Some Factors affecting the Response of Muscle to Insulin

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1. The effect of various changes in the composition of the supporting medium on the capacity of isolated rat diaphragm to incorporate amino acids into its protein has been studied. 2. Replacement of most of the normal ionic constituents by sucrose is inhibitory towards protein synthesis, as is also substitution of choline or K^+ for Na^+. 3. The capacity of the tissue to respond to a stimulatory effect of insulin is impaired in the sucrose media and under certain conditions in the absence of Na^+, particularly when Na^+ is replaced by K^+ and the ^{14}C-labelled amino acid is presented at a relatively high concentration. 4. Cutting of the tissue before incubation also decreases incorporating capacity and markedly decreases responsiveness to insulin. 5. In abnormal media the cellular content of ATP falls sharply. 6. The ATP content of the tissue also declines in the presence of 2-deoxyglucose. This change is prevented by the addition of glucose but not of pyruvate and succinate. 7. Although affecting the rate of amino acid incorporation the ATP content is not thought generally to limit sensitivity to insulin.

Although insulin *in vitro* will very consistently stimulate a number of metabolic activities of isolated rat diaphragm muscle, particularly uptake of sugar, incorporation of amino acids into protein and accumulation of amino acids (for review see Manchester, 1965), there have been a number of papers recently describing conditions of incubation under which normal effects of insulin are no longer seen.

Bhattacharya (1959*a*,*b*, 1961) found that replacement of much of the normal ionic composition of the incubation medium for diaphragm by sucrose interfered with the stimulation by insulin of glucose uptake. However, Mg^{2+} appeared to possess a unique role of preserving sensitivity to insulin. Manchester (1961*a*) showed that replacement of Na^+ by K^+ interfered with incorporation of amino acids into protein of diaphragm muscle and its stimulation by insulin. However, Kostyo (1964), in a study of the action of pituitary growth hormone on diaphragm, found that when Na^+ was replaced by choline, although the amount of amino acid incorporation declined, stimulation of incorporation by insulin was still observable. In a slightly different context work by Fong, Silver, Popenoe & Debons (1962) and others (Cadenas, Kaji, Park & Rasmussen, 1961; Carlin & Hechter, 1962; Mirsky & Perisutti, 1962; Edelman, Rosenthal & Schwartz, 1963) has suggested that a primary point of interaction of insulin with responsive tissues is through thiol groups and the response of a tissue to insulin can be modified by agents affecting such groups. DeSchepper, Toyoda & Bessman (1965) have reported the inhibition of amino acid incorporation into protein of diaphragm, and its stimulation by insulin, by the addition of 2-deoxyglucose to the incubation medium.

A determination of the precise factors in an intact tissue necessary for it to be capable of responding to insulin would assist in understanding how precisely insulin regulates in muscle the transport of sugars and the accumulation and incorporation of amino acids into protein. In particular, it would be valuable to know the degree of derangement of structure and organization of a tissue that can be tolerated before its ability to respond to insulin is lost. The present paper examines some of the parameters regulating the capacity of muscle to respond to the stimulating influence of insulin on the incorporation of amino acids into protein.

MATERIALS AND METHODS

Diaphragm muscle was taken from non-starved female rats, about 100g. body wt., and incubated with shaking for 2hr. at 37° as described by Manchester (1961*b*). The intact diaphragm preparation (retaining the rib-cage) was that of Kipnis & Cori (1957). The medium for incubation was Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) or various modifications. The composition of the buffers is stated in Table 1. Replacement of Na^+ by K^+ means that the Krebs–Ringer bicarbonate buffer contained KCl in place of NaCl and $KHCO_3$ instead of $NaHCO_3$. Choline buffer had choline chloride in place of NaCl and $KHCO_3$ instead of $NaHCO_3$. In potassium methyl sulphate buffer,

potassium methyl sulphate replaced NaCl, calcium acetate replaced $CaCl_2$ and $KHCO_3$ replaced $NaHCO_3$. Unless otherwise stated no glucose was added to the media. All solutions were gassed with $CO_2 + O_2$ (5:95) and this was the gas phase throughout the incubation. Radioactive amino acids, from The Radiochemical Centre, Amersham, Bucks., were all L-isomers and uniformly labelled with ^{14}C except for [1-^{14}C]glycine. They were added to the medium unless otherwise stated at about 1 μc/6·5 ml. (Tables 4–7) or at about 1 μl./12·5 ml. (Tables 2 and 9), either undiluted or diluted with unlabelled amino acid to a concentration of 1 mM.

At the end of the incubation the tissue was placed in 10% (w/v) trichloroacetic acid and protein samples were prepared for counting as described by Manchester (1961*b*) except for a minor modification in the plating technique. After washing the protein with acetone the material was either suspended in ether and poured on to tared aluminium planchets (experiments in Tables 2 and 9) or dissolved in formic acid and half the available material pipetted on to a concentric ringed stainless-steel planchet (experiments in Tables 4–6 and 8). The results in the different Tables are therefore not strictly comparable. The content of radioactivity was assessed in a Nuclear–Chicago thin end-window gas-flow counter having an efficiency of about 25%. Since the sample weights in any given experiment were fairly constant no corrections for self-absorption were made in calculating specific activities.

Accumulation of amino acids was measured as described by Manchester & Young (1960).

ATP was measured by an enzymic method based on phosphoglycerate kinase (EC 2.7.2.3). The tissue was ground in ice-cold 5% (v/v) $HClO_4$ and the $HClO_4$ neutralized and precipitated by the addition of a mixture of KOH and potassium acetate. A sample of supernatant was added to an assay medium consisting of tris (0·1 M), EDTA (10 mM), $MgSO_4$ (9 mM), hydrazine sulphate (3·5 mM), phosphoglycerate (8·5 mM) and NADH (0·2 mM), final pH 7·5. The decrease in the extinction at 340 mμ was followed after the addition of 100 μg. of glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) (to remove diphosphoglycerate), followed by 40 μg. of phosphoglycerate kinase. Both enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

RESULTS

Role of Mg^{2+}. The first experiments were carried out to see whether Mg^{2+} affects the stimulation by insulin of incorporation of amino acids into protein of diaphragm muscle as it influences sugar transport (Bhattacharya, 1959*a*,*b*, 1961). Incorporation of radioactivity in the absence of insulin was as great in Krebs–Ringer phosphate buffer (II) or Stadie–Zapp medium (III) (Stadie & Zapp, 1947) as in Krebs–Ringer bicarbonate buffer (I) (Table 2). Replacement of Krebs–Ringer bicarbonate buffer by a sodium chloride–bicarbonate buffer (IV) brought about some diminution in glycine incorporation. The addition of insulin (0·1 unit/ml.) to each buffer brought about a marked stimulation of incorporation. Replacement of Mg^{2+} and Ca^{2+} by Na^+ (buffer V) decreased incorporation slightly, though replacement of only Mg^{2+} by Na^+ (buffer VI) had no similar effect. Neither change resulted in diminution of response to insulin. Replacement of all Na^+ by K^+ (buffer VII) markedly decreased incorporation and abolished the effect of insulin, but partial reintroduction of Na^+ (buffer VIII) led to restoration of the rate of incorporation and to recovery of sensitivity to insulin. Incorporation and its enhancement by insulin was markedly curtailed when diaphragm was incubated in a sucrose–bicarbonate medium (IX) similar to that used by Bhattacharya (1961) or in a similar medium compounded of sorbitol buffered with sodium hydrogen carbonate or potassium hydrogen carbonate (not shown in Table 2). As Bhattacharya (1961) showed for the uptake of glucose, sensitivity to stimulation by insulin of incorporation of amino acids into protein is enhanced by Mg^{2+} when the tissue is incubated in a buffer containing substantial amounts of sucrose. However, in buffers of more physiological ionic composition a normal response

Table 1. *Composition of buffers used for the incubation of isolated rat diaphragm*

Further details are given in the text. These buffers were used in the experiments described in subsequent Tables.

Buffer	Composition (μmoles/ml.)								
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	$H_2PO_4^-$–HPO_4^{2-}	HCO_3^-	SO_4^{2-}	Sucrose
I	143	6	1·2	2·5	128	1·2	25	1·2	—
II	151	5	1·2	2·5	133	16	—	1·2	—
III	140	—	5	—	80	40	—	—	—
IV	154	—	—	—	128	—	26	—	—
V	147	6	—	—	128	1·2	25	1·2	—
VI	144	6	—	2·5	128	1·2	25	1·2	—
VII	—	150	1·2	2·5	128	1·2	25	1·2	—
VIII	25	124	1·2	2·5	128	1·2	25	1·2	—
IX	25	—	—	—	—	—	25	—	250

Table 2. *Effect of alteration of ionic composition of the incubation medium on the incorporation of [^{14}C]glycine into the protein of isolated rat diaphragm and the stimulation of this process by insulin*

Incubation was for 2 hr. in the media indicated whose composition is described in Table 1. [1-^{14}C]Glycine was present at a concentration of 1 mM and about 80 μc/m-mole. Each result is the mean ± S.E.M. of the number of observations indicated.

Incubation buffer	No. of observations	Radioactivity in diaphragm protein (counts/min./mg.) No insulin added	Insulin added (0·1 unit/ml.)	Percentage increase	P
1 (a) Krebs–Ringer bicarbonate buffer (I)	6	18·7 ± 1·05	31·4 ± 2·48	69	< 0·001
(b) Krebs–Ringer phosphate buffer, pH 7·2 (II)	6	18·9 ± 1·79	30·4 ± 2·53	61	< 0·001
(c) Stadie–Zapp buffer, pH 7·2 (III)	6	18·2 ± 1·19	31·6 ± 2·31	74	< 0·001
2 (a) Krebs–Ringer bicarbonate buffer (I)	12	22·8 ± 1·36	36·9 ± 2·48	62	< 0·001
(b) NaCl–$NaHCO_3$ buffer (IV)	12	19·2 ± 0·38 (a)–(b) $P < 0·02$	29·6 ± 0·91 (a)–(b) $P < 0·001$	54	< 0·001
3 (a) Krebs–Ringer bicarbonate buffer (I)	6	16·0 ± 0·74	—		
(b) Buffer (I) with all Mg^{2+} and Ca^{2+} replaced by Na^{+} (V)	6	13·2 ± 1·24	22·3 ± 0·72	69	< 0·001
4 (a) Krebs–Ringer bicarbonate buffer (I)	4	14·6 ± 0·76	—		
(b) Buffer (I) with all Mg^{2+} replaced by Na^{+} (VI)	4	15·5 ± 0·43	20·7 ± 2·10	34	= 0·05
5 (a) Krebs–Ringer bicarbonate buffer (I)	6	19·7 ± 1·69	—		
(b) Buffer (I) with all Na^{+} replaced by K^{+} (VII)	6	6·8 ± 0·52	6·5 ± 0·42	−4	
(c) Buffer (I) with 118 m-equiv. of Na^{+} replaced by K^{+} (VIII)	6	11·9 ± 0·49 (a)–(b) $P < 0·001$ (a)–(c) $P < 0·01$ (c)–(b) $P < 0·001$	15·7 ± 1·15 (c)–(b) $P < 0·001$	32	= 0·02
6 (a) Krebs–Ringer bicarbonate buffer (I)	12	20·2 ± 1·24	31·8 ± 2·12	57	< 0·001
(b) Sucrose–$NaHCO_3$ buffer (IX) + NaCl (1 mM)	12	11·7 ± 0·51	12·8 ± 0·42	9	
(c) Sucrose–$NaHCO_3$ buffer (IX) + $MgCl_2$ (1 mM)	12	14·7 ± 0·99 (a)–(b) $P < 0·001$ (a)–(c) $P < 0·01$ (c)–(b) $P < 0·02$	20·0 ± 1·18 (a)–(b) $P < 0·001$ (a)–(c) $P < 0·001$ (c)–(b) $P < 0·001$	36	< 0·01
7 (a) Sucrose–$NaHCO_3$ buffer (IX) + NaCl (1 mM)	12	10·9 ± 0·59	—		
(b) Sucrose–$NaHCO_3$ buffer (IX) + KCl (1 mM)	12	10·3 ± 0·57	—		
(c) Sucrose–$NaHCO_3$ buffer (IX) + $MgCl_2$ (1 mM)	12	15·2 ± 0·85	—		
(d) Sucrose–$NaHCO_3$ buffer (IX) + $CaCl_2$ (1 mM)	12	12·8 ± 0·91	—		
(e) Sucrose–$NaHCO_3$ buffer (IX) + $MnCl_2$ (1 mM)	12	8·8 ± 0·41	—		
(f) Sucrose–$NaHCO_3$ buffer (IX) + $ZnCl_2$ (1 mM)	12	9·0 ± 0·55 (c)–(a) $P < 0·001$	—		

to insulin is observed in the absence of added Mg^{2+} and there seems no reason to suppose that under these conditions this is especially involved in the response of muscle to the hormone. The results of Table 2, for the incorporation of amino acid into protein, reveal what is not obvious from the study of glucose uptake, namely the inadequacy of a sucrose or sorbitol–bicarbonate medium as a supporting medium for diaphragm muscle as judged by the lower rate of amino acid incorporation. Moreover, as shown in Table 3, incubation in iso-osmotic sucrose leads to shrinkage of the diaphragm. The

addition of Mg^{2+} appeared to restrain the onset of rigor during incubation in sucrose–bicarbonate medium and it seems probable that the effect of the ion on the amount of incorporation and sensitivity to insulin is a consequence of this maintenance of the condition of the tissue and its influence on the ATP content (discussed below and Table 10). The effect of Mg^{2+} on incorporation was not reproduced by other bivalent ions (Table 2).

Role of Na^{+}. The effect of replacement of Na^{+} by K^{+} is more drastic than replacement by choline because increasing the concentration of K^{+} both depolarizes the cell membrane and brings about swelling of the tissue (Table 3), effects that are not seen with choline. The complexity of the system is indicated in Table 4; the loss of response to insulin in K^{+} buffer occurs only when a relatively high concentration of ^{14}C-labelled amino acid is added but not when a trace concentration is used. A stimulation by insulin is always seen when the amino acid is added to the medium at a concentration of about 10–20μM, but at 1mM concentration of amino acid K^{+} brings about a repression of the response to the hormone whereas choline does not. Incorporation of [^{14}C]glycine is decreased to a similar degree in K^{+} or choline buffer (Table 5), whereas incorporation of [^{14}C]leucine is less severely decreased in choline than in K^{+} buffer. In the latter medium the degree of inhibition for leucine appears to be comparable with that for glycine.

Table 3. *Effect of composition of the supporting medium on the weight of rat diaphragm muscle during incubation*

The composition of Krebs–Ringer bicarbonate buffer (I) and the various modifications is described in the Materials and Methods section, and that of the sucrose–$NaHCO_3$ buffer (IX) in Table 1. Incubation was for 2hr. Each result is the mean ± S.E.M. of the number of observations indicated.

Incubation buffer	No. of observations	Final wet wt. (% of initial wt.)
Krebs–Ringer bicarbonate buffer (I)	6	97·3 ± 0·41
Buffer (I) with Na^{+} replaced by choline	6	99·5 ± 1·46
Buffer (I) with Na^{+} replaced by K^{+}	12	120 ± 1·66
Buffer (I) with Na^{+} replaced by K^{+} and Cl^{-} by $MeSO_4^{-}$	12	108 ± 0·82
Sucrose–$NaHCO_3$ buffer (IX)	6	83·3 ± 0·95
Sucrose–$NaHCO_3$ buffer (IX) + $MgCl_2$ (1mM)	6	86·5 ± 2·26

Table 4. *Effect of insulin on the incorporation of* ^{14}C*-labelled amino acids into the protein of diaphragm incubated in buffers containing no* Na^{+}

The incubation buffer had the same composition as Krebs–Ringer bicarbonate buffer (I) except that all the Na^{+} had been replaced by choline or K^{+} as described in the Materials and Methods section. Incubation was for 2hr. Each result is the mean ± S.E.M. of six observations.

Na^{+} of incubation buffer replaced by	Amino acid	Radioactivity in diaphragm protein (counts/min./mg.): No insulin added	Radioactivity in diaphragm protein (counts/min./mg.): Insulin added (0·1 unit/ml.)	Percentage increase
Choline	Glycine (1·0mM)	14·9 ± 0·47	20·9 ± 1·04‡	40
	Glycine (20μM)	31·2 ± 1·12	36·8 ± 1·88*	18
	Proline (1·0mM)	16·8 ± 0·56	23·0 ± 1·78†	37
	Proline (13μM)	99 ± 4·2	126 ± 7·2†	27
	Phenylalanine (1·0mM)	18·1 ± 0·94	22·4 ± 0·84†	24
	Phenylalanine (21μM)	41·4 ± 2·34	53·4 ± 2·80†	23
K^{+}	Glycine (1·0mM)	14·7 ± 1·31	15·7 ± 2·07	7
	Glycine (20μM)	23·8 ± 1·13	30·5 ± 1·50†	28
	Proline (1·0mM)	18·6 ± 1·12	19·4 ± 1·48	4
	Phenylalanine (1·0mM)	20·2 ± 0·60	20·8 ± 1·20	3
	Phenylalanine (21μM)	57·0 ± 1·88	64·4 ± 2·90*	31
	Leucine (1·0mM)	70·6 ± 3·48	69·6 ± 4·60	−2
	Leucine (19μM)	332 ± 27	436 ± 31*	31

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

Table 5. *Effect of various changes in the composition of the incubation medium on the basal rate of incorporation of amino acids into the protein of isolated rat diaphragm*

The composition of Krebs–Ringer bicarbonate buffer (I) and the various modifications is described in the Materials and Methods section. Incubation was for 2hr. Each result is the mean ± S.E.M. of six observations.

Amino acid	Incubation buffer	Radioactivity in diaphragm protein (counts/min./mg.)	
Leucine (19 μM)	Krebs–Ringer bicarbonate buffer (I)	827 ± 77	$P < 0.01$
	Buffer (I) with Na^+ replaced by choline	530 ± 22	
	Buffer (I) with Na^+ replaced by K^+	312 ± 22	$P < 0.001$
Leucine (1·0 mM)	Buffer (I) with Na^+ replaced by choline	113 ± 4·3	$P < 0.001$
	Buffer (I) with Na^+ replaced by K^+	76 ± 10·0	
Glycine (20 μM)	Buffer (I) with Na^+ replaced by choline	29·0 ± 1·42	
	Buffer (I) with Na^+ replaced by K^+	28·5 ± 1·30	
Glycine (1·0 mM)	Buffer (I) with Na^+ replaced by choline	16·3 ± 1·55	
	Buffer (I) with Na^+ replaced by K^+	15·7 ± 0·93	

Table 6. *Effect of potassium methyl sulphate as a supporting salt on the incorporation of amino acids into protein by isolated rat diaphragm*

Incubation buffers had the same composition as Krebs–Ringer bicarbonate buffer (I) except where indicated that Na^+ had been replaced by K^+, and/or Cl^- by $MeSO_4^-$, as described in the Materials and Methods section. The final concentration of amino acid added was 1 mM; incubation was for 2hr. Each result is the mean ± S.E.M. of six observations.

Amino acid	Incubation buffer	Radioactivity in diaphragm protein (counts/min./mg.) No insulin added	Insulin added (0·1 unit/ml.)	Percentage increase
Glycine	Buffer (I) with Na^+ replaced by K^+	16·7 ± 2·85	16·5 ± 1·16	−1
	Buffer (I) with Na^+ replaced by K^+ and Cl^- by $MeSO_4^-$	31·4 ± 2·83	36·3 ± 1·24	12
Leucine	Krebs–Ringer bicarbonate buffer (I)	261 ± 11·1	300 ± 4·4	15
	Buffer (I) with Na^+ replaced by K^+ and Cl^- by $MeSO_4^-$	158 ± 12·2	162 ± 8·4	2·5

Table 7. *Effect of replacement of* Na^+ *by* K^+ *or choline on the accumulation of amino acids by isolated rat diaphragm*

Incubation buffers had the same composition as Krebs–Ringer bicarbonate buffer (I) except where indicated that Na^+ had been replaced by choline or K^+ as described in the Materials and Methods section. Incubation was for 2hr. Each result is the mean ± S.E.M. of six observations.

Amino acid	Incubation buffer	Radioactivity of tissue water (counts/min./ml.) / Radioactivity of medium (counts/min./ml.) No insulin added	Insulin added (0·1 unit/ml.)
Glycine (1·0 mM)	Krebs–Ringer bicarbonate buffer (I)	3·60 ± 0·14	4·40 ± 0·37
	Buffer (I) with Na^+ replaced by K^+	1·03 ± 0·029	1·08 ± 0·024
Leucine (1·0 mM)	Krebs–Ringer bicarbonate buffer (I)	3·66 ± 0·20	3·65 ± 0·16
	Buffer (I) with Na^+ replaced by K^+	1·08 ± 0·019	1·10 ± 0·021
Glycine (20 μM)	Buffer (I) with Na^+ replaced by choline	1·38 ± 0·023	1·42 ± 0·025

The swelling of muscle on incubating in a high-K^+ medium can be decreased somewhat, though not completely, by replacing Cl^- by the methyl sulphate anion (Table 3). In such buffer the basal level of incorporation is higher than in the presence of potassium chloride, but is still depressed by comparison with normal conditions (Table 6). In the presence of potassium methyl sulphate there is an equivocal response to insulin (Table 6).

If, as suggested by Parrish & Kipnis (1964), active transport of amino acids in muscle is dependent on and linked to the 'sodium pump', it is reasonable to suppose that interference with its functioning would bring about a decrease in the amount of ^{14}C-labelled amino acid available in the cell for incorporation into protein. Table 7 shows that replacement of Na^+ by K^+ or choline does indeed decrease the amount of accumulation of both glycine and leucine in the tissue water.

That retardation of accumulation can lessen incorporation (see Kostyo, 1964) can be demonstrated with the intact diaphragm preparation (where the tissue is retained in the rib-cage during incubation). Accumulation of glycine and aminoisobutyric acid occurs more slowly in this preparation than in the isolated hemidiaphragm (Kipnis & Noall, 1958; Manchester & Young, 1960) and in consequence (Table 8) incorporation of [^{14}C]glycine is less in the former preparation than in the latter. For leucine and phenylalanine, as opposed to glycine, whose rates of accumulation are more comparable in the two preparations (Manchester & Young, 1960), the rate of incorporation is less in the isolated hemidiaphragm, i.e. a damaged preparation, than in the rib-cage preparation. This trend is perhaps the more normal, for cutting diaphragm into strips and small pieces leads to a substantial diminution in the capacity to incorporate amino

Table 8. *Incorporation of ^{14}C-labelled amino acids into protein by the 'intact' and isolated hemidiaphragm muscle preparations*

Incubation was for 2hr. in Krebs–Ringer bicarbonate buffer (I). Each amino acid was present at a concentration of 1 mM and about 0·125 μc/ml. Each result is the mean ± S.E.M. of the number of observations indicated.

		Radioactivity in diaphragm protein (counts/min./mg.)	
Amino acid	No. of observations	Diaphragm contained in rib-cage	Isolated hemidiaphragm
Glycine	6	45 ± 2·7	61 ± 3·9†
Leucine	6	214 ± 2·4	195 ± 7·0*
Phenylalanine	12	81 ± 2·6	64 ± 3·2‡

* $P < 0·05$; † $P < 0·01$; ‡ $P < 0·001$.

Table 9. *Effect of cutting isolated rat diaphragm on its ability to incorporate ^{14}C-labelled amino acids into protein and its response to an effect of insulin on this process*

Incubation was for 2hr. in Krebs–Ringer bicarbonate buffer (I). The ^{14}C-labelled amino acids were present at about 0·08 μc/ml. Each result is the mean ± S.E.M. of six observations.

		Radioactivity in diaphragm protein (counts/min./mg.)		
Amino acid	Treatment	No insulin added	Insulin added (0·1 unit/ml.)	Difference
Glycine (1·0 mM)	Isolated hemidiaphragm	23·0 ± 0·73	32·5 ± 3·55*	9·5
	Each hemidiaphragm cut into three longitudinal strips	13·3 ± 0·61	16·1 ± 0·89*	2·8
	Each hemidiaphragm cut into small pieces about 1 mm.^{2}	10·7 ± 1·21	9·4 ± 1·23	−1·3
Phenylalanine (1·0 mM)	Isolated hemidiaphragm	52 ± 2·45	79 ± 3·71†	27
	Each hemidiaphragm cut into small pieces	14·2 ± 0·68	14·1 ± 0·91	−0·1
Glycine (10 μM)	Each hemidiaphragm cut into small pieces	57 ± 5·0	58 ± 5·0	1

* $P < 0·05$; † $P < 0·001$.

Table 10. *Effect of incubation in various media on the ATP content of rat diaphragm muscle*

The composition of Krebs–Ringer bicarbonate buffer (I) and the various modifications is described in the Materials and Methods section, that of the sucrose–$NaHCO_3$ buffer (IX) in Table 1. Incubation was for 2hr. Each result is the mean ± S.E.M. of six observations. (The ATP content of diaphragm muscle as prepared for incubation but before incubation is 2·5–3·0 μmoles/g. wet wt.).

Incubation buffer	Concn. of ATP (μmoles/g. wet wt.)
Krebs–Ringer bicarbonate buffer (I)	2·48 ± 0·23
Buffer (I) with Na^+ replaced by choline	1·32 ± 0·14
Buffer (I) with Na^+ replaced by K^+	0·61 ± 0·054
Buffer (I) with Na^+ replaced by K^+ and Cl^- by $MeSO_4^-$	1·45 ± 0·29
Sucrose–$NaHCO_3$ buffer (IX)	0·72 ± 0·040
Sucrose–$NaHCO_3$ buffer (IX) + $MgCl_2$ (1mM)	1·24 ± 0·13

Table 11. *Effect of 2-deoxyglucose and other materials on the ATP content of isolated rat diaphragm*

Incubation was for 2hr. in Krebs–Ringer bicarbonate buffer (I). Each result is the mean ± S.E.M. of six observations. The concentrations of metabolites are those used by DeSchepper *et al.* (1965).

Addition(s) to incubation buffer	Concn. of ATP (μmoles/g. wet wt.)
None	2·35 ± 0·069
Deoxyglucose (30mM)	1·15 ± 0·056
Deoxyglucose (30mM) + pyruvate (8·5mM) and succinate (7·4mM)	1·21 ± 0·061
Deoxyglucose (30mM) + glucose (30mM)	2·16 ± 0·203

acids, for both glycine and phenylalanine (Table 9). As the tissue becomes increasingly broken up the capacity to respond to insulin is progressively lost, and this is in conformity with the generally held view that *in vitro* actions of insulin are not readily seen in homogenates.

Effect of media on ATP content. Another factor that severely regulates the rate of incorporation of amino acids into protein is the availability of ATP. Thus agents that interfere with the respiratory chain bring about substantial inhibition of incorporation by diaphragm (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Manchester & Young, 1959). Table 10 indicates that many of the buffers used in the present study lead to a fall in the cellular content of ATP, the most severe losses being seen after incubation in the potassium chloride or sucrose–bicarbonate media. The factors responsible for these changes in ATP content are not known, but it seems likely that the diminutions are responsible for the lower rates of amino acid incorporation observed. It is noteworthy how the presence of Mg^{2+} in the sucrose–bicarbonate buffer restrains the fall in the ATP content under these conditions, an observation that correlates with the increased rate of amino acid incorporation (Table 2).

Changes in the cellular content of ATP also appear to be the explanation of the inhibition of incorporation found on the addition of 2-deoxyglucose (Wool, 1960) (Table 11), for the presence of the sugar brings about a substantial fall in the nucleotide concentration. DeSchepper *et al.* (1965) found in addition that the presence of deoxyglucose interfered with the stimulation of incorporation by insulin, but that its inhibitory effect in this respect was overcome by the addition of glucose, though not of a mixture of pyruvate and succinate. The special property of glucose not shared by succinate and pyruvate is that it appears to be able to reverse the diminution of the cellular ATP content brought about by deoxyglucose (Table 11).

DISCUSSION

Since the results of Parrish & Kipnis (1964) suggest that insulin can influence amino acid accumulation by muscle only when active transport is involved, the fact that insulin can influence incorporation in the absence of Na^+ (Table 4) emphasizes the point that the influence of insulin on incorporation of amino acids into protein in muscle is not dependent on its effect on amino acid accumulation, even though the two effects may appear to be closely linked (for review see Manchester, 1965). There are now numerous aspects of the metabolism of muscle that show responses to insulin and that so far as can be ascertained respond independently of each other (Manchester, 1965). Whether the insulin effect in each situation starts from a common point is still unknown.

The present results do not provide much support for the concept (Bhattacharya, 1959*a*,*b*, 1961) that Mg^{2+} has a specific function in facilitating the response of muscle to insulin, for the capacity of the tissue to respond to insulin is well maintained under the different conditions studied except (*a*) when the tissue is broken up, (*b*) the ions of the medium are replaced by sucrose and (*c*) when Na^+ is replaced by K^+ and the amino acid is present in a high concentration. One of the aims of the present work was the hope of defining critical factors that determine whether or not a muscle preparation will respond to insulin. The diminution of response to the hormone on cutting the tissue emphasizes the importance of the integrated cell structure for insulin to be able to bring about its action. Whether it is specifically the intact membrane that is necessary for the hormonal response or certain features of the

intracellular cytoskeleton (Peters, 1956; Krahl, 1957) is not known, but clearly the physical state of the tissue has an important bearing on its responsiveness and it is presumably these same considerations that prevent the effects of the hormone in cell-free systems from being seen. It is generally accepted that the capacity of subcellular fragments to incorporate amino acids is much less than that of the intact cell (Hendler, 1962), and this is borne out by the severe diminution in incorporation seen on cutting diaphragm into pieces (Table 9). Hendler (1962) has suggested that protein synthesis near the membrane may be a very significant proportion of the total synthetic activity. If so, physical damage to membranes may be the chief factor involved in the diminution of incorporation when the tissue is cut up.

The loss of insulin response with K^+ buffers might be correlated with the swelling the tissue undergoes (Table 3) and the physical damage to mitochondria and membranes that can be observed under these conditions (Harris, 1961). Incubation in choline buffer, in which swelling does not occur, does not diminish the insulin response (Table 4). Although the results with 2-deoxyglucose might suggest that a decline in the internal ATP content could become a factor limiting the extent of a stimulation of incorporation by insulin, the fact that effects of insulin are readily seen in the choline buffer despite the fall in ATP content (Table 10) argues against the importance of this possibility. Indeed, its interference in the presence of deoxyglucose with an effect of insulin in promoting incorporation is probably a special case arising from the simultaneous stimulation by the hormone of the entry of the sugar into the cell (Kipnis & Cori, 1959), which will aggravate the inhibition of anabolic processes. Normal insulin effects on incorporation expressed as percentage changes can be seen in the presence of metabolic inhibitors or inhibitors of protein synthesis (Manchester, 1961*b*; Wool, Castles & Moyer, 1965).

The decline in the ATP content on the addition of 2-deoxyglucose presumably arises because the sugar can be phosphorylated by diaphragm though not further metabolized (Kipnis & Cori, 1959), and the sugar will thus utilize 'energy-rich' phosphate without leading to its eventual replacement. The presence of deoxyglucose might be expected to promote oxygen consumption, but this does not appear to be so (Woodward & Hudson, 1954; K. L. Manchester, unpublished work). Likewise, the lowered ATP content of diaphragm in all the abnormal buffers (Table 10) may lead to increased oxygen consumption, and this has been noted by Harris (1965) for frog sartorius muscle in choline buffer, but in K^+ medium there did not appear to be much change. If glucose uptake of muscle is inversely related to ATP content (Smith, Randle & Battaglia, 1961), the low ATP content of muscle incubated in sucrose medium would explain the tendency towards high basal glucose uptakes observed by Bhattacharya (1961). Why oxidation of glucose, but not of succinate and pyruvate, can restore towards normal the decline in ATP content in the presence of deoxyglucose is not clear, for both pyruvate and succinate are readily oxidized by diaphragm (Villee, White & Hastings, 1952; K. L. Manchester, unpublished work). DeSchepper *et al.* (1965) showed that the effect of glucose was not attributable to a decrease in the rate of entry of deoxyglucose in the presence of the other sugar as a result of competition for transport. 'Compartmentation' phenomena between cytoplasmic generation of ATP from glucose as opposed to mitochondrial generation from citric acid-cycle intermediates might be of significance since it is uncertain how readily ATP moves into and out of the mitochondrion (Siekevitz & Potter, 1955). Why the addition of pyruvate and succinate promotes any extra oxygen consumption by the tissue at all is problematical, since in the absence of added substrate the basal oxygen consumption of the incubated tissue appears to be adequate to maintain the ATP concentration near its initial level. The addition of substrates to the perfused heart does not influence oxygen consumption (Fisher & Williamson, 1961; Williamson & Krebs, 1961). However, in the presence of pyruvate and succinate, aerobically promoted reduction of the type described by Krebs and co-workers (Kulka, Krebs & Eggleston, 1961; Krebs, Eggleston & d'Alessandro, 1961) might lead to an added requirement for ATP, a factor that would both promote oxygen uptake and be consistent with failure of pyruvate and succinate to raise the ATP content in the presence of deoxyglucose.

The variability of responsiveness to insulin on the substitution of K^+ for Na^+ depending on whether the amino acid is present in a high or low concentration is not easily explained. Although under normal conditions of incubation increasing the concentration of added amino acid diminishes the effects of the hormone on transport phenomena, insulin still observably affects the amount of incorporation even when the amino acid is added at concentrations as high as 20mM (Manchester, 1961*b*; Parrish & Kipnis, 1964). It is not yet known in what way the pool of ^{14}C-labelled amino acids used in protein synthesis is related to the total pool of amino acids in the tissue (Kipnis, Reiss & Helmreich, 1961; Manchester & Wool, 1963). The possibility of a large proportion of the cell's protein synthesis taking place near the membrane would be very germane to this point. If there were a small amino acid pool near the membrane effectively distinct from the

general pool of the tissue, it is possible that with the depolarization and partial destruction of the membrane consequent on substitution of K^+ for Na^+ the action of insulin to influence amino acid accumulation in this area would be less strongly exerted and rendered insignificant more readily as the concentration of added amino acid rose.

The greater diminution in the incorporation of ^{14}C from glycine than from leucine seen in choline buffer (Table 5) is noteworthy in the context of the evidence of Oxender & Christensen (1963) of the factors regulating the entry of amino acids into ascites-tumour cells. They found that the kinetic parameters of the accumulation of amino acids like alanine and glycine were very different from those like leucine and phenylalanine. The former entered the cell more slowly and largely by active accumulation, whereas for the latter group exchange reactions predominated. Qualitatively the characteristics of amino acid accumulation by the perfused rat heart (Manchester & Wool, 1963) seem very similar to those of the ascites-tumour cells and it is probably not unreasonable to extrapolate from these cells to muscle. Thus, if the primary inhibitory influence of Na^+ deprivation is on the active accumulation of amino acids, a more severe diminution of incorporation of glycine than of leucine in the absence of Na^+ is to be expected. A contrast between the behaviour of glycine and of leucine and phenylalanine also shows itself in the comparison of their accumulation and incorporation into protein in the rib-cage preparation and the isolated hemidiaphragm (Manchester & Young, 1960; Table 8). It seems at first sight paradoxical that the active type of accumulation of glycine and aminoisobutyric acid should occur more slowly in the rib-cage preparation than in the isolated hemidiaphragm, for it would be expected that damage to the tissue would interfere with rather than facilitate active accumulation. However, the differences become understandable if the active accumulation of amino acids is linked either to the entry of Na^+ into the cell or to the exit of K^+ (Vidaver, 1964), for both these processes occur to a greater extent in isolated hemidiaphragm than in the rib-cage preparation. The observation of Peckham & Knobil (1962) that crushing diaphragm can under certain conditions enhance the accumulation of aminoisobutyric acid is explicable in a like manner.

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REFERENCES

Bhattacharya, G. (1959*a*). *Nature, Lond.*, **183**, 324.
Bhattacharya, G. (1959*b*). *Nature, Lond.*, **184**, 1401.
Bhattacharya, G. (1961). *Biochem. J.* **79**, 369.
Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1950). *J. biol. Chem.* **186**, 309.
Cadenas, E., Kaji, H., Park, C. R. & Rasmussen, H. (1961). *J. biol. Chem.* **236**, PC 63.
Carlin, H. & Hechter, O. (1962). *J. biol. Chem.* **237**, PC 1371.
DeSchepper, P. J., Toyoda, M. & Bessman, S. P. (1965). *J. biol. Chem.* **240**, 1670.
Edelman, P. M., Rosenthal, S. L. & Schwartz, I. L. (1963). *Nature, Lond.*, **197**, 878.
Fisher, R. B. & Williamson, J. R. (1961). *J. Physiol.* **158**, 102.
Fong, C. T. O., Silver, L., Popenoe, E. A. & Debons, A. F. (1962). *Biochim. biophys. Acta*, **56**, 190.
Harris, E. J. (1961). *J. biophys. biochem. Cytol.* **9**, 502.
Harris, E. J. (1965). *J. Physiol.* **177**, 355.
Hendler, R. W. (1962). *Nature, Lond.*, **193**, 821.
Kipnis, D. M. & Cori, C. F. (1957). *J. biol. Chem.* **224**, 681.
Kipnis, D. M. & Cori, C. F. (1959). *J. biol. Chem.* **234**, 171.
Kipnis, D. M. & Noall, M. W. (1958). *Biochim. biophys. Acta*, **28**, 226.
Kipnis, D. M., Reiss, E. & Helmreich, E. (1961). *Biochim. biophys. Acta*, **51**, 519.
Kostyo, J. L. (1964). *Endocrinology*, **75**, 113.
Krahl, M. E. (1957). *Perspectives Biol. Med.* **1**, 69.
Krebs, H. A., Eggleston, L. V. & d'Alessandro, A. (1961). *Biochem. J.* **79**, 537.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Kulka, R. G., Krebs, H. A. & Eggleston, L. V. (1961). *Biochem. J.* **78**, 95.
Manchester, K. L. (1961*a*). *Proc. 4th Congr. int. Diabetes Fed.*, p. 610. Geneva: Éditions Médicine et Hygiène.
Manchester, K. L. (1961*b*). *Biochem. J.* **81**, 135.
Manchester, K. L. (1965). In *On the Nature and Treatment of Diabetes: Proc. 5th Congr. int. Diabetes Fed.*, p. 101. Exerpta Medica Foundation.
Manchester, K. L. & Wool, I. G. (1963). *Biochem. J.* **89**, 202.
Manchester, K. L. & Young, F. G. (1959). *J. Endocrin.* **18**, 381.
Manchester, K. L. & Young, F. G. (1960). *Biochem. J.* **75**, 487.
Mirsky, I. A. & Perisutti, G. (1962). *Biochim. biophys. Acta*, **62**, 490.
Oxender, D. L. & Christensen, H. N. (1963). *J. biol. Chem.* **238**, 3686.
Parrish, J. E. & Kipnis, D. M. (1964). *J. clin. Invest.* **43**, 1994.
Peckham, W. D. & Knobil, E. (1962). *Biochim. biophys. Acta*, **63**, 207.
Peters, R. A. (1956). *Nature, Lond.*, **177**, 426.
Siekevitz, P. & Potter, V. R. (1955). *J. biol. Chem.* **215**, 237.
Smith, G. H., Randle, P. J. & Battaglia, F. C. (1961). *Mem. Soc. Endocrin.* **11**, 124.
Stadie, W. C. & Zapp, J. A. (1947). *J. biol. Chem.* **170**, 55.
Vidaver, G. A. (1964). *Biochemistry*, **3**, 795.
Villee, C. A., White, V. K. & Hastings, A. B. (1952). *J. biol. Chem.* **195**, 287.
Williamson, J. R. & Krebs, H. A. (1961). *Biochem. J.* **80**, 540.
Woodward, G. & Hudson, M. (1954). *Cancer Res.* **14**, 599.
Wool, I. G. (1960). *Amer. J. Physiol.* **198**, 57.
Wool, I. G., Castles, J. J. & Moyer, A. N. (1965). *Biochim. biophys. Acta*, **107**, 333.