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Metabolism of Rat-Liver Cell Suspensions

2. FATTY ACID OXIDATION AND KETONE BODIES

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It has been shown (Exton, 1964) that rat-liver cell suspensions respire and form ketone bodies endogenously, the ratio acetoacetate/β-hydroxybutyrate being greater than 1. When the cells oxidize added citric acid-cycle substrates the ratio acetoacetate/β-hydroxybutyrate becomes less than 1 but the total quantity of ketone bodies is substantially unaltered. Since the liver cells oxidize

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added palmitate or stearate and accumulate 'extra' ketone bodies, it seemed desirable to investigate the metabolism of long-chain fatty acids in relation to the citric acid cycle. Generally ^{14}C-labelled palmitate was chosen as the substrate for study.

MATERIALS AND METHODS

Procedures for preparation and incubation of rat-liver cell suspensions, isolation and estimation of metabolites, degradation of ^{14}C-labelled compounds and the technique for counting ^{14}C have been described (Exton, 1964).

Generally ^{14}C-labelled palmitic acid, [G-^{14}C]palmitic acid, in benzene solution was obtained from The Radiochemical Centre, Amersham, Bucks. A stock solution was made by diluting this with benzene and adding sufficient unlabelled palmitic acid to give a specific activity 2 μc/μmole. To prepare sodium [G-^{14}C]palmitate solution a portion of the stock solution was evaporated to dryness and redissolved in ethanol. In most cases unlabelled palmitic acid was added to give a specific activity 0·1 μc/μmole. A slight excess of NaOH was added, the solution evaporated to dryness, the residue dissolved in water and the pH adjusted to 7.

Albumin–[^{14}C]palmitate complex was prepared by adding sodium [G-^{14}C]palmitate to rat blood serum as described by Bragdon & Gordon (1958).

Gas–liquid chromatography. The fatty acids extracted from a saponified cell suspension were converted into methyl esters with anhydrous methanolic HCl solution (James, Lovelock, Webb & Trotter, 1957). The esters were separated on a column of Apiezon L (Metropolitan Vickers Ltd., England) on Celite no. 545 (100–120 mesh; Shandon Scientific Co., London) in a gas chromatograph (Shandon Scientific Co.) fitted with an ionization detector (Lovelock, 1958). The chromatograph was operated at 197° with argon at a flow rate of 33 ml./min. and an inlet pressure of 8 lb./in.^{2}.

Methyl esters were identified by their retention volumes, methyl palmitate (chromatographically pure, Mann Research Laboratories Inc., New York, U.S.A.) being used as a reference standard. Radioactive samples were collected in tubes packed with glass wool moistened with toluene (Meinertz & Dole, 1962). Satisfactory recovery (92–95%) of methyl [G-^{14}C]palmitate was obtained.

Fractionation of lipids. Lipids were extracted with chloroform-methanol (2:1, v/v) according to Folch, Lees & Sloane-Stanley (1957), fractionated by silicic acid chromatography (Borgström & Olivecrona, 1961) and determined by titration (Albrink, 1959).

RESULTS

Palmitate oxidation. Palmitate (0·25–2·0 mM) increased respiration and acetoacetate production in rat-liver cell suspensions whereas higher concentrations were inhibitory. When 10 μmoles of [G-^{14}C]palmitate were added to a cell suspension, 87·5% of the ^{14}C was recovered in the metabolic products examined and in the residual fatty acids extracted from the cells and from the medium (Table 1). The distribution of ^{14}C amongst the products shows that 1·3 μmoles of [^{14}C]palmitate were oxidized to carbon dioxide, 3·6 μmoles to acetoacetate, 1·7 μmoles to β-hydroxybutyrate and 2·1 μmoles were recovered in the fatty acid fraction.

Calculations based on the specific activities of the metabolic products show that about one-third of the respiratory carbon dioxide is derived from [^{14}C]-palmitate and about two-thirds from endogenous sources (Table 2). Slightly more ketone bodies are formed from [^{14}C]palmitate than from endogenous fatty acids. Although the addition of [^{14}C]-palmitate increased the total ketone-body formation from 25·5 μmoles to 39·1 μmoles, it depressed the endogenous ketogenesis from 25·5 μmoles to 17·9 μmoles.

To test the extent of possible radiochemical contamination of the substances isolated, a control experiment was carried out with a heated (100° for 10 min.) cell suspension to which [G-^{14}C]palmitate (84 000 counts/min.) was added. After incubation

Table 1. *Oxidation of* [G-^{14}C]*palmitate by a liver-cell suspension*

Warburg vessels (200 ml. capacity) contained cells (357 mg. of trichloroacetic acid-insoluble dry wt.) suspended in 20 ml. of 0·1 M-KCl medium containing 0·02 M-tris–HCl buffer, pH 7·4. Added substrate, sodium [G-^{14}C]-palmitate (10 μmoles, 140 000 counts/min.). Gas, O_2. Temp., 30°. Time, 1 hr.

	No added substrate	[G-^{14}C]Palmitate		
	Amounts (μmoles)	Amounts (μmoles)	^{14}C recovered (%)	Sp. activity (counts/min./μmole of C)
Uptake of O_2	—	106	—	—
Metabolic products				
CO_2	61	61	13·1	304
Acetoacetate	16·4	26·6	35·9*	473
β-Hydroxybutyrate	9·1	12·5 (mg.)	17·1†	478†
Fatty acids	—	59·1	21·4	34
			Total 87·5	

* The recovery of ^{14}C in C-1 was 9·0% and that in C-2 + C-3 + C-4 was 26·9%.
† Calculated on the assumption that the ^{14}C content of C-1 was one-third that of C-2 + C-3 + C-4.

Table 2. *Sources of carbon dioxide and ketone bodies in the experiment of Table* 1

	Metabolites formed (μmoles)			
	CO_2	Acetoacetate	β-Hydroxybutyrate	Total ketone bodies
Endogenous substrates (in the absence of added palmitate)	61	16·4	9·1	25·5
Endogenous substrates (in the presence of added palmitate)	40	12·2	5·7	17·9
Added palmitate	21	14·4	6·8	21·2

30 μmoles of acetoacetate and 15 μmoles of β-hydroxybutyrate were added. More than 98 % of the counts were recovered in the fatty acid fraction, 0·4 % in the ketone bodies and none in the carbon dioxide. Thus the adsorption of [^{14}C]-palmitate on to the acetone–mercury complex used for the separation of ketone bodies did not introduce a significant error.

The results of the experiment given in Table 1 are typical of those obtained in identical experiments with liver-cell suspensions prepared from eight rats. In these experiments the mean stoicheiometric proportions between oxygen consumption and the products formed from [G-^{14}C]palmitate were found to be:

$$1\ \text{Palmitate} + 8{\cdot}6\ O_2 \rightarrow 2{\cdot}2\ CO_2 + 2{\cdot}2\ \text{acetoacetate} + 1{\cdot}2\ \beta\text{-hydroxybutyrate} + 4{\cdot}6\ H_2O$$

Thus ketone bodies are the major products of fatty acid oxidation and the R.Q. (mean, 0·26) is correspondingly low.

There is little doubt that the terminal oxidation of [^{14}C]palmitate takes place in the citric acid cycle. Direct evidence of incorporation of ^{14}C into citric acid-cycle intermediates was obtained by the simultaneous addition of labelled palmitate and non-isotopic α-oxoglutarate. Under these conditions cell suspensions accumulate a substantial amount of malate and lesser quantities of other citric acid-cycle intermediates (Exton, 1964). Table 3 shows that ^{14}C derived from [G-^{14}C]palmitate is incorporated into malate and pyruvate–oxaloacetate with about twofold dilution of the ^{14}C.

In the presence of malate, succinate or pyruvate slightly less [G-^{14}C]palmitate is oxidized to $^{14}CO_2$ than in their absence. With malate and succinate this is probably due to the accumulation of ^{14}C in citric acid-cycle intermediates. There is also a small diminution in ketogenesis (Tables 4 and 5) but this is not due to the sparing of fatty acid oxidation since the ^{14}C content of the residual fatty acids is not increased by addition of any of the three substrates. Accordingly the main effect of pyruvate and the citric acid-cycle substrates is reduction of acetoacetate, leading to inversion of the acetoacetate/β-hydroxybutyrate ratio. The effect applies almost equally to the ketone bodies formed from [^{14}C]palmitate and to those arising endogenously. In consequence the specific activities of the acetoacetate and β-hydroxybutyrate are practically unaltered. In each experiment the specific activity of the $^{14}CO_2$ is diminished by dilution with $^{12}CO_2$ much of which is probably released during the oxidation of the added non-isotopic substrate.

