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THE EFFECT OF 2,4-DINITROPHENOL ON NEUROMUSCULAR TRANSMISSION

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In 1957 Kraatz and Trautwein carried out a detailed electrophysiological investigation to clarify the role of oxidative phosphorylation in the neuromuscular transmission of the frog. These authors demonstrated that 2,4-dinitrophenol (DNP) decreases end-plate potential (e.p.p.) height and increases miniature end-plate potential (m.e.p.p.) frequency, leaving their amplitude unaffected. Both effects were reversed by hyperpolarization of the motor nerve endings, suggesting that DNP affects the electrosecretory coupling and the spontaneous neurosecretion through reduction of transmembrane potential in nerve terminals. The prejunctional effect of DNP did not seem to depend on the exhaustion of transmitter stores because the effect was rapid in onset and the m.e.p.p.s maintained their amplitude, although their frequency greatly increased.

However, the metabolic damage to the nerve (Abood, Koketsu & Noda, 1961) could, perhaps, not only impair the transmembrane ionic equilibrium, but also reduce the acetylcholine available for release, especially during prolonged nerve stimulation when the transmitter requirement and its synthesis rate increase considerably (MacIntosh, 1963). In view of this hypothesis, the effect of DNP on the mammalian neuromuscular junction has been re-examined, comparing the electrophysiological findings with the changes in acetylcholine release and stores in the presence of the drug.

A preliminary account of some of these results has already been published (Beani, Bianchi & Ledda, 1963).

METHODS

The experiments were performed *in vitro* using phrenic nerve-diaphragms obtained from guinea-pigs of either sex, weighing between 300–350 g, or from adult male (Wistar strain) albino rats, weighing between 200–250 g.

Guinea-pig diaphragm. The left and right hemi-diaphragms of the same animal were set up in two separate baths each containing 4 ml. oxygenated Tyrode solution of the following composition: NaCl 137 mM ; $CaCl_2$ 1.8 mM ; NaH_2PO_4 0.3 mM ; $MgCl_2$ 0.1 mM ; $NaHCO_3$ 11.9 mM ; glucose 11 mM.

One hemi-diaphragm was treated with DNP, the other was kept as a control. Contractions were recorded on smoked paper with an isotonic lever ; amplification 1:7, load 2 g. The stimulating rectangular pulses were of 0.1 msec duration for the indirect stimulation and 2 msec for the direct stimulation, which was performed with platinum electrodes applied to the costal margin of the muscle. The effect of DNP on the release of acetylcholine and on total tissue acetylcholine of Dyflos-pretreated preparations, at rest and indirectly stimulated at various frequencies, was examined

by methods described previously (Beani, Bianchi & Ledda, 1964); the biossay of acetylcholine was performed using the isolated ileum of the guinea-pig. In some experiments neostigmine 1×10^{-5} g/ml. was employed, instead of dyflos (Straughan, 1960) and choline 1×10^{-5} g/ml. was added to the bath solution; leech dorsal muscle was then used for the bioassay (Murnaghan, 1958).

The tissue ATP content was determined by the method of Lamprecht & Trautschold (1963). The extraction of nucleotide was made as suggested by Abood *et al.* (1961).

The effect of DNP on choline acetyltransferase obtained from freshly excised homogenized hemi-diaphragms was determined by the method described by Hebb, Krnjević & Silver (1964); the rectus abdominis muscle was used for the bioassay.

Rat diaphragm. The intracellular recording from the end-plates of rat hemi-diaphragms was made with KCl-filled glass capillary microelectrodes (resistance 5–10 MΩ), using conventional techniques (Fatt & Katz, 1951). The preparation was placed in a chamber similar to that described by Boyd & Martin (1956) at 33° C in oxygenated Tyrode solution; the Tyrode solution flowed through the bath at a rate of 2 ml./min. The motor nerve endings were easily located in the transilluminated diaphragm with the aid of a binocular microscope (magnification × 60).

The phrenic nerve was stimulated with rectangular pulses of 0.03 msec duration. Tubocurarine $0.5–1 \times 10^{-6}$ g/ml. or $MgCl_2$ 10 mM were used to block neuromuscular transmission. When the $MgCl_2$ concentration was increased, NaCl was reduced to keep the solution isomolar. Potentials were recorded by photographing oscilloscope double-beam traces, the first one being a d.c. record at low amplification to follow the transmembrane potential values, the second an a.c. record (time constant 0.5 sec) at higher amplification, to analyse m.e.p.p. and e.p.p. height. Only impalements giving a stable resting potential of 70–80 mV were selected.

M.e.p.p.s were recorded, before and during DNP treatment, from diaphragms kept in normal or $MgCl_2$-enriched solution. In some experiments the preparations were preincubated with dyflos 500 μg/ml. for 1 hr, the anticholinesterase agent then being washed out. The e.p.p.s were recorded, before and during DNP treatment, at different stimulation rates, either from curarised or $MgCl_2$-treated diaphragms; in some cases the electrical activity of the same end-plate was followed throughout the experiment. In the preparations blocked by $MgCl_2$ 10 mM, quatum content M and quantum size q of sequential e.p.p.s (Del Castillo & Katz, 1954; Martin, 1955; Elmquist & Quastel, 1965b) were calculated. The presence of m.e.p.p.s enabled a comparison to be made of the agreement between the value of the mean m.e.p.p. and that of q, calculating the latter as $\frac{\text{variance of e.p.p.s}}{\text{mean e.p.p.}}$ q did not usually exceed a 10% increase of the mean m.e.p.p. value; M was, therefore, determined from the ratio: $\frac{\text{mean e.e.p.}}{\text{mean m.e.p.p.}}$ All the values for the e.p.p. and the m.e.p.p. amplitude were corrected for the non-linearity of the post-synaptic response, according to Martin (1955).

