence has thus been obtained about the probable overall character of the route of incorporation. A brief preliminary account of this work was given by Dickens (1958).

EXPERIMENTAL

Materials

Radioactive substances. These were obtained from The Radiochemical Centre, Amersham, Bucks, with the following approximate specific activities: generally labelled [G- ^{14}C]glucose, 10 mc/m-mole; [1- ^{14}C]glucose and [6- ^{14}C]glucose, 2 mc/m-mole; L-[3- ^{14}C]serine, 3 mc/m-mole; sodium [3- ^{14}C]pyruvate, [2- ^{14}C]pyruvate and [1- ^{14}C]pyruvate, 1 mc/m-mole. They were diluted for use as required (see tables) with non-isotopic material.

Lithium hydroxypyruvate. This was prepared and crystallized as already described (Dickens & Williamson, 1958a). The [1- ^{14}C]-, [2- ^{14}C]- and [3- ^{14}C]-hydroxypyruvate were also prepared in this way for use in most experiments, but in some, particularly where a high specific activity was needed, the corresponding bromo[^{14}C]pyruvate was used directly after conversion into a solution of hydroxypyruvate by neutralization with 2 equivalents of alkali (Sprinson & Chargaff, 1946). Although this avoided the considerable loss of radioactive material accompanying crystallization, such solutions contain traces of bromopyruvate as well as the NaBr formed on neutralization. They were found, however, to be suitable for injection into the whole animal,

when the results were checked by the use of the purified material.

In the preparation of bromo[^{14}C]pyruvate by bromination of sodium [1- ^{14}C]pyruvate and added unlabelled pyruvic acid (Dickens & Williamson, 1958a), very thorough mixing of these substances is essential before bromination, otherwise undissolved particles of sodium salt may escape

Fig. 1. Column separation of pyruvate and hydroxypyruvate. An acidified aqueous solution containing non-isotopic pyruvate (50 μ moles) and hydroxy[3- ^{14}C]pyruvate (90 μ moles) was adsorbed on Celite and the column was eluted successively with chloroform and then with chloroform containing the stated percentage (v/v) of butan-1-ol. Titration of 8 ml. fractions is shown by the curve; the dotted line indicates level of H_2SO_4 in eluates and the solid curve total acidity (keto acid plus H_2SO_4). Peak I, pyruvate (39.5 μ moles, 79% recovery). Peak II, hydroxypyruvate (70 μ moles, 78%; after vacuum-concentration and removal of indicator, overall recovery 60%). For details see text.

bromination, resulting in the possible appearance of highly active pyruvate in the final preparation. Although this impurity can be removed by column chromatography (see below) it was subsequently found preferable to dissolve the sodium [^{14}C]pyruvate in a little water and to mix it with pyruvic acid and dry thoroughly *in vacuo* before bromination. With this procedure contamination of the hydroxypyruvate with [^{14}C]pyruvate was negligible.

Chromatographic separation of pyruvate and hydroxypyruvate. These two substances proved difficult to separate but the following method (based on that of Swim & Utter, 1957) gave clearly defined fractions and reasonable recovery of materials.

Celite 535 (Johns Manville Co. Ltd., London, S.E. 11) was washed in the dry state on a large column (100 g.) with ether (1 l.) and air-dried. Quantities (10 g.) of Celite, intimately mixed with 5 ml. of 0.2N- H_2SO_4 , were made into a slurry by addition of CHCl_3 (which had previously been shaken with 0.2N- H_2SO_4) and packed closely into a chromatographic column, 2 cm. diam. $\times$ 15 cm. The mixture for separation was brought to pH 2 with H_2SO_4 , mixed with twice its volume of the washed Celite 535 and added to the top of the column. The column was eluted successively with CHCl_3 and with increasing concentrations of butan-1-ol in CHCl_3 (Fig. 1): all solvents were equilibrated with 0.2N- H_2SO_4 . In all 60–70 fractions (8 ml. each) of eluate were collected mechanically.

The fractions were immediately titrated in a stream of N_2 to phenol red with 0.01N- NaOH , after the addition to each of 5 ml. of CO_2 -free water. For isolation purposes the aqueous layers from the appropriate fractions were combined, adjusted to pH 4 and concentrated to 5–10 ml. *in vacuo* (bath temp. below 40°), after removal of phenol red by shaking with 50 mg. of Norit and filtering on a micro-filter.

Fig. 1 shows a typical separation of hydroxy[3- ^{14}C]pyruvate (90 μmoles) from unlabelled pyruvate (50 μmoles) by this method. Although butanol- CHCl_3 (60:40, v/v) elutes some H_2SO_4 together with the hydroxypyruvate, the presence of a little sulphate, formed after neutralization, is not objectionable for many biological purposes.

Analytical

Hydroxypyruvate. This was determined either colorimetrically or enzymically (Dickens & Williamson, 1958b): the diphosphopyridine nucleotide (DPN)-linked reductase from parsley, used in the method, reacts only with hydroxypyruvate and glyoxylate.

Pyruvate. This (plus hydroxypyruvate if present) was determined by crystalline muscle lactic dehydrogenase (cf. Holzer & Holldorf, 1957).

Formaldehyde. This was liberated from C-3 of serine, hydroxypyruvate or glycinate (and also from C-6 of glucose if present) by HIO_4 oxidation and was estimated with chromotropic acid (Elwyn *et al.* 1957): the separate determination requires preliminary fractionation as described below. For determination of ^{14}C liberated as formaldehyde in this reaction, the weighed carrier (serine or recrystallized lithium hydroxypyruvate) was added where required and the formaldehyde was distilled, precipitated as the dimedon derivative and transferred to a planchet for counts of radioactivity.

Glucose. This was estimated by the method of Nelson (1944). Glycogen (10–100 mg.) was weighed and hydrolysed

to glucose by 2N- H_2SO_4 (2 ml.) at 100° for 2 hr., neutralized to pH 7 with cold saturated $\text{Ba}(\text{OH})_2$ and the solution centrifuged to remove BaSO_4 .

Total carbon of radioactive compounds. These were converted into CO_2 by persulphate oxidation in the presence of AgNO_3 and counted as BaCO_3 (Katz, Abraham & Baker, 1954).

Carboxyl carbon, of pyruvate and hydroxypyruvate. This was liberated by oxidation with ceric sulphate (cf. Krebs & Johnson, 1937); the CO_2 was collected in NaOH as described above and converted into BaCO_3 . A similar method applied to [2- ^{14}C]pyruvic acid 2:4-dinitrophenylhydrazone was found by Wriston, Lack & Shemin (1955) to give only slight contamination from C-2.

Determinations of radioactivity. These were made at infinite thickness, with 1 cm.² compressed disks of material on polythene planchets, by means of a thin mica (2.3 mg./cm.²) end-window Geiger-Müller tube and automatic scaler (Ekco Electronics Ltd.). With this arrangement a 1 cm.² methacrylate standard (10^{-3} $\mu\text{C}/\text{mg.}$) gave 760 counts/min. Full details of counting and precautions to ensure sufficient accuracy were similar to those reported by Campbell & Greengard (1958). In a few instances, either where low counts were concerned or as a general control on the accuracy of counting, Mr J. K. Whitehead, of the B.E.C.C. Radiochemical Laboratory, Middlesex Hospital Medical School, very kindly carried out $^{14}\text{CO}_2$ determinations by gas-counting for us. Satisfactory agreement was obtained.

