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THE EFFECTS OF ADRENALINE AND OTHER DRUGS AFFECTING CARBOHYDRATE METABOLISM ON CONTRACTIONS OF THE RAT DIAPHRAGM

BY

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The ability of several drugs to restore directly elicited twitches of the rat diaphragm depressed by excess potassium chloride has been studied. The drugs found to be effective were sympathomimetic amines, insulin, glucagon, caffeine, theophylline, calcium chloride and hexosephosphates. The effects of the sympathomimetic amines and glucagon were blocked by β-receptor blocking agents. Phloridzin blocked the effect of insulin and depressed that of glucagon. The increase in twitch tension still occurred under anaerobic conditions and was not abolished by the glycolytic inhibitor, iodoacetate. All of the effective drugs are known to affect carbohydrate metabolism and the suggestion by Ellis (1955) that the effect on contractions may be a result of increased intracellular hexosephosphate levels is discussed.

Adrenaline and some other sympathomimetic amines have long been known to cause an increase in the contractions of fast-contracting skeletal muscles, stimulated directly or through their motor nerves (see Bowman, Goldberg & Raper, 1962, for references). The mechanism underlying this potentiation is unknown, but Ellis, Davis & Anderson (1955) demonstrated that the relative potencies of a series of sympathomimetic amines in potentiating the contractions of the isolated diaphragm muscle of the rat and in stimulating glycogenolysis in this tissue were similar, suggesting that the two effects might be interrelated. Ellis & Beckett (1954) had previously shown on the same tissue that adrenaline retained its twitch-potentiating effect under anaerobic conditions and in the presence of the glycolytic inhibitor, iodoacetate. These results indicated that the action of adrenaline on the muscle contractions was dependent neither on oxidative metabolism, nor on the energy-yielding steps of the Embden-Myerhof pathway which, according to Bloom, Stetten & Stetten (1953), is the main pathway for carbohydrate metabolism in this tissue. Ellis (1955) therefore concluded that if the effect of adrenaline on contractions is related to stimulation of glycogenolysis, it must depend on the stage in which the intracellular content of hexosephosphates is increased. Ellis (1955) then showed that the addition of glucose-6-phosphate and fructose-1,6-diphosphate to the fluid bathing the isolated diaphragm muscle produced an increase in twitch tension like that produced by adrenaline, but glycero-β-phosphate did not. The suggestion was made by Ellis (1959) that the effect of adrenaline on the contractions might be the

result of a change in the physicochemical environment of the contractile system brought about by a change in the cellular content of hexosephosphates.

In the experiments described in this paper this suggestion has been further tested by comparing the effects of sympathomimetic amines with those of a number of other drugs known to influence carbohydrate metabolism. The experiments have been carried out on isolated diaphragm preparations of rats, in most cases depressed by excess potassium chloride, since in this condition the muscle is very sensitive to the potentiating action of adrenaline (Knox, McDowall & Montagu, 1951).

METHODS

Phrenic nerve–hemidiaphragm preparations from random-bred Wistar rats weighing 200 to 300 g were set up in a 50 ml. organ-bath containing Krebs-Henseleit solution at 32° C, according to the method of Bülbring (1946). The composition of the Krebs-Henseleit solution was as follows (in g/l.): NaCl 6.95 ; KCl 0.34 ; $CaCl_2$ 0.28 ; KH_2PO_4 0.162 ; $MgSO_4$ 0.294 ; $NaHCO_3$ 2.1 ; and dextrose 2. In most experiments the mixture was continually gassed with 95% oxygen and 5% carbon dioxide, but when anaerobic conditions were required the oxygen was replaced by nitrogen. Each preparation was initially stimulated via the phrenic nerve with rectangular pulses of 100 μsec duration and of three or four times the strength necessary to produce a maximal twitch. Tubocurarine, in a concentration sufficient to block neuromuscular transmission completely (6 μg/ml.) was then added to the reservoir of Krebs-Henseleit solution and the muscle was stimulated directly for the remainder of the experiment. Direct stimulation was applied between two silver pins embedded in the muscle near its origin in the ribs. The pins were also used to anchor the diaphragm to the electrode block. Twitches were elicited by stimulation at a frequency of 6 shocks/min with rectangular pulses of 1 msec duration and of a strength to produce contractions equal in amplitude to, or slightly greater than, maximal twitches produced by indirect stimulation. Stronger direct stimuli often produced greater contractions, probably because repetitive responses were elicited in an increasing number of muscle fibres. The electrodes on the phrenic nerve were left in position and indirect stimulation was occasionally applied throughout the experiment to check that curarization was complete. Contractions of the muscle were recorded on smoked paper by attaching the central tendon to a spring-loaded lever.

Before studying the effects of drugs, the contractions of the diaphragm were usually partially depressed by the addition of 0.4 to 0.6 ml. of a 5% solution of potassium chloride. This produced an initial potentiation of the twitches followed by a slowly developing depression (Hajdu & McDowall, 1949). The drug being tested was added when the twitches were depressed by 50 to 90%. After observing the effect of the drug, the bath fluid was changed two or three times. Normal contractions were then recorded for 10 to 15 min before a further depression was produced with potassium chloride solution and the effects of the same or of another drug were determined. In preparations used to study the effects of anoxia or of treatment with sodium iodoacetate, the optimal conditions outlined by Ellis & Beckett (1954) were used ; the bath temperature was lowered to 27° C and the glucose concentration of the bath fluid was increased from 0.2 to 0.38%. Anaerobic conditions were established by stimulating the preparation at a frequency of 6 shocks/min while the bath fluid was gassed with 95% nitrogen and 5% carbon dioxide for at least 30 min before any drugs were added to the bath.

