of different ages, a peak value occurring on the eighth day. Our quantitative results also suggest that the metabolic mechanism may not be the same throughout the whole of the period of growth, since it was found that most of the acetic acid appeared during the first 6 days, while succinic acid was most actively formed during the later stages.

The metabolic pathways by which these substances are produced, and the differences between the metabolism of the flagellate and that of the intracellular stages of the parasite, must remain conjectural until more information is available. In particular, it would be interesting to know the part played by phosphorylation reactions and carbon dioxide fixation, and also the relationship between carbohydrate and protein metabolism which has been claimed by Salle & Schmidt (1928). Our findings, however, indicate the formation of several substances, the presence of which is not inconsistent with metabolic cycles known to occur in some other cells.

SUMMARY

1. A study of the biochemical activities of *Leishmania donovani* has been made *in vitro*.

2. During the period of growth glucose disappears from the culture medium, the pH of which falls in a manner related to the growth of the organisms.

3. Carbon dioxide, acetic, pyruvic, succinic, and probably lactic acids were produced, and their quantitative production during the growth of cultures was followed.

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Tricresyl Phosphates and Cholinesterase

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It has been stated that tri-*o*-cresyl phosphate (TOCP) is an inhibitor of cholinesterase *in vitro* (Hottiger & Bloch, 1943; Bloch, 1943; Earl & Thompson, 1952*a*). As a general rule, however, such stable organophosphorus compounds are poor inhibitors. Amongst a series of related compounds, a relationship between stability to hydrolysis and inhibitory power has been demonstrated (Aldridge & Davison, 1952*a*, *b*). Since TOCP is a stable substance, it was considered unlikely that it would have the inhibitory activity attributed to it, and this view was reinforced by the demonstration by Myers & Mendel (1953) that the inhibitory activity of TOCP against the hydrolysis of tributyrin by rat serum varied considerably from sample to sample. The experiments described in this paper show that most of the inhibitory activity of at least one specimen of TOCP is due to an impurity.

TOCP has the interesting property of causing weakness and ataxia in the legs of chickens due to the demyelination of certain tracts of the spinal cord (Smith & Lillie, 1931). After feeding TOCP to chickens, a profound lowering of the pseudo cholinesterase at various sites is always obtained (Earl & Thompson, 1952*b*). Using a purified specimen of TOCP possessing little *in vitro* inhibitory activity,

experiments have now been carried out which show that TOCP is undoubtedly converted into another, more inhibitory compound *in vivo* in chickens, rabbits and rats. Further work with chickens has shown that the tri-*m*- and tri-*p*-cresyl phosphates do not inhibit cholinesterase *in vivo*, do not produce paralysis, and do not, within the limits of experimental observation produce more than traces of demyelination.

EXPERIMENTAL

Methods of analysis. Cholinesterase activity was determined manometrically using as substrates 0·015 M acetylcholine chloride or 0·03 M butyrylcholine perchlorate, and tributyrinase activity using a suspension of 3–4 mg./ml. tributyrin. All determinations were carried out in a buffered solution containing 0·0357 M-$NaHCO_3$, 0·164 M-NaCl and 0·1% (w/v) gelatin, and gassed with 5% CO_2 in N_2.

o-Cresol was determined using the following modification of the method of Gottlieb & Marsh (1946). Reagents. (1) 4-Aminoantipyrine, 0·05% (w/v) in Sørensen's 0·0667 M phosphate buffer, pH 7·4. (2) Potassium ferricyanide, 0·4% (w/v) in Sørensen's 0·0667 M phosphate buffer, pH 7·4. Procedure. To 4 ml. of a solution containing *o*-cresol (0–60 μg.) at approx. pH 7·4 were added 2 ml. 4-aminoantipyrine reagent; the solution was mixed and 2 ml. potassium ferricyanide reagent were added. This too was mixed and left at room temperature for 5 min., 2 ml. ethanol were then added and the colour intensity was measured at 510 mμ., using a Unicam D.G. spectrophotometer. There is a linear relationship between optical density and concentration of *o*-cresol. The ethanol was added so that when the hydrolysis of TOCP was being studied, any unchanged TOCP was brought into solution. The method is unaffected by the ethanol or by salts produced by neutralizing up to 1 ml. of 2 N ethanolic KOH. By this method *o*- and *m*-cresols can be estimated, but not *p*-cresol. The total combined *o*-cresol was determined by refluxing with 50% (w/v) solution of KOH in methyl cellosolve (Haslam & Squirrell, 1952). Total P was determined by the colorimetric phosphomolybdate method (King, 1946), after digestion of a sample by a reagent containing molybdate, H_2SO_4, $HClO_4$, and HNO_3 (Simmons & Robertson, 1950).

Materials used. Tri-*o*-cresyl phosphate (TOCP) was obtained from Geigy Co. Ltd. (Found: P, 8·3; combined *o*-cresol 88·0. Calc. for $C_{21}H_{21}O_4P$: P, 8·4, combined *o*-cresol, 88·0%.) Tri-*m*- and tri-*p*-cresyl phosphates (TMCP and TPCP) were obtained from A. Boake, Roberts & Co. TPCP was recrystallized from absolute ethanol. (M.p. 76°; Verhoek & Marshall (1939) reported m.p. 76·4–76·7°. Found: P, 8·4.) The TMCP was a liquid, difficult to solidify when received, but was twice recrystallized at −5° from a solution of 120 g. in 300 ml. 17%(w/v) ethyl ether in light petroleum (b.p. <40°). This material was stored at −5°. (M.p. 25°; Breusch & Keskin (1942) reported m.p. 25–6°. Found: P, 8·5.)

Purification of TOCP. The impurity which gives to specimens of TOCP its *in vitro* activity against cholinesterase can be differentially hydrolysed by alkali. In view of the insolubility of TOCP in water, the following method had been developed using ethanolic NaOH. TOCP (40 ml.) and 0·2 N ethanolic NaOH (40 ml.) were separately cooled to 5°. The solutions were mixed and left at 5° for 1 hr. $CHCl_3$ (200 ml.) and water (400 ml.) were added and the aqueous layer was washed with a further 100 ml. $CHCl_3$. The $CHCl_3$ extracts were combined and washed four times with 200 ml. portions of water. Any emulsion in the $CHCl_3$ layer was broken by the addition of a little NaCl. After any droplets of water had been filtered off, the $CHCl_3$ layer was dried over anhydrous Na_2SO_4 for 2 days. The dry solution was then poured through an alumina column (Peter Spence, Type H) to remove any free *o*-cresol. This was followed by 100 ml. dry $CHCl_3$, and then the column was allowed to drain dry. The solvent was further removed *in vacuo* at 50° and then the last traces at 80° by a stream of air. This purified material contained 8·4% P (theoretical 8·4%) and as a further check that the compound had not been chemically altered, it was shown that the rate of hydrolysis and the turbidity of the aqueous suspensions were, within experimental error, the same for both the purified and unpurified materials.

Solubility test for inhibitory impurities in tricresyl phosphates. When a substance is pure, a saturated solution will contain the same amount of substance no matter how much solute was present in excess during the preparation of the solution. For the detection of impurities in tricresyl phosphates, suspensions of 20 μg./ml. and 2 mg./ml. were prepared. The excess of solute was removed by centrifuging and the clear supernatants were incubated with human serum cholinesterase or rat serum tributyrinase for 30 min. and the residual activity was determined.

Table 1. *Solubility test for inhibitory impurity in samples of tricresyl phosphates*

(The suspensions of TOCP were prepared by vigorous shaking in buffer solutions containing $NaHCO_3$ (0·0357 M) and NaCl (0·164 M) and gassed with 5% CO_2 in N_2. Excess of TOCP was centrifuged off after the solution had stood for 10 min. The clear supernatant (0·5 ml.) was incubated for 30 min. at 37° with either of twice diluted, citrated human plasma (0·5 ml.) or five times diluted rat serum (0·5 ml.). After incubation, the residual enzymic activities were determined in duplicate using as substrates acetylcholine perchlorate (0·015 M) and tributyrin (3–4 mg./ml.), respectively.)

Supernatant from (mg./ml.)	Enzymic activity (as % of original): Hydrolysis of acetylcholine by human plasma	Enzymic activity (as % of original): Hydrolysis of tributyrin by rat serum
Unpurified tri-*o*-cresyl phosphate		
0·02	88, 88	100, 103
2·0	36, 36	75, 73
Purified tri-*o*-cresyl phosphate		
0·02	96, 97	98, 101
2·0	103, 96	96, 90
Tri-*m*-cresyl phosphate		
0·04	99, 101	—
2·0	102, 97	—
Tri-*p*-cresyl phosphate		
0·02	98, 101	—
2·0	102, 103	—

Table 2. *Comparison of the inhibition of cholinesterases* (1) *by TOCP administered to living animals and* (2) *incubated* in vitro *in blood from the same animals*

(For the calculation of the *in vivo* concentration in blood, blood volumes of rabbit and chicken have been taken as 70 ml./kg. (Levine *et al.* 1941). It was assumed that all the drug would be in the circulating blood. All compounds were injected intravenously as solutions in absolute ethanol. Cholinesterase activity was determined on whole blood using acetylcholine (AcCh, 0·015M) and butyrylcholine (BuCh, 0·03M) as substrates. Heparin was used as an anticoagulant.)

Animal	Dose (mg./kg.)	Time after injections or of incubation (min.)	Inhibition (%) AcCh hydrolysis *In vivo*	AcCh hydrolysis *In vitro*	BuCh hydrolysis *In vivo*	BuCh hydrolysis *In vitro*
Rabbit	6·8	30	41	3	81	26
		90	58	0	78	29
Rabbit	1·61	30	9	0	43	20
		103	13	0	39	11
Chicken	6·8	30	—	—	65	8
		80	—	—	64	22

RESULTS

The inhibitory activity of TOCP against esterases in vitro

Using the solubility test on an unpurified sample of TOCP, it was found that the inhibitory activity of a saturated solution is dependent upon the concentration of the suspension used to prepare it. This was demonstrated using as the test enzymes both human serum cholinesterase and rat serum tributyrinase (Table 1). Another specimen of TOCP kindly supplied by Dr Myers gave a similar result. These experiments indicated that these samples of TOCP contain an impurity which is an inhibitor of cholinesterase and of tributyrinase. A saturated solution of the Geigy material purified as described above showed a negligible activity (Table 1). It should be noted that this sample might still not have been free from the impurity, but the concentration of the latter was below the threshold of the methods of detection.

The inhibitory activity of TOCP in vivo

An experiment has been described (Aldridge & Barnes, 1952) to show that various organophosphorus compounds are converted into more active inhibitors *in vivo*. In this method the compound is injected intravenously into the animal and samples of blood are removed at various times for the determination of enzymic activity. Some of the animal's normal blood is incubated with that concentration of substance which would be present if all of the injected substance were circulating in the blood stream (this is clearly a much higher concentration than would actually be present). In this way a comparison may be made of the *in vivo* and *in vitro* effects after various time intervals. The results given in Table 2 show that both in the rabbit and the chicken the inhibition of cholinesterase is more than can be accounted for by the *in vitro* activity of the TOCP. It seems, therefore, that TOCP is converted into a more active inhibitor of cholinesterase *in vivo*.

Table 3. *Inhibitory power of solutions of TOCP after incubation with rat-liver slices*

(Approx. 4 g. crude rat-liver slices of thickness approx. 0·5 mm. were added to a suspension of 30 μg. TOCP/ml. in buffer solution containing $NaHCO_3$, 0·0357M; NaCl, 0·164M; and gassed with 5% CO_2 in O_2. After various times, a 5 × diluted supernatant (0·5 ml.) was incubated with 6 × diluted horse serum (0·5 ml.). After a further incubation at 37° for 30 min. the residual cholinesterase activity was determined manometrically using acetylcholine chloride (0·015M) as substrate. Incubation of undiