An improved method for the isolation of barium phosphoarginine

The purpose of this communication is to outline the general principles involved in a new method for the preparation, in good yield, of barium phosphoarginine and to draw attention to the fact that the resultant compound has a structure considerably different from that hitherto described.

Phosphoarginine was first isolated from crayfish muscle by MEYERHOF AND LOHMANN[1] who used a procedure which involved precipitation of the phosphagen from the soluble Ba salts of a protein-free extract first as a sulphate and then as a barium salt. The compound isolated corresponded to the formula $(C_6H_{14}O_5N_4P)_2Ba.2H_2O$.

In connection with studies of the enzyme, arginine phosphokinase, it was necessary to prepare pure arginine phosphate, and the method of MEYERHOF AND LOHMANN[1] was modified in such a way as to avoid the large losses inherent in the older procedure.

Briefly the new method consists of the following steps; the insoluble barium salts were precipitated at pH 9.0 from a protein-free extract of crayfish muscle. The water-soluble barium salts present in the supernatant were then precipitated by the addition of 3 vols. of ethanol and collected by centrifugation. The phosphoarginine contained therein was recovered in quantitative yield as a Cu salt which was then converted into and precipitated as phosphoarginine hydrochloride after removal of the Cu as CuS. The hydrochloride which on elementary analysis corresponded to the formula $(C_6H_{15}O_5N_4P)HCl$ was then converted into a Ba salt.

The conditions under which this final precipitation was carried out were similar to those described by MEYERHOF AND LOHMANN[1] but the yield was about 30 times greater (10 g/kg fresh muscle).

It was anticipated that the compound would have the same structure as that described by MEYERHOF AND LOHMANN[1], but its elementary analysis and chemical properties corresponded to the formula $(C_6H_{13}O_5N_4P)Ba.H_2CO_3 \cdot H_2O$

The above procedure has been repeated many times and the final precipitation of the barium salt carried out at pH 7.0 and 8.0, and on each occasion a compound identical in nature to that described above was obtained. The failure to obtain a compound similar in constitution to that described by MEYERHOF AND LOHMANN[1] is difficult to explain with certainty, but the formula suggested would perhaps be anticipated havig regard to the pK of phosphoric acid moiety[2] and to basicity of the compound.

The complete details of this work will be submitted for publication elsewhere.

A. H. ENNOR
J. F. MORRISON
H. ROSENBERG*

Department of Biochemistry, John Curtin School of Medical Research, The Australian National University, Canberra (Australia)

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* Australian National University Research Scholar.

Factors influencing the oxidation of dl-β-hydroxybutyrate by tumour mitochondria

In a preceding publication[1] the adenosine triphosphate (ATP) dependent oxidation of octanoate by liver mitochondria was used to collect information on the ATP-splitting activity (ATPase) of tumour mitochondria, when it was found that addition of the latter to the former mitochondria abolished the oxygen consumption of the fatty acid oxidation process completely. As a result of those and similar experiments[2] high ATPase activities could be attributed to mitochondrial preparations from a number of mouse and rat tumours; direct measurements of the amount of inorganic phosphate liberated from ATP by the tumour mitochondrial suspensions using the *tris*-KCl medium of CHAPPELL AND PERRY[3] led to the same results[4].

The ATP-splitting activities interfere with the capacity of tumour mitochondria *per se* to oxidize fatty acids. The mitochondria from five tumours, which were otherwise inactive but whose ATPase activities could be lowered sufficiently, by inclusion of versene into the medium, were found to oxidize octanoate in the presence of a "sparker" and diphosphopyridine nucleotide (DPN); the mitochondria from the other tumours studied did not show this oxidation, even in a number of cases in which the ATPases were low[2].

In search for other factors which govern the oxidative behaviour of tumour mitochondria towards fatty acids, the oxidation of *dl*-β-hydroxybutyrate (BHB) has been studied. Both *d*- and

l-BHB are known to be oxidized by a number of normal tissues[5]. LEHNINGER AND GREVILLE[5] concluded from their experiments with rat liver preparations that the oxidation of the *d*-isomer proceeds after the formation of its coenzyme A(CoA) derivative, and thus depends on an initial supply of ATP. The *l*-isomer on the other hand, is oxidized to acetoacetate without the intervention of CoA and, accordingly, this oxidation is independent of ATP.

