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A COMPARISON OF THE ACTION OF TRIETHYLTIN WITH OTHER DRUGS ON CREATINE PHOSPHATE LEVELS IN RAT BRAIN AND DIAPHRAGM PREPARATIONS

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Abstract—The effect of triethyltin sulphate on the oxygen consumption and creatine phosphate synthesis of brain slices has been measured. The results have been compared with those obtained using chlorpromazine, dinitrophenol (DNP) and pentachlorophenol. In the presence of triethyltin and chlorpromazine, creatine phosphate synthesis was inhibited to a greater extent than oxygen uptake and creatine leaked from the slice into the medium. DNP and pentachlorophenol stimulated oxygen uptake and inhibited creatine phosphate synthesis, but did not cause a leakage of creatine from the slice. Identical experiments using diaphragm segments gave essentially similar results except for triethyltin which was without effect.

Experiments using a phrenic-nerve diaphragm preparation confirmed that in the presence of DNP and pentachlorophenol there is a failure in the response to electrical stimuli and a depletion of creatine phosphate in the diaphragm. In the presence of triethyltin the preparation also failed to respond normally to electrical stimuli but the creatine phosphate of the diaphragm was not lowered.

DURING the past ten years a wide variety of drugs and poisons have been tested for their ability to uncouple oxidative phosphorylations. Such tests have usually been made on isolated mitochondrial preparations, particularly from rat liver. The positive findings have been used in many instances to help explain the *in vivo* effects even though the response of animals to the different drugs often varied widely in character.

Several of the compounds examined appear to have a selective action *in vivo* on central nervous tissue; but when tested *in vitro* against liver mitochondrial preparations they are also active.[1, 2, 3]

McIlwain and co-workers[4] have shown that it is possible to measure the synthesis of creatine phosphate in cerebral cortex slices incubated in a saline medium containing glucose. The action of several drugs on such preparations has already been examined.[5, 6] In a similar manner it is possible to measure the creatine phosphate content of incubated segments of rat diaphragm, and a few studies of drug action have been made on this preparation.[7, 8]

In the present work a direct comparison has been made between the action of triethyltin sulphate, chlorpromazine, dinitrophenol and pentachlorophenol on the levels of creatine and creatine phosphate in rat cerebral cortex slices and segments of rat diaphragm. In this way it has been possible to compare the action of these compounds on nervous and non-nervous tissue under virtually identical conditions.

A few experiments have also been made using a rat phrenic nerve-diaphragm preparation.

METHODS

Adult male albino rats weighing about 200 g were used. Brain cortex slices were prepared and used as previously described.[9] Segments of diaphragm approximately 7 × 7 mm were cut with scissors. For experiments with diaphragm the conditions were identical to those used for brain slices except that the concentration of Ca^{++} in the incubation medium was lowered by a factor of 3 to give a final concentration of 0·001 M. Higher and more consistent values for creatine phosphate were obtained using this modified Krebs–Ringer phosphate medium, and the concentration of Ca^{++} approximated more closely that of Tyrode's solution used for the phrenic nerve–diaphragm experiments.

At the end of the incubation period the pieces of tissue were dropped quickly into 4·0 ml of ice-cold 10% (w/v) trichloroacetic acid, homogenized and centrifuged at −5 °C. Free and total creatine were determined on aliquots of the supernatant by the colorimetric method of Eggleton, Elsden and Gough[10] as used by Ennor and Rosenberg[11]. The difference between the two was taken as a measure of creatine phosphate. After removing the tissue from the Warburg flasks 1·0 ml of 30% (w/v) trichloroacetic acid was added to the incubation fluid and aliquots were taken for total creatine estimations.

The isolated rat phrenic nerve–diaphragm preparation was that of Bülbring[12]. It was suspended in a bath of 60 ml capacity containing Tyrode's solution[13] at 37 °C and aerated with a mixture of 95 per cent O_2 and 5 per cent CO_2. Stimulation was by means of rectangular impulses of 200 μsec duration. The routine procedure adopted was as follows: The preparation was mounted in position and left for 30 min while the nerve was stimulated once every 5 sec. This was followed by a series of tetanic stimulations of 50, 100 and 200/sec for 5 sec duration at 1 min intervals. A dose of the inhibitor was then added to the bath and a series of tetanic stimulations applied at 15 and 30 min. Exactly 5 min after the last tetanus, the preparation was plunged into 10% trichloroacetic acid at −5 °C, homogenized, centrifuged, and aliquots of supernatant taken for free and total creatine estimations as described above.

