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EFFECTS OF 2,4-DINITROPHENOL AMYLOBARBITONE AND CERTAIN OTHER DRUGS ON THE RATE OF OXYGEN CONSUMPTION AND FORCE OF CONTRACTION OF ISOLATED CURARIZED DIAPHRAGM MUSCLE OF THE RAT

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1 A technique has been developed for studying over periods of 10 min or longer the effects of drugs on both the force of electrically-induced contractions and the oxygen consumption of an isolated, curarized, mammalian, skeletal muscle preparation.

2 The resting oxygen consumption of the muscle was increased substantially by 2,4-dinitrophenol in concentrations (0.02 mM and higher) that eventually produced contracture. Two other uncoupling agents, 4,6-dinitro-*o*-cresol and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, behaved similarly.

3 The oxygen consumption over 10 min of the stimulated muscle was also increased by 2,4-dinitrophenol (0.05 mM), although the strength of the 'maximal' contractions was lessened.

4 Amylobarbitone increased the strength of contraction at a concentration (0.2 mM) that did not affect oxygen consumption significantly. Amylobarbitone and pentobarbitone also increased it at a concentration (1 mM) that depressed oxygen consumption. They decreased both strength of contraction and oxygen consumption at a concentration of 5 mM. Phenobarbitone had a weaker action.

5 S-*n*-decyl-thiouronium increased oxygen consumption when given at a concentration (1 mM) that diminished strength of contraction and eventually produced contracture of the muscle.

6 Both S-methyl-thiouronium (1 mM) and 4-aminopyridine (0.1 mM and 0.5 mM) increased strength of contraction without increasing oxygen consumption. Neither strength of contraction nor oxygen uptake was affected by ouabain (up to 0.01 mM) or by phenformin (0.1 mM).

7 It is concluded that the response to 2,4-dinitrophenol is due mainly, if not wholly, to its known ability to uncouple oxidative phosphorylation; that the response to the barbiturates is due to a combination of a known metabolic action (viz., blocking of the respiratory chain) and a stimulant action on muscle; and the response to S-*n*-decyl-thiouronium to a disruptive action on cell membranes. The disproportionate actions of 4-aminopyridine and S-methyl-thiouronium on strength of contraction relative to oxygen consumption are also attributed to a non-metabolic action.

Introduction

Many of the drugs that depress muscular activity probably do so almost exclusively by direct interference with excitation or contraction, but since prolonged muscular activity is dependent upon the production of 'energy-rich' phosphates, one would expect it to be depressed also by inhibitors of oxidative phosphorylation. Various compounds have been shown to inhibit aerobic metabolism *in vitro*. However, this action might be insignificant *in vivo* for such reasons as the inability of a drug to penetrate cells or its possession of other and more powerful actions. It remains uncertain even with such well-known drugs as the barbiturates as to what extent their metabolic actions contribute to their effects on muscle and nerve (Goodman & Gilman, 1975).

In the hope of obtaining decisive information, we have utilized the technique recently described by Fastier & Sullivan (1977) for measuring simultaneously over periods of 10 min or more the strength of contractions and oxygen consumption of an isolated,

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electrically stimulated, curarized, skeletal muscle preparation. Part of this work was briefly described at a meeting of the Australian Physiological and Pharmacological Society (Bills & Fastier, 1977) and another part at a meeting of the British Pharmacological Society (Beresford, 1978).

Methods

The technique was based on that described by Fastier & Sullivan (1977), except in some control experiments with 4-aminopyridine, in which the technique was that of Bülbring (1946). It involves mounting curarized rat diaphragm muscle in a small closed chamber containing both an oxygen electrode and stimulating electrodes.

The organ bath

This was cut out of a thick 'Perspex' block. It was approximately 45 mm deep, 35 mm long, and 10 mm wide. To facilitate setting up the preparation in so narrow a bath, both the mounting hooks and the stimulating electrodes were attached to the block of Perspex which was used to plug the bath. Leakage was minimized by placing a well-greased gasket between the top of the bath and the electrode 'plug'. The bath had a shallow well to accommodate a small plastic-covered iron rod, which stirred the contents of the bath when the apparatus was placed on a magnetic stirrer. To permit insertion of an oxygen electrode, another cylindrical portion was cut out of the bath block just broad enough to fit the greased polythene holder of the electrode. The membrane of the electrode was so placed that the stirrer directed against it a stream of the Krebs-Henseleit solution which filled the bath. Krebs-Henseleit solution was introduced through a tube at the bottom of the bath and removed by overflow through a tube at the top.

