The Stimulatory Effect by Insulin on the Incorporation of ^{32}P Radioactive Inorganic Phosphate into Intracellular Inorganic Phosphate, Adenine Nucleotides and Guanine Nucleotides of the Intact Isolated Rat Diaphragm

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Summary. The aim of these investigations has been to contribute to an understanding of the mechanism of action of insulin in promoting increased turn over of ^{32}P inorganic phosphate into ATP and other mononucleotides in the rat diaphragm. During incubation of the intact rat diaphragm in Krebs Ringer bicarbonate medium without addition of substrate, no effect of insulin on the content of adenine nucleotides and guanine nucleotides including cyclic 3',5' AMP and cyclic 3',5' GMP has been observed. When ^{32}P radioactive inorganic phosphate was present in the medium, insulin increased the specific activity of intracellular inorganic phosphate and promoted an increased incorporation of ^{32}P into ATP and GTP. The effect on ADP was less pronounced. It has been shown that these effects of insulin depend upon the ionic composition of the medium and on ion membrane-transport. The insulin-promoted increase of specific activity of intracellular inorganic phosphate and of increased ^{32}P labelling of ATP (and GTP) was to a great extent inhibited by ouabain. During incubation in media where sodium chloride had been iso-osmotically replaced by potassium chloride or choline chloride, the above mentioned effects by insulin were abolished. The results indicate that the increased labelling of ATP (and GTP) promoted by insulin can be primarily attributed to events occurring at the cell membrane. The increased specific activity of intracellular inorganic phosphate in the presence of insulin can be explained by increased uptake of ^{32}P inorganic phosphate from the medium. Apparently the increased ^{32}P labelling of ATP (and GTP) occur as a secondary consequence. It is suggested that this insulin effect is primarily concerned with an effect on membrane enzymes, particularly the Na^+ , K^+ , Mg^{2+} activated ATP-ase.

Effet stimulant de l'insuline sur l'incorporation du phosphate inorganique radioactif marqué au ^{32}P , dans le phosphate inorganique intracellulaire, les adénine-nucléotides et les guanine-nucléotides du diaphragme intact, isolé du rat

Résumé. Le but de ces investigations a été de contribuer à la compréhension du mécanisme d'action de l'insuline, en provoquant un turn-over accru du phosphate inorganique marqué au ^{32}P , en ATP et autres mononucléotides, dans le diaphragme du rat. Pendant l'incubation du diaphragme intact de rat dans un milieu bicarbonaté de Krebs Ringer, sans addition de substrat, on n'a pas observé d'effet de l'insuline sur le contenu en adénine-nucléotides, en guanine-nucléotides, y compris le 3',5' AMP cyclique et le 3',5' GMP cyclique. Quand du phosphate inorganique radioactif marqué au ^{32}P était présent dans le milieu, l'insuline augmentait l'activité spécifique du phosphate inorganique intracellulaire et provoquait une incorporation accrue du ^{32}P dans l'ATP et le GTP. L'effet sur l'ADP était moins prononcé. Il a été montré que ces effets de l'insuline dépendent de la composition ionique du milieu et du transport membranaire des ions. L'augmentation due à l'insuline de l'activité spécifique du phosphate inorganique intracellulaire et du marquage

accru au ^{32}P de l'ATP (et du GTP) était en grande partie inhibée par l'ouabaine. Pendant l'incubation dans les milieux où le chlorure de sodium a été remplacé iso-osmotiquement par le chlorure de potassium ou de choline, les effets de l'insuline décrits ci-dessus étaient abolis. Les résultats indiquent que le marquage accru de l'ATP (et du GTP) provoqué par l'insuline, peut être en premier lieu attribué à des phénomènes survenant au niveau de la membrane cellulaire. L'augmentation de l'activité spécifique du phosphate inorganique intracellulaire en présence d'insuline, peut s'expliquer par une captation accrue du phosphate inorganique marqué au ^{32}P , à partir du milieu. Apparemment le marquage accru au ^{32}P de l'ATP (et du GTP) est une conséquence secondaire. On suggère que cet effet de l'insuline est d'abord en rapport avec un effet sur les enzymes de la membrane, en particulier sur l'ATP-ase activée par Na^+ , K^+ et Mg^{2+} .

