Metabolism of Rat-Liver Cell Suspensions

1. GENERAL PROPERTIES OF ISOLATED CELLS AND OCCURRENCE OF THE CITRIC ACID CYCLE

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Berry (1959, 1962) has demonstrated that cells isolated from mouse liver possess an endogenous respiration, accumulate acetoacetate and oxidize a variety of added substances when the cells are suspended in a favourable medium. The present work on rat-liver cell suspensions attempts to define some properties and limitations of this preparation and to test the validity of the hypothesis that a citric acid cycle operates in the isolated cells.

MATERIALS AND METHODS

Animals. Male rats (230–270 g.) of the Wistar albino strain were supplied by the Animal Department, University of Otago Medical School.

Enzymes and cofactors. Alcohol dehydrogenase, lactate dehydrogenase and malate dehydrogenase (analytical reagent grade) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; glucose 6-phosphate dehydrogenase, NAD^+, NADH, $NADP^+$, NADPH and cytochrome *c* were from Sigma Chemical Co., St Louis, Mo., U.S.A.

^{14}C-labelled compounds. [2,3-$^{14}C_2$]Succinic acid and sodium [3-^{14}C]pyruvate were obtained from The Radiochemical Centre, Amersham, Bucks.

Chemicals. Sodium acetoacetate was prepared by the method of Krebs & Eggleston (1945) and standardized according to Edson (1935). Sodium DL-β-hydroxybutyrate was obtained from British Drug Houses Ltd., Poole, Dorset, L-malic acid from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and palmitic acid and stearic acid (chromatographically pure) from Mann Research Laboratories Inc., New York, U.S.A. Solutions of palmitate or stearate were prepared by dissolving the acids in ethanol, adding a slight excess of KOH or NaOH, evaporating to dryness *in vacuo*, dissolving the salt in water and adjusting the pH to 7·0. Potassium pyruvate was prepared by neutralizing redistilled pyruvic acid with $KHCO_3$ and standardized by the method of Long (1942).

2,5-Diphenyloxazole was obtained from British Drug Houses Ltd.; *p*-bis-(5-phenyloxazol-2-yl)benzene and 1·0M-hyamine hydroxide [(*p*-di-isobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide] in methanolic solution were obtained from Nuclear Enterprises, Winnipeg, Canada.

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Preparation of liver cells. Rat liver was perfused with 50 ml. of 0·4M-sucrose containing 0·04M-tris–HCl buffer, pH 7·4, and dispersed in 50 ml. of this medium at 37° by the method of Branster & Morton (1957). All subsequent procedures were carried out at 0–4°. The crude suspension was filtered through bolting nylon and centrifuged at 300*g* for 2 min. The supernatant was removed, the cell pellet suspended in 3 vol. of 0·2M-KCl containing 0·04M-tris–HCl buffer, pH 7·4, and centrifuged again at 300*g* for 2 min. The washed cell pellet was suspended in 1 vol. of the buffered KCl medium. In most experiments the suspension was diluted with an equal volume of water or substrate solution. Thus the routine medium was 0·1M-KCl buffered with 0·02M-tris–HCl, pH 7·4, and the residual sucrose concentration was not more than 0·025M.

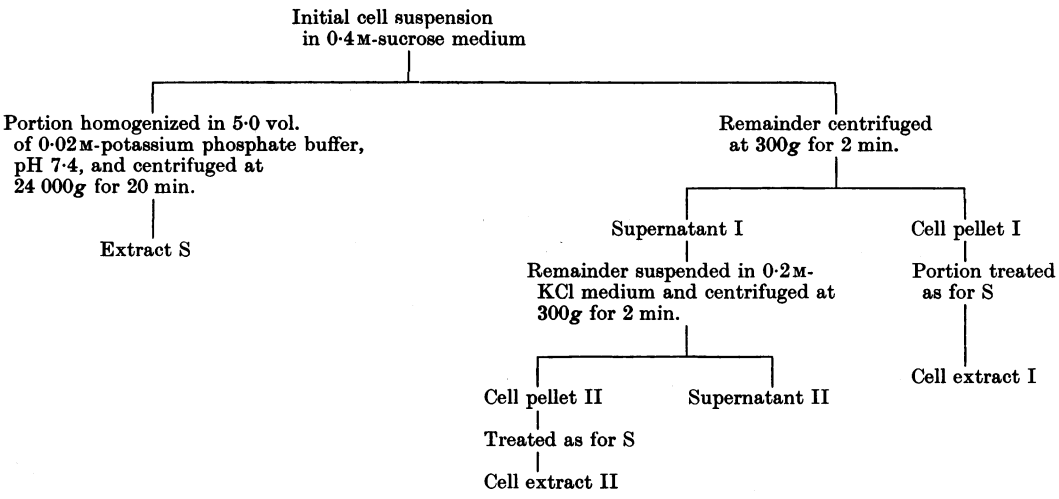
Electron microscopy. Cell pellets were prepared and examined by the methods of Berry & Simpson (1962).

Manometry. Oxygen uptake of cell suspensions was measured at 30° by the conventional Warburg techniques described by Umbreit, Burris & Stauffer (1957). Carbon dioxide production was measured by the direct method (Umbreit *et al.* 1957).

Tissue dry weight. Dry weights of cell suspensions were determined as trichloroacetic acid-insoluble dry matter according to Werkheiser & Bartley (1957).

Enzyme assays. To investigate the effect of dispersion and washing on the enzymic activities of liver cells assays were carried out on fractions of the suspension. For aldolase the procedure of Berry (1962) was followed. In the assay of lactate, malate and L-iditol dehydrogenases, liver cells were dispersed in 0·4M-sucrose medium as usual and the suspension was fractionated at 0–4° according to Scheme 1. The volume of each fraction was measured and a sample taken for protein estimation by the method of Layne (1957). Lactate and malate dehydrogenases were assayed by the methods of Delbrück, Zebe & Bücher (1959) and L-iditol dehydrogenase by the method of Smith (1962).