A transmitter equilibrium potential of 15 mV inside negative was assumed. In the same preparations the post-synaptic chemosensitivity was also tested, using applications of ACh rapidly injected into the bath, to give a final concentration of 2×10^{-4} g/ml.

Drugs used were 2,4-dinitrophenol (Merck), Dyflos (Diisopropylfluoro-phosphonate, DFP, Boots), Acetylcholine chloride (Roche), Tubocurarine chloride (Wellcome), Nicotine bitartrate, the chemicals commercially available for extraction of tissue acetylcholine, Boëhringer reagents, substrate and enzymes for the estimation of tissue ATP and choline acetyltransferase.

All the concentrations given refer to the appropriate salt.

RESULTS

At 28°, 33° or 38° C the contraction of hemi-diaphragms to direct and indirect stimulation at 15/min were inhibited to the same degree after a 30 min incubation in DNP 1×10^{-5}, 3×10^{-5} or 1×10^{-4} M (six pairs of preparations for each experimental condition). DNP 1×10^{-4}M produced contracture at all three temperatures; at 28° C, DNP 1×10^{-5}M

had no effect. Tubocurarine 5×10^{-6} g/ml. was added 10 min before starting with the direct stimulation. Only in the case of higher stimulation frequencies was the contraction to indirect stimulation more reduced than that to direct stimulation: e.g. at 33° C after 30 min stimulation at 100/min in the presence of DNP 3×10^{-5}M, the indirect contraction fell to $38.5 \pm 15\%$ ($\pm$S.D.) and the direct contraction to $61.5 \pm 5.7\%$ ($\pm$S.D.) ($P<0.01$, $n=9$).

However, at each temperature and drug concentration the responses to short tetanic stimulation at 20/sec and 50/sec, although reduced in size, were well sustained even when post-tetanic potentiation was decreased or absent. (Fig. 1).

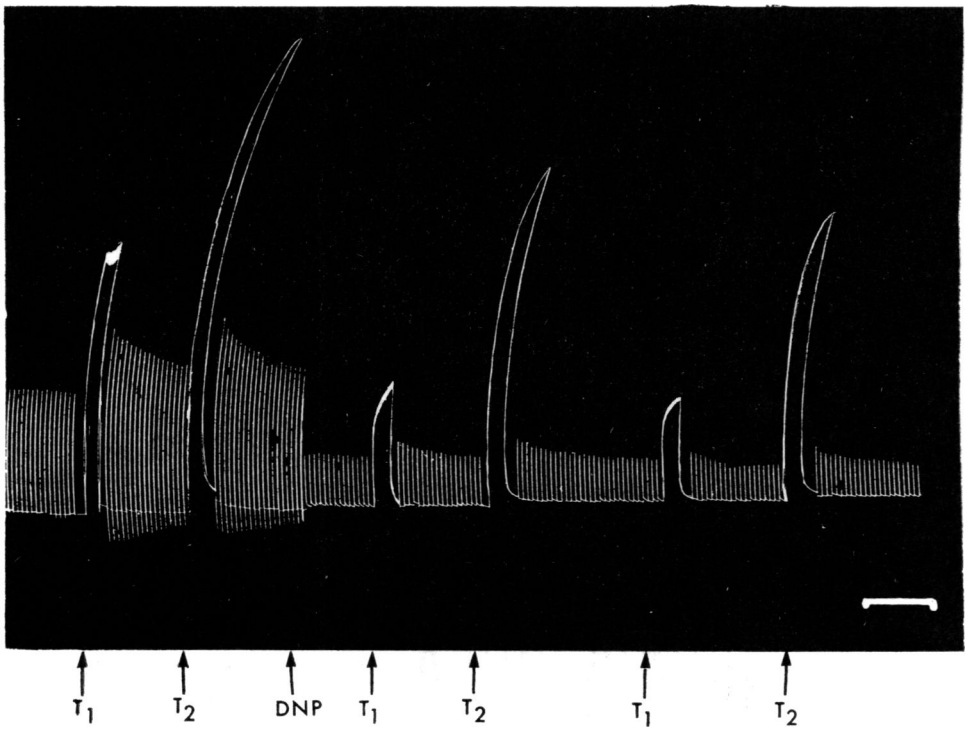

Fig. 1. Guinea-pig phrenic nerve-diaphragm preparation, indirect stimulation at 15/min: temperature, 38° C. At T_1 and T_2 the nerve was stimulated at 20/sec and 50/sec for 15 sec. Contraction height is halved 30 min after adding dinitrophenal 3×10^{-5}M (DNP), but the shape of tetanic responses is normal; post-tetanic potentiation is reduced. Time mark=1 min.

The time required by nicotine 5×10^{-5} g/ml. and tubocurarine 1×10^{-6} g/ml. to halve the indirect contraction (TI_{50}) in DNP-treated hemi-diaphragms (stimulation rate 15/min; temperature 33° C) was determined, taking into account the reduction of the contraction brought about by DNP alone. The blocking time was significantly shorter in the preparations preincubated with DNP 3×15^{-5}M for 30 min. The average TI_{50} after nicotine was 422 ± 167 sec ($\pm$S.D.) in the controls and 194 ± 89 sec ($\pm$S.D.) in the DNP treated preparations ($P<0.05$, $n=10$); after tubocurarine it was $1{,}223 \pm 138$ sec ($\pm$S.D.) and 289 ± 115 sec, respectively, ($P<0.01$, $n=10$). Therefore, DNP reduces the safety factor

of neuromuscular transmission, regardless of the mechanism of action of the blocking agent.

