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The Effects of Potassium Ions and Denervation on Protein Synthesis and the Transport of Amino Acids in Muscle

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1. The effects of varying concentrations of K^+ during incubation, of denervation and of various drugs on the accumulation of ^{14}C-labelled amino acids, their incorporation into protein and the stimulation of these processes by insulin in rat diaphragm preparations were studied. 2. The accumulation of glycine and aminoisobutyrate and incorporation of glycine into protein was less in tissue incubated in K^+-free buffer or 20mM-K^+ than with 5–10mM-K^+. Incorporation of leucine was unaffected. 3. Incorporation into protein of amino acids by diaphragm that had been denervated 3 days previously was elevated. Accumulation of both glycine and aminoisobutyrate was also raised but that of phenylalanine was unaffected. 4. Accumulation of glycine by diaphragm and extensor digitorum longus muscle was decreased by a number of agents including cocaine and mepyramine. 5. The stimulation of incorporation by insulin was unaffected by changes in K^+ or in the presence of cocaine and mepyramine. Denervated tissue was markedly less responsive to insulin than its control. 6. The results are discussed in the context of the relation of amino acid accumulation to operation of the Na^+ pump and the influence of insulin thereon.

There are now many observations that the accumulation of amino acids by tissues, both by the intestinal mucosa from the lumen and by cells from their extracellular medium, is related in some way to active movement of cations (Csaky, 1961; Lahiri & Lajtha, 1964; Fox, Thier, Rosenberg & Segal, 1964; Johnstone & Scholefield, 1965). One can distinguish several possibilities: (*a*) amino acids enter against a concentration gradient as a direct consequence of extrusion of Na^+; (*b*) amino acids enter instead of the K^+ that normally replaces emergent Na^+; (*c*) the amino acid enters along with K^+; (*d*) entry of amino acid is coupled to emergence of K^+. In addition, in experiments *in vitro*, entry of labelled amino acid may result from exchange with unlabelled acid already in the cell pool.

The complexity of the problem is aggravated in that different amino acids do not all share the same uptake characteristics, and therefore necessarily the same mechanism of transport into the cell. Christensen and colleagues (Christensen, Akedo, Oxender & Winter, 1962; Oxender & Christensen, 1963) have distinguished those whose uptake appears to be primarily due to an active process and those for which exchange reactions predominate. Glycine and the unnatural acid aminoisobutyric acid are good examples of amino acids in the former category, and leucine and phenylalanine in the latter.

Insulin enhances the accumulation of some amino acids, particularly glycine and aminoisobutyrate (Kipnis & Noall, 1958; Manchester & Young, 1960; Wool, 1964), and stimulates the incorporation into protein of all those capable of being incorporated (Manchester & Young, 1958; Wool & Krahl, 1959). Insulin has also been shown to assist in exclusion of Na^+ from rat muscle (Creese, D'Silva & Northover, 1958) and to increase the resting potential of muscle fibres (Zierler, 1959*a*,*b*). Hence it is reasonable to infer that active Na^+ extrusion can be stimulated by the hormone. Although these facts taken together point to a coupling between amino acid uptake and active Na^+ movement, it remains true that insulin will stimulate protein synthesis, though not amino acid accumulation, even in the complete absence of added Na^+ (Manchester, 1966). It was thus thought of value to examine treatments that alter K^+ permeability to see whether the apparent relation of amino acid transport with Na^+ pumping stems from the complementary character of Na^+ and K^+ movements that normally holds in muscle (Dydynska & Harris, 1966).

The permeability of frog muscle cell membrane

to K^+ is decreased by a number of anaesthetics (Shanes, 1950), and the effects of various of these compounds on amino acid accumulation and incorporation have been investigated. Permeability of muscle to K^+ is also decreased by denervation (Nicholls, 1956; Harris & Nicholls, 1956; Hubbard, 1963; Thesleff, 1963). Moreover, the sensitivity of denervated muscle to insulin with regard to sugar uptake is also much decreased after denervation (Buse & Buse, 1959, 1961). We have therefore looked at the effect of denervation on the accumulation and incorporation into protein of amino acids in muscle. While this work was in progress Buse, McMaster & Buse (1965) showed that incorporation of [^{14}C]leucine is elevated by denervation and, in common with the glucose metabolism, at the same time loses its sensitivity to insulin. Our results, obtained with other amino acids, confirm and extend their findings.

METHODS

Diaphragm muscle was taken from non-starved albino rats (about 100g. body wt.) and incubated with shaking, usually for 2hr., at 37°, as described by Manchester (1961). Where isolated hemidiaphragms were used (Tables 1–3, 5 and 7) the diaphragm muscle dissected from the rib-cage was further dissected into the individual hemidiaphragms on a piece of hardened filter paper (Whatman no. 40) and the pieces of tissue were placed for a few minutes before incubation in beakers of buffer at room temperature. The intact diaphragm preparation (retaining the rib-cage) of Kipnis & Cori (1957) was placed for a few minutes after dissection in K^+-free buffer at 37° through which O_2+CO_2 (95:5) was bubbled (Tables 5–7). Leg muscle (extensor digitorum longus) was also placed after dissection and before incubation in oxygenated buffer at 37° (Table 8).

The medium for incubation was Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) or various modifications as stated (Tables 2, 3 and 6). No glucose or oxidizable substrate was added to the medium unless otherwise stated. All solutions were gassed with O_2+CO_2 (95:5), and this was the gas phase throughout the incubation. Each hemidiaphragm or extensor muscle was normally incubated in 1ml. of solution, each intact diaphragm preparation in 10ml. Because of the leakage of K^+ from the damaged intercostal muscle, intact diaphragms were incubated in buffer from which the KCl had been omitted. At the end of 2hr. incubation the concentration of K^+ in the medium was 5–8mM. 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, either undiluted or diluted with unlabelled amino acid, at the specific activities and concentrations stated in the Tables.

At the end of the incubation, hemidiaphragms were dissected from the intact diaphragm preparation. Each piece of tissue was weighed and placed in 2ml. of water in a boiling-water bath. Accumulated ^{14}C-labelled amino acid in the tissue, i.e. amino acids that are not incorporated in protein but are readily extractable from the tissue contents, was measured as described by Manchester & Young (1960) and its incorporation into protein as described by Manchester (1961, 1966). Accumulation is expressed as the ratio of radioactivity in the tissue to radioactivity in the medium, and incorporation as counts/min./mg. of dried tissue protein after extraction.

K^+ and Na^+ were determined by flame photometry. The ATP content of tissue was estimated enzymically as described by Manchester (1966). For estimation of the inulin space, the tissue was incubated for 2hr. with medium containing inulin (2%, w/v) and the inulin extracted from the tissue in boiling water. The inulin content of samples of the medium and tissue extract was determined as fructose by the method of Pogell (1954).

Denervation. Left unilateral phrenicotomy of diaphragm was carried out under ether anaesthesia, the phrenic nerve being exposed through a thoracotomy incision. As noted by others (Sola & Martin, 1953; Stewart, 1955; Stewart & Martin, 1956; Buse & Buse, 1959, 1961), a substantial increase in weight of the denervated tissue occurred within 3 days, and an increase in the water content was evident [dry wt. $17{\cdot}7\pm0{\cdot}13$ and $20{\cdot}1\pm0{\cdot}16$% of wet wt. (means ±S.E.M. of 12 observations) for denervated and control hemidiaphragms respectively after incubation as intact diaphragm ($P<0{\cdot}001$)]. Buse & Buse (1961) noted a decrease in the thiosulphate space of the isolated hemidiaphragm after denervation; we found a slight though not significant decrease in the inulin space of the intact preparation [$15{\cdot}9\pm0{\cdot}61$ and $17{\cdot}7\pm0{\cdot}82$ml./100g. wet wt. (means ±S.E.M. of 12 observations) for denervated and controls respectively]. Some expansion of the intracellular fluid as a result of denervation therefore seems likely.

RESULTS

Role of K^+ *ions.* Incubation of diaphragm in a medium containing no added K^+ does not significantly affect its capacity to incorporate amino acids into protein (Table 1), but, since during incubation K^+ accumulates in the medium in the limited volume of fluid normally used, the medium soon ceases to be K^+-free. If the tissue is first incubated for 30min. in a K^+-free medium and is then transferred to fresh medium, containing initially no K^+, the uptake of [^{14}C]glycine from this latter medium and its incorporation into protein are less than in controls incubated in

Table 1. *Effect of omission of* K^+ *on the incorporation of* [^{14}C]*glycine into the protein of isolated rat diaphragm*

Incubation was for 2hr. Each value is the mean ±S.E.M. of six observations. Glycine was present at a concentration of 12μM and about 0·080μc/ml.

Diaphragm incubated in	Radioactivity in diaphragm protein (counts/min./mg.)
Krebs–Ringer bicarbonate	$78\pm5{\cdot}5$
Krebs–Ringer bicarbonate with K^+ replaced by Na^+	$73\pm5{\cdot}0$

Table 2. *Effect of incubation in K+-free medium on the accumulation and incorporation into protein of [14C]glycine by isolated rat diaphragm muscle*

Hemidiaphragms were preincubated for 30min., then incubated for 2hr. in the presence of [14C]glycine in either Krebs–Ringer bicarbonate or Krebs–Ringer bicarbonate in which K+ had been replaced by Na+. Preincubation was in 10ml. per hemidiaphragm and subsequent incubation in 1ml. of fluid. In Expt. 1 each result is the mean ± S.E.M. of three observations and in Expts. 2 and 3 of four values. In Expt. 1 radioactivity was added to a concn. of about 0·16μc/ml., in Expt. 2 about 0·25μc/ml. and in Expt. 3 about 0·12μc/ml.

Expt. no.	K+ added to medium during: Preincubation	K+ added to medium during: Incubation	Concn. of glycine (μM)	Concn. of ions in tissue at end of incubation (μequiv./g. wet wt.): K+	Concn. of ions in tissue at end of incubation (μequiv./g. wet wt.): Na+	Radioactivity in tissue (counts/min./g.) / Radioactivity in medium (counts/min./ml.)	Radioactivity in diaphragm protein (counts/min./mg.)	
1	+	+	20	—	—	—	243±19	$P<0.01$
	−	−	20	—	—	—	143± 7·0	
	+	+	1000	—	—	—	78± 7·5	$P<0.05$
	−	−	1000	—	—	—	49± 5·5	
2	+	+	30	52	70	2·78±0·18	416±60	$P<0.001$
	−	−	30	26	93	1·55±0·09	310±44	
	−	+	30	48	71	2·69±0·17	405±52	$P<0.001$
3	−	−	15	24	99	1·28±0·07	102± 5·7	$P<0.05$
	−	−*	15	22	100	1·44±0·16	144±14	

* Insulin added (0·1 unit/ml.).

medium with 5mM-K+ throughout (Table 2). If, however, after incubation for 30min. in K+-free buffer the tissue is returned to medium containing K+, accumulation and incorporation is normal (Table 2). Incubation in initially K+-free buffer results in depletion of cellular K+ and increase in tissue Na+ (Table 2). Under these conditions the rate of incorporation of 14C-labelled amino acid, although less than in the control medium with 5mM-K+, is still enhanced by insulin (Table 2, Expt. 3).

Not only K+ deprivation, but also K+ in excess (at 20mM), interferes with accumulation and incorporation into protein of [14C]glycine. Table 3 (and Table 4, an analysis of variance) shows that the optimum concentration of K+ in the medium is between 5 and 10mM. Similar effects of the concentration of K+ in the medium on the accumulation of aminoisobutyric acid were also noted, particularly in the presence of insulin, but these changes were not seen on the accumulation and incorporation of [14C]leucine. Changes in the concentration of K+ did not modify the response of the tissue to insulin (Table 3), i.e. the effect of insulin is seen on the accumulation of glycine and aminoisobutyrate, though not on accumulation of leucine, as previously found (Manchester & Young, 1960). There is a stimulation of incorporation into protein of both glycine and leucine by insulin, as is normally seen (Manchester & Young, 1958).

As is well known (Creese, 1954), the isolated hemidiaphragm loses K+ during incubation, and this is reflected in the low concentration of K+ in the tissue at the end of incubation (Tables 2 and 3) by comparison with that found in the intact preparation (Tables 5–7). Although insulin tends to assist K+ retention by tissue in K+-containing media it also leads to a higher Na+ content after exposure to the low-K+ media. This could result either from an increased extracellular space or from a greater 'leakiness' to Na+; the latter explanation accords with the observations of Otsuka & Ohtsuki (1965) on the effects of insulin on resting potential in K+-free media. Since any changes in K+ concentrations found in further experiments seemed more meaningful if they referred to intact tissue, most of the subsequent experiments used either the intact diaphragm preparation or the extensor digitorum longus muscle, which is dissected without cutting any of the fibres.

Effect of denervation. The accumulation and incorporation into protein of [14C]glycine by diaphragm that has been denervated 2–3 days previously is enhanced by comparison with its unoperated control (Table 5). This increased incorporation is demonstrable in the rib-cage-retained preparation and equally with the conventional hemidiaphragm, and is seen with [14C]-phenylalanine as well as with glycine. Accumulation of aminoisobutyrate is also enhanced.

After denervation the concentration of Na+ in the tissue is not perceptibly changed, but there is

Table 3. *Effect of various* K^+ *concentrations on the accumulation and incorporation into protein of* ^{14}C*-labelled amino acids by the isolated rat diaphragm*

Insulin when present was at a concentration of 0·1 unit/ml. Each hemidiaphragm was first incubated in 10 ml. for 30 min., then incubated for 2 hr. in 1 ml. of medium containing the ^{14}C-labelled amino acid. Each result is the mean ± S.E.M. of three observations. Radioactivity was added to the medium at about 0·16 μc/ml.

