linoleic acids rather than in stearic acid. Thus the amount of saturated fatty acids in the liver phospholipids decreased.

The liver phospholipids were fractionated by silicic acid chromatography. After $CCl_4$ treatment, the arachidonic acid content in the cephalin and lecithin fractions of liver decreased from 24.7 % to 17.6 % and from 25.5 to 15.2 %, respectively, while the saturated fatty acids were unaffected. After ethionine treatment, the palmitic acid content in the cephalin and lecithin fractions decreased from 17.1 % to 11.6 % and from 26.6 % to 17.8 %, respectively; however, the arachidonic acid level was not changed significantly.

The triglycerides which accumulate in the liver after $CCl_4$ and ethionine administration are synthesized primarily from fatty acids mobilized from the depots. In addition these two agents case qualitative changes in the composition of the liver lipids. $CCl_4$ lowers the arachidonic acid content and correspondingly raises the oleic and linoleic acid content of liver phospholipids; ethionine lowers the palmitic acid content of both liver triglycerides and phospholipids. Because arachidonic acid is an essential fatty acid, its decrease may be of particular significance in the action of $CCl_4$ on the liver.

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## The mechanism of the reduction of mitochondrial DPN+ coupled with the oxidation of succinate

There is now considerable evidence[1–5] in support of CHANCE's original suggestion[6] that the reduction of mitochondrial DPN+ brought about by the addition of succinate to aerobic mitochondria in State 4 (*i.e.* rate of respiration limited by ADP concentration) is due to a reversal of the respiratory chain.

We have studied this reaction in rat-liver mitochondria by coupling it to the synthesis of glutamate in the presence of α-ketoglutarate and $NH_3$, with arsenite added to prevent the oxidation of α-ketoglutarate. Aspartate was found as well as glutamate; it must have been synthesised from malate (derived from succinate) according to Reactions 1 and 2.

$$\text{malate} + \text{DPN}^+ \leftrightharpoons \text{oxaloacetate} + \text{DPNH} + \text{H}^+ \quad (1)$$

$$\text{glutamate} + \text{oxaloacetate} \leftrightharpoons \alpha\text{-ketoglutarate} + \text{aspartate} \quad (2)$$

Since in State 4 the oxidation of DPNH by oxygen through the respiratory chain is almost completely inhibited, the DPNH formed in Reaction 1 must be oxidized by α-ketoglutarate and $NH_3$ (Reaction 3).

$$\text{DPNH} + \alpha\text{-ketoglutarate} + \text{NH}_3 + \text{H}^+ \leftrightharpoons \text{DPN}^+ + \text{glutamate} \quad (3)$$

Reaction 4, the sum of Reactions 1–3,

$$\text{malate} + \text{NH}_3 \rightleftharpoons \text{aspartate} \quad (4)$$

describes the synthesis of aspartate and is a measure of the amount of $DPN^+$ reduced by malate. The extent of reduction of $DPN^+$ by succinate is given by the amount of glutamate synthesised.

Table I shows that the synthesis of glutamate was increased by oligomycin (*cf.* ERNSTER[4]) and inhibited by dinitrophenol, antimycin, Amytal or malonate. The remainder of the experiments described in this paper were carried out in the presence of oligomycin.

Oligomycin inhibits the formation of ATP from a dinitrophenol-sensitive high-energy intermediate of oxidative phosphorylation[9,10]. The considerable stimulation by oligomycin of the synthesis of glutamate and the inhibition by dinitrophenol (even in the presence of oligomycin which inhibits the dinitrophenol-induced ATPase[9]) shows clearly that the dinitrophenol-sensitive intermediate (or another intermediate in equilibrium with it) is involved in the reduction of $DPN^+$ by succinate. The utilization of the energy of a high-energy intermediate implies that it will be split. Thus, the reduction of $DPN^+$ would be expected to "uncouple" the system and to relieve the inhibition of respiration by oligomycin, in much the same way as dinitrophenol (*cf.* ERNSTER[4]). Fig. 1 shows, indeed, that the addition of α-ketoglutarate and $NH_3$, which will promote the succinate-linked reduction of $DPN^+$ by removing DPNH,