The effect of DNP on ACh release and acetylcholine stores. In the first group of experiments, DFP 500 μg/ml. pretreatment (1 hr) was used to inhibit the cholinesterases; DFP was then washed out and both hemi-diaphragms from the same animal were submitted to four 10 min periods of stimulation, each of which was followed by a 20 min period of rest. DNP was added to only one hemi-diaphragm after the second period of rest; it was kept in contact for 30 min before the start of the third stimulation period and remained until the end of the experiment. Details of calculation and biological estimation are fully described in our previous work (Beani *et al.*, 1964).

At no concentration and at no temperature was the resting release of acetycholine modified by DNP. The average release of control groups during each of the four 10 min rest periods was about 2–3 ng at 28° C, 4–5 ng at 33° C, 7–8 ng at 38° C; the release of DNP-treated preparations was almost the same.

On the other hand, the acetylcholine released during nerve stimulation was reduced by DNP. The effect was directly related to temperature, drug concentration and stimulation rate. The influence of temperature was investigated in hemi-diaphragms stimulated at

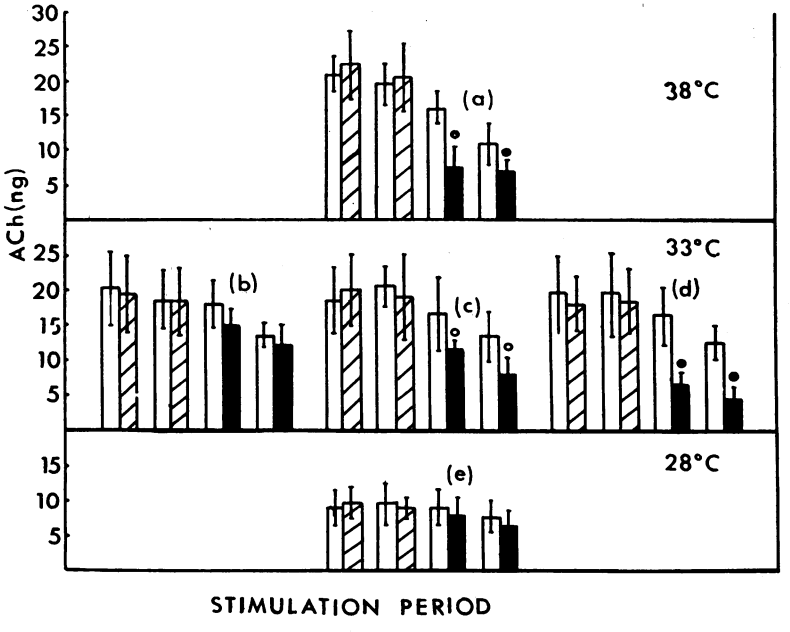

Fig. 2. Acetylcholine release (ng±S.D., vertical lines) in four successive periods of 10 min nerve stimulation from control and DNP-treated diaphragms. Stimulation rate 6/sec; a, 38° C, DNP 1×10^{-5}M, six experiments; b, 33° C, DNP 3×10^{-6}M, six experiments; c, 33° C, DNP 1×10^{-5}M, seven experiments; d, 33° C, DNP 3×10^{-5}M, six experiments; e, 28° C, DNP 1×10^{-5}M, seven experiments. Clear rectangles, release of ACh, control group; hatched, release before DNP treatment; black, release during DNP treatment. □, significantly different ($P<0.01$) from the release of ACh in the control group in the same period; O, significantly different ($P<0.05$) from the release in the control group in the same period.

6/sec and treated with DNP 1×10^{-5}M. At 28° C the average release in the third and fourth stimulation period was reduced to 87% of the control value; at 33° C to 64.6%; and at 38° C to 48.1%. The difference in ACh output between treated and control group was statistically significant at 33° C and 38° C (Fig. 2).

The effect of DNP concentration was determined in preparations stimulated at 6/sec and kept at 33° C: the acetylcholine release was reduced to 92.7% of the control values after DNP 3×10^{-6}M; to 64.6% after DNP 1×10^{-5}M; and to 41.2% after DNP 3×10^{-5}M. The difference in acetylcholine output between treated and control groups was statistically significant only in the preparations treated with DNP 1×10^{-5}M and 3×10^{-5}M (Fig. 2).

The influence of the stimulation rate was investigated at 33° C, in preparations incubated with DNP 1×10^{-5}M. In the DNP-treated group stimulated at 100/min the release was slightly lower than in the corresponding control group (88.3%), at 6/sec and 20/sec it was 64.6% and 64%, respectively; at 50/sec it was 45.8%. The acetylcholine release in the DNP-treated preparations was significantly lower than that in controls, at the stimulation rates of 6/sec, 20/sec and 50/sec (Fig. 3).

The total tissue acetylcholine was estimated at the end of the fourth stimulation period in experiments carried out at 33° C and a stimulation rate of 6/sec. DNP reduced tissue

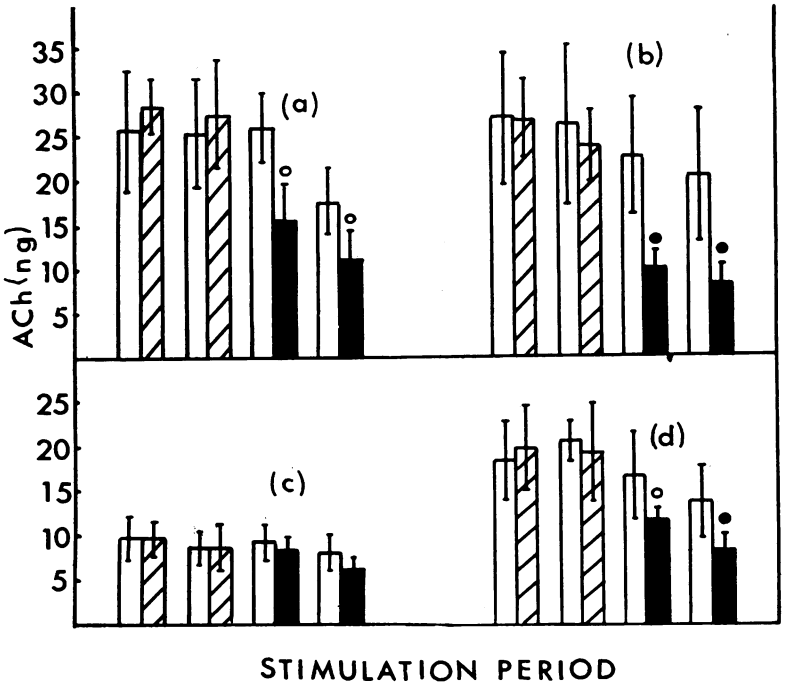

Fig. 3. Acetylcholine release (ng ± S.D., vertical lines) in four successive periods of 10 min nerve stimulation from control and DNP-treated preparations. Temperature 33° C, DNP 1×10^{-5}M. a, stimulation rate 20/sec, six experiments; b, 50/sec, six experiments; c, 100/min, six experiments; d, 6/sec, seven experiments. Clear rectangles, release of acetylcholine, control group; hatched, release before DNP treatment; black, release during DNP treatment; ●, significantly different ($P<0.01$) from the release of ACh in the control group in the same period. O, significantly different ($P<0.05$) from the release of ACh in the control group in the same period.

TABLE 1

EFFECT OF 2,4-DINITROPHENOL ON TOTAL ACETYLCHOLINE CONTNET OF GUINEA-PIG HEMI-DIAPHRAGMS

Measurements were made at the end of the fourth period of stimulation at 6/sec, lasting 10 min; temperature 33° C; dyflos μg 500/ml pretreatment. The % reduction of acetylcholine release during stimulation at 6/sec is given for comparison. ●; significantly different ($0.05 > P > 0.02$) from the control group. ●●; significantly different ($P < 0.001$) from the control group.

DNP Concentration (M)	No. Expts	Hemi-diaphragm weight ± S.D.	Acetylcholine ng/hemi-diaphragms ± S.D.	Acetylcholine stores %	Acetylcholine release %
—	16	274·0 ± 29·0	122·3 ± 28·0	100	100
3×10^{-6}	10	229·0 ± 38·0	103·1 ± 15·6	91·8	92·7
1×10^{-5}	10	255·0 ± 27·0	87·3 ± 24·0●	77·7	64·4
3×10^{-5}	12	276·0 ± 38·0	71·4 ± 12·8●●	63·5	41·2

acetylcholine only at the higher concentrations (Table 1). It is evident that the percentage reduction in acetylcholine stores is lower than the reduction in acetylcholine release under the same experimental conditions, but it is again a function of DNP concentration.

In the second group of experiments, in which neostigmine 1×10^{-5} g/ml. was added to the bath solution, both hemi-diaphragms were stimulated at 15/min for 1 hr. After renewing the Tyrode solution, they were stimulated at 6/sec for 15 min: 2 min after the end of stimulation the solution was removed for bioassay. Subsequently DNP was added to both preparations and kept in contact with them until the end of the experiment. The stimulation at 15/min was started again for 43 min; then a second period of stimulation at 6/sec for 15 min took place. After the second period of tetanic stimulation, choline 1×10^{-5} g/ml. was added to one hemi-diaphragm and, after a further 43 min stimulation at 15/min, the last tetanus was performed. The ACh output during the first tetanus was between 15–20 ng (about 1 ng/min), a little lower than that detected in the DFP-pretreated preparations under the same experimental conditions. In the second period (i.e., after adding DNP) the output was halved and remained the same in the third tetanus even in choline-treated hemi-diaphragms (Fig. 4).

Therefore, DNP reduces acetylcholine output to the same extent both in DFP-pretreated and in neostigmine-treated diaphragms and its effect is unaffected by choline.

In three pairs of normal preparations it was ascertained that the acetylcholine output was constant during successive stimulation periods, as found by Straughan (1960).

The effect of DNP on tissue ATP. So as to obtain some information on the degree of metabolic inhibition of diaphragms by DNP, their ATP content was estimated at the end of the fourth stimulation period at 6/sec, 33° C. The average nucleotide content of diaphragms in the absence of DNP (18 experiments) was mM/g 0.92 ± 0.28 (±S.D.) of fresh tissue. In the preparations treated with DNP (6 for every drug concentration) the ATP content fell to 70.1 ± 8.4; 56.0 ± 12.4; 44.6 ± 11.6, per cent after DNP 3×10^{-6}, 1×10^{-5}, 3×10^{-5}M, respectively. The reduction was statistically significant ($P < 0.01$) in the groups treated with DNP 3×10^{-5} and 1×10^{-5}M.

The ATP content of untreated preparations was considerably lower than that found in freshly excised hemi-diaphragms (mM/g 5.63 ± 0.62, $n = 6$). This reduction depends on the long-lasting experimental procedure, according to Barnes, Duff & Threlfall (1955), who found that ATP concentration in rat diaphragms (kept in Tyrode solution at 37° C) falls, after 2 hr incubation, to mM 1.42/g of fresh tissue.

