SYNTHESIS VIA THE KREBS' CYCLE IN THE UTILIZATION OF ACETATE BY RAT LIVER SLICES*

by

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In a preceding paper we presented a method for studying the simultaneous incorporation of the ^{14}C of acetate-1-^{14}C into more than 15 compounds by liver slices[1]. A substantial fraction of the ^{14}C was recovered in glucose, glutamate, lactate, and alanine. Incorporation of acetate into some of these compounds was observed, *in vivo* as well as *in vitro*, by other workers[2,7], and it is now generally agreed that such incorporation is linked with the operation of the Krebs' cycle.

The present report deals with the extent and significance of the incorporation of both of the acetate carbons into the above-mentioned compounds by liver. Our findings indicate that, in rat liver slices, the Krebs' cycle serves not only as an oxidative but also as a major synthetic pathway.

EXPERIMENTAL

Rats of the Long-Evans strain, weighing 200–300 g, were used. They were raised on Purina Laboratory Chow and, unless otherwise stated, were fed this diet until they were sacrificed.

The acetate-1-^{14}C was prepared by carboxylation of the appropriate Grignard compound with $^{14}CO_2$. The acetate-2-^{14}C was obtained from Dr. B. M. TOLBERT to whom our thanks are due.

The radiochromatographic procedures used here were described in an earlier report[1]. The incorporation, by rat liver slices, of the ^{14}C of the labeled acetates into (1) CO_2, (2) lipides, (3) protein, and (4) a water-soluble, nonvolatile fraction was measured. The latter fraction usually contained about 15 compounds, and its composition was determined chromatographically.

To test the reliability of our procedure, the recoveries of ^{14}C from acetate-1-^{14}C and lactate-2-^{14}C in the glucose spot on the chromatograms were compared with those obtained by isolation of glucosazones[7]. The results of the two methods rarely differed by more than 20%, and in six out of 13 experiments the differences were less than 10%.

RESULTS

Utilization of acetate by rat liver slices

Data on total acetate utilization by this tissue and on the incorporation of its carboxyl and methyl carbons into lipides, proteins, CO_2, and the water-soluble fraction are recorded in Table I. Typical chromatograms prepared from the water-soluble fraction

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References p. 101.

TABLE I

RECOVERY OF ACETATE-^{14}C IN VARIOUS FRACTIONS PREPARED FROM INCUBATION MIXTURE

Unless otherwise designated (see Expt. 4), the slices were incubated in a Krebs-Ringer-bicarbonate buffer; the gas phase was 95% oxygen and 5% CO_2. In experiments 1 and 2, 1 gram of liver slices was incubated with 5 ml of buffer. In experiments 3, 4, and 5, 0.5 gram of liver slices was incubated with 3 ml of buffer. Incubation at 37° for 3 hours.

Expt.	Rat No.	Treatment	Label of acetate	Acetate added to medium		Per cent of added ^{14}C recovered in					
				Micromoles	c.p.m. × 10^{6}*	Steam volatile fraction	CO_2	Non-volatile fraction	Lipide fraction	Protein fraction	Not accounted for
1	7A	Fed	COOH	49	5.65	3	33.7	22.2	4.5	2.9	34
			CH_3	56	1.96	8	10.7	45.8	4.1	4.5	27
	7B	Fasted**	COOH	49	5.65	5	27.2	22.0	0.3	1.4	34
			CH_3	56	1.96	9	10.2	36.2	0.7	2.7	34
2	11A	Fed	COOH	48	4.12	73	12.5	10.5	5.0	0.6	8
			CH_3	51	2.70	60	4.3	15.6	6.5	0.9	3
	11B	Fasted**	COOH	48	4.12	38	20.9	18.6	0.3	0.9	31
			CH_3	51	2.70	43	8.8	34.7	0.7	1.4	22
	11C	Fed	COOH	48	4.12	47	17.8	20.8	4.7	1.3	8
3	14	Fasted**	COOH	48	4.12	32	22.5	13.4	0.2	0.9	31
4	17	Fed	COOH***	30	2.25	7	52	26	3.8	2.9	9
			COOH	30	2.25	7	54	23	4.1	2.7	9
			CH_3	31	2.19	14	22	48	6.2	5.8	4
5	18	Fed	COOH	25	1.95	15	41.7	19.7	12.8	3.9	12

* One microcurie is approximately $2 \cdot 10^5$ c.p.m.
** Deprived of food for 24 hours.
*** Slices incubated in a Krebs-Ringer* phosphate buffer with 100% O_2.

are shown in Fig. 1. The composition of this water-soluble fraction and the amount of ^{14}C incorporated into some 15 compounds are given in Table II.

The values presented in Table I and II are typical of the range obtained with about 30 rat livers. In most cases the comparison between the utilization of the methyl and that of the carboxyl carbon of acetate was made with separate portions of the same liver. The results for a given nutritional state were quite uniform when several experiments were carried out on a single day. Considerable variation was observed, however, in the amounts of acetate utilized by liver slices prepared from rats in the same nutritional state when the experiments were conducted on different days. This day-to-day variability was also noted by other investigators in this laboratory even though the treatment of the animals and the incubation conditions were standardized. In view of this variability in acetate utilization, it is preferable to express the ^{14}C distribution among the various compounds as a percentage of the *utilized* ^{14}C-*acetate* rather than as a percentage of the total ^{14}C added to the medium. Results so expressed are much more uniform (Table II). Acetate utilization was measured by determining the ^{14}C in the residual volatile fraction.

The compounds studied accounted for 65–90% of the added ^{14}C. The incorporation into acetoacetate—one of the major products of acetate metabolism—was not determined. In a series of experiments with acetate-1-^{14}C (not reported here), the ^{14}C in-

References p. 101.

corporation into acetoacetate was also determined, and in those experiments, the total recoveries ranged from 90–105% of the added ^{14}C. It is thus probable that the ^{14}C unaccounted for, in Table I, represents mostly acetoacetate.