The drugs used were: (−)-adrenaline (B.D.H.), (−)-noradrenaline bitartrate (Light & Co.), (−)-isoprenaline bitartrate (Wyeth), (±)-noradrenaline hydrochloride (Sterling Winthrop), (±)-isoprenaline sulphate (Bayer), (+)-adrenaline (Light & Co.), (+)-noradrenaline bitartrate (Sterling Winthrop), (±)-*N*-ethylnoradrenaline hydrochloride (Sterling Winthrop), dopamine hydrochloride (Light & Co.), *N*-isopropyldopamine hydrochloride (Sterling Winthrop), tyramine hydrochloride (Light & Co.), phenylethylamine (Winthrop), (−)-ephedrine hydrochloride (Light & Co.), (±)-amphetamine sulphate (Light & Co.), dichloroisoprenaline hydrochloride (Lilly), phentolamine (Ciba), pronethalol (I.C.I.), isopropylmethoxamine (Burroughs Wellcome,

61-43), methoxamine hydrochloride (B.W. & Co.), phloridzin (Light & Co.), caffeine (B.D.H.), theophylline (B.D.H.), glucagon (Lilly), insulin (Burroughs Wellcome), disodium glucose-6-phosphate (Light & Co.), disodium glucose-1-phosphate (Light & Co.), monosodium fructose-1, 6-diphosphate (Light & Co.), L-thyroxine sodium (Light & Co.), 5-hydroxytryptamine creatinine sulphate (May & Baker), hydroxy-L-proline (Light & Co.) and L-lysine (Light & Co.).

The doses of the sympathomimetic amines and of 5-hydroxytryptamine refer to the base. Doses of all other compounds refer either to the base or to the salt as indicated in the above list. Caffeine and theophylline were dissolved in 0.1 N-hydrochloric acid, and phloridzin was made up as a suspension. The phloridzin dissolved completely when added to the bath fluid. In all experiments control injections of the diluent alone were completely without effect.

RESULTS

Adrenaline is known to exert two independent effects on skeletal muscle contractions, one on the neuromuscular junction and one on the muscle fibres themselves (Bowman *et al.*, 1962). These experiments were concerned with the latter action and, to avoid the complications of simultaneous actions at the neuromuscular junction, all drugs were studied during direct stimulation of the fully curarized preparation. Both potassium chloride and adrenaline exert anticurare actions (Wilson & Wright, 1936) and, since the effects of the drugs were studied in the presence of excess potassium chloride, it was necessary to ascertain that neuromuscular transmission remained blocked throughout. Fig. 1 illustrates an initial

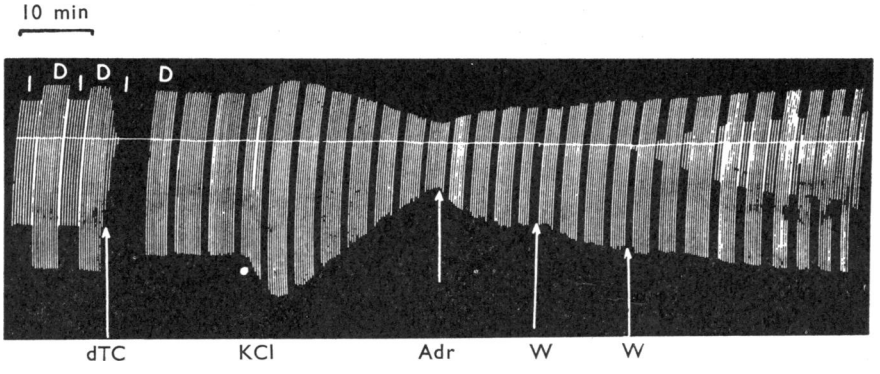

Fig. 1. Twitches of the diaphragm elicited once every 10 sec. by alternate periods of indirect (I) and direct (D) stimulation. Contractions downwards. At dTC, tubocurarine (6 μg/ml.), at KCl, potassium chloride (0·5 mg/ml.) and at Adr, (—)-adrenaline (20 ng/ml.) were added. At W, the bath was washed three times with normal Krebs-Henseleit solution. Tubocurarine completely abolished the responses to indirect stimulation and these were not restored by potassium chloride or by adrenaline. Twitches in response to indirect stimulation reappeared only after the second washing period. Time calibration in this and the remaining Figures: 10 min.

experiment in which this was done. Throughout this experiment alternate periods of direct and indirect stimulation were applied. Tubocurarine (6 μg/ml.) selectively and completely abolished the contractions elicited by indirect stimulation and these remained blocked in spite of the subsequent addition of potassium chloride and adrenaline, showing that the amount of tubocurarine used was too great to be antagonized by these agents. In all the remaining experiments, indirect stimulation was occasionally applied throughout the experiment and was found to be without

effect. It can therefore be concluded that the effects described are independent of events occurring at the neuromuscular junction.

Other workers (Brown, Bülbring & Burns, 1948 ; Goffart, 1952) have shown, and we have confirmed, that adrenaline potentiates the directly elicited twitches of the unfatigued diaphragm muscle in the absence of depression by potassium chloride. However, under these conditions the effect is small and requires relatively large doses (approximately 1 μg/ml. of bath fluid). In order to obtain some quantitative comparison of the effects of the drugs it was therefore decided to use the potassium-depressed preparation in which the effect of adrenaline is very much greater and is produced by very small concentrations. The following results indicated that the effect on the sensitive potassium-depressed preparation was an exaggeration of the same effect as that occurring in the absence of excess potassium chloride. (1) The order of potency of adrenaline, noradrenaline and isoprenaline was the same in both potassium-depressed and normal diaphragms, (−)-isoprenaline being slightly more potent than (−)-adrenaline, and (−)-noradrenaline weaker. (2) The twitch-potentiating action of the amines in both preparations was unaffected by phentolamine in a concentration of 6 μg/ml. (3) The effect of the amines in both preparations was abolished by the previous addition of pronethalol (1 μg/ml.) or dichloroisoprenaline (1 μg/ml.). Similar results on the directly stimulated chronically-denervated diaphragm have recently been reported by Paterson (1963), and the amines exhibit the same order of potency and susceptibility to the blocking agents in their direct actions on skeletal muscles of the cat (Bowman *et al.*, 1962).

