Phosphate-Transfer Reactions of Adenosine and Inosine Nucleotides

BY H. A. KREBS AND R. HEMS

*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry,
University of Oxford*

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It was shown in a previous paper (Krebs & Hems, 1953) that animal tissues contain an enzyme catalysing a rapid transfer of phosphate between adenosine- and inosine-di- and -tri-phosphates according to the scheme

where ATP and ADP stand for adenosine tri- and di-phosphates, and ITP and IDP for inosine tri- and di-phosphates respectively. Berg & Joklik (1953, 1954) found this reaction simultaneously and independently in yeast. The present paper is concerned with another phosphate-transfer reaction of animal tissues:

where AMP stands for adenosine monophosphate. While the experiments establish the occurrence of reaction (2), they leave the mechanism of the phosphate transfer undecided. Reaction (2) is analogous to that catalysed by adenylate kinase (myokinase).

but purified adenylate kinase of muscle tissue does not catalyse reaction (2). It is possible that the enzyme is identical with the adenylate kinase of other tissues, which, according to Kotelnikova (1949, 1950) and Barkulis & Lehninger (1951), differs from the muscle-adenylate kinase. It is also possible that the transfer is the result of the combined action of reactions (3) and (1). In this case catalytic quantities of ADP and ATP would participate in the reaction. No detectable quantities of these reagents were present at the beginning of the incubation period, but this does not rule out the presence of catalytic amounts.

MATERIALS AND METHODS

ATP was prepared according to LePage (1949) and ITP according to Kleinzeller (1942). ADP was obtained from Schwarz Laboratories Inc., New York, and AMP from Roche Products Ltd., Welwyn Garden City; IDP was prepared by deamination of ADP with nitrous acid, the directions given by Kleinzeller (1942) for the deamination of ATP being followed. ATP and ITP contained ADP and IDP respectively. The quantities of these impurities present at zero time are stated in the tables.

ATP containing ^{32}P in the β - and γ -positions was prepared according to Hems & Bartley (1953). β -Labelled ADP was a by-product in the preparation of labelled ATP and was separated chromatographically. γ -Labelled ITP was prepared by the procedure of Hems & Bartley, by including ITP as well as ATP among the added reagents and separating the products by paper chromatography. About 92% of the isotope was in the γ -phosphate and about 8% in the β -phosphate of ITP. The sample also contained some β -labelled IDP. Labelled AMP was prepared according to Eggleston (1954). The specific activity of the nucleotide phosphorus was of the order of 10^6 counts/min./ μg . The substances could therefore be diluted about 1000-fold with carrier material.

Sheep-liver mitochondria were prepared by homogenizing fresh liver with 3 vol. of 0.25M-sucrose in the apparatus of Potter & Elvehjem (1936) made of stainless steel. The supernatant (30 ml.) obtained on centrifuging at 150 g for 15 min. was mixed with 1.5M-KCl (4 ml.) and this was centrifuged for 15 min. at 2500 g. The sediment of mitochondria was resuspended in 0.5% KCl and centrifuged at 2500 g for 7 min. This suspension was again centrifuged and the residue was suspended in 5 vol. of water. The suspension was frozen at about -15° . After thawing, the material was centrifuged at 24000 g and the supernatant was used as enzyme preparation. It was stored at -15° .

Acetone-dried powder of sheep brain was obtained by freezing brain tissue in solid CO_2 as soon as possible after the death of the animal. The solid material was broken up into smaller pieces in a cooled mortar and then disintegrated in a Waring Blender with 10 vol. of acetone at -15° . The insoluble material was filtered off and washed three times with 10 vol. of cold acetone. The powder was finally dried in a desiccator over H_2SO_4 and stored *in vacuo* in the cold. Aqueous extracts of the powder were made by homogenizing it with 20 vol. of water and centrifuging it in the cold at 20000 g. The pink, almost clear supernatant served as enzyme solution.