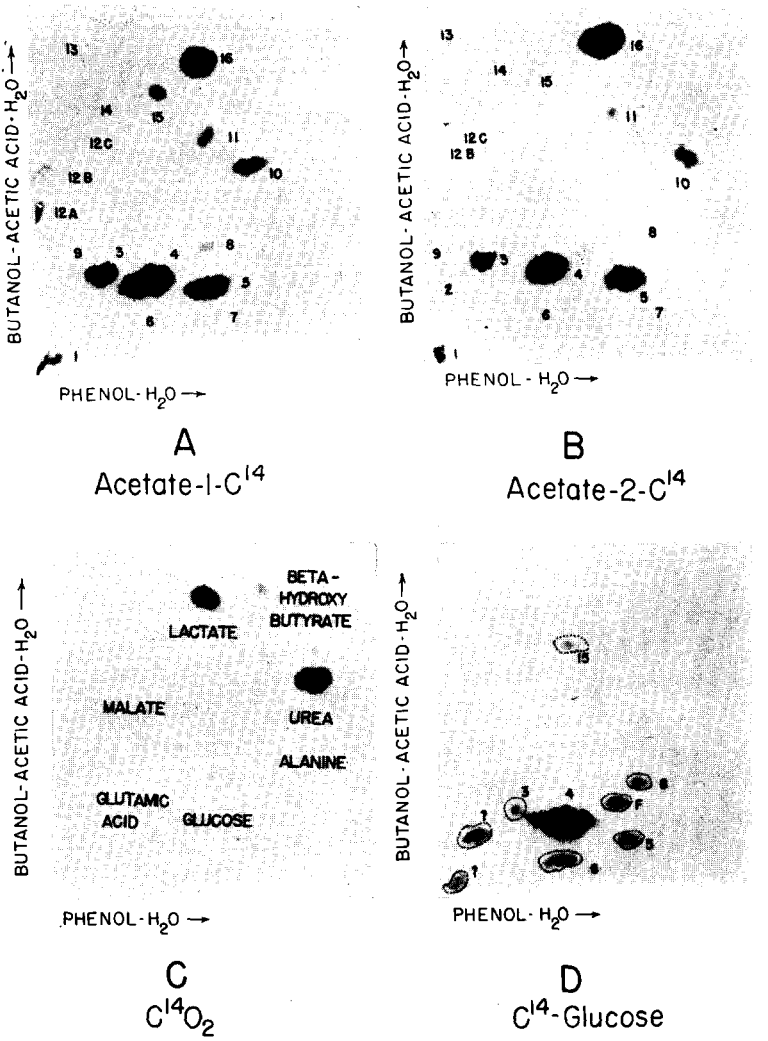

Fig. 1. Radioautographs of paper chromatograms. Incorporation of ^{14}C-labeled substrate by liver slices of fed and fasted rats into the water-soluble fraction which had been electrolytically desalted. The chromatograms represent extracts prepared from 50–70 mg of slices. Exposed 6–12 days. A and B represent results obtained with acetate-1-^{14}C. In the case of A, the rat was fed. In the case of B, the rat was fasted for 24 hours. A and B represent results with rats 7A and 7B, respectively (Table II). Fig. C shows the results of the $^{14}CO_2$ fixation experiment with a fed rat. Fig. D shows the results with evenly-labeled ^{14}C-glucose; the rat in this experiment was fasted for 48 hours. Compounds: (1) glutathione, (2) aspartic acid, (3) glutamic acid, (4) glucose, (5) glutamine, (6) and (7) glucosyl-amines, (8) alanine, (9) and (12A) citric acid, (10) urea, (11) pyrrolidone carboxylic acid, (12B) malate and α-ketoglutarate, (12C) unidentified acid, (13) fumarate, (14) succinate, (15) lactate, (16) β-hydroxybutyrate. In D, the glucose spot contains about 95% of the ^{14}C activity of the paper. The unnumbered spots in D were not identified.

References p. 101.

TABLE

DISTRIBUTION OF ^{14}C AMONG COMPOUNDS

All values were calculated from ^{14}C assay of chromatograms. All values represent

Rat number	7A		7B		11A	
Condition	Fed		Fasted		Fed	
Label	COOH	CH_3	COOH	CH_3	COOH	CH_3
Compound on chromatogram					Per cent of utilized	
Glutathione	0.9	1.0	0.7	0.9	0.2	0.4
Glutamate**	6.6	13.5	6.1	11.1	4.2	5.5
Glucose	5.3	20.7	5.0	16.8	3.7	8.9
Alanine	0.4	1.3	0.3	0.4	0.7	1.7
Lactate	0.8	3.1	0.2	0.3	0.7	3.4
Aspartate	trace	0.1	0.1	0.2	0.2	0.3
Citrate and malate***	1.5	2.1	0.1	0.1	1.3	1.8
Succinate	0.3	0.6	0.1	0.2	0.5	0.8
Fumarate	0.1	trace	--	--	0.2	0.2
Hydroxybutyrate	5.6	6.5	8.9	9.4	16.3	15.8
Urea	1.2	0.3	1.0	0.3	0.4	0.2
Others§	0.2	0.6	0.6	0.1		
Total	22.9	49.8	23.1	39.8	28.4	39.0

* Phosphate buffer used.
** This group includes ^{14}C found in glutamine, glutamic acid, and pyrrolidone carboxylic acid.

Relative incorporation of the two acetate carbons into various compounds

Although the total utilization of each of the two acetate carbons was, within experimental error, the same, the incorporation of these carbons into the various fractions and compounds differed considerably (Table I). Thus the oxidation of the carboxyl carbon to CO_2 was two to four times that of the methyl carbon, but this difference in carbon oxidation was associated with a greater incorporation of the methyl carbon into the non-volatile fraction and into proteins. The incorporation of the methyl carbon into lipides was, with few exceptions, somewhat higher than that of the carboxyl. This fraction is, of course, not homogenous, for it contains fatty acids, cholesterol, glycerol, *etc.*

The most pronounced difference in utilization of the two acetate carbons was observed in the case of glucose. The incorporation of the methyl carbon into glucose was two to four times that of the carboxyl (Table II). The glutamate-^{14}C recoveries in the experiments with the methyl-labeled acetate were about one and one-half to two times those found with the carboxyl carbon. About equal amounts of the two carbons were recovered in β-hydroxybutyric acid. The relative incorporation of each of the two carbons into CO_2 and glucose, as observed here, confirms earlier reported findings[7,8].

