is completely dependent on the presence of ATP, taurine, cholic acid and CoA. The mechanism of the synthesis is discussed.

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Glucose Oxidation by Brain Mitochondria

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Christie, Judah & Rees (1953), working with rat-brain mitochondrial preparations, measured the rates of oxidation of substrates of the tricarboxylic acid cycle and the process of oxidative phosphorylation. These measurements were employed as indicators of the survival of mitochondria as effective units. The survival time is short, but may be prolonged by the addition of a number of soluble factors including adenosine triphosphate, coenzyme I, glutathione, cobalt, and what was believed to be a new cofactor present in liver and yeast autolysates. In the present paper, experiments are described which show that the activity observed in tissue autolysates was due to glucose or glycolytic intermediates. It was found that rat-brain mitochondrial preparations, in contrast to liver and kidney mitochondrial preparations, are capable of oxidizing glucose completely to carbon dioxide and water.

METHODS

Enzyme preparations. Mitochondria were prepared in 0.25M sucrose (Schneider, 1948).

Reagents. Adenosine triphosphate (ATP) was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). Adenylic acid was a commercial preparation, recrystallized from hot water. All adenine nucleotides were at least 95% pure as determined by a spectrophotometric method (Kalckar, 1947). Cytochrome *c* was prepared by a method of Keilin & Hartree (1937) and dialysed against distilled water. Coenzyme I (CoI) was prepared by an unpublished method of Ochoa and coenzyme II by the method of LePage & Mueller (1949). Glutathione and DL-isocitric acid were commercial preparations.

Methods of analysis. α -Oxoglutarate and pyruvate were estimated by the method of Friedemann & Haugen (1943).

Lactate was determined by the method of Barker & Summerson (1941), and citrate by the method of Weil-Malherbe & Bone (1949).

Oxygen uptakes were measured by Warburg manometers with KOH solution to absorb CO_2 .

Unless specified the following *standard medium* was used, the concentrations given being those in the final mixture in the Warburg flasks: adenylic acid 0.001M; ATP 0.001M; MgSO_4 0.0067M; KCl 0.025M; sodium phosphate buffer pH 7.4, 0.0133M; cytochrome *c* 2×10^{-5} M; glutathione 6.6×10^{-4} M; enzyme was added in 0.25M sucrose, followed by water to 3 ml. total volume. Gas phase, air; temperature, 38°.

In experiments where citrate analyses were carried out, the enzyme preparation was added in 0.5 ml. of 0.15M-KCl, and KCl was omitted from the standard medium.

All the experiments reported were carried out in duplicate and the results given are mean values.

Preparation of yeast extract. Yeast was autolysed for 6 hr. at 38° in the presence of toluene. The proteins were precipitated by the addition of equal volumes of 0.3M- Ba(OH)_2 and 5% (w/v) ZnSO_4 solution. The protein precipitate was removed by filtration. The filtrate was placed in the cathode chamber of a three-compartment electro-dialysis apparatus and the other compartments were filled with water. After electro-dialysis the required activity was located in the centre compartment, and every 10 g. of fresh yeast yielded on freeze-drying 5.5 mg. of solid material. In the experiments described in the text 150 μg . of this material in 0.5 ml. of water was added to each Warburg flask.

RESULTS

In an earlier publication (Christie *et al.* 1953) it had been observed that the oxidation of L-glutamate by brain mitochondrial preparations fell off after a period of 1.5 hr. This decline in respiration was prevented by preparations from autolysed liver or brain or yeast.

In the present investigation a yeast autolysate was used as a source of the active material. This was tested with all the substrates of the tricarboxylic acid cycle, and it was found that with citrate oxidation an immediate increase of the respiration occurred, whereas with the other substrates the effect was the same as that observed with L-glutamate (Table 1). The same result was obtained when these substrates were tested in one-step oxidations, e.g. when the oxidation of citrate was carried out in the presence of arsenite.