For the present experimental design mitochondria were prepared from mouse livers with (0.25 *M*) sucrose[6] and from tumours with (0.25 *M*) sucrose containing (0.001 *M*) versene (pH 7.4). After two washings mitochondrial suspensions, prior to incubation, were also made in these media[1,2].

The liver mitochondria obtained by this procedure are capable of oxidizing *dl*-BHB to acetoacetate without the necessity for adding a "sparker" (α-oxy caproate[7]) or DPN, which is the coenzyme of both the *l*-BHB and the *d*-BHByl-CoA dehydrogenase.

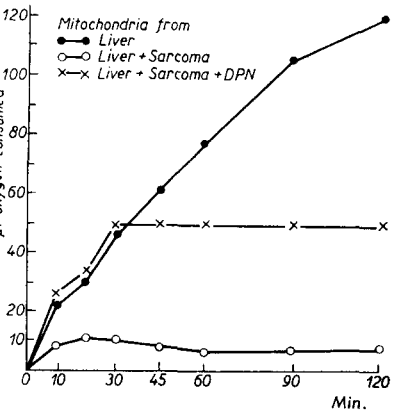

Fig. 1. Oxidation of *dl*-β-hydroxybutyrate by mouse liver mitochondria, and by a combination of liver and sarcoma mitochondria in the absence and in the presence of DPN. Additions: *dl*-BHB (0.0075 *M*), Mg^{2+} (0.005 *M*), ATP (0.0007 *M*), cytochrome *c* (0.00001 *M*) in a total volume of 1.6 ml KCl (0.06 *M*) -phosphate (0.013 *M*) buffer (pH 7.4), including 0.3 ml mitochondrial suspension from mouse liver (containing 1.56 mg N) prepared in 0.25 *M* sucrose, with or without 0.3 ml mitochondrial suspension from tumour (containing 0.87 mg N) prepared in 0.25 *M* sucrose-versene (0.001 *M*) (pH 7.4). DPN was added in a final concentration of 0.001 *M*. Incubation at 27°C with shaking in air. Oxygen uptake as the difference between O_2 consumption in the presence and absence of BHB under the particular conditions.

When, however, mitochondria from the transplanted mouse sarcoma UV 256 were added to the liver mitochondria, oxidation of *dl*-BHB to any significant extent did not take place (Fig. 1). In this combined system DPN appeared to be a limiting factor since addition of DPN resulted in an enhanced oxygen uptake, which, however, stopped after 30 minutes indicating that another oxidation process was inhibited. DPN did not stimulate the O_2 uptake of the liver mitochondria alone and, hence, it was concluded from the DPN-effect on the combined system that the sarcoma mitochondria possessed an active DPNase[8,9,10] which destroyed the DPN functioning as hydrogen carrier in the oxidation of BHB by the liver mitochondria. Addition of extra DPN at minute 45 to the combination of liver and tumour mitochondria incubated in the presence of DPN had no further effect, demonstrating that in the latter system DPN was no limiting factor anymore. The second process inhibited in the presence of the tumour mitochondria was thought to be an ATP dependent one, since the sarcoma mitochondria were found earlier to possess an active ATPase, even when prepared in sucrose-versene, which abolished the ATP dependent oxidation of octanoate by the liver mitochondria, irrespective of the fact whether DPN was added or not[2]. This view was strengthened by the fact that in a similar experiment, addition of fluoride (0.01 *M*) to the *dl*-BHB containing medium resulted in a greater oxygen consumption than when DPN was added alone. According to these arguments, it seems probable that by the combination of liver plus tumour mitochondria incubated in the presence of DPN, only the *l*-isomer of *dl*-BHB, being not dependent on ATP for its oxidation, is converted.

Mitochondria prepared from an adrenalcortex carcinoma (T 17572), from a sarcomatoid ovarian tumour (T 20212) and from butteryellow-induced adenocarcinomas of the rat liver had similar high ATPase and DPNase activities under the present conditions. The high ATPase activities in the sucrose-versene medium were also found earlier[2]. Mitochondria from the lymphosarcoma (T 86157) which had been found to possess low ATPase activities when prepared in sucrose-versene[2], exhibited marked DPNase activity in the BHB oxidation experiments. In this case liver plus tumour mitochondria plus DPN had the same rate of oxygen uptake as the liver mitochondria, thus confirming the absence of ATP-splitting activities influencing BHB oxidation. (Oxygen consumptions after 120 min. by mitochondria from liver: 116 μl, liver + tumour: 26 μl, liver + tumour + DPN: 130 μl).