More of the methyl than of the carboxyl carbon was incorporated into lactate, alanine, glutathione, and di- and tricarboxylic acids (Table II). However, the ratio of the ^{14}C of acetate-2-^{14}C to that of acetate-1-^{14}C incorporated into each of these compounds could not be determined accurately since their activity was too low.

References p. 101.

II

IN THE WATER-SOLUBLE FRACTION

percentages of the utilized ^{14}C. For experimental details, see Table I and text.

11B		11C	14		17		18
Fasted		Fed	Fasted		Fed		Fed
COOH	CH_3	COOH	COOH	COOH*	COOH	CH_3	COOH
^{14}C recovered in							
0.2	0.5	0.7	0.3	0.9	0.6	1.1	1.0
5.8	12.4	12.9	5.3	6.5	8.7	15.9	8.1
4.7	18.9	6.5	2.4	8.0	6.7	22.5	4.4
0.2	0.7	1.0	0.1	0.7	0.6	2.6	0.7
0.2	0.2	0.6	0.1	0.6	0.7	3.1	1.0
trace	0.1	0.1	0.3	0.2	trace	0.2	0.2
trace	0.3	1.7	0.1	0.5	0.7	1.4	1.0
0.1	0.3	1.1	0.1	0.2	0.5	1.2	0.7
—	—	0.2	—	0.1	—	0.3	0.1
14.4	17.3	14.7	10.5	7.8	5.2	5.9	3.9
0.4	0.3	0.4	0.4	2.1	0.6	0.4	0.5
—	0.2	0.1	0.1	0.5	0.9	1.1	1.6
26.0	51.2	40.0	19.7	28.1	25.2	55.7	23.2

*** Also includes small amounts of ^{14}C in α-ketoglutarate.
§ Glucosyl amines, origin, and unidentified compounds.

Only in the case of urea was more activity incorporated from the carboxyl than from the methyl carbon, and this no doubt reflects the higher specific activity of the CO_2 in the experiments with carboxyl carbon. Urea carbon is derived solely from CO_2; hence the ^{14}C incorporated into urea can serve as an index for the extent of CO_2 fixation.

CO_2 Fixation by rat liver slices

The ^{14}C recovered in the compounds dealt with here need not have been derived *directly* from acetate, but may have arisen indirectly from respiratory CO_2. However, when bicarbonate media are used, the $^{14}CO_2$ that is formed mixes with relatively large amounts of CO_2 present both in solution and in the gas phase, and thus the specific activity of the respired CO_2 is greatly diluted.

To evaluate the extent of incorporation of ^{14}C into various compounds by CO_2 fixation, liver slices were incubated, in the usual manner, with radioactive bicarbonate. A chromatograph of the results is shown in Fig. 1,C. The pattern of CO_2 fixation is similar to that reported by DAUS *et al.*[6]. In four experiments, from 4 to 10% of the ^{14}C of the added $^{14}CO_2$ was fixed. Over 90% of that fixed was recovered in the water-soluble, nonvolatile fraction, and the rest in the tissue residue. The ^{14}C on the chromatogram was distributed as follows: 40–60% in urea; 0–25% in lactate; 10–20% in glucose; 5–15% in alanine; 5–10% in glutamate; and a few per cent in β-hydroxybutyric and in di- and tricarboxylic acids.

About one-half of the fixed $^{14}CO_2$ was found in urea. Since urea rarely accounted

References p. 101.

TABLE

^{14}C BALANCE OF ACETATE-1-^{14}C AND ACETATE-2-^{14}C

The data for rats A, B, and C were calculated from experiments reported earlier[1]. The rest

Rat No. and condition	7A Fed		7B Fasted		11A Fed		11B Fasted	
^{14}C label	COOH	CH_3	COOH	CH_3	COOH	CH_3	COOH	CH_3
Per cent of the utilized ^{14}C metabolized via Krebs' cycle***	55	60	44	45	48	37	42	48
					^{14}C Distribution among Krebs-cycle-			
Glutamate group§	19.0	32.5	19.0	33.5	13.0	23.5	17.5	30.0
Pyruvate group§§	12.0	42.5	12.0	39.5	10.5	38.5	12.0	41.5
Di- and tricarboxylic acids	3.5	4.5	0.5	0.5	4.0	7.5	0.5	1.0
Others§§§	0.5	0.5	1.5	0.5	0.5	0.5	0.0	0.0
CO_2†	65.0	20.0	67.0	26.0	72.0	30.0	70.0	27.5
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* In experiments A and B, incorporation into proteins was not determined; hence the actual values are higher.

** Phosphate buffer.

*** Represents the ^{14}C in CO_2 plus proteins plus the nonvolatile, aqueous extract minus the ^{14}C in β-hydroxybutyric acid.

for more than 1% of the ^{14}C utilized in our experiments with carboxyl-labeled acetate (bicarbonate buffer), it can be concluded that the contribution of CO_2 in the acetate experiments was small. Appropriate corrections can be made from parallel experiments with radioactive bicarbonate. Such corrections will be small and, in the case of methyl-labeled acetate, the correction for CO_2 fixation is negligible. When a phosphate buffer is used, the urea spot on the chromatogram is more prominent, and in this case the corrections for CO_2 fixation become somewhat more important.

Incorporation of acetate into compounds via the Krebs' cycle

The majority of the compounds derived from acetate can be classified into two groups: (1) those formed by direct condensation, such as ketone bodies and fatty acids, and (2) those formed via the Krebs' cycle, which include CO_2, amino acids, and organic acids.

The extent of incorporation of the two acetate carbons into compounds of the first group should be the same. This was demonstrated for fatty acids by FELTS *et al.*[8]. A compound belonging to this group is represented by β-hydroxybutyric acid and, as can be seen from Table II, the incorporation of the methyl and carboxyl carbons of acetate into this acid is the same within experimental error.

Most of the radioactive compounds formed belong to the second group, and these are characterized by incorporation into them of unequal amounts of the two acetate carbons. The carbons of acetate can be incorporated, according to the known reactions

References p. 101.

III

UTILIZATION VIA THE KREBS' CYCLE

were calculated from the data given in Tables I and II. All values rounded off to nearest 0.5%.