The result of a complete balance study of the oxidation of citrate to α -oxoglutarate in the presence of arsenite is shown in Table 2. It will be observed that the citrate disappearance, the accumulation of keto acid and the consumption of oxygen agree well with the requirements of the reaction, and that an increase in rate was found in the presence of the yeast preparations. An experiment with DL-isocitrate as substrate is included in the table, and it will be seen that a similar effect is obtained with this substrate. In other experiments the yeast preparation was tested on a soluble isocitric dehydrogenase prepared from brain, but on this system the yeast preparation had no effect.

We had observed that the addition of calcium ions stimulated the oxidation of citrate by brain mitochondria; we therefore examined the yeast preparation and found that free calcium ions were present in all samples. Keilin & Hartree (1949) have shown that the oxidation of succinate by heart preparations is stimulated by the addition of calcium, and that the same effect can be obtained by increasing the phosphate concentration of the medium. In the presence of high phosphate concentration, calcium no longer increases the oxygen uptake. We obtained identical results in brain mitochondrial preparations oxidizing citrate (Table 3).

When the yeast preparation was tested with optimum phosphate concentrations it was found still to show a stimulatory action on the oxidation of citrate (Table 3, Expt. 3). The removal of calcium ions by an ion-exchange resin (Zeo-Karb 215) left a considerable residue of activity in the yeast preparation.

Table 1. *Effect of a yeast preparation on the oxidation of citrate by brain mitochondria*

System: standard medium and 30 μ moles of citrate. Arsenite (0.007M) was added as indicated in the table. Brain mitochondria were added equivalent to 150 mg. of fresh tissue. 0.5 ml. of yeast preparation was added as indicated in the table. Duration of the experiment was 50 min.

Additions	O ₂ uptake (μ g. atoms)
Citrate	6.9
Citrate and yeast preparation	12.5
Citrate and arsenite	2.3
Citrate and arsenite and yeast preparation	7.9

Table 2. *Effect of the yeast preparation on the one-step oxidation of citrate and isocitrate by brain mitochondria*

System: standard medium, 15 μ moles of substrate as indicated in the table. Final concentration of arsenite was 0.007M. Brain mitochondria were added equivalent to 250 mg. of fresh tissue, added in 0.5 ml. of 0.15M-KCl. Duration of experiment was 60 min.

Additions	O ₂ utilized (μ g. atoms)	Citrate utilized (μ moles)	Keto acid formed (μ moles)
Citrate (15 μ moles)	1.5	1.5	1.3
Citrate (15 μ moles) + yeast preparation	4.5	4.2	4.5
isoCitrate (15 μ moles)	1.2	—	1.3
isoCitrate (15 μ moles) + yeast preparation	3.6	—	3.5

Table 3. *Effect of calcium and phosphate at various concentrations on the oxidation of citrate by brain mitochondria*

System: standard medium; sodium phosphate buffer pH 7.4, concentration as indicated in the table; 30 μ moles of citrate as substrate. Calcium concentration as indicated in the table. Brain mitochondria were added equivalent to 150 mg. of fresh tissue. Duration of the experiment was 60 min.

Expt. no.	Yeast preparation	Phosphate (final concn.) (M)	Calcium (final concn.) (M)	O ₂ uptake (μ l.)
1	Nil	0.013	Nil	55
	Nil	0.013	5.4×10^{-4}	73
	Nil	0.08	Nil	73
2	Nil	0.013	Nil	65
	Nil	0.013	3.3×10^{-4}	96
	Nil	0.08	Nil	107
	Nil	0.08	10^{-3}	108
	Nil	0.08	3.3×10^{-4}	110
3	Nil	0.08	Nil	60
	0.5 ml.	0.08	Nil	116

Table 4. *Effect of decalcified yeast preparation on the oxidation of citrate by brain mitochondria*

System: standard medium; 30 μ moles of citrate were added as indicated in the table; 0.5 ml. of decalcified yeast preparation was added where indicated; arsenite at final concn. of 0.0077 M. Brain mitochondria were added equivalent to 250 mg. of fresh tissue, prepared in KCl and added to the Warburg flask in 0.5 ml. of 0.15 M-KCl. Duration of the experiment was 60 min. Quotient of absorptions of 2:4-dinitrophenylhydrazones at 420 and 520 $m\mu$. for pyruvate was 1.20 and for α -oxoglutarate was 1.40.