Adrenaline still exerted its twitch-potentiating action both when the bath fluid was gassed with nitrogen and carbon dioxide (Fig. 2), and also under aerobic conditions in the presence of 33 μg/ml. of sodium iodoacetate (Fig. 2). These results confirm those of Ellis & Beckett (1954). When the bath fluid was gassed with nitrogen and carbon dioxide in the presence of iodoacetate, the muscle rapidly went into rigor

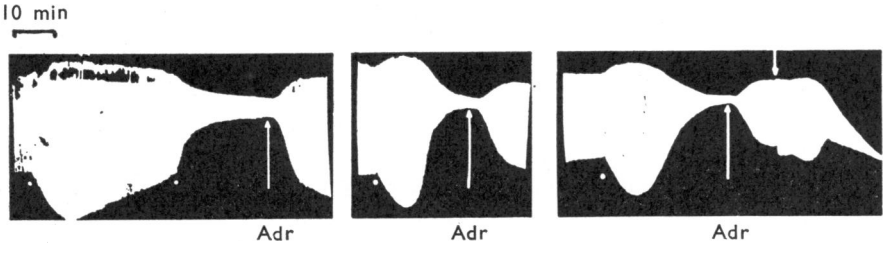

Fig. 2. Twitches of a fully-curarized diaphragm elicited by direct stimulation once every 10 sec. (Drum speed much slower than for Fig. 1.) At the white dots, potassium chloride was added to the bath and at Adr, (—)-adrenaline, to make a final concentration of 13 ng/ml., was added. The first panel shows the response to adrenaline under aerobic conditions. The middle panel shows the response to adrenaline under anaerobic conditions after bubbling the bath solution for 30 min with 95% nitrogen and 5% carbon dioxide. The third panel shows the response to adrenaline after aerobic conditions (95% oxygen + 5% carbon dioxide) had been restored but in the presence of sodium iodoacetate (1 in 30,000). The upper arrow in the third panel shows the effect of replacing the oxygen by nitrogen in the presence of iodoacetate. After an initial potentiation of the twitches the diaphragm went into rigor. Note that in the first two panels of this Figure and in all the remaining Figures the records are terminated at the time when the drug under test was washed out of the bath.

(Fig. 2), indicating that sufficient iodoacetate was present to block the glycolytic chain (Ellis & Beckett, 1954).

Twitch-potentiating agents

In addition to adrenaline, the substances listed in Table 1 were tested, each on between eight and thirty preparations, and found to be effective in increasing the tension of the potassium-depressed twitches. Table 1 gives the effective concen-

TABLE 1

EFFECTIVE CONCENTRATIONS OF TWITCH-POTENTIATING AGENTS AND THE TIMES OF ONSET OF THEIR ACTIONS

The concentrations are those required in the organ-baths to produce approximately 100% potentiation of the potassium-depressed twitches. The times of onset are the times from addition of the drug to the first detectable reversal of the potassium-depression.

Drug	Concentration	Time of Onset (min.)
(—)-Adrenaline	3–10 ng/ml.	0·5–3
(—)-Noradrenaline	80–400 ng/ml.	0·75–3
(—)-Isoprenaline	2–10 ng/ml.	0·5–2
(±)-Noradrenaline	200–800 ng/ml.	0·75–3·5
(±)-Isoprenaline	3–10 ng/ml.	0·5–2
(+)-Adrenaline	10–20 μg/ml.	1–3
(+)-Noradrenaline	10–20 μg/ml.	1–3·5
(±)-Ethylnoradrenaline	200–400 ng/ml.	0·5–2
Dopamine	2–4 μg/ml.	1–3·5
N-Isopropyldopamine	0·4–2 μg/ml.	1–2
Tyramine	40–200 μg/ml.	1–6
(—)-Ephedrine	10–30 μg/ml.	1–3
Glucose-6-phosphate	0·4–0·8 mg/ml.	2–3·5
Glucose-1-phosphate	0·4–0·8 mg/ml.	1·5–3
Fructose-1,6-diphosphate	0·4–0·8 mg/ml.	1–3
Caffeine	0·2–0·8 mg/ml.	1·5–2
Theophylline	0·4–1 mg/ml.	0·5–1·5
Calcium chloride	0·2–0·6 mg/ml.	1–3
Insulin	0·15–0·40 units/ml.	1·5–7
Glucagon	2–4 μg/ml.	1–6

trations of these substances and Figs. 3 and 4 illustrate their effects under aerobic conditions. Although the hexosephosphates were active, glucose (0.5 mg/ml.), sodium dihydrogen phosphate and disodium hydrogen phosphate (0.5 mg/ml.) were without effect. In the absence of an effective drug, the depressant action of potassium chloride eventually led to permanent abolition of the contractions (first panel of Fig. 3). Table 1 gives the range of times of onset of the effects for the concentrations shown. The higher the concentration of drug, the shorter the time of onset and the quicker the rate of increase in twitch tension. However, even with the largest concentrations used, the time of onset for all drugs was never less than 0.5 min.

With the exception of glucagon and the hexosephosphates, all the drugs were active in all experiments in which they were studied. Glucagon was ineffective in six out of twenty-eight experiments. Seven different samples of glucagon were used in the experiments and the preparations in which it was found inactive were all treated with the same sample. Unfortunately, owing to the smallness of the samples, the inactive one was exhausted before it could be compared with a different