Die stimulierende Wirkung von Insulin auf den Einbau von mit ^{32}P markiertem anorganischen Phosphat in das intrazelluläre anorganische Phosphat und die Adenin- und Guaninnucleotide des intakten isolierten Rattenzwerchfells

Zusammenfassung. Ziel dieser Untersuchungen war es, zu einem besseren Verständnis der stimulierenden Wirkung von Insulin auf den Einbau von anorganischem ^{32}P Phosphat in ATP und andere Mononucleotide im Rattenzwerchfell beizutragen. Während der Inkubation intakter Rattenzwerchfelle in Krebs-Ringer-Bicarbonat Puffer ohne Substratzusatz konnte kein Insulineffekt auf den Gehalt an Adenonucleotiden und Guaninnucleotiden, sowie von cyclischem 3',5' AMP und cyclischem 3',5' GMP beobachtet werden. In Gegenwart von anorganischem ^{32}P Phosphat steigerte Insulin die spezifische Aktivität von intrazellulärem anorganischen Phosphat und bewirkte einen verstärkten Einbau von ^{32}P in ATP und GTP. Die Wirkung auf ADP war weniger ausgeprägt. Es konnte gezeigt werden, daß diese Insulineffekte von dem Ionen-Gehalt des Mediums und dem Membrantransport der Ionen abhängen. Die Steigerung der spezifischen Aktivität des anorganischen Phosphates und der Markierung von ATP (und GTP) mit ^{32}P ließ sich weitgehend durch Ouabain wieder aufheben. Bei Inkubation in Pufferlösungen, die statt NaCl isosmolare Mengen von KCl oder Cholinchlorid enthielten, waren die oben erwähnten Insulineffekte nicht mehr nachweisbar. Die Resultate sprechen dafür, daß die verstärkte Markierung von ATP (und GTP) unter Insulin in erster Linie auf Vorgängen an der Zellmembran beruht. Die erhöhte spezifische Aktivität des intrazellulären anorganischen Phosphates in Gegenwart von Insulin läßt sich durch einen verstärkten Einstrom von ^{32}P Phosphat aus dem Medium erklären. Augenscheinlich kommt es dadurch sekundär zu einer gesteigerten ^{32}P Markierung von ATP (und GTP). Wir möchten annehmen, daß dieser Insulineffekt vorwiegend über die Membranenzyme erfolgt und sich vor allem auf die Na^+ , K^+ und Mg^{++} aktivierbare ATPase erstreckt.

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It has previously been reported that insulin enhances the incorporation of ^{32}P -labelled inorganic phosphate into ATP¹ and ADP in the cut diaphragm incubated in the absence of substrate (CLAUSER et al., 1962). No definite explanation for this insulin effect has, however, been given. In particular, no effect of insulin on the specific activity of intracellular inorganic phosphate was observed. The effect could not be attributed to an effect on protein synthesis, since the increased labelling of ATP and ADP was seen also in the presence of puromycin and actinomycin-D (EBOUÉ-BONIS et al., 1963).

In the present study, an increased ^{32}P labelling of nucleotides during incubation with insulin has been confirmed on the intact diaphragm in the absence of added substrate. Further investigations have been performed in order to elucidate the mechanism of this effect. It has been particularly considered whether this insulin effect is related to events occurring at the cell membrane. Actually, increased specific activity of intracellular inorganic phosphate has been demonstrated as the result of insulin action. In further experiments, the dependence of the insulin effect on the ion composition of the medium, and on membrane ion transport has been investigated. The results have been discussed in relation to an insulin effect on the membrane enzymes, particularly adenyl-cyclase and sodium-potassium-activated ATP-ase.

Experimental procedure

Incubation technique. Female rats weighing 200–250 g from a local strain (originally Wistar) were used. The animals were fasted for 18 hours, and killed by a blow on the head and decapitated. The diaphragms were dissected out, chilled in ice-cold medium, and the so called "intact preparation" according to KIPNIS and CORI (1957) was prepared. Before incubation the preparation was divided into two halves according to KONO and COLOWICK (1961). One half was used as control, the other half incubated with addition of hormone. Two or three hemidiaphragms together were incubated in 100 ml Erlenmeyer flasks. Soaking of the diaphragms was done for 15 minutes in 50 ml Krebs Ringer bicarbonate medium without substrate at 37°C, aerated with 95% O_2 /5% CO_2 in a Dubnoff metabolic shaking incubator. Thereupon the diaphragm preparations were transferred to a second 100 ml incubation flask containing 50 ml fresh medium of the same composition. To the medium had been added 400 μC radioactive phosphate ($\text{NaH}_2\text{ }^{32}\text{PO}_4$) contained in a volume of 400 μl . Addition of insulin was made to give a final concentration of 0.1 U/ml. As shown in the legends to the tables, some experiments were performed in sodium-

free media, where sodium chloride had been replaced by iso-osmotic concentrations of potassium chloride or choline chloride. (See details in legends to tables). The incubation time was usually 10 min, with continuous aeration with 95% O_2 /5% CO_2 and constant shaking. Thereupon the preparations were transferred to chilled media, and the diaphragms rapidly dissected out and frozen in liquid nitrogen. For the determination of intracellular inorganic phosphate the time of incubation were 30, and 60 min respectively. The diaphragms were then rapidly transferred to a chilled plate, blotted, frozen and weighed.

Extraction procedure. The frozen diaphragms were weighed on a torsion balance, and mechanically powdered in a stainless steel mortar cooled with liquid nitrogen. The frozen powder from two or three hemidiaphragms weighing 600–900 mg was homogenized in 5 ml 10% perchloric acid (PCA) for 5 min in an all glass Potter Elvehjem homogenizer cooled in an ice bath. The homogenate was centrifuged for 10 min at 10000 g in a Sorvall SS centrifuge at +2°C. The residue was reextracted twice with 2 ml 10% perchloric acid, and the combined supernatants were subjected to the purification procedure.

Purification of extracts. The diaphragm extracts containing the acid-soluble nucleotides were purified on charcoal, essentially according to the procedure of Tsuboi and Price (1959). Thereupon the nucleotides were separated by thin layer chromatography (TLC) according to RANDERATH and RANDERATH (1964). The main steps were the following: 10–20 mg Nuchar C was added to 8 ml PCA-extract in an 50 ml Erlenmeyer flask. The Nuchar C had been previously washed twice with 0.1 N HCl and three times with distilled water. A stock solution of Nuchar C, containing 10 g in 100 ml H_2O was made. After dilution of the stock solution with H_2O (1:6), 2 ml was added to the PCA extract. After shaking for 15–30 min in the cold (+4°C), adsorption of purine nucleotides as well as pyrimidine nucleotides on to Nuchar C was complete. The washings of the particles and the adjustment of pH for proper elution were made according to GORSKI and MUELLER (1963), except for the use of phosphate buffer. In our hands, several washings with water were preferred for the removal of perchloric acid and perchlorates, and the pH was adjusted by a final washing with 0.05 M acetate buffer, pH 4.5. Elution of nucleotides from Nuchar C was achieved by shaking with 6 ml 2.5% pyridine in 50% ethanol in Erlenmeyer flasks for 2 h at 37°C in a water bath. Nuchar C was removed by centrifugation and filtration through a sintered-glass filter. The eluting solvents were evaporated *in vacuo* over night.

Thin layer chromatography. The residues were dissolved in 170 μl H_2O and appropriate volumes were applied on polyethylene-imine-cellulose plates. Two dimensional, thin-layer chromatography (LiCl at three concentrations 0.3 M, 0.8 M and 2.0 M in the first dimension and sodium formate at four different concen-

¹ Abbreviations:

ATP, ADP, AMP: Adenosine tri-, di-, mono-phosphate.
Cyclic 3',5'-AMP: Adenosine 3',5' cyclophosphate.
GTP, GDP, GMP: Guanosine tri-, di-, mono-phosphate.
Cyclic 3',5' GMP: Guanosine 3',5' cyclophosphate.

trations 0.25 M, 1.0 M, 2.0 M and 4.0 M in the second dimension) was done essentially according to RANDE-RATH and RANDE-RATH (1964). The plates were dried, and the nucleotide spots were located by visualization of UV absorption with a UV light lamp at 2537 Å (The chromatolight, Hanovia, Slough, England). Guanine nucleotides were visualized by their fluorescence in UV light after exposing the plates to conc. HCl vapour for 5 min. The powder from each spot was removed with a small spatula, transferred to alumina plates and counted in a windowless, methane flow counter (Frieske and Hoepfner).

Phosphorus determination. After counting, the powder samples containing the nucleotide spots were extracted with 500 μl N H_2SO_4 by gentle stirring with a glass rod for 30 min at room temperature. After centrifugation, 400 μl of the supernatant was ashed with 100 μl 5N H_2SO_4 at 170°C, followed by boiling with H_2O_2 . Phosphorus was determined by the method of FISKE and SUBBABOW (1925). Powder "blank spots" from each plate were extracted and treated in the same way as the nucleotide spots. Phosphorus content of the "blank spots" did not exceed 0.01 μg , and was found to be rather constant. The results are expressed as cpm/ μg P of nucleotides (specific activity), or as percentage of control specific activity. The determination of inorganic phosphorus in muscle extracts was done on aliquots by the principle of BERENBLUM and CHAIN (1938), as modified by WEIL-MALHERBE and GREEN (1951). Determination of inorganic phosphorus in incubation media was done after protein precipitation with 4 per cent perchloric acid (1:5). After centrifugation, phosphorus was determined on aliquots of the supernatant by the same method.

Recovery of nucleotides. A near quantitative recovery (85%–95%) of ATP, UTP and CTP was obtained when these compounds were added as "internal standards" to PCA extracts and carried through the whole procedure of TLC. However, recovery of the guanine nucleotides (GTP, GDP and GMP) added as "internal standards" to PCA extracts and carried through the procedure was only 50%–60%. The recovery of standards of guanine nucleotides subjected to TLC without charcoal treatment was 92%–100%. The loss, therefore, could be attributed to the purification step with charcoal. Although the adsorption of guanine nucleotides on to charcoal was as complete as for the other nucleotides, it was shown that the elution from Nuchar C was incomplete. However, in repeated experiments a constant value of 50%–60% recovery was found. This is in accordance with PLAISTED and REGGIO (1963), who found a loss of 47% when guanine nucleotides were adsorbed on to and eluted from charcoal. The values of guanine nucleotides presented, are corrected for the loss which occurs during the purification procedure. It has previously been shown that during incubation of rat diaphragm *in vitro*, a considerable breakdown of nucleoside triphosphates takes place (ALERTSEN et al., 1958). Part of the nucleoside diphos-

phates observed on the chromatograms are therefore derived from dephosphorylation of the nucleoside triphosphates during incubation. The same is true for the relatively large amounts of IMP observed.

Calculation of specific radioactivities

The specific activity of the γ -phosphate of ATP has been calculated on the assumption that the β -phosphate of ATP is in isotopic equilibrium with the terminal phosphate in ADP. This is in accordance with the work of FLECKENSTEIN and JANKE (1957), HARTH and MANDEL (1961) and CLAUSER et al. (1962). During the short incubation period used in the present study the specific activity of the terminal phosphate in ADP was much lower than that of the γ -phosphate in ATP. This indicates that a considerable part of ADP does not take part in the adenylate kinase reaction. On the other hand, the specific activities of the terminal phosphate in GDP and the γ -phosphate in GTP were of the same order of magnitude. The labelling of the α -phosphate in AMP and GMP was very low, and no correction for the isotopic labelling of the α -phosphates has been made in the calculation of specific activities of the nucleoside di- and triphosphates.

As will be presented below, the uptake of radioactive phosphate from the medium into the intact diaphragm is rather slow. However, after an incubation period of 30 min the content of inorganic phosphate in the medium was increased by 10–20 per cent, while the specific activity decreased by 20–30 per cent. This can be attributed to a rapid exchange of phosphate between the medium and the damaged muscle fibres of the tissue preparation along the rib cage. The large and somewhat variable changes in the phosphate content and in the specific activity of the medium complicate the determination of the specific activity of intracellular inorganic phosphate. The phosphate content and the specific activity of the medium at the end of the incubation period were used as an expression for these figures in the extracellular space of the diaphragm. An extracellular space of 18 per cent in the intact diaphragm was used in the calculations (KIPNIS and CORI, 1957).

Materials

Insulin from Novo, Copenhagen (10 $\times$ recrystallized ox insulin) was dissolved in 0.6 per cent acetic acid to a concentration of 50 U/ml, freshly made. 100 μl of this stock solution was added to 50 ml medium.

Radioactive phosphate $\text{NaH}_2\text{}^{32}\text{PO}_4$ at a concentration of 1 mCi/ml was obtained from Institutt for Atomenergi, Kjeller, Norway. 400 μCi of the neutralized solution was added to each incubation flask, containing 50 ml medium.

Ouabain, caffeine and nucleotide standards were obtained from Sigma Chemical Company, Saint Louis, Mo., U.S.A.

Guanosine 3',5' cyclophosphate (cyclic 3',5' GMP) was synthesized according to the method of SMITH et al. (1961). On thin layer chromatography the purified compound was identical with a sample of cyclic 3',5' GMP synthesized by Dr. GEORGE I. DRUMMOND, University of British Columbia, Vancouver.

Charcoal used for nucleotide adsorption was *Nuchar C*, Vegetable Carbon from Chemical Division, West Virginia Pulp and Paper Co., New York. The material for thin layer chromatography was *Cellulose* powder MN 300 from Macherey, Nagel & Co., Germany, and *Polyethyleneimine* (PEI) from Badische Anilin und Sodafabrik, Germany.

Results

In Fig. 1 the chromatographic separation of nucleotides from an extract of rat diaphragm is presented. The different spots were identified by co-chromatography of appropriate standards. In addition, the

Fig. 1. Separation of acid soluble nucleotides in PCA extract of rat diaphragm by thin layer chromatography according to Randerath and Randerath (1964)

different spots were eluted and identified by their absorption spectra at acid and alkaline pH, and by determination of phosphorus content. Cyclic 3',5' AMP and cyclic 3',5' GMP were further identified by thin layer chromatography in a second two-dimensional system (RANDERATH, 1964), which specifically separates nucleoside monophosphates. A total of four guanosine monophosphates were separated on the chromatogram. Two of these compounds are unknown and not identical with 5'-GMP, cyclic 3',5' GMP, 2'-GMP or 3'-GMP.

The concentrations of adenine nucleotides and guanine nucleotides in the diaphragm are reported in Table 1. It appears that the concentration of GTP amounted to 2 per cent of the ATP concentration. The concentrations of UTP and CTP were even less. These latter compounds were not easily separated from ATP by the chromatographic procedure. It is seen that insulin has no effect on the concentration of nucleotides during the short incubation time used. In particular, it should be pointed out that no effect of insulin on the

content of cyclic 3',5' AMP and cyclic 3',5' GMP could be observed.

Table 2 shows the specific activity of adenine nucleotides and guanine nucleotides after incubation with ^{32}P -radioactive inorganic phosphate. The specific activity of the γ -phosphate group in ATP was about twice as high as the specific activity of the terminal phosphate group in ADP, which is in equilibrium with the β -phosphate group in ATP (FLECKENSTEIN and JANKE, 1957; HART and MANDEL, 1961; CLAUSER et al., 1952). The specific activity of the γ -phosphate group in GTP was slightly higher than the γ -phosphate in ATP. The specific activity of the terminal phosphate group in GDP and the β -phosphate group in GTP were of the same order of magnitude as the γ -phosphate in GTP. Incorporation of ^{32}P into the phosphate group of AMP and GMP was very low under these conditions of incubation *in vitro* with radioactive phosphate.

Insulin gave a pronounced increase (mean value 74%) in the specific activity of the γ -phosphate group in ATP, whereas the labelling of the terminal phosphate group in ADP was only slightly increased (mean value 34%). Insulin also increased the labelling of the terminal phosphate groups in GTP and GDP to the same extent as the γ -phosphate in ATP. No effect of insulin on the incorporation of ^{32}P into AMP and GMP was observed.

Fig. 2 shows that the specific activity of ATP during incubation with ^{32}P increased in linear correlation with time up to 30 min. During this incubation period the percentage stimulatory effect of insulin was constant. Similar curves were obtained for the incorporation of ^{32}P into GTP. For the subsequent experiment a short incubation time of 10 min was chosen.

Fig. 2. The effect of insulin on the specific activity of ATP¹ and intracellular inorganic phosphate. Intact rat diaphragm incubated in Krebs Ringer bicarbonate medium without substrate

—○— : Inorganic P.
—●— : ATP

¹ Refers to the γ -P of ATP.

The increased incorporation of ^{32}P into ATP and GTP produced by insulin may be due to an increase of ^{32}P uptake from the medium, or to an increased turnover with more rapid equilibration of ATP (and GTP)

with ^{32}P intracellular inorganic phosphate. In order to distinguish between these possibilities measurements of the specific activity of intracellular inorganic phosphate were made. Owing to the rather slow penetration

measurements were made with incubation times of 30 and 60 min. Table 3 shows that the specific activity of intracellular inorganic phosphate was significantly increased by insulin after 60 min incubation. From Fig. 2

Table 1. Acid soluble adenine nucleotides and guanine nucleotides in the intact rat diaphragm after incubation for 10 min in the absence of substrate, in the absence and the presence of insulin

Group	ATP	ADP	5'-AMP	Cyclic 3',5' AMP	GTP	GDP	5'-GMP	Cyclic 3',5' GMP
	μ moles per g wet tissue				μ moles per g wet tissue			
Control (6)	2.36 ± 0.15	0.46 ± 0.04	0.051 ± 0.011	0.0060 ± 0.0005	0.058 ± 0.004	0.032 ± 0.007	0.094 ± 0.012	0.0062 ± 0.0015
Insulin (6)	2.30 ± 0.24	0.45 ± 0.05	0.057 ± 0.013	0.0058 ± 0.0012	0.060 ± 0.003	0.026 ± 0.002	0.092 ± 0.014	0.0058 ± 0.0012

$\pm$ Standard error of the mean.

(): Number of experiments.

Table 2. The effect of insulin on the incorporation of inorganic radioactive phosphate ^{32}P into adenine nucleotides and guanine nucleotides of the intact isolated rat diaphragm incubated in the absence of substrate in Krebs Ringer bicarbonate medium. Experiments performed in the presence of 0.2 mM ouabain or 1 mM caffeine without and with insulin are also presented. The specific activity of ATP and GTP refers to the γ -phosphate group. The specific activity of ADP and GDP refers to the terminal phosphate group. $\pm$ Standard error of the mean. P: Probability of the significance of the difference of controls versus insulin

Group	ATP	ADP	5'-AMP	GTP	GDP	5'-GMP
Specific activity						
Control (9)	746 $\pm$ 37	394 $\pm$ 20	35 $\pm$ 8	867 $\pm$ 55	923 $\pm$ 74	69 $\pm$ 4
Insulin (6)	1293 $\pm$ 198	528 $\pm$ 55	46 $\pm$ 15	1263 $\pm$ 113	1430 $\pm$ 209	42 $\pm$ 25
P	0.005	0.02	> 0.10	0.001	< 0.01	> 0.10
Ouabain (7)	797 $\pm$ 107	386 $\pm$ 36	34 $\pm$ 3	1052 $\pm$ 95	862 $\pm$ 115	87 $\pm$ 26
Insulin + ouabain (7)	926 $\pm$ 100	423 $\pm$ 23	83 $\pm$ 29	1127 $\pm$ 29	1008 $\pm$ 168	127 $\pm$ 34
P	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10	> 0.10
Caffeine (4)	580 $\pm$ 62	341 $\pm$ 20	72 $\pm$ 29	898 $\pm$ 42	835 $\pm$ 104	117 $\pm$ 30
Insulin + caffeine (6)	1061 $\pm$ 89	434 $\pm$ 30	48 $\pm$ 15	1452 $\pm$ 134	1270 $\pm$ 172	119 $\pm$ 24
P	< 0.005	0.05	> 0.10	0.02	0.10	> 0.10

(): Number of experiments.

Table 3. The effect of insulin on the specific activity of intracellular inorganic phosphate. Intact rat diaphragm incubated for 60 min in different incubation media without substrate

Incubation medium	Control		Insulin		
	mg P/g wet weight	Specific activity (% extracellular P)	mg P/g wet weight	Specific activity (% extracellular P)	P ^b
Krebs Ringer bicarbonate (4)	0.35 $\pm$ 0.02	3.0 $\pm$ 0.3	0.39 $\pm$ 0.03	6.3 $\pm$ 0.8	< 0.001
Krebs Ringer bicarbonate + 0.2 mM ouabain (6)	0.33 $\pm$ 0.03	4.0 $\pm$ 0.8	0.36 $\pm$ 0.02	4.6 $\pm$ 0.8	> 0.10
Choline Chloride medium ^a (3)	0.26 $\pm$ 0.02	4.6 $\pm$ 0.9	0.28 $\pm$ 0.03	4.2 $\pm$ 0.8	> 0.10

^a Sodium chloride replaced isoosmotically by choline chloride.

^b P refers to insulin effect on specific activity.

of ^{32}P into the intact diaphragm (after 10 min incubation the specific activity of intracellular inorganic phosphate was less than 1 per cent of the specific activity of inorganic phosphate in the medium) mea-

it also appears that the specific activity of ATP was so high that a nearly complete equilibration of the terminal phosphate group of ATP (and GTP) with the bulk intracellular inorganic phosphate had taken place

both in the absence and the presence of insulin. As discussed below, these results indicate that the increased labelling of ATP produced by insulin is due to increased uptake of ^{32}P from the medium rather than a more rapid phosphorylation of ADP with intracellular inorganic phosphate. In these experiments no significant effect of insulin on the concentration of intracellular inorganic phosphate could be shown (Table 3).

In further experiments the dependency of the insulin effect upon the ion composition of the medium and on ion transport in the diaphragm was studied. This was done partly by addition of ouabain, which is known to inhibit sodium and potassium transport by an effect on the Na^+ , K^+ -activated membrane ATP-ase (SKOU, 1957), and partly by incubation in a sodium-free medium. Table 2 shows the results when ouabain was present during the final incubation period of 10 minutes. In the absence of insulin, no effect by ouabain on the incorporation of ^{32}P into nucleotides was observed. However, insulin stimulation of the specific activity of nucleotides was greatly depressed when ouabain was present. In the absence of ouabain, the stimulatory effect of insulin on the specific activity of ATP amounted to 74% (mean value), whereas the corresponding effect of insulin in the presence of ouabain was only 18% (not significant). It also appears that ouabain inhibited the insulin effect on the labelling of ADP, GTP, GDP to the same extent as for ATP.

The specific activity of the intracellular inorganic phosphate was also studied in diaphragms incubated in media of different ionic composition (Table 3). In the absence of insulin, the specific activity of the intracellular inorganic phosphate of diaphragms incubated in media containing ouabain, or in media where sodium had been replaced by choline chloride, was slightly higher compared with the values obtained by incubation in usual Krebs Ringer media, but the difference was not significant. On the other hand, the insulin effect on the labelling of intracellular inorganic phosphate was completely abolished when sodium was absent, or when ion transport was inhibited by ouabain. This gives additional support to the concept that the insulin effect on ^{32}P labelling of nucleotides is dependent upon an insulin stimulation of ^{32}P phosphate uptake.

It has been shown that under certain conditions insulin action on tissues results in a decreased level of cyclic 3',5' AMP (BUTCHER et al., 1966). The possibility that the insulin effect is counteracted when the level of cyclic 3',5' AMP tends to increase was therefore investigated. The results are given in Table 2 in experiments with addition of caffeine, which is known to be an inhibitor of phosphodiesterase (SUTHERLAND and RALL, 1960), thereby increasing the level of cyclic 3',5' AMP in tissues. It is, however, seen that the labelling of nucleotides in the absence and the presence

Table 4. The influence of insulin on the incorporation of radioactive phosphate ^{32}P into mononucleotides of intact isolated rat diaphragm in the absence of substrate. Sodium chloride in incubation medium iso-osmotically replaced by potassium chloride or choline chloride. The specific activity of ATP and GTP refers to the γ -phosphate group. The specific activity of ADP and GDP refers to the terminal phosphate group

Group	Medium	ATP	ADP	5'-AMP	IMP	GTP	GDP	5'-GMP
		Specific activity						
Control (4)	Potassium	498 $\pm$ 40	475 $\pm$ 31	— ^a	65 $\pm$ 10	876 $\pm$ 84	998 $\pm$ 49	118 $\pm$ 55
Insulin (4)	chloride	474 $\pm$ 22	408 $\pm$ 15	— ^a	64 $\pm$ 11	973 $\pm$ 108	946 $\pm$ 96	201 $\pm$ 60
Control (4)	Choline	350 $\pm$ 17	240 $\pm$ 41	17 $\pm$ 10	—	410 $\pm$ 82	376 $\pm$ 144	100 $\pm$ 44
Insulin (4)	chloride	310 $\pm$ 31	216 $\pm$ 10	18 $\pm$ 7	—	316 $\pm$ 48	380 $\pm$ 122	134 $\pm$ 62

^a 5'-AMP was almost absent. Instead considerable amount of IMP (inosinic acid) was present.