Glycogen determination. Glycogen was determined at various stages during the routine preparation of cells. With the rat under ether anaesthesia a portion of the liver was rapidly removed and weighed in a tared centrifuge tube containing 30% (w/v) KOH solution. The liver remaining *in situ* was perfused at 37° with buffered 0·4M-sucrose medium. After removal of the distended liver another sample was taken and weighed in the same way. The remainder of the liver was weighed and a washed-cell suspension prepared in buffered 0·1M-KCl medium in the routine manner. Samples were transferred to 30% (w/v) KOH solution at each stage and the final cell suspension was incubated at 30° for 40 min. before it was inactivated



Scheme 1. Fractionation of the initial cell suspension for enzyme assays. Operations were carried out at 0-4°.

in the KOH solution. Glycogen was estimated by the method of Seifter, Dayton, Novic & Muntwyler (1950).

Determination of lipids. Lipids were extracted from liver and cell suspensions with chloroform–methanol (2:1, v/v) by the method of Folch, Lees & Sloane-Stanley (1957). Extracts were fractionated into free fatty acids, neutral lipids and phospholipids by chromatography on a silicic acid (100 mesh; Mallinckrodt Chemical Works, U.S.A.) column according to Borgström (1952). Each fraction was evaporated to dryness *in vacuo* and the dry weight determined after standing for 12 hr. over P_2O_5 and shredded paraffin wax.

The total fatty acids in cell suspensions were determined by saponifying samples in 5 vol. of 30% (w/v) KOH solution for 1 hr. at 60–80°. An equal volume of ethanol was added and heating continued for 1 hr. under reflux. The saponified mixture was washed three times with 20 ml. of light petroleum (b.p. 60–80°) to remove non-saponifiable lipids before acidification (to Congo red) with HCl and extraction of the fatty acids with three 20 ml. portions of light petroleum. The combined extracts were evaporated to dryness *in vacuo* over P_2O_5 and paraffin wax and weighed.

The phosphorus content of lipid fractions was determined by the method of Fiske & Subbarow (1925).

Nicotinamide–adenine dinucleotides. These were determined by the method of Bassham, Birt, Hems & Loening (1959). Fluorescence was measured in a single-sided fluorimeter (Locarte Co., London). The primary filter was Chance OX1 (transmission 320–390 mμ) and the secondary filters were a combination of Ilford 622 and Chance OY18 to exclude transmission below 440 mμ. The instrument was checked before and after use against a solution of quinine sulphate (0·1 mg./l. of 0·1 N-H_2SO_4).

Procedure in metabolic experiments. In general the apparatus described by Sakami (1955) was used for gravimetric estimation of CO_2 and for wet combustion of ^{14}C-labelled compounds with the reagent of Van Slyke & Folch (1940). Acetoacetate was determined by the method of Walker (1954) and β-hydroxybutyrate by a minor modification of the method of Hird & Symons (1959). The modification was necessary to eliminate interference by sucrose and acetoacetate (cf. Kulka, Krebs & Eggleston, 1961).

The cell suspension (10 ml.) was mixed with 8·0 ml. of water in a large Warburg vessel (200 ml. capacity) standing on ice. Substrate solution or water (2·0 ml.) was placed in one side arm and 2·0 ml. of 2 N-HCl in the other. The inseal contained 2·0 ml. of carbonate-free 2 N-NaOH and filter paper. The vessel was gassed with O_2 and equilibrated at 30° for 10 min., the substrate added and uptake of O_2 measured for 1 hr. The acid was then tipped into the main compartment and shaking continued for 15 min. The vessel was placed on ice and the contents of the inseal were quickly transferred along with four washings (1 ml. each of CO_2-free water) to the apparatus (Sakami, 1955), in which CO_2 was liberated and trapped in a bubbler filled with carbonate-free 2 N-NaOH and determined gravimetrically, giving the total CO_2 (free and bound). The bound CO_2 initially present in an unincubated control was measured manometrically.

A small portion of the acidified cell suspension was reserved for immediate determination of acetoacetate and the remainder was used for other operations, which were carried out at 0–4° unless stated otherwise.

The residual cell suspension (20 ml.) was centrifuged at 1600*g* for 15 min. The cell pellet was saponified with 10 vol. of ethanolic 15% (w/v) KOH at 60–80° for 2 hr. and the cell fatty acids were isolated and estimated gravimetrically as described above (Determination of lipids). After the volume of the supernatant A had been measured it was neutralized by addition of a measured volume of 2 N-NaOH. Protein and adsorbed lipids were precipitated by adding 2·0 ml. of 0·3 N-$Ba(OH)_2$ and 2·0 ml. of 5% (w/v) $ZnSO_4,7H_2O$ solution and allowing the mixture to stand for 10 min. It was centrifuged at 1600*g* for 15 min., giving supernatant B and a precipitate. The fatty acids in the precipitate were determined gravimetrically after saponification and extraction as before.

Portion of supernatant B (20·0 ml.) was treated with 4·0 ml. of 20% (w/v) $CuSO_4,5H_2O$ and 4·0 ml. of 10% (w/v) $Ca(OH)_2$ suspension and allowed to stand for 30 min. with frequent stirring to remove sucrose. It was then centrifuged

at 1600*g* for 10 min. giving supernatant C. In isotopic experiments a portion (10·0 ml.) of supernatant C was mixed in a conical absorption flask (50 ml. capacity) with 150 μmoles of unlabelled sodium acetoacetate and 10·0 ml. of Denigès's reagent (3·5 vol. of 10%, w/v, $HgSO_4$ solution mixed with 1 vol. of 50%, w/v, H_2SO_4 solution). 2N-Sodium hydroxide (1·0 ml.) was placed in the centre well, the flask stoppered, evacuated and heated in an oven at 80° for 2 hr. The alkaline carbonate solution in the centre well was diluted to 4·0 ml. with 1% (w/v) unlabelled Na_2CO_3 solution and plated as $Ba^{14}CO_3$. This represents the carboxyl carbon atom of acetoacetate. The acetone–Hg complex precipitated in the main compartment was collected on a sintered-glass filter, washed with 100 ml. of water, dried at 105° for 1 hr., weighed and subjected to combustion, giving the ^{14}C content of C-2 + C-3 + C-4 of acetoacetate.