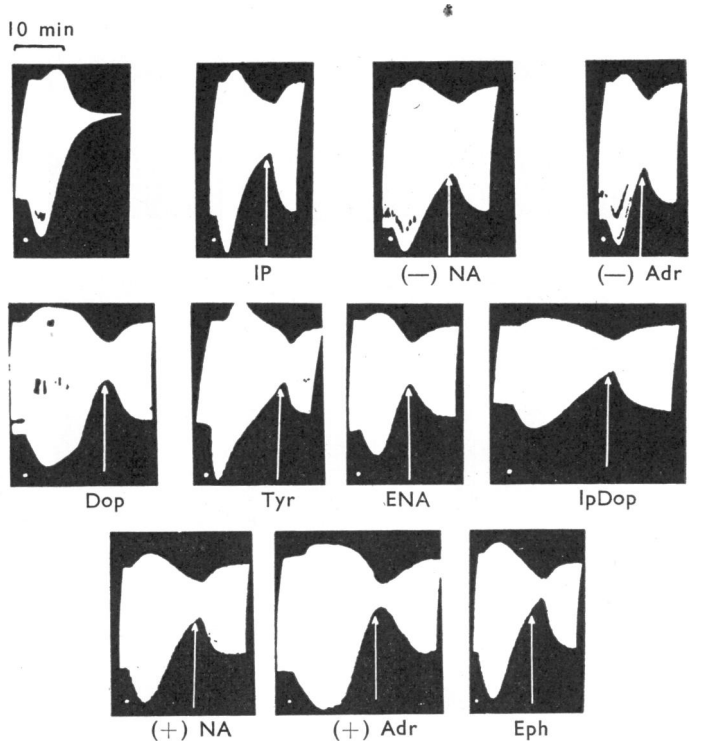

Fig. 3. Recordings as in Fig. 2 but aerobic conditions throughout. The responses in the top row are from the same preparation; each of the remaining responses is from a different preparation. The first panel shows the complete abolition of contractions which occurred when potassium chloride alone was added to the bath. At the arrows, the following drugs were added to give the final bath concentrations indicated: (—)-isoprenaline (IP, 20 ng/ml.), (—)-noradrenaline ((—) NA, 0·4 μg/ml.), (—)-adrenaline ((—) Adr, 20 ng/ml.), dopamine (Dop, 10 μg/ml.), tyramine, (Tyr, 100 μg/ml.), (±)-*N*-ethylnoradrenaline (ENA, 0·6 μg/ml.), *N*-isopropyldopamine (IpDop, 1 μg/ml.), (+)-noradrenaline, (+) NA, 20 μg/ml.), (+)-adrenaline ((+) Adr, 20 μg/ml), and (—)-ephedrine (Eph 20 μg/ml).

sample on the same preparation. However, since the rats used were of the same strain throughout, the inactivity of the glucagon sample appeared to be due to a difference in the sample rather than in the experimental conditions. The same samples of hexosephosphates were inactive in four out of twenty experiments in which they were tested.

The approximate relative potencies of the sympathomimetic amines used can be seen from comparisons of their effective concentrations in Table 1. More accurate comparisons between two or three closely related amines were obtained by determining equipotent concentrations in the same preparation. These results gave the following estimates of relative potency on a weight basis. (1) (−)-Adrenaline and (−)-noradrenaline were 500- to 1,000-times more potent than the respective (+)-isomers. Concentrations of the (+)-isomers sufficient to give maximal responses (about 20 μg/ml.) temporarily reduced the responses to subsequent additions of the (−)-isomers. Similarly, very large concentrations of the (−)-isomer (20 μg/ml.)

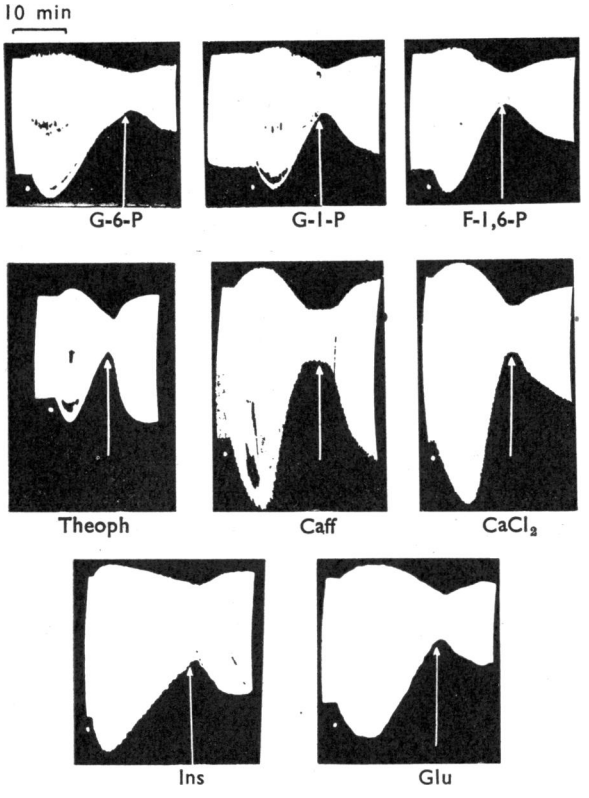

Fig. 4. Recordings as in Fig. 3. The responses in the top row are from the same preparation; each of the remaining responses is from a different preparation. At the arrows the following drugs were added to give the final bath concentrations indicated : glucose-6-phosphate (G-6-P, 0·5 mg/ml.), glucose-1-phosphate (G-1-P, 0·5 mg/ml.), fructose-1,6-diphosphate (F-1,6-P, 0·5 mg/ml.), theophylline (Theoph, 0·4 mg/ml.), caffeine (Caff, 0·4 mg/ml.), calcium chloride ($CaCl_2$, 0·3 mg/ml.), insulin (Ins, 0·2 U/ml.) and glucagon (Glu, 2 μg/ml.).

reduced the responses to previously effective small concentrations of the same isomer. (2) (−)-Noradrenaline was 20- to 25-times more active than dopamine. (3) (−)-Isoprenaline was 1.5- to 2-times more active than (−)-adrenaline. (4) (−)-Adrenaline was about 10-times more active than (−)-noradrenaline. (5) Isopropyldopamine was 5- to 8-times more active than dopamine. (6) (±)-Ethylnoradrenaline was 3- to 5-times more active than (±)-noradrenaline. (7) Dopamine was 10- to 20-times more active than tyramine. (8) Phenylethylamine and (±)-amphetamine were completely inactive in all concentrations used (up to 0.6 mg/ml.).

