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Effects of Metal Ions on the Utilization of Glucose and on the Influence of Insulin on it by the Isolated Rat Diaphragm

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Insulin causes an increased uptake of glucose by the isolated rat diaphragm (Gemmill, 1940), although the mechanism by which insulin exerts its effect has not yet been explained. On the basis of the evidence presented by Levine & Goldstein (1955), Park, Bornstein & Post (1955), Park & Johnson (1955), Park, Johnson, Wright & Batsel (1957) and others, it is, however, now generally agreed that insulin, both *in vivo* and *in vitro*, exerts its effect by increasing the rate of entry of glucose from the extracellular fluid to the interior of the cell, presumably by some effect on the cell membrane. Nothing, however, is so far known regarding the nature of the effect of insulin on the cell membrane.

This paper describes the results of experiments on the effects of physiologically important cations, and of certain other related ions, on the uptake of glucose and on the influence of insulin on it by the rat diaphragm *in vitro*. It is well known that these ions, in addition to their functions of osmotic regulation of tissue fluids and of activation of various enzymes, play essential roles in the preservation of integrity, and the regulation of permeability properties, of cell membranes. Preliminary reports of some of the experiments described in this paper have been published (Bhattacharya, 1959*a, b*).

MATERIALS AND METHODS

Incubation media. The standard incubation medium was the bicarbonate buffer of Gey & Gey (1936). Various other incubation mixtures were used. These latter mixtures

generally consisted of a suitable buffer in iso-osmotic sucrose (0.25 M) or in a mixture of iso-osmotic sucrose and iso-osmotic solutions of the chlorides of certain univalent and bivalent metals. 0.154 M-solutions of the chlorides of the univalent metals and 0.11 M-solutions of the chlorides of bivalent metals were considered to be iso-osmotic. In most instances the pH of the medium was adjusted to 7.4. The buffers used and their concentrations in the medium were usually as follows: LiHCO₃, NaHCO₃ and KHCO₃, 0.02 M; Na₂HPO₄, 0.01 M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0.025 M. Glucose when present was added to give a final concentration of about 250 mg./100 ml.

Insulin. Crystalline insulin (Wellcome Foundation or Boots Pure Drug Co. Ltd.) was dissolved in 0.033 N-HCl to give a concentration of 10 units/ml. This stock insulin was added to the incubation medium to give the required concentration just before each experiment. The final insulin concentration was usually about 0.1 unit/ml.

¹²⁵I-marked insulin. This was prepared according to the method of Bournsnel, Coombs & Rizk (1953) as modified by Bournsnel (1958). The final stock preparation was obtained in 0.05 M-Na₂HPO₄ buffer, pH 7.4. The insulin concentration was about 0.5 mg./ml. No inactivation of the insulin, as measured by its effect on increased uptake of glucose by the isolated rat diaphragm, could be demonstrated.

Animals. Diaphragm muscle was obtained from female albino Wistar rats (wt. 100–150 g.) which had been starved 20–24 hr. before use.

Preparation of cut diaphragm. The rat was killed by decapitation and bled. The diaphragm was then removed and put into a beaker containing freshly gassed buffer. After the required number (usually six) of diaphragms had been obtained, each diaphragm was gently blotted and cut into halves, and each half was transferred to a small conical flask containing 1 ml. of the medium. One-half of a diaphragm served as the control and the other was used to determine the effect of insulin or of other treatment. The flasks were then gassed with the required gas mixture (see Table 1), sealed with rubber stoppers and incubated at 38°

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in a Dubnoff shaker usually for 60 or 90 min. After incubation the hemidiaphragms were taken out, blotted and weighed on a torsion balance, and the medium was analysed.

Preparation of intact diaphragm. The rat was killed by decapitation and bled, and the intact diaphragm was removed as described by Kipnis & Cori (1957). The diaphragm was washed in freshly gassed medium and then slid into a 50 ml. conical flask containing 15 ml. of the medium. The flasks were gassed with the required gas mixture, sealed with rubber stoppers and incubated at 38° in a Dubnoff shaker for 1 hr. After incubation the tissue was taken out of the flask and the diaphragm (excepting the posterior portion) excised from the rib cage. It was then blotted, weighed on a torsion balance and analysed. The medium in the flask was also analysed.

Measurement of pH. This was done with the glass electrode.

Glucose uptake. This was determined by measuring the disappearance of glucose from the incubation medium. Samples of the medium were deproteinized with BaSO₄ (Somogyi, 1945), and the sugar content of the protein-free filtrate was determined either by the Somogyi (1945) modification of Nelson's method or by the method of Hagedorn & Jensen (1923), as found convenient.

Xylose. For estimating xylose in the diaphragm after incubation, the muscle was rapidly made into a paste with 20 vol. of water by grinding with sand in a mortar and

deproteinizing with BaSO₄ (Somogyi, 1945). The protein-free filtrate was analysed for xylose by the *p*-bromoaniline method of Roe & Rice (1948). The xylose content of the medium was also similarly determined.