General technique for stepwise degradation of glucose and collection of $^{14}\text{CO}_2$

We are much indebted to Dr Helen Porter, F.R.S., and to Dr J. Edelman for details of the method, which is essentially that used in their Laboratory, and also for an initial culture of *Leuconostoc mesenteroides*. All degradative stages were carried out in special conical absorption flasks (50 ml. capacity) with ground-off (rimless) necks, having a fused-in central absorption well (about 6 ml.) and closed by rubber seals (Simplex no. 8; Britton Malcolm and Co., 38 Southwark Bridge Road, London, S.E. 1). By puncture of the stoppers with a hypodermic needle connected to a filter pump the flasks were evacuated after addition of the main contents. Further additions, as required, including the suspension of organisms, were made by hypodermic syringe. A general account of this technique is given by Abraham & Hassid (1957, p. 550 *et seq.*). CO_2 was absorbed, where required, in 2 ml. of CO_2 -free N- NaOH in the centre well, which was afterwards transferred with 10 ml. of CO_2 -free water to a stoppered tube. M- BaCl_2 solution (1 ml.) was added with a drop of phenolphthalein, the solution was neutralized by titration with 0.2N- HCl and the BaCO_3 was collected by centrifuging and washed with aqueous ethanol (20:80, v/v), absolute ethanol and ether, and dried *in vacuo*.

Leuconostoc mesenteroides fermentation. Glucose was degraded by fermentation to completion at pH 6.2 with *L. mesenteroides*, strain 39, as described originally by Gunsalus & Gibbs (1952). Various details of technique are given by Edelman, Ginsberg & Hassid (1955), Abraham & Hassid (1957) and Bernstein & Wood (1957), and only special points are described in detail below. In this fermentation, C-1 of glucose is directly liberated as CO_2 , C-2 and C-3 form the methyl and carbinol groups of ethanol and C-4, C-5 and C-6 of glucose form the C-1, C-2 and C-3

atoms of lactic acid. The ethanol is distilled from the fermentation mixture after collection of the CO_2 , oxidized to acetate and degraded by NaN_3 in H_2SO_4 . The lactic acid produced in the fermentation remains in the residue from which the ethanol has been distilled, and this is concentrated to about 0.5 ml., mixed with 20 g. of anhydrous powdered KHSO_4 , made into a column and eluted by 50 ml. of ether (Edelman *et al.* 1955). After the addition of a few drops of water and complete removal *in vacuo* of the ether, the aqueous residue is titrated with N-NaOH , aqueous phenol red being used as indicator. This solution is oxidized in the special absorption flasks by heating at 80° for 0.5 hr. with 2 ml. of 10% (w/v) KMnO_4 and 2 ml. of $4\text{N-H}_2\text{SO}_4$, the CO_2 (C-4 of glucose) being collected in 1 ml. of N-NaOH in the centre well and, by the usual method, converted into BaCO_3 and counted. The acetate remaining is degraded by NaN_3 in the usual way.

When this method was tested with glucose of known specific labelling, it gave nearly quantitative recoveries of ^{14}C if larger quantities of [^{14}C]glucose (about 100 mg.) were used; with the smaller amounts which were available in some experiments recoveries were somewhat lower, especially in C-3 and C-6 of glucose, no doubt owing to slight contamination with non-radioactive CO_2 during manipulation (cf. Edelman *et al.* 1955). For control purposes [$\text{G-}^{14}\text{C}$]glucose and [^{14}C]- and [$6\text{-}^{14}\text{C}$]glucose were used: the former showed good uniformity and the latter had negligible activity except in C-1 and C-6. In all degradations a control with [$\text{G-}^{14}\text{C}$]glucose was run simultaneously and, where necessary, corrections were applied based on the recovery of each carbon atom in these controls. These corrections were never large enough to affect materially the general interpretation of the results (cf., for example, the two uncorrected results in Table 3).

Yeast fermentations. This method has been used by Barnet & Wick (1950) but without detailed description. It was applied here only in experiments with liver slices, where the total radioactivity of the glucose was insufficient for accurate determination by the above-mentioned complete degradation. The glucose solutions were fermented to completion in the above-mentioned absorption flasks at 37° in N_2 by washed baker's yeast (The Distillers Co. Ltd.), the activity of which was checked manometrically by parallel fermentations in the Warburg apparatus. The CO_2 liberated, by fermentation with 150–200 mg. fresh wt. of yeast in 10 ml. of $0.2\text{M-KH}_2\text{PO}_4$, was collected in NaOH as usual and converted into BaCO_3 for counting (sum of C-3 and C-4 of glucose). The ethanol remaining in the flasks was distilled quantitatively as described above, oxidized to acetic acid, which was distilled, and degraded by the Schmidt reaction, giving CO_2 (sum of C-2 and C-5) and methylamine. The latter was oxidized by hot alkaline KMnO_4 to CO_2 , which was collected in NaOH , converted into BaCO_3 and the radioactivity counted (sum of C-1 and

C-6 of glucose). When applied to [$\text{G-}^{14}\text{C}$]glucose the method gave nearly quantitative (93–97%) recoveries of C-3 and C-4 and less good recoveries of the other carbon atoms, but sufficiently so for the minor part played by these carbon atoms in this part of the work. With [$6\text{-}^{14}\text{C}$]glucose, contamination of the other carbon atoms was also negligible (Table 1). A similar result for fermentation of [$6\text{-}^{14}\text{C}$] and [$1\text{-}^{14}\text{C}$]glucose by *Saccharomyces cerevisiae* is reported by Blumenthal, Lewis & Weinhouse (1954).

Check on radioactive labelling of compounds used. The labelling of D-[$\text{G-}^{14}\text{C}$]glucose was checked by degradation by both methods. Radioactivity of L-[$3\text{-}^{14}\text{C}$]serine and hydroxy[$3\text{-}^{14}\text{C}$]pyruvate was measured by the activity of formaldehyde liberated (from C-3) by HIO_4 . Hydroxy-[$1\text{-}^{14}\text{C}$]pyruvate was decarboxylated by $\text{Ce}_2(\text{SO}_4)_3$ and the $^{14}\text{CO}_2$ counted as BaCO_3 .

Procedure with intact rats

Albino rats of Wistar strain bred in the Institute, of body wt. approx. 200–250 g., were kept in metabolism cages, with separate collection of urine. After withdrawal of food, usually for 18 hr., they were injected subcutaneously with labelled materials as stated below and at once provided with food *ad lib.* (rat cubes; North Eastern Agriculture Co-operative Society, Aberdeen). In some experiments a second injection was given 3 hr. after the first, and in either case the animals were killed 3 hr. after the last injection.

The livers were excised immediately after death and used for the isolation of glycogen, and in some experiments for other fractions also, as described by Bernstein (1953). In this method glycogen is precipitated by alcohol from the trichloroacetic acid extract, weighed and purified by reprecipitation. The fractionation of the washed trichloroacetic acid-insoluble material was carried out only as far as the separation of lipid, protein and mixed sodium nucleate fractions. Counts of radioactivity were made, and, with glycogen, hydrolysis to glucose and stepwise degradation to BaCO_3 also, as already described.