RESULTS

Interaction between AMP and ITP in lysed liver mitochondria

The reaction mixture contained 10% (v/v) of the lysed mitochondria preparation, 0.003M- CaCl_2 , 0.035M- NaHCO_3 and initially the amounts of AMP, ITP and IDP stated in Table 1. The ITP sample contained ^{32}P in the terminal phosphate group, and small amounts also in the β -phosphate. Three parallel mixtures were set up, the first containing both AMP and ITP, with some IDP, the

Table 1. Reaction between AMP and ITP (labelled with ³²P in terminal P) in sheep-liver mitochondria

Substance to which results refer	AMP, ITP (and IDP) added					ITP (and IDP) added					AMP added				
	Counts/min./25 μ l.					P found (μ g./25 μ l.)					P found (μ g./25 μ l.)				
	Change ($m\mu$ moles)					Change ($m\mu$ moles)					Change ($m\mu$ moles)				
	0 min.	60 min.	Specific activity (counts/min./ μ g. P)	0 min.	60 min.	0 min.	60 min.	0 min.	60 min.	Change ($m\mu$ moles)	0 min.	60 min.	0 min.	60 min.	Change ($m\mu$ moles)
ITP	4.78	2.27	1788	8548	3901	1720	4.71	4.03	~0	-7.3	3.81	3.49	~0	~0	-10.3
IDP	2.17	1.29	299	649	356	276	2.21	1.22	~0	-16.0	0.06	0.22	~0	~0	+5.2
IMP	0	1.38	0	0	205	148	0	0.91	~0	+29.3	—	—	~0	~0	—
ATP	0	0.21	0	0	670	3190	—	~0	~0	—	—	—	~0	~0	—
ADP	0	1.05	0	0	2563	2440	—	~0	~0	—	—	—	~0	~0	—
AMP	3.83	3.13	0	3	68	21	—	~0	~0	—	—	—	~0	~0	—
Inorganic P	0.36	1.74	440	159	1375	790	0.49	1.48	~0	+31.9	—	—	—	—	—

For conditions see text.

second ITP with some IDP, and the third AMP. The samples were saturated with CO₂ + N₂ (5:95) at 30°. For analysis a 0.5 ml. sample was mixed with 0.05 ml. of 30 % (w/v) trichloroacetic acid and 25 μ l. were chromatographed according to Krebs & Hems (1953).

As will be seen in Table 1, the initial quantities of AMP, ITP and IDP present in the first mixture decreased on incubation and ATP, ADP and IMP (inosine monophosphate) were formed. Two types of reaction can be visualized as causing a formation of ATP and ADP. Either an amino group is transferred from AMP to the inosine phosphates (transamination) or phosphate is transferred from inosine phosphates to AMP (transphosphorylation). In transamination the specific activities of ATP and ITP and ADP and IDP would be the same. In fact the specific activity of ATP was about twice that of ITP and that of ADP a multiple of that of IDP. These findings disprove the occurrence of transamination, but are in accordance with the assumption that transphosphorylation occurs, the radioactive phosphate of ITP providing the β - and γ -phosphates of ATP and the β -phosphate of ADP. The quantitative changes agree with the assumption that the reactions listed in Table 2 take place. The procedure for obtaining the data in Table 2 was as follows. As ATP participates in reaction *b* only, the amounts reacting according to *b* are equal to the amount of ATP formed. ADP participates in

Table 2. Reactions accounting for the changes on addition of AMP, ITP and IDP recorded in Table 1

Reactions	Quantities reacting in 60 min./25 μ l. of soln. ($m\mu$ moles)
<i>a</i> ITP + AMP = IDP + ADP	20
<i>b</i> ITP + ADP = ATP + IDP	2.3
<i>c</i> ITP = IDP + P	4.7
<i>d</i> IDP = IMP + P	41.3
<i>e</i> AMP = IMP + NH ₃	3

Table 3. Comparison of changes observed (see Table 1), and calculated from Table 2

The values refer to 25 μ l. of solution and 60 min.; AMP, ITP and IDP added.

Substances	Changes	
	Calc. ($m\mu$ moles)	Obs. ($m\mu$ moles)
ITP	-27.0	-27.0
IDP	-14.5	-14.2
IMP	+44.3	+44.5
ATP	+2.3	+2.3
ADP	+17.7	+17.0
AMP	-23.0	-22.6
Inorganic P	+46.0	+44.5

a and *b* only and so, if *b* is known, the amount of *a* can be calculated from the amount of ADP formed. Similarly, *c*, *d* and *e* can be calculated. Since the number of values obtained experimentally is greater than the number of quantities to be solved for, there is a choice of procedures for calculating *a*, *b*, etc.; and since the values are subject to experimental error, the values obtained will differ according to the procedure chosen. Accordingly one procedure was chosen arbitrarily to give a set of values of *a*, *b*, etc., as a first approximation, and these values were then finally adjusted to give the best general agreement between the observed and predicted changes in the quantities of the various substances (Table 3). A statistical method of solution might have been used, but would not have been profitable since the weights to be attached to the experimental values were not known.