Tetanic stimulation of skeletal muscle temporarily augments the contractile responses to subsequent single shocks, and there are some similarities between the effects of a tetanus and the effects of adrenaline on the twitches of skeletal muscles (Ellis, 1955 ; Bowman *et al.*, 1962). A tetanus was found not to increase twitch tension in the potassium-depressed diaphragm, possibly because the potassium

released from the tetanized muscle added to the excess already present and masked the effect. The effects of direct tetanic stimulation were therefore studied in the curarized preparation suspended in normal Krebs-Henseleit solution. A tetanus also exerts a decurarizing action (Boyd, 1932) and frequent checks were therefore made to ascertain that neuromuscular transmission remained blocked.

The increase in twitch tension produced by a previous tetanus still occurred under anaerobic conditions (95% nitrogen and 5% carbon dioxide for 30 min) and under aerobic conditions in the presence of iodoacetate (33 μg/ml.). It was not abolished by pronethalol (1 μg/ml.) or by phloridzin (1.3 mg/ml.).

When changing from aerobic to anaerobic conditions the twitch tension of the diaphragm in normal Krebs-Henseleit solution was initially increased. This potentiating action of nitrogen was not seen in the diaphragm when the twitches were depressed with excess potassium.

Drugs antagonizing twitch-potentiating agents

The twitch-potentiating actions of the sympathomimetic amines were unaffected by the previous addition of phentolamine (6 μg/ml.) to the bath fluid (Fig. 5, *a*) but were abolished by the addition of 1 μg/ml. of pronethalol (Fig. 5, *b*), 1 μg/ml. of dichloroisoprenaline, 2.5 μg/ml. of *N*-isopropylmethoxamine (Fig. 5, *c*), or 3 μg/ml. of methoxamine. After dichloroisoprenaline and pronethalol, the responses to the sympathometic amines returned slowly after several washings (Fig. 5, *b*).

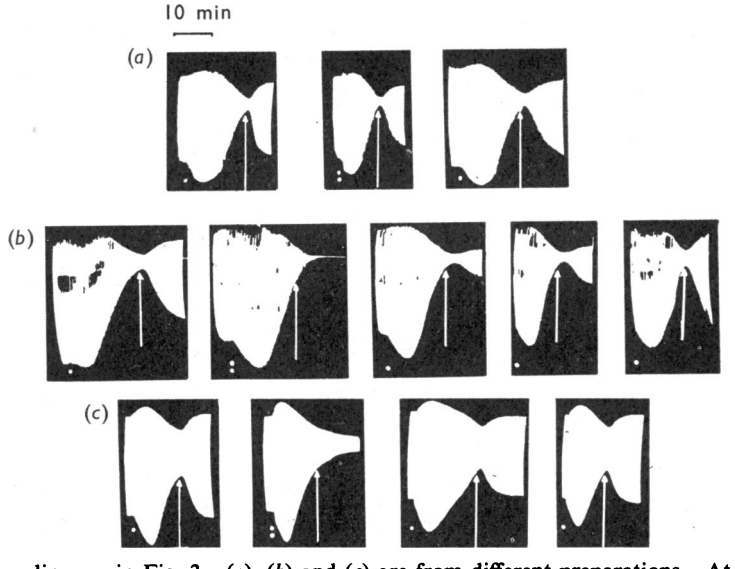

Fig. 5. Recordings as in Fig. 3. (*a*), (*b*) and (*c*) are from different preparations. At the arrows, (—)-adrenaline was added to give a final bath concentration in (*a*) of 6·6 ng/ml., in (*b*) of 3·3 ng/ml. and in (*c*) of 10 ng/ml. The second panel in each row shows the effect of adrenaline in the presence of an antiadrenaline drug which was added at the double white dots together with the potassium chloride. The antiadrenaline drugs are: in (*a*) phentolamine (6 μg/ml.), in (*b*) pronethalol (1 μg/ml.) and in (*c*) *N*-isopropylmethoxamine (2·6 μg/ml.). The preparations were washed once before each of the remaining panels.

However, with the concentrations of methoxamine and *N*-isopropylmethoxamine used, responses to the sympathomimetic amines returned after a single change of the bath fluid (Fig. 5, *c*). To demonstrate the antagonistic action of these drugs they were added to the bath fluid before or together with the potassium chloride. None of these drugs had an adrenaline-like action when added during the potassium-depression in the above concentrations.

Pronethalol was also tested for its ability to antagonize the other drugs mentioned in Table 1. In a concentration of 1 μg/ml. it abolished the twitch potentiating action of glucagon (Fig. 6) but was without effect on the responses to insulin (Fig. 7, *c*), hexosephosphates, calcium chloride, caffeine and theophylline.

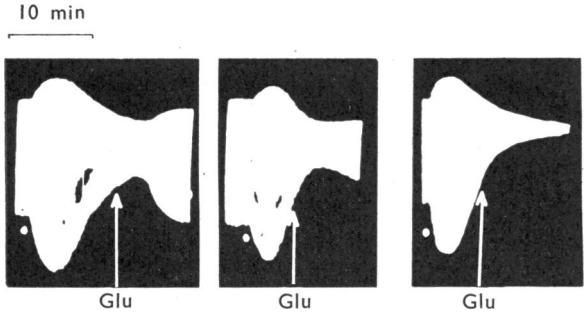

Fig. 6. Recordings as in Fig. 3. All records from the same experiment. At the arrows glucagon (Glu), to give a final concentration of 2 μg/ml., was added to the bath. The first panel shows a control response to glucagon. The middle panel shows the depressed response to glucagon in the presence of phloridzin (1·3 mg/ml.) which was added together with the potassium chloride. The third panel shows block of the response to glucagon in the presence of pronethalol (1 μg/ml.) added together with the potassium chloride. After washing out the phloridzin, the response to glucagon returned to the control level (not shown).

