The Fate of Isotopic Carbon in Kidney Cortex Synthesizing Glucose from Lactate

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1. When slices of kidney cortex are incubated with lactate and acetoacetate, lactate is almost quantitatively converted into glucose whereas acetoacetate provides a major part of the fuel of respiration. 2. In apparent contrast with these findings a large fraction of ^{14}C -labelled acetoacetate appears in glucose and a large fraction of ^{14}C -labelled lactate appears in the carbon dioxide. 3. The findings can be explained by: (a) the participation of oxaloacetate as an intermediate of both gluconeogenesis and respiration; (b) the fact that the carbon dioxide formed in the course of one turn of the tricarboxylic acid cycle is exclusively derived from oxaloacetate. 4. As a result there is a 'crossing over' of the carbon of the substrate of respiration to the pathway of gluconeogenesis and of the carbon of the glucogenic precursors to the pathway of respiration. 5. In the given situation the fate of the label does not allow predictions to be made about the net fate of the labelled metabolites. 6. The implications of the findings on the interpretation of isotopic data are discussed.

Acetoacetate is known to promote the synthesis of glucose in slices of rat kidney cortex (Krebs, Speake & Hems, 1965). The evidence indicates that this stimulation does not represent a conversion of acetoacetate into carbohydrate. The effect can be explained partly as a sparing action, acetoacetate serving as a fuel of respiration in place of gluconeogenic precursors and partly as an activation of pyruvate carboxylase [pyruvate-carbon dioxide ligase (ADP); EC 6.4.1.1] by acetyl-CoA formed from acetoacetate, the latter enzyme being rate-limiting in gluconeogenesis (Krebs, 1964).

It was thought that it could be directly demonstrated with the help of isotopic substrates that acetoacetate is not converted into glucose. Accordingly two types of experiment were carried out to investigate the fate of carbon atoms of acetoacetate and of lactate in kidney-cortex slices. Slices were incubated in phosphate-buffered saline with either L-[U- ^{14}C]lactate and unlabelled acetoacetate or with [4- ^{14}C]acetoacetate and unlabelled L-lactate. The net changes in the amounts of metabolites (oxygen, glucose, acetoacetate, β -hydroxybutyrate and carbon dioxide) were measured and the radioactivity of glucose and carbon dioxide was determined. [4- ^{14}C]Acetoacetate was used because it retains the label longer than [1- ^{14}C]- or [3- ^{14}C]-acetoacetate. Owing to special circumstances (the

most important of which is the fact that intramitochondrial oxaloacetate is an intermediate in both the combustion of acetoacetate and in gluconeogenesis from lactate) the fate of isotopic carbon supplies no decisive information on the question of the conversion of ketone bodies into carbohydrate. The experiments forcibly demonstrate that the incorporation of carbon atoms into a product of metabolism does not necessarily indicate a net conversion, a fact emphasized before, especially by Weinman, Strisower & Chaikoff (1957), but all too often neglected.

EXPERIMENTAL

Incubation procedure. Kidney-cortex slices from rats maintained on a low-carbohydrate diet were incubated as described by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963). The medium was the phosphate-buffered saline of Krebs & de Gasquet (1964). To collect the CO_2 formed, small test tubes (2 cm. $\times$ 0.6 cm.) containing 0.2 ml. of freshly prepared 2N-NaOH (as low in carbonate as possible) were placed inside the centre wells of the conical manometer vessels. At the end of a 2 hr. incubation period 0.5 ml. of 0.1N-HCl was added from the side arms to the main compartments and the manometers were shaken for a further 15 min. Complete absorption of the CO_2 set free from the medium was indicated by the cessation of pressure changes. The cups were detached from the manometers and the tubes containing the alkali were dropped into 5 ml.

glass-stoppered test tubes containing 4.8 ml. of CO₂-free water. The CO₂ content of this mixture was determined manometrically. The CO₂ content of all reagents was measured in a control cup without slices. In another control vessel without tissue the extent of non-enzymic decarboxylation of acetoacetate under the test conditions was measured.

The tissue was removed from the incubation medium, lightly blotted on hardened filter paper and transferred to weighing bottles for the determination of the dry weight. This value was corrected for the loss of weight (approx. 20%) occurring during the incubation. The correction factor was found by measuring the wet weight/dry weight ratio of non-incubated and incubated slices. The medium was treated with HClO₄ and prepared for analysis as described by Gevers & Krebs (1966).