Procedure with liver slices

Freshly excised liver from Wistar rats was cut into slices in the apparatus of Stadie & Riggs (1944). Batches of 1 g. wet wt. of slices were incubated at 37° with shaking in the conical absorption flasks provided with rubber seals and centre wells described above. The gas space was filled with $\text{O}_2 + \text{CO}_2$ (95:5) and the suspension medium was 10 ml. of Krebs–Ringer bicarbonate solution, pH 7.4 (Umbreit, Burris & Stauffer, 1949); other general conditions, including the incubation time (90 min.), were similar to those adopted by Elwyn *et al.* (1957).

After incubation with the substrates as stated below, the decanted medium plus the water washings of the slices

Table 1. Degradation of [^{14}C]glucose by yeast fermentation

For details see text: 32 mg. of glucose was taken. Results for [$\text{G-}^{14}\text{C}$]glucose are means of eight experiments $\pm$ s.d.

Substance	Total activity (μC)	Percentage of total radioactivity in carbon atoms			Total recovery (%)
		C-3 + C-4	C-2 + C-5	C-1 + C-6	
D-[$\text{G-}^{14}\text{C}$]Glucose	0.0805	31.7 ± 0.7	26.2 ± 2.6	23.5 ± 3.5	81.4
D-[$6\text{-}^{14}\text{C}$]Glucose	0.0920	0.4	0.2	76.0	76.6
	0.0905	0.7	0.2	79.0	79.9

were made to measured volume (20 ml.). If the washed slices were to be analysed (Elwyn *et al.* 1957) they were homogenized in water and the proteins, together with lipids and nucleic acids, precipitated by making to 2% trichloroacetic acid, centrifuging, washing and drying. A measured fraction of the incubation medium plus washings was used for determination of total CO₂ liberated on acidification with trichloroacetic acid (2% final concn.) in absorption flasks, by collection in NaOH followed by conversion into BaCO₃ for weighing and counting. A small correction was applied, based on the amount of total (free + bound) CO₂ present in the flasks, as determined in parallel experiments (cf. section *b* below).

The main part of the medium plus washings was also deproteinized by addition of trichloroacetic acid to 2% final concentration. Measured portions of the supernatant were used for estimations of glucose, hydroxypyruvate, pyruvate and groups which formed formaldehyde on periodate oxidation as already described. The remainder was fractionated as follows.

(a) *Hydroxy[3-¹⁴C]pyruvate or L-[3-¹⁴C]serine as substrates.* Amino acids (together with other basic materials) were adsorbed by passing the solution (about 20 ml.) through a column (1 cm. diam. x 6 cm.) of Amberlite IR 120 (H⁺ form: British Drug Houses Ltd.), and washing through with 10 ml. of water (eluate A). The column was then eluted with aq. 2*N*-NH₃ soln. (20 ml.), the NH₃ distilled off *in vacuo* and the residue taken to dryness, then dissolved in water for determination of radioactive formaldehyde liberated by treatment with HIO₄. This value is referred to as 'serine', since good recoveries of added serine were obtained and this is probably the main formaldehydogenic substance likely to be present in this fraction, the acidic and neutral substances passing into eluate A. The latter was freed from ionized material by shaking with 4 g. of Bio-Deminrolit G (The Permutit Co. Ltd.; see Dickens & Williamson, 1956*a*) and its glucose content measured by the Nelson method; after concentration to 10 ml. and the addition of a weighed amount of glucose (30–50 mg.) the phenylsazone was prepared, weighed and its radioactivity measured before and after recrystallization.

(b) *Hydroxy[1-¹⁴C]pyruvate and NaH¹⁴CO₃ as substrates.* When hydroxy[1-¹⁴C]pyruvate was incubated with slices the general procedure was similar to that described above, but, after incubation for 90 min., 2 ml. of NaOH was injected into the centre well of the vessels, followed by the injection of 2 ml. of 20% (w/v) trichloroacetic acid into the main part of the flasks. The total CO₂ thus liberated was absorbed in the NaOH and converted into BaCO₃ in the usual way. The protein precipitate was centrifuged off, and the total supernatant fluid was deionized directly with Bio-Deminrolit G. After estimation of glucose in the resulting solution, carrier glucose was added and glucosazone prepared as before.

RESULTS

Metabolism of hydroxypyruvate in the intact rat

The data based on nine rats which received subcutaneous injections of hydroxy[¹⁴C]pyruvate are summarized in Table 2, together with one example from a rat given a control injection with [1-¹⁴C]-glucose. As a regular procedure an approximately

Table 2. *Metabolism of hydroxy[¹⁴C]pyruvate in the intact rat*

Rat	No.	Wt. (g.) (Adult)	Material injected	Total dose		Period of fasting (hr.)	Period after last dose (hr.)	Liver glycogen		
				(μmoles)	(μC)			Wt. (mg.)	¹⁴ C (μmc)	¹⁴ C (% of dose)
I	I	277	Lithium hydroxy[3- ¹⁴ C]pyruvate	200	0.21	17	6	188	4.3	2.0
II	II	140	Lithium hydroxy[3- ¹⁴ C]pyruvate	200	0.21	18	3	223	10.5	5.0
III	III	286	Lithium hydroxy[3- ¹⁴ C]pyruvate	500*	0.53*	—	2	40	7.6	1.5
IV	IV	209	Lithium hydroxy[3- ¹⁴ C]pyruvate	300	0.32	20	2	213	14.9	4.7
V	V	220	Sodium hydroxy[3- ¹⁴ C]pyruvate	200	6.20	24	3	134	268	4.5
VI	VI	310	Sodium hydroxy[3- ¹⁴ C]pyruvate	200	6.20	24	3	188	212	3.4
VII	VII	290	Sodium hydroxy[2- ¹⁴ C]pyruvate	200	6.00	24	3	265	265	4.4
VIII	VIII	255	Sodium hydroxy[2- ¹⁴ C]pyruvate	200	6.00	24	3	35	111	1.9
IX	IX	250	Sodium hydroxy[2- ¹⁴ C]pyruvate	200	6.00	24	3	98	190	3.2
X	X		[1- ¹⁴ C]Glucose	550	1.00	18	3	150	39	3.8

* As five injections at 1 hr. intervals.

18 hr. fast was followed by giving food and injection, and killing the rats 3 hr. later; this was adopted in the later experiments on the basis of comparison of the percentage incorporation of ^{14}C in rats I–IV, Table 2. With this procedure, on the whole, fairly consistent incorporation was observed in liver glycogen, amounting to 3–5 % of the dose; lower figures for rats III and VIII may have been due to little or no food being taken, as suggested by the low total glycogen in the livers of these animals. The fate of the greater part of the ^{14}C dose has not yet been studied, but preliminary experiments showed that the urinary excretion accounted for a variable but often high proportion of the dose. In view of this loss of material the percentage incorporation into liver glycogen is quite high, as compared with that of [^{14}C]glucose given under closely similar conditions (rat X).

In a few experiments, measurement of total radioactivity of liver protein, nucleic acids and lipids was also made. Liver nucleic acids from rats I, II and V had less than 0.06 % of the dose, de-fatted protein in rats V and VI 1.75 and 1.25 %, and total lipids showed rather low incorporation and were not accurately measured. No check on the optimum period for these incorporations was made.

