Effect of phosphate on the respiratory inhibition caused by thiol reagents in mitochondrial preparations

Following a recent study[1] on the inhibitory effect of fluoropyruvate on the oxidation of various substrates of the citric acid cycle in guinea-pig liver mitochondria, it was observed that the inhibition by fluoropyruvate depended markedly on the buffer used. Further investigation of this phenomenon, with glutamate as the substrate, showed that in phosphate buffer the extent of inhibition was much lower than in all other buffers tested (tris(hydroxymethyl)aminomethane (Tris) chloride, Tris sulfate, sodium pyrophosphate, and sodium borate). Arsenate which in many respects resembles phosphate showed no protective effect. The nature of the cation had no influence.

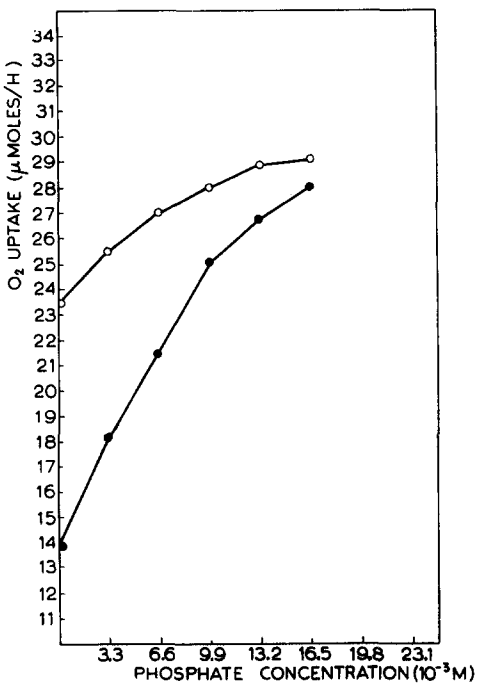

Fig. 1. Tris phosphate, pH 7.4, was added as indicated. The concentration of the Tris was brought to $2 \cdot 10^{-2}$ *M* by the addition of Tris chloride, pH 7.4. Fluoropyruvate, $3 \cdot 10^{-4}$ *M*. Other conditions were the same as mentioned in Table I. Control: ○; with fluoropyruvate: ●.

When using mixtures of Tris chloride and phosphate, the decrease in inhibition became the more pronounced the higher the concentration of the phosphate (Fig. 1). Phosphate exerted its protective action only at concentrations of fluropyruvate which caused in Tris chloride an inhibition not exceeding about 90 %.

Since FP was shown[1] to act as an –SH reagent on mitochondrial enzyme systems, the "phosphate effect" was also examined with other thiol reagents serving as inhibitors (Table I). The thiol-alkylating iodoacetate and the mercaptide-forming *p*-chloromercuribenzoic acid were found to behave like fluoropyruvate. On the other hand, the inhibition caused by sodium arsenite was observed to be independent of the buffer used. Arsenite differs from the other thiol reagents mentioned, in that it

TABLE I

EFFECT OF VARIOUS INHIBITORS ON THE OXIDATION OF L-GLUTAMATE BY LIVER MITOCHONDRIA IN TRIS AND PHOSPHATE BUFFERS

Each Warburg flask contained $2 \cdot 10^{-2}$ *M* Tris brought to pH 7.4 with either HCl (Tris chloride) or with H_3PO_4 (Tris phosphate) as indicated; 0.25 *M* sucrose; $1.3 \cdot 10^{-2}$ *M* KCl; $2.6 \cdot 10^{-3}$ *M* adenosine triphosphate; $1.3 \cdot 10^{-3}$ *M* $MgSO_4$, $1 \cdot 10^{-2}$ *M* L-glutamate; 0.5 ml guinea-pig-liver mitochondria (prepared according to SCHNEIDER[4]) equivalent to 4 mg N. Total vol., 3 ml. Center well, 20% KOH. Incubation at 37°. Gas phase, air. Readings were taken at intervals of 10 min for a period of 60 min.

Inhibitor	*Concentration (M)*	*Inhibition of respiration (%)*	
		Tris chloride	*Tris phosphate*
Fluoropyruvate	$3 \cdot 10^{-4}$	43	6
Iodoacetate	$3 \cdot 10^{-4}$	51	4
p-Chloromercuribenzoate	$3 \cdot 10^{-5}$	46	19
Arsenite	$3 \cdot 10^{-5}$	36	36
Urethane	$6 \cdot 10^{-5}$	37	34
Nembutal	$9 \cdot 10^{-4}$	48	54

TABLE II

COMPARISON OF THE "PHOSPHATE EFFECT" IN MITOCHONDRIA FROM DIFFERENT SOURCES

Liver mitochondria, 0.5 ml (4 mg N); kidney mitochondria (prepared by the same method as liver mitochondria), 0.5 ml (2 mg N); rat-heart sarcosomes (prepared according to CLELAND AND SLATER[5]), 0.5 ml (2 mg N) were added to the cups as indicated. All the other conditions were the same as described in Table I.

