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PHOSPHATE ACCEPTOR SPECIFICITY DURING OXIDATIVE PHOSPHORYLATION IN SUBMITOCHONDRIAL PARTICLES

IVAR VALLIN AND PER LUNDBERG
The Wenner-Gren Institute, University of Stockholm, Stockholm (Sweden)
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SUMMARY

The absolute specificity for ADP as phosphate acceptor during oxidative phosphorylation is lost upon sonic treatment of mitochondria.

1. The specificity varies with the substrate and thus with the number of phosphorylation sites involved.

2. K_m values for IDP and ADP as phosphate acceptors with various substrates and at optimal pH have been determined.

3. Uncouplers, monovalent ions and oligomycin all affect the phosphorylation of IDP more strongly than that of ADP.

4. The nonspecific reaction is not due to the involvement of a nucleoside diphosphate kinase reaction.

The ability to phosphorylate the various nucleoside diphosphates is considered to be dependent on the affinity between these nucleotides and the phosphorylating enzyme and may reflect the endogenous condition when the translocase has lost its physiological rôle.

INTRODUCTION

The absolute requirement for an adenosine part in nucleotides involved in energy-linked reactions in mitochondria is due to the enzyme translocase, which exchanges endogenous nucleotides with nucleotides supplied to the system[1,2]. The translocase inhibitor atractyloside has been shown[3] to bind preferentially to the inner membrane of the mitochondrion. During sonic treatment of the mitochondria the inner membrane is inverted[4–6] and the affinity for atractyloside is either greatly reduced or lost[7,8]. Since the enzymes involved in the phosphorylation process in these submitochondrial particles are turned towards the medium, as evidenced by the negative staining technique[9], there seems to be no need for a translocase function preceding the transfer of phosphate bound energy on to the nucleoside diphosphate. The phosphate acceptor specificity in submitochondrial systems would thus possibly reflect the situation of the primary phosphorylation event. It can be demonstrated

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

that submitochondrial particles obtained by sonic disintegration of mitochondria from beef heart, rat heart or rat liver all show a decreased specificity for the nucleoside diphosphate used as phosphate acceptor during oxidative phosphorylation. On the other hand digitonin particles, with the inner membrane oriented similar to intact mitochondria, have an absolute specificity for ADP in oxidative phosphorylation[10].

In our beef heart submitochondrial particles it is thus possible to use other phosphate acceptors than ADP[7]. The degree of specificity for the phosphate acceptor varies with the substrate added to the system and increases with the number of phosphorylation sites involved. The capacity to phosphorylate for example IDP in comparision to ADP with a particular substrate is similar whether submitochondrial particles from beef heart or rat liver are investigated.

The degree of specificity is further dependent on the pH of the reaction medium to a variable extent depending on the substrate used. Monovalent ions, uncouplers or oligomycin have generally a more pronounced effect on phosphorylation of IDP compared to that of ADP. The nonspecific phosphorylation is not due to the involvement of a nucleoside diphosphate kinase reaction.

MATERIALS

All nucleotides used, alcohol dehydrogenase (EC 1.1.1.1) and DL-β-hydroxybutyric acid (sodium salt) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Hexokinase (EC 2.7.1.1) was a standard preparation from Boehringer and Soehne GmbH (Mannheim, Germany). *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine (TMPD) was bought from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was a gift from Dr P. G. Heytler, Du Pont de Nemours, Wilmington, Del., U.S.A. ^{32}P was obtained from the Radiochemical Centre, Amersham, U.K. *N*-Tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (TES) was bought from Calbiochem, Los Angeles, Calif., U.S.A. All other chemicals were reagent grade.

METHODS

The preparation of beef heart mitochondria and the sonic treatment of these to yield submitochondrial particles has been published earlier[11,12]. Rat liver mitochondria were prepared according to the method of SCHNEIDER AND HOGEBOOM[13]. Submitochondrial particles from rat liver were prepared essentially according to the methods of MCMURRAY *et al.*[14] and HOPPEL AND COOPER[15], except that the mitochondria were disintegrated in the presence of 1 mg/ml bovine serum albumin in a 10-kcycles Raytheon Sonic Oscillator.

Protein content of particle suspensions was determined according to GORNALL *et al.*[16]. Oxygen consumption was measured polarographically with a Clark oxygen electrode (Yellow Springs Instrumental Co. Inc., No 4004) in a thermostable electrode chamber at 30°.

Basic medium: (beef heart particles) 50 mM glycylglycine buffer (pH 7.5), $MgCl_2$ as indicated, 0.15 M sucrose, 3 mM phosphate buffer (pH 7.5), 2 mM nucleoside diphosphate as indicated, 20 mM glucose, hexokinase in excess and 0.3 mg of submitochondrial particle protein per ml. 0.6 mM NAD^+, 0.06 mg of crystalline alcohol dehydrogenase powder per ml, 43 mM ethanol and 3 mM semicarbazide were added

when NADH was the substrate. Otherwise 3 mM sodiumsuccinate or 5 mM sodium ascorbate *plus* 0.3 mM TMPD were used.

Basic medium: (rat liver particles) 20 mM TES buffer (pH 6.8), 5 mM phosphate buffer (pH 6.8), 4.5 mM nucleoside diphosphate as indicated, $MgCl_2$ as indicated, 1 mg/ml bovine serum albumin, 20 mM glucose, hexokinase in excess and 1 mg of submitochondrial particle protein per ml. As substrate 20 mM DL-β-hydroxybutyric acid (sodium salt) *plus* 1.2 mM NAD^+ or 1.2 mM NAD^+ in combination with 0.12 mg of crystalline alcohol dehydrogenase powder per ml, 86 mM ethanol and 6 mM semicarbazide or 10 mM sodiumsuccinate or 5 mM sodium ascorbate *plus* 0.3 mM TMPD were used.

Final volume in all cases was 1.0 ml. Esterification of phosphate was determined according to the ^{32}P-distribution method described by LINDBERG AND ERNSTER[17].

RESULTS

The ability to replace ADP as phosphate acceptor varied with the structural configuration of the nucleoside diphosphate added[7] and IDP has been used in this study not because of an assumed physiological rôle but simply as the structural analogue of ADP which was found to be phosphorylated at a comparatively high rate.

Nucleoside specificity with various substrates

The capacity of the particulate system to distinguish between IDP and ADP as phosphate acceptor during the process of oxidative phosphorylation differed with the substrate added. The degree of specificity for the nucleoside part of the phosphate acceptor could be expressed as the quotient of μmoles of IDP esterified per min and mg protein *versus* μmoles of ADP esterified per min and mg protein. Mean values from a number of experiments with three different substrates are given in Table I. With NADH as the substrate the P/O ratios obtained with IDP as phosphate acceptor were approx. 20 % of those with ADP. With succinate as the substrate these ratios were increased to about 40 % and they were between 50 and 60 % when ascorbate *plus* TMPD were used as electron donors.

The oxidative phosphorylation activities with the latter substrate varied to a certain degree in different particle preparations and the specificity was usually

TABLE I

PHOSPHORYLATION OF IDP COMPARED TO THAT OF ADP

The electrode chamber contained in a final volume of 1.0 ml; 50 mM glycylglycine buffer (pH 7.5), 0.15 M sucrose, 3 mM phosphate buffer (pH 7.5), 2 mM nucleosidediphosphate, 20 mM glucose, hexokinase in excess and 0.3 mg of submitochondrial particle protein per ml. 3 mM $MgCl_2$ was used with NADH and 6 mM with succinate or ascorbate *plus* TMPD as the substrates. Substrates were: 0.6 mM NAD^+, 0.06 mg alcohol dehydrogenase per ml, 43 mM ethanol and 3 mM semicarbazide (= NADH), 3 mM succinate or 5 mM ascorbate *plus* 0.3 mM TMPD.