11C Fed	14 Fasted		17 Fed		18 Fed	A Fed	B Fed	C Fed
COOH	COOH	COOH**	COOH	CH_3	COOH	COOH	COOH	COOH
61	65	88	90	82	85	50	64	79
derived compounds (per cent)								
26.5	16.0	12.0	14.5	29.0	19.0	9.0*	8.0*	16.5
13.5	6.0	10.5	9.5	34.0	8.5	10.0	13.0	8.5
4.5	0.5	1.0	1.5	3.5	2.5	4.5	2.0	3.0
0.5	1.0	0.5	1.0	2.0	2.5	2.0	1.5	2.0
55.0	76.0	76.0	73.5	31.5	67.5	74.5	75.5	70.0
100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

§ The glutamate fraction consists of glutathione, glutamine, and glutamate in soluble form and in proteins.

§§ This fraction consists of glucose, alanine, and lactate.

§§§ These include aspartate, unidentified compounds, and artifacts; all are assumed to be formed via the Krebs' cycle.

† The activity in urea was added to that in the CO_2.

of the Krebs' cycle and glycolysis, into glucose, alanine, lactate, and the di- and tricarboxylic acids. For convenience, these compounds have been divided into several groups according to their mode of formation. These groups are: (1) respiratory CO_2 and urea; (2) the pyruvate group which consists of glucose, alanine, and lactate; (3) the glutamate group which includes glutathione, glutamine, and free and protein-bound glutamic acid; and (4) all of the di- and tricarboxylic acids.

The compounds represented by these 4 groups are herein designated *Krebs'-cycle-derived compounds*. The ^{14}C recovered in these compounds varied from 40 to 90% of the ^{14}C-acetate utilized, but the ^{14}C distribution among them was fairly constant. This is brought out in Table III where the incorporation into each group mentioned above is expressed as a percentage of the total ^{14}C revovered in the Krebs'-cycle-derived compounds.

The largest incorporation of the methyl carbon occurred in the pyruvate group, notably into glucose. In the experiments with acetate-1-^{14}C, however, the recoveries of ^{14}C in the glutamate group exceeded those in the pyruvate group. Lesser amounts of ^{14}C were incorporated into the di- and tricarboxylic acids. It is of interest that *$^{14}CO_2$ accounts for only about half of the total acetate carbon metabolised through the Krebs' cycle* (Table III).

The incorporation of ^{14}C into members of the pyruvate and glutamate groups was not appreciably affected by fasting. The recoveries of ^{14}C in di- and tricarboxylic acid were, however, drastically reduced in that state (Fig. 1,B; Table II).

References p. 101.

The constancy of the CO_2 ratio

It has been observed by a number of investigators that the carboxyl carbon of acetate is oxidized to CO_2 to a greater extent than is the methyl carbon[7]. The values for the CO_2 ratio (defined as $\frac{^{14}CO_2 \text{ derived from acetate-1-}^{14}C}{^{14}CO_2 \text{ derived from acetate-2-}^{14}C}$) reported in the literature vary from 2 to 5. Those obtained by us ranged from 2.4 to 3.8.

It was of interest to learn whether the ratios varied or remained constant during the period of incubation. Liver slices were incubated in a phosphate buffer with 50 micromoles of acetate labeled in the carboxyl or methyl carbon. Experiments were terminated at the various intervals shown in Fig. 2, and the CO_2 was collected in the usual manner.

The specific activity of CO_2 reached maximal values in less than half an hour, and from then on the values remained almost constant. The CO_2 ratio also changed little, decreasing from 2.4 in the first half hour to 2.1 at the end of the second hour.

This constancy in the CO_2 ratio was confirmed in another type of experiment in which the labeled acetates were added to the medium at various intervals after the start of the experiment. The following CO_2 ratios were observed: 3.5 for 0–30 minutes;

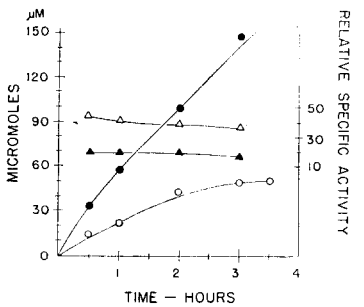

Fig. 2. The rate of CO_2 formation by liver slices incubated with acetate-1-^{14}C and acetate-2-^{14}C. One gram of rat liver slices was incubated at 37° with 52 μM of acetate in 5 ml of Krebs-Ringer-phosphate buffer in an oxygen atmosphere. The incubations were stopped at the times indicated. The relative specific activities of the $^{14}CO_2$ are expressed as

$$\frac{\text{per cent applied } ^{14}C \text{ in } CO_2 \times 100}{\mu M \text{ of } CO_2}$$

● Cumulative respiratory CO_2; ○ Acetate utilized; △ Relative specific activity of CO_2 from acetate-1-^{14}C; ▲ Relative specific activity of CO_2 from acetate-2-^{14}C.

3.9 for 30–60 minutes; 3.4 for 60–120 minutes; and 2.8 for 120–180 minutes. This observation indicates that a stable isotope distribution of the major products in the liver slices is reached quite early.

It is of interest that the rate of CO_2 formation and acetate utilization did not change appreciably throughout the experiment until the added acetate was practically exhausted (Fig. 2).

The catabolism of glucose

Since glucose and glutamate are the two major compounds into which acetate carbon is incorporated, it was of interest to determine to what extent the reactions leading to incorporation of ^{14}C into these compounds are reversible. In the case of glucose, this was studied by incubating evenly-labeled ^{14}C-glucose of high specific activity with liver slices, and preparing chromatograms as described above.

Liver slices prepared from normal rats contain little glucose at the start, but during their incubation, glucose accumulates in the medium because of glycogen breakdown. In view of the large and uncertain dilution of the added labeled glucose by endogenous sources of glucose, it is difficult to estimate the absolute amount of glucose utilized from ^{14}C recoveries. To minimize this difficulty, we made use of fasted rats because their

References p. 101.

glycogen stores are depleted. Slices prepared from their livers were incubated with 0.1 mg of uniformly-labeled glucose containing about 10 microcuries of ^{14}C. In three hours, 2 to 4% of the glucose-^{14}C was recovered in CO_2, about 1% in the insoluble residue (probably mostly glycogen), and the rest in the water-soluble fraction. A chromatograph of this water-soluble fraction is shown in Fig. 1,D. In addition to glucose, which accounted for about 95% of the activity on the paper, some 7 to 8 radioactive spots were detected. Roughly 0.5% each was found in lactate, alanine, glutamic acid, and glutamine, and the rest in unidentified compounds.