Neither of these five tumours yielded particles which, when incubated separately, were capable of oxidizing *dl*-BHB in the presence of DPN (0.001 *M*) and α-oxy caproate (5 μM). The mitochondria from a granulosa cell tumour of the mouse ovary (T 5441) exhibited no DPNase activity. ATPase activity as measured by the inhibition of octanoate or BHB oxidation of the liver

mitochondria was also absent. Under the conditions in which the aforementioned tumour mitochondria failed, the mitochondria from this tumour showed sometimes the same O_2 uptake with *dl*-BHB as liver mitochondria.

Four other tumours (three transplanted hepatomas and one testis tumour) yielded mitochondria which oxidized various amounts of *dl*-BHB, in general a little less than half the amount oxidized by the liver mitochondria. These mitochondria showed insignificant DPNase and no ATPase activities. The present results, as far as the ATPase activities are concerned, confirm the earlier data[2]. The conditions governing the DPNase activities in relation to the ATPases of the tumour mitochondria are being studied further. A full account of our work will be published later.

Department of Biochemistry, Antoni van Leeuwenhoek-huis, P. EMMELOT
The Netherlands Cancer Institute, Amsterdam (Netherlands) C. J. BOS

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Inhibition of immune hemolysis by di*iso*propyl fluorophosphate

The demonstration that Ca^{++} and Mg^{++}, or other divalent cations, such as Co^{++} or Ni^{++}, are essential[1,2] for hemolysis of sheep erythrocytes (*E*) by rabbit antibody (*A*) and guinea pig complement (*C'*) suggests an enzymic mechanism, the postulate reaction scheme[3] being as follows:

$$E + A \rightleftharpoons EA \qquad (1)$$

$$EA + C'1,4 \xrightarrow{Ca^{++}} EAC'1,4 \xrightarrow[37^\circ]{\text{slow}} \text{inactive} \qquad (2)\ (2')$$

$$EAC'1,4 + C'2 \xrightarrow[(Co^{++} \text{ or } Ni^{++})]{Mg^{++}} EAC'1,4,2 \xrightarrow[37^\circ]{\text{rapid}} \text{inactive} \qquad (3)\ (3')$$

$$EAC'1,4,2 + C'3 \longrightarrow E^* \qquad (4)$$

$$E^* \longrightarrow \text{ghost} + \text{hemoglobin} \qquad (5)$$

where E^* refers to a damaged cell which undergoes further transformation with release of hemoglobin and *C'*1,2,3 and 4 refer to components of *C'*.

In the course of an investigation of chemical inhibitors of immune hemolysis, it was found that di*iso*propyl fluorophosphate (DFP) inhibited the immune hemolytic reaction. Inhibitory concentrations of DFP were less than the concentrations of DFP shown to be without effect on nonesterolytic enzymes[4,5].

Sheep erythrocytes, *A*, *C'*, *EA*, *EAC'*1,4, *EAC'*1,4,2, *C'*2,3, veronal buffer, and ethylenediaminetetraacetate (EDTA)-veronal buffer, used as diluent, were prepared as described in previous papers[6,7]. DFP was first diluted in *iso*propyl alcohol and kept as a stock solution. Aliquots were emulsified in 0.12 *M* sodium bicarbonate to a concentration of 0.125 *M* DFP and subsequent dilutions of the 0.125 *M* DFP were made in veronal buffer. All DFP emulsions were made from the stock DFP-*iso*propyl alcohol (2.5 *M* DFP) just before addition to the reaction mixtures. Control experiments showed that the alcohol itself and the alcohol-bicarbonate solution in the amounts used, did not affect the hemolytic reaction.

The effect of DFP on reaction steps (3) and (4) and on the overall reaction, *EA* + *C'*, was determined in the following manner: DFP was added to reaction mixtures containing *EA* or *EAC'* 1,4 in veronal buffer, or *EAC'*1,4,2 in EDTA-veronal buffer at 0°C immediately followed by the