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LIPID BIOSYNTHESIS AND CHEMICALLY INDUCED PARALYSIS IN THE CHICKEN

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Dyflos (di*iso*propylphosphorofluoridate, DFP) retards the synthesis of lipid in the sciatic nerve of the chicken *in vivo*. A similar inhibition occurs *in vitro*, but only at relatively high concentrations (10^{-3}M) of the drug. The major portion of the ^{14}C supplied as acetate, and incorporated into lipid, is found with the fatty acids. Attempts to separate the lipids involved by paper chromatography were unsuccessful.

The anticholinesterase properties of substances which are capable of producing "ginger" paralysis in man and experimental animals have led some workers to the belief that a direct correlation exists between these two properties. Bloch (1941) and Hottinger and Bloch (1943) considered that the neurotoxic syndromes resulting from triorthocresol phosphate (TOCP) were due to inhibition of true-cholinesterase at motor end-plates. It was subsequently shown, however, that TOCP is a highly specific inhibitor of pseudo-cholinesterase, an enzyme which does not occur at motor end-plates (Mendel and Rudney, 1944; Earl and Thompson, 1952a).

Earl and Thompson (1952b) followed cholinesterase levels in the nervous system of chickens poisoned with TOCP. They concluded that the biochemical lesion which led ultimately to paralysis was the prolonged depression of pseudo-cholinesterase activity, particularly in the spinal cord. As supporting evidence they pointed out that all known compounds which produce "ginger" paralysis are selective inhibitors of pseudo-cholinesterase. Davison (1953) and Austin and Davies (1953) examined a wide range of anticholinesterases, many of them highly selective inhibitors of the pseudo-enzyme. Further, Austin and Davies (1953) maintained the pseudo-cholinesterase activity of chicken spinal cord at a low level for 21 days with methyl*iso*propylphosphonofluoridate, a non-paralysing anticholinesterase, but this treatment did not lead to paralysis.

The biosynthesis of phospholipid in the nervous system of the chicken has been shown by Webster (1954) to be unaffected by TOCP. Strickland, Thompson, and Webster (1956) found that dyflos (di*iso*propylphosphorofluoridate (DFP) and NN'-di*iso*propylphosphorodiamidic fluoride (mipafox) inhibit oxygen uptake and oxidative phosphorylation in chicken brain slices only at very high concentrations of the drug, and they concluded that these effects were unlikely to play a part in degeneration of the nerve fibres.

The insecticide mipafox has been shown by Barnes and Denz (1953) to cause paralysis in chickens. The repeated injection of mipafox into rats results in an abnormal metabolism of the sciatic nerves (Majno and Karnovsky, 1955). When these nerves were incubated with ^{14}C acetate the rate of oxygen consumption was depressed 16% and of ^{14}C incorporation into lipid 50%.

Majno and Karnovsky (1955) confined their studies to animals which had been exposed to mipafox for a period of three to five weeks. Although rats are not susceptible to nerve degeneration produced in this way, the period of time between the initial administration of the drug and the examination of the nerve tissue is such that changes in metabolism found could be of a secondary nature and not necessarily directly connected with the primary biochemical lesion.

A similar study has been made here of some aspects of the metabolism of sciatic nerve of the fowl, when poisoned with dyflos, and results similar to those of Majno and Karnovsky obtained.

METHODS

The Formation of Nerve Lipids.—Both sciatic nerves were dissected from normal or dyflos-treated birds as quickly as possible after death and adhering

tissues and fat removed by stroking with a scalpel blade. The nerves were quickly weighed and placed in an ice-cold solution of phosphate buffer (Krebs and Henseleit, 1932) until used. After drying with filter paper, the nerves were placed in Warburg vessels.

When the effect of dyflos was being studied *in vivo*, 1.5 ml. of Krebs–Henseleit phosphate containing ^{14}C acetate was added to the flask. For *in vitro* studies, 1.0 ml. of the buffer containing ^{14}C acetate was added, after the addition of 0.5 ml. of buffer containing dyflos. To all flasks was added 0.3 ml. of a solution containing 60 μM./ml. of glucose and 24 μM./ml. of sodium acetate. The centre well contained rolls of No. 40 Whatman paper saturated with 0.2 ml. of 30% potassium hydroxide, and the side arm 0.3 ml. of 5 N sulphuric acid. The vessels were gassed with oxygen and incubated for 3 hr. at 37°.