The total liver glycogen from each of nine injected rats was hydrolysed to glucose, which was degraded by *L. mesenteroides* as described in the Methods section. The results (Table 3) were in satisfactory general agreement whether recrystallized lithium hydroxypyruvate or the solution of sodium salt (uncrystallized) was used, though they are likely to be more reliable with the latter owing to the higher specific activities available in this form. With hydroxy [$3\text{-}^{14}\text{C}$]pyruvate, incorporation was almost symmetrical and was mainly in C-1 and C-6 (average 35 % of total radioactivity of glucose in each carbon atom), secondarily in C-2 and C-5

(average 11 % each) and with very little activity (4 %) in C-3 or C-4.

With hydroxy[$2\text{-}^{14}\text{C}$]pyruvate a closely similar type of distribution occurred, labelling being mainly in C-2 and C-5, in which the average was 31 and 23 % respectively, but the latter figure may be too low owing to an aberrant and possibly erroneous low recovery in C-5 of rat VIII. C-1 and C-2 averaged 13 and 10 %, with again only small amounts (4–5 %) in C-3 and C-4. The control experiment (rat X) with [$1\text{-}^{14}\text{C}$]glucose showed a distribution of radioactivity almost identical with that described after a 1 hr. period in an otherwise generally similar experiment by Hers (1955); Hers's animals received an intracardiac injection of labelled glucose instead of a subcutaneous one, as in our experiments, and the latter may have resulted in prolongation of the absorption period with less randomization in the 3 hr. period adopted by us than in the 3 hr. experiments which Hers also reports. Hers accompanied the [^{14}C]glucose injection with 0.75 g. of unlabelled glucose by stomach tube and this may also partly account for the higher percentage incorporation (12.2 % of injected ^{14}C) into liver glycogen observed in his rats.

Metabolism of hydroxypyruvate in rat-liver slices

Hydroxy[$3\text{-}^{14}\text{C}$]pyruvate. Table 4 shows the result of comparative experiments in which liver slices were incubated aerobically with either hydroxy[$3\text{-}^{14}\text{C}$]pyruvate or L-[$3\text{-}^{14}\text{C}$]serine; also included are the results of Elwyn *et al.* (1957) on the utilization of D- and L-[$3\text{-}^{14}\text{C}$]serine by rat-liver slices. The table shows that hydroxy[$3\text{-}^{14}\text{C}$]pyruvate contributes about as much to CO_2 (about 5.5 %) as does similarly labelled L-serine in our experiments, though both figures are lower than that given by Elwyn *et al.* for L-serine (14.4 %).

Table 3. *Degradation of glucose from hydrolysis of liver glycogen*

Samples (Table 2) were degraded by *Leuconostoc mesenteroides* (see text).

Rat no.	Material injected	Percentage of total counts in glucose carbon atoms					
		C-1	C-2	C-3	C-4	C-5	C-6
III	Lithium hydroxy[$3\text{-}^{14}\text{C}$]pyruvate	30	15	6	2	14	33
IV	Lithium hydroxy[$3\text{-}^{14}\text{C}$]pyruvate	35	5	4	4	15	40
V*	Sodium hydroxy[$3\text{-}^{14}\text{C}$]pyruvate	33	10	3.5	5	9	28
VI*	Sodium hydroxy[$3\text{-}^{14}\text{C}$]pyruvate	36	9	2.5	4	10	28
Mean III–VI	Hydroxy[$3\text{-}^{14}\text{C}$]pyruvate	35	10	4	4	12	35
VII	Sodium hydroxy[$2\text{-}^{14}\text{C}$]pyruvate	13	37	4	4	27	13
VIII	Sodium hydroxy[$2\text{-}^{14}\text{C}$]pyruvate	12	28	5	6	12	5
IX	Sodium hydroxy[$2\text{-}^{14}\text{C}$]pyruvate	15	30	5	7	30	12
Mean VII–IX	Hydroxy[$2\text{-}^{14}\text{C}$]pyruvate	13	32	5	6	23	10
X	[$1\text{-}^{14}\text{C}$]Glucose	93	3	2	1	1	1

* Percentage recoveries in these two experiments are the uncorrected values, owing to accidental loss of the [$\text{G-}^{14}\text{C}$]glucose control.

On the other hand, our results, based on the radioactive counts in recrystallized glucosazone, show that hydroxypyruvate is very much more readily incorporated into glucose of the medium (15.7%) than is L-serine (1.6%). The latter figure agrees well with those (1.2%) found for both L- and D-serine by Elwyn *et al.*

In considering the total formaldehyde liberation on periodate oxidation in Table 4, account has to be taken of this large difference in the radioactivity of the glucose, since each mole of glucose gives 1 mole of formaldehyde (from C-6) with HIO_4 (cf. Jackson, 1944). The total formaldehydogenic substance found after incubation also includes any unchanged starting material, and whereas with L-serine this is stated to be the only or main formaldehydogenic substance present (Elwyn *et al.* 1957; but cf. Table 4), with hydroxypyruvate as substrate direct enzymic estimation showed that very little of it (about 5%) remains unchanged at the end of the experiment. These points are further brought out in Table 4 by comparison of the values for formaldehydogenic substance before and after resin-adsorption of ions and elution of amino acids (see Methods section). The results indicate that whereas more than 70% of the formaldehydogenic substance in the serine experiment is probably unchanged serine, most of the hydroxypyruvate has been converted into substances which no longer yield formaldehyde with HIO_4 , especially when the formaldehyde due to the 15.7% conversion into glucose has been subtracted. Consequently, the eluted (amino acid) fraction has only 3.7% of the initial H^{14}CHO -yielding material, and this is to be taken as an upper limit of the average formation of serine from hydroxypyruvate in these

experiments. It would be interesting to carry out a similar separation for the high total formaldehydogenic substance (89%) which Elwyn *et al.* found after incubation with D-[3- ^{14}C]serine, but this isomer was not available to us. However, from the very small amount of D-serine utilized (11%) Elwyn *et al.* found as much glucose formed as from the much larger amount (37%) of L-serine taken up by the slices. This they attribute as possibly due to transamination or oxidative deamination of D-serine to give hydroxypyruvate as the first product. Such a conclusion would agree with our finding that the latter is a very effective glucose-forming substance in liver slices.

Hydroxy[1- ^{14}C]pyruvate and $\text{NaH}^{14}\text{CO}_3$. Table 5 shows the results of similar experiments in which rat-liver slices were incubated aerobically with carboxyl-labelled hydroxypyruvate. In two experiments, as a control $\text{NaH}^{14}\text{CO}_3$ was used instead of hydroxypyruvate, with total radioactivity five times as great, as an attempt to provide a check on the extent of uptake of $^{14}\text{CO}_2$ which might have occurred through a primary decarboxylation of the hydroxypyruvate and subsequent CO_2 fixation into glucose. The CO_2 fixation into glycogen is known to be low in a high-sodium medium such as that used here (cf. Hiatt, Goldstein, Lareau & Horecker, 1958). The incorporation of $^{14}\text{CO}_2$ into glucose of the medium is also low, of the order of 0.1% of the total amount present (Table 5) with our conditions.

On the other hand, hydroxy[1- ^{14}C]pyruvate is readily incorporated into the glucose molecule (average in recrystallized glucosazone equivalent to 12% of that added), as is shown in Table 5. During the same (90 min.) period, 29% of the carboxyl appears as CO_2 , but this would correspond

Table 4. *Comparison of the metabolism of hydroxy[3- ^{14}C]pyruvate and L-[3- ^{14}C]serine by rat-liver slices*

Liver slices (1 g.) from a fed rat were incubated for 90 min. in $\text{O}_2 + \text{CO}_2$ (95:5) at 37° in 10 ml. of Krebs-Ringer bicarbonate medium containing 10 μmoles of either hydroxy[3- ^{14}C]pyruvate (0.05 μC) or L-serine (1.0 μC). Figures represent percentages of total counts added occurring in the named fractions of the medium, $\pm$ S.D. For details of fractionation see text. Results of Elwyn *et al.* (1957) are included for comparison and are $\pm$ the average deviation. The numbers of experiments are shown in parentheses.

Authors	Analysis (^{14}C)	Substrate added	
		Hydroxy-[3- ^{14}C]pyruvate (%)	L-[3- ^{14}C]Serine (%)
This paper	Total formaldehydogenic substance*	24 $\pm$ 3.8 (8)	54 $\pm$ 16.9 (5)
	Total CO_2	5.6 $\pm$ 0.2 (8)	5.5 $\pm$ 3.8 (5)
	Glucose (as glucosazone)	15.7 $\pm$ 2.1 (8)	1.6 $\pm$ 1.0 (5)
	Formaldehydogenic substance† (basic fraction)	3.7 $\pm$ 1.2 (8)	39 $\pm$ 17.5 (5)
Elwyn <i>et al.</i> (1957)		D-[3- ^{14}C]Serine	L-[3- ^{14}C]Serine
	Total formaldehydogenic substance*	89 $\pm$ 6.5 (4)	63 $\pm$ 5.3 (6)
	Total CO_2	0.4 $\pm$ 0.1 (8)	14.4 $\pm$ 5.4 (10)
	Glucose	1.2 $\pm$ 0.4 (8)	1.2 $\pm$ 0.6 (10)

* This fraction represents the total H^{14}CHO liberated from the medium after incubation, on treatment with HIO_4 .

† This fraction represents the H^{14}CHO liberated by HIO_4 from the basic fraction of the medium after column separation as detailed in text (mainly serine).

with only about $0.1 \mu\text{C}$ liberated, compared with $2.5 \mu\text{C}$ added as $\text{NaH}^{14}\text{CO}_3$ in the above-mentioned control experiments.

For two rats in this series, duplicate batches of liver slices were incubated with hydroxypyruvate; the deproteinized, deionized medium was then evaporated and the glucose contained in the residue was degraded by fermentation with baker's yeast (see Methods section). The results (Table 5) show that nearly all (93.5%) of the ^{14}C of $^{14}\text{CO}_2\text{H}$ from hydroxypyruvate appears in C-3 and C-4 of the glucose molecule, whereas C-2 and C-5 have 4%, and C-1 and C-6 2%, only.

In this series of experiments identification of glucose was confirmed by paper chromatography, when the total radioactivity was contained in the single spot corresponding to a glucose marker. Although the yield of total C by persulphate oxidation of the neutral fraction obtained from the deionized medium shows the presence of some carbon compounds other than glucose, they were either non-fermentable by yeast or non-radioactive, or both, and therefore did not interfere with the degradation mentioned above.

DISCUSSION

Hydroxypyruvate has frequently been suggested (cf. Friedmann, Levin & Weinhouse, 1956) as a possible intermediate in the glycolytic metabolism of serine, but hitherto very few direct studies on hydroxypyruvate metabolism have been made. The natural occurrence of small amounts of hydroxypyruvate in animal tissues, including tumours, has been detected chromatographically (Linko & Virtanen, 1957) and enzymically (Holzer & Holldorf, 1957). It is also reported as a constituent of green plants (Virtanen & Alfthan, 1955), although in green algae Milhaud, Benson & Calvin (1956) could find no evidence of the incorporation of hydroxy[$2\text{-}^{14}\text{C}$]pyruvate into carbohydrate but

only of its oxidative transformation, possibly after transketolase action, into [$1\text{-}^{14}\text{C}$]glycollic acid. In higher plants evidence has been provided by Hanford & Davies (1958) that serine may be formed via phosphoserine from 3-[^{32}P]phospho-D-glycerate by a pathway resembling that described for rat liver by Ichihara & Greenberg (1957a). The following metabolic route from D-glyceric acid [which is phosphorylated by adenosine triphosphate and a kinase shown by Ichihara & Greenberg (1957b) to be present in liver extracts] or D-3-phosphoglyceric acid is postulated by these authors: D-glyceric acid $\rightarrow$ D-3-phosphoglyceric acid $\rightarrow$ 3-phosphohydroxypyruvic acid $\rightarrow$ L-3-phosphoserine $\rightarrow$ L-serine.

Free hydroxypyruvate has also been shown by Sallach (1956) to be converted into L-serine by a different transamination reaction in which L-alanine is the amino-group donor, glutamate being almost inert; this transaminase, which also occurs in liver, may or may not be the same as that concerned in Ichihara & Greenberg's (1957a) experiments. Slight activation by pyridoxal phosphate was observed by Sallach, who also demonstrated the reversibility of the reaction:

Hydroxypyruvate + L-alanine

$\rightleftharpoons$ L-serine + pyruvate.

Other pathways of hydroxypyruvate metabolism in animal tissues may include transketolase action in the presence of thiamine pyrophosphate as coenzyme. Among the appropriate acceptor aldehydes, the presence of which is necessary for this well-known reaction to proceed, we must now include formaldehyde (Dickens & Williamson, 1958c), which yields dihydroxyacetone, with corresponding decarboxylation of the hydroxypyruvate. Crystalline muscle L-lactic dehydrogenase and reduced diphosphopyridine nucleotide yield L-glyceric acid readily from hydroxypyruvate (Meister, 1952; Stafford, Magaldi & Vennesland,

Table 5. Comparison of metabolism of hydroxy[$1\text{-}^{14}\text{C}$]pyruvate and $\text{NaH}^{14}\text{CO}_3$ by rat-liver slices

Liver slices (1 g.) from a fed rat were incubated for 90 min. in $\text{O}_2 + \text{CO}_2$ (95:5) at 37° in 10 ml. of Krebs-Ringer bicarbonate medium; $10 \mu\text{moles}$ of hydroxy[$1\text{-}^{14}\text{C}$]pyruvate ($0.4 \mu\text{C}$) or $\text{NaH}^{14}\text{CO}_3$ ($2.5 \mu\text{C}$) was added as shown. Figures represent percentages of total counts added occurring in the total (bound + free) CO_2 and glucose of the medium after incubation ($\pm$ s.d.).

Substrate added	...	...	...
Fraction	Hydroxy- [$1\text{-}^{14}\text{C}$]pyruvate (%; mean of five experiments)	$\text{NaH}^{14}\text{CO}_3$ (%; two experiments)	
Total CO_2	29 ± 3.5	—	
Glucose (as glucosazone)	12 ± 1.2	0.09, 0.075	
Distribution of radioactivity* in glucose carbon atoms			
(two experiments) { C-3 + C-4	93, 94	—	
{ C-2 + C-5	4.5, 4	—	
{ C-1 + C-6	2.5, 2	—	

* Total activity in glucose taken was about $0.03\text{--}0.05 \mu\text{C}$. Degradation was by yeast fermentation.

1954). In green plants a similar enzyme yields D-glyceric acid (Stafford *et al.* 1954): in both reactions the equilibrium strongly favours reduction. In yeast extracts, crude carboxylase preparations containing transketolase yield L-erythrulose (Dickens & Williamson, 1956*b*) but purified yeast carboxylase gives only glycolaldehyde (Dickens, 1958).