TABLE I

EFFECT OF INHIBITORS ON GLUTAMATE SYNTHESIS COUPLED TO SUCCINATE OXIDATION

Reaction mixture (1.0 ml) contained: 15 m*M* KCl, 2 m*M* EDTA, 50 m*M* Tris–HCl, 20 m*M* $KH_2PO_4$–$K_2HPO_4$, 5 m*M* $MgCl_2$, 20 m*M* $NH_4Cl$, 10 m*M* (Expts. 71 and 78) or 20 m*M* (Expts. 90 and 104) α-ketoglutarate, 60 m*M* succinate, 0.1 m*M* ADP, 1 m*M* arsenite, 25 m*M* sucrose and 3.9–5.5 mg mitochondrial protein. The pH was 7.5. Reaction carried out in Warburg vessels at 25°, with KOH in centre well. Reaction time, 15 min (Expts. 71 and 78) or 20 min (Expts. 90 and 104). Glutamate was determined with glutamate decarboxylase[7], and aspartate by the method of PFLEIDERER *et al.*[8].

| *Expt.* | *Addition* | *ΔGlu (μmoles)* | *ΔAsp (μmoles)* |
|---|---|---|---|
| 71 | none | 0.94 | 0.29 |
| | oligomycin (1.6 μg/mg protein) | 2.84 | 1.61 |
| | dinitrophenol (50 μ*M*) | 0.35 | 0.13 |
| | oligomycin + dinitrophenol | 0.29 | 0.17 |
| 78 | none | 0.69 | 0.24 |
| | oligomycin (1.5 μg/mg protein) | 3.24 | 1.44 |
| | Amytal (2 m*M*) | 0.69 | 0.38 |
| | Amytal + oligomycin | 0.54 | 0.90 |
| 90 | oligomycin (1.1 μg/mg protein) | 6.39 | 3.09 |
| | oligomycin + malonate (20 m*M*) | 0.43 | 0.09 |
| 104 | oligomycin (2.5 μg/mg protein) | 4.22 | 2.42 |
| | oligomycin + antimycin (0.5 μg/mg protein) | 0.49 | 0 |

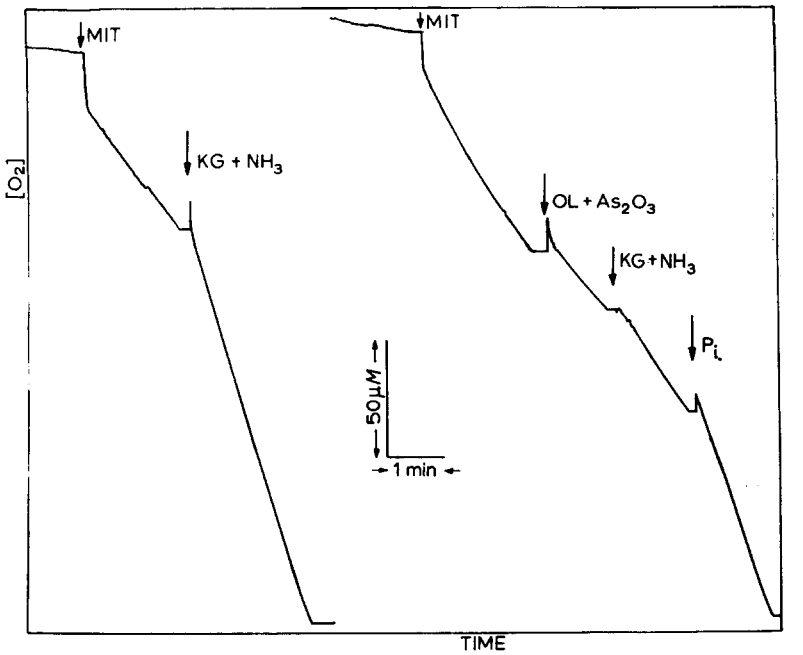


Fig. 1. Effect of α-ketoglutarate + $NH_3$ on succinate oxidation. Tracings of G.M.E. Oxygraph records. Mitochondria (4.3 mg protein) added to solution containing: 15 m*M* KCl, 2 m*M* EDTA, 5 m*M* $MgCl_2$, 50 m*M* Tris–HCl, 0.1 m*M* ADP, 10 m*M* glutamate, 20 m*M* glucose, hexokinase, 30 m*M* succinate and (left-hand trace only) 1 m*M* arsenite, 10 μg oligomycin and 5 m*M* $P_i$. Additions made as indicated by the arrows. Abbreviations: MIT, mitochondria; KG + $NH_3$, 18 μmoles α-ketoglutarate + 18 μmoles $NH_4Cl$; OL + $As_2O_3$, 10 μg oligomycin + 2 μmoles arsenite; $P_i$, 10 μmoles $P_i$. Final volume, 2.0 ml; pH, 7.5; temperature, 25°.

stimulates the $O_2$ uptake, but only in the presence of inorganic phosphate. Other experiments showed that phosphate or arsenate is also required for the synthesis of glutamate under these conditions.