Xylose space. The space was calculated as follows:

Space (ml./100 g.)

$$= \frac{\text{muscle content (g./100 g. of wet muscle)}}{\text{medium concentration (g./ml.)}}$$

Measurement of binding of insulin by diaphragm. Diaphragms were incubated for 0.5 hr. at 38° under the desired conditions in the presence of ¹²⁵I-marked insulin. One hemidiaphragm was suspended in 1 ml. of the medium containing about 0.1 unit of insulin. After incubation the hemidiaphragms were removed, washed in appropriate media for 10 min. with three changes, blotted, weighed and spread on aluminium planchets. They were then dried under an infrared lamp and counted with a thin end-window Geiger-Müller tube. Individual hemidiaphragms gave 400–1100 counts/min. and at least 1000 counts were recorded for each sample.

Statistical treatment of results. In most cases experiments were done in groups of six and the significance of the differences between the means was assessed on the basis of Student's *t* test. A value of *P* (probability that the difference was due to chance) less than 0.05 was considered to be significant.

Table 1. Influence of insulin on glucose uptake by rat diaphragm from iso-osmotic sucrose in the presence or absence of small amounts of metal ions

Hemidiaphragms were incubated in iso-osmotic sucrose (1 hemidiaphragm/ml.) buffered with NaHCO₃, KHCO₃ or LiHCO₃ (0.02 M), Na₂HPO₄ (0.01 M) or tris (0.025 M). The gas phase was O₂ + CO₂ (95:5) with the bicarbonate buffers, and O₂ with phosphate and tris buffers. Incubation was for 1.5 hr. at 38°.

Medium	Buffer	Glucose uptake (mean ± s.e.m.) (mg./g. of wet tissue/hr.)		<i>P</i>
		Without insulin	With insulin (0.1 unit/ml.)	
Gey's salt mixture	NaHCO ₃	3.16 ± 0.14	7.67 ± 0.44	< 0.001
Sucrose	NaHCO ₃	4.15 ± 0.15	4.18 ± 0.21	> 0.05
Sucrose	KHCO ₃	4.11 ± 0.28	4.64 ± 0.26	< 0.01
Sucrose	LiHCO ₃	6.49 ± 0.20	6.61 ± 0.21	> 0.05
Sucrose	Na ₂ HPO ₄	4.58 ± 0.19	4.87 ± 0.15	> 0.05
Sucrose	Tris	4.43 ± 0.22	4.68 ± 0.15	> 0.05

Table 2. Effect of alkali-metal ions on the influence of insulin in causing increased uptake of glucose by rat diaphragm

Experimental details were the same as under Table 1. Iso-osmotic solutions of sucrose and of the different metal chlorides were mixed in various proportions to give the desired concentration of the salts in the medium.

Buffer	Electrolyte added and its concn. in the medium (mM)	Glucose uptake (mean ± s.e.m.) (mg./g. of wet tissue/hr.)		<i>P</i>
		Without insulin	With insulin (0.1 unit/ml.)	
NaHCO ₃	NaCl (32)	4.45 ± 0.20	6.95 ± 0.17	< 0.001
NaHCO ₃	NaCl (62)	5.16 ± 0.28	8.04 ± 0.40	< 0.001
Na ₂ HPO ₄	NaCl (77)	3.84 ± 0.30	6.12 ± 0.27	< 0.001
Tris	NaCl (77)	4.46 ± 0.12	7.18 ± 0.06	< 0.001
KHCO ₃	KCl (32)	2.99 ± 0.25	5.59 ± 0.18	< 0.001
KHCO ₃	KCl (64)	2.82 ± 0.22	4.81 ± 0.17	< 0.001
LiHCO ₃	LiCl (64)	7.95 ± 0.31	9.10 ± 0.31	< 0.001
Tris	LiCl (77)	6.80 ± 0.24	7.93 ± 0.40	< 0.001
Tris	RbCl (51)	3.24 ± 0.21	5.52 ± 0.33	< 0.001
Tris	CsCl (51)	3.30 ± 0.13	6.33 ± 0.37	< 0.001

RESULTS

Influence of moderate amounts of alkali-metal ions on the uptake of glucose and on the influence of insulin on it by rat diaphragm. Diaphragm incubated in iso-osmotic sucrose (0.25M), buffered at pH 7.4 with tris (0.025M), Na_2HPO_4 (0.01M) or NaHCO_3 or KHCO_3 (0.02M), removed glucose from the medium in amounts comparable with those removed from the bicarbonate buffer of Gey & Gey

Table 3. Comparison of glucose uptake by rat diaphragm in media containing equivalent amounts of the alkali-metal ions

Experiments were done in pairs by using hemidiaphragms from the same rat and then comparing two of the metal chlorides in turn. The experimental details were otherwise the same as in Table 2.