Preparation of tissue

All except the preliminary experiments were performed with rat quarter-diaphragms. Female Wistar rats weighing about 200 g were killed by stunning them and breaking their necks. The right hemi-diaphragm was removed rapidly and divided into two. One of the quarter-diaphragms was stored in oxygenated Krebs-Henseleit solution and the other was prepared for immediate mounting on the platinum pins attached to the base of the stimulating electrode holder. The apex of the tissue was attached to a cotton thread connected to a force displacement transducer through a seal in the bath plug. A resting tension of 20 mN was applied to the muscle. Tension changes induced in it by supramaximal shocks were recorded through one channel of a Devices M2 chart recorder.

A train of 100 V pulses (duration 1 ms) was then administered to the muscle at a frequency of 1 Hz for 10 s. To minimize electrolytic effects the pulses were given biphasically. This sequence of pulses was given every 30 s, usually for 10 min, but sometimes for a longer period. After each period of stimulation fresh Krebs-Henseleit solution was introduced into the bath and the tissue was left unstimulated for 10 min. After a second 10 min recovery period the fluid was replaced by fresh Krebs-Henseleit solution containing the drug. The stimulation and recovery cycle was then repeated.

Several such cycles could be completed satisfactorily with most of the drugs tested. The preparation was discarded if it failed to return to within 10% of its pre-drug level of oxygen consumption and strength of contraction.

All experiments were carried out at room temperature (20–24°C).

Measurement of oxygen consumption

The uptake of oxygen by resting tissue was determined initially by measurement of the reduction in the partial pressure of oxygen contained in the closed bath by means of a gas analyser (Beckman). However, this direct technique was replaced in later experiments by a comparative one. The Po_2 of the chamber fluid was monitored continuously with an oxygen electrode (Yellow Springs) inserted into the bath. The signal was amplified and displayed on the second channel of the Devices recorder.

The eventually 'steady' rate of reduction of Po_2 during stimulation of the drug-treated muscle was compared with that obtained during the stimulation period immediately preceding drug treatment.

Drugs and solutions

The Krebs-Henseleit solution had the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 2.54, $MgSO_4$ H_2O 1.18, KH_2PO_4 1.19, $NaHCO_3$ 24.99 and glucose 66.7. Tubocurarine (13 μM) was added to the solution to prevent indirect stimulation of the muscle. The solution was then bubbled with 5% CO_2 in O_2.

The following drugs were obtained from commercial sources: 2,4-dinitrophenol (BDH); 4,6-dinitro-*o*-cresol (Hopkins & Williams); carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (Boehringer); amylobarbitone sodium (M & B); pentobarbitone sodium (Abbott); phenobarbitone sodium (BDH); S-*n*-decylthiouronium chloride (ICN); S-methyl-thiouronium hemisulphate (Sigma); 4-aminopyridine (Sigma); phenformin (Warner-Lambert); and ouabain (Nati-

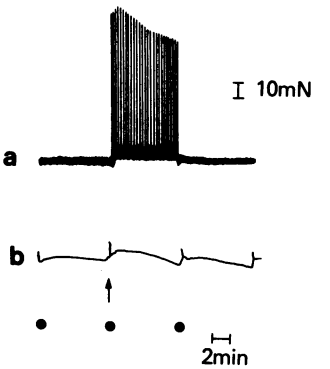

Figure 1 Simultaneous measurement of (a) electrically-induced isometric contractions of rat diaphragm muscle and (b) the P_{O_2} of the surrounding Ringer solution before, during, and after supramaximal stimulation, which began at the arrow. Fresh Krebs-Henseleit solution was introduced at points marked with dots.

velle). The sample of Δ^9-tetrahydrocannabinol was prepared by our colleague, Mr D.G. Ferry.

Statistical methods

Student's t test was used to assess the probability of difference between mean errors arising by chance. The measure of variability used was the standard error.