Another portion (12·0 ml.) of supernatant C was subjected to a second precipitation with the same volume of Cu–lime reagent and centrifuged at 1600*g* for 10 min. giving supernatant D, which was transferred to a test tube. A drop of H_2SO_4 (sp.gr. 1·84) and a few alundum chips were added. The tube was weighed and placed in a water bath at 100° for 20 min. to decompose acetoacetate and remove the acetone. The original weight was restored with water. A portion (15·0 ml.) of the now acetone-free supernatant D was transferred to a 40 ml. Clin-Britic vaccine bottle and mixed with 4·0 ml. of 10N-H_2SO_4 and 4·0 ml. of 0·4% (w/v) $K_2Cr_2O_7$ solution. The bottle was sealed with a rubber cap and autoclaved at 15 lb./in.^{2} for 40 min. The acetone content of the autoclaved solution and the percentage conversion of β-hydroxybutyrate were determined as described by Hird & Symons (1959). Another portion (18·0 ml.) of the autoclaved sample was transferred to a conical absorption flask to which 150 μmoles of non-isotopic acetone and 10·0 ml. of Denigès's reagent were added. The acetone–Hg complex was treated as before to estimate the ^{14}C content of C-2 + C-3 + C-4 of the β-hydroxybutyrate.

For smaller-scale experiments in which 2·0 ml. of cell suspension was used with non-isotopic substrates, β-hydroxybutyrate was estimated in heated supernatant D by the modified method of Hird & Symons (1959), the volumes of the reagents being scaled down appropriately.

Measurement of radioactivity. Respiratory CO_2 and the CO_2 obtained in chemical degradations were plated in triplicate as $Ba^{14}CO_3$. The plates were counted (minimum of 5000 counts) under a thin-end-window Geiger–Müller tube and the counts corrected for background and self-absorption (to infinite thinness). The values given are the mean counts of three plates differing by not more than ±2%.

Samples with a low ^{14}C content were counted in an Ekco N 664A scintillation counter (Ekco Electronics, Essex). Fatty acids were dissolved in 10 ml. of scintillation fluid composed of 0·4% (w/v) 2,5-diphenyloxazole and 0·005% (w/v) *p*-bis-(5-phenyloxazol-2-yl)benzene in toluene and counted in 16 ml. vials. Samples other than fatty acids were converted into CO_2, which was collected in 2N-NaOH. Portions (1·0 ml.) of the alkaline carbonate solution were placed in the main compartment of conical absorption flasks (30 ml. capacity), the centre well of which contained 0·5 ml. of 1·0M-hyamine hydroxide in methanolic solution. The flasks were stoppered with gas-tight rubber bungs (Simplex no. 8; Britton Malcolm Co. Ltd., London) through which 1 ml. of 5N-H_2SO_4 was injected into the main compartment. The flasks were shaken at 30° for 2 hr. and the hyamine solution, together with two washings (each 1·0 ml. of scintillation fluid), was transferred to vials containing 8·0 ml. of scintillation fluid. At least 5000 counts were taken for each sample and corrected for background and for the quenching effect of methanol when present.

Chromatographic analysis of organic acids. Organic acids were extracted from cell suspensions and separated by the method of Swim & Utter (1957). A column (20 cm. × 2 cm.) of Celite no. 545 (Johns-Manville Corp., U.S.A.) was employed and fractions (10 ml.) were titrated with 0·02N-NaOH with phenol red as indicator.

The validity of the separation was tested with a mixture of acids made up in the proportions likely to be encountered in experiments with the cell suspensions. The recoveries of pyruvic acid, fumaric acid, succinic acid, malic acid, citric acid and isocitric acid were in the range 91–103%; for α-oxoglutaric acid the value was 84%. Because β-hydroxybutyrate and fumarate are not resolved by the procedure, fumarate was determined by measuring β-hydroxybutyrate independently and subtracting it from the β-hydroxybutyrate–fumarate peak. Acetoacetate is completely broken down during the procedure and oxaloacetate is largely (about 80%) decomposed to pyruvate. Since the pyruvate fraction obtained in experiments contains therefore an uncertain amount of oxaloacetate it is designated pyruvate–oxaloacetate in the Tables.

Other estimations. Oxaloacetate, malate, pyruvate and lactate were estimated by the enzymic methods of Hohorst, Kreutz & Bücher (1959), ammonia and urea by the methods of Conway (1947).

Metabolic quotients. These were defined as: q_{O_2} = μl. of O_2 uptake/mg. of trichloroacetic acid-insoluble dry wt. of tissue/hr.; q_{Acac} = μm-moles of acetoacetate formed/mg. of trichloroacetic acid-insoluble dry wt. of tissue/hr.

RESULTS

Morphology of the isolated cells

Isolated rat-liver cells suspended in 0·1M-potassium chloride medium show essentially the same alterations in ultrastructure as those described by Berry & Simpson (1962) in mouse-liver cells. The chief abnormal features are partial disruption of the cell membrane and vesicular distortion of the mitochondria.

Loss of cytoplasmic constituents

Enzymes. The observed damage to the cell membrane could permit leakage of enzymes that are located predominantly in the cell sap. Several authors (Henley, Sorensen & Pollard, 1959, Zimmerman, Devlin & Pruss, 1960; Berry, 1959) have reported evidence consistent with this possibility.

In the present work leakage of aldolase and lactate dehydrogenase was confirmed, 97% of the original aldolase activity and 90% of the original lactate-dehydrogenase activity being lost from twice-washed cells. Table 1 shows that 95% of the

L-iditol-dehydrogenase activity that can be extracted from the cells with phosphate buffer is lost, whereas 59% of the malate dehydrogenase is retained, probably in the mitochondria. Table 1 also indicates the extent to which protein escapes from the cells.

Codehydrogenases. In Table 2 the amounts of the nicotinamide–adenine dinucleotides in fresh rat liver are compared with those present in cell preparations that have been washed twice with 0·1M-potassium chloride medium to eliminate sucrose, which otherwise interferes with the estimation of the nucleotides. A third washing did not alter the values significantly. Only 18% of the total nicotinamide nucleotides present in the fresh tissue are recovered in the cells. The NAD/NADP ratio of the cells is much lower than in the fresh tissue and resembles more closely that found in isolated rat-liver mitochondria (Birt & Bartley, 1960*a*, *b*; Christie & Le Page, 1962). Although the low recovery of the nucleotides may be attributed partly to cell destruction or to enzymic breakdown, it may also be due to leakage through the broken cell membranes, since a high proportion of the nicotinamide nucleotides of liver homogenate is located in the supernatant fraction (Glock & McLean, 1956; Jacobson & Kaplan, 1957; Birt & Bartley, 1960*b*).