Substrate	*Mean value* (%)	*S.D.*	*Number of experiments*
NADH	21.9	± 7.0	11
Succinate	40.6	± 5.1	15
Ascorbate, TMPD	56.1	± 16.5	13

more pronounced when high P/O ratios were obtained in the presence of ADP. The degree of specificity for the phosphate acceptor was thus increased with the number of phosphorylation sites involved. This general tendency was found also when submitochondrial particles from other tissues were investigated and seemed to be a general pattern for particulate systems obtained by sonic disintegration.

Measured phosphorylative capacities and P/O ratios in a typical experiment with beef heart particles and NADH, succinate or ascorbate *plus* TMPD as substrates are given in Table II. The oxygen consumption with a given substrate was essentially the same whether ADP or IDP was used as phosphate acceptor. No substantial change in P/O ratio was obtained upon addition of hexokinase and glucose where ascorbate plus TMPD was the substrate. Phosphorylation of IDP with NADH as the substrate was similarly unaffected by the glucose hexokinase trap.

In Table III phosphorylative capacity as well as P/O ratios with submitochondrial particles from rat liver are demonstrated. It is noteworthy that the activities with succinate as the substrate were very low. The respiratory rate with each particular substrate was of the same magnitude regardless if IDP or ADP was the phosphate acceptor. It was obvious that the presence of Mg^{2+} played an important rôle as

TABLE II

IDP OR ADP AS PHOSPHATE ACCEPTOR AND THE EFFECT OF HEXOKINASE ON OXIDATIVE PHOSPHORYLATION

Experimental conditions as in Table I but $MgCl_2$ concentration was 9 mM with succinate and ascorbate *plus* TMPD as the substrates. Glucose and hexokinase present only when indicated.

Substrate	*Phosphorylative capacity (P/O ratio) (μmoles P_i esterified per min per mg protein)*			
	With ADP		*With IDP*	
	+ Hexokinase	*— Hexokinase*	*+ Hexokinase*	*— Hexokinase*
NADH	0.464 (1.68)	0.291 (1.11)	0.056 (0.24)	0.062 (0.26)
Succinate	0.226 (1.31)	0.184 (1.14)	0.090 (0.53)	0.065 (0.39)
Ascorbate, TMPD	0.406 (0.66)	0.360 (0.63)	0.231 (0.38)	0.213 (0.37)

TABLE III

EFFICIENCY OF IDP OR ADP AS PHOSPHATE ACCEPTOR IN RAT LIVER PARTICLES

The electrode chamber contained in a final volume of 1.0 ml; 20 mM TES buffer (pH 6.8), 5 mM phosphate buffer (pH 6.8), 4.5 mM nucleosidediphosphate, 5 mM $MgCl_2$, 1.0 mg bovine serum albumin per ml, 20 mM glucose, hexokinase in excess and 1.0 mg of submitochondrial particle protein per ml. Substrates were: 20 mM DL-β-hydroxybutyrate *plus* 1.2 mM NAD^+, 10 mM succinate or 5 mM ascorbate *plus* 0.3 mM TMPD.

Substrate	*Phosphorylative capacity (P/O ratio) (μmoles P_i esterified per min per mg protein)*	
	With ADP	*With IDP*
DL-β-Hydroxybutyrate, NAD^+	0.260 (1.26)	0.060 (0.28)
Succinate	0.055 (1.19)	0.024 (0.47)
Ascorbate, TMPD	0.193 (0.71)	0.120 (0.46)

far as the specificity was concerned. In contrast to HOPPEL AND COOPER[15] we found no specificity with DL-β-hydroxybutyrate or NADH as substrates without Mg^{2+} added to the reaction medium (Table IV) and further an increase in magnesium concentration favoured the phosphorylation of ADP with a higher specificity as a consequence.