Analytical methods. The metabolites were determined spectrophotometrically by enzymic methods: glucose by the method of Krebs *et al.* (1963), L-lactate by the method of Hohorst (1963a), pyruvate by the method of Bücher, Czok, Lamprecht & Latzko (1963), acetoacetate and β -hydroxybutyrate by the method of Williamson, Mellanby & Krebs (1962), glutamate by the method of Bernt & Bergmeyer (1963) and malate by the method of Hohorst (1963b).

Preparation of labelled substrates. L-[U-¹⁴C]Lactate is not commercially available. It was prepared from sodium [U-¹⁴C]pyruvate obtained from The Radiochemical Centre, Amersham, Bucks., by reducing it with NADH₂ in the presence of lactate dehydrogenase and regenerating the NADH₂ with ethanol and alcohol dehydrogenase. To the ampoule containing sodium [U-¹⁴C]pyruvate (0.1 mc) were added 0.02 M-phosphate buffer, pH 8.0 (0.6 ml.), 0.01 M-NAD (0.1 ml.), ethanol (0.05 ml.), lactate dehydrogenase (0.1 ml. of the Boehringer stock suspension) and alcohol dehydrogenase (0.05 ml. of the Boehringer stock suspension). The reaction was completed in 30 min. at room temperature. After heating the ampoule in boiling water for 2 min. the material was chromatographed on Whatman no. 1 paper previously washed five times in water. The solvent system was diethyl ether-formic acid (3:2, v/v) run in the ascending direction. The paper was dried in a stream of air for 24 hr. and the position of the L-[U-¹⁴C]-lactate was located by radioautography on Kodirex X-ray film (Kodak Ltd., London, W.C. 2). The lactate band was cut out and eluted with approx. 1 ml. of water. To remove ammonia (contributed by the enzyme suspensions), the lactate solution was brought to pH 12 with NaOH and gassed vigorously with N₂ for 1 hr., until nesslerization of a small sample gave a negative reaction. After adjustment of the pH to 7.0 the solution was stored in the deep freeze. The radiochemical purity of the lactate was checked by subjecting a small sample (10 μ l.) of the final solution to descending chromatography on Whatman no. 1 paper with butan-1-ol-propionic acid-water (47:22:31, by vol.). The position of the lactate was ascertained by radioautography. Under these conditions the lactate moved as a single spot that contained more than 98% of the radioactivity recovered from the paper.

[4-¹⁴C]Acetoacetate was prepared from its ethyl ester obtained from The Radiochemical Centre. To the ampoule containing the ester (0.1 mc in 0.01 ml.) were added 2N-NaOH (0.045 ml.) and water (0.032 ml.) and the solution was incubated at 40° for 1 hr. (Krebs & Eggleston, 1945).

The product was removed from the ampoule by washing the latter with 10 vol. of water. The solution was freeze-dried to remove ethanol and water and then dissolved in 2 ml. of water. The radiochemical purity was checked by decarboxylating a sample in a boiling-water bath for 10 min. at pH 2 and determining the radioactivity of the solution as described below. More than 96% of the radioactivity was removed under these conditions. This was confirmed by the method of Mayes & Felts (1966) in which acetone and CO₂ were collected separately in hydrazine-lactic acid and Hyamine hydroxide after decarboxylation of acetoacetate in a sealed ampoule. By this method 95.5% of the original radioactivity was recovered and 4.4% of the radioactivity was found in the 1-position of acetoacetate.

Separation of radioactive metabolites. Glucose and other metabolic products not decomposing on paper were separated by two-dimensional paper chromatography and radioautography as described by Gevers & Krebs (1966). The data of Crowley, Moses & Ullrich (1963) were used in identifying the labelled metabolites. The identity was further checked by co-chromatography with authentic compounds and by specific sprays on duplicate chromatograms. Spots containing the labelled compounds were cut out and eluted with 0.5 ml. of water. By this procedure glucose, maltose, hexose phosphates, lactate, pyruvate, malate, succinate, α -oxoglutarate and several amino acids were separated. With lactate more than 30% was lost from the chromatogram. A correction was made for this loss on the basis of the known amount of lactate placed on the paper and the specific activity of the recovered material.

Paper chromatography was not suitable for separating acetoacetate as the loss by decomposition was substantial. Acetoacetate, acetone and β -hydroxybutyrate were therefore separated for counting by the method of Mayes & Felts (1966).

Counting procedure. The substances separated by chromatography were applied directly on to glass-fibre filter paper (Pinter, Hamilton & Miller, 1963), dried for 30 min. in a warm air stream and counted in 8 ml. of scintillator fluid prepared by dissolving 3 g. of 2,5-diphenyloxazole and 0.1 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 l. of sulphur-free A.R. toluene. Of the eluate recovered from the chromatogram, 0.1 ml. was added to each counting vial on four separate glass-fibre filter-paper squares and the counts were measured in a liquid-scintillation counter. Each determination was carried out in duplicate.

The bicarbonate contained in the NaOH solution recovered from the centre wells was applied to glass-fibre filter-paper squares that had previously been treated with 0.07 ml. of newly prepared barium acetate solution (10%, w/v). This made certain that no labelled bicarbonate was lost during the preparation of the samples for counting. Glass-fibre filter-paper squares impregnated with unlabelled BaCO₃ had no quenching effect on solutions of known radioactivity. The ¹⁴C-labelled acetone recovered in hydrazine-lactic acid and the ¹⁴CO₂ recovered in Hyamine hydroxide (1M) by the method of Mayes & Felts (1966) were counted in a scintillation fluid consisting of 5 ml. of the toluene solution described above plus 3 ml. of ethanol. The results were corrected for quenching by comparing the counts obtained from a standard ¹⁴C solution in toluene under these conditions with those obtained in the original scintillation fluid.

RESULTS

Experiments with L-[U-¹⁴C]lactate and unlabelled acetoacetate

Net changes of metabolites. As in previous experiments (Krebs *et al.* 1965), the presence of acetoacetate increased, in this case more than doubled, the yield of glucose when added together with lactate (Table 1). The amounts of lactate used were more than sufficient to account for the formation of glucose, the ratio lactate used/glucose formed being 5.3 when lactate alone was added and 2.48 when lactate and acetoacetate were added together. As for the fuel of respiration, with both acetoacetate and lactate present, acetoacetate accounted for 73%, lactate for 13% and endogenous substrates for 14%. These data indicate that the added substrates inhibited the endogenous respiration by 71%. In making these calculations pyruvate formation from lactate has been taken into account; the contribution of glutamate, because the extra formation is small, has been ignored. The formation of glucose in the control cup (no added substrate) has not been deducted because there is no evidence that this is justifiable. The contribution made by acetoacetate to the fuel of respiration was calculated as described by Williamson & Krebs (1961).

When acetoacetate alone was added, it accounted for 69% of the respiratory fuel and the endogenous respiration was inhibited by 61%. When lactate alone was added the surplus lactate not accounted for by the formation of glucose and pyruvate could contribute 48.0% (i.e. 939 μ moles) to the respiratory fuel. This is not much more than the extra oxygen uptake on the addition of lactate (867 μ -

moles), which implies that the suppression of the endogenous respiration by lactate was only moderate.

The net changes observed thus suggest that lactate was the precursor of glucose and that acetoacetate, when present, was the predominant fuel of respiration. Accordingly, the label of lactate would be expected to appear mainly in glucose, especially in the presence of acetoacetate, when lactate makes a very small contribution to the respiratory fuel, and the label of acetoacetate would appear in the respiratory carbon dioxide but not in the glucose.

Isotope distribution. The isotope-distribution data (Table 2) are not in accordance with this expectation, especially when both lactate and acetoacetate were added together. In this case the glucose formed contained 32.7% and the carbon dioxide 56.8% of the radioactivity not recovered in lactate. The specific activity of the glucose carbon was only 39.9% of that of the added lactate carbon, though the expected value would lie between 66.7 and 100%, depending on the amount of carboxyl carbon of lactate (C-3 and C-4 of glucose) replaced by unlabelled carbon dioxide as a result of the 'dicarboxylic acid shuttle' (Topper & Hastings, 1949; Lorber, Lifson, Wood, Sakami & Shreeve, 1950). This implies that a large part of the glucose carbon was derived from sources other than lactate or carbon dioxide. The specific activity of the respiratory carbon dioxide was 0.83×10^5 counts/min./ μ g. atom of C, although lactate contributed no more than 13% to the respiratory fuel, in which case the specific activity of the lactate carbon should have been diluted 6.6-fold. In fact,

Table 1. *Net changes on addition of L-[U-¹⁴C]lactate and unlabelled acetoacetate to rat kidney-cortex slices*

For full experimental details see the text. The specific activity of L-[U-¹⁴C]lactate was 251×10^3 counts/min./ μ g. atom of C and the total radioactivity added per cup was 7543×10^3 counts/min. Each cup contained 8–10 mg. dry wt. of tissue. The formation of metabolites is indicated by a + sign and disappearance by a – sign. Acetoacetate 'oxidized' refers to the fraction of acetoacetate not accounted for by β -hydroxybutyrate.

Substrate added.....	None	Acetoacetate (5 mm)	L-[U- ¹⁴ C]Lactate (2.5 mm)	L-[U- ¹⁴ C]Lactate (2.5 mm) + acetoacetate (5 mm)
Metabolic changes (μ moles/g. of tissue/2 hr.)				
O ₂	– 1090	– 1368	– 1957	– 2230
CO ₂	+ 1058	+ 1466	+ 1820	+ 2180
Respiratory quotient	0.97	1.07	0.93	0.98
Glucose	+ 12	+ 10	+ 96	+ 200
Lactate			– 527	– 516
Pyruvate		+ 3	+ 22	+ 19
Glutamate		+ 10	+ 16	+ 17
Acetoacetate		– 321		– 522
β -Hydroxybutyrate		+ 79		+ 105
Acetoacetate 'oxidized'		– 242		– 417

Table 2. *Isotope distribution on addition of L-[U-¹⁴C]lactate and unlabelled acetoacetate to rat kidney-cortex slices at the end of incubation for the experiment recorded in Table 1*

Substrates added.....	L-[U- ¹⁴ C]Lactate			L-[U- ¹⁴ C]Lactate + unlabelled acetoacetate		
	10 ⁻² × Radio-activity recovered (counts/min.)	Percentage of recovered radioactivity (not including lactate)	10 ⁻⁵ × Specific activity (counts/min./μg.atom of C)	10 ⁻² × Radio-activity recovered (counts/min.)	Percentage of recovered radioactivity (not including lactate)	10 ⁻⁵ × Specific activity (counts/min./μg.atom of C)
Lactate	33716	—	2.51	32207	—	2.51
Pyruvate	407	1.1	—	270	0.7	—
CO ₂	27105	71.4	1.37	20738	56.8	0.83
Glucose	6967	18.4	1.42	11917	32.7	1.00
Maltose	607	1.6	—	693	1.9	—
Hexose phosphates	517	1.4	—	323	0.9	—
Glycogen, protein	317	0.8	—	200	0.5	—
Aspartate	203	0.5	—	93	0.3	—
Glutamate	610	1.6	0.91	227	0.6	0.20
Glutamine, glycine and serine	353	0.9	—	540	1.5	—
Alanine	673	1.8	—	1310	3.6	—
Malate	153	0.4	2.26	103	0.3	1.52
Succinate	73	0.2	—	73	0.2	—
Total radioactivity recovered	71701	—	—	68694	—	—

it was diluted only 3.0-fold. Lactate carbon thus contributed a much greater share to the respiratory carbon dioxide than that expected on the basis of the net changes.

When L-[U-¹⁴C]lactate alone was added, the glucose formed contained 10% and the carbon dioxide 71% of the radioactivity not recovered in lactate. The specific activity of the carbon dioxide was 55% of that of the added lactate carbon, i.e. within the expected range, as the net changes indicate that lactate accounted for 48% of the respiratory fuel. The specific activity of the glucose carbon was distinctly lower than the lowest expected value on the improbable assumption that C-3 and C-4 of glucose were all derived from unlabelled carbon dioxide. On this assumption the specific activity should have been 1.67×10^5 compared with the value of 1.37×10^5 counts/min./μg.atom of C observed (Table 2). These results show that with lactate as the sole substrate some of the glucose carbon was derived from precursors other than lactate.

Experiments with [4-¹⁴C]acetoacetate and unlabelled lactate

Net changes of metabolites. The net changes in an experiment with [4-¹⁴C]acetoacetate and unlabelled lactate were similar to those given in Table 1. Differences (Table 3) that illustrate the variation obtained from experiment to experiment are listed in Table 4.

The only major difference concerns the fate of lactate. In the experiment given in Table 3 the glucose formed was 5% more than could be accounted for by the disappearance of lactate. Thus all the lactate removed seems to have been used for the synthesis of glucose. In addition, a small amount of glucose must have been formed from endogenous sources. No lactate was available as a fuel of respiration and a correspondingly greater contribution to the respiratory fuel was made by endogenous substrates. The net changes thus again suggest that lactate served as a precursor of glucose and that acetoacetate was used as a fuel of respiration.

Isotope distribution. The isotope data again failed to conform with these conclusions. When acetoacetate alone was added, it is true, the specific activity of the carbon dioxide was as expected, i.e. 68% of that of acetoacetate carbon, which contributed 69% to the respiratory fuel. But the small amount of glucose formed was strongly labelled and had virtually the same specific activity as the respiratory carbon dioxide. When both acetoacetate and lactate were added the specific activity of the carbon dioxide was much lower than that of acetoacetate carbon (0.47×10^5 against 0.93×10^5 counts/min./μg.atom of C) and, unexpectedly, much radioactivity was found in glucose. The specific activity of the glucose carbon was higher than that of the carbon dioxide carbon and was about two-thirds of that of the acetoacetate carbon. Minor quantities of labelled carbon,

Table 3. *Net changes on addition of [4-¹⁴C]acetoacetate and unlabelled L-lactate to rat kidney-cortex slices*

For full experimental details see the text. The specific activity of [4-¹⁴C]acetoacetate was 112×10^3 counts/min./ $\mu\text{g. atom of C}$ and the total radioactivity added per cup was 8920×10^3 counts/min. For other details see Table 1.

Substrate added..... Metabolic changes ($\mu\text{moles/g. of tissue/2 hr.}$)	None	[4- ¹⁴ C]Acetoacetate (5 mm)	L-Lactate (2.5 mm)	[4- ¹⁴ C]Acetoacetate (5 mm) + L-lactate (2.5 mm)
O ₂	- 1278	- 1722	- 2219	- 2590
CO ₂	+ 1025	+ 1684	+ 1968	+ 2455
Respiratory quotient	0.80	0.98	0.89	0.95
Glucose	+ 17	+ 24	+ 113	+ 250
Lactate	—	—	- 531	- 480
Pyruvate	—	+ 5	+ 21	+ 17
Glutamate	+ 7	+ 23	+ 29	+ 19
Acetoacetate	—	- 405	—	- 555
β -Hydroxybutyrate	—	+ 111	—	+ 121
Acetoacetate 'oxidized'	—	- 294	—	- 434

Table 4. *Contribution of added substrates to the respiratory fuel*

For the method of calculation see the text.

Substrate added	Expt. no.	Contribution to fuel of respiration (%)				Inhibition of endogenous respiration by added substrates (%)
		Lactate used Glucose formed	Endogenous substrates	Aceto- acetate	Lactate	
Acetoacetate (5 mm)	1	—	31	69	Nil	61
	2	—	34	66	Nil	54
Lactate (2.5 mm)	1	5.3	52	Nil	48	7
	2	4.5	62	Nil	38	0
Lactate (2.5 mm) + acetoacetate (5 mm)	1	2.5	14	73	13	71
	2	1.9	34	66	Nil	36

introduced through acetoacetate, were found in hexose phosphate, lactate, pyruvate, aspartate, glutamate, malate, α -oxoglutarate and a few amino acids (Table 5). Of the added radioactivity about 90% was recovered when acetoacetate alone was added and 95% when acetoacetate and lactate were added.