The agreement between the expected and observed changes shown in Table 3 is satisfactory and testifies to the reliability of the analytical methods. A further check on the reliability of the results is afforded by the balance of the changes as shown in Table 4. The agreement is again satisfactory. As expected from Table 2 there is a small loss (3.3 μ moles) of adenosine nucleotide and an equivalent gain of inosine nucleotide.

only discrepancy concerns inorganic phosphate, where the observed value is too low by 229 counts. This may be due to imperfect separation of the phosphates and in particular to contamination of IMP with inorganic phosphate. Most of the missing counts (205) were found in the IMP area, though it is unlikely that IMP itself was radioactive.

The changes observed in the control containing ITP, with some IDP, as recorded in Table 1, are in agreement with the assumption that both substrates undergo dephosphorylation. The quantitative aspects are recorded in Tables 6 and 7. There is a discrepancy between the IMP value calculated and observed, but this is within the limits of error of the analytical methods ($\pm 4\%$). The change of ITP is probably not very accurate as it is a relatively small difference value. The rate of dephosphorylation of ITP is much slower than that of IDP.

The changes in the control containing only AMP do not balance satisfactorily, in that the AMP removed is not accounted for by the substances which were determined. Other substances such as adenosine or inosine must have been formed. Dresel (1953) has demonstrated the instability of nucleotides in liver preparations.

Table 4. *Balance of changes on incubation (experiment of Table 1)*

The values refer to 25 μ l. of solution.

Total nucleotides	...	63.8 μ moles formed; 63.8 μ moles removed
Adenosine phosphates	...	19.3 μ moles formed; 22.6 μ moles removed
Inosine phosphates	...	44.5 μ moles formed; 41.2 μ moles removed
Total P	...	11.14 μ g. at 0 min.; 11.07 μ g. at 60 min.
Counts/min.	...	9359 at 0 min.; 9138 at 60 min.

Table 5. *Analysis of radioactive counts*

The calculated values are based on the assumption that the reactions listed in Table 2 took place, and refer to counts/min. in 25 μ l. of solution.

Substance	Changes in counts	
	Calc.	Obs.
ADP	+2560	+2563
ATP	+ 666	+ 760
Inorganic P	+1604	+1375

The conclusions formulated in Table 2 are confirmed by the quantitative analysis of the distribution of radioactivity. The numbers of counts expected in ADP, ATP and phosphate have been calculated on the assumption that the reactions listed in Table 2 take place in the quantities stated in the last column, and that the specific activity of the β -phosphate of ITP was identical with that of IDP. The agreement between the calculated and observed counts is again very good (Table 5). The

Table 6. *Reactions accounting for changes on addition of ITP and IDP recorded in Table 1*

Reactions	Quantities reacting in 60 min./25 μ l. of soln. (μ moles)
ITP = IDP + P	7.3
IDP = IMP + P	23.3

Table 7. *Comparison of changes observed (see Table 1), and calculated from Table 6*

The values refer to 25 μ l. of solution and 60 min.; ITP and IDP added.

Substance	Changes	
	Calc. (μ moles)	Obs. (μ moles)
ITP	- 7.3	- 7.3
IDP	-16.0	-16.0
IMP	+23.3	+29.3
Inorganic P	+30.6	+31.9

*Interaction between AMP and ITP
in brain extract*

A similar experiment in which the liver preparation was replaced by an aqueous extract of acetone-dried sheep brain is shown in Table 8. The compo-

sition of the incubated solution was the same as in the previous experiment, except for the source of the enzyme. The results are in good agreement with the assumption that essentially the same five reactions are responsible for the changes in brain and in liver. The quantitative aspects are stated in

Table 8. *Reaction between AMP and ITP (labelled with ^{32}P in terminal P)
in sheep-brain acetone powder extract*

For conditions see text.

Substance to which results refer	AMP and ITP (and IDP) added						
	P found ($\mu\text{g.}/25\ \mu\text{l.}$)		Change (μmoles)	Counts/min.		Specific activity (counts/min./ $\mu\text{g. P}$)	
	0 min.	120 min.		0 min.	120 min.	0 min.	120 min.
ITP	5.36	2.17	-34.3	8795	3157	1640	1453
IDP	2.29	4.05	+28.4	946	817	413	202
IMP	0	1.58	+51.0	0	241	0	152
ATP	0	0.63	+6.8	0	1624	0	2580
ADP	0	1.32	+21.3	0	3032	0	2295
AMP	4.13	1.85	-73.3	13	71	0	38
Inorganic P	0.63	1.10	+11.9	465	800	738	726

Table 9. *Reactions accounting for the
changes recorded in Table 8*

Reactions	Quantities reacting in 120 min./25 $\mu\text{l.}$ of soln. (μmoles)
ITP + AMP = IDP + ADP	28
ITP + ADP = ATP + IDP	6.7
ITP = IDP + P	1
IDP = IMP + P	7
AMP = IMP + NH_3	45

Table 10. *Comparison of changes observed (see
Table 8) and changes calculated from Table 9*

The values refer to 25 $\mu\text{l.}$ of solution and 120 min.; AMP, ITP and IDP added.