Glycogen. Much of the glycogen present in a fresh liver disappeared during perfusion (Table 3). The glycogen content of the perfused liver dropped from 265 to 212 mg. during dispersion of the cells and only 98 mg. of glycogen was recovered in the washed cells. This quantity was diminished further during incubation at 30° and glucose accumulated in the medium.

Lipids. A portion of perfused liver was removed, its lipid content determined and the remainder converted into a cell preparation. Samples of washed cells and of the combined supernatants were analysed for lipids.

Table 4 shows that most of the lipids originally in the perfused liver were retained by the cells but the distribution of lipids and lipid phosphorus between the cellular fraction and the supernatant washings indicates that triglyceride is lost from the cells more rapidly than phospholipid.

Endogenous metabolism

Despite the morphological abnormalities and the loss of some cytoplasmic constituents, isolated rat-liver cells consume oxygen and produce acetoacetate when they are suspended in sucrose or saline media. This confirms the work of Berry (1962) on mouse-liver cells. The mean q_{Acac} of cells prepared from the livers of 14 fed rats and incubated in potassium chloride medium at 30° is 62 ± 1 (S.E.M.) and is not significantly different from the mean q_{Acac} of cells prepared from eight rats starved for 24 hr. (61 ± 2). The rate of acetoacetate production is thus of the same order as that found by Edson (1935) with liver slices prepared from starved rats and incubated at 37° in phosphate saline (Krebs, 1933). The mean respiratory quotient for 11 cell preparations was 0·55 ± 0·06 (S.E.M.), as against 0·8 for rat-liver

Table 1. *Loss of L-iditol and malate dehydrogenases from rat-liver cells*

Experimental details are given in the Materials and Methods section. Liver fractions refer to Scheme 1. The protein content is that of each fraction before extraction with phosphate buffer. For malate dehydrogenase 1 unit of enzyme is the amount that oxidizes 1·0 μmole of NADH/min. under the conditions of Delbrück, Zebe & Bücher (1959).

Liver fraction	Protein (mg.)	L-Iditol dehydrogenase (units)	Malate dehydrogenase (units)
Extract S	1705	87·3	231
Supernatant I	890	66·1	70
Cell extract I	810	16·9	166
Supernatant II	196	12·5	14
Cell extract II	610	4·0	136

Table 2. *Loss of nicotinamide–adenine dinucleotides from rat-liver cells*

Nucleotides were determined in cell suspensions prepared in the routine way but washed twice with buffered 0·1M-KCl medium, pH 7·4. Values given for cell suspensions are means ±S.E.M. for eight preparations.

	Nicotinamide-adenine dinucleotides (μm-moles/g. wet wt. of liver)	
	Fresh liver*	Cell suspensions
NAD^+	513	77·7 ±6·2
NADH	211	21·9 ±2·5
$NADP^+$	59	22·3 ±3·9
NADPH	283	65·7 ±3·4
NADH/NAD^+	0·41	0·28±0·05
NADPH/$NADP^+$	4·80	2·95±0·14
NAD/NADP	2·12	1·14±0·19

* Only one liver was analysed as the concentrations found were very similar to those reported by Bassham, Birt, Hems & Loening (1959).

Table 3. *Glycogen content of rat-liver fractions*

Procedure is described in the Materials and Methods section. Incubated cells were suspended in 0·1M-KCl medium containing 0·02M-tris–HCl buffer, pH 7·4, and incubated aerobically at 30° for 40 min.

Fraction	Glycogen (mg./liver)
Unperfused liver	441
Perfused liver	265
Initial suspension	212
Supernatant from initial suspension	108
Washed cells	98
Washed cells (after incubation)	53

Table 4. *Lipid content of isolated rat-liver cells*

Experimental details are given in the text. Values (expressed as mg./g. of liver) are means for four rats. The supernatant from the initial suspension was pooled with that from the cell washing to give combined supernatants.

Liver fraction	Total lipid	Neutral lipid	Phospho-lipid	Lipid P	Total fatty acid
Perfused liver	44·7	13·7	31·0	1·06	—
Washed cells	32·0	6·4	25·6	0·92	13·8
Combined supernatants	11·2	6·8	4·4	0·16	—

slices (Baker, 1938). These facts suggest that fatty acid is the major endogenous metabolite in isolated liver cells.

No significant quantity of free amino acids could be detected in incubated cell suspensions by paper chromatography with phenol–water (4:1, w/v), no urea was formed and little ammonia was liberated (1·2 μm-moles/mg. of trichloroacetic acid-insoluble dry weight/hr.). Thus it is clear that significant amounts of protein are not used as fuel for the endogenous metabolism of the cells.

Composition of the medium. Table 5 shows that 0·1 M-potassium chloride medium permits endogenous respiration and ketogenesis to take place at maximum or near-maximum rates. The addition of 0·5–2·0 mM-Mg^{2+} ions had no significant effect but the addition of 1–6 mM-phosphate or 1 mM-Ca^{2+} ions caused a depression of the metabolism. The addition of the oxidized and reduced forms of NAD or NADP (0·1–0·2 mM) with or without cytochrome *c* (0·2 mM) did not increase oxygen uptake.

Effects of added substrates

Carbohydrates. Zimmerman *et al.* (1960) have demonstrated that glucose is not metabolized aerobically or anaerobically in isolated rat-liver cells. Addition of glucose, fructose, sorbitol or glycerol to cell suspensions caused no significant alteration in the rates of oxygen uptake and acetoacetate production (Table 6). On the other hand glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and DL-glycerol 1-phosphate depressed both oxygen consumption and acetoacetate formation.

The failure of the hexoses and polyols to raise the oxygen consumption above the endogenous level can be explained by the loss of the glycolytic enzymes and dehydrogenases already mentioned. The inhibitory effect of hexose phosphates and glycerol 1-phosphate can be explained by the release of inorganic phosphate by a powerful phosphatase, which is known to be active in rat-liver cell suspensions (McLean, 1962).