Readings of oxygen consumption were taken at 30 min. intervals, and the uptake was linear over 3 hr. Enzyme action was stopped by tipping the acid from the side arm. The flasks were left overnight in the cold room, after which the nerves were transferred to stoppered test tubes, and washed 20 times with 2 ml. portions of 0.01 M sodium acetate. They were finally washed with 0.01 N hydrochloric acid and dried in a desiccator over sodium hydroxide and calcium chloride, or alternatively they were freeze dried. The final acid washing and subsequent drying was performed to ensure removal of all acetate.

When dry, the nerves were homogenized in a Potter–Elvehjem homogenizer (Potter, 1945) in a 2:1 chloroform:methanol mixture. Water, equal to one-fifth of the volume of solvent, was added, and the tube contents thoroughly mixed (Folch, Lees, and Sloane-Stanley, 1954).

The chloroform layer was centrifuged down and transferred to a clean vessel. The solution was made up to a volume of 10 ml. from which 2 ml. aliquots were removed to an oxidizing vessel (Calvin, Heidelberger, Reid, Tolbert, and Yankwick, 1949).

After removal of the solvent on the water bath, the lipid extracts were oxidized to carbon dioxide (Calvin *et al.*, 1949), which was collected in a saturated solution of barium hydroxide. The barium carbonate was washed twice in the centrifuge with carbon-dioxide-free water and once with acetone. It was suspended in ethanol and transferred with a Pasteur pipette to a weighed 1 in. diameter stainless steel planchette and dried slowly under an infra-red lamp. When dry it was weighed and counted in a Tracerlab gas flow Geiger counter. All counts were corrected to infinite thinness in a sample area of 4.7 sq. cm. by multiplying by the appropriate factor determined from the sample weight. The filter paper rolls were removed from the Warburg flasks and placed in a 25 ml. standard flask. The centre well of the Warburg vessel was washed out six times with small quantities of carbon-dioxide-free distilled water and transferred to the volumetric flask.

The flasks were made up to volume and allowed to stand overnight. 5 ml. aliquots were placed in a centrifuge tube and to this was added 0.5 ml. of 0.29 M potassium carbonate as carrier, and 2 ml. of 0.2 M barium chloride. The precipitate barium carbonate was washed, plated, and counted as before.

The Separation of Lipids.—An attempt was made to separate the extracted lipids by a modification of the spot test method of Hack (1953), who used filter paper disks. Strips of Whatman No. 3 paper, 28 cm. by 7.5 cm., were washed with a 4:1 chloroform:methanol mixture and dried. 1 ml. of the lipid extract containing approximately 2 mg. of lipid was applied slowly, while being dried with a current of hot air, to a line 4 cm. from the bottom of the filter paper strip. The paper was suspended in a jar containing 4:1 chloroform:methanol for half an hour. Filter paper strips, saturated with solvent, lined the walls of the jar to maintain a saturated solvent vapour within the jar. After equilibration the lower end of the paper was immersed in the solvent and the solvent front allowed to run to a pencil line 5 cm. from the origin. The paper was dried and the process repeated twice. Acetone was run in a similar manner to a line 20 cm. from the origin. Two more runs were carried out with acetone, the paper being dried between each run. Finally four runs were made with methanol to a line 15 cm. from the origin.

The initial papers were cut into strips longitudinally and the following tests applied: (1) The paper was exposed to acid ammonium molybdate, followed by H_2S vapour (Hanes and Isherwood, 1949). A blue band showed up phosphorus against a buff background. (2) 0.01% ninhydrin in butanol was sprayed on the paper which was subsequently dried at 105°. Amine lipids produced a typical purple ninhydrin colour. (3) The paper was dipped in a cooled 1:1 sulphuric acid:acetic anhydride mixture. A pink to blue stain showed the presence of cholesterol. (4) Exposure to osmic acid vapour produced a brown to black band at the site of unsaturated fatty acids. Staining with fat stains such as Nile blue and Sudan IV was too unspecific to be of any value. Hack (1953) stated that the exposure of choline lipids to iodine shows the presence of choline as a transient yellow-brown colour. While this has been found to be so, Brante (1949) has shown that the test is not specific for choline.

For ^{14}C measurement the papers were cut into strips transversely corresponding to the positions of the lipids as shown by the spot tests. These strips were cut up finely with scissors and extracted four times with 4:1 chloroform:methanol. The extracts were filtered into Van Slyke oxidizing vessels where the solvent was evaporated. Lipids extracted from paper corresponding to the acetone front were saponified with 5 ml. of 10% KOH in 50% ethanol at 100° overnight. The ethanol was removed by evaporation and non-saponifiable lipids removed by extraction with four portions of petroleum ether (60 to 80°), each of 4 ml. The aqueous layer was extracted, after acidification, with six 4 ml. lots of ether. The extracted lipids were then oxidized and

the resulting barium carbonate washed, plated, and counted.

The Isolation of Fatty Acids.—The distribution of isotopic carbon was studied in another way, namely by a separation of the lipids into non-saponifiable matter, fatty acids, and fat alcohols. The saponification technique of Masoro, Chernick, Chaikoff, and Felts (1950) was applied to the lipid extracts. The petroleum ether extracts of non-saponifiable matter and the ether extracts of fatty acids were dried on the water bath, while the aqueous solution remaining after precipitation of fatty acids was freeze dried. All three samples were oxidized and counted as before.

RESULTS

Majno and Karnovsky (1955) confined their studies to the metabolic state of nerves after prolonged exposure to mipafox. It is important, however, to learn what changes occur during the latent period which, in the fowl, extends up to fourteen days after dyflos ingestion. Groups of birds were injected subcutaneously with dyflos 1 mg./kg. and atropine, and, from these, individuals were killed at intervals. At the same time a comparable number of normal animals were killed as controls. The sciatic nerves were removed and the rate of oxygen consumption, carbon dioxide production and lipid synthesis measured. Table I summarizes the results.

TABLE I
THE METABOLISM OF FOWL PERIPHERAL NERVE AFTER DYFLOS INTOXICATION *IN VIVO*

Time of Killing (Days)	No. of Nerves/ Group	O_2 Consumption/ 100 mg. of Nerve μl. O_2/hr.		$^{14}CO_2$ Production/ 100 mg. of Nerve Counts/min.		Lipid Synthesis/ 100 mg. of $BaCO_3$ Counts/min.	
		Control	Dyflos	Control	Dyflos	Control	Dyflos
1	13	12·24 ±0·65	11·48 ±0·43	13,520 ±960	13,420 ±1,040	444 ±74	266 ±28
5	8	7·1 ±0·25	8·4 ±0·42	7,766 ±515	8,560 ±802	341 ±27	319 ±21
17	5	7·7 ±0·69	7·7 ±0·84	3,789 ±129	4,140 ±468	175 ±21·6	134 ±22·5

Oxygen consumption is expressed as μl./100 mg. of fresh nerve/hr., and carbon dioxide production as counts/100 mg. of barium carbonate/min. Since Folch *et al.* (1954) have found that the chloroform layer of the lipid extract contains all lipids, and that no other material is extracted, lipid isotope content has been referred to the mass of barium carbonate formed from the oxidation of the lipid, rather than to dry weight of lipid. The rate of lipid synthesis then is expressed as counts/min./100 mg. of barium carbonate.

Twenty-four hours after the administration of dyflos there is a significant fall in the rate of formation of lipid from acetate. At 5 and 17 days the rate is still lower in the experimental group than in the control group.

The action of dyflos on lipid biosynthesis by sciatic nerve is shown in Table II.

TABLE II
THE METABOLISM OF FOWL PERIPHERAL NERVE AFTER DYFLOS INTOXICATION *IN VITRO*

Dyflos Conc. M	No. of Nerves/ Group	O_2 Consumption/ 100 mg. of Nerve μl. O_2/hr.		$^{14}CO_2$ Production/ 100 mg. of Nerve Counts/min.		Lipid Synthesis/ 100 mg. of $BaCO_3$ Counts/min.	
		Control	Dyflos	Control	Dyflos	Control	Dyflos
10^{-2}	2	11·28	5·68	15,500	3,135	1,182	228
10^{-3}	11	7·84 ±0·53	7·31 ±0·52	8,935 ±1,162	7,950 ±1,064	349 ±22·4	230 ±21·2
10^{-4}	5	7·90 ±0·72	10·00 ±0·56	7,905 ±1,800	8,800 ±774	422 ±84	360 ±118

A concentration of 10^{-2} M dyflos reduces very considerably the rate of lipid synthesis, but at the same time markedly inhibits the rate of oxygen consumption and of carbon dioxide production of the nerve tissue.