Substance	Changes	
	Obs. (μmoles)	Expected (μmoles)
ITP	-34.3	-35.7
IDP	+28.4	+28.7
IMP	+51.0	+52
ATP	+6.8	+6.7
ADP	+21.3	+21.3
AMP	-73.3	-73
Inorganic P	+11.9	+8

Tables 9-12. They confirm the agreement between the various independent checks of the results. There are some differences in the relative activities of the enzymes in the brain and liver preparations. In liver the dephosphorylation of IDP was a more prominent reaction than in brain. The deamination of AMP was much more rapid in brain than in liver. It should be noted that the enzyme preparation did not comprise all the enzymes of the original tissue and the comments on the relative activities therefore refer only to the preparations used.

The distribution of ^{32}P shows some unexpected features which may be due to experimental errors, e.g. the fall of specific activity of IDP on incubation (Table 8). This is probably due to an error in the count for the IDP phosphorus at 0 min. As the same specimen was used as in the experiment of Table 1, the ratio of the counts of ATP and ADP should be the same in both experiments. Another doubtful value is the radioactivity count in IMP after incubation. The reactions listed in Table 2 do not account for this. Whether additional reactions take place or whether the chromatographic separation is imperfect remains to be investigated.

Table 11. *Balance of changes on incubation for the experiment of Table 8*

The values refer to 25 $\mu\text{l.}$ of solution.

Total nucleotides	...	107.5 μmoles formed; 107.6 μmoles removed
Adenosine nucleotides	...	28.1 μmoles formed; 73.3 μmoles removed; loss, 45.2 μmoles
Inosine nucleotides	...	79.4 μmoles formed; 34.3 μmoles removed; gain, 45.1 μmoles
Total P	...	12.41 $\mu\text{g.}$ at 0 min.; 12.70 $\mu\text{g.}$ at 120 min.
Counts/min.	...	10 219 at 0 min.; 9742 at 120 min.

Reversibility of interaction between ITP and AMP

The reversibility of the reaction

is shown by an experiment in which IDP and ADP were added to lysed liver mitochondria. The experimental conditions were the same as in the experiment shown in Table 1 except that the substrates added were ADP, labelled with ^{32}P in the terminal phosphate group, and IDP. Some IMP and AMP also were initially present as contaminations of the IDP and ADP preparations used; the initial

Table 12. *Analysis of radioactive counts for the experiment of Table 8*

The calculated values are based on the assumption that the reactions listed in Table 9 took place and that the specific activity for the β -P of IDP and ITP was 202 counts/min./ μg . P. The values are for counts/min. in 25 μl . of solution.

Substance	Changes in counts	
	Calc.	Obs.
ADP	+2995	+3032
ATP	+1880	+1624
Inorganic phosphate	+784	+800

Table 13. *Reaction between IDP and ADP (labelled with ^{32}P in β -P) in sheep-liver mitochondria*

Substrates added: ADP, IDP and small amounts of IMP; 30°; for other conditions see text.

Substance to which results refer	P found ($\mu\text{g./}25\ \mu\text{l.}$)		Change ($\text{m}\mu\text{moles}$)	Counts/min.		Specific activity (counts/min./ $\mu\text{g. P}$)	
	0 min.	60 min.		0 min.	60 min.	0 min.	60 min.
ITP	~ 0	1.95	+ 21.0	39	555	—	265
IDP	8.47	5.74	- 44.1	94	101	—	—
IMP	0.38	0.81	+ 13.8	53	80	—	—
ATP	<0.05	1.85	+ 19.9	1602	1524	—	822
ADP	6.41	2.69	- 59.9	2551	1691	397	628
AMP	0.42	1.47	+ 33.9	10	6	—	—
Inorganic P	0.44	0.91	+ 15.1	392	613	890	675

quantities of IMP and AMP are stated in Table 13 under zero time.