The insulin effect was also investigated in a sodium-free medium, where sodium had been iso-osmotically replaced by potassium (Table 4). Under these conditions, labelling of nucleotides by ^{32}P in the absence of insulin was only slightly decreased compared with experiments in usual Krebs-Ringer medium (Table 2). However, the stimulatory effect by insulin was completely abolished in the sodium-free medium. Table 4 also demonstrates the results when the diaphragm was incubated in a medium where sodium chloride had been iso-osmotically replaced by choline chloride. This procedure gave a general reduction of phosphorylations as shown by the low level of specific activity of the nucleotides in the absence of insulin. Under these conditions also insulin did not give any stimulation of the ^{32}P labelling of adenine and guanine nucleotides.

of insulin is not significantly different from the results without caffeine present.

Discussion

Insulin effect on nucleotides and inorganic phosphate. The observation made by CLAUSER et al. (1962) that insulin promotes enhanced labelling of ATP and ADP with radioactive inorganic phosphate when the isolated diaphragm is incubated in the absence of added substrate, has been confirmed in the present study. Similar observations have recently been reported on isolated fat cells (HEPP et al., 1968).

The present work has also demonstrated that insulin gives an increased specific activity of intracellular inorganic phosphate, indicating that insulin increases

the transport of radioactive phosphate from the medium across the cell membrane. An insulin stimulation of phosphate uptake in muscle is in accordance with the results reported by KAJI and PARK (1961) and by SARKAR and OTTAWAY (1962) in experiments on the isolated perfused rat heart. In red blood cells a rapid effect of insulin on phosphate transport has also been shown (DORMANDY, 1965).

Such an effect of insulin was not seen by CLAUSER et al. (1962) in the cut diaphragm preparation used in their studies. However, this discrepancy may be explained by the much slower uptake of radioactive phosphate from the medium into the intact diaphragm compared with the cut diaphragm preparation. The intracellular inorganic phosphate in the intact preparation attains a specific activity of below 2 per cent of extracellular phosphate after 30 min incubation, whereas in the cut preparation the specific activity amounts to 18–19 per cent. Therefore, the rapid uptake of phosphate from the cut surfaces in the experiments of CLAUSER et al. (1962) may have masked an insulin effect on the membrane transport of phosphate.

The present work indicates an insulin effect primarily concerned with increased uptake of radioactive phosphate into the cell, and with increased labelling of nucleoside triphosphates as a secondary consequence.

Thus the increased specific activity of ATP and GTP given by insulin may be attributed to the equilibration of the nucleotide pool with inorganic phosphate of a higher specific activity. Our results, therefore, indicate that the insulin effect on the ^{32}P labelling of nucleotides is linked to events occurring at the cell membrane rather than to a primary effect on the intracellular metabolism. This view is supported by the experiments where ouabain was added during incubation, and also when incubation was performed in sodium-free media.

Mechanism of insulin action. In studies concerned with the mechanism of insulin action it has been repeatedly suggested that an insulin effect on transphosphorylation reactions may explain many secondary effects of insulin on metabolism. It may therefore be of interest to discuss the insulin effect on ^{32}P labelling of nucleoside phosphates reported here in relation to an effect by insulin on transphosphorylating enzymes. Of particular interest in this respect are the enzymes nucleoside diphosphokinase, adenyl cyclase and Mg^{++} , Na^+ , K^+ -activated ATP-ase.

Relation to nucleoside diphosphokinase. In a recent theory KLACHKO (1966) has proposed that the increased rate of synthesis and storage in the cell, produced by insulin, might be explained by an effect on the nucleoside diphosphokinase. According to this author, an effect by insulin on this enzyme might result in an accelerated transfer of high energy phosphate from ATP to all other nucleoside diphosphates, resulting in an increased level of GTP, UTP and CTP at the expense of ATP. It should be pointed out that no evidence for this theory has been obtained in the present work. No

influence of insulin on the concentration of GTP and ATP has been shown, and the specific activity of GTP and ATP were increased to the same extent by insulin.