Lactic acid was estimated by the technique of Barker and Summerson[14] and pyruvic acid by the method of Friedemann and Haugen[15] with the modification for increasing the sensitivity previously described.[16] Triethyltin was estimated as described earlier.[9]

RESULTS

During the preparation of slices of brain cortex it has already been shown,[4] and confirmed by us, that the creatine phosphate content falls to low or zero levels. On incubation in a suitable medium containing glucose, creatine phosphate is re-synthesized reaching a fairly constant level of 1·6 μmoles/g. In Table 1 the effect of triethyltin sulphate and chlorpromazine hydrochloride together with those of dinitrophenol and pentachlorophenol (classical uncouplers of oxidative phosphorylation), on oxygen consumption, free and total creatine levels in brain slices is given. With low concentrations of triethyltin the oxygen consumption was slightly stimulated while the level of creatine phosphate was lowered. At concentrations of triethyltin which inhibited oxygen consumption between 15 and 55 per cent creatine phosphate synthesis was inhibited between 90 and 100 per cent, and at the same time there was a leakage of creatine from the slice into the medium. Chlorpromazine produced similar effects, although the difference between the percentage inhibition of oxygen consumption

and creatine phosphate synthesis was less marked, but the leakage of creatine from the slice to the medium was greater than in the presence of triethyltin. Dinitrophenol and pentachlorophenol on the other hand stimulated the consumption of oxygen and inhibited the synthesis of creatine phosphate. Even when creatine phosphate synthesis was completely inhibited creatine did not leak into the medium.

TABLE 1. EFFECT OF TRIETHYLTIN AND OTHER COMPOUNDS ON CREATINE LEVELS IN BRAIN SLICES

Compound	Concentration (M)	Tissue				Medium
		O_2 uptake	Free Creatine	Total Creatine	Creatine phosphate	Total Creatine
—	—	2·09–2·21(4)	3·49–4·76(4)	4·97–6·45(4)	1·48–1·69(4)	5·17–5·96(4)
Triethyltin sulphate	$2{\cdot}62 \times 10^{-7}$	2·38	4·46	5·53	1·06	5·61
	$3{\cdot}65 \times 10^{-7}$	1·86	4·4	4·46	0·06	6·67
	$5{\cdot}5 \times 10^{-7}$	1·26	4·0	3·89	0	7·36
Chlorpromazine-hydrochloride	$9{\cdot}7 \times 10^{-5}$	2·09	4·42	5·92	1·5	7·0
	$2{\cdot}9 \times 10^{-4}$	1·5	2·21	2·85	0·64	10·75
Dinitrophenol	$1{\cdot}1 \times 10^{-5}$	2·89	4·79	5·34	0·55	5·72
Pentachlorophenol	$2{\cdot}5 \times 10^{-6}$	3·46	6·34	6·18	0	5·92

Each flask contained 3 ml of Krebs–Ringer phosphate with 0·011 M glucose, triethyltin or other compounds as indicated, tissue slice of 50–60 mg wet wt and 0·2 ml of 20% (w/v) KOH in the centre well for absorption of CO_2. The manometers were placed in a bath at 37°, gassed for 5 min with 100% O_2, equilibrated for a further 10 min and O_2 uptake was measured at intervals up to 75 min. Results are the arithmethical mean of duplicate experiments except for the controls where the range of individual values are given followed by the number of experiments in parenthesis. O_2 uptake is expressed as μl of O_2/mg wet wt tissue per hr and creatine as μmoles/g wet wt tissue.

TABLE 2. EFFECT OF TRIETHYLTIN AND OTHER COMPOUNDS ON CREATINE LEVELS IN DIAPHRAGM SEGMENTS

Compound	Concentration (M)	Tissue				Medium
		O_2 uptake	Free Creatine	Total Creatine	Creatine phosphate	Total Creatine
—	—	0·9–1·25(5)	5·07–6·81(5)	14·02–17·25(5)	8·6–10·95(5)	10·71–13·7(5)
Triethyltin sulphate	$4{\cdot}9 \times 10^{-6}$	1·2	8·09	14·1	6·01	11·73
	$7{\cdot}9 \times 10^{-6}$	1·18	6·37	14·51	8·14	14·3
Chlorpromazine-hydrochloride	$1{\cdot}9 \times 10^{-4}$	1·39	6·44	10·81	4·37	16·7
	$3{\cdot}2 \times 10^{-4}$	1·16	3·72	3·45	0	21·1
Dinitrophenol	$5{\cdot}6 \times 10^{-6}$	1·65	8·5	11·28	2·68	11·06
	$1{\cdot}1 \times 10^{-5}$	1·87	9·3	7·52	0	13·8
Pentachlorophenol	$8{\cdot}3 \times 10^{-7}$	1·81	7·26	11·39	4·13	10·0
	$1{\cdot}05 \times 10^{-6}$	1·9	13·11	16·76	3·65	12·4

For details see Methods and Table 1.

Similar experiments were carried out using cut segments of rat diaphragm. Unlike cerebral cortex slices, diaphragm segments only lose a small amount of creatine phosphate during the preparative procedure. The level of creatine phosphate at the time of

placing the tissue in the flasks was about 85 per cent of that found after incubation for 75 min. The action of the four drugs on diaphragm segments is shown in Table 2. Triethyltin, even at high concentrations compared with those active against brain slices, had little or no effect on oxygen consumption or creatine phosphate levels.

TABLE 3. DISTRIBUTION OF TRIETHYLTIN BETWEEN TISSUE AND MEDIUM WITH BRAIN AND DIAPHRAGM

Tissue	Concentration (M) of triethyltin		R
	Added in medium	Found in tissue	
Brain slice	$5{\cdot}3 \times 10^{-5}$	$10{\cdot}4 \times 10^{-4}$	20
	$1{\cdot}8 \times 10^{-5}$	$2{\cdot}7 \times 10^{-4}$	15
Diaphragm segment	$4{\cdot}9 \times 10^{-5}$	$7{\cdot}6 \times 10^{-4}$	16
	$1{\cdot}6 \times 10^{-5}$	$2{\cdot}9 \times 10^{-4}$	18

The amount of triethyltin found in the tissue after 75 min incubation was calculated as the molar concentration in the tissue fluid taking the tissue fluid as 80 per cent of the wet weight.

R is the quotient: molar concentration of triethyltin in tissue fluid/ molar concentration added in medium.

TABLE 4. CREATINE LEVELS IN BRAIN SLICES AND DIAPHRAGM SEGMENTS FROM RATS INJECTED WITH TRIETHYLTIN SULPHATE

Tissue	Animal	Tissue				Medium
		O_2	Free creatine	Total creatine	Creatine phosphate	Total creatine
Brain slice	Control	2·15	4·24	5·85	1·61	5·38
	Injected	1·34	3·72	3·85	0·13	6·94
Diaphragm segment	Control	1·11	5·91	15·77	9·87	11·8
	Injected	1·43	8·08	17·6	9·52	12·3

Conditions were identical to those described in Tables 1 and 2.

Since earlier work[9] had shown that after incubating brain slices in a medium containing triethyltin the concentration found in the slice was 17 times greater than that in the medium at the beginning of the experiment, it was possible that the lack of effect of triethyltin on diaphragm segments was due to a lower penetration of the drug into this tissue. However, values given in Table 3 show that equal amounts of triethyltin entered diaphragm segments as entered brain cortex slices.

Chlorpromazine did not inhibit the oxygen consumption of diaphragm segments but lowered the creatine phosphate level, and, as with brain slices, caused a marked increase in the amount of creatine in the medium. Dinitrophenol and pentachlorophenol affected diaphragm segments in the same way as they affected brain slices at similar concentrations.

It has already been demonstrated that it is possible to show an altered carbohydrate metabolism in brain slices prepared from rats injected with triethyltin.[9] Measurements were therefore made of the oxygen consumption and creatine levels in brain slices and diaphragm segments prepared from animals killed 2 hr after an

intraperitoneal injection of triethyltin sulphate 10 mg/kg. The results given in Table 4 show that brain slices were affected in the same way as when triethyltin was added *in vitro*, whereas diaphragm segments did not vary from the controls.

A few experiments were carried out using phrenic-nerve-diaphragm preparations. The procedure adopted is fully described under Methods. In the presence of the highest concentration of triethyltin used the preparation failed to respond normally to electrical stimulation but the level of creatine phosphate was not lowered (Table 5).

TABLE 5. EFFECT OF TRIETHYLTIN AND PENTACHLOROPHENOL ON RESPONSE TO STIMULI AND CREATINE LEVELS OF PHRENIC-NERVE DIAPHRAGM PREPARATION

Compound	Concentration (M)	Response to stimuli	Creatine phosphate μmoles/g
—	—		8·5
Triethyltin sulphate	$2{\cdot}5 \times 10^{-6}$	Failure to hold a tetanus of 100 and 200 p.s.	6·8
	5×10^{-6}	Failure to hold a tetanus of 100 and 200 p.s.	8·58
	1×10^{-5}	Failure to hold a tetanus of 100 and 200 p.s. and reduced response to single stimuli	8·0
Pentachlorophenol	$1{\cdot}88 \times 10^{-6}$	Contracture developed and a failure to hold a tetanus	0

For details see Methods.

TABLE 6. EFFECT OF TRIETHYLTIN AND OTHER COMPOUNDS ON AEROBIC AND ANAEROBIC GLYCOLYSIS OF BRAIN SLICES

Compound	Concentration (M)	Aerobic			Anaerobic
		Q_{O_2}	lactic acid μg/flask	pyruvic acid μg/flask	lactic acid μg/mg dry wt
—	—	12·4	186	11·4	46·3
Triethyltin sulphate	$1{\cdot}3 \times 10^{-6}$	7·4	440	5·5	27·5
Chlorpromazine HCl	$2{\cdot}4 \times 10^{-4}$	12·1	242	7·5	31·5
DNP	3×10^{-5}	—	—	—	45·0

Each flask contained 3 ml of Krebs–Ringer phosphate with 0·011 M glucose and triethyltin or other compounds as indicated. Average wet wt of tissue slice was 56 mg. The centre-well contained 0·2 ml of 20% (w/v) KOH. For aerobic experiments the gas phase was O_2 and for anaerobic experiments N_2 was used. After incubation for 75 min at 37° slices were removed and 3 ml of 18% (w/v) TCA was added. Samples of the flask contents were taken for lactic and pyruvic acid determinations. Q_{O_2} is expressed as μl of O_2/mg dry wt/hr, the aerobic lactic and pyruvic acids as μg of each acid/flask and anaerobic lactic acid as μg/mg dry wt of brain tissue.

After the addition of pentachlorophenol the preparation went into a state of contracture, failed to hold tetanic stimulations and creatine phosphate was depleted completely. These results were virtually identical to those of Barnes, Duff and Threlfall using dinitrophenol.[13] Addition of chlorpromazine hydrochloride at a concentration found to be effective in the Warburg experiments caused a white cloudy precipitate which interfered with the experiments.

DISCUSSION

As mentioned in the introduction a large number of studies have been devoted to testing the effect of drugs on the oxidative-phosphorylation capacity of mitchondrial preparations. All four compounds used in the present work have been shown to affect this process in rat liver mitochondria.[1, 2, 17] Although triethyltin and chlorpromazine inhibit both phosphate uptake and oxygen consumption in such preparations, the P : O ratio is lowered by both compounds. Dinitrophenol and pentachlorophenol increase oxygen consumption and decrease phosphate uptake. Fewer and sometimes conflicting results have been reported for similar experiments using brain mitochondria. This is probably due to the difficulties of preparing brain mitochondria which are metabolically stable.[18] Bernsohn, Namajuska and Cochrane[19] found that chlorpromazine inhibited oxygen and phosphate uptake of brain mitochondria, while Berger, Strecker and Waelsch[2] found that although chlorpromazine inhibited oxidative phosphorylation in liver mitochondria it was inactive against brain mitochondria. Dawkins, Judah and Rees[20], on the other hand, showed that there appeared to be no special difference between brain and liver mitochondrial preparations in their sensitivity to chlorpromazine.

The measurement of creatine phosphate in brain slices and diaphragm segments provides an alternative method for testing the effects of drugs on energy-rich phosphate compounds. The results with triethyltin sulphate show that it is an active inhibitor of creatine phosphate synthesis in brain slices (Table 1). Furthermore the synthesis of creatine phosphate appears to be more sensitive to triethyltin than oxygen consumption. In agreement with previous findings[9] triethyltin had virtually no effect on diaphragm segments. It could be shown that equal amounts of triethyltin entered both diaphragm and brain (Table 3). Thus triethyltin showed a selective action against metabolic processes of nervous tissue under the experimental conditions used. Chlorpromazine showed a definite inhibition of creatine phosphate synthesis in brain slices at a concentration of $2{\cdot}9 \times 10^{-4}$ M. Lindan, Quastel and Sved[21] showed that chlorpromazine inhibited the incorporation of glycine-^{14}C into proteins of brain slices at this concentration whereas oxygen consumption was less sensitive.