Results

Much experimentation was needed before reproducible results could be obtained. Since the oxygen uptake of the muscle is very small, bath size was reduced as far as possssible to provide easily measurable changes in Po_2. It was found important to use thick blocks of Perspex for the bath; otherwise warping, which was exacerbated by repeatedly screwing together the two sections of the bath, soon led to serious leakage. To minimize oxygen uptake by bath components, an important source of error (Kushmerick & Paul, 1976), we aimed, with apparent success, at establishing near-equilibrium conditions before the testing of a drug. It was found useful at the beginning of an experiment to see whether the Po_2 of the bath fluid fell appreciably before the muscle had been introduced but after the bath had been temporarily sealed and then filled with well-oxygenated Krebs-Henseleit solution. Normally the fall in Po_2 was insignificant. Like Weeks & Chenoweth (1952), we found that electrical stimulation could itself lead to substantial oxygen loss unless suitable precautions were taken.

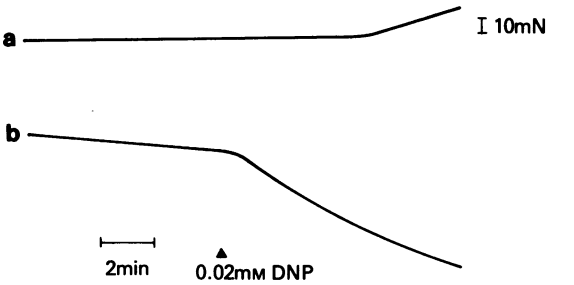

Figure 2 Effect of 2,4-dinitrophenol (DNP 0.02 mM) on unstimulated rat diaphragm. Simultaneous measurement of (a) muscle tone and (b) the P_{O_2} of the surrounding Ringer solution in the absence of electrical stimulation. The drug was added at (▲).

In 9 experiments in which absolute measurements were made by gas analysis, the average value for the oxygen uptake of the resting tissue was 1.3 μl mg^{-1} dry wt. h^{-1}, a figure which, given the differing experimental techniques involved, does not differ unduly from that of Weeks & Chenoweth (1952). Upon stimulation of the muscle, oxygen consumption rose to 2.1 to 3.5 μl mg^{-1} dry wt. h^{-1}. In the much larger number of experiments in which only relative measurements were made, electrical stimulation of the muscle produced within a minute or two, as can be seen in Figure 1, a significantly steeper fall in Po_2 than that obtained before stimulation (160 observations; $P < 0.001$).

The delay between onset of stimulation and maximum rate of Po_2 decline might be related to the time required to use existing ATP stores. We think it significant that, after a period of electrical stimulation, the oxygen consumption of the resting muscle took several minutes to decline to a steady level. In the course of 1 to 2 h the oxygen consumption of both resting and stimulated muscle slowly declined.

The force of the isometric twitch diminished with prolonged and fairly rapid stimulation. After 10 min it had declined by 19 ± 1% of the maximal value (160 observations). The same phenomenon has been noted by other workers (Brown, Bülbring & Burn, 1948; Weeks & Chenoweth, 1952).

Drug effects on resting muscle

In the presence of 2,4-dinitrophenol (DNP), in a concentration of 0.02 mM or more, a state of contracture developed after several minutes (Figure 2). An increased rate of oxygen consumption, indicated by a rapid fall in Po_2 preceded and accompanied the contracture.

Similar effects were obtained with 4,6-dinitro-*o*-

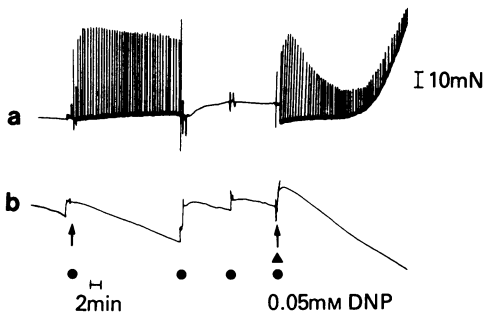

Figure 3 Effect of 2,4-dinitrophenol (DNP, 0.05 mM) on (a) the force of contraction of rat diaphragm muscle and (b) the P_{O_2} of the surrounding Ringer solution. Electrical stimulation was begun at each arrow. Fresh Krebs–Henseleit solution was introduced at the points marked with dots and the drug at (▲).

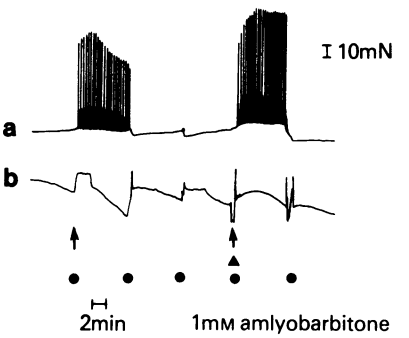

Figure 4 Effect of amylobarbitone (1 mM) on (a) the force of contraction of rat diaphragm muscle and (b) the P_{O_2} of the surrounding Ringer solution. Electrical stimulation was begun at each arrow. Fresh Krebs-Henseleit solution was introduced at the points marked with dots and the drug at (▲).

cresol (3 μM) and with carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (0.1 μM).