Fatty acids and amino acids. The effects of adding *n*-fatty acids (C_2–C_{18}) are shown in Table 7. The inhibitory effect of the C_2–C_8 acids is unexpected; that of the higher homologues reaches a maximum

Table 5. *Effects of the composition of the medium on respiration and acetoacetate production of isolated rat-liver cells*

Warburg vessels contained cells (34–51 mg. of trichloroacetic acid-insoluble dry wt.) suspended in 2·0 ml. of media containing 0·02 M-tris–HCl buffer, pH 7·4. Gas, O_2. Temp., 30°. Time, 45–60 min.

Expt. no.	Suspending medium: Sucrose (M)	Suspending medium: KCl (M)	q_{O_2}	q_{Acac}
1	0·10	0	2·9	51
	0·20	0	3·3	46
	0·25	0	1·2	10
	0·30	0	0·8	3
	0·40	0	0·4	3
2	0	0·05	5·7	48
	0	0·10	5·8	71
	0	0·15	5·0	71
	0	0·20	1·2	14
3	0·20	0	2·0	27
	0·16	0·02	3·8	49
	0·12	0·04	5·1	66
	0·08	0·06	5·3	67
	0·04	0·08	5·1	66

Table 6. *Effects of carbohydrates and their phosphate esters on the metabolism of isolated rat-liver cells*

Warburg vessels contained cells (32–45 mg. of trichloroacetic acid-insoluble dry wt.). Time, 40–60 min. Other conditions were as given in Table 5. Glycerol 1-phosphate was added as the sodium salt, other acidic substrates as salts of potassium.

Expt. no.	Additions	Concn. (mM)	q_{O_2}	q_{Acac}
1	None	0	4·9	51
	D-Glucose	5	5·0	52
	D-Fructose	5	5·3	50
	D-Sorbitol	10	5·1	50
	Glycerol	5	4·6	56
	DL-Glycerol 1-phosphate	5	4·0	36
2	None	0	4·5	60
	D-Glucose 1-phosphate	2	2·7	25
	D-Glucose 6-phosphate	2	1·4	22
	D-Fructose 6-phosphate	2	2·9	27
	D-Fructose 1,6-diphosphate	1	2·9	35
	D-Fructose 1,6-diphosphate	5	1·9	16

with dodecanoate and is analogous to their action on rat-liver slices (Ahmed & Scholefield, 1961). In contrast 1 mM-palmitate and 1 mM-stearate increase oxygen uptake and acetoacetate formation (Table 7). Alanine has no effect probably because the cells have lost alanine aminotransferase (Henley, Wiggins, Pollard & Dullaert, 1958). Glutamate and aspartate increase oxygen uptake but decrease acetoacetate formation.

Citric acid-cycle intermediates and related substances

Berry (1962) found that citric acid-cycle intermediates and some related substances increased the oxygen uptake but diminished the acetoacetate production of mouse-liver cells. Table 8 shows the same phenomenon in rat-liver cells. The Table also shows that the fall in acetoacetate is counterbalanced by an equivalent increase in the formation of β-hydroxybutyrate. The failure of citric acid-cycle substrates to diminish the formation of ketone bodies raises the question of the intactness of the citric acid cycle in isolated cell preparations.

Tables 9 and 10 illustrate a series of experiments in which single citric acid-cycle substrates were added to cell suspensions and the intermediary metabolites determined by column chromatography (Table 9) or by enzymic methods (Table 10). When no substrate was added malate was the only citric acid-cycle metabolite present in measurable amount. Except for malate and pyruvate the added substrates, including the utilizable isomer of isocitrate, were almost completely removed after 1 hr. In the presence of each substrate there was an increased oxygen consumption and, except with pyruvate, a large quantity of malate accumulated together with small amounts of fumarate, succinate and pyruvate–oxaloacetate. The same changes were observed whether oxygen or air was used as the gas phase. The experiments indicate that the citric acid cycle is operating in the cells and this conclusion is confirmed by the typical blocking effects exerted by the well-known selective inhibitors of the cycle reactions (Table 9).

The accumulation of malate during the oxidation of citric acid-cycle intermediates is always accompanied by reduction of endogenous acetoacetate to β-hydroxybutyrate (Table 8). This suggests that the electrons required for the reduction of acetoacetate to β-hydroxybutyrate have been made available by a substantial increase in the ratio NADH/NAD$^+$. The mean NADH/NAD$^+$ ratio of four cell suspensions rose from 0·24 ± 0·07 (± S.E.M.)

Table 7. *Oxidation of fatty acids and amino acids by liver-cell suspensions*

Warburg vessels contained cells (43–48 mg. of trichloroacetic acid-insoluble dry wt.). Time, 40–60 min. Other conditions were as given in Table 5. Substrates were added as salts of potassium.

Expt. no.	Substrate	Concn. (mM)	q_{O_2}	q_{Acac}
1	None	0	5·1	54
	Acetate	5	4·1	35
	n-Butyrate	2	2·0	16
	n-Hexanoate	1	2·5	24
	n-Octanoate	1	2·2	18
	n-Decanoate	1	1·3	14
	n-Dodecanoate	1	1·2	10
	n-Tetradecanoate	1	3·1	32
	Palmitate	1	7·6	70
	Stearate	1	7·2	73
2	None	0	4·9	57
	DL-Glutamate	10	6·5	39
	DL-Aspartate	10	6·6	45
	L-Alanine	10	4·8	56

Table 8. *Effects of citric acid-cycle intermediates and related substances on respiration and ketone-body formation*

Conditions were as given in Table 5. Time, 1 hr. Substrates were added as salts of potassium. DL-Lactate and DL-isocitrate, 5·0 mM; pyruvate, succinate and malate, 0·5 mM; others, 2·5 mM.