Metal ion and its concn. in the medium (mm)	Buffer	Glucose uptake (mean \pm S.E.M.) (mg./g. of wet tissue/hr.)	P
Li^+ (64)	Bicarbonate	7.48 \pm 0.26	< 0.05
Na^+ (64)	Bicarbonate	5.83 \pm 0.43	
Na^+ (64)	Bicarbonate	5.74 \pm 0.37	< 0.01
K^+ (64)	Bicarbonate	3.08 \pm 0.18	
Li^+ (77)	Tris	7.37 \pm 0.45	< 0.001
Na^+ (77)	Tris	4.26 \pm 0.27	
Na^+ (77)	Tris	4.14 \pm 0.27	< 0.01
K^+ (77)	Tris	2.55 \pm 0.21	
K^+ (77)	Tris	2.30 \pm 0.14	> 0.05
Rb^+ (77)	Tris	2.44 \pm 0.16	
Rb^+ (77)	Tris	2.05 \pm 0.16	< 0.02
Cs^+ (77)	Tris	1.56 \pm 0.10	
Li^+ (20)	Bicarbonate	5.40 \pm 0.15	< 0.001
Na^+ (20)	Bicarbonate	3.73 \pm 0.15	
Na^+ (20)	Bicarbonate	3.69 \pm 0.13	< 0.001
K^+ (20)	Bicarbonate	2.97 \pm 0.14	

Table 4. Comparison of glucose uptake by rat diaphragm which had been previously incubated in media containing equivalent amounts of Li^+ , Na^+ and K^+ ions

Hemidiaphragms were incubated for 0.5 hr. at 38° in iso-osmotic sucrose (1 hemidiaphragm/ml. of the medium without any added glucose) containing LiHCO_3 , NaHCO_3 or KHCO_3 (0.02M). The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). The hemidiaphragms were then incubated for 1 hr. at 38° in Gey's buffer containing about 250 mg. of glucose/100 ml. and the uptake of glucose was measured.

Preincubation	Glucose uptake (mean \pm S.E.M.) (mg./g. of wet tissue/hr.)	P
LiHCO_3	6.22 \pm 0.39	< 0.01
NaHCO_3	4.40 \pm 0.14	
KHCO_3	3.07 \pm 0.16	< 0.01

(1936). Insulin, however, had little or no effect on the uptake of glucose under such conditions (Table 1). With LiHCO_3 (0.02M) as the buffer, there was also little effect of insulin, although in this case the basal uptake of glucose was considerably higher. The glucose uptake given in this Table and elsewhere represents an extent of uptake rather than a rate. It has been shown (see Khral, 1951) that the basal uptake is highest if measured for the first 0.5 hr. of incubation, less if measured for a 1 hr. period and still less for a 2 hr. period of measurement. Thus the uptake does not proceed linearly over the usual period (1-2 hr.) of measurement. The observed effects with insulin and ions reported in this paper also represent an extent rather than a rate.

Addition of moderate amounts of the chlorides of sodium or potassium or of the other related univalent metals, lithium, rubidium and caesium, to the inactive sucrose medium, however, resulted in the appearance of an insulin effect (Table 2). The response to insulin in media containing Li^+ ions was small. This apparently resulted from the high basal uptake of glucose from such media (Table 2). The basal uptake of glucose in fact varied in a regular way in the presence of equivalent amounts of the different metal ions in the medium. A comparison of the glucose uptake by the diaphragm in the presence of equivalent amounts of the alkali-metal ions in the medium is given in Table 3, which shows that the uptake was highest with Li^+ ions and lowest with Cs^+ ions and followed the order $\text{Li}^+ > \text{Na}^+ > \text{K}^+ = \text{Rb}^+ > \text{Cs}^+$. This influence of the alkali-metal ions in regulating the basal glucose uptake by the rat diaphragm was exerted even when the diaphragms were preincubated (without glucose) for a short period in media containing the different ions, the final incubation during which the uptake of glucose was measured being carried out in Gey's buffer (Table 4).

Influence of small amounts of bivalent metal ions on the insulin-sensitivity of the rat diaphragm. Low concentrations of Mg^{2+} ions promoted increased uptake of glucose due to insulin from the inactive sucrose medium buffered with NaHCO_3 (0.02M) or Na_2HPO_4 (0.01M) (Table 5). Mg^{2+} ions also had a tendency to increase the basal uptake itself. Of the other bivalent ions, Ca^{2+} and Zn^{2+} ions had clearly no effect. Mn^{2+} ions might have had some effect, but it was not very clear-cut. Ca^{2+} ions in fact counteracted the influence of Mg^{2+} ions in promoting insulin response to the tissue (Table 6). This inhibitory effect of Ca^{2+} ions could be overcome by increasing the concentration of Mg^{2+} ions in the medium.

Also Ca^{2+} ions had a strong depressing effect on the basal glucose uptake itself (Table 6).

Table 5. *Effects of the addition of low concentration of bivalent metal ions on the influence of insulin in causing increased uptake of glucose by rat diaphragm from inactive sucrose media*

Hemidiaphragms were incubated in iso-osmotic sucrose (1 hemidiaphragm/ml.) buffered with NaHCO_3 (0.02M) or Na_2HPO_4 (0.01M) for 1 hr. at 38° under the appropriate gas phase as given in Table 1.