No obvious effect was produced by Δ^9-tetrahydrocannabinol (THC) when it was tested in up to a saturation concentration (3 μM) or by ethanol in the amount needed to dissolve the THC introduced into the reservoir.

Drug effects on contracting muscle

The chief results are summarized in Table 1, which shows that certain drugs can affect oxygen consumption and strength of contraction disproportionately. DNP (0.05 mM) increased the rate of oxygen consumption some 6 fold although the muscle contractions obtained in the presence of the drug were smaller than those obtained in plain Ringer (Figure 3). In all 9 experiments contracture developed within 8 to 10 min. When its concentration was lowered to 0.01 mM, DNP was without effect on either oxygen consumption or strength of evoked contractions.

Barbiturates had complex effects, as indicated in Table 1. Amylobarbitone and pentobarbitone in high concentrations (5 mM) depressed both the strength of muscle contractions and the oxygen uptake of the tissue. If the concentration of either barbiturate was

Table 1 Oxygen uptake of rat diaphragm during electrically-induced activity: effects of various drugs

Drug treatment (No. of experiments)	*Concentration* (m*M*)	P_{O_2} *decline in stimulated muscle*		*Force of muscle contractions after 10 min (mN)*	
		Control	*Drug-treated*	*Control*	*Drug-treated*
2,4-Dinitrophenol (8)	0.01	1.00(0.13)	1.04(0.11)	48.0(5.4)	42.0(5.0)
2,4-Dinitrophenol (9)	0.05	0.38(0.02)	0.65(0.02)**	24.0(5.0)	12.6(2.0)*
S-*n*-decylthiouronium (8)	0.5	1.59(0.23)	1.56(0.18)	38.0(1.3)	31.4(2.1)
S-*n*-decylthiouronium (6)	1	1.18(0.02)	1.63(0.03)*	47.6(4.0)	15.6(0.3)**
Amylobarbitone (8)	0.2	0.38(0.03)	0.33(0.02)	42.9(1.9)	51.0(2.2)*
Amylobarbitone (8)	1	0.78(0.05)	0.61(0.04)*	24.7(1.2)	34.0(2.2)*
Amylobarbitone (9)	5	0.55(0.06)	0.25(0.04)**	23.3(0.9)	10.1(2.5)**
Pentobarbitone (12)	1	0.98(0.09)	0.74(0.04)*	25.0(1.8)	32.0(1.8)*
Pentobarbitone (8)	5	0.66(0.08)	0.36(0.04)*	17.2(0.9)	11.1(1.6)*
Phenobarbitone (8)	1	0.81(0.10)	0.73(0.07)	25.1(3.0)	31.6(3.4)
Phenobarbitone (10)	5	1.10(0.09)	1.12(0.11)	24.5(2.3)	38.5(3.5)*
4-Aminopyridine (9)	0.1	1.38(0.05)	1.46(0.05)	44.2(3.0)	56.7(3.0)*
4-Aminopyridine (13)	0.5	0.47(0.02)	0.44(0.01)	46.3(4.2)	88.2(8.8)*
S-methylthiouronium (15)	1	0.43(0.02)	0.39(0.02)	27.8(2.4)	40.4(3.6)*

All results are expressed as mean (±s.e. mean).
Significance of results (as determined by Student's *t* test) denoted by * for $P < 0.05$ and by ** for $P < 0.01$.

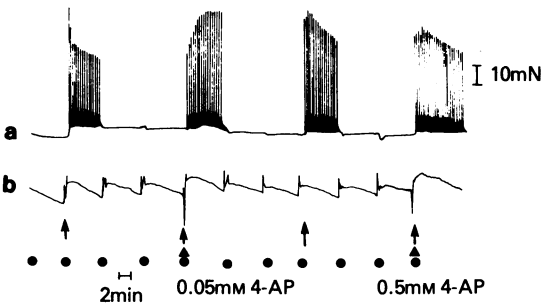

Figure 5 Effect of 4-aminopyridine (4-AP, 0.5 mM) on (a) the force of contraction of rat diaphragm muscle and (b) the P_{O_2} of the surrounding Ringer solution. Electrical stimulation was begun at each arrow. Fresh Krebs-Henseleit solution was introduced at the points marked with dots and the drug at (▲).

lowered to 1 mM, the strength of muscle contractions was increased (Figure 4) although oxygen consumption remained depressed. In another 8 experiments with amylobarbitone in which the concentration was reduced to 0.2 mM, the strength of muscle contractions was increased significantly but the oxygen consumption was unchanged. Phenobarbitone (1 mM) did not affect either muscle contractions or oxygen consumption. The response to a higher concentration (5 mM) of phenobarbitone resembled that to 0.2 mM of amylobarbitone.

S-*n*-decyl-thiouronium (S-10) in high concentration (1 mM) sharply increased muscle tone. As contracture developed, the strength of the electrically-evoked contractions soon declined. Throughout the period of exposure to the drug, oxygen consumption was increased significantly. Lower concentrations of S-10 did not increase either the muscle contractions or the oxygen consumption.

A significant increase in the strength of contractions was produced by S-methyl-thiouronium (S-1), a short-chain homologue of S-10. The rate of oxygen consumption of the tissue was not altered.

The effect of 4-aminopyridine (4-AP) on the strength of muscle contractions resembled that of S-1. At 0.1 mM the increase was slight but statistically significant. At five times this concentration (0.5 mM) the force of contraction was roughly doubled (Figure 5). The stimulant effect of the drug persisted for 1 or 2 h even when the preparation was washed repeatedly with plain Ringer solution. A further large dose of 4-AP had little additional stimulant effect. Neither concentration altered the rate of oxygen consumption.

To confirm that a 13 μM concentration of tubocurarine was sufficient to maintain neuromuscular blockade in the presence of 4-AP, a known anticurare agent (Bowman, Harvey & Marshall, 1976), control experiments were performed with the Bülbring (1946) innervated rat diaphragm preparation. Tubocurarine, added to the bath to give a 13 μM concentration, suppressed the contractions evoked by electrical stimulation of the phrenic nerve. This suppression of indirect stimulation of the muscle could not be counteracted by 4-AP, even when given in the highest concentration (0.5 mM) in which it was used in our direct stimulation experiments.

Neither strength of contraction nor rate of oxygen consumption was affected by phenformin (1 mM) or by ouabain (tested in concentrations of up to 0.01 mM).

Discussion

Oxygen consumption has been established as a good indicator of total energy turnover in mammalian smooth muscle (Bülbring & Golenhofen, 1967; Weston, 1972, Kumar, 1977). Drug effects on the oxygen consumption of skeletal muscle have received less attention (Stainsby & Barclay, 1972). We have used a curarized skeletal muscle preparation because we wished to ascertain whether direct metabolic actions can play an important part in determining the effects on muscle of such drugs as the barbiturates and it was therefore important to minimize other possible actions. Our technique shares certain features with those described by Novotny & Bianchi (1967) and by Lechner, Siess & Hoffman (1970).

Although the energy needed for muscle contraction is supplied directly by 'high-energy' phosphates, other compounds are needed in turn as fuel. A valid measure of their rate of consumption under aerobic conditions is supplied by recovery oxygen consumption (Kushmerick & Paul, 1976). However, this measure must be used with caution. Steady state conditions will not be approached until stores of high-energy phosphates have been used up. Likewise, oxygen consumption will remain above the level for resting muscle for some time after a period of stimulation. In our experiments the slope of the P_{O_2} trace gradually increased during the first few minutes of stimulation (Figures 1,3,4,5). It was for this reason that we have employed the steady slope (i.e., that obtained from about the 4th min onwards) to measure the rate of oxygen uptake.

We tested DNP first because some of its effects on rat diaphragm have already been studied. Like Weeks & Chenoweth (1952), we found that DNP can increase the oxygen consumption of the resting muscle even without the development of contracture. When contractions were produced by electrical stimulation, their strength was subnormal in the presence of a concentration of DNP which increased oxygen consumption. This would be expected of a compound which acted largely by preventing oxidative reactions in mitochondria being used to phosphorylate adeno-

sine diphosphate. The uncoupling action for DNP (Slater, 1967) might be sufficient to impair ATP production even when oxidative activity is excessive. Barnes, Duff & Trelfall (1955) have shown by chemical analysis that a dose of DNP which produces contracture of the isolated rat diaphragm causes profound falls in the concentration of energy-rich phosphates. Lechner *et al.* (1970) did not observe an increased oxygen uptake when DNP was given in a dose which depressed atrial contractions, but this dose was higher than that used by either Weeks & Chenoweth (1952) or ourselves. The difference in dose may be significant in that high concentrations of DNP have been shown to inhibit the oxygen uptake of isolated mitochondria (Chance, Williams & Hollunger, 1963; van Dam, 1967).