Responses to insulin became smaller with successive additions to the bath fluid. This tachyphylaxis to insulin always developed at similar rates in both hemidiaphragms of the same rat. When the effects of blocking drugs were studied, the contractions of both hemidiaphragms from the same rat were recorded simultaneously in separate baths. Insulin was added to both preparations but the blocking drug was added only to one. The twitch potentiating action of insulin was abolished by the previous addition of 1.3 mg/ml. of phloridzin to the bath fluid (Fig. 7, *b*). The same concentration of phloridzin reduced but did not abolish the action of glucagon (Fig. 6) and was without effect on the action of the remaining compounds listed in Table 1.

Insulin was without effect on the potassium-depressed twitches of diaphragms bathed in glucose-free Krebs-Henseleit solution (Fig. 7, *a*).

Factors augmenting the action of twitch-potentiating agents

In four out of five experiments, the addition of L-thyroxine (10 μg/ml.) to the bath fluid for 1 hr, augmented the twitch-potentiating action of adrenaline, thus confirming the results of de Visscher & Dijkmans (1950) who obtained a similar

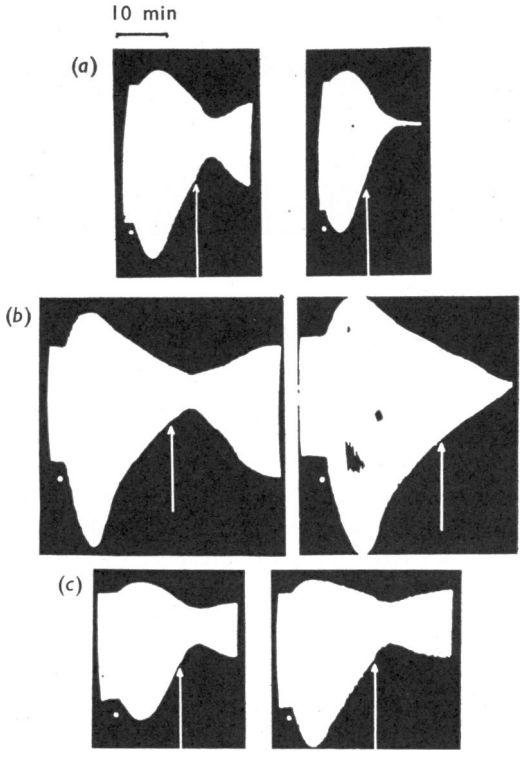

Fig. 7. Recordings as in Fig. 3. (*a*), (*b*) and (*c*) are from different preparations. Records on the left are from the left hemidiaphragm and those on the right are from the corresponding right hemidiaphragm made simultaneously. At the arrows in all cases, insulin was added to give a final bath concentration of 0·2 U/ml. The left-hand records show control responses. The right-hand records show, in (*a*) the absence of effect in glucose-free Krebs-Henseleit solution, in (*b*) the absence of effect in the presence of phloridzin (1·3 mg/ml.) and in (*c*) the effect in the presence of pronethalol (1 μg/ml.).

effect in rat diaphragms bathed in a medium containing a normal potassium concentration. The twitch potentiating action of (−)-adrenaline was potentiated by caffeine and by theophylline. Fig. 8 shows the potentiation of the response to a small dose of (−)-adrenaline produced by a subeffective dose of caffeine.

Inactive substances

A number of other substances known to exert some action on carbohydrate metabolism were tested and found to be inactive. These substances were 5-hydroxytryptamine (1 mg/ml.) (Leonard & Day, 1960), sodium fluoride (0.4 to 0.5 mg/ml.) (Sutherland & Rall, 1960), L-lysine (117 μg/ml.) and hydroxy-L-proline (131 μg/ml.). The two amino acids have been shown to be active in influencing carbohydrate metabolism in the diaphragm at 20° C (Borrebaek & Walaas, 1963), but they were found inactive on the contractions of the diaphragm both at 20 and at 32° C. Sodium fluoride was without effect itself and either abolished or depressed the twitch-potentiating action of adrenaline.

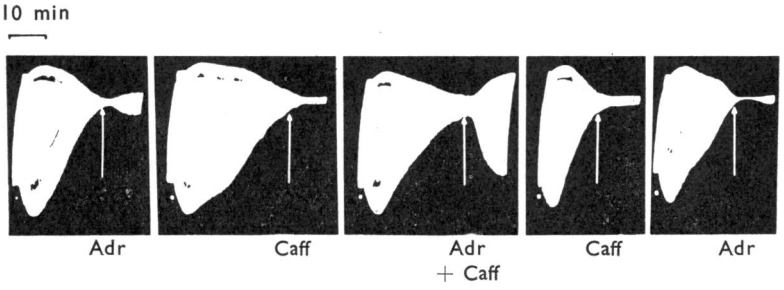

Fig. 8. Recordings as in Fig. 3. All responses from the same preparation. At Adr, adrenaline (final bath concentration 0·4 ng/ml.) and at Caff, caffeine (final bath concentration 0·2 mg/ml.) were added. The centre panel shows the effect of adding both drugs together.

The ions, magnesium and barium, were tested to see if they exerted an action like calcium. Barium chloride (up to 0.6 mg/ml.) was without effect and magnesium chloride (up to 0.6 mg/ml.) caused only a slight depression of the twitches like that often preceding the potentiating action of calcium chloride. The potentiating action of calcium chloride was not reduced in the presence of excess magnesium chloride (0.6 mg/ml.).

DISCUSSION

Of the sympathomimetic amines studied, isoprenaline was the most potent in reversing the potassium-induced depression of the muscle contractions. The effects of the amines were blocked by the β-receptor blocking agents, dichloroisoprenaline and pronethalol, and were unaffected by the α-receptor blocking agent, phentolamine. These results indicate that, like their actions on directly elicited contractions of non-fatigued muscles *in vivo* (Bowman *et al.*, 1962), the actions of the sympathomimetic amines in reversing the potassium-depression correspond, on Ahlquist's (1948) classification, to β-receptor effects. The fact that the (−)-isomers were most potent and that, of the directly acting amines studied, the order of potency resembled that on sympathetically innervated structures such as the heart, suggests that their mechanism of action on skeletal muscle contractions may be closely related to some of their sympathomimetic actions. Tyramine and ephedrine were relatively much less active than they are on sympathetically innervated structures, and β-phenylethylamine and amphetamine were inactive. However, it is now established that the action of these four amines is largely indirect through the release of noradrenaline (Burn & Rand, 1958, 1962). Their potency will therefore depend upon the amount of noradrenaline stored in the tissue and this is known to be very small in skeletal muscle.

The slow onset of action of the sympathomimetic amines in skeletal muscle, together with the duration of their effects, which persist *in vivo* long after the time that the amines themselves have been inactivated, suggests that a metabolic action is the underlying cause of their effects on contractions. All of the drugs found to reverse the potassium-depression also affect carbohydrate metabolism, and drugs known to antagonize or potentiate the effect on carbohydrate metabolism were seen to produce corresponding changes in the effect of the drug on contractions.

Thus, adrenaline and other sympathomimetic amines have been shown to stimulate glycogenolysis in skeletal muscle under both aerobic and anaerobic conditions. This effect is associated with accumulation of adenosine-3′,5′-phosphate and activation of phosphorylase, and results in increased levels of hexosephosphates (Hegnauer & Cori, 1934 ; Ellis, McGill & Anderson, 1957 ; Sutherland & Rall, 1960 ; Hornbrook & Brody, 1963). Furthermore, dichloroisoprenaline and pronethalol prevent the action of sympathomimetic amines on carbohydrate metabolism in the heart and skeletal muscle (Mayer, Moran & Fain, 1961 ; Murad, Chi, Rall & Sutherland, 1962 ; Hornbrook & Brody, 1963) as well as their action on potassium-depressed muscle contractions. Methoxamine and its *N*-isopropyl derivative were also found to abolish the effects of the amines on contractions. Little is known about the effects of methoxamine and its derivative on muscle carbohydrate metabolism but *N*-isopropyl-methoxamine prevents hyperlacticacidaemia produced by adrenaline (Salvador, Colville & Burns, 1963). These two drugs were without adrenaline-like action on the depressed contractions of the diaphragm and, in the heart, they have been shown to be without effect both on phosphorylase and on contractions (Kukovetz, Hess, Shanfield & Haugaard, 1959).

Insulin increases the transport of glucose across the cell membranes of the isolated diaphragm (Park, Bornstein & Post, 1955 ; Borrebaek & Walaas, 1963) and treatment with insulin results in increased levels of hexosephosphates (Haugaard, Marsh & Stadie, 1951 ; Chain, 1962 ; Borrebaek & Walaas, 1963). The dependence on glucose of the insulin-effect on contractions was illustrated by its inactivity in the absence of glucose. Phloridzin prevents the action of insulin on glucose transport in the heart (Park, Morgan, Henderson, Regen, Cadenas & Post, 1961) and was found to abolish its action on contractions of the diaphragm.

The action of glucagon on muscle carbohydrate metabolism is not clearly understood. Unlike its action in the liver, glucagon has no effect on adenosine-3′,5′-phosphate formation or glycogenolysis in skeletal muscle (Sutherland, 1951 ; Rall & Sutherland, 1958). However, some workers have found that it increases the peripheral utilization of glucose as measured from arterio-venous differences (Elrick, Hlad & Witten, 1955 ; Van Itallie, Morgan & Dotti, 1955 ; Stunkard, Van Itallie, & Reis, 1955), and it increases phosphorylation of glucose in the heart (Park *et al.*, 1961). The fact that phloridzin depressed the action of glucagon in the potassium-depressed diaphragm suggests that part of its action may be connected with transport and hence utilization of glucose. The action of glucagon also resembled that of sympathomimetic amines to the extent that it was blocked by β-receptor blocking agents. The adrenaline-like action of glucagon on the contractions of the heart has also been shown to be blocked by dichloroisoprenaline (Farah & Tuttle, 1960).

Several workers have studied the effects of caffeine, theophylline and aminophylline on carbohydrate metabolism in the heart and skeletal muscle. These drugs stimulate glycogenolysis, adenosine-3′,5′-phosphate formation, phosphorylase activation and hexosephosphate formation (Gemmill, 1947 ; Hess & Haugaard, 1958 ; Rall & Sutherland, 1958 ; Belford & Feinleib, 1962). Theophylline potentiates the action of adrenaline on phosphorylase activation in the heart and in skeletal muscle (Hess, Hottenstein, Shanfield & Haugaard, 1963) and both theophylline and

caffeine potentiate the positive inotropic action of noradrenaline in the heart (Rall & West, 1963). These drugs are believed to act by inhibiting the phosphodiesterase responsible for the breakdown of adenosine-3′,5′-phosphate (Rall & Sutherland, 1959 ; Butcher & Sutherland, 1962).

Studies with calcium ions on skeletal muscle have shown that it activates dephosphophosphorylase and therefore mimics the effect of adenosine-3′,5′-phosphate (Krebs, Graves & Fischer, 1959). In the heart, treatment with excess calcium ions results in increased levels of hexosephosphates (Belford & Feinleib, 1962).

In the diaphragm bathed in normal Krebs-Henseleit solution it was observed that replacement of oxygen with nitrogen resulted in an increase in twitch tension which was not abolished in the presence of iodoacetate. Anoxia has been shown to increase glucose uptake by the isolated diaphragm and by the heart (Randle & Smith, 1958 ; Park *et al.*, 1961) and to increase glucose phosphorylation in the heart (Park *et al.*, 1961).

A tetanus causes a temporary increase in twitch tension and stimulates muscle carbohydrate metabolism with the result that there is an increase in the cellular content of hexosephosphates. The time courses of the two effects coincide (Cori & Cori, 1933, 1934). The effect of a tetanus on the twitch tension still occurred under anaerobic conditions and was not abolished by iodoacetate.