Expt. no.	Amino acid	Concn. of K^+ in medium (mM)	Concn. of ions in tissue at end of incubation (μequiv./g. wet wt.)				Radioactivity in tissue (counts/min./g.) / Radioactivity of medium (counts/min./ml.)		Radioactivity in diaphragm protein (counts/min./mg.)	
			K^+		Na^+					
			Insulin absent	Insulin present	Insulin absent	Insulin present	Insulin absent	Insulin present	Insulin absent	Insulin present
1	Glycine (20 μM)	0	34	26	90	92	1·33 ± 0·06	1·51 ± 0·08	160 ± 11	222 ± 17
		5	46	44	86	80	1·84 ± 0·12	2·35 ± 0·12	204 ± 19	303 ± 59
		10	59	64	63	60	2·04 ± 0·15	2·55 ± 0·51	197 ± 23	316 ± 27
		20	59	62	88	84	1·58 ± 0·08	2·25 ± 0·31	141 ± 18	212 ± 36
2	Glycine (1·0 mM)	0	32	35	99	110	1·37 ± 0·06	1·74 ± 0·07	63 ± 6·7	92 ± 7·6
		5	55	60	77	81	1·70 ± 0·07	2·67 ± 0·27	67 ± 2·2	27 ± 6·8
		10	—	—	—	—	1·75 ± 0·15	2·72 ± 0·27	79 ± 6·2	117 ± 9·0
		20	—	—	—	—	1·50 ± 0·16	1·89 ± 0·07	59 ± 8·5	79 ± 11
3	Aminoisobutyrate (67 μM)	0	37	36	106	106	1·36 ± 0·06	1·96 ± 0·12	—	—
		5	58	64	68	62	1·25 ± 0·15	3·15 ± 0·31	—	—
		10	66	66	80	80	1·36 ± 0·08	3·47 ± 0·08	—	—
		20	79	78	72	73	1·18 ± 0·06	2·84 ± 0·39	—	—
4	Aminoisobutyrate (1·0 mM)	0	31	38	98	118	1·51 ± 0·25	1·90 ± 0·20	—	—
		5	55	58	77	80	1·70 ± 0·15	2·68 ± 0·37	—	—
		10	60	54	82	85	1·52 ± 0·02	2·64 ± 0·31	—	—
		20	60	54	104	91	1·46 ± 0·18	1·95 ± 0·21	—	—
5	Leucine (19 μM)	0	32	30	93	100	1·40 ± 0·04	1·35 ± 0·04	422 ± 37	520 ± 89
		5	37	42	91	88	1·66 ± 0·16	1·46 ± 0·01	348 ± 64	532 ± 32
		10	53	62	85	77	1·41 ± 0·08	1·67 ± 0·15	357 ± 7	610 ± 40
		20	50	51	98	90	1·50 ± 0·15	1·60 ± 0·18	348 ± 14	465 ± 33

Table 4. *Analysis of variance of results in Table 3*

Degrees of freedom ...		Effect of K^+ 3/16		Effect of insulin 1/16	
		F	*P*	*F*	*P*
1	Glycine				
	Accumulation	5·9	<0·01	9·1	<0·01
	Incorporation	4·3	<0·05	19·1	<0·001
2	Glycine				
	Accumulation	7·1	<0·01	27·1	<0·001
	Incorporation	5·7	<0·01	37	<0·001
3	Aminoisobutyrate				
	Accumulation	5·8*	<0·01	147	<0·001
4	Aminoisobutyrate				
	Accumulation	2·3	<0·2	20	<0·001
5	Leucine				
	Accumulation	1·2	>0·2	0·2	>0·2
	Incorporation	1·1	>0·2	24	<0·001

* Substantial *F* for interaction, i.e. K^+ effect is linked to insulin effect. For Expts. 3 and 4 together, taking values in the presence of insulin only, *F* for K^+ effect is 5·2, $P<0·01$.

a small decrease in the K^+ content, which was 79·0 μequiv./g. in the controls and fell by a mean of 3·4 μequiv./g. (mean of five groups each of six pairs: P 0·05). Taken with the decreased concentration of protein and the slightly decreased extracellular space this implies that there is an appreciable decrease in the internal K^+ concentration.

A striking feature of the denervated preparation is its lack of response to the usual stimulant effect of insulin on amino acid incorporation (Table 5). Although insulin raises the amount of incorporation by control diaphragms, the incorporation remains less than that which occurs in the denervated tissue, despite the fact that incorporation by the latter is not increased with insulin.

If the tissue is denervated 10 days before its incubation, then the above changes are not seen, the rate of incorporation is similar to that of control tissue and an increase in incorporation is brought about by addition of insulin (Table 5).

Buse & Buse (1959) considered the possibility that incubation of denervated tissue with a neuromuscular transmitter agent might restore insulin sensitivity. However, they did not observe any effect on adding acetylcholine. We have studied the effect of adding acetyl- or succinyl-choline with varying results. On two occasions (Table 6, Expts. 1 and 2) the presence of acetyl- or succinyl-choline decreased the incorporation and accumulation by the denervated diaphragm to that of its control. There was in Expt. 2 the suggestion of an increased incorporation with insulin after application of succinylcholine to the denervated tissue. However, in two subsequent tests (Expts. 3 and 4) no effect of succinylcholine was discernible. The reason for this variability is not known. Likewise, since denervated tissue is characterized by a greater and more generalized sensitivity to neuromuscular transmitter agents (Miledi, 1962), we looked for a possible effect of curare on activity of denervated tissue, but the results were again equivocal (Table 6). The control results do, however, support those already given (Table 5), showing increased accumulation and incorporation by the denervated tissue.