Expt. no.	Trichloroacetic acid-insoluble dry wt. of cells (mg.)	Substrate	Uptake of O_2 (μmoles)	Acetoacetate formed (μmoles)	β-Hydroxybutyrate formed (μmoles)
1	52	None	11·8	2·88	0·40
		Fumarate	14·6	0·80	2·25
		α-Oxoglutarate	14·2	1·11	2·13
		DL-Isocitrate	13·5	1·04	2·15
		cis-Aconitate	14·4	0·76	2·31
		Citrate	15·3	1·16	2·19
		DL-Lactate	13·0	1·29	2·28
2	42	None	9·7	2·79	1·56
		Pyruvate	12·0	1·55	3·17
3	43	None	9·7	2·51	0·75
		Succinate	10·4	0·70	2·38
4	45	None	11·1	2·78	1·40
		L-Malate	11·9	0·36	3·12

Table 9. *Amounts of citric acid-cycle intermediates after incubation of cell suspension with various substrates and inhibitors*

Each Warburg vessel (200 ml. capacity) contained cells suspended in 16·0 ml. of 0·1 M-KCl medium containing 0·02 M-tris–HCl buffer, pH 7·4. Substrates were added as salts of potassium. DL-Isocitrate, 80 μmoles; palmitate, 8 μmoles; other substrates, 40 μmoles. Gas, O_2 (except with fumarate and pyruvate, when air was used). Temp., 30°. Time, 1 hr. Metabolites were determined by column chromatography. Values are given on the basis of 300 mg. of trichloroacetic acid-insoluble dry wt. of cells.

				Metabolites found (μmoles)					
Substrate	Inhibitor	Concn. of inhibitor (mM)	Uptake of O_2	Pyruvate–oxalo-acetate	Malate	Fumarate	Succinate	α-Oxo-glutarate	Citrate + iso-citrate + *cis*-aconitate
None	None		53·5	0	0·2	0	0	0	0
L-Malate	None		59·1	1·5	29·3	5·0	0·4	0·5	0
L-Malate	Malonate	10	35·2	1·1	10·8	2·8	11·5	0	0·1
L-Malate	Fluoroacetate	10	44·5	1·2	25·2	4·5	1·2	0·4	5·8
L-Malate	Arsenite	0·1	30·6	2·2	23·1	3·9	1·4	1·3	0
Oxaloacetate	None		82·2	3·9	23·2	2·1	1·8	0·1	0
Oxaloacetate	Malonate	10	17·6	3·5	14·8	2·0	16·5	0	0
Citrate	None		90·7	1·0	21·9	2·6	1·3	0	0
Citrate	Arsenite	0·1	21·6	1·0	1·6	2·1	0	32·5	1·3
DL-Isocitrate	None		87·4	1·5	31·2	4·1	1·3	0	45·2
α-Oxoglutarate	None		71·4	3·2	23·3	3·9	1·2	1·0	0·1
Fumarate	None		—	0·7	21·5	4·8	0·8	0	0
Pyruvate	None		—	2·2	0·3	0	0	0	0·1
Palmitate	None		99·5	0	0·2	0·1	0	0	0

Table 10. *Metabolites found after incubation of cell suspensions with malate, oxaloacetate and pyruvate*

Conical flasks contained cells suspended in 16·0 ml. of 0·1 M-KCl medium containing 0·02 M-tris–HCl buffer, pH 7·4. Substrates were added as salts of potassium. Gas, air. Temp., 30°. Time, 1 hr. Values were determined enzymically (Hohorst *et al.* 1959) and are given on the basis of 300 mg. of trichloroacetic acid-insoluble dry wt. of cells.

Substrate added (40 μmoles)	Metabolites found (μmoles)			
	Malate	Oxaloacetate	Pyruvate	Lactate
None	0·3	0	0	0
L-Malate	28·2*	0·05*	0·85*	—
Oxaloacetate	29·7	0·07	0·53	0·15
Pyruvate	0·4	0·01	12·20	3·81

* Mean value of four expts.

to 1·77 ± 0·35 during aerobic incubation for 0·5 hr. in the absence of added substrate and the mean NADPH/$NADP^+$ ratio from 2·06 ± 0·25 to 9·6 ± 2·9. These findings, which differ from those obtained when rat-liver mitochondria are incubated without the addition of substrate (Birt & Bartley, 1960*b*, *c*), can be attributed to a greater supply of endogenous substrates in the cell suspensions. The reduction of the nucleotides occurred to a greater degree when the suspensions were incubated with added citric acid-cycle intermediates or with pyruvate and lactate but not with palmitate (Table 11). Similar findings were observed with oxygen as the gas phase instead of air. During incubation there was little change in the total quantity of the coenzymes, showing that the increase of the ratio reduced form/oxidized form was not due to decomposition of the oxidized form.

Succinate. Table 12 compares the endogenous metabolism of a cell suspension with that of an equal portion to which 10 μmoles of [2,3-$^{14}C_2$]-succinate were added. At this low concentration (0·5 mM) the increase in the oxygen uptake was small (7 μmoles). About 41 % of the ^{14}C was recovered in respiratory carbon dioxide, the residue being in the protein-free supernatant prepared from the cells. The bulk of the residual ^{14}C appeared in malate (34 %) and in fumarate (9·6 %). The ketone bodies contained a negligible amount (0·5 %) and none was found in the fatty acids. Like other citric acid-cycle intermediates, succinate inverted the ratio acetoacetate/β-hydroxybutyrate with little change in the total amount of ketone bodies formed.

The yield of $^{14}CO_2$ corresponds to the oxidation of 8·2 μg.atoms of the methylene carbon atoms of [2,3-$^{14}C_2$]succinate and there has been a net loss of 9·4 μg.atoms of carbon from the citric acid-cycle intermediates and pyruvate. It is probable that the net loss of carbon is largely due to the removal of pyruvate formed from oxaloacetate during incubation (see Table 10). However, the metabolism of succinate by the decarboxylation pathway alone cannot account for the amount of $^{14}CO_2$ formed nor can it explain why the specific activities of malate and fumarate are only 63–64 % of that of the added succinate. It therefore seems that succinate

has also been oxidized in the citric acid cycle without being converted into pyruvate.

Addition of succinate increased the endogenous carbon dioxide production by 17 μmoles and, as well as increasing the oxygen uptake by 7 μmoles, provided approximately 34 μequiv. of hydrogen for the reduction of acetoacetate. Since the conversion of succinate into the intermediates which accumulate would require only 3·9 μmoles of oxygen, it appears that succinate has increased the production of carbon dioxide and hydrogen from endogenous substrates as well as from any pyruvate formed during its metabolism. The most likely explanation for the findings is that succinate has increased the rate of oxidation of endogenous and ^{14}C-labelled acetyl-CoA in the citric acid cycle. This would also account for the small decrease in ketone-body production caused by succinate.