Table 3. *Oxidation of* [G-^{14}C]*palmitate by a rat-liver cell suspension in the presence of* α*-oxoglutarate*

A Warburg vessel (200 ml. capacity) contained 696 mg. of trichloroacetic acid-insoluble dry wt. of cells suspended in 30 ml. of 0·1 M-KCl medium containing 0·02 M-tris–HCl buffer, pH 7·4, 0·5 mM-sodium [G-^{14}C]palmitate (210 000 counts/min.) and 1 mM-potassium α-oxoglutarate. Gas, O_2. Temp., 30°. Time, 1 hr. Malate, fumarate, succinate and pyruvate–oxaloacetate were isolated by column chromatography (Exton, 1964).

	Amounts (μmoles)	% of added ^{14}C	Sp. activity (counts/min./μmole of C)
Uptake of O_2	180	—	—
Metabolic products			
CO_2	93	6·8	154
Malate	15·0	11·2	392
Fumarate	3·7	—	—
Succinate	1·6	—	—
Pyruvate–oxaloacetate	1·8	2·0	570

Other aspects of palmitate metabolism. From the data of Tables 1 and 4 it is evident that 10–20 % of the [^{14}C]palmitate presented to a liver-cell suspension could be available for incorporation into the cell lipids. An experiment designed to test the possibility of esterification is shown in Table 6. Since the [G-^{14}C]palmitate had a much higher specific activity than that used earlier, the risk of radiochemical contamination of the fatty acid fractions was increased. It is seen that after incubation 45 % of the added ^{14}C was recovered in the free fatty acid fraction of the cells, where it was diluted with an approximately equal quantity of endogenous fatty acid carbon; of the [^{14}C]palmitate present in the cells 3·2 % was recovered in the fatty

acids of the triglycerides and 1·2 % in those of the phospholipids. These results show that, if esterification occurs at all, it is barely significant.

To determine whether [G-^{14}C]palmitate is transformed into other fatty acids during incubation of the suspension, the fatty acids were extracted from the cells after 1 hr. and converted into methyl esters, which were analysed by gas–liquid chromatography. The only fatty acid showing a significant concentration of ^{14}C was palmitic acid. Of the radioactivity found in the fatty acids, 90 % was present in palmitic acid, 3 % in stearic acid, 3 % in oleic acid and linoleic acid and 1 % in palmitoleic acid. The experiment shows that the cell suspension does not convert a significant amount of palmitic acid into any other fatty acid.

Metabolism of cells containing endogenous ^{14}C-labelled fatty acids. The liver lipids of two rats were labelled with ^{14}C by administration of albumin–[G-^{14}C]palmitate complex. Under anaesthesia induced by intraperitoneal sodium pentobarbitone each rat was injected with complex (3 μmoles of

Table 4. *Effects of malate, succinate and pyruvate on the oxidation of* [G-^{14}C]*palmitate by liver-cell suspensions*

Warburg vessels contained cells suspended in 20 ml. of 0·1 M-KCl medium containing 0·02 M-tris–HCl buffer, pH 7·4, and 0·5 mM-sodium [G-^{14}C]palmitate (140 000 counts/min.). Gas, O_2. Temp., 30°. Time, 1 hr. Substrates added (10 μmoles, potassium salt) were: Expt. 1, L-malate; Expt. 2, succinate; Expt. 3, pyruvate. Trichloroacetic acid-insoluble dry wt. of cells were: Expt. 1, 449 mg.; Expt. 2, 440 mg.; Expt. 3, 455 mg.

		Substrate absent			Substrate present		
Expt. no.		Amounts (μmoles)	Recovery of ^{14}C (%)	Sp. activity (counts/min./ μmole of C)	Amounts (μmoles)	Recovery of ^{14}C (%)	Sp. activity (counts/min./ μmole of C)
1	Uptake of O_2	111	—	—	119	—	—
	Production of CO_2	51	9·2	256	61	6·6	151
	Acetoacetate	26·0	32·7	440	3·6	5·2	458
	β-Hydroxybutyrate	25·9 (mg.)	36·6	495	45·3 (mg.)	55·0	425
	Fatty acids	78	12·4	—	76	10·9	—
			Total 90·9			Total 77·7	
2	Uptake of O_2	118	—	—	120	—	—
	Production of CO_2	93	16·3	245	101	12·2	168
	Acetoacetate	38·1	60·0	554	6·9	10·8	547
	β-Hydroxybutyrate	7·3 (mg.)	11·4	545	35·9 (mg.)	56·2	548
	Fatty acids	82	12·2	—	81	11·0	—
			Total 99·9			Total 90·2	
3	Uptake of O_2	117	—	—	135	—	—
	Production of CO_2	69	14·9	303	111	12·5	158
	Acetoacetate	21·5	37·8	614	2·4	4·2	615
	β-Hydroxybutyrate	18·5 (mg.)	33·0	624	37·1 (mg.)	62·7	590
	Fatty acids	84	13·2	—	87	10·2	—
			Total 98·9			Total 89·6	