Effect of anaesthetics and substances that increase membrane resistance. Muscle permeability to K^+ is decreased by several drugs (Shanes, 1950). If accumulation of amino acids is related to movement of K^+, it is possible that materials affecting movement of K^+ will also influence the former process. Cocaine is an example of a local anaesthetic that decreases K^+ permeability. In parallel experiments made on frog sartorius muscle in a Cl^--free medium the electrical resistance was increased threefold in the presence of 1 mM-cocaine (E. J. Harris, unpublished work). The antihistamine mepyramine at 1 mM led to as much as a fourfold increase in resistance. Accordingly we chose these two substances as typical of those that decrease K^+ permeability to see whether there was a parallel effect on the penetration of amino acids. There appeared to be some diminution in the accumulation of glycine in the presence of 1 mM-cocaine, both with the intact diaphragm and the isolated hemidiaphragm preparation studied at two different concentrations of glycine and in the presence and absence of insulin. The differences were small and for the most part not individually significant, but an analysis of variance for an effect of cocaine on all the values

Table 5. *Effect of denervation on the accumulation and incorporation into protein of* ^{14}C*-labelled amino acids by rat diaphragm muscle*

Each value is the mean ± S.E.M. of the number of observations indicated. Insulin when added was at a concentration of 0·1 unit/ml.

Diaphragm preparation	Days after denervation	^{14}C-labelled amino acid	No. of observations	Radioactivity in tissue (counts/min./g.) / Radioactivity in medium (counts/min./ml.)				Radioactivity in diaphragm protein (counts/min./mg.)			
				Denervated		Control		Denervated		Control	
				Insulin absent	Insulin present	Insulin absent	Insulin present	Insulin absent	Insulin present	Insulin absent	Insulin present
Intact	3	Glycine (1 mM, 0·07 μc/ml.)	6	—	1·35±0·18	—	1·15±0·13	—	55±8·8	—	39±5·6
Intact	3	Glycine (1 mM, 0·12 μc/ml.)	4	2·22±0·21	1·95±0·16	1·18±0·05	1·54±0·05	102±15	100±7·0	46±8·8	70±3·7
Intact	3	Aminoisobutyrate (100 μM, 0·25 μc/ml.)	2	1·28	—	0·86	—	—	—	—	—
Intact	3	Phenylalanine (1 mM, 0·25 μc/ml.)	3	0·72±0·03	0·77±0·08	0·70±0·04	0·71±0·06	134±23	128±8·0	70±6·5	115±11
Hemi-diaphragm	3	Glycine (32 μM, 0·25 μc/ml.)	2	1·91	—	1·48	—	407	—	255	—
Intact	10	Glycine (1 mM, 0·12 μc/ml.)	4	1·02±0·03	1·20±0·10	1·18±0·07	1·35±0·18	36±3·7	68±5·4	39±4·7	100±5·9
Intact	10	Glycine (1 mM, 0·08 μc/ml.)	4	0·96±0·06	1·22±0·10	1·26±0·07	1·84±0·14	37±2·7	56±4·0	42±2·7	79±5·5

Table 6. *Effect of various neuromuscular agents on the accumulation and incorporation of [^{14}C]glycine by denervated rat diaphragm*

In Expts. 1–4 buffer contained only half the normal Ca^{2+} content and incubation was for 1 hr. [^{14}C]Glycine was added at a concentration of 1 mM and a specific activity of about 0·08 μc/ml. Succinylcholine, acetylcholine, curare and Tensilon (edrophonium chloride) were all added to a concentration of 10 p.p.m., insulin to 0·1 unit/ml.

Expt. no.	Additions to the medium	No. of observations	Radioactivity in tissue (counts/min./g.) / Radioactivity of medium (counts/min./ml.)		Radioactivity in diaphragm protein (counts/min./mg.)	
			Denervated	Control	Denervated	Control
1	No addition	3	1·25±0·07	1·02±0·06	21·3±1·1	15·7±1·3
	Acetylcholine+Tensilon	4	0·99±0·07	1·15±0·09	15·2±1·0	15·9±1·0
	Succinylcholine	3	0·92±0·02	1·09±0·13	16·3±1·3	15·4±2·2
2	Succinylcholine	6	1·15±0·04	1·23±0·05	20·9±0·9	20·6±4·2
	Succinylcholine+insulin	6	1·22±0·04	1·66±0·14	25·0±2·1	27·4±3·9
3	Succinylcholine	6	1·31±0·06	0·87±0·05	28·3±2·9	15·5±0·6
	Succinylcholine+insulin	6	1·52±0·11	1·43±0·08	30·5±3·7	26·0±1·9
4	Succinylcholine	6	1·38±0·07	1·00±0·04	23·2±1·0	15·2±1·6
	Succinylcholine+insulin	6	1·42±0·06	1·30±0·09	23·0±1·2	18·5±1·3
5	No addition	7	1·81±0·20	1·30±0·06	50±8·6	32±3·6
	Curare	7	1·67±0·13	1·35±0·09	45±4·6	35±5·5
	Curare+insulin	7	2·31±0·08	2·17±0·23	58±4·9	54±5·7

presented in Table 7 gives $P < 0·01$. Cocaine did not prevent stimulation of uptake of insulin. The presence of mepyramine (1 mM) also inhibited uptake of glycine. No clear effects of either mepyramine or cocaine on the incorporation of glycine into protein were seen (Table 7). As Kostyo (1964) pointed out, changes in the rate of accumulation are not always reflected in the amount of incorporation.

In parallel with many of the above experiments, we have studied the accumulation and incorporation into protein of [^{14}C]glycine by extensor digitorum longus muscle. The inhibitory effect of mepyramine on accumulation is again seen (Table 8), though any effect on incorporation is variable. With neither diaphragm nor extensor muscle was there any consistent effect on K^+ concentrations in the tissue. Chlorpromazine, tetracaine (*p*-butylaminobenzoyl-2-dimethylaminoethanol hydrochloride), Phenergan (promethazine) and phenolphthalein all appeared to be inhibitory, both with respect to accumulation and incorporation of glycine and to maintenance of K^+. Tetrodotoxin, isoprenaline and Ba^{2+} at the concentrations used had no observable effect.

One of the expected virtues of the extensor digitorum preparation is that, being an intact tissue, it will, like the intact diaphragm preparation, retain its internal K^+. However, for reasons that are unknown, in different experiments the extensor muscle retained its K^+ to very varying degrees, as shown in the controls in Table 8. A similar phenomenon was observed by Manery, Gourley & Fisher (1956). Addition of albumin (0·2–4%) did not influence the retention of K^+. The extracellular space *in vivo*, as far as we know, has not been measured. Sorbitol and inulin spaces *in vitro* fluctuated between 24 and 48% of the wet weight, and varied inversely with the tissue K^+ content. Probably low tissue K^+ concentration in the controls indicates a high extracellular space *in vitro*.

DISCUSSION

Effect of denervation. Our finding that the capacity of muscle to incorporate amino acids is elevated shortly after denervation is similar to that of Ferdman (1963) and Buse *et al.* (1965). Buse *et al.* (1965) showed that incorporation of [^{14}C]leucine was accelerated after denervation. Our results (Table 5) show similar changes with [^{14}C]glycine and [^{14}C]phenylalanine, and similar changes would probably be seen with most of the amino acids. Buse *et al.* (1965) used the isolated hemidiaphragm preparation; most of our results are with the intact preparation, but the effect of denervation is (as indeed are most of the responses of diaphragm) qualitatively similar in either preparation. Like Buse *et al.* (1965), we find that 10 days after denervation there is no longer an enhancement of incorporation, and this would probably fit with the fact that the hypertrophy of the tissue, so marked after

Table 7. *Effect of various drugs on the accumulation and incorporation of* [^{14}C]*glycine into protein by the intact rat diaphragm*

Each value is the mean ±S.E.M. of four results. Insulin when present was at 0·1 unit/ml. Radioactivity was added to a concn. of about 0·125 μc/ml.

Diaphragm preparation	Concn. of glycine (μM)	Addition to the medium	Concn. of K^+ in tissue at end of incubation (μequiv./g. wet wt.)		Radioactivity in tissue (counts/min./g.)		Radioactivity in diaphragm protein (counts/min./mg.)	
			Insulin absent	Insulin present	Radioactivity in medium (counts/min./ml.)			
					Insulin absent	Insulin present	Insulin absent	Insulin present
Intact	1000	No addition	72	76	1·24±0·11	2·20±0·17	55±5·9	86±9·4
		Cocaine (1 mM)	77	80	1·08±0·07	1·82±0·16	46±4·7	99±9·5
Hemidiaphragm	16	No addition	64	—	2·19±0·17	—	102±14	—
		Cocaine (1 mM)	60	—	1·58±0·12	—	81±9·7	—
	1000	No addition	59	—	1·53±0·13	—	39±1·5	—
		Cocaine (1 mM)	57	—	1·24±0·11	—	37±1·5	—
Intact	1000	No addition	81	81	1·50±0·04	2·15±0·08	30±4·0	42±2·4
		Mepyramine (1 mM)	83	80	1·31±0·03	1·55±0·04	36±1·9	42±2·6

3 days, is by 10 days slackening off. The enhancement of incorporation can therefore be related to the rate of hypertrophy of the tissue, except that Buse *et al.* (1965) found a return of the enhanced rate of incorporation 5–6 weeks after denervation, by which stage the tissue had atrophied considerably.