TABLE IV

THE EFFECT OF $MgCl_2$ ON PHOSPHATE ACCEPTOR SPECIFICITY IN RAT LIVER PARTICLES

Experimental conditions as in Table III. Substrate was: 20 mM DL-β-hydroxybutyrate *plus* 1.2 mM NAD^+ or 1.2 mM NAD^+, 0.12 mg alcohol dehydrogenase per ml, 86 mM ethanol and 6 mM semicarbazide (= NADH) or 10 mM succinate. The specificity expressed as IDP phosphorylated in % of ADP phosphorylated.

Substrate	*$MgCl_2$ (mM)*	*Specificity*
DL-β-Hydroxybutyrate, NAD^+	—	132.5
	1	64.9
	3	53.2
NADH	—	119.4
	1	116.7
	3	44.9
Succinate	—	31.9
	1	34.7
	3	60.8
	6	47.9

pH-dependence

The nucleoside specificity was not only variable in proportion to the number of phosphorylation sites involved but showed further individual features in relation to the pH of the reaction medium. When succinate was used as the substrate the optimum for ADP phosphorylation differed considerably from that of IDP (Fig. 1). Furthermore the phosphorylation with ADP was constant in the alkaline pH range tested while phosphorylation of IDP decreased with higher pH values. On the other hand when ascorbate *plus* TMPD was used as the electron source, phosphorylation of ADP and IDP had almost the same pH optimum and both activities became less pronounced with increase in pH (Fig. 2). When using the more specific system where NADH was the substrate a pattern corresponding to that with ascorbate *plus* TMPD was obtained with an optimum of the phosphorylative capacity of both nucleoside diphosphates around pH 7.25.

It should be mentioned that when a glycylglycine buffer of pH 7.5 was added to the system the final pH value before the reaction started was less than 7.5. Thus when ADP was used the real pH was 7.12 and when IDP was utilized it was 7.28. Unless otherwise stated these were the final pH values of the systems. pH values indicated in Figs. 1 and 2 were measured immediately before start of reaction.

The affinity for phosphate acceptors

In Table V are given the K_m-values obtained from Lineweaver–Burk plots where the reciprocal of the phosphorylative capacity is put *versus* the reciprocal of

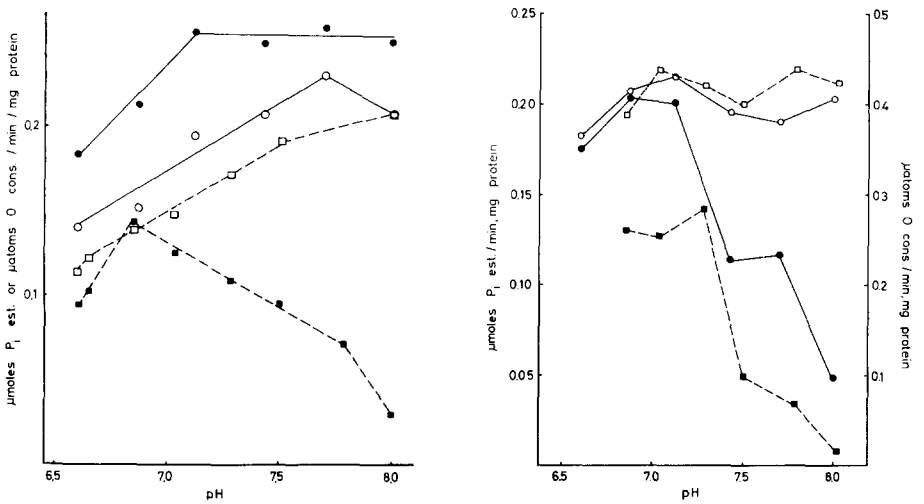


Fig. 1. The effect of pH on oxidative phosphorylation with succinate as the substrate. Experimental conditions as in Table I. pH values varied with glycylglycine buffer. ○—○, µatoms oxygen consumed with ADP as phosphate acceptor; □---□, µatoms oxygen with IDP; ●—●, µmoles phosphate esterified with ADP, ■---■, µmoles phosphate with IDP.

Fig. 2. The effect of pH on oxidative phosphorylation with ascorbate *plus* TMPD as the substrate. Experimental conditions as in Table I. Symbols as in Fig. 1.