Buffer	Addition		Glucose uptake (mean \pm s.e.m.) (mg./g. of wet tissue/hr.)	
	Salt	Concn. (mM)	Without insulin	With insulin (0.1 unit/ml.)
NaHCO_3	None	—	4.16 \pm 0.17	4.86 \pm 0.14
NaHCO_3	MgCl_2	0.2	4.40 \pm 0.10	6.12 \pm 0.11
NaHCO_3	MgCl_2	1.0	5.53 \pm 0.31	7.97 \pm 0.12
NaHCO_3	CaCl_2	0.2	4.84 \pm 0.20	5.00 \pm 0.18
NaHCO_3	CaCl_2	1.0	3.36 \pm 0.16	3.92 \pm 0.21
NaHCO_3	MnCl_2	0.2	4.85 \pm 0.18	5.93 \pm 0.14
NaHCO_3	MnCl_2	1.0	4.82 \pm 0.26	6.15 \pm 0.18
NaHCO_3	ZnSO_4	0.2	4.25 \pm 0.18	4.50 \pm 0.24
NaHCO_3	ZnSO_4	1.0	4.03 \pm 0.15	4.45 \pm 0.24
Na_2HPO_4	None	—	4.25 \pm 0.13	4.56 \pm 0.17
Na_2HPO_4	MgCl_2	1.0	5.01 \pm 0.71	8.50 \pm 0.67

Table 6. *Antagonism between Ca^{2+} and Mg^{2+} ions with respect to increased uptake of glucose due to insulin by the rat diaphragm in vitro*

The basal incubation medium was iso-osmotic sucrose buffered with NaHCO_3 (0.02M). The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). Incubation was for 1 hr. at 38° .

Additions	Glucose uptake (mean \pm s.e.m.) (mg./g. of wet tissue/hr.)	
	Without insulin	With insulin (0.1 unit/ml.)
1 mM- MgCl_2	5.94 \pm 0.22	8.22 \pm 0.16
1 mM- MgCl_2 + 5 mM- CaCl_2	3.40 \pm 0.22	5.35 \pm 0.30
5 mM- MgCl_2 + 5 mM- CaCl_2	4.46 \pm 0.30	7.69 \pm 0.32

Table 7. *Influence of Na^+ and Mg^{2+} ions on the increased uptake of glucose due to insulin by rat diaphragm from an iso-osmotic sucrose medium buffered to pH 7.0 with 5.9 mM-sodium bicarbonate*

Hemidiaphragms were incubated in the above medium (1 hemidiaphragm/ml. of medium) for 1 hr. at 38° in $\text{O}_2 + \text{CO}_2$ (95:5).

Additions	Glucose uptake (mean \pm s.e.m.) (mg./g. of wet tissue/hr.)	
	Without insulin	With insulin (0.1 unit/ml.)
14 mM- NaCl	3.85 \pm 0.23	4.97 \pm 0.16
1 mM- MgCl_2	4.90 \pm 0.23	5.39 \pm 0.29
14 mM- NaCl + 1 mM- MgCl_2	3.79 \pm 0.08	6.30 \pm 0.24

Although the presence of 1 mM- Mg^{2+} considerably increased the uptake of glucose due to insulin from an iso-osmotic sucrose medium containing 0.02M- NaHCO_3 (Table 5), there was no response to insulin in a medium containing 5.9 mM- NaHCO_3 , pH 7.0, in $\text{O}_2 + \text{CO}_2$ (95:5), even in the presence of

the same concentration of Mg^{2+} ions (Table 7). Addition of NaCl to this medium so as to make the concentration of Na^+ ions about 0.02M led to the appearance of an insulin effect. Addition of NaCl (about 0.02M) alone (in the absence of added Mg^{2+} ions) produced only a small response to insulin.

Influence of ethylenediaminetetra-acetic acid on the increased uptake of glucose due to insulin from sucrose-sodium chloride medium. Addition of ethylenediaminetetra-acetic acid (EDTA) to a concentration of 2 mM largely abolished the increased uptake of glucose due to insulin from a sucrose medium containing 0.077M- NaCl and buffered with tris (0.025M) (Table 8). This inhibitory effect of EDTA was fully counteracted by the presence of 2 mM- Mg^{2+} in the medium.

Role of Mg^{2+} ions in maintaining insulin-sensitivity of the isolated rat diaphragm. The results of the foregoing experiments showed that a small amount of Mg^{2+} ions was critically important for insulin-responsiveness of the rat diaphragm. Some idea of the nature of this action of Mg^{2+} ions was obtained in a series of experiments in which diaphragms were first incubated for 0.5 hr. in media with or without added Mg^{2+} ions and without any added glucose. The effect of this preincubation on the insulin-sensitivity of the tissue, as measured by increased uptake of glucose in the presence of insulin from Gey's buffer, was then tested. The results showed that insulin caused the usual increase in the uptake of glucose by diaphragm which had been preincubated for 0.5 hr. in Gey's buffer either at room temperature or at 38° . The ability of the tissue to respond to insulin was also left unimpaired when it was preincubated for 0.5 hr. at room temperature in iso-osmotic sucrose buffered with NaHCO_3 (0.02M). Preincubation for 0.5 hr. at 38° in the sucrose-bicarbonate buffer,

however, caused a marked loss of the insulin-sensitivity of the diaphragm; this did not occur in the presence of a low concentration of Mg^{2+} ions (1 mM). Ca^{2+} ions in suitable concentrations counteracted the protective effect of Mg^{2+} ions, and the action of Ca^{2+} could be overcome by increasing the concentration of Mg^{2+} ions in the medium. Mn^{2+} ions could not protect the tissue against loss of insulin-sensitivity due to the preincubation.

Stadie, Haugaard & Vaughan (1952, 1953) showed that rat diaphragm which had been previously incubated with insulin in appropriate media showed increased glycogen synthesis during subsequent incubation without any added insulin. The influence of ions in this phenomenon was studied. It was observed that a hemidiaphragm which had been preincubated for 0.5 hr. in the presence of 0.1 unit of insulin/ml. in iso-osmotic sucrose buffered with either $NaHCO_3$ (0.02M) or tris (0.025M) showed increased uptake of glucose during subsequent incubation in Gey's buffer. The increment was comparable with those obtained when the preincubation was done in Gey's buffer itself. EDTA (5 mM) in the sucrose medium during the

preincubation, however, depressed the increased uptake of glucose considerably during subsequent incubation. This inhibitory effect of EDTA could again be fully counteracted by Mg^{2+} ions (5 mM) in the preincubation medium.

Effect of anaerobiosis on the increased uptake of glucose due to insulin. The experiments so far reported were all carried out under aerobic conditions. When some of them were repeated under anaerobic conditions (Table 9), there was little or no increased uptake of glucose due to insulin even when the medium contained the required ions in sufficient amounts. With bicarbonate buffer, the basal uptake of glucose, however, tended to be higher under anaerobic conditions than under aerobic conditions. This was particularly true for the sucrose-NaCl medium (Expt. no. 1, Table 9). Anaerobiosis has a similar effect on the basal uptake of glucose by rat diaphragm from Gey's bicarbonate buffer (Randle, 1956).

In a few preincubation experiments under anaerobic conditions it was observed that preincubation of the diaphragm with insulin in Gey's medium (without glucose) under anaerobic conditions did not lead to any increased uptake of glucose during subsequent incubation either under anaerobic or under aerobic conditions. Preincubation under anaerobic conditions also led to the abolition of the increased uptake of glucose under aerobic conditions.

Influence of lack of ions and of oxygen on the binding of insulin by rat diaphragm. Incubation of diaphragm at 38° under anaerobic conditions in Gey's buffer or under aerobic conditions in iso-osmotic sucrose buffered with 0.02M- $NaHCO_3$ led to a loss of insulin-sensitivity of the tissue. The effect of such preincubations on the binding of insulin by the tissue (Stadie *et al.* 1952, 1953) was studied with ^{131}I -marked insulin (Table 10). It was observed that incubation in the sucrose-bicarbonate medium under aerobic conditions largely increased the amount of insulin bound by the tissue as compared

Table 8. *Influence of ethylenediaminetetra-acetic acid on the increased uptake of glucose due to insulin from sucrose-sodium chloride medium buffered with tris*

Hemidiaphragms were incubated in an iso-osmotic sucrose-NaCl mixture in which the concentration of NaCl was 0.077M and which was buffered with tris (0.025M) at pH 7.4. Incubation was for 1 hr. at 38° in O_2 .

Additions	Glucose uptake (mean \pm s.e.m.) (mg./g. of wet tissue/hr.)	
	Without insulin	With insulin (0.1 unit/ml.)
None	4.46 \pm 0.12	7.18 \pm 0.06
0.5 mM-EDTA	3.00 \pm 0.17	7.17 \pm 0.35
2 mM-EDTA	4.07 \pm 0.24	5.82 \pm 0.22
2 mM-EDTA + 2 mM-MgCl ₂	3.58 \pm 0.24	7.86 \pm 0.22

Table 9. *Influence of insulin on glucose uptake by rat diaphragm incubated in iso-osmotic sucrose containing moderate amounts of Na^+ or K^+ ions under anaerobic conditions*

Hemidiaphragms were incubated in mixtures of iso-osmotic sucrose and iso-osmotic NaCl or KCl buffered with bicarbonate (0.02M), Na_2HPO_4 (0.01M) or tris (0.025M) to pH 7.4. One hemidiaphragm was suspended in 1 ml. of the medium. Incubation was carried out under anaerobic conditions, $N_2 + CO_2$ (95:5) with bicarbonate buffers and N_2 with phosphate or tris buffer, for 1.5 hr. at 38°.

Expt. no.	Metal ion and its concn. in the medium (mM)	Buffer	Glucose uptake (mean \pm s.e.m.) (mg./g. of wet tissue/hr.)		P
			Without insulin	With insulin (0.1 unit/ml.)	
1	Na^+ (64)	$NaHCO_3$	7.91 \pm 0.22	8.39 \pm 0.25	> 0.05
2	Na^+ (77)	$NaHPO_4$	3.32 \pm 0.12	3.52 \pm 0.12	> 0.05
3	Na^+ (77)	Tris	2.52 \pm 0.09	2.81 \pm 0.07	< 0.02
4	K^+ (64)	$KHCO_3$	3.93 \pm 0.25	4.40 \pm 0.21	< 0.05

Table 10. *Binding of insulin by rat diaphragm in iso-osmotic sucrose buffered with sodium bicarbonate (0.02M) under aerobic conditions and from Gey's buffer both under aerobic and under anaerobic conditions*

One hemidiaphragm of a rat was incubated in 1 ml. of Gey's buffer containing 0.1 unit of ^{131}I -marked insulin/ml. under aerobic conditions at 38° for 0.5 hr. The corresponding hemidiaphragm was similarly incubated in iso-osmotic sucrose buffered with NaHCO_3 (0.02M) under aerobic conditions, and the amount of insulin bound was determined.