At a concentration of 10^{-3} M, however, when t is calculated on the basis of a paired experiment, the difference in the rates of lipid synthesis between control and experimental groups is significant at the 0.1% level. No difference was found between the rates of oxygen uptake or carbon dioxide output in the control or experiment groups, when submitted to a t test. At 10^{-4} M the trend towards reduction of lipid synthesis is still apparent, although no longer statistically significant.

The extent of chromatographic separation of lipids may be seen in Table III. A complete

TABLE III
THE REACTION OF LIPIDS AT SOLVENT FRONTS TO VARIOUS SPOT TESTS
The probable lipids present shown in the last column are based on the known solubilities of the lipids indicated.

Solvent Front	Phosphate	Ninhydrin	Cholesterol	Osmic Acid	Probable Lipids Present
Origin	Trace	Small	Nil	Small	
$CHCl_3+CH_3OH$ ..	Intense	Intense	,,	Intense	Sphingomyelin, phosphatidyl-serine, diphosphoinositide gangliosides, sulphatides
CH_3OH	Medium	,,	,,	Medium	Lecithin, plasmalogens, phosphatidyl-ethanolamine
Acetone	Trace	Nil	Intense	Intense	Cholesterol, neutral fat, free fatty acids

separation was not possible by this technique, but a useful differentiation into lipid classes was obtained.

The recovery of isotopic carbon after the application of this technique was poor, however, only 37 to 54% recovery being obtained (Table IV).

TABLE IV
% RECOVERY OF ISOTOPIC CARBON IN LIPIDS SEPARATED BY VARIOUS SOLVENTS ON PAPER

Origin	$CHCl_3$+ CH_3OH	CH_3OH	Acetone		Total Recovery
			Non-sapon.	Fatty Acids	
0	8·2	10·3	7·5	12·3	38·3
0	8·7	6·3	9·3	12·9	37·2
0	13·4	5·0	11·6	24·6	54·6
0	10·2	6·6	15·7	13·3	45·8
0	11·3	8·9	11·3	16·0	47·2

About half of the lipid, in terms of ^{14}C content, was found to be firmly bound to the paper. This was demonstrated in two ways. A control sample of 0.4 ml. of the original chloroform: methanol extract of nerve was transferred to an oxidizing vessel and the lipid solute oxidized. First 0.4 ml. of extract was allowed to spread on Whatman No. 3 paper and, immediately after drying, the lipid was extracted from the paper with chloroform: methanol. Secondly 0.4 ml. was dried on to a line on a filter paper strip as before and chromatogrammed with chloroform: methanol. The lipid which travelled with the solvent front was extracted with chloroform: methanol. Of the added ^{14}C, 45.4% was recovered from the unchromatogrammed sample and 50.5% from the chromatogrammed lipid. A further 5.6% of the added isotope was recovered with hydrochloric acid from the unchromatogrammed sample, but neither acidic nor basic solvents were capable of extracting more isotope (Table V).

TABLE V
% RECOVERY OF ISOTOPIC CARBON IN LIPIDS ABSORBED ON VARIOUS REGIONS OF FILTER PAPER
The lipid stain on paper was not chromatogrammed.

Eluent	Lipid Stain on Paper	$CHCl_3$+CH_3OH Front	Origin
$CHCl_3$+CH_3OH (4:1)	45·4	50·5	—
1 N HCl in CH_3OH+C_2H_5OH (1:1)	5·6	0	0
Pyridine	0	—	0
Total recovery ..	51	50·5	—

When the lipids were saponified, and fatty acids obtained by soxhlet extraction of the filtered precipitate resulting from acidification of saponified material, very poor recovery of the ^{14}C was obtained. Extraction of the precipitated fatty acids with ether in a separating funnel, however, resulted in a correct balance sheet, as is shown in Table VI. This suggests that a considerable amount of the label is being lost during the Masoro procedure probably in the form of low molecular weight fatty acids.