This pattern differs considerably from that obtained with ^{14}C-acetate in fasted rats (Table II). Thus the value for the ratio $\frac{\text{glutamate-}^{14}C}{\text{alanin-}^{14}C + \text{lactate-}^{14}C}$ was about 1 in the experiment with ^{14}C-glucose, about 15 to 20 in that with acetate-2-^{14}C, and as high as 30 in that with acetate-1-^{14}C. Such large differences in ratios speak against extensive reversibility in the conversion of oxalacetate to glucose by fasted liver.

Glucose oxidation contributes only a small portion to the total respiration of liver slices. For instance, liver slices prepared from fasted rats contained, at the end of the incubation period (3 hours), about 4 to 6 mg of glucose per g wet weight, and during that time about 3% of the ^{14}C-labeled glucose was converted to CO_2. Thus, no more than 6 micromoles of CO_2 could have been derived from glucose. On the other hand, endogenous CO_2 formation by such slices was about 80 to 100 micromoles (per g in 3 hours), and upon the addition of 50 to 100 micromoles of acetate it increased to about 150 micromoles.

DISCUSSION

1. *On the incorporation of the acetate carbons into the di- and tricarboxylic acids, and the effect of fasting*

The recoveries of ^{14}C in the di- and tricarboxylic acid fraction were small (Table II). The ^{14}C recovered in this fraction was about 5% of the ^{14}C metabolized via the Krebs' cycle in the experiments with livers of fed rats, and only 0.5 to 1% in those with fasted rats (Table III). The incorporation from the methyl was greater than from the carboxyl carbon. The ^{14}C was distributed principally in citric, malic, and succinic acids, and incorporation into each of these acids rarely exceeded 1% of the utilized acetate. Smaller amounts of ^{14}C were found in fumaric acid. The low incorporation into fumarate is of interest. Its activity was frequently barely detectable, and was less than one-fifth of the activity present in either malate or succinate. According to FROEHMAN *et al.*[9], the concentration of fumarate in rat liver is equal to or greater than that of succinate or malate, and thus the low activity of fumarate indicates an incomplete equilibration of activity among the acids of the Krebs' cycle.

Lack of equilibration between the various intermediates of the Krebs' cycle, and even between endogenous and added acids, was reported by other workers[10]. This does not imply that the Krebs' cycle is not operating, but rather that the bulk of di- and tricarboxylic acids in the cells does not participate in that operation. The reactions of the Krebs' cycle are localized primarily in mitochondria, and some of the components are enzyme or coenzyme bound. Thus the extent of the total incorporation of ^{14}C into any of the di- and tricarboxylic acids may bear no relation to the actual operation of the Krebs' cycle.

References p. 101.

Several workers have noted that fasting for one to two days does not depress the oxidation of *added* acetate to CO_2 by rat liver slices[11,12], and this finding is confirmed here. Fasting of the animals for one or two days did not affect significantly the incorporation of acetate carbon into CO_2, glucose, and amino acids (Tables II and III), but fasting did depress greatly the incorporation into di- and tricarboxylic acids and into lactate. This depression could be due either to a decrease in the concentration of these acids in the tissue or to a slower rate of exchange between the acids in mitochondria and those in cytoplasm.

2. *On the operation of the Krebs' cycle in rat liver slices*

As noted above, the methyl and carboxyl carbons of acetate are incorporated unequally into each of the following: CO_2, glucose, glutamate, and several other compounds. The ratio for the relative incorporation of these two carbons into a given compound is fairly constant. Thus the ratio, $\frac{^{14}CO_2 \text{ derived from acetate-2-}^{14}C}{^{14}CO_2 \text{ derived from acetate-1-}^{14}C}$ is approximately 0.35, and the corresponding ratios for glucose and glutamate are 3 and 1.4, respectively. Any explanation of our findings must, of course, account for these experimentally-obtained ratios.

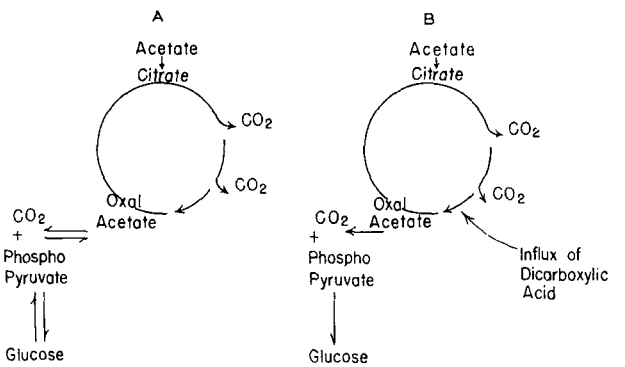

Fig. 3. Operation of the Krebs' cycle and acetate incorporation into glucose. A. No glucose synthesis occurs, and the net reaction can be represented as $CH_3COOH \longrightarrow 2\,CO_2$; B. An inflow of a dicarboxylic acid occurs with the resultant synthesis of glucose. The stoichiometry of the reaction will depend on the amount and point of inflow in the Krebs' cycle. When the inflow is a dicarboxylic acid, and the isotope dilution is 50%, the reaction may be expressed by

$$2 \text{ acetate} + 2 \text{ dicarboxylic acid} \longrightarrow 1 \text{ glucose} + 6\,CO_2.$$

The Krebs' cycle is conventionally depicted as an oxidative mechanism[13–16], and its operation is expressed by the net reaction:

$$1 \text{ acetate} \longrightarrow 2CO_2.$$

Such a cycle is shown in Fig. 3,A.* No net synthesis of glucose or amino acids occurs in this system, and the incorporation of acetate carbon into glucose is represented as an *exchange* between the carbons of oxalacetate and those of pyruvate and CO_2.

The above scheme is based on the assumption that the steady state equilibrium in the reaction

$$\text{oxalacetate} + \text{ATP} \rightleftharpoons \text{phosphopyruvate} + CO_2 + \text{ADP}$$

is such that the rates of reaction in both directions are *exactly equal*, and that no change

* In the scheme depicted in Fig. 3 the decarboxylation of phosphopyruvate to acetate (or rather acetyl-CoA) is not considered. If such decarboxylation did occur, the resulting acetyl fragment would be evenly labeled in the case where acetate-2-^{14}C was used as substrate, and not labeled at all where acetate-1-^{14}C was the substrate; in that case more ^{14}C would have been recovered as fatty acids and ketone bodies from the acetate-2-^{14}C than from the acetate-1-^{14}C. But as noted above the incorporation of 1-^{14}C and 2-^{14}C-acetate into fatty acids and betahydroxybutyrate was about the same. Thus, decarboxylation of phosphopyruvate and recycling of acetyl fragments probably occurs only to a minor extent in liver slices and may therefore be neglected here.

References p. 101.

in the amounts of reactants and products occurs. There is, however, no evidence to support this assumption, and the existence of such a special case of reversibility is questionable. The equilibrium constant for the decarboxylation of oxalacetate lies far to the right and, as discussed by KREBS[17], it is unlikely that the reaction is readily reversible.Furthermore, the assumption that the equilibrium between oxalacetate and phosphopyruvate is such that the net change in the amount of reactants and products is zero is not consistent with the conversion to glucose of a compound like succinate.

The scheme shown in Fig. 3,A cannot adequately explain the ratios referred to above. If this scheme did hold, the ratios for the relative incorporation of the two acetate carbons into CO_2, glucose, and glutamate would be 1, 5, and 2.3, respectively, values that differ considerably from those obtained here experimentally.*

3. *The dual role of the Krebs' cycle in rat liver*

Krebs, in discussing the physiological significance of the cycle, pointed out that it has two functions, one concerned with oxidation and the other with synthesis[18,19]. He proposed the view "that in some organisms the tricarboxylic acid cycle supplies energy, in others intermediates, and in again others both". Evidence that the cycle serves as a synthetic mechanism has been recently provided by ROBERTS, ABELSON, and their association in experiments with *E. coli*[20,21]. According to ROBERTS *et al.*[20], the cycle in this organism supplies more than 50 % of the carbon required for protein synthesis. The model proposed by these workers for the flow of metabolites into and out of the cycle is similar to the one presented earlier by STRISOWER *et al.*[7] for the operation of the Krebs' cycle in rat liver slices. The scheme of STRISOWER *et al.*[7] is depicted in Fig. 3,B, and will account for the observed CO_2 and glucose ratios. In this diagram, for the sake of simplicity, only one source of inflow and only a single outflow are considered, but an inflow and outflow can proceed at several points. Thus, during acetate oxidation, appreciable amounts of Krebs'-cycle intermediates are removed—for example, by the amination of α-ketoglutarate to glutamate and by decarboxylation of oxalacetate to pyruvate which in turn gives rise to lactate, alanine, and glucose. When cycle intermediates are so removed, an inflow of some dicarboxylic acid into the Krebs' cycle must occur to maintain a steady state. Hence, for each molecule of some carboxylic acid flowing into glutamate, lactate, or other compound, another molecule of some dicarboxylic acid must enter the cycle from another metabolic pool.

It has been amply demonstrated in recent years that the Krebs' cycle is involved in the conversion of lactate and pyruvate to liver glycogen. TOPPER AND HASTINGS[22] found that, compared with the total amounts of their carbons incorporated into glycogen,

* These ratios can be obtained by following the incorporation of acetate carbon through the Krebs' cycle[2]. When the specific activity of acetate is taken as 1, the specific activities of glucose and glutamate derived from acetate-1-^{14}C will be

$$\overset{\frac{1}{2}}{C}—C—\overset{\frac{1}{2}}{C}—C—C—C = 1 \text{ and } \overset{\frac{1}{2}}{C}—C_{NH_2}—C—C—\overset{1}{C} = 1.5.$$

For acetate-2-^{14}C the specific activities will be:

$$\overset{1}{C}—\overset{1}{C}—\overset{\frac{1}{2}}{C}—\overset{\frac{1}{2}}{C}—\overset{1}{C}—\overset{1}{C} = 5 \text{ and } \overset{\frac{1}{2}}{C}—\underset{NH_2}{\overset{1}{C}}—\overset{1}{C}—\overset{1}{C}—\overset{0}{C} = 3.5$$

From the above, the proper ratios are obtained. In the case of the CO_2 ratio, since the net reaction is acetate $\longrightarrow$ 2 CO_2, the ratio is obviously 1. These values are also obtained by substituting the value O for x in equations (2)–(4).

References p. 101.

the conversion of lactate and pyruvate to that compound by direct phosphorylation and condensation is small. We have also shown, with the aid of ^{14}C-labeled lactates, that a major pathway in liver for the incorporation of lactate into glucose is via formation of a dicarboxylic acid and passage through the cycle. Furthermore, FELTS AND OSBORNE* found that malonate and transaconitate inhibit the incorporation of the ^{14}C of pyruvate-2-^{14}C and of malate-3-^{14}C into glucose in liver slices. These observations suggest that a functioning Krebs' cycle is required for carbohydrate synthesis. Thus, in rat liver slices, the available experimental evidence is consistent with the concept that the Krebs' cycle plays a dual role, as both an oxidative and a synthetic mechanism.

In a recent discussion of the path of glucose synthesis from lactate[17], Krebs has also stressed the unlikelihood that such synthesis involves simply the reversal of the reactions of glycolysis. On the basis of several lines of evidence, he concluded that lactate (or pyruvate) is converted to glucose by the carboxylation of pyruvate to malate, the oxidation of malate to oxalacetate, and the latter's conversion to phosphopyruvate which subsequently is converted into glucose or glycogen. Expressed in our terms, this means that there is an inflow of malate into the Krebs' cycle and an outflow of oxalacetate.

4. *Some quantitative aspects in the operation of the Krebs' cycle*

The quantitative aspects of the operation of a cycle in which ^{14}C-acetate is being metabolized by liver slices were dealt with by STRISOWER *et al.*[7]. They showed that the specific activities of the compounds formed and consequently, the isotope distribution among the products derived from the cycle are all functions of the extent of the inflow (or outflow), and can be expressed by a number of simple formulas.

The term *isotope dilution* was used to express the extent of the inflow of unlabeled compounds into the cycle. If the extent of this dilution is designated by x, then

$$x = \frac{100\,y}{1 + y} \tag{1}$$

where x is expressed in per cent; the relative rate of the cycle flux (*i.e.*, the rate at which acetate condenses with oxalacetate) is designated as 1; and the diluting rate of influx (or outflow) is designated by y.

The equations relating the CO_2 and glucose ratios to the diluiton, x, were derived by STRISOWER *et al.*[7] as follows:

$$CO_2 \text{ ratio} = \frac{^{14}CO_2 \text{ derived from acetate-1-}^{14}C}{^{14}CO_2 \text{ derived from acetate-2-}^{14}C} = \frac{100 + x}{100 - x} \tag{2}$$

$$\text{glucose ratio} = \frac{^{14}C \text{ incorporated into glucose from acetate-2-}^{14}C}{^{14}C \text{ incorporated into glucose from acetate-1-}^{14}C} = \frac{500 - x}{100 + x} \tag{3}$$

Glutamate is one of the main labeled products observed when ^{14}C-acetate is incubated with liver slices, and the glutamate ratio is given by:

$$\frac{^{14}C \text{ incorporated into glutamate from acetate-2-}^{14}C}{^{14}C \text{ incorporated into glutamate from acetate-1-}^{14}C} = \frac{70{,}000 - 400\,x + x^2}{30{,}000 + 200\,x - x^2}\text{**}. \tag{4}$$

The values for the CO_2 ratio observed in 13 experiments are recorded in Table IV. The degree of dilution, x, calculated from these ratios, varied from 41 to 58%.

* J. M. FELTS AND M. J. OSBORNE, unpublished observations.

** Equation (4) is obtained in an analogous way as equation (3) derived by STRISOWER *et al.*[7]. Taking the specific activity of acetate as 1, the specific activities of the carboxyl carbons of oxalacetate

References p. 101.

TABLE IV

COMPARISON OF THE CALCULATED AND EXPERIMENTALLY OBTAINED GLUCOSE AND GLUTAMATE RATIOS

Rat No.	*Observed CO_2 ratio*	*Per cent isotope dilution, x, calculated from equation (2)*	*Glucose ratio*		*Glutamate ratio*	
			Calculated from equation (3)	*Observed*	*Calculated from equation (4)*	*Observed*
7A	3.1	51	3.0	4.1	1.4	2.2
7B	2.7	45	3.2	3.4	1.5	1.8
11A	2.9	48	3.1	2.4	1.4	1.3
11B	2.4	41	3.3	4.0	1.5	2.1
17	2.4	41	3.3	3.0	1.5	1.6
D	2.7	45	3.2	3.4	1.5	1.3
E	3.0	50	3.0	3.7	1.4	1.5
F	3.8	58	2.8	2.1	1.3	1.3
G	2.9	48	3.1	2.7	1.4	1.2
H	3.7	57	2.8	2.9	1.3	1.1
I	2.5	43	3.2	3.2	1.5	2.0
J	3.2	52	2.9	3.3	1.3	1.6
K	3.0	50	3.0	3.3	1.4	1.3
Avg.	3.0	50	3.0	3.2	1.4	1.4

The experimental results for Expts. 7–17 are given in Tables I and II. The rest of the ratios are taken from another series of experiments not described here. The observed CO_2 ratios were obtained from Table I. The observed glucose and glutamate ratios were obtained from Table II. The calculated ratios for the glucose and glutamate ratios were obtained by using values for x given in Column 3 above.

In columns 5 and 7 of Table IV, the experimentally-determined values for glucose and glutamate ratios are presented. Values for these ratios were also calculated from equations (3) and (4), using the value for dilution, x, derived from the CO_2 ratio, and these calculated values are given in columns 4 and 6. In most cases the agreement between the calculated and observed values is good. The experimentally-determined ratios are consistent with the operation of the Krebs' cycle shown in Fig. 3,B. It should be noted again that if no influx had occurred (see Fig. 3,A), the CO_2, glucose, and glutamate ratios should be 1, 5, and 2.3, respectively.

The derivation of equations (2)–(4), was based on the assumption that the Krebs' cycle operates in our liver slices at a steady state. That a steady state was rapidly

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can be expressed as

$$\frac{100 - x}{200} \text{ and } \frac{(100 - x)^2}{200\,(100 + x)} \text{ for acetate-1-}^{14}\text{C and -2-}^{14}\text{C,}$$

respectively, and the specific activity of the two center carbons derived from acetate-2-^{14}C is expressed by $(100 - x)/(100 + x)$. The specific activity of the glutamate derived from acetate-1-^{14}C is

$$\overset{1}{\text{C}}\text{—}\overset{0}{\text{C}}\text{—}\overset{0}{\text{C}}\text{—}\overset{0}{\text{C}}\text{—C}\quad \overset{\frac{100 - x}{200}}{}$$
$$\text{NH}_2$$

and from acetate-2-^{14}C,

$$\overset{0}{\text{C}}\text{—}\overset{1}{\text{C}}\text{—}\quad \overset{\frac{100 - x}{100 + x}}{\text{C}}\quad \text{—}\quad \overset{\frac{100 - x}{100 + x}}{\text{C}}\quad \text{—}\quad \overset{\frac{(100 - x)^2}{200\,(100 + x)}}{\text{C}}$$
$$\text{NH}_2$$

By adding up the activities for each compound and taking the respective ratios, equation (4) is obtained.

References p. 101.

established in our experiments is shown in Fig. 2; the maximum specific activity of the CO_2 was attained in less than half an hour, and during the next three hours there was little change in the specific activities of the CO_2 and in the CO_2 ratios.