It is evident therefore that a wide range of metabolic pathways is possible for hydroxypyruvic acid (cf. Fig. 3). As a first approach to deciding which routes are in fact followed in animal tissues, we have studied the metabolism of specifically labelled hydroxy[¹⁴C]pyruvate in the intact rat and in liver slices, and particularly the incorporation of ¹⁴C into carbon atoms of glucose.

Metabolism of hydroxy[¹⁴C]pyruvate in the whole rat. ¹⁴C of labelled hydroxypyruvate, administered subcutaneously, is incorporated into liver glycogen to about the same extent as ¹⁴C of glucose in our experiments (Table 2). Such a comparison, of course, takes no account of dilution, extra hepatic utilization or of excretion. Table 3 gives the results of degradation of the glycogen obtained in these experiments, and in Table 6 these mean values for distribution of ¹⁴C in carbon atoms of glucose from liver glycogen are compared with those of other authors on some related glycogenic compounds in the rat. In our experiments, with both hydroxy-[3-¹⁴C]- and hydroxy[2-¹⁴C]-pyruvate the type of pattern of radioactivity of glucose was essentially similar: main labelling was in C-1 and C-6 of glucose when the [3-¹⁴C]compound was given and in C-2 and C-5 of glucose after giving the hydroxy-[2-¹⁴C]pyruvate. In both cases, some randomiza-

tion occurred to the adjacent end carbons (C-2 and C-5 with 3-¹⁴C and C-1 and C-6 with 2-¹⁴C). In neither case did C-3 or C-4 of glucose become significantly labelled, indicating little fixation of ¹⁴CO₂ which might have arisen from complete oxidation (or from transfer of 3-¹⁴C of hydroxypyruvate to form a metabolite which itself became oxidized): it is well known that ¹⁴CO₂ is fixed in positions 3 and 4 almost exclusively (Wood, Lifson & Lorber, 1945; Topper & Hastings, 1949). The minor part played by carbon dioxide fixation is supported by our experiments with liver slices, discussed below, in which the carboxyl carbon of hydroxy[1-¹⁴C]pyruvate is shown to enter almost exclusively into C-3 and C-4 of glucose, and with only slightly lower percentage incorporation than that found for hydroxy[3-¹⁴C]pyruvate.

The overall pattern therefore strongly suggests the entry of C-1, C-2 and C-3 of hydroxypyruvate mainly as an intact (or at least isotopically identically reconstituted) carbon chain into glucose in the following nearly symmetrical manner:

Hydroxypyruvate C atoms no.	3	2	1	1	2	3
Glucose C atoms no.	1	2	3	4	5	6

This is, of course, the well-known distribution of the main labelling of glucose carbon atoms resulting from administration of C₃ intermediates, such as lactate and pyruvate (cf. Table 6). Further inspection of Table 6 shows, however, that there is much more randomization to adjacent carbon atoms (C-1 ⇌ C-2 and C-5 ⇌ C-6) with lactate and pyruvate than that observed by us for hydroxypyruvate. With the latter, the ratios of activity of the main labelling to the adjacent secondary

Table 6. Incorporation of ¹⁴C from labelled hydroxypyruvate and some related compounds into glucose carbon atoms of liver glycogen in the previously fasted rat

Compound	¹⁴ C incorporated (% of dose)	Specific activity of glucose carbon atoms (%)						Reference
		C-1	C-2	C-3	C-4	C-5	C-6	
Hydroxy[3- ¹⁴ C]pyruvate	1.5-5.0	35	10	4	4	12	35	This paper (mean)
Hydroxy[2- ¹⁴ C]pyruvate	1.9-4.4	13	32	5	6	23	10	This paper (mean)
DL-[3- ¹⁴ C]Serine	4.7	34	12	5	5	12	32	Friedmann, Levin & Weinhouse (1956)
[3- ¹⁴ C]Pyruvate	4.5	24	18	7	8	22	22	Friedman, Levin & Weinhouse (1956)
[2- ¹⁴ C]Pyruvate*	—	16	21	12	12	21	19	Landau, Hastings & Nesbett (1955)
[3- ¹⁴ C]Lactate†	4.9-8.9	22	21	6	6	21	22	Lorber, Lifson, Wood, Sakami & Shreeve (1950)
[2- ¹⁴ C]Glycolaldehyde	2.2	21	26	6	7	22	19	Friedmann, Levin & Weinhouse (1956)
[2- ¹⁴ C]Glycine	3.9	20	26	5	6	24	20	Friedmann, Levin & Weinhouse (1956)

* For liver slices in high K⁺ ion medium. Other data are for intact rats.
† Carbon atoms were determined in pairs (C-1 and C-6, C-2 and C-5, C-3 and C-4) and these calculated values are based on an assumed symmetrical distribution.

labelling is approximately 2.3:3.5:1. With lactate and pyruvate the corresponding ratios are only about 1:1.5:1. The high degree of randomization with these latter substrates is attributed to 'shuttling' through rapid interconversion with symmetrical C_4 intermediates of the tricarboxylic acid cycle (Topper & Hastings, 1949; Lorber, Lifson, Wood, Sakami & Shreeve, 1950). The probable inference is that hydroxypyruvate is more nearly situated with relation to phosphoglyceric acid in the glycolytic chain than are pyruvate and lactate. If hydroxypyruvate enters by a more rapid or more direct pathway into 3-phosphoglycerate for resynthesis of hexose, it will obviously stand less chance of randomization in the process. It is currently thought that at least part of the incorporation of pyruvate proceeds by a carboxylation reaction involving malate or oxaloacetate or both, which are in equilibrium with fumarate, thus accounting for the randomization of carbon atoms occurring with pyruvate and lactate during hexose resynthesis (cf. Krebs & Kornberg, 1957). Presumably therefore this type of 'shuttling' occurs, if at all, to a much less extent with hydroxypyruvate.

Friedmann *et al.* (1956) have presented data for the incorporation of ^{14}C of DL-[3- ^{14}C]serine into rat-liver glycogen, which are also included in Table 6. The very close parallelism of isotope distribution with that observed by us with hydroxy[3- ^{14}C]pyruvate is striking. These authors, also, conclude that the deamination product from serine may be a C_3 acid which is more directly incorporated into glyceric acid than is pyruvate, and tentatively suggest that this unknown intermediate may be hydroxypyruvate. We are in agreement with these authors inasmuch as our direct experiments with labelled hydroxypyruvate agree so closely with theirs for serine. Since, however, Friedmann *et al.* used only DL-serine it is possible (as they mentioned) that the natural L-isomer might behave differently, and their experiments need to be repeated with isotopic L-serine.

This is particularly necessary since D-serine is the isomer mainly or exclusively attacked to yield hydroxypyruvate when incubated aerobically with rat-kidney slices (Sprinson & Chargaff, 1946; Holzer & Holldorf, 1957). In unpublished work we have confirmed these observations and have extended them to rat-liver slices, which, however, even in the presence of arsenite, failed to accumulate significant amounts of hydroxypyruvate under the same conditions. We have also found that preparations of D-amino acid oxidase, purified from rat kidney as far as stage 3 as described by Negelein & Brömel (1939), readily oxidized DL-serine to hydroxypyruvate, which we identified colori-

metrically and by the specific dehydrogenase (cf. Dickens & Williamson, 1958*b*).