Table II shows some aspects of the stoicheiometry. The following points are worthy of attention: (i) in the absence of oligomycin, the addition of α-ketoglutarate and $NH_3$ led to a definite lowering of the P:O ratio, confirming that the synthesis of glutamate brings about an uncoupling of oxidative phosphorylation; (ii) the increased

TABLE II

STOICHEIOMETRY OF OXYGEN UPTAKE, PHOSPHORYLATION AND GLUTAMATE SYNTHESIS

Experimental conditions as in Table I, with addition of 20 m*M* glucose and hexokinase. Reaction time, 20 min. Esterified phosphate was determined by Method I of SLATER[11].

| *Oligomycin (μg/mg protein)* | *α-Keto-glutarate (mM)* | *$NH_3$ (mM)* | *ΔO (μatoms)* | *Δest. P (μmoles)* | *ΔGlu (μmoles)* | *ΔAsp (μmoles)* | *P:O* | *Glu:O* |
|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 10.1 | 14.4 | 0 | 0.04 | 1.43 | 0 |
| 0.2 | 0 | 0 | 3.9 | 1.1 | — | — | 0.28 | — |
| 2.1 | 0 | 0 | 4.6 | 0 | — | — | 0 | — |
| 21.0 | 0 | 0 | 5.2 | 0 | — | — | 0 | — |
| 0 | 20 | 20 | 9.6 | 10.6 | 2.36 | 2.20 | 1.11 | 0.25 |
| 0.2 | 20 | 20 | 6.8 | 0.8 | 4.84 | 2.82 | 0.12 | 0.71 |
| 2.1 | 20 | 20 | 7.0 | 0 | 4.33 | 2.84 | 0 | 0.62 |
| 21.0 | 20 | 20 | 6.5 | 0 | 4.30 | 2.18 | 0 | 0.66 |

oxygen uptake brought about by the addition of α-ketoglutarate + $NH_3$ in the presence of oligomycin was not associated with any phosphorylation; (iii) the ratio glutamate:O in the presence of oligomycin was about one-half the P:O ratio measured in the absence of oligomycin, α-ketoglutarate and $NH_3$. (The mean glutamate:O ratio in 11 experiments was 0.73.)

This last-mentioned finding strongly suggests that only one of the two dinitrophenol-sensitive intermediates formed during the oxidation of succinate is involved in the reduction of $DPN^+$. Phosphate (or arsenate) presumably in some way uncouples the other phosphorylation step. This must be unrelated to the normal phosphorylation reaction, since no phosphorylation was found.

Formulating the succinate chain thus

$$\text{succinate} \longrightarrow A \overset{\sim}{\longrightarrow} B \overset{\sim}{\longrightarrow} C \longrightarrow O_2$$

we propose the mechanism given in Reactions 5–12 to describe the reduction of mitochondrial $DPN^+$ coupled with the oxidation of succinate.

$$2 \text{ succinate} + 2 A \longrightarrow 2 \text{ fumarate} + 2 AH_2 \quad (5)$$

$$AH_2 + B + I \leftrightharpoons A \sim I + BH_2 \quad (6)$$

$$A \sim I + H_2O \longrightarrow A + I \quad (7)$$

$$BH_2 + C + I' \leftrightharpoons B \sim I' + CH_2 \quad (8)$$

$$CH_2 + O \longrightarrow C + H_2O \quad (9)$$

$$B \sim I' + DPN^+ + AH_2 \leftrightharpoons B + I' + DPNH + A + H^+ \quad (10)$$

$$DPNH + H^+ + \alpha\text{-ketoglutarate} + NH_3 \leftrightharpoons DPN^+ + \text{glutamate} \quad (11)$$

*Sum:* $2 \text{ succinate} + O + \alpha\text{-ketoglutarate} + NH_3 \longrightarrow 2 \text{ fumarate} + \text{glutamate}$ (12)

Reaction 7 is promoted by phosphate or arsenate.

Our conclusions differ in some respects from those made by ERNSTER[4] from experiments on the succinate-induced reduction of acetoacetate. These differences will be discussed in detail elsewhere.

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