A point requiring comment is the lower specific activity of the three ketone bodies after the 2 hr. incubation, decreasing from 1.12×10^5 to 1.02×10^5 and 0.93×10^5 counts/min./ $\mu\text{g. atom of C}$. This difference is rather greater than the experimental error and suggests that the added acetoacetate has been diluted by newly formed acetoacetate. Incubation of kidney-cortex slices without substrates in fact showed that ketone bodies were released from endogenous sources in sufficient amounts to account for the observed dilution of specific activity (H. A. Krebs, R. Hems, M. J. Weidemann & R. N. Speake, unpublished work).

DISCUSSION

The data on the net changes suggest that in the presence of lactate and acetoacetate the two main metabolic processes in the kidney slices were respiration, with acetoacetate as the major fuel:

and glucose synthesis from lactate:

At first sight the distribution of isotopic carbon, introduced either as [4-¹⁴C]acetoacetate or as [U-¹⁴C]lactate, does not appear to be in accordance with this interpretation because much of the glucose carbon was derived from acetoacetate and much of the respiratory carbon dioxide from lactate. However, the following analysis shows that 'crossing over' of carbon atoms from respiration to gluconeogenesis and from gluconeogenesis to

Table 5. *Isotope distribution on addition of [4-¹⁴C]acetoacetate and unlabelled L-lactate to rat kidney-cortex slices at the end of incubation for the experiment recorded in Table 3*

Substrate added	[4- ¹⁴ C]Acetoacetate			[4- ¹⁴ C]Acetoacetate plus L-lactate		
	10 ⁻² × Radio- activity recovered (counts/min.)	Percentage of recovered radioactivity (excluding ketone bodies)	10 ⁻⁵ × Specific activity (counts/min./ μg. atom of C)	10 ⁻² × Radio- activity recovered (counts/min.)	Percentage of recovered radioactivity (excluding ketone bodies)	10 ⁻⁵ × Specific activity (counts/min./ μg. atom of C)
Acetoacetate	56310	—	1.02	39648	—	0.93
Acetone	4683	—	1.02	6094	—	0.93
β-Hydroxybutyrate	4493	—	1.02	7000	—	0.93
CO ₂	12700	77.3	0.70	15750	49.4	0.47
Glucose	1233	7.5	0.68	13017	40.8	0.63
Hexose phosphates	120	0.7		200	0.6	
Glycogen, protein	103	0.6		100	0.3	
Lactate	233	1.4		813	2.6	
Pyruvate	253	1.5		234	0.8	
Aspartate	143	0.9		167	0.5	
Glutamate	607	3.7	0.36	543	1.7	0.37
Glutamine, glycine and serine	540	3.3		513	1.6	
Alanine	290	1.8		283	0.9	
Malate	117	0.7	0.70	137	0.4	0.81
Succinate	43	0.3		17	0.05	
α-Oxoglutarate	53	0.3		103	0.3	
Total radioactivity recovered	81921			84628		

respiration is to be expected on account of the metabolic role and behaviour of oxaloacetate.

If [¹⁴C]acetoacetate were the sole substrate of the tricarboxylic acid cycle functioning in isolation, with oxaloacetate acting as an ideal catalyst, the specific activity of the carbon atoms of acetoacetate and carbon dioxide would be identical. However, isolation, i.e. separation from other processes, does not exist in the kidney slice synthesizing glucose from lactate where oxaloacetate is not only a catalyst in the tricarboxylic acid cycle but is also an intermediate of gluconeogenesis. As the oxaloacetate molecules formed in the two processes arise within the same cell compartment, the mitochondrion, they mix in a common pool, and this implies that the carbon atoms of gluconeogenesis and respiration mix in a manner that can be exactly predicted if all the parameters are known. This does not apply to the respiring slice where the required information is incomplete and predictions are therefore necessarily of a qualitative kind.

An important feature bearing on the interpretation of the isotope data is the fact that the acetyl carbon atoms entering the tricarboxylic acid cycle after one turn of the cycle appear in oxaloacetate, and that the carbon atoms of the 2 molecules of carbon dioxide formed during one turn are entirely derived from oxaloacetate (see Weinman *et al.*

1957). The oxaloacetate pool therefore becomes labelled when the substrate of respiration (e.g. acetoacetate) is labelled, and this is bound to cause an appearance of the carbon atoms of acetoacetate in glucose. At the same time, the lactate carbon atoms, having formed oxaloacetate, can take part in the tricarboxylic acid cycle and supply carbon atoms of respiration although, on balance, all of the lactic acid may be converted into glucose, and all the respiratory carbon dioxide may be derived from acetoacetate. In other words there is a 'crossing over' of carbon atoms from acetoacetate to glucose and from lactate to carbon dioxide, as illustrated in Scheme 1.

