Oxidative phosphorylation in a bacterial extract

Attempts to demonstrate a coupling between substrate oxidation and phosphate esterification in bacterial extracts have been negative or have yielded very low ratios of phosphate esterified to oxygen consumed[1]. That a coupling similar to that of mammalian systems does exist in microorganisms has, however, been indicated by LIPMANN[2] in his study of the mechanism of oxidation of pyruvate by *Lactobacillus delbrückii*. More recently FRENKEL[3] reported that light induced phosphorylation in extracts of a photosynthetic microorganism. In the present communication we wish to report the existence in crude extracts of *Mycobacterium phlei* of a system which can couple oxidation to phosphorylation.

When cell-free extracts were incubated with succinate as electron donor, and with glucose, yeast hexokinase, and AMP as a phosphate acceptor system, there was a pronounced disappearance of orthophosphate (Table I). This esterification of phosphate was dependent on the addition of the phosphate acceptor system and Mg^{++}. Since preparations from this organism contained ATPase, it was essential to add fluoride. Only 43% inhibition of ATPase could be obtained by the addition of fluoride; therefore the values reported for phosphate disappearance probably represent a minimum. Nevertheless, it was possible to obtain P/O ratios greater than 1.0 with succinate, malate, α-ketoglutarate, β-hydroxybutyrate and pyruvate as electron donors. Further evidence that this phosphorylation occurred above the substrate level was obtained by using uncoupling agents. Gramicidin and 2,4-dinitrophenol (DNP) $5 \cdot 10^{-5}$ *M*, inhibited the disappearance of orthophosphate with all substrates investigated.

At the end of such experiments aliquots of the supernatant were chromatographed in the methanol ammonia solvent described by BANDURSKI AND AXELROD[5]. When the systems contained only AMP as phosphate acceptor, there accumulated U.V. adsorbing material having the same R_F as ATP. Acceptor systems containing AMP, hexokinase and glucose showed material corresponding to ADP. Control systems did not contain any detectable ATP or ADP.

TABLE I

System	Oxygen (μ atoms)	Δ Pi (μM)	P/O
No additions	0.35	0	0
Acceptor system	0.55	0.3	
Succinate	3.26	1.0	0.3
Succinate + acceptor system	3.2	4.9	1.53
Succinate + acceptor system + DNP	3.6	0.2	0.05

The incubation mixture contained 0.7 ml crude sonic extract pH 7.2 (19 mg protein), 15 μM PO_4, 15 μM $MgCl_2$, 25 μM KF. The following additions were added as indicated: 50 μM succinate, 2.5 μM AMP, 1 mg of yeast hexokinase, 20 μM glucose, $5 \cdot 10^{-5}$ *M*, DNP, and H_2O to volume 1.3 ml.

Reactions were carried out at 30° for 10 minutes after addition of substrate, then stopped by the addition of 10% TCA and phosphate analyzed by the method of FISKE AND SUBBAROW[4].

TABLE II

System	Oxygen (μ atoms	Δ Pi (μM)	P/O	P_γ (μM)
No additions	0.43	0	0	0.16
β-Hydroxybutyric + AMP	3.9	3.9	1.0	1.16
β-Hydroxybutyric + AMP + yeast hexokinase, glucose	4.0	4.9	1.20	0.67
β-Hydroxybutyric + AMP + yeast hexokinase, glucose + DNP	3.9	1.6	0.41	0

The incubation system contained 0.7 ml crude sonic extract, pH 7.2 (15 mg protein), 10 μM of PO_4, 15 μM $MgCl_2$, 25 μM KF, 50 μM β-hydroxybutyric acid where indicated, 2.5 μM AMP, 1 mg of yeast hexokinase, glucose, $5 \cdot 10^{-5}$ *M* DNP, and H_2O to volume 1.3 ml. The reaction was carried out at 30° for 10 min, and stopped by the addition of 10% TCA. An aliquot was removed and Pi determined. Activated norit was added to another aliquot, mixed well, then centrifuged and washed 4 times with distilled water. The adenosine polyphosphates adsorbed on the norit were hydrolyzed in *N* HCl and the P_γ determined.