Table 11. *Effects of added substrates on the nicotinamide–adenine dinucleotides of liver cell suspensions incubated aerobically*

Conical flasks contained 624–792 mg. of trichloroacetic acid-insoluble dry wt. of cells suspended in 16·0 ml. of buffered 0·1 M-KCl medium, pH 7·4. Gas, air. Temp., 30°. Time, 30 min. Substrates were added as salts of potassium. Palmitate, 0·5 mM; DL-lactate, 5 mM; other substrates, 2·5 mM.

		Nicotinamide–adenine dinucleotides after incubation			
		NAD	NADP	NADH/NAD$^+$	NADPH/NADP$^+$
Expt. no.	Substrate	(μm-moles/mg. of trichloroacetic acid-insoluble dry wt.)			
1	None	1·53	1·19	2·05	4·71
	Fumarate	1·47	1·06	6·76	8·38
2	None	1·70	1·22	1·78	6·15
	L-Malate	1·59	1·19	5·17	9·90
3	None	1·50	1·38	2·65	15·1
	Succinate	1·41	1·43	6·68	19·7
	Citrate	1·39	1·48	5·55	27·2
4	None	1·48	1·54	1·20	6·0
	α-Oxoglutarate	1·40	1·56	2·19	10·7
5	None	1·33	1·45	1·03	11·3
	Pyruvate	1·23	1·44	4·04	20·4
6	None	1·30	1·44	0·94	2·11
	DL-Lactate	1·28	1·44	1·03	18·0
	Palmitate	1·26	1·45	2·15	2·20

Table 12. *Oxidation of* [2,3-$^{14}C_2$]*succinate by a rat-liver cell suspension*

Each Warburg vessel (200 ml. capacity) contained cells (434 mg. of trichloroacetic acid-insoluble dry wt.) suspended in 20 ml. of buffered 0·1 M-KCl medium, pH 7·4. Gas, O_2. Temp., 30°. Time, 1 hr. The [2,3-$^{14}C_2$]-succinate (10 μmoles) contained 380 000 counts/min. Organic acids were analysed by chromatography on Celite (see the Materials and Methods section).

	No added substrate	[2,3-$^{14}C_2$]Succinate		
	Amounts (μmoles)	Amounts (μmoles)	^{14}C recovered (%)	Sp. activity (counts/min./μmole of C)
Uptake of O_2	97	104	—	—
Metabolic products				
CO_2	60	77	41·1	2030
Acetoacetate	25·1	7·5	0·5	—
β-Hydroxybutyrate	7·0	23·8		
Malate	0·3	5·4	33·8	5950
Fumarate	0	1·5	9·6	6080
Pyruvate–oxaloacetate	0	0·6	1·1	—
Succinate	0	0·6	1·4	—
Undetermined compounds in supernatant*	—	—	6·6	—
Cell fatty acids	—	—	0	—
			Total 94·1	

* Citrate, isocitrate, *cis*-aconitate and α-oxoglutarate were not detected in the supernatant.

Table 13. *Oxidation of* [3-^{14}C]*pyruvate by a rat-liver cell suspension*

Each Warburg vessel contained liver cells (419 mg. of trichloroacetic acid-insoluble dry wt.) suspended in 20 ml. of buffered 0·1 M-KCl medium (pH 7·4). Gas, O_2. Temp., 30°. Time, 1 hr. The [3-^{14}C]pyruvate (10 μmoles) contained 311 000 counts/min.

	No added substrate	[3-^{14}C]Pyruvate		
	Amounts (μmoles)	Amounts (μmoles)	^{14}C recovered (%)	Sp. activity (counts/min./ μmole of C)
Uptake of O_2	97	120	—	—
Metabolic products				
CO_2	61	76	37·3	1530
Acetoacetate	27·9	15·5	10·3	517
β-Hydroxybutyrate	15·6	31·7	19·3	462
Undetermined compounds	—	—	33·5	—
Cell fatty acids	—	—	0·02	—
			Total 100·4	

Oxidation of pyruvate and lactate

The oxidation of [3-^{14}C]pyruvate by a cell suspension is illustrated in Table 13. Calculations from the radiochemical yields show that 3·7 μmoles of [3-^{14}C]pyruvate have been oxidized to $^{14}CO_2$ and 3·0 μmoles have been oxidized and condensed to give ketone bodies. This will account for 14·1 μmoles out of the 15 μmoles of 'extra' carbon dioxide formed in the presence of the added pyruvate. The incorporation of ^{14}C into the cell fatty acids was not significant, showing that fatty acid synthesis from pyruvate does not occur in cell suspensions. Residual pyruvate, lactate (cf. Table 10) and other undetermined substances containing 33·5 % of the added ^{14}C will account for the rest of the isotope.

As expected, inversion of the ketone body involving about 32 μequiv. of hydrogen has occurred and the total ketone-body formation is slightly greater (3·7 μmoles) in the presence of pyruvate than in its absence. The 'extra' oxygen uptake (23 μmoles) is twice that required (11 μmoles) for the oxidation of pyruvate to carbon dioxide and water and the formation of 'extra' ketone bodies from it. The unexplained difference is too large to be an experimental error.

The results are consistent with the hypothesis that pyruvate is oxidized in the cells by a citric acid-cycle mechanism. It is probable that lactate is oxidized by the same mechanism. Since most of the lactate dehydrogenase has been lost from the cytoplasmic matrix, it is likely that lactate oxidation (Table 8) is catalysed in the cells by a mitochondrial lactate dehydrogenase (Walker & Seligman, 1963).

DISCUSSION

Compared with liver slices, rat-liver cell suspensions exhibit abnormalities of composition and metabolism that limit their usefulness as an experimental preparation. Some of the abnormalities, e.g. the loss of glycolytic enzymes, Na^+ and K^+ ions (Little & Exton, 1963) and neutral lipid, can be attributed to disruption of the cell membrane (Berry & Simpson, 1962), whereas others, e.g. the high rate of endogenous ketone-body production and the low rate of oxygen consumption, appear to be due to disordered mitochondrial functions. In spite of their distorted mitochondrial morphology isolated liver cells oxidize a wide variety of substrates, including palmitate and stearate, without requiring supplements of adenosine triphosphate, Mg^{2+} ions or phosphate ions.