The steady-state rates of supply of oxaloacetate into the common pool from the two sources and the rates of 'feeding' oxaloacetate into gluconeogenesis and the cycle can be approximately calculated from the rates of respiration and of gluconeogenesis. For each molecule of glucose synthesized there is an intermediate formation of 2 molecules of oxaloacetate. The rate of oxaloacetate formation through the tricarboxylic acid cycle is one-half of the rate of the oxygen uptake when acetoacetate is the fuel of respiration, one-third for lactate and a little more than one-third for long-chain fatty acids. On the basis of these values and those given in Tables 1 and 3 it can be calculated that in the presence of

Scheme 1. Diagram illustrating the 'crossing over' of carbon atoms of the tricarboxylic acid cycle and of gluconeogenesis. The pathway on the left is that of gluconeogenesis. The circle on the right represents the tricarboxylic acid cycle. Oxaloacetate is 'fed' into, and removed from, a pool common to both processes. Moreover, after one turn of the cycle, as a consequence of the asymmetrical behaviour of citrate, the acetate carbon appears quantitatively in oxaloacetate and the respiratory CO_2 is quantitatively derived from oxaloacetate:

Hence acetate carbon appears in glucose and lactate carbon in respiratory CO_2 .

both lactate and acetoacetate approximately one of the 3.6 molecules of oxaloacetate used was converted into glucose and 2.6 entered the tricarboxylic acid cycle. In other words each molecule of oxaloacetate has a two to three times greater chance to re-enter the cycle than to form phosphoenolpyruvate and glucose. This accounts for the substantial degree of 'crossing over' observed.

The principles of a quantitative analysis of this kind of situation have been elaborated by Weinman *et al.* (1957) in connexion with their studies of the incorporation of fatty acid carbon into carbohydrate, but the information available in the present experiments is insufficient for a detailed quantitative evaluation. It should be emphasized that the basic principles involved have been clearly stated by several other authors (Searle & Chaikoff, 1952; Searle, Strisower & Chaikoff, 1954; Allweis, Gainer & Chaikoff, 1960; Steele, Wall, de Bodo & Altszuler, 1956; Annison & Lindsay, 1961; Ashby, Heath & Stoner, 1965), but papers still appear in which they have not been adequately appreciated (e.g. Vrba, 1964; Reading, 1963; Bryant, Smith & Hines, 1963; Chain, Pocchiari & Reading, 1962; Vrba, Gaitonde & Richter, 1962; Gaitonde, Dahl & Elliott, 1965).

In the system investigated, the fate of the label in the substrates, because of the 'crossing-over'

phenomenon, differs from the net fate of the substrate. There was an almost quantitative net conversion of lactate into glucose but carbon atoms of lactate appeared as carbon dioxide; there was a net combustion of acetoacetate but carbon atoms of acetoacetate appeared in glucose. The inference is that in certain situations it is not possible to predict the fate of the labelled carbon atoms from the net metabolic changes alone; and if the label does not appear in the predicted places (predicted on the basis of inadequate premises), it is equally impossible, from the isotope evidence alone, to draw valid conclusions about the net fate of the labelled metabolites.

A transfer of isotopic atoms to another molecule without net conversion can thus be brought about by two different mechanisms. There are the simple exchange reactions that result from the oscillations of a reversible system, e.g. the appearance of the isotopes of hydrogen in the carboxyl groups of organic acids when these are dissolved in water containing deuterium oxide or tritium oxide, or the exchange of the carbon atoms of glutamate and α -oxoglutarate brought about by the transaminase half-reactions (see Haslam & Krebs, 1963). The term 'isotope exchange' might be reserved for this type of mechanism. The mechanisms of isotope incorporation discussed in the present paper are not due to simple reversibility. They are characterized by the involvement of irreversible reactions such as the tricarboxylic acid cycle. It may be useful to distinguish these mechanisms from isotope exchanges caused by reversibility by referring to them as 'metabolic incorporation of isotopes'.

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