The supernatants from the systems were also treated with norit to adsorb any ATP or ADP formed and the P_7* assayed from the thoroughly washed norit. With AMP as the sole acceptor system, ATP was formed and detected in the P_7 fraction (Table II). No ATP was formed in the presence of DNP. The amount of P_7 was reduced 50% in the presence of a complete acceptor system (AMP, hexokinase, and glucose). The lower value for P_7 in the complete system was due in part to the formation of glucose-6-phosphate, which was detected by Zwischenferment.

By the addition of ^{32}P labeled orthophosphate to the reaction mixture, the incorporation of phosphate into the P_7 fraction eluted from norit could be demonstrated in the presence of AMP as acceptor system. In this system 9% of the total activity of the ^{32}P was incorporated into P_7 whereas less than 1% was incorporated in the presence of dinitrophenol.

We wish to thank Dr. J. H. HANKS for advice, Dr. M. L. KARNOVSKY for making available facilities of the Biophysical Laboratory, and Mrs. TOBEY CANINO, Miss MILDRED LANGE, and Mr. ROBERT THOMAS for technical assistance.

ARNOLD F. BRODIE
CLARKE T. GRAY**

Leonard Wood Memorial, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass. (U.S.A.)

1 G. B. PINCHOT, *J. Biol. Chem.*, 205 (1953) 65.
2 F. LIPMANN, *Nature*, 143 (1939) 281.
3 A. FRENKEL, *J. Am. Chem. Soc.*, 76 (1954) 5568.
4 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
5 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.

Received March 8th, 1955

* P_7, orthophosphate liberated by 7 minute hydrolysis in *N* HCl at 100° after treatment with activated norit.

** This investigation was supported (in part) by a research grant (E-827) from the National Microbiological Institute of the National Institutes of Health, U.S. Public Health Service.

Paper chromatography of mixtures of amino acids containing glutamic or aspartic acid

In a recent paper BECK AND ÉBREY[1] record having observed "interesting phenomena inhibiting the exact qualitative and quantitative evaluation of amino acid chromatograms". The observation is that when certain mixtures of glycine and glutamic acid are subjected to paper chromatography, using phenol as the solvent, the glycine sweeps along an appreciable amount of the glutamic acid leading to incorrect quantitative results. With butanol-acetic acid as solvent three spots appear. They report that other pairs of amino acids behave similarly. Their explanation is that the "amino acids react with each other, depending upon the ratio of their concentration and medium, thus the chromatograms show not only the spots corresponding to the free amino acids, but also those of the products of the indicated reaction". If this explanation is correct then the interpretation of paper chromatograms would become extremely complicated, results of earlier workers might have to be reassessed and the usefulness of paper chromatography for the quantitative and qualitative analysis of amino acids would be considerably reduced.

From the information published we estimate that they are using amino acid concentrations of up to something like 0.05 molar. In an endeavour to repeat their work the following chromatograms were run in 80% aqueous phenol by the ascending technique:

0.0133 *M* glycine + 0.0125 *M* glutamic acid, 0.0667 *M* glycine + 0.0125 *M* glutamic acid and 0.0133 *M* glycine + 0.0625 *M* glutamic acid using 5 μl spots

(estimated to be half that used by BECK AND ÉBREY) and Whatman No. 1 filter paper. The R_F values of the spots as detected by ninhydrin are given in Table I, experiments 1, 2 and 3. The spot of R_F 0.40 is that given by glycine, while the remaining spots, which overlapped, are given by the glutamic acid. Of the glutamic acid spots in experiment 3 that of R_F 0.35 is by far the most intensely coloured. There was no difference between the intensities of the colour of the glycine spots in experiments 1 and 3 nor between the intensities of the colour of the glutamic acid spots (R_F 0.22) in experiments 1 and 2. The other results given in the table serve to explain the results given in the first three experiments. From the results of experiments 1 to 7 it is clearly seen that there is no interaction between the glycine and the glutamic acid as the spot occupying the position of serine (our R_F 0.35), claimed by BECK AND ÉBREY to be the reaction product, is found when glutamic acid is run alone. Experiments 8 to 15 indicate that the spot of R_F 0.22 observed in experiments 1 to 7 is given by glutamate (although the free base of glutamic acid gives the same R_F value) and the spot of R_F 0.27 is given