TABLE V

K_m VALUES FOR ADP AND IDP IN OXIDATIVE PHOSPHORYLATION

Experimental conditions as in Table I except that pH optima from Fig. 1 were used.

Phosphate acceptor	K_m *value (M)*		
	NADH	*Succinate*	*Ascorbate, TMPD*
ADP	$7.4 \cdot 10^{-5}$	$8.3 \cdot 10^{-5}$	$9.5 \cdot 10^{-5}$
ADP + glucose, hexokinase	$3.6 \cdot 10^{-5}$	$2.1 \cdot 10^{-5}$	$3.0 \cdot 10^{-5}$
IDP	$2.2 \cdot 10^{-4}$	$1.1 \cdot 10^{-3}$	$1.3 \cdot 10^{-3}$
IDP + glucose, hexokinase	$5.6 \cdot 10^{-4}$	$5.7 \cdot 10^{-4}$	$1.6 \cdot 10^{-4}$

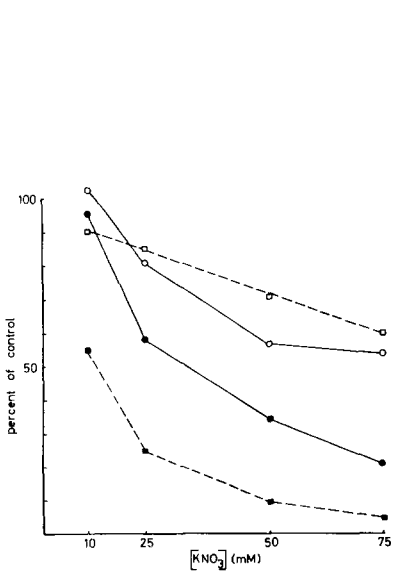
the nucleoside diphosphate concentration. pH of the media were those found optimal for each particular nucleoside diphosphate and substrate (*cf.* Figs. 1 and 2). K_m values for a complex reaction sequence like the oxidative phosphorylation mechanism may be of limited value but the table seems to indicate that the affinity for IDP as phosphate acceptor was considerably lower than that for ADP. The further addition of hexokinase and glucose increased the affinity between ADP and the phosphorylative enzyme. BYGRAVE AND LEHNINGER[18] have reported a K_m value of 300 µM for ADP in oxidative phosphorylation in sonicated beef heart submitochondrial particles with NADH as the substrate. PAPA *et al.*[19] suggested this value too high depending on presence of monovalent cations and obtained in absence of K^+ when utilizing β-hydroxybutyrate a K_m value of 40 µM. This value corresponds rather well with our findings and the K_m value for ADP showed only minor variations depending

on the substrate used. When IDP was the phosphate acceptor, however, the K_m values turned out quite differently. With all phosphorylation sites involved the affinity between IDP and the phosphorylation enzyme was about 5 times higher than when succinate or ascorbate *plus* TMPD were the substrates. Upon addition of glucose and hexokinase this affinity surprisingly decreased 2.5 times in the case where NADH was the substrate, increased 2 times where succinate was added and about 10 times if ascorbate *plus* TMPD was the source of electrons. A low K_m value for IDP was thus obtained only when energy fixation was restricted to the cytochrome oxidase region of the respiratory chain.

Effect of ions and uncouplers

PAPA *et al.*[19] and CHRISTIANSEN *et al.*[20] have shown that the presence of monovalent ions decreased the phosphorylative capacity and the P/O ratios in submitochondrial particulate systems. As an example of this effect we have added KNO_3 to beef heart particles where succinate was the substrate. The ability to phosphorylate IDP as well as ADP was considerably decreased by increasing concentrations of the salt (see Fig. 3). The phosphorylation of IDP was, however, much more sensitive and the response to variations in salt concentrations seemed to be one of the few features where a clear distinction could be seen in phosphorylation activity of the two nucleoside diphosphates.