Thus all of the various agents shown to increase the twitches of the diaphragm also affect carbohydrate metabolism and the diagram of Fig. 9 summarizes their probable sites of action. Although the end-effects of different drugs may be very different (for example, with insulin and adrenaline), one stage, an increased level of

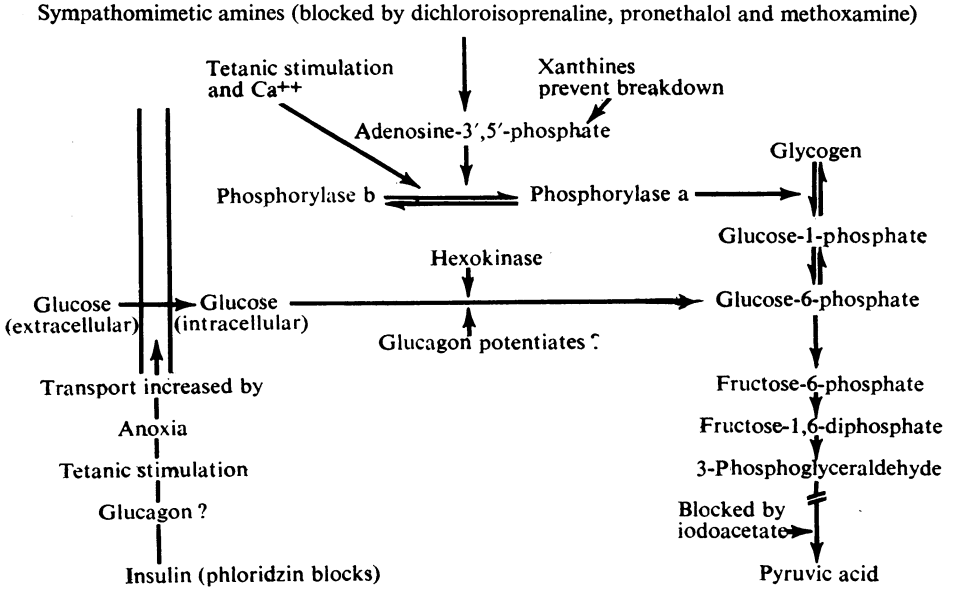

Fig. 9. Summary of probable sites of action of the agents which increase twitches of the diaphragm and affect carbohydrate metabolism.

intracellular hexosephosphates, appears to be common to all. Furthermore, the stages in the glycolytic chain which occur after the formation of hexosephosphates did not appear to be important, since, as shown also by Ellis & Beckett (1954), the effects on the contractions were not abolished by the glycolytic inhibitor, iodoacetate. Finally, the addition of large concentrations of hexosephosphates itself usually caused an increase in contractions resembling that produced by adrenaline. These experiments therefore confirm the results and support the conclusions of Ellis and his co-workers (see review by Ellis, 1959) that the effect of adrenaline on potassium-depressed skeletal muscle contractions is in some way a consequence of its action in increasing the intracellular concentration of hexosephosphates.

Excess potassium ions in the extracellular fluid will lower the concentration gradient for this ion across the muscle cell membranes. Consequently, the tendency for potassium ions to diffuse out of the cells, carrying positive change to the outside, is reduced and the muscle cell membranes become depolarized. The depression of the contractions in the presence of excess potassium is therefore a consequence of depolarization of the muscle fibre membranes. Large intra-arterial doses of adrenaline have been shown to increase the demarcation potential of cat skeletal muscles *in vivo* and this has been interpreted as evidence of a hyperpolarizing action (Brown, Goffart & Vianna Dias, 1950). We have confirmed this finding in cat muscles and shown that very small intravenous doses are also effective. However, Krnjević & Miledi (1958), using intracellular electrodes, failed to observe any consistent effect of adrenaline on the resting membrane potential of rat skeletal muscle either *in vivo* or *in vitro*. If adrenaline does exert a hyperpolarizing action, it may well be more pronounced when the fibre membranes are depolarized, and such an action would probably be sufficient to account for reversal of potassium-depression. Zierler (1959) found that insulin (0.1 U/ml.) caused a small and slowly developing hyperpolarization in the rat excised extensor digitorum longus muscle. As yet we have no information concerning the effect of the remaining active drugs on the membrane potential of skeletal muscles, but it is possible that all of them owe their effect to a hyperpolarizing action on the muscle cell membranes. Therefore, an increase in the level of cellular hexosephosphates may in some way influence ionic shifts across the cell membranes with the result that the membranes become hyperpolarized. Goffart & Perry (1951) found, in cat skeletal muscle, that adrenaline caused an increased retention of ^{42}K, and that the time course of this effect coincided with the increases in twitch tension and in demarcation potential recorded by Brown *et al.* (1950).

A few of the substances tested were without effect on the potassium-depressed twitches despite their having been shown to influence muscle carbohydrate metabolism. These substances were: (1) 5-hydroxytryptamine, which decreases the glycogen content of the rat diaphragm but has no effect on phosphorylase activity (Leonard & Day, 1960); (2) sodium fluoride (0.01 M) which produces maximal activation of adenosine-3′,5′-phosphate formation in broken cell preparations of skeletal muscle so that in its presence adrenaline has little additional effect (Sutherland & Rall, 1960); (3) the amino acids, L-lysine and hydroxy-L-proline which increase glucose transport and the intracellular concentration of hexosephosphates

in the rat diaphragm. These results might be taken as evidence against the possibility that the effect of adrenaline is a consequence of its action on carbohydrate metabolism. However, in some experiments the conditions used for studying the effects on carbohydrate metabolism are necessarily very different from those used to study effects on contractions, and the time course of the metabolic effect may be such that it does not result in an increase in contractions when studied under the conditions of the present experiments. In some instances, other actions of the drug might be responsible for preventing any increase in contractions. Evidence that this might be so with sodium fluoride was provided by the fact that it depressed the response of the diaphragm to adrenaline. Although 5-hydroxytryptamine has been shown to decrease the glycogen content, there is no evidence that the concentration of hexosephosphates is at any time increased ; it may be that increased utilization of hexosephosphates keeps pace with the increased production.

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