TABLE VI
TYPICAL RECOVERIES OF ^{14}C IN FRACTIONS OBTAINED FROM SAPONIFIED LIPID, EXPRESSED AS COUNTS/MIN. CALCULATED TO INFINITE THINNESS

	Total	Non-saponifiable	Fatty Acids	Alcohols	Sum of Fractions
Fatty acids soxhlet extracted ..	550	38	208	25	271
Fatty acids extracted in separating funnel ..	262	42	216	—	258

DISCUSSION

In this type of chronic poisoning, the observed symptoms are only secondary. In the case of dyflos poisoning, very little free dyflos is present in tissues, even 20 min. after its administration, whereas the first signs of chronic poisoning take from 8 to 14 days to appear. The biochemical change which finally produces nerve degeneration probably occurs then shortly after dyflos absorption. Smith and Lillie (1931) thought that the delay in the onset of symptoms produced by TOCP intoxication was due to a slow release of the compound from lipoidal tissue in which it was dissolved, but this now appears to be unlikely.

The lipid components of nerve amount to more than 50% of the dry weight of that tissue, and consequently any interference with the maintenance of this lipid fraction could profoundly affect the overall function as well as structure of the nerve. The ingestion of dyflos by fowls leads to a significant fall in the rate of synthesis of lipid during the first day of poisoning. There is still a trend towards a lower rate of synthesis at 5 and at 17 days. Since the fall is most marked shortly after dyflos absorption, and the effect is produced on such an important component of nerve, namely lipid, it may well be that this depression of lipid synthesis is the initial cause of the degenerative changes. Although a recovery of the synthetic mechanism is seen, the nerve may not be able to repair the damage sufficiently to prevent the ultimate result of complete degeneration of nerve structure.

In vitro, dyflos inhibits lipid synthesis. This inhibition is most marked at a dyflos concentration of 10^{-2} M, but there is also a very considerable depression of respiration by the nerve, and hence no conclusion can be reached regarding the action

of dyflos, at this concentration, on lipid synthesis. When lower dyflos concentrations are used, however, there is still a marked depression of the rate of lipid synthesis. The difference between means at 10^{-3} M dyflos, as shown in Table II, is significant at the 0.1% level when subjected to a *t* test, while no difference exists between the means of the controls or the experimental groups in the columns showing the rates of oxygen consumption and carbon dioxide production.

When the concentration of dyflos is reduced to 10^{-4} M an inhibition is still evident, although small. These results support those obtained from the series of experiments *in vivo*.

Although organo-phosphorus anticholinesterase drugs are commonly considered as substances which inhibit cholinesterase at extremely low concentrations *in vitro*, and thus exert their toxic action *in vivo*, this high affinity for enzymes need not always be necessary for an effect to be produced *in vivo*. A discrepancy exists between *in vivo* and *in vitro* results which cannot be adequately explained at present. It is perhaps significant in this regard that a relatively high dosage of dyflos must be administered to the animals to produce the neuritis. The dosage required for fowls is so high that death would result in the majority of the animals if atropine were not given together with the dyflos. If an even distribution of dyflos throughout the fowl tissues is assumed, the concentration would be no higher than 5.5×10^{-6} M. This concentration is too low to produce any substantial effect on lipid synthesis *in vitro*, but *in vivo* a 1 mg./kg. dose results in a significant inhibition.

Separation of the extracted lipid by paper chromatography was unsuccessful. The results obtained suggest that about half of the labelled lipid is firmly bound to filter paper. The absorbed material may be largely kephalin, since Hack (1953) states that kephalin is strongly absorbed on filter paper, and he suggests that all extracts should be filtered through sintered glass, for this reason.

The incomplete recovery of ^{14}C in the fractions separated after saponification is probably due to loss of ^{14}C incorporated into fatty acids of low molecular weight. Such fatty acids would remain in solution after the acidification of the saponified material, and would subsequently be removed during the freeze-drying operation. Table VI shows that at least 80% of the total counts are due to ^{14}C in fatty acids. About 10% of the label appears in the non-saponifiable fraction and less than 5% with the alcohols. In view of this, dyflos must depress lipid synthesis during the formation of fatty acids.

It is perhaps significant that a similar effect can be obtained with fowls, after one dose of a paralysing agent, to that which results when rats are subjected to repeated doses of such an agent. Rats, which are not susceptible to this form of paralysis, require prolonged treatment with the drug to produce any degenerative changes in nerve tissue.

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