Buse *et al.* (1965) also found that the accumulation of leucine in the tissue pool was not increased by denervation, but that the accumulation of aminoisobutyrate was. In our experiments (Table 5) the accumulation of glycine rises after denervation, whereas that of phenylalanine is unchanged. There is a striking similarity between the above results of denervation and the response of diaphragm to insulin. Thus changes in the accumulation of both glycine and aminoisobutyrate are seen in the presence of insulin (Kipnis & Noall, 1958; Manchester & Young, 1960) and after denervation, whereas the accumulation of phenylalanine and leucine is not affected in either case (Manchester & Young, 1960; Table 5). But both denervation and insulin stimulate the incorporation into protein of all the amino acids studied. The possible significance of these differences in response are discussed below.

Like Buse *et al.* (1965) we find that, at 3 days after denervation, when the elevation of incorporation is most marked, there is no response to addition of insulin. Buse & Buse (1959, 1961) found that denervated tissue did not respond to stimulating effects of insulin on uptake of glucose or accumulation of xylose, but there was the marked difference in their observations from the present situation in that the glucose uptake and xylose penetration remained at the low non-insulin control values instead of being, as we have found with amino acid accumulation and incorporation, already elevated. The reason for this loss of response to insulin, and its at least partial reappearance at later stages (Table 5), is not known. The possibility that the tissue might have become hypersensitive to the hormone and so be already fully activated by insulin *in vivo* before removal, or that other factors might limit the maximum possible rate of incorporation and so prevent a response to insulin, would appear to be ruled out by the low basal glucose uptake and xylose accumulation of denervated tissue. Buse & Buse (1959) considered the possibilities that the diminished response to insulin of the paralysed muscle could be due either to lack of activity or denervation. Park & Johnson (1955) found rhythmically contracting muscle to be in some ways more responsive to insulin than gastrocnemius, possibly because of better supply of substrates. However, we find extensor muscle to respond readily to the hormone (Table 8 and unpublished work). Miledi (1962) has shown that

Table 8. *Effect of various drugs on the accumulation and incorporation of* [^{14}C]*glycine into protein by rat extensor digitorum longus muscle*

Radioactivity was added in Expts. 1 and 3 to about 0·25 μc/ml. and in Expts. 2, 4 and 5 to about 0·14 μc/ml.

Expt. no.	Concn. of [^{14}C]glycine (μM)	Additions to the medium	No. of observations	Concn. of K+ in tissue at end of incubation (μequiv./g. wet wt.)	Radioactivity in tissue (counts/min./g.) / Radioactivity of medium (counts/min./ml.)	Radioactivity in diaphragm protein (counts/min./mg.)
1	1000	No addition	4	44	1·16±0·03	14·7±0·56
		Insulin (0·1 unit/ml.)		42	1·34±0·08	24·5±3·4
		Mepyramine (1 mM)		45	1·06±0·04	15·7±1·47
		Mepyramine+insulin		37	1·13±0·05	22·5±1·33
2	24	No addition	6	69	1·66±0·07	58·2±2·9
		Mepyramine (1 mM)		52	1·14±0·10	31·0±2·3
	1000	No addition		63	1·32±0·08	24·0±0·5
		Mepyramine (1 mM)		65	1·04±0·07	15·0±0·8
3	32	No addition	4	64	1·67±0·07	103±9·3
		Tetracaine (1 mM)		45	0·89±0·06	25±3·2
		Phenergan (1 mM)		21	0·68±0·01	3±0·5
		Tetrodotoxin (0·4 μg./ml.)		65	1·94±0·10	118±9·6
4	24	No addition	6	48	1·40±0·07	66±6·8
		Isoprenaline (1 mM)		59	1·54±0·06	46±5·6
		Phenolphthalein (0·13 mM)		33	1·25±0·14	24±4·8
5	24	No addition	6	65	1·87±0·15	124±2·5
		Chlorpromazine (1 mM)		12	0·84±0·01	3·9±0·7
		Ba^{2+} (5 mM)		63	1·37±0·04	115±4·7

after denervation the cell membrane becomes increasingly sensitive to neuromuscular transmitter agents and it seems likely that there are modifications in the structure of the membrane that lie at the root of the changed responsiveness of the tissue to insulin. We tried therefore to reverse the effect of denervation by blocking the sensitivity to acetylcholine with curare or to see whether contractile and electrical activity induced by acetyl- and succinyl-choline would lead to a more normal response to insulin, but without any obvious success (Table 6).