On incubation the concentrations of IDP and ADP decreased and ATP, AMP, ITP and IMP were formed, as well as inorganic phosphate (Table 13). The balance of the changes of phosphate and nucleotides shows deficits which are above the limits of error and indicate that substances in addition to those estimated were formed, e.g. purines, ribosides, ribose phosphate and pyrophosphate. The results are roughly in agreement with the assumption that the reactions listed in Table 14 take place. It will be seen from Table 15 that some changes of IDP and IMP remain unaccounted for; the balance of the substances removed and recovered not being perfect (see Table 16), it is impossible to formulate the changes as satisfactorily as in the previous two experiments.

The radioactivity measurements qualitatively confirm the reactions formulated in Table 14. ^{32}P appeared in ITP, the gain being 516 counts. The expected gain was 897 counts. ADP lost 864 counts but the specific activity rose, which indicates the formation of ADP from a highly active ^{32}P donor. This was a trace of ATP present in the radioactive ADP in quantities which could

Table 14. *Reactions accounting for the changes on addition of ADP and IDP recorded in Table 13*

Reactions	Quantities reacting in 60 min./25 μl . of soln. ($\text{m}\mu\text{moles}$)
ADP + IDP = ITP + AMP	21
2ADP = ATP + AMP	40 and 20 respectively
IDP = IMP + P	15
AMP = IMP + NH_3	7

Table 15. *Comparison of changes observed (see Table 13), and calculated from Table 14*

The values refer to 25 μl . of solution and 60 min.; ADP and IDP added.

Substances	Changes	
	Calc. ($\text{m}\mu\text{moles}$)	Obs. ($\text{m}\mu\text{moles}$)
ITP	+21	+21.0
IDP	-36	-44.1
IMP	+22	+13.8
ATP	+20	+19.9
ADP	-61	-59.9
AMP	+34	+33.9
Inorganic P	+15	+15.1

Table 16. *Balance of changes on incubation for the experiment of Table 13*The values refer to 25 μ l. of solution.

Total nucleotides	...	88.6 μ moles formed; 104.0 μ moles removed
Adenosine phosphates	...	53.8 μ moles formed; 59.9 μ moles removed
Inosine phosphates	...	34.8 μ moles formed; 44.1 μ moles removed
Total P	...	16.17 μ g. at 0 min.; 15.42 μ g. at 60 min.
Counts/min.	...	4741 at 0 min.; 4570 at 60 min.

not be determined as phosphate, but which, owing to its high specific activity (10^5 – 10^6 counts/min./ μ g. P), added significantly to the radioactivity of the solution. If allowance is made for the conversion of ATP phosphorus into ADP phosphorus and the liberation of inorganic phosphate from ATP, the initial counts in ATP should have fallen from 1602 to 760. The number of counts found in ATP at the end of the incubation was greater by 764 counts. This is somewhat less than expected (996) but of the right order of magnitude.

Reaction between ATP and IMP

Brain extract was incubated with ATP (containing small amounts of ADP) and IMP under the conditions applying to Table 8. No interaction took place, although AMP and ITP readily interact under these conditions. ATP reacted in the presence of IMP in the same way as in its absence, i.e. a slow dephosphorylation occurred, and once AMP was formed the adenylate kinase reaction took place. No conditions in which IMP was converted into other nucleotides have been found. When it disappeared, it was by hydrolysis to inosine and possibly hypoxanthine.

Effects of inhibitors

The phosphate-transfer reactions described in this paper are, like those catalysed by ATPase and adenylate kinase, completely inhibited by 0.01M ethylenediaminetetraacetate (EDTA). NaF (0.05M) also causes inhibition. Additions of Mg or Ca ions are not necessary, but CaCl_2 (0.003M) inhibits the liberation of inorganic phosphate from nucleotides. It was added because it stabilizes the preliminary products of phosphate transfer.

DISCUSSION

The results leave no doubt of the occurrence, in brain and liver extracts, of the reversible reaction:

As the material may have contained trace quantities of ATP this reaction could have been the result of the adenylate kinase reaction:

combined with the reaction previously demonstrated:

There is no direct evidence in support of this mechanism. Addition of catalytic quantities of ATP failed to accelerate the reaction; a more direct transfer of phosphate from ITP to AMP is an alternative mechanism. It is noteworthy that ATP and IMP do not interact under conditions where ITP and AMP react.