Additions	O ₂ utilized (μ g. atoms)	Citrate utilized (μ moles)	'420/520 $m\mu$.' quotient	Pyruvate formed (μ moles)	Corrected '420/520 $m\mu$.' quotient*	α -Oxo-glutarate formed (μ moles)
Nil	0.3	—	—	Nil	—	—
Yeast preparation (0.5 ml.)	4.9	—	1.2	1.6	—	Nil
Citrate (30 μ moles)	8.3	8.5	1.39	Nil	1.43	7.9
Citrate (30 μ moles) + yeast preparation (0.5 ml.)	12.1	8.1	1.35	1.6	1.43	8.3

* The 420 and 520 $m\mu$. absorptions of the 2:4-dinitrophenylhydrazones of the blanks, i.e. 'nil' and the yeast preparation alone, were subtracted from the absorptions obtained with citrate alone and citrate + yeast preparation respectively. The 420/520 $m\mu$. quotient was then determined.

Table 5. *Oxidation of glucose and glucose 6-phosphate by brain mitochondria*

System: standard medium; substrate added as indicated in the table; brain mitochondria added equivalent to 150 mg. of fresh tissue. Duration of experiment was 4 hr.

Additions	O ₂ required for complete oxidation (μ g. atoms)	O ₂ utilized* (μ g. atoms)
Glucose (2 μ moles)	24	23.2
Glucose 6-phosphate (2 μ moles)	24	22.8

* The O₂ uptake of the control (without added substrate) has been deducted from these figures.

Investigation of the decalcified material showed a considerable change in its effect on the one-step oxidation of citrate in the presence of arsenite. A balance study revealed that, although oxygen uptake and keto acid accumulation were increased, the removal of citrate was unchanged (Table 4). In other words, it was certain that a substrate other than citrate was being oxidized. By the use of the Friedemann & Haugen (1943) method it was possible to show that the keto acid accumulating in the presence of the yeast preparation alone was probably pyruvic acid, because of the characteristic ratio of the absorption at 420 $m\mu$. to the absorption at 520 $m\mu$. of its 2:4-dinitrophenylhydrazone. Glucose or glycolytic intermediates would serve as a precursor of pyruvate in such a system provided that the glycolytic enzymes were present. We tested glucose and glucose 6-phosphate and found that both were oxidized aerobically (Table 5). In these experiments oxygen uptake was measured until it ceased. After subtraction of the control without substrate the figures for the oxygen uptake agree well with those theoretically required for the complete oxidation of the substrate to CO₂

and water. Glucose and fructose 1:6-diphosphate were both glycolysed under anaerobic conditions (Table 6) by brain mitochondrial preparations.

Under aerobic conditions in the presence of arsenite pyruvate accumulated, and the addition of fluoride to the system prevented the formation of pyruvate (Table 7). In the absence of either inhibitor a strong oxidation proceeded without significant accumulation of keto acid.

Table 6. *Anaerobic glycolysis by brain mitochondria*

System: adenylic acid, 0.0017 M; MgSO₄, 0.0066 M; KCl, 0.025 M; inorganic phosphate, 0.0133 M; sodium bicarbonate, 0.025 M; pyruvate, 0.0066 M; CoI, 0.00033 M; ATP, 0.001 M. Mitochondrial suspension, washed three times by recentrifuging, was added in amount equivalent to 250 mg. of fresh tissue. Gas phase N₂ + CO₂ (95:5). Duration of the experiment was 65 min.