Behaviour of hydroxy[^{14}C]pyruvate in rat-liver slices. The metabolism of hydroxypyruvate has been studied in isolated liver slices as a stage towards the localization of the glycogenic action found by us in the whole animal, and also because Elwyn *et al.* (1957) have recently made a valuable and detailed study of the metabolism of both D- and L-[3- ^{14}C]serine in rat-liver slices. The medium used in our experiments (Krebs-Ringer bicarbonate) corresponds with the 'Ringer bicarbonate' medium of these workers, except that it contains 1.2 mM-phosphate and 1.2 mM- Mg^{2+} ions, less bicarbonate (25 instead of 40 mM) and slightly more Ca^{2+} ions. It is therefore a 'high sodium medium', in which, owing to activation of phosphorylase, little glycogen deposition occurs, and any newly formed carbohydrate appears mainly as glucose in the medium (Ashmore, Cahill, Hastings & Zottu, 1957). This is a practical convenience in isolating the glucose formed.

Results on the incubation with liver slices of hydroxy[3- ^{14}C]pyruvate and L-[3- ^{14}C]serine may therefore be directly compared with those of Elwyn *et al.*, also included in Table 4. Like these authors, we found a large (about 40%) disappearance of L-serine during incubation, based on the total formaldehydogenic material on periodate treatment; moreover, our results based on column fractionation after incubation suggest that not all the liberation of residual formaldehyde was due to serine (Table 4). We also found a similar rate of incorporation of ^{14}C from L-serine (about 1.5% of total added radioactivity). Elwyn *et al.* noted that the much lower utilization of D-serine by liver slices (11% of total radioactivity compared with 40% for L-serine) was accompanied by a relatively much higher incorporation, expressed as a percentage of the serine utilized, into glucose (11.2% for D-serine against 3.4% for the L-isomer).

These results may be compared with those reported in this paper for hydroxypyruvate (Table 4). The rate of incorporation of hydroxypyruvate into glucose is very high (15.7% in 90 min. incubation), a value clearly sufficient to support the possible role of hydroxypyruvate as an intermediate in the metabolic conversion into glucose of both D- and L-serine. If it is in fact an intermediate, the formation of hydroxypyruvate from both isomers of serine would appear to be the limiting stage in the conversion of serine into carbohydrate in liver.

The total activity of formaldehydogenic substance remaining after incubation with hydroxypyruvate (24% of that added) consisted of unchanged hydroxypyruvate (5%), formaldehydogenic amino acid (serine; 3.7%) and about 7% due

to glucose (total 16 % of that added accounted for). The almost complete disappearance of hydroxypyruvate shows extensive metabolic utilization by still undetermined pathways. But the high glucose formation from hydroxypyruvate agrees with that found by us in the intact rat.

The results of Table 5 show that in comparison with hydroxy[3- ^{14}C]pyruvate the hydroxy[1- ^{14}C]pyruvate gives considerably more $^{14}\text{CO}_2$, as might be expected since this substance undergoes some slight decarboxylation at pH 7 even spontaneously and it is a substrate for transketolase. However, nearly 20 % of the hydroxypyruvate ^{14}C not lost as carbon dioxide or remaining unchanged (total $29 + 5 = 34\%$) appears as glucose in the medium. This is again a very high rate of incorporation. By comparison, added $\text{NaH}^{14}\text{CO}_3$ (Table 5) gives only a very low percentage incorporation.

Degradation of the purified glucose by the yeast method (Table 5) indicates again the same predominant type of incorporation as that described above for hydroxy-[3- ^{14}C]- and -[2- ^{14}C]-pyruvate in the whole animal, since C-3 and C-4 between them have 93–94 % of the total radioactivity of the glucose formed:

Hydroxypyruvate C atoms no.	3	2	1*	1*	2	3
Glucose C atoms no.	1	2	3*	4*	5	6

Fig. 2 illustrates diagrammatically the percentage distribution of labelled carbon among the products observed with liver slices, and some probable conclusions from these data are suggested.

Pathways of metabolism of hydroxypyruvate and serine. Since the incorporation into glucose of

radioactivity is so much higher with labelled hydroxypyruvate than with either L- or D-serine in liver-slice experiments, it is unlikely that serine lies on the pathway from hydroxypyruvate to glucose, although as already stated the converse may apply, especially for the D-isomer.

With L-[^{14}C]serine and hydroxy[^{14}C]pyruvate the possible effect of dilution with the metabolic pool of the unlabelled substances has to be considered. L-Serine is converted into 3-phosphoserine in rat liver, in which both of these substances are normally present to the extent of 1.1 μmole of free serine and 0.04 μmole of phosphoserine/g. of wet liver (Nemer & Elwyn, 1957). The amount of hydroxypyruvate found in rat-liver tissue is $2.2 \pm 0.9 \mu\text{moles/g.}$ (Holzer & Holldorf, 1957). Since 10 μmoles of labelled hydroxypyruvate or L-serine/g. of liver slices was regularly added in our experiments, and the greater part of this was metabolized, differences in any dilution with the metabolic pool of these substances can hardly have had much effect on the observed relative rates of incorporation into glucose, even though the turnover rate of the pools themselves should also be considered. Consequently the much higher rate of glucose formation from hydroxypyruvate appears to be a genuine finding, and on this basis hydroxypyruvate could very well be an intermediate in the conversion of serine into glucose.

Such a pathway involving entry as an intact C_3 unit would require either a transamination or deamination to hydroxypyruvate, or else the action of liver serine dehydrase (Chargaff & Sprinson, 1943), which converts L-serine (but not

Fig. 2. Metabolic products incorporating 1- ^{14}C (denoted °) and 3- ^{14}C (denoted *) of hydroxypyruvate in rat-liver slices. [* The evidence presented suggests that this is predominantly directly incorporated as the carboxyl of a C_3 chain, and not as free CO_2 , to the extent of 13 % in C-3 + C-4 of glucose. † Randomization of C-1 and C-6 with C-2 and C-5 has not been directly studied with liver slices and is provisionally assumed to be the same as in rat liver *in vivo*. The total 3- ^{14}C of hydroxypyruvate entering C-1, C-2, C-5 and C-6 of glucose is 17%. The extent (4 %) to which this figure exceeds that for incorporation of ^{14}C could indicate that this amount of ^{14}C from decarboxylated hydroxypyruvate enters the glucose molecule either as free glyceraldehyde (cf. Friedmann, Levin & Weinhouse, 1956, in Table 6) or as a result of the various possible transketolase-transaldolase transformations of 'active glyceraldehyde'.]

the D-isomer) into pyruvate (Sayre & Greenberg, 1956). As already discussed, the randomization of the terminal pairs of glucose carbon atoms is much less when ^{14}C -labelled hydroxypyruvate is the substrate than with labelled pyruvate. If pyruvate formation by this route lies on the pathway of the L-serine-glucose transformation it would therefore be expected to account for only that small part of the serine metabolized which would correspond to the slight degree of randomization observed.

Other possible metabolic routes of serine and hydroxypyruvate metabolism are outlined in Fig. 3. The improbability of a main pathway of hydroxypyruvate metabolism via serine to glucose (reactions I, II, V, VI and VIII, Fig. 3) has already been discussed. In addition, the reductive pathway of enzyme VI shown by the broken arrow (3-phosphohydroxypyruvate dehydrogenase) has not yet been experimentally demonstrated, although evidence of the oxidative reaction has been provided for liver by Ichihara & Greenberg (1957a).