Relation to adenyl cyclase. The possibility that insulin exerts an effect on adenyl cyclase, resulting in a decreased formation of cyclic 3',5' AMP, has recently been the focus of much interest. Evidence for such an influence has in fact been put forward by BUTCHER et al. (1966), who reported that insulin in the fat pad decreased the level of cyclic 3',5' AMP, which had first been elevated by large doses of adrenaline. Insulin alone, or insulin with lower doses of adrenaline had no effect. In experiments on muscle, LARNER et al. (1968), however, did not find any evidence for an effect by insulin on an adenyl cyclase. Insulin, *in vitro* as well as *in vivo*, did not decrease the level of cyclic 3',5' AMP in muscle in the presence or in the absence of adrenaline. In the present study no evidence for any effect by insulin on adenyl cyclase was obtained either. The level of cyclic 3',5' AMP as well as cyclic 3',5' GMP, were of the same order of magnitude in the absence and in the presence of insulin. In addition, caffeine was without any inhibitory action on the insulin-stimulated phosphate turnover in ATP and GTP. In a recent report, no evidence has been obtained that cyclic nucleotides other than cyclic 3',5' AMP act as mediators of insulin action (WALAAS et al., 1968). This refers to experiments on glycogen synthetase and its kinase from leg muscle. The known D $\rightarrow$ I conversion of glycogen synthetase promoted by insulin (VILLAR-PALASI and LARNER, 1960) could not be attributed to cyclic 3',5' GMP, cyclic 3',5' UMP nor to cyclic 2',3' GMP or cyclic 2',3' UMP as mediators.

Relation to Na^+ , K^+ , Mg^{++} -ATPase. The observation that the insulin effect is dependent on the ion composition of the medium and is inhibited by ouabain, may indicate a relationship between the effect of insulin and the activity of the sodium-potassium-activated ATP-ase. This enzyme system, first isolated from a microsomal fraction of nerve tissue by SKOU (1957) has been found in muscle and a number of other tissues (BONTING et al., 1961). The enzyme system is considered to be involved in the mechanism of sodium and potassium transport across cellular membranes (SKOU, 1965; POST et al., 1965), and to be related to the restoration of the membrane potential (KEYNES, 1965). This ATP-ase activity, which is dependent upon the presence of sodium and potassium, is inhibited by ouabain, and it is indicated that the splitting of ATP provides the energy for ion transport across cell membranes. It has been pointed out (BLOND and WHITTMAN, 1964) that this ATP-dependent, directional flow of ions in part controls the intracellular concentration of ADP, which in turn controls the rate of mitochondrial phosphorylations.

An effect of insulin on the transport of ions across the cell membrane is well established. ZIERLER (1959) reported that insulin increased the concentration of potassium in muscle also in the absence of glucose in

the medium. It was further shown that insulin decreased the influx of potassium more than the efflux (ZIERLER, 1960). In addition ZIERLER (1966) has reported that the active efflux of sodium is increased by insulin. GOURGLEY (1961) observed that ouabain caused a net loss of potassium from the isolated frog muscle, and that the insulin stimulation of potassium uptake by the muscle was completely blocked by ouabain.

A number of effects of ouabain on membrane transport and metabolism have recently been reported. KIPNIS and PARRISH (1965) demonstrated that ouabain inhibits the transport of α -aminoisobutyric acid in the intact diaphragm in the absence and the presence of insulin. In a recent work, LETARTE and RENOLD (1967) observed that the insulin stimulation of glucose metabolism in isolated fat cells exhibits a marked dependency on sodium. A number of studies have shown that ouabain can exert metabolic effects that are similar to, but not identical with insulin. In the rat diaphragm ouabain induces increased glycogen synthesis and inhibition of lactic acid formation (CLAUSEN, 1965, 1966). In the fat pad ouabain has an antilipolytic effect, and stimulates glucose uptake and the oxidation of glucose to CO_2 (HO and JEANRENAUD, 1967).

From these reports it is obvious that changes in the cation milieu, or in conditions leading to inhibition of the active $\text{Na}^+ \text{K}^+$ transport can interfere with the action of insulin on diaphragm muscle (BHATTACHARYA 1961) and adipose tissue (MOSINGER and KUJALOVA, 1966; CLAUSEN et al., 1968; RODBELL, 1968).

The present work has demonstrated an insulin effect on the uptake of inorganic phosphate and an increased labelling of ATP (and GTP) in the diaphragm. These effects are inhibited by ouabain and by omitting sodium from the incubation medium. The results may indicate an effect of insulin directly on the membrane sodium-potassium ATP-ase. Such an effect has, however, not yet been demonstrated (ZIERLER, 1966). An alternative explanation could be an effect of insulin on the physico-chemical state of membrane lipids, the alterations of which will result in interference with movements of critical inorganic ions. In this connection it is of interest that insulin-responsive cells treated with phospholipases (RODBELL and JONES, 1966), or proteases (KUO et al., 1967) acquire insulin-like activity. Whatever the mechanism of the insulin action may be, the present work clearly indicates that the increased labelling of nucleotides caused by insulin in the absence of added substrate is due to increased transport of phosphate, and is dependent upon the cation distribution within the membrane.

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