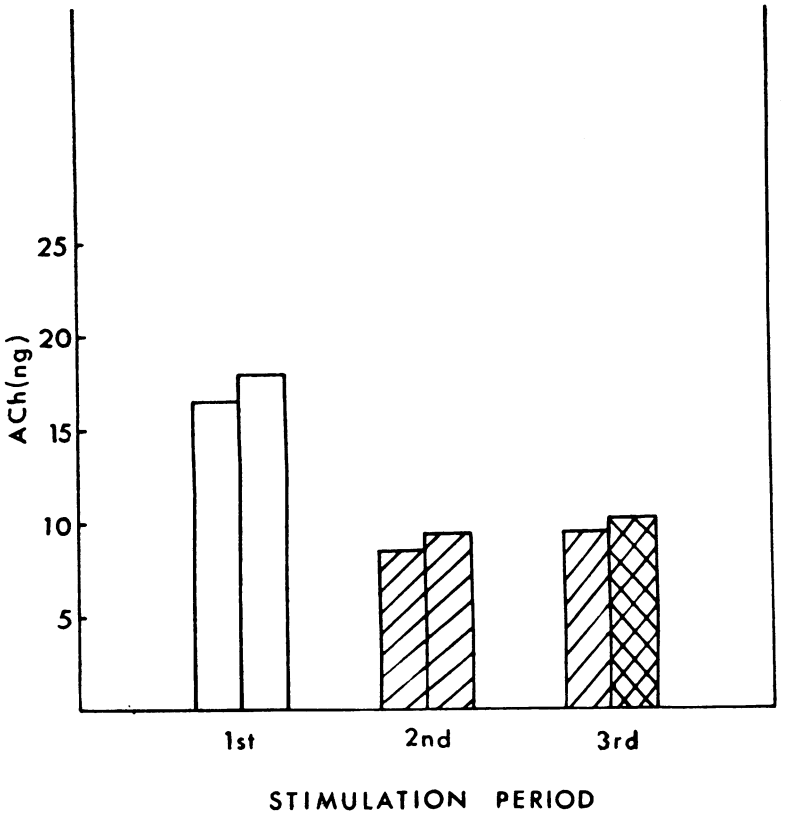

Fig. 4. Effect of 2,4-dinitrophenol 3×10^{-5}M and choline 1×10^{-5} g/ml. on the average acetylcholine release from four pairs of hemi-diaphragms stimulated at 6/sec for 15 min in the presence of neostigmine 1×10^{-5} g/ml. Temperature 33° C. Clear rectangles, normal ACh release; hatched, release during DNP treatment; double hatched, release during DNP treatment plus choline.

The effect of DNP on choline acetyltransferase. From the results described above it is clear that: firstly DNP reduces acetylcholine stores and release in the stimulated preparations; secondly the effect of DNP is independent of the anticholinesterase drug employed and is insensitive to choline treatment; and thirdly the concentrations of DNP used were sufficient to inhibit phosphorylation.

It was therefore of interest to establish whether the drug interfered with the final step of acetylcholine synthesis.

In three experiments the reaction mixture for testing choline acetyltransferase activity was prepared both with and without different DNP concentrations and incubated for 15 min at 37° C. Subsequently 0.1 ml. cysteine-sucrose homogenate, prepared from freshly excised diaphragms (concentration: 100 mg muscle/ml.) was added and incubation at 37° continued. The enzymatic reaction was stopped after 1 hr. The amount of acetylcholine formed was unaffected by DNP: the mean value of choline acetyltransferase activity (expressed as ACh μg/hr/g, of fresh tissue) was 129.4 without DNP), 122 with DNP 3×10^{-6}M; 132 with DNP 1×10^{-5}M and 142 with DNP 3×10^{-5}M.

The effect of DNP on the m.e.p.p. frequency and amplitude. As shown in Table 2, the control values of m.e.p.p. amplitude and frequency in normal Tyrode solution are well in agreement with the observations of other authors. Cholinesterase inhibition produced a 30% increase in the m.e.p.p. amplitude and frequency (Boyd & Martin, 1956). Owing to the well-known post-synaptic effect, the rise in $MgCl_2$ concentration halved the m.e.p.p. amplitude, but also decreased slightly their frequency (Hubbard, 1961). A few minutes after starting perfusion with normal Tyrode solution containing DNP 1×10^{-5}M, the m.e.p.p. frequency greatly increased in most of the end-plates, but their amplitude remained the same; the effect lasted throughout the experimental period (60–90 min).

On the other hand, the increase in m.e.p.p. frequency was absent or slight in preparations preincubated with DFP or perfused with $MgCl_2$-enriched solutions.

TABLE 2

EFFECT OF 2,4-DINITROPHENOL 1×10^{-5}M ON M.E.P.P. FREQUENCY AND AMPLITUDE IN GUINEA-PIG HEMI-DIAPHRAGMS

The diaphragms were either kept in normal Tyrode solution, pretreated with dyflos μg 500/ml for 1 hr, or blocked with $MgCl_2$ 10mM. In brackets, the number of diaphragms

	Normal Tyrode solution (8)		Normal Tyrode solution, plus dyflos pretreatment (4)		$MgCl_2$-enriched Tyrode solution (12)	
	Controls	Dinitrophenol	Controls	Dinitrophenol	Controls	Dinitrophenol
No. of end-plates	18	19	13	13	18	18
No. of m.e.p.p.s.	992	1055	780	811	981	1071
Frequency/min	130±54	684(90–2400)	218±94	210±150	107±69	185±40
Amplitude mV	0·89±0·42	0·89±0·45	1·19±0·50	1·18±0·46	0·40±0·14	0·43±0·15

It is worth noting that in the presence of DNP m.e.p.p. amplitude does not change after prolonged nerve stimulation at 10–50/sec in $MgCl_2$-blocked preparations and post-tetanic potentiation of spontaneous discharge frequency, up to two–three times the control values, is still present (Liley, 1956).