Apart from some ability to break down glycogen (Table 3) the cell suspensions appear to be unable to metabolize carbohydrates (Table 6). The high rate of production of endogenous ketone bodies, which is the same whether the cells are prepared from fed or starved rats, could well be a consequence of this defect. This situation offers an advantage by providing an extreme case of ketogenesis for experimental study. Addition of pyruvate or of citric acid-cycle substrates does not increase the oxidation of endogenous fatty acids significantly, but causes reduction of acetoacetate to β-hydroxybutyrate without change in the total quantity of ketone bodies formed (Tables 8, 12 and 13). A similar formation of β-hydroxybutyrate from acetoacetate under aerobic conditions has been observed in rat-liver homogenates (Leloir & Muñoz, 1939; Krebs, Eggleston & d'Alessandro, 1961).

Convincing evidence for the occurrence of the citric acid cycle in liver-cell suspensions has been presented (Table 9). Although it is difficult to make valid comparisons between different tissue preparations, it is probable that the turnover of the cycle is lower in cell suspensions than in liver slices. The incomplete oxidation of palmitate (Exton, 1964) suggests that such a defect exists.

Oxidation of any citric acid-cycle substrate leads to accumulation of malate (Table 9) but malate-dehydrogenase activity does not appear to be impaired since reduction of oxaloacetate to malate occurs readily when the cycle is blocked by malonate. It is well known that the equilibrium of the malate-dehydrogenase reaction greatly favours the reduction of oxaloacetate. For the reaction to proceed in the direction of malate oxidation the concentration of oxaloacetate must be maintained extremely low and this is accomplished under normal conditions by removal of oxaloacetate in the citrate-synthase reaction, the equilibrium of which favours citrate formation. Attempts to assay citrate synthase directly gave no conclusive evidence of inactivation of this enzyme. An alternative means of disposal of oxaloacetate is decarboxylation to pyruvate. There is evidence that this process occurs in the cell suspension but not excessively (Table 12).

No oxaloacetate was detected in cell suspensions incubated without added substrate and the amount found when citric acid-cycle substrates were added was 3–4 μM. This concentration is about half that found in fresh liver (Kalnitsky & Tapley, 1958; Hohorst *et al.* 1959; Hohorst, Kreutz, Reim & Hübener, 1961; Wieland, Löffler, Weiss & Neufeldt, 1960; Löffler & Wieland, 1963) and is probably below the level required to saturate citrate synthase in liver. The saturating concentration for purified pig-heart citrate synthase is about 0·4 mM (Stern, Shapiro, Stadtman & Ochoa, 1951). It seems therefore that a low concentration of oxaloacetate may limit the rate of turnover of the citric acid cycle in isolated liver cells.

A possible explanation for lowering of the oxaloacetate concentration may lie in the oxidation–reduction state of the NAD system to which the malate-dehydrogenase system is coupled. The malate/oxaloacetate ratio in cell suspensions is 360–690, as compared with an equilibrium ratio 657 (25°, pH 7·55) found with the purified malate dehydrogenase of muscle (Burton & Wilson, 1953) and 55–71 for the malate/oxaloacetate ratio in fresh liver (Hohorst *et al.* 1959, 1961). In view of these facts it would not be surprising if the oxidation–reduction potentials of the NAD systems to which the malate-dehydrogenase reaction is linked in cell suspensions were more negative than those in whole liver. The mean $NADH/NAD^+$ ratio of cell suspensions incubated without substrate is 1·7 and it is 4·5 in the presence of citric acid-cycle substrates. These values are much higher than those reported for fresh rat liver (Helmreich, Holzer, Lamprecht & Goldschmidt, 1954; Spirtes & Eichel, 1954; Glock & McLean, 1955; Jedeikin & Weinhouse, 1955; Jacobson & Kaplan, 1957; Bassham *et al.* 1959; Christie & Le Page, 1962).

A significant feature of the metabolism of isolated liver cells is the ready aerobic reduction of acetoacetate in the presence of hydrogen donors such as pyruvate and citric acid-cycle intermediates. The formation of β-hydroxybutyrate reflects an increased supply of NADH consistent with the high $NADH/NAD^+$ ratio found by analysis.

The experimental results do not provide an explanation of the increased $NADH/NAD^+$ ratio. It is suggested that a defect in the respiratory chain might limit the rate of oxidation of NADH. If this were considered to be the primary defect, the consequences could be a strong reducing potential and limitation of the activity of the citric acid cycle.

SUMMARY

1. Cells isolated from rat liver showed disruption of the plasma membrane with loss of protein, neutral lipids and certain soluble enzymes. Glycolysis was greatly impaired.

2. The cells consumed oxygen and produced large quantities of ketone bodies when incubated at 30° in various media. Respiration and acetoacetate production were inhibited by Ca^{2+} and phosphate ions and media of high osmolarity.

3. The cells oxidized citric acid-cycle intermediates with the accumulation of malate, fumarate, succinate and pyruvate–oxaloacetate. Malonate, fluoroacetate and arsenite produced changes consistent with the operation of a citric acid cycle.

4. Citric acid-cycle intermediates and related substrates promoted the aerobic reduction of acetoacetate with little effect on total ketone-body production by the cells.

5. Studies with [2,3-$^{14}C_2$]succinate and [3-^{14}C]-pyruvate confirmed the operation of a citric acid cycle in the cells. Pyruvate was oxidized to carbon dioxide and ketone bodies and partly reduced to lactate.

6. About one-fifth of the total nicotinamide nucleotides present in fresh liver was recovered in the cells. Incubation caused large increases in the $NADH/NAD^+$ and $NADPH/NADP^+$ ratios, especially in the presence of oxidizable substrates.

7. It is suggested that a high $NADH/NAD^+$ ratio accounts for the ready aerobic reduction of acetoacetate and the apparently low activity of the citric acid cycle in isolated liver cells.

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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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