A striking difference between triethyltin and chlorpromazine became apparent when creatine levels of diaphragm segments were measured. Chlorpromazine lowered creatine phosphate at the same concentrations as it inhibited creatine phosphate synthesis in brain slices. It did not therefore share the selective effect on nervous tissue shown by triethyltin.

A common finding with triethyltin and chlorpromazine was that they both caused a leakage of creatine from brain slices into the medium. In a recent study of amino acid levels in brain slices,[22] it was shown that chlorpromazine caused an increase in the amount of glutamic acid and γ-amino-butyric acid appearing in the incubation fluid when glucose was added as substrate. The authors suggested that certain psychotropically active compounds tested by them, including chlorpromazine, may have two distinct modes of action, one on certain phases of the respiratory chain and the other on the membrane permeability. The present findings would lend support to such a view since in the presence of dinitrophenol and pentachlorophenol creatine phosphate levels in brain slices could be reduced to zero without there being an accompanying leakage of creatine into the membrane. Both compounds are known to act on the respiratory chain. The separate effects on membrane permeability may in some way

help to explain the seemingly contradictory results on aerobic and anaerobic glycolysis of brain slices with triethyltin and chlorpromazine. Both compounds bring about an increase in lactic acid formation under aerobic conditions at concentrations which, under anaerobic conditions inhibit lactic acid formation (Table 6). Dinitrophenol did not inhibit anaerobic glycolysis of brain slices. It is highly probable that anaerobiosis is detrimental to the cells[23] so that the structural integrity of brain slices under anaerobic conditions may become more sensitive to certain agents. Neither chlorpromazine[19] nor triethyltin[16] inhibit anaerobic glycolysis of brain homogenates, which also suggests that the inhibition seen in brain slices is related to some structural alteration only apparent when whole-cell preparations are used.

Some pharmacological studies with triethyltin have already been reported.[24] Experiments using a phrenic-nerve diaphragm preparation showed that triethyltin at $2{\cdot}5 \times 10^{-6}$ M brought about a failure to hold a tetanus and at higher concentrations a reduced response to single stimuli. The response of the diaphragm to direct stimuli was not affected by triethyltin at 1×10^{-5} M.[24] The level of creatine phosphate was not lowered in the nerve-diaphragm preparation even after it failed to respond to stimuli in the presence of triethyltin. The results are quite dissimilar to those with DNP[13] or with pentachlorophenol (Table 6) where failure to respond to stimuli is accompanied by a large fall in creatine phosphate. Although no measurements have been made of the metabolism of isolated nerves in the presence of triethyltin it is possible that the phrenic nerve is sensitive to triethyltin since it has been shown that glucose metabolism by brain cortical grey and white matter and pieces of spinal cord are all inhibited in a similar manner.[9]

During the course of studies carried out in these laboratories on the mechanism of triethyltin intoxication attempts have been made to correlate the results of experiments carried out *in vitro* with changes occurring in the whole animal. Some of the difficulties arising have already been pointed out.[25] There seems little doubt that the predominant features of triethyltin poisoning are indicative of a central nervous effect.[26] Considerable progress towards understanding the production of an oedematous lesion in the brain by triethyltin has been made by Parsons[27]. By using ^{24}Na he was able to show that the earliest change produced by triethyltin *in vivo* is a stagnation of the sodium pools of the cerebrospinal fluid and brain. It is suggested by the same author that triethyltin may inhibit the supply of energy necessary to maintain the mechanism which controls cerebral salt and water balance. From results of experiments with triethyltin *in vitro* using either brain mitochondria or brain slices this would seem a possibility. However, no lowering of creatine phosphate or adenosine triphosphate (ATP) was found in brains of rats injected with triethyltin sulphate.[25] Similarly chlorpromazine, well-known for its psychotic action, does not cause a lowering of ATP in brains of injected animals.[28] On the other hand when 3:5 dinitro-O-cresol (a compound which closely resembles the nitro- and halo-phenols) was administered to rats *in vivo* it caused a marked decrease in the creatine phosphate and ATP content of brain.[29] ATP was also shown to be lowered in heart and diaphragm muscle but creatine phosphate values are not given.[29]

It would seem that a close agreement exists between the known effects of certain substituted phenol compounds on enzyme systems *in vitro* and their effect on the metabolism of animals *in vivo*. With triethyltin and chlorpromazine many discrepancies still exist between the results of studies made *in vivo* and *in vitro* and it is as

yet impossible to explain the predominance of central nervous effects by these two compounds in the living animal.

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