The uncoupling effect of CCCP on oxidative phosphorylation in submitochondrial particles obtained by sonication and with NADH or succinate used as the substrate has previously been demonstrated[12]. Fig. 4 shows the effect on the succinate



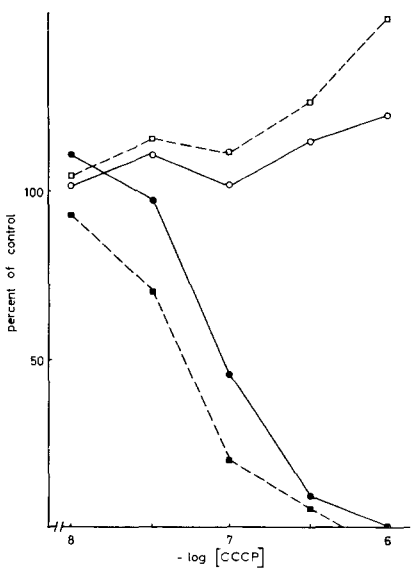


Fig. 3. The effect of KNO_3 on oxidative phosphorylation with succinate as the substrate. Experimental conditions as in Table I. Symbols as in Fig. 1.

Fig. 4. The effect of CCCP on oxidative phosphorylation with succinate as the substrate. Experimental conditions as in Table I. Symbols as in Fig. 1. [CCCP] is expressed in M.

oxidase system of various concentrations of CCCP with ADP and IDP respectively as phosphate acceptor. The stimulation of respiration caused by the uncoupler was somewhat more pronounced when IDP was used. Furthermore 50 % of the phosphorylative capacity with IDP as phosphate acceptor was eliminated at a concentration of about $5 \cdot 10^{-8}$ M CCCP. At this concentration of the uncoupler the phosphorylation with ADP still remained to about 75 % of the control value. As was the case in the presence of high concentrations of ions, IDP with its lower affinity for the phosphate-bound energy was a less effective competitor when primary higher energy intermediates were gradually drained off with increasing concentrations of the uncoupler.

Effect of oligomycin

Oligomycin acts on oxidative phosphorylation in submitochondrial particles from beef heart in two ways. At low concentrations the P/O ratios are increased but higher concentrations of oligomycin cause an inhibition. This effects have been demonstrated on EDTA particles by LEE AND ERNSTER[21]. On the other hand they found that oligomycin had little or no increasing effect on the P/O ratio and did not inhibit the respiration of phosphorylating particles prepared in the presence of Mg^{2+} and ATP. The effect of oligomycin on Mg^{2+}, Mn^{2+} submitochondrial particles prepared according to BEYER[22] is demonstrated in Fig. 5. The phosphorylation was slightly stimulated with 0.1 μg oligomycin per mg of protein but the phosphorylative capacity was rapidly decreased with higher concentrations of oligomycin. When IDP was used as the phosphate acceptor a stimulatory effect of oligomycin was hardly observed. With IDP a 50 % inhibition was achieved with an oligomycin concentration which was about two thirds of that needed when ADP was the phosphate acceptor. The same effects were found in ordinary Mg^{2+}, ATP particles with succinate as the substrate. It should be noted that the specificity of the particles prepared according to BEYER[22] was similar to that of ordinary particles, the IDP/ADP phosphorylation quotient with succinate as substrate in Fig. 5 being 0.39.

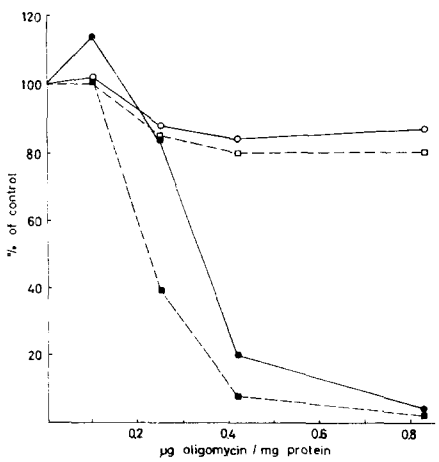


Fig. 5. The effect of oligomycin on oxidative phosphorylation with succinate as the substrate. Submitochondrial particles obtained by sonication with Mn^{2+}, Mg^{2+} and ATP in the medium according to BEYER[22]. Experimental conditions as in Table I. Symbols as in Fig. 1.

Nucleoside diphosphate kinase activity

Attempts were made to determine the extent of nucleoside diphosphate kinase (EC 2.7.4.6) activity in our beef heart particle preparation. A system similar to that of HELDT AND SCHWALBACH[23] was used where 0.1 mg particle protein was added, GTP substituted by ITP and the rate of $NADP^+$ reduction was followed spectrophotometrically. Under optimal conditions with equal concentrations of ITP and ATP (0.5 mM) the exchange rate was of the order of 0.015 μmole per min and mg particle protein. The rate of ITP–ADP exchange was much too low to correspond to the rate of P_i uptake with IDP as the phosphate acceptor. The same conclusion was made by HOPPEL AND COOPER[15].

The ATP–IDP exchange was studied by use of ATP labelled by ^{32}P in the γ-position. 1 mM ATP was added to a system containing glycylglycine buffer, $MgCl_2$, sucrose, submitochondrial particles and IDP in the same concentrations as in the oxidative phosphorylation systems and KCN was further present to get anaerobic conditions. Extracts were subject to thin-layer chromatography on a DEAE-cellulose-MN-cellulose layer with 0.6 M HCl as eluant. Nucleotide standards were run parallel to the labelled samples. The distribution of ^{32}P was obtained by scanning the chromatogram with a Geiger–Müller tube supplied with a narrowslitted cover. The amount of ITP formed during these optimal conditions was 0.016 μmoles/min and mg particle protein corresponding to less than 10 % of that formed on oxidative phosphorylation with IDP as the phosphate acceptor under least favourable conditions.

Samples taken from oxidative phosphorylation media without hexokinase and glucose present and where IDP was the phosphate acceptor were subject to the same type of thin-layer chromatography as above. Only one ^{32}P-labelled nucleotide peak appeared. The R_F value for this peak coincided well with that found for the ultraviolet light absorbing spot achieved from ITP added to the same chromatogram.

These three types of experiments clearly indicated that ITP was the only nucleotide formed during ordinary oxidative phosphorylation where IDP was used as the phosphate acceptor. It seemed also evident that the amount of ITP obtained during these circumstances was caused mainly by a direct binding of P_i to IDP and only to a small extent by the nucleoside diphosphate kinase activity.

Variations of the sonication medium

Modifications of the nucleotide addition to the standard media where mitochondria were sonically disintegrated (*cf.* METHODS) could yield particles where

TABLE VI

PHOSPHATE ACCEPTOR SPECIFICITY IN THREE TYPES OF BEEF HEART PARTICLES

Experimental conditions as in Table I. Differences between the three types of particles described in the text. The specificity expressed as IDP phosphorylated in percent of ADP phosphorylated. Substrates as indicated.

Type of particle	*Specificity*		
	NADH	*Succinate*	*Ascorbate, TMPD*
"3 × ATP particles"	15.5	34.7	57.9
"ITP particles"	12.3	38.4	39.0
Ordinary particles	9.4	32.9	47.0

P/O ratios as well as specificity for the phosphate acceptor differed from what was routinely observed. A 3-times increase in ATP concentration or a replacement of ATP by an equal concentration of ITP gave rise to particle preparations where the NADH as well as the succinic oxidase activities were decreased. The corresponding phosphorylative activities were usually less affected and thus increased P/O ratios were obtained. The ratio between IDP/ADP phosphorylated was generally increased (Table VI) and again it could be observed that a decrease in the activities of oxidative phosphorylation was followed by reduced specificity.

DISCUSSION

The data given above indicate that a decreased specificity for nucleoside diphosphates as phosphate acceptor during oxidative phosphorylation is inherent with submitochondrial particles obtained by sonic disintegration of mitochondria. It is also evident that the degree of this specificity varies with the substrate added to the particulate system and thus to the number of phosphorylation sites involved.