The effect of DNP on the e.p.p.s of curarized preparations. In many experiments the e.p.p.s were recorded in three successive 5-sec periods of nerve stimulation at 1, 10 and 100/sec, both before and 30 min after starting DNP 1×10^{-5} perfusion, at 33° C. Different end-plates were impaled before and during drug treatment. DNP significantly decreases e.p.p. height to the same extent at any stimulation rate, thus not altering the inverse relationship between amplitude and frequency (Table 3).

TABLE 3

EFFECT OF 2,4-DINITROPHENOL ON E.P.P. HEIGHT (±S.D.) OF RAT CURARIZED DIAPHRAGMS

d-Tubocurarine 1×10^{-6} g/ml present throughout stimulation at 1, 10 and 100/sec for 5 sec; temperature 33° C. Only one end-plate was impaled before and after dinitrophenol in each of 28 preparations. The reductions at the higher stimulation rates are given as a percentage of the value at 1/sec. ● = significantly different ($P < 0{\cdot}001$) from the control group

	Controls		Dinitrophenol 1×10^{-5}M	
Frequency of stimulation	Amplitude mV	%	Amplitude mV	%
1/sec	2·62±0·54	100	2·10±0·31●	100
10/sec	1·89±0·45	72·1	1·43±0·27●	68·0
100/sec	1·33±0·32	50·9	0·98±0·26●	46·0

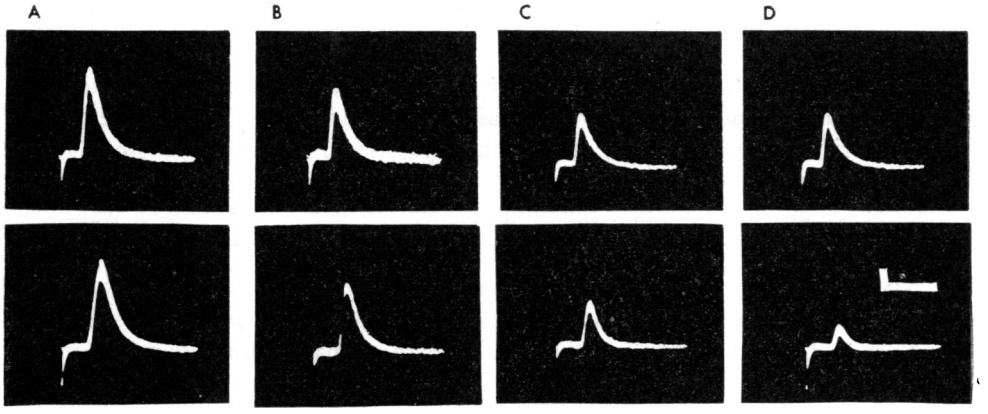

Fig. 5. Intracellular superimposed records of e.p.p. from the same end-plate after 30 sec (A), 3 min (B), 6 min (C), and 10 min (D), of nerve stimulation at 10/sec before (upper tracing) and 30 min after starting DNP 1×10^{-5}M perfusion (lower tracing); temperature 33° C. Tubocurarine 1×10^{-6} g/ml. present throughout. Voltage-time scale=1 mV–5 msec. Transmembrane potential=70 mV.

In other experiments, the e.p.p.s of the same end-plate were recorded, both before and 30 min after DNP, during a 10 min period of nerve stimulation at 10/sec. After 10 min stimulation the e.p.p. amplitude of the control recording was about one-half that at the beginning; whereas after DNP treatment the reduction at the end of the stimulation period was greater (Fig. 5).

Assuming that the post-synaptic sensitivity is unaffected by the drug, as judged by maintained m.e.p.p. amplitude (see above), the conclusion may be reached that the amount of acetylcholine released from the stimulated nerve is reduced by DNP, above all when the nerve stimulation is long lasting.

The effect of DNP on the e.p.p. quantum content. In six hemi-diaphragms perfused with Tyrode solution containing $MgCl_2$ 10 mM the e.p.p.s. were recorded during a 10 min period of stimulation at 10/sec before and after DNP 1×10^{-5}M.

The corrected values of e.p.p. amplitude and quantum content are given in Table 4.

In contrast with the experiments made with curarized preparations, the e.p.p. height in control recording with the $MgCl_2$-blocked diaphragm did not fall after 10 min. Even in the presence of DNP the fall was small and the reduction in quantum content was not significant.

TABLE 4

EFFECT OF 2,4-DINITROPHENOL ON E.P.P. HEIGHT AND QUANTUM CONTENT OF $MgCl_2$-BLOCKED ($MgCl_2$ 10mM) RAT DIAPHRAGMS

Stimulation at 10/sec for 10 min; temperature 33° C. Only one end-plate was impaled before and after dinitrophenol in each of six preparations. The mean cumulative sum (S) of quanta released is given at each time. Quantum content, M

	Controls			Dinitrophenol 1×10^{-5}M		
Time from start of stimulation	Amplitude mV	M	S	Amplitude mV	M	S
20 sec	3·76±1·27	8·70±2·53	1740	3·39±0·95	7·55±1·79	1510
10 min	3·65±1·22	8·19±1·66	50640	2·71±0·64	6·30±2·00	41520

Our results are in agreement with those obtained by Kraatz & Trautwein (1957) who established that $MgCl_2$ antagonizes the e.p.p. reduction caused by DNP.