Table 5. *Sources of carbon dioxide and ketone bodies in the experiments of Table 4*

		Amounts are expressed as μmoles.			
	Source	CO_2	Acetoacetate	β-Hydroxybutyrate	Total ketone bodies
Malate absent	Endogenous	36	12·9	11·3	24·2
	[^{14}C]Palmitate	15	13·1	14·6	27·7
Malate present	Endogenous + malate	50	1·7	23·3	25·0
	[^{14}C]Palmitate	11	1·9	22·0	23·9
Succinate absent	Endogenous	67	13·9	2·8	16·7
	[^{14}C]Palmitate	26	24·2	4·5	28·7
Succinate present	Endogenous + succinate	82	2·6	13·4	16·0
	[^{14}C]Palmitate	19	4·3	22·5	26·8
Pyruvate absent	Endogenous	45	6·4	5·3	11·7
	[^{14}C]Palmitate	24	15·1	13·2	28·3
Pyruvate present	Endogenous + pyruvate	91	0·7	12·0	12·7
	[^{14}C]Palmitate	20	1·7	25·1	26·8

[G-^{14}C]palmitate) via the internal saphenous vein. After 20 min. livers were removed and cell suspensions made by the routine procedure were pooled (30 ml.). Lipids were extracted from 10 ml. of the suspension and separated by silicic acid chromatography. A further 10 ml. portion was incubated aerobically at 30° for 1 hr. The incubated suspension was fractionated as described by Exton (1964). The lipids present in the cell pellet were not saponified but were extracted and separated chromatographically. The results are shown in Table 7. The ^{14}C recovered in the pooled liver-cell suspension was 15·5 % of the quantity administered ($9·78 \times 10^6$ counts/min.). Before incubation the fatty acids of the phospholipid fraction contained 60 % and the triglyceride fatty acids 36 % of the ^{14}C that had been incorporated into the cell lipids.

Table 6. *Esterification of* [G-^{14}C]*palmitate by a liver-cell suspension*

A conical flask contained 310 mg. of trichloroacetic acid-insoluble dry wt. of cells suspended in 20 ml. of 0·1 M-KCl medium containing 0·02 M-tris–HCl buffer, pH 7·4, and 0·5 mM-sodium [G-^{14}C]palmitate. After incubation in air at 30° for 40 min. the lipids were extracted and separated by chromatography on silicic acid.

	Amount (μequiv.)	$10^{-3} \times$ Radioactivity (counts/min.)	Sp. activity (counts/min./μequiv.)
Palmitate added	10·0	2650	265 000
Fatty acid recovered after incubation as			
Free fatty acid	10·4	1184	114 000
Triglyceride	19·1	39·6	2 070
Phospholipid	77	15·1	196

The labelling pattern found after incubation (Table 7) is similar to that found when [G-^{14}C]-palmitate is added to cell suspensions. About 8 % of the ^{14}C previously incorporated into the lipids is recovered in carbon dioxide and in the two ketone bodies, which are in isotopic equilibrium with each other. More ^{14}C is recovered in the ketone bodies and carbon dioxide than was initially present in the free fatty acid fraction of the cells. It is clear that esterified ^{14}C-labelled fatty acids have been liberated to increase the ^{14}C in the free fatty acid fraction and to provide the ^{14}C that appears in the oxidation products. Changes in the levels of the three lipid fractions run parallel with the changes in the ^{14}C content and so the specific activities of the fractions have not been altered significantly.

This experiment confirms the view expressed by Exton (1964) that lipids provide the chief fuel for endogenous respiration in isolated liver cells.

DISCUSSION

When [G-^{14}C]palmitate is added to rat-liver cell suspensions at a concentration comparable with that of endogenous free fatty acids in the intact liver, it is oxidized at a mean rate (eight experiments) of 21 μm-moles/mg. of trichloroacetic acid-insoluble dry wt. of cells/hr. The chief products are the ketone bodies; only two to three of the 16 carbon atoms in palmitic acid are oxidized to carbon dioxide (Table 1). The R.Q. (0·26) is correspondingly low. No significant changes in the form of esterification or conversion into other fatty acids could be detected.