Hydroxypyruvate conversion into 3-phospho-D-glyceric acid by a fairly direct route, followed by

synthesis of glucose from the latter by the well-known glycolytic pathway, would appear to be the most probable explanation and would be consistent with the pattern of labelling of glucose observed in these experiments. Although transketolase reactions (XI) *in vitro* occur with hydroxypyruvate in the presence of suitable acceptor aldehydes, we have seen that the almost symmetrical labelling of the glucose produced, and also the retention of most of the carboxyl carbon in the glucose molecule, indicate that *in vivo* this pathway to glucose, if it occurs, must be a minor one for hydroxypyruvate. So, also, must be the part played by formation of free glycolaldehyde (cf. Table 6). Likewise, formation of formaldehyde and glycine by serine transhydroxymethylase acting together with tetrahydrofolic acid (reaction IV), and subsequent metabolism of these two products to glycogen by oxidative routes, do not appear to be consistent with the pattern of glucose labelling observed.

It is tentatively suggested that either (a) hydroxypyruvate may be reduced to D-glyceric acid by

Fig. 3. Possible pathways of metabolism of serine and hydroxypyruvate in liver. I, L-Serine with pyruvate and transaminase (Sallach, 1956), or D-serine with D-amino acid oxidase. II and III, Serine phosphokinase [(?), see Nemer & Elwyn, 1957], or a phosphatase (probably non-specific, Ichihara & Greenberg, 1957a). IV, Serine aldolase (serine transhydroxymethylase). V, Transaminase (Ichihara & Greenberg, 1957a). VI, A dehydrogenase (DPN-linked?), (Ichihara & Greenberg, 1957a). VII, D-Glycerate kinase (Ichihara & Greenberg, 1957b). VIII, Embden-Meyerhof glycolytic reactions. IX, Unknown phosphokinase for hydroxypyruvate. X, D-Glyceric dehydrogenase [DPN-linked, in plant tissues (cf. Stafford *et al.* 1954)]. L-Lactic dehydrogenase of animal tissues, if concerned here, would require a racemase for conversion of the product L-glyceric acid into D-glyceric acid (see text). XI, Transketolase (+ acceptor aldehyde). XII, Serine dehydrase (Chargaff & Sprinson, 1943; Sayre & Greenberg, 1956).

a dehydrogenase (reaction X), which could then be phosphorylated by the D-glyceric acid kinase of Ichihara & Greenberg (1957*b*), or (b) hydroxypyruvate could first be phosphorylated by an as yet unknown phosphokinase reaction (IX) to give 3-phosphohydroxypyruvate, which is subsequently reduced to 3-phosphoglyceric acid, this substance being then incorporated into glucose. The former route (a) also involves an enzyme system as yet unidentified in animal tissues, producing D-glyceric acid from hydroxypyruvate. This reaction does, however, occur with plant extracts (Stafford *et al.* 1954), and enzyme systems which metabolize D- α -hydroxy acids, especially liver-mitochondrial systems which may involve racemases (cf. Huenekens, Mahler & Nordman, 1951), have been described. Their mechanism of action, however, is as yet uncertain and D-glyceric acid does not appear to have been studied as a substrate with such preparations. A further possibility would be the intermediate formation of (optically active) tartronic semialdehyde, which might arise enzymically from hydroxypyruvate and which appears to be a precursor of glyceric acid in *Aerobacter* (McRorie & Novelli, 1958). It is intended to investigate the metabolism of D- and L-[^{14}C]glyceric acid in animal enzyme systems and in intact liver with a view to clarifying this at present obscure part of the pathway of metabolism of hydroxypyruvate.

SUMMARY

1. The metabolism of specifically ^{14}C -labelled hydroxypyruvate, and particularly its conversion into glucose, has been studied in the intact rat and in rat-liver slices.

2. In the fasting rat, radioactivity of subcutaneously injected hydroxy[3- ^{14}C]pyruvate and hydroxy[2- ^{14}C]pyruvate appears in the liver glycogen. This incorporation amounted to 1.9–5.0 % of the dose given, whereas with [1- ^{14}C]glucose under similar conditions incorporation was 3.8 %.

3. Stepwise degradation of the glucose obtained on hydrolysis of the liver glycogen showed that with either hydroxy[3- ^{14}C] or hydroxy[2- ^{14}C]pyruvate a nearly symmetrical pattern of distribution occurred. With the former, main labelling was in C-1 and C-6 of glucose; with the latter, it was in C-2 and C-5. Randomization of radioactivity to the adjacent end carbons (C-1 $\rightleftharpoons$ C-2 and C-6 $\rightleftharpoons$ C-5) was observed, but it was much less than has been reported for similarly labelled lactate or pyruvate, though with hydroxy[3- ^{14}C]pyruvate it was almost identical with that reported for DL-[3- ^{14}C]serine.

4. When incubated aerobically in Krebs–Ringer bicarbonate solution with liver slices (from fed rats), 95 % of specifically labelled hydroxypyruvate is

metabolized. The utilization of substrate and incorporation of radioactivity into glucose were much higher with hydroxy[3- ^{14}C]pyruvate (average 15.7 % of total radioactivity incorporated) than with L-[3- ^{14}C]serine (average 1.6 %), the latter percentage being similar to that reported for either L- or D-[3- ^{14}C]serine by Elwyn *et al.* (1957). In similar experiments with hydroxy[1- ^{14}C]pyruvate incorporation was almost exclusively in C-3 and C-4 of glucose and amounted to 12 % of the total ^{14}C ; parallel experiments with $\text{NaH}^{14}\text{CO}_3$ showed relatively slight incorporation into glucose.

5. These experiments are considered to show that the rat *in vivo* and rat-liver tissue *in vitro* are able to incorporate readily all three carbon atoms of hydroxypyruvate into the glucose molecule, the evidence suggesting that this occurs symmetrically with combination of two unbroken C_3 chains by a fairly direct route not passing through L-serine and probably not through pyruvate as intermediates. The possible bearing of these observations on serine metabolism is discussed.

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Adaptation to Form Bacteriochlorophyll in *Rhodopseudomonas spheroides*: Changes in Activity of Enzymes concerned in Pyrrole Synthesis

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Rhodopseudomonas spheroides, like other members of the Athiorhodaceae, is able to grow either anaerobically in the light or aerobically in the dark (van Niel, 1944). Organisms cultured under the former conditions are heavily pigmented and are rich in bacteriochlorophyll and carotenoids, while aerobically grown organisms contain only traces of these pigments (van Niel, 1944; Cohen-Bazire, Sistrom & Stanier, 1957).

Growth under anaerobic conditions occurs only in the light and is therefore dependent upon the intracellular pigments concerned in photosynthesis

(photopigments), in particular bacteriochlorophyll. Kinetic studies with exponentially growing cultures of *R. spheroides* have shown that transfer of aerobically growing organisms to anaerobic conditions in the light results in the adaptation of all these organisms in the inoculum to form the photopigments, and synthesis of these substances precedes cell division (Cohen-Bazire *et al.* 1957).

Cultures of *R. spheroides* grown anaerobically in the light contain 40–100 times as much bacteriochlorophyll as those grown aerobically. The concentration of haematin compounds is similar in both types of organism, but is only a small fraction (1–5%) of the amount of bacteriochlorophyll

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