On the other hand, the antagonism by $MgCl_2$ could only depend on the fact that it hinders the release of acetylcholine, above all, at low stimulation rates. Therefore the electrical activity of the end-plates of six other diaphragms was followed for a 10 min period at a higher stimulation rate, i.e. at 50/sec, before and during DNP treatment. Under these conditions the e.p.p. quantum content of control recordings was greater than at 10/sec and slightly increased throughout the stimulation period.

The post-activation potentiation clearly prevails over the interference produced by $MgCl_2$ on quanta recruitment. This observation is well in agreement with the findings of Elmquist & Quastel (1965a) at the end-plate of a magnesium-blocked diaphragm stimulated for a long time at 10.5/sec.

In the presence of DNP the value of M slightly increased only in the first two to four min of stimulation, after which it fell below the initial value (Table 5). Despite the decrease in M, DNP did not modify the quantum size: this pattern of action differs from that of HC3 (Elmquist & Quastel, 1965a).

TABLE 5

EFFECT OF 2,4-DINITROPHENOL ON E.P.P. HEIGHT AND QUANTUM CONTENT OF DIAPHRAGMS BLOCKED WITH $MgCl_2$ 10 mM

Stimulation at 50/sec for 10 min; temperature 33° C. Only one end-plate was impaled before and after dinitrophenol in each of six preparations. The mean cumulative sum (S) of quanta released is given. The values 10 min after dinitrophenol are the average of only three experiments, owing to conduction failure.

Time from start of stimulation	Controls			Dinitrophenol 1×10^{-5}M		
	Amplitude mV	M	S	Amplitude mV	M	S
20 sec	5·45±1·28	14·74±3·50	14740	4·64±1·10	12·25±3·03	12250
2 min	5·49±1·01	14·85±2·58	88740	5·09±1·13	13·38±2·42	76050
6 min	6·28±2·57	16·76±5·63	278340	4·42±1·25	11·34±1·42	224410
10 min	6·72±2·90	17·95±6·03	486540	4·11 (4·13–3·70)	9·30 (11–7·94)	348250

In three of the six end-plates, conduction failure developed after seven–eight min of nerve stimulation. A brief recovery of conduction could be obtained when the preparation was left at rest for 30–60 sec. In these same six preparations the depolarization to acetylcholine was tested at the end of the 10 min periods of stimulation. It was the same before and after DNP, not only in absolute value (9.8 ± 2.7mV and 10 ± 1.8mV respectively), but also in onset and time course (10″–15″). This finding, together with the maintained height of m.e.p.p.s confirms the absence of any effect of DNP on the post-synaptic membrane.

DISCUSSION

In preliminary experiments it was demonstrated that in DNP-treated diaphragms the neuromuscular block developed before the failure of direct contraction only at high stimulation rates (100/min). At lower rates (15/min), the reduction in muscular efficiency seemed to be the prevailing factor, but, even in these conditions, the safety factor of the neuromuscular junction is impaired by DNP, as shown by the enhanced blocking effect of tubocurarine and nicotine. The drug, however, does not impair the transmission of

high frequency impulses, because the indirect tetanic contraction is well sustained ; the reduced or abolished post-tetanic potentiation suggests that the site of action is pre-synaptic (Standaert & Adams, 1965). The demonstration of this effect is offered by the reduced acetylcholine output from stimulated preparations. The reduction is directly related to DNP concentration, incubation temperature and stimulation rate ; it develops either in dyflos- or neostigmine-treated diaphragms and is not reversed by choline.

The enhancement of the effect, both at higher temperatures and at higher stimulation rates, is in agreement with the hypothesis of metabolic damage at the motor nerve endings. The concurrent exhaustion of acetylcholine content after stimulation suggests that the reduced output is due, at least partly, to the lower levels of neurotransmitter available. The finding that the percentage reduction of release is greater than that of stores (see Table 1), does not argue against the above suggestion because the available acetylcholine is only a part of the total amount present in the tissue, above all during esterase inhibition (MacIntosh, 1963 ; Hebb *et al.*, 1964). Therefore the percentage reduction of acetylcholine " actually " available may be much higher than that calculated. The depletion in acetylcholine stores seems to result from the uncoupling effect of the oxidative phosphorylation rather than from a direct inhibition of the last steps of acetylcholine synthesis: DNP, in fact, lowers ATP levels in the hemi-diaphragms, but it does not change choline acetyltransferase activity and its effect on acetylcholine release is unaffected by choline.

In contrast with the reduced acetylcholine output from the stimulated preparations is the ineffectiveness of DNP on the resting release. The lack of any increase in the resting release apparently contradicts the enhanced frequency of m.e.p.p.s described by Kraatz & Trautwein (1957). The disagreement, however, is easily explained in the light of two arguments: firstly DNP does not increase m.e.p.p. frequency in DFP-treated diaphragms ; and secondly loss of acetylcholine during the rest is only partly due to m.e.p.p. frequency (Krnjević, 1963). Therefore, the possible slight increase in m.e.p.p.s by DNP cannot be detected by bioassay.

The electrophysiological findings offer further explanations as to the mechanism of action of DNP. Unlike HC3, the metabolic inhibition does not reduce the m.e.p.p. height (Elmquist & Quastel, 1965a) even after prolonged nerve stimulation at high frequency: in other words, the quantum size remains the same, in full agreement with previous findings (Kraatz & Trautwein, 1957). On the other hand, the behaviour or the m.e.p.p. frequency seems to differ from that described in the frog neuromuscular junction, for DNP increases the junctional spontaneous activity in rat diaphragms kept in normal Tyrode solution. The increase is slight or absent in $MgCl_2$-blocked or DFP-pretreated preparations. In the former conditions, $MgCl_2$ may be suspected of hindering the spontaneous neurosecretion (Hubbard, 1961), in the latter, a convincing explanation cannot be advanced. It may only be noted that some end-plates failed to show increased m.e.p.p. frequency even in experiments carried out by Kraatz & Trautwein in the presence of neostigmine 1×10^{-6} and with a higher DNP concentration.