Incubation	Amount of insulin bound (mean \pm s.e.m.) (counts/min./g. of wet tissue)	P
In Gey's medium, aerobic	4 952 \pm 188	< 0.001
In iso-osmotic sucrose- NaHCO_3 medium, aerobic	12 228 \pm 712	
In Gey's medium, anaerobic	4 879 \pm 207	> 0.05
In Gey's medium, anaerobic	4 121 \pm 205	

Table 11. *Xylose-spaces of diaphragms incubated in the presence of Li^+ , Na^+ and K^+ ions (77 mM)*

Intact diaphragms were incubated in mixtures of iso-osmotic sucrose, and iso-osmotic chlorides and bicarbonates of the alkali metals so that the bicarbonate concentration was 0.02M. The incubation medium contained 150 mg. of D-xylose/100 ml. The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). Incubation was for 1 hr. at 38° .

Metal ion	Xylose-space (mean \pm s.e.m.) (ml. of water/100 g. of wet tissue)	P
Li^+	56 \pm 2.3	< 0.01
Na^+	47 \pm 0.9	
K^+	35 \pm 2.5	< 0.01

with that bound from Gey's buffer under similar conditions. There was, however, no difference in the amounts of insulin bound under aerobic or anaerobic conditions from Gey's buffer.

Xylose-spaces of diaphragms incubated in the presence of equivalent amounts of Li^+ , Na^+ and K^+ ions. Table 11 gives a comparison of the xylose-spaces of intact diaphragms incubated in bicarbonate buffers containing equivalent amounts of Li^+ , Na^+ or K^+ ions. The results show that the xylose-space was highest in the medium containing Li^+ ions and lowest in the medium containing K^+ ions. The xylose-spaces of the diaphragm in the different media were thus directly proportional to the glucose uptake by the diaphragm from such media (Table 3).

DISCUSSION

The experiments described in this paper show that insulin increases the uptake of glucose by the

isolated rat diaphragm only in the presence of certain metal ions. Moderate concentrations of any of the different alkali-metal ions are effective in this respect but, of the bivalent-metal ions tested, only Mg^{2+} ions are effective.

The Mg^{2+} ion is of particular interest in this respect. The presence of a low concentration of Mg^{2+} ions enabled the diaphragm to respond to insulin under ionic conditions in which otherwise there was little or no response (Table 5). The results in Tables 7 and 8, however, indicate that two ions, small concentrations of Mg^{2+} ions and moderate concentrations of Na^+ ions (or of some other alkali-metal ions), are possibly required for a proper insulin action. A medium containing 0.02M-sodium bicarbonate plus 1 mM-magnesium chloride, pH 7.4, showed considerable insulin effect, but there was little effect in a medium containing 5.9 mM-sodium bicarbonate and 1 mM-magnesium chloride, pH 7.0, although raising the Na^+ ion concentration to 0.02M re-established the insulin stimulation (Table 7). The presence of 2 mM-EDTA largely abolished the increased uptake of glucose due to insulin from a sucrose medium containing only 0.077M-sodium chloride (buffered with tris) and this inhibitory effect was overcome by the addition of 2 mM-magnesium chloride (Table 8). This indicates that the effect of large amounts of Na^+ ions (and possibly of the other alkali-metal ions) in promoting insulin-sensitivity to the tissue is perhaps associated with a simultaneous sparing action of the alkali-metal ions on the magnesium present in the tissue *in situ*.

The nature of the action of Mg^{2+} ions in maintaining insulin-sensitivity of the tissue is not clear from the present data. It seems, however, that Mg^{2+} ions possibly act by controlling the activity of some enzyme in the system. This is indicated by the antagonism between Mg^{2+} and Ca^{2+} ions with respect to the insulin-sensitivity of the tissue, and also by the sharp loss of insulin-sensitivity at 38° but not at room temperature when the tissue is incubated in iso-osmotic sucrose-sodium bicarbonate (0.02M) buffer. It is also indicated by the observation previously reported (Bhattacharya, 1959b) that when diaphragms are put into iso-osmotic sucrose buffered with 0.02M-sodium bicarbonate (and also with 0.01M-disodium hydrogen phosphate) they begin to contract vigorously after a few minutes and that addition of Mg^{2+} ions in concentrations which preserve the insulin-sensitivity of the tissue also stops the contraction. The bivalent metal ions, Ca^{2+} or Zn^{2+} , which are ineffective in maintaining the tissue sensitive to insulin, are also ineffective in stopping the contraction.

The loss of insulin-sensitivity of the diaphragm in the inactive sucrose medium at 38° is associated

with an increased binding of insulin by the tissue. Assuming that the binding of insulin takes place on cell surfaces, as postulated by Stadie (1954, 1956), the increased binding of insulin by the diaphragm at 38° in the inactive sucrose medium possibly indicates some profound change in the nature of the cell membranes. It is this change in the nature of the cell membranes that is possibly responsible for the loss of sensitivity to insulin of the tissue. When diaphragms are incubated anaerobically in Gey's buffer, they lose their sensitivity to insulin. However, there is no difference in the amounts of insulin bound by the diaphragm due to incubation under aerobic or anaerobic conditions from Gey's buffer.