Source of mitochondria	*Inhibition of respiration (%)*					
	Tris chloride			*Tris phosphate*		
	Fluoropyruvate (M)			*Fluoropyruvate (M)*		
	$3 \cdot 10^{-4}$	$6 \cdot 10^{-4}$	$9 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	$6 \cdot 10^{-4}$	$9 \cdot 10^{-4}$
Guinea-pig liver	35	65	85	0	21	47
Guinea-pig kidney	54	67	80	0	26	42
Rat heart	65	90	97	45	74	91

combines with *two* –SH groups in the same molecule[2]. Other inhibitors tested, upon which phosphate had no effect, were the narcotic agents nembutal (sodium 5-ethyl-5-(1-methylbutyl) barbiturate) and urethane. These compounds were examined because it had been shown[3] that their inhibitory pattern on mitochondrial respiratory-enzyme systems resembled that of fluoropyruvate. Essentially similar results were obtained when, instead of L-glutamate, α-ketoglutarate or L-malate were used as substrates.

A "phosphate effect", analogous to that in liver mitochondria, could also be demonstrated in kidney mitochondria. In rat-heart sarcosomes, however, this effect was less pronounced (Table II). Preincubation of the mitochondria with phosphate at 0° or at 37°, or addition of phosphate after the fluoropyruvate had interacted with the mitochondria, caused no change in the extent of inhibition.

It has been reported recently[6] that phosphate protects thiol groups of yeast fumarase from the inhibitory action of melarsene (3 melaminylphenylarsenoxide) and iodosobenzoate; alternatively, protection could be effected by raising the concentration of the substrate. It was concluded that phosphate and fumarate combine with

the active area of the enzyme containing the essential thiol groups. The case studied here was found to be different, since glutamate within the limits of concentrations 10^{-3} to 10^{-2} *M* had no protective effect. Thus, while it is possible that also in the present case phosphate and thiol reagents compete for an active site of the enzyme surface, this site does not appear to be identical with the one which binds the substrate.

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Received September 4th, 1958

Is oxidized bacteriochlorophyll an intermediate in bacterial photosynthesis?

DUYSENS *et al.*[1] have suggested that intracellular bacteriochlorophyll in an excited electronic state may react to form a reductant and an oxidized form of bacteriochlorophyll analogous to the product formed[2,3] when a methanol solution of bacteriochlorophyll is treated with $FeCl_3$. Intracellular oxidation of bacteriochlorophyll was inferred on the basis of absorption increases at 432 mμ and 790 mμ and an absorption decrease near 890 mμ observed upon illumination of *Rhodospirillum rubrum* suspended in aerated water[1,4]. CHANCE AND SMITH[5] also observed the appearance of the absorption band at 432 mμ upon irradiation of aerobic suspensions of *Rsp. rubrum* in the presence of phenylmercuric acetate. Similar absorption changes have also been observed in *Chromatium*[1,6]. The present study shows that in *Chromatium* the absorption change at 432 mμ is *not* correlated with the absorption changes in the near infrared.

Chromatium, strain D, was grown in a liquid inorganic medium[7] containing sulfide, thiosulfate, and bicarbonate as substrates. Cultures were illuminated in a light cabinet. The bacteria were concentrated by centrifugation and resuspended in aerated supernatant liquid.

An earlier version of the spectrophotometer has been described previously[8]. A detailed description of the new split-beam arrangement is in preparation[9]. The monochromatic measuring beam from the monochromator is split into two parts, one of which passes through a sample cuvette and the other of which passes through an optical-density wedge. Both parts of the beam are focused onto a photomultiplier. The light from a super-high-pressure mercury lamp is filtered and focused on the sample cuvette to provide actinic irradiation in the region 500 to 600 mμ. By means of two concentric rotating discs with appropriate openings the intervals during which the actinic light beam irradiates the sample cuvette and the intervals during which the two measuring beams strike the photomultiplier are separated in a fixed time sequence as shown in Fig. 1. An actinic light flash lasting 2 msec is given every