The reduction in the membrane potential of the motor nerve endings may be accepted as one of the causes of the DNP effect on spontaneous neurosecretion, taking into account the reversal by hyperpolarization. The fall of membrane potential is further substantiated

by the conduction failures, which frequently develop during long-lasting stimulation at higher rates, according to similar observations made after treatment with inhibitors of the membrane ionic pump (Birks, 1963) and by the reduction in the e.p.p amplitude, detected in poisoned preparations even during short periods of stimulation, suggesting reduced recruitment (Hubbard & Willis, 1962) and not exhaustion of acetylcholine stores.

On the other hand, the inverse relationship between e.p.p. height and stimulation rate (Lundberg & Quilisch, 1953) seems to be normal to judge by the parallel decline in e.p.p.s at higher frequencies both before and during DNP treatment. This fact suggests that the recovery cycle in the motor nerve endings is unaffected by DNP. The first conclusion to be drawn is that DNP 1×10^{-5}M probably produces a small decline in the transmembrane potential of mammalian motor nerve endings as it does at the amphibian neuromuscular junction. The second and, in our opinion, more interesting aspect of the effects of DNP concerns the availability of quanta. The long-lasting stimulation at 10/sec in d-tubocurarine-blocked diaphragms shows the greater and faster decline in e.p.p. height during DNP. This effect, explained by Kraatz & Trautwein as a reduced recruitment ensuing from the depolarization of motor nerve endings, is in similar experimental conditions associated with a remarkable depletion in acetylcholine stores (see Table 1). The exhaustion of available acetylcholine may therefore be the prevailing cause of the reduced release.

When the experiments are performed in $MgCl_2$-blocked preparations it is possible to demonstrate that the lower e.p.p. height during long-lasting stimulation is due to the reduction in the number of quanta the e.p.p.s contain.

During a 10 min tetanus at 10/sec the e.p.p. quantum content remains about 8 and does not vary throughout the stimulation period. In DNP-poisoned diaphragms the quantum content progressively decreases without, however, reaching levels of statistical signficance. Evidently, in this condition, the 48,000 quanta released in 10 min (Table 4) are too few to disclose any effect on their rate of formation.

When, on the other hand, the stimulation rate is higher (50/sec), the quantum content of normal e.p.p.s progressively increases from 14 to 17 in ten min, giving a total release of about 300,000 quanta in six min (Table 5). The number of quanta released in six min is close to that released after a 10 min stimulation at 6/sec in normal Tyrode solution assuming a quantum content of about 50–100 (Liley, 1956). Moreover, this value corresponds to the quantum content of a normal motor nerve ending at rest (Elmquist & Quastel, 1965*a*).

As the synthesis rate greatly increases during nerve stimulation, to compensate for the loss of neurotransmitter (MacIntosh, 1963), the quantum content of the e.p.p.s at the end of the stimulation period can be close to the initial one, only when the replenishment is efficient. This ability is lost by DNP-poisoned nerve endings. The second conclusion, therefore, may be as follows: DNP impairs the resynthesis rate of normal size quanta in the course of high rates of stimulation. In other words, the damage to oxidative phosphorylation limits the availability of the quanta. Bearing in mind the hypothesis advanced by Elmquist & Quastel (1965a), it may be suggested that this effect is linked to the unavailability of material required for the normal quanta formation. The selective inhibition of acetylcholine synthesis reduces the size, but not the number of neurosecretory units.

In contrast with the complex action of DNP on the motor nerve endings is the ineffectiveness of the drug on the post-junctional membrane as shown by firstly, the unaltered degree and time course of acetylcholine depolarization at the end of the tetanic stimulation, and, secondly, the maintained m.e.p.p. height and e.p.p. height, shape and duration. The uncoupling of oxidative phosphorylation clearly does not interfere with the development and summation of post-synaptic potentials.

SUMMARY

1. The effects of 2,4-dinitrophenol (DNP) on the neuromuscular junction of guinea-pig and rat diaphragms has been investigated.

2. The neuromuscular block exerted by DNP developed before failure of muscular contraction only in the case of high stimulation frequencies. The safety factor of the transmission was reduced by DNP also at lower frequencies, as shown by the enhanced blocking effect of tubocurarine and nicotine. Tetanic responses were, however, well sustained.

3. Acetylcholine release from indirectly stimulated diaphragms was lowered by DNP. The effect was directly related to drug concentration, stimulation rate and temperature. The resting release of acetylcholine was unaffected. Concomitantly with the reduction in transmitter output, the acetylcholine tissue stores were depleted, suggesting that the reduced output may be related to the exhaustion of acetylcholine available for release.

4. DNP reduced the ATP content of hemi-diaphragms, but did not affect choline acetyltransferase activity and its effect on release was not reversed by choline. Therefore the drug does not affect the last step of neurotransmitter synthesis.

5. M.e.p.p. frequency was increased by DNP in diaphragms kept in normal Tyrode solution and their amplitude remained the same both before and after long-lasting nerve stimulation which considerably reduced e.p.p. amplitude in poisoned preparations.

6. The quantum content of e.p.p.s recorded in Mg-blocked diaphragms was decreased by DNP at stimulation frequencies which promoted a substantial neurotransmitter release. Under these conditions the post-junctional chemosensitivity to acetylcholine remained normal.

7. The results are discussed in the light of the hypothesis that the uncoupling of oxidative phosphorylation restrains the rapid formation of normal size quanta.

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