The experiments described in this paper stress the importance of an aerobic environment for insulin action. Insulin causes increased uptake of glucose only under aerobic conditions. This has been demonstrated by a number of workers (Walaas & Walaas, 1952; Demis & Rothstein, 1954). The dependence of insulin action on an aerobic environment has had various explanations. Demis & Rothstein (1954) suggest that this indicates that the action of insulin is dependent on the presence of some cellular constituent which is present in adequate amounts only under aerobic conditions. Randle & Smith (1958), on the basis of their experiments on the effects of anaerobiosis and of certain metabolic inhibitors including 2,4-dinitrophenol on glucose uptake by the isolated diaphragm, consider that oxidative phosphorylation is involved in the process, although possibly in an indirect way. The present author, however, believes that oxidative phosphorylation is not involved in the phenomenon. The dependence of insulin action on an aerobic environment does not necessarily implicate oxidative phosphorylation. Assuming that insulin interacts with certain constituents (possibly protein in nature) of the cell surface, thus changing their nature and the permeability properties of the surface, the presence of oxygen might be necessary merely for the interaction to take place in the right way. Indeed, it is not clear to the author why some enzymic or transport mechanism must necessarily be invoked (as is the present tendency) in attempts to explain insulin action. Many important biological reactions, such as antigen-antibody reactions, are only protein-protein reactions without the involvement of any enzyme. There seems to be no reason why insulin action cannot be thought to be one of such biological reactions.

The alkali metals affected the basal uptake of glucose by the diaphragm in a regular way, apparent both in the concentrations at which they sustained the insulin-sensitivity of the tissue and in the concentrations at which they failed to do so,

and the tissue lost permanently its ability to respond to insulin. This latter phenomenon shows that the ways in which the metal ions and insulin influence the glucose uptake are possibly separate and distinct from each other, one operating independently of the other. Insulin possibly acts by affecting certain magnesium-dependent sites on the cell surface. The metal ions, on the other hand, possibly act by influencing other sites. There are, however, interesting similarities between the action of insulin and the action of the alkali-metal ions in regulating the uptake of glucose by the diaphragm. The xylose-spaces of the diaphragm in media containing equivalent amounts of the alkali-metal ions (Table 11) are directly proportional to the glucose uptake by the diaphragm from such media (Table 3). Thus the varied uptake of glucose under the influence of the different alkali-metal ions is possibly brought about by a control of the rate of entry of the sugar from the extracellular fluid to the interior of the cell. This is the way in which insulin also is now considered to increase the utilization of glucose by the muscle. Further, the ions varied the basal uptake in a regular way even when the diaphragms were preincubated for a short period in media containing the different ions and subsequently incubated in Gey's buffer with glucose (Table 4). This effect of preincubation with the metal ions is again strikingly similar to the effect of preincubation of the diaphragm with insulin on increased uptake of glucose during subsequent incubation without any added insulin, first observed by Stadie *et al.* (1952, 1953) and confirmed in the present work. Stadie (1954, 1956) has interpreted this result with insulin to mean that during the preincubation insulin combines with the diaphragm and that it is only in the bound state that insulin exerts its customary physiological action. In view of the similar effects of preincubation of the tissue with the alkali-metal ions, it is possible that the real significance of these preincubation effects lies in a binding of the agents to the cells. Undoubtedly there is some interaction between insulin or the cations and certain constituents of the cell surface. These results may indicate that insulin or the cations bring about some sustained change in the cell surface, thus making it more permeable to certain solutes such as glucose, and not that insulin or the cations exert their effects only in a bound state. During the preincubation the binding necessary for subsequent action during the final incubation is achieved.

The order in which the alkali metals affect the uptake of glucose by the diaphragm (Table 3) is the same as the order of these ions in the so-called lyotropic or Hofmeister series. It is generally agreed that the series has its origin in the intensity of the electrostatic field around the ions, the small

ions of the same valency having a more intense field than the large ones. In biological reactions, in which the importance of water cannot be over-emphasized, it seems that the property of the ions which is the immediate cause of the series arises from a difference in hydration of the ions as a result of the difference of the intensity of the field due to their different sizes. Thus with the univalent ions in question here, the Li^+ ion has the smallest radius, the most intense field, and maximum ionic hydration, whereas the Cs^+ ion has the largest radius, the weakest field and the smallest hydration, if any. It is tempting to correlate the uptake of glucose in the presence of equivalent amounts of the above ions with the amount of water that the ions possibly carry to some active sites on the cell surface, thus altering in a regular way the permeability of those sites to glucose and to other similar solutes.

In conclusion it seems that two separate and distinct processes (one, insulin-independent and the other, insulin-dependent) are possibly involved in the uptake of glucose by the isolated diaphragm of the rat. The process independent of insulin, although markedly influenced by ionic environments, can nevertheless function in the absence of metal ions in the medium. It can also function both under aerobic and under anaerobic conditions. The process dependent on insulin, however, seems to operate best only under aerobic conditions and in the presence of certain metal ions in the incubation medium. There are certain apparent similarities between the action of insulin and of the alkali-metal ions in regulating the glucose metabolism of the diaphragm. It seems, however, that the ways in which they exert their effects are different, two different sites of the cell surface being possibly involved in the two processes. Insulin may act by affecting certain magnesium-dependent sites on the surface, whereas the ions act on certain other sites.

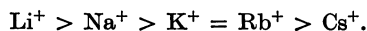
SUMMARY

1. Insulin increases the uptake of glucose by the isolated rat diaphragm only in the presence of certain metal ions in the added medium. It appears that simultaneous participation of Mg^{2+} ions and an alkali-metal ion such as Na^+ is required for the maximum effect of insulin.

2. Mg^{2+} ions seem to be essential for maintaining the tissue sensitive to insulin. Magnesium possibly exerts this effect by preserving the integrity of some components of the cell surface by controlling the activity of some enzyme in the system. Calcium antagonizes magnesium in this respect.

3. The alkali-metal ions have a striking regulatory effect on the basal uptake of glucose by the

diaphragm of the rat. In the presence of equivalent amounts of the ions the uptake is highest with Li^+ ions and follows the order



In the presence of appropriate amounts of Li^+ ions the uptake is almost as high as that due to insulin from Gey's buffer. The ions exert their regulatory effect even when the tissue has permanently lost its ability to respond to insulin.

4. Preincubation of the diaphragm with the alkali-metal ions leads to the uptake of different amounts of glucose when the tissue is subsequently incubated in Gey's medium, and the uptake follows the order mentioned above.

5. In the presence of equivalent amounts of the alkali-metal ions, the xylose-space of the diaphragm followed the order $\text{Li}^+ > \text{Na}^+ > \text{K}^+$. This suggests that the regulation of the utilization of glucose by the alkali-metal ions is possibly brought about by a regulation of the rate of entry of the sugar from the extracellular fluid to the interior of the cell.

6. It is suggested that two separate and distinct processes (one, insulin-independent and the other, insulin-dependent) are possibly involved in the utilization of glucose by the rat diaphragm.

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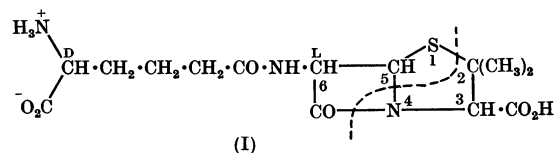
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The Structure of Cephalosporin C

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The isolation of cephalosporin C was described by Newton & Abraham (1956). Degradative studies by Abraham & Newton (1956*a*) indicated that cephalosporin C was structurally related to cephalosporin N[(D-4-amino-4-carboxy-*n*-butyl)penicillin; I] (Abraham, Newton & Hale, 1954; Newton & Abraham, 1954; Abraham & Newton, 1954). This paper presents chemical evidence for a definitive structure for cephalosporin C.



The molecular formula proposed for cephalosporin C ($C_{18}H_{21}O_8N_3S$) had two carbon atoms and two oxygen atoms more than the formula of cephalosporin N (Newton & Abraham, 1956). Cephalosporin C behaved as a monoaminodicarboxylic acid. It contained a residue of D- α -aminoadipic acid, linked to the rest of the molecule through its δ -carboxyl group, and gave 1 mole of carbon dioxide on hydrolysis with hot acid. It yielded L-alanine, valine and glycine, as well as D- α -aminoadipic acid, when hydrolysis was preceded by vigorous hydrogenolysis with Raney nickel. The infrared spectrum of its sodium salt showed a strong band at 5.62μ , similar to that shown by the penicillins and attributed in the latter to the C=O stretching vibration of the lactam group in the fused β -lactam-thiazolidine ring system (Fig. 1). In these properties cephalosporin C resembled cephalosporin N. Cephalosporin C showed a high degree of resistance to inactivation by penicillinase, but it proved to be a competitive

inhibitor of penicillinase (Abraham & Newton, 1956*b*) and an inducer of the synthesis of the enzyme by *Bacillus cereus* and *B. subtilis* (Pollock, 1957).

The close similarities of cephalosporin C and cephalosporin N were accompanied by several striking differences. Cephalosporin C yielded no penicillamine (D- β -mercaptovaline) on hydrolysis and gave 2 moles of ammonia when hydrolysed under conditions in which only 1 mole of ammonia was liberated from cephalosporin N or benzylpenicillin. The valine obtained after treatment of cephalosporin C with Raney nickel was racemic, whereas the valine-yielding fragment of cephalosporin N had the D-configuration. The ultraviolet-absorption spectrum of cephalosporin C sodium salt showed a band with λ_{max} at $260 m\mu$ ($\log \epsilon$ 3.95).

We first considered the hypothesis that cephalosporin C contained the skeleton of the characteristic ring system of the penicillins. The infrared-absorption spectrum of cephalosporin C and the ability of the substance to act as a competitive

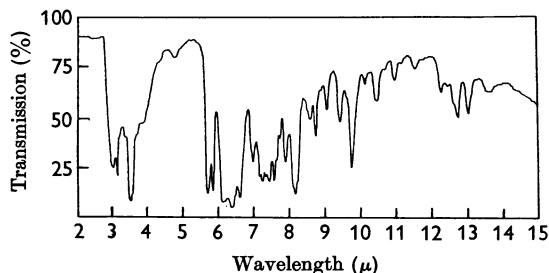


Fig. 1. Infrared-absorption spectrum of cephalosporin C sodium salt (Nujol paste).