The occurrence of analogous phosphate-transfer reaction has already been demonstrated in the intact animal by Potter, Herbert, Takagi, Siekevitz & Brumm (1954), who demonstrated the phosphorylation of uridine mono- and di-phosphate in the presence of ATP. They did not investigate phosphate-transfer reactions involving inosine phosphates.

The presence of radioactivity in the β -phosphate of IDP and ITP prepared from respiring heart-muscle preparations after incubation with inosine phosphates and radioactive inorganic phosphate cannot be explained on the basis of the known reactions. Formation of labelled IDP might be due to dephosphorylation of β - γ -labelled ATP to ADP and deamination of the ADP by the enzyme described by Webster (1953) and by Deutsch & Nilsson (1953). However, in this case the specific activities of ADP and IDP should be equal, whereas in fact that of IDP was much lower. This suggests the formation of IDP by phosphorylation of IMP.

SUMMARY

1. Extracts of acetone-dried sheep brain and lysed mitochondria of sheep liver have been shown to bring about the phosphate-transfer reaction: $\text{ITP} + \text{AMP} \rightarrow \text{ADP} + \text{IDP}$. The evidence rests on paper-chromatographic separation and quantitative determinations of the reactants and on the distribution of radioactive phosphate.

2. The reaction was found to be reversible in sheep-liver mitochondria.

3. The reaction is inhibited by ethylenediaminetetraacetate and sodium fluoride.

4. ATP and IMP did not interact under conditions where ITP and AMP interacted.

5. Apart from interacting as stated under (1), ITP and AMP enter other reactions. The observed

changes in the concentrations of nucleotides taking place on addition of ITP and AMP can be accounted for by the transphosphorylations, dephosphorylations and deamination listed in Table 2. The changes taking place on addition of ADP and IDP are approximately, but not fully, accounted for by the reactions listed in Table 14.

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The Effect of Insulin on the Anaerobic Metabolism of Rat Diaphragm

By J. H. OTTAWAY

Department of Biochemistry, University College, London

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The mechanism by which insulin increases the rate of glucose utilization by muscle has not yet been established. Cori put forward the hypothesis that insulin activates muscle hexokinase (Price, Cori & Colowick, 1945), and the work of Chaikoff and his collaborators (Chernick & Chaikoff, 1951) on glucose metabolism in liver supports this view; but the difficulty of demonstrating activation of the enzyme by insulin in tissue extracts has led to some loss of confidence in the theory. Much recent work (reviewed by Stadie, 1954) has been based on a hypothesis that insulin facilitates the transport of glucose across the cell membrane. This facilitation has sometimes been visualized as the removal of a 'permeability barrier' to glucose which is presumed to exist in the membrane. A third hypothesis, which has been suggested in various forms by Polis, Polis, Kerrigan & Jedeikin (1949), Russell (1951), Haugaard, Marsh & Stadie (1951), and others, is that insulin increases glucose utilization by making more adenosine triphosphate (ATP) available to hexokinase, possibly by increasing the efficiency of oxidative phosphorylation.

It is difficult to provide experimental evidence to confirm any one of these hypotheses, assuming that one of them is, in fact, correct. In all probability no one line of attack will provide decisive evidence, but investigation of the effectiveness of insulin in muscle kept anaerobic was thought likely to be profitable for the following reasons. If either the Cori hypothesis or the 'permeability' hypothesis is correct, exclusion of oxygen from the muscle should

not itself affect insulin activity, so long as more than enough ATP remains in the tissue to saturate hexokinase. ATP is regenerated, in anaerobic conditions, by glycolysis and by transfer of phosphate from phosphocreatine, so that the ATP concentration might be expected to remain above saturation level for some time after exclusion of oxygen. If the hypothesis that insulin increases the efficiency of oxidative phosphorylation is correct, however, exclusion of oxygen from the system should severely impair or abolish insulin activity. For this type of work the *in vitro* incubation of the isolated rat diaphragm was regarded as very suitable, and this paper reports the results of experiments using this technique. The effects of other methods of reducing ATP synthesis have been included for comparison.

No conclusions about the mechanism of insulin action can be drawn if glucose utilization is impaired because the ATP concentration falls below saturation level early in the experiment. It was therefore important to be certain that ATP was not, in these experiments, the limiting factor in glucose uptake. Two methods of checking this point have been used in two independent laboratories, with almost identical results. The concentration of ATP in anaerobic diaphragm muscle has been estimated directly by Walaas & Walaas (1952), who were also interested in the relationship between insulin effectiveness and ATP levels in muscle. They found that the ATP concentration in the aqueous phase of the muscle remained above