The addition of [G-^{14}C]palmitate depresses endogenous ketogenesis and carbon dioxide production

Table 7. *Oxidation of endogenous lipids of isolated liver cells labelled by prior administration of albumin–*[G-^{14}C]*palmitate complex*

Details of administration of the palmitate complex are given in the text. The cell suspension (420 mg. of trichloroacetic acid-insoluble dry wt.) was suspended in 20 ml. of 0·1 M-KCl medium containing 0·02 M-tris–HCl buffer, pH 7·4, and incubated in a Warburg vessel in O_2 at 30° for 1 hr. Lipids were analysed as described in the text.

	Before incubation			After incubation		
	Amount (μequiv.)	Radioactivity (counts/min.)	Sp. activity (counts/min./μequiv.)	Amount (μequiv.)	Radioactivity (counts/min.)	Sp. activity (counts/min./μequiv.)
Free fatty acids	10·0	21 100	2 110	14·8	29 800	2 010
Triglyceride fatty acids	55·6	182 000	3 280	44·4	155 000	3 500
Phospholipid fatty acids	206	302 000	1 460	194	281 000	1 530
Total	272	505 000	1 860	253	465 000	1 840
						(counts/min./μmole of C)
Metabolic products				(μmoles)		
CO_2	—	—	—	49	7 100	145
Acetoacetate	—	—	—	16·8	21 100	313
β-Hydroxybutyrate	—	—	—	9·0	11 700	325
					Total 39 800	

in the same proportion. A possible explanation for the preferential utilization of the added palmitate is diminution of the rate of release of free fatty acids from endogenous triglycerides and phospholipids.

The metabolism of ^{14}C-labelled fatty acid incorporated into the liver lipids 20–30 min. before the cell suspensions were prepared is virtually the same as that of added [G-^{14}C]palmitate (Table 7). The stoicheiometric relationships between the ^{14}C-labelled products of oxidation are similar; but the specific activity of the $^{14}CO_2$ is much lower than that of the ketone bodies, showing that about half the carbon dioxide is produced from endogenous substrates other than fatty acids. The source of this carbon is not clear.

The use of [G-^{14}C]palmitate has confirmed the conclusion (Exton, 1964) that addition of pyruvate or a citric acid-cycle substrate does not promote the oxidation of endogenous fatty acid to any significant degree. With all the substrates examined the decrease in acetoacetate was found to be almost entirely due to its reduction to β-hydroxybutyrate.

The low rate of oxidation of fatty acid to carbon dioxide indicates that the turnover rate of the citric acid cycle is low in isolated liver cells. It has been suggested (Exton, 1964) that this might be due to a low concentration of oxaloacetate. However, if this is the case it is surprising that malate and succinate exert such little effect on the oxidation of palmitate in the citric acid cycle. Since citric acid-cycle intermediates increase the NADH/NAD^+ ratio of the cells (Exton, 1964) and cause inversion of the acetoacetate/β-hydroxybutyrate ratio, it is probable that they also increase the malate/oxaloacetate ratio. This would partly explain why malate, succinate and other intermediates produce only a slight decrease in ketone-body production by the cells.

SUMMARY

1. Palmitate at concentrations below 2 mM increased the oxygen uptake and carbon dioxide production of cells isolated from rat liver.

2. [G-^{14}C]Palmitate added *in vitro* was utilized in preference to endogenous substrates. It was oxidized largely to ketone bodies and was not converted into other fatty acids or esterified to a significant extent.

3. Succinate, malate and pyruvate promoted the reduction of acetoacetate formed from [G-^{14}C]-palmitate and endogenous substrates. The total ketone-body production was little altered by these substrates.

4. About 16% of the ^{14}C injected in the form of albumin–[G-^{14}C]palmitate complex was recovered in the fatty acids of the liver cells isolated 20–30 min. later. During incubation of the cells the isotope content of the phospholipids and triglycerides fell while that of the free fatty acids rose. The ^{14}C which disappeared from the lipids was equal to that which appeared in the carbon dioxide and ketone bodies.

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Blood Lipids

5. THE LIPIDS OF SHEEP PLASMA*

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Until fairly recently there was a dearth of information on the nature and fatty acid composition of the lipids in the plasma of ruminant animals. With respect to the ox, however, work in this Laboratory has established the overall pattern of lipids in the plasma of cows during pregnancy and lactation (Lough & Garton, 1957; Garton, Duncan & Lough, 1961; Duncan & Garton, 1963) and in the plasma of non-lactating non-pregnant heifers (Duncan & Garton, 1962). As far as we know, no detailed study

* Part 4: Lough (1964).