The proportion of IDP to ADP phosphorylated was rather constant from preparation to preparation (Table I) as long as the same substrate was used. It seems not possible to confer the unspecific phosphorylation to one single site although the unspecificity is most pronounced when the cytochrome oxidase part of the respiratory chain is exclusively utilized for generation of energy.

The activity to phosphorylate IDP was very low when NADH was used as the substrate and this may be an effect of the particular phosphorylation sites involved but is certainly also affected by the pyridine nucleotide added. We have shown earlier[24] that addition of NAD^+ or NADH to particles utilizing succinate or ascorbate *plus* TMPD as the substrate limits the possibility to phosphorylate IDP whereas phosphorylation of ADP is unaffected. This effect of pyridine nucleotides on the specificity of the phosphorylation process will be dealt with in more detail in a later article.

The phosphorylative capacity of the submitochondrial preparation was relatively sensitive to changes of pH in the reaction medium and especially with succinate as the substrate a distinct difference in pH optima for IDP and ADP phosphorylation, respectively, could be demonstrated. There were thus prominent differences in nucleotide specificity depending on the particular phosphorylation sites involved.

It was argued by HOPPEL AND COOPER[15] that the loss of membrane-bound divalent cations, especially Mg^{2+}, would be the main reason for the unspecific phosphorylation reaction and also that the unspecificity is increasing with the concentration of divalent ions added to the particulate medium. In our hands, however, the specificity of a rat liver particle system prepared essentially according to these authors increased with higher concentrations of Mg^{2+} in the medium (Table IV).

Our particles from rat liver were thus characterized by a very high IDP/ADP phosphorylation ratio in the absence of added Mg^{2+} and this quotient decreased upon enhancement of this ion concentration in the medium. However, beef heart particles become less specific with increasing concentrations of Mg^{2+} and the phosphorylation quotient of particles from both sources turned out to be equal when Mg^{2+} concentrations optimal for the phosphorylation of ADP were used (*cf.* Tables I and III).

A further addition of Mn^{2+} (ref. 22) besides $MgCl_2$ and ATP to the sonication

medium yields beef heart particles with a 60-times increase in membrane-bound Mg^{2+} (ref. 4). We found that such particles had a nucleoside specificity very similar to ordinary Mg^{2+}, ATP particles (Fig. 5). Neither the amount of Mg^{2+} bound to the membrane nor the Mg^{2+} concentration of the medium seem thus to constitute the ultimate regulatory factor as far as the unspecific phosphorylation reaction is concerned.

In accordance with the findings of HOPPEL AND COOPER for rat liver particles[15], the activity of nucleoside diphosphate kinase in our beef heart particle preparations was much too low to account for more than a few percent of the phosphorylative activities found when IDP was the acceptor. The fact that the phosphorylation of IDP stands in no simple relationship to the activity to phosphorylate ADP but varied with the substrate employed, further argues against a simple transfer of high-energy phosphate from endogenous ATP to IDP via a nucleoside diphosphate kinase reaction. We consider a direct phosphorylation of IDP to be the pertinent mechanism and that the affinity between this nucleotide and the phosphorylation enzyme varies according to the K_m values obtained with the different substrates.

Agents which affect the phosphorylative capacity had generally a stronger influence on phosphorylation of IDP than of ADP. This phenomenon can be related back to the difference in affinity between the two nucleoside diphosphates and the phosphorylation enzyme as indicated by the difference in K_m values. IDP competes less successfully with energy dissipating reactions for energy generated than does ADP and this circumstance is further stressed by the fact that the glucose hexokinase trap is less efficient when IDP is the phosphate acceptor.

The reported observations with individual specificity features when a different number of phosphorylation sites are involved have made us consider the limited nucleoside specificity in oxidative phosphorylation of submitochondrial systems to be of endogenous nature and disclosed when the adenosine specific translocase activity has lost its physiological function.

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