Additions	CO ₂ evolved (μ moles)	Lactate formed (μ moles)
Nil	2.6	1.7
Glucose (30 μ moles)	9.3	9.5
Glucose (30 μ moles) + hexose diphosphate (6 μ moles)	10.0	12.5

Table 7. *Oxidation of glucose by brain mitochondria in the presence of arsenite and sodium fluoride*

System: standard medium; NaF where added, final concentration 0.014 M; arsenite, final concentration 0.007 M. Duration of experiment was 135 min.

Additions	O ₂ utilized (μ g. atoms)	Keto acid formed (μ moles)
Glucose (90 μ moles)	41.0	α -Oxoglutarate (0.6)
Glucose (90 μ moles) + As ₂ O ₃	7.6	Pyruvate (3.5)
Glucose (90 μ moles) + As ₂ O ₃ + NaF	8.2	Nil
Glucose (90 μ moles) + NaF	19.2	Nil

It would therefore appear that brain mitochondrial preparations can carry out a complete oxidation of glucose or of glycolytic intermediates.

As a matter of interest we tested rat-liver and rat-kidney mitochondrial preparations for glycolytic ability. No appreciable activity could be detected, aerobically or anaerobically.

DISCUSSION

In a previous paper Christie *et al.* (1953) described what was thought to be a new cofactor. However, Sir Rudolph Peters (personal communication) pointed out that it might be an unknown substrate, and this we admitted at the time though we did not consider it likely. In the present work we have resolved this 'cofactor' into its components. We find that in the presence of low concentrations of inorganic orthophosphate the oxidation of citrate is increased by the presence of calcium ions. Calcium was present in the 'cofactor' preparations. This effect of calcium could be simulated by increasing the inorganic orthophosphate concentration. This finding is in agreement with that reported by Keilin & Hartree (1949) for the oxidation of succinate by a purified system. We were surprised that we obtained this effect in our preparations, as calcium ions are generally regarded as being inhibitory in freshly prepared mitochondrial systems which are oxidizing substrates other than succinate. The presence of calcium explains why we were able to draw up balance sheets for the one-step oxidation of citrate to α -oxoglutarate that showed an increase in disappearance of citrate and an accumulation of α -oxoglutarate as compared with a control system.

The activating effect of calcium described by Keilin & Hartree (1949) was due to the formation of calcium phosphate gel. In these brain preparations an alternative explanation may be possible. The slow rate of oxidation by brain preparations might be due to the fact that citrate does not penetrate the intact mitochondrion very well. Calcium is known to bring about the rapid swelling of mitochondria suspended in isotonic media, presumably by increasing the permeability of the mitochondrial membrane to solutes, and phosphate behaves similarly. Alternatively the complex of calcium and citrate might penetrate more readily than citrate. In view of our findings that endogenous citrate (i.e. citrate produced during the oxidation of pyruvate) is oxidized rapidly, it would seem that these latter explanations based on permeability would be more applicable here.

The results in Table 2 show a good balance between citrate utilized and α -oxoglutarate formed in the presence of the yeast preparation. The increased rates observed must be due in this experi-

ment to the action of calcium and not glucose. With the increased rate of citrate oxidation, glucose would not be utilized.

The yeast preparation also contained another substance or substances that gave an increase of oxygen uptake when citrate was being oxidized. Balance studies of this system showed clearly that the extra oxygen uptake was due to oxidation of substrates present in the yeast preparations which gave rise to a keto acid, probably pyruvate. The whole of this effect could be duplicated by the addition of glucose to the Warburg flask. In addition, we have been able to show that isolated brain mitochondria can carry out the anaerobic conversion of glucose into lactate; in the presence of oxygen a complete oxidation to CO_2 and water can take place. It is interesting that rat-liver and rat-kidney mitochondria are unable to carry out this series of reactions.