The respiratory chain is inhibited by various barbiturates and long-chain amidines (Slater, 1967). This action would explain why oxygen consumption was depressed when amylobarbitone or pentobarbitone was given in a concentration of 1 mM or higher. It does not explain why these barbiturates initially increased strength of contraction. Since this effect was obtained in curarized preparations, it is probably due to a direct action on muscle, possibly one involving the release of calcium from the sarcoplasmic reticulum. The higher concentration of phenobarbitone required to produce an action on the preparation may be a consequence of its slower rate of penetration into tissues.

Several amidines have been found capable of inhibiting the respiratory chain at phosphorylating sites I, II or III (Chance & Hollunger, 1963; Slater, 1967; Papa, Tuena de Gomez-Puyou & Gomez-Puyou, 1975; Tuena de Gomez-Puyou & Beigel, 1976). These amidines include synthalin (decamethylenediguanide) and phenformin (2-phenylethyl-diguanide), which have been used clinically as hypoglycaemic agents. We found synthalin too insoluble for satisfactory testing. Phenformin did not affect the preparation at a concentration of 1 mM. S-10, which would be expected on structural grounds (Fastier, 1962) to be mainly a site I inhibitor like the corresponding alkyl-guanidine, resembled DNP in that it increased oxygen consumption and eventually produced muscle contracture when tested at a 1 mM concentration. The concentration of S-10 was critical, lower doses being without effect on the preparation. A comparable dose-dependency has been found with S-10 when this compound has been tested on other preparations. For example, when incubated with erythrocytes *in vitro*, S-10 stabilizes the membrane at low concentrations but lyses it when given in concentrations of 1 mM or more (Beresford, 1976). It has been suggested (Green & Baum, 1970) that compounds which damage the cell membrane might eventually rupture repeating units in the membrane. These ruptured units would then act as sinks for the energized state, uncoupling respiration within the cell, a state of affairs comparable with that produced by freezing and rapid thawing.

Several drugs were tested to see whether it might be possible, at least in the short run, to obtain increased strength of contractions without a commensurate increase in oxygen consumption. Since rat muscle is relatively insensitive to cardiac glycosides (Goodman & Gilman, 1975), it is not surprising that ouabain had no obvious effect on the preparation. Experiments were then performed with S-1 and 4-AP, two of a number of small cations known to share such pharmacological properties as an ability to increase the 'maximal' twitch of rat diaphragm (Fastier, 1962). Both compounds were able to increase strength of contraction without increasing significantly the rate of oxygen consumption. It has been shown recently (Pelhate & Pichon, 1974; Wagner & Ulbricht, 1975; Gillespie, 1977) that 4-AP can block potassium channels in various tissues. This action would be expected to prolong the action potential and hence increase strength of contraction. S-1 may act similarly.

The lack of response to THC, which is now believed to be the chief active constitutent of cannabis, seems ironical in that the preparation was devised primarily in the hope that it might be used to show that THC can uncouple oxidative phosphorylation, not only in isolated mitochondria (Mahoney & Harris, 1972; Bino, Chari-Bitron & Shahar, 1972), but also in a relatively intact preparation. Since it has been observed by Parker, Barnes & Denz (1951) that rigor mortis develops very rapidly in animals poisoned by uncoupling agents of the DNP type, we think it significant that contracture can be provoked more readily in muscles taken from rats which have been fed large amounts of THC over a day or two than in the muscles taken from control animals (Bills & Fastier, unpublished observations). THC is known to be strongly bound by certain proteins and other tissue constituents (Paton, 1975). Its inactivity under the conditions of our experiments might therefore be attributable to its being taken up at sites of loss to such an extent that insignificant amounts reach mitochondria within a few minutes. Whatever the explanation it is clear that the bioavailability of the drug under test can be an important consideration. However, this is not necessarily a disadvantage of the preparation. The results of experiments carried out with mitochondrial preparations may give little indication of what would happen *in vivo* for the very reason that some normal permeability barriers have been removed.

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