5. A comparison of acetate metabolism in the intact animal and in liver slices

The conclusions presented here on acetate metabolism were derived from experiments with liver slices. The pattern of acetate metabolism in the slice appears to differ, however, in several aspects, from that in the intact animal. Thus, in the latter, the bulk of administered acetate is rapidly expired as CO_2, and only a small percentage of the administered carbon is incorporated into glucose or amino acids. Furthermore, the pattern of isotope distribution in glucose conforms with a process of "exchange" (Scheme 3,A) rather than of one involving synthesis via the Krebs' cycle.

The metabolism of acetate by a variety of tissue slice preparations has been examined in this laboratory with the same techniques as those used for liver slices. Kidney slices were found to oxidize acetate more readily than do liver slices, and the incorporation into glucose and amino acids was appreciable. On the other hand, heart, diaphragm, lungs, spleen, intestines, testis, and brain oxidized acetate to a variable extent, and the incorporation of acetate carbon into glutamate and organic acids by these tissues was much less than that observed with either kidney or liver. No incorporation of acetate carbon into glucose was observed with tissues other than liver and kidney.

Thus, in the intact animal, administered acetate is oxidized mainly in extrahepatic tissues. In this case, acetate metabolism might show little resemblance to the metabolism of acetate in the isolated liver. In the experiments with liver slices, the products formed (mainly glucose and probably glutamate) accumulate, but in the intact animal, breakdown and recycling of labeled intermediates will occur, and if such recycling is extensive, the isotope patterns in glucose and other products will approximate those expected from "exchange" reactions.

It is conceivable that, in conditions where intermediates are drained off (glycosurias, lactation, *etc.*), the importance of recycling in the intact animal is reduced. In these cases, the patterns of distribution of acetate carbons might approach those observed with liver slices.

SUMMARY

1. The incorporation of ^{14}C of acetate-1-^{14}C and -2-^{14}C, by liver slices prepared from fed and fasted rats, into CO_2, lipides, protein, β-hydroxybutyric acid, glucose, glutamate, glutathione, alanine, lactate, urea, and di- and tricarboxylic acids was studied.

2. Approximately half of the utilized acetate was metabolized via the Krebs' cycle. About 40 to 50% of the acetate carbon metabolized via the Krebs' cycle was recovered as CO_2, and about 20% each in glucose and glutamate.

3. Fasting for 24–48 hours did not affect the conversion of ^{14}C of ^{14}C-acetate to CO_2, glucose, and glutamate. It did, however, greatly depress acetate incorporation into the di- and tricarboxylic acids.

4. The carboxyl carbon of acetate was oxidized to CO_2 three times as rapidly as was the methyl carbon. On the other hand, the incorporation of the methyl carbon into glucose was three times as great as that of the carboxyl carbon, and the incorporation into glutamate, one and one-half times as great.

5. A model is presented for the operation of the Krebs' cycle that will account for the above-mentioned ratios. It is concluded that, in rat liver slices, the Krebs' cycle acts as a synthetic as well as an oxidative mechanism.

References p. 101.

RÉSUMÉ

1. L'incorporation du ^{14}C de l'acétate-1-^{14}C ou -2-^{14}C, par des coupes de foie préparées à partir de rats nourris ou à jeun dans le CO_2, les lipides, les protéines, l'acide β-hydroxybutyrique, le glucose, le glutamate, le glutathion, l'alanine, le lactate, l'urée et les acides di- et tri-carboxyliques a été étudiée.

2. A peu près la moitié de l'acétate utilisé est métabolisé par l'intermédiaire du cycle de Krebs. Environ 40 à 50% du carbone de l'acétate métabolisé par le cycle de Krebs se retrouvent sous forme de CO_2, et environ 20% dans le glucose et 20% dans le glutamate.

3. Un jeûne de 24 à 48 heures n'a pas d'influence sur la transformation du ^{14}C de l'acétate-^{14}C en CO_2, glucose et glutamate. Mais il diminue considérablement l'incorporation de l'acétate dans les acides di- et tricarboxyliques.

4. Le carbone carboxylique de l'acétate est oxydé en CO_2 trois fois plus vite que le carbone méthylique. D'autre part, l'incorporation du carbone méthylique dans le glucose est trois fois plus grande que celle du carbone carboxylique, et l'incorporation dans le glutamate, une fois et demi aussi grande.

5. Un modèle de fonctionnement du cycle de Krebs, qui rendrait compte des rapports mentionnés plus haut, est présenté. Les auteurs concluent que, dans les coupes de foie de rat, le cycle de Krebs joue à la fois un rôle synthétique et un rôle oxydatif.

ZUSAMMENFASSUNG

1. Die Einverleibung von ^{14}C aus Azetat-1-^{14}C und -2-^{14}C, durch Leberschnitten, welche aus gefütterten und fastenden Ratten gewonnen worden waren, in CO_2, Lipide, Proteine, β-Hydroxybuttersäure, Glukose, Glutamat, Glutathion, Alanin, Laktat, Harnstoff, sowie Di- und Trikarbonsäuren wurde untersucht.

2. Ungefähr die Hälfte des verbrauchten Azetates wurde durch den Krebs-Zyklus metabolisiert. Ungefähr 40–50% des durch den Krebszyklus metabolisierten Azetatkohlenstoffes wurde in Form von CO_2 wiedergewonnen, sowie je ungefähr 20% in Form von Glukose und Glutamat.

3. 24–48 stündiges Fasten hatte keine Wirkung auf die Umwandlung von ^{14}C aus ^{14}C-Azetat in CO_2, Glukose und Glutamat. Es zeigte sich jedoch eine stark deprimierende Wirkung auf die Einverleibung von Azetat in Di- und Trikarbonsäuren.

4. Karboxylkohlenstoff aus Azetat wurde dreimal so schnell wie Methylkohlenstoff in CO_2 verwandelt. Andererseits war die Einverleibung von Methylkohlenstoff in Glukose dreimal so gross wie die Einverleibung von Karboxylkohlenstoff; die Einverleibung von Methylkohlenstoff in Glutamat war anderthalbmal so gross wie diejenige von Karboxylkohlenstoff.

5. Es wird ein Operationsschema für den Krebs-Zyklus vorgeschlagen, welches den obigen Verhältnissen Rechnung trägt. Es wird der Schluss gezogen, dass der Krebs-Zyklus in Rattenleberschnitten sowohl als synthetischer, als auch als oxydativer Mechanismus funktioniert.

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