The oxidation of added citrate by brain mitochondria was low in comparison with the oxidation of other tricarboxylic acid-cycle intermediates. It would appear that this is the reason why an immediate effect of the 'cofactor' was observed. This may be contrasted with the effect of the 'cofactor' on the oxidation of L-glutamate. With this substrate no effect was observed until late in the experiment. Until the rate of oxidation was reduced adenosine diphosphate would not be available to permit the oxidation of glucose. Why these brain preparations should oxidize citrate so poorly is as yet not clear. The addition of pyridine nucleotides had no effect on the rate, and even stimulation by calcium or by high phosphate concentration failed to bring the rate of oxidation to the level observed with other tricarboxylic acid-cycle intermediates. These low rates are found only with added citrate (cf. Peters & Wakelin, 1953); thus when the oxidation of pyruvate was studied we were unable to find any significant accumulation of citrate although pyruvate was being oxidized at a considerable rate.

SUMMARY

1. A study of rat-brain mitochondria has shown that these preparations possess properties not found in the mitochondria of liver, kidney and heart.

2. Rat-brain mitochondria have been found capable of oxidizing glucose and glycolytic intermediates completely to carbon dioxide and water.

3. Calcium, which is inhibitory to freshly prepared liver and kidney mitochondrial preparations, stimulates the oxidation of citrate by brain mitochondrial preparations.

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Observations on some Nutritional Factors that Influence the Lipotropic Activity of Methionine

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The discovery that fatty infiltration of the liver is a sign, not only of a choline deficiency, but also of certain amino acid deficiencies (Singal, Hazan, Sydenstricker & Littlejohn, 1953; Harper, Monson, Benton & Elvehjem, 1953; Cole & Scott, 1954) emphasized the need for a re-investigation of the lipotropic action of protein. Subsequently it was shown, in experiments in which there was only one variable, that two distinct lipotropic effects of protein can be demonstrated (Harper, Monson, Benton, Winje & Elvehjem, 1954*c*; Harper, Benton, Winje & Elvehjem, 1954*b*). In animals fed on diets deficient in choline, the methionine contained in the dietary protein serves as a precursor for the synthesis of choline (which was previously well known) and, in animals fed on diets low in protein, additional amounts of well-balanced proteins prevent partial deficiencies or imbalances of essential amino acids which cause an accumulation of liver fat. These observations, which explained many of the inconsistencies in earlier studies on the lipotropic action of protein, have been confirmed and similar conclusions have been drawn by Lucas & Ridout (1955). The papers of Harper, Benton, Winje & Elvehjem (1954*a*), Lucas & Ridout (1955) and Best, Hartroft, Lucas & Ridout (1955) contain references to much of the early literature. The two types of fatty infiltration can also be differentiated histologically (Shils &

Stewart, 1954; Nino-Herrera, Harper & Elvehjem, 1954; Cole & Scott, 1954; Best *et al.* 1955).

Subsequently, when these effects were being studied in greater detail, an anomalous and complicating situation was encountered. It had been shown previously (Singal *et al.* 1953; Harper *et al.* 1953) that a supplement of threonine, gelatin or casein decreased the deposition of liver fat in rats receiving a diet low in protein (casein) and containing choline. Threonine and casein were found to have similar though less pronounced effects when the choline in the diet was replaced by methionine (Harper *et al.* 1954*b*), but gelatin, surprisingly, was without effect under these conditions. The original object of the investigation reported in this paper was to explain this unexpected observation; the end result, however, has been a study of some factors that influence the lipotropic activity of methionine.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain, 21 days old and weighing 40–50 g., were used throughout this investigation. Each group consisted of six rats maintained in individual, suspended cages with $\frac{1}{2}$ in. mesh screen bottoms. The average initial weights for the groups within each experiment did not differ by more than 1 g. The rats were fed *ad lib.* and were weighed weekly.

The percentage composition of the basal diet was as follows: casein, 9.0; sucrose, 82.0; corn oil, 5.0; salts (Hegsted, Mills, Elvehjem & Hart, 1941), 4.0. Vitamins were included to provide (in mg./100 g. of ration): thiamine

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