Effect of K+ *ions.* The results of Tables 2 and 3 demonstrate that deprivation of K+ can decrease the accumulation and incorporation into protein of some amino acids, leaving leucine unchanged. Again it is the amino acids (glycine and amino-isobutyrate) whose accumulation by muscle can be influenced by insulin that are influenced by the concentration of K+, whereas the accumulation of leucine is unaffected by both factors. Since insulin and denervation affect the capacity of muscle to incorporate leucine into protein, whereas K+ does not, K+ appears to affect primarily the accumulation of the responsive amino acids, any effect on incorporation possibly arising indirectly. We thought that the inhibitory influence of 20 mM-K+, which also occurs on uptake of histidine by brain slices

Table 9. *ATP content of isolated hemidiaphragms after incubation in buffers of various* K+ *composition*

Each value is the mean ±S.E.M. of eight results. Each hemidiaphragm was first incubated for 30 min. in 10 ml. of the appropriate buffer and then transferred to 1 ml. of fresh medium and incubation continued for a further 2 hr., as in Table 3.

Concn. of K+ in medium (mM)	ATP in muscle (μmoles/g. wet wt.)
0	2·19±0·10
5	2·23±0·08
20	2·08±0·11

(de Almeida, Chain & Pocchiari, 1965), could result from an increased demand for ATP to maintain a raised metabolism (Hill & Howarth, 1957), leaving less ATP as an energy source for the accumulation of amino acid. However, this concentration of K+ did not increase the oxygen consumption of hemidiaphragms (unpublished work) or affect the ATP content of the tissue (Table 9). Whether the crucial factor with the low-K+ solution is the low external K+ concentration or the decreased intracellular value is uncertain, though Kuchler & Marlowe-Kuchler (1965) found that extracellular

K^+ is required for aminoisobutyrate uptake by mouse fibroblast cells. The deficiency is, however, speedily corrected on restoration of the extracellular ion (Table 2).

Any attempt to link the concentrations or movements of K^+ and Na^+ in muscle with the accumulation of amino acids must contend with the observation of Kostyo & Schmidt (1963) that ouabain can change the tissue concentrations of these ions in conditions under which uptake of amino acids is not affected. However, the gross flux of Na^+ is not necessarily decreased, for although we may have a lower rate of turnover of the whole pool, the combination of a lower rate of turnover with a larger pool of Na^+ could still lead to the same value for the total flux. A coupling of amino acid movement with Na^+ entry (Vidaver, 1964) would explain the greater uptake of amino acid by the hemidiaphragm than by the intact preparation (Manchester, 1966) and would meet the requirement for an ouabain-insensitive step.

The suggestion that intracellular K^+ concentration controls the glycine uptake of ascites cells (Riggs, Walker & Christensen, 1958) was not confirmed by Heinz (1962). In the present example the low intracellular K^+ concentration of the damaged preparation favours amino acid uptake (Manchester, 1966, and Table 3). Such observations might be explicable if amino acid uptake is linked to K^+ turnover. Highly permeable cells readily accumulate glycine and aminoisobutyrate; but drugs that decrease the permeability of muscle to K^+, and hence decrease K^+ flux, also lessen amino acid transfer. This idea has, however, to meet the case of the increased uptake of amino acid found in denervated tissue, which has a decreased K^+ permeability. A further assumption has to be made, e.g. that the activity of the ribosomes of the denervated tissue has been increased.

The entry of labelled amino acid into intracellular water of a tissue *in vitro* can obviously occur as the result of several possible processes. If the external concentration of added amino acid is greater than the intracellular concentration and the membrane is permeable, then passive diffusion will take place. When the concentration of added amino acid is much less than that in the cell, net uptake of free amino acid presumably occurs as the result of some active process, which the evidence suggests is linked with operation of the Na^+ pump. However, accumulation of radioactivity in the tissue could result either from exchanges between labelled molecules derived from the extracellular medium and unlabelled molecules derived from the cell ('exchange diffusion'), or as a result of a mixing of the internal labelled and unlabelled pools due to mutual diffusion. In practice, entry of a labelled amino acid into the cell probably results from a combination of several processes, and it is difficult to distinguish experimentally between these possibilities. The studies of Christensen *et al.* (1962) and Oxender & Christensen (1963) suggested that the uptake of labelled leucine and phenylalanine by the ascites-tumour cell is largely by exchange reactions in distinction to uptake of labelled glycine and aminoisobutyrate, which are active processes. The accumulation characteristics of these respective amino acids in the perfused heart are consistent with the same distinction (Manchester & Wool, 1963). Our results can thus be put into two classes in a similar way; the effects of K^+ concentration and denervation are only seen on uptake of those acids (e.g. glycine and aminoisobutyric acid) whose accumulation is related to an active process. Where incorporation is affected without a change in accumulation, as seen with leucine after denervation, it is necessary to assume either 'compartmentation' or a slow equilibration of molecules in different parts of the pool (Kipnis, Reiss & Helmreich, 1961), or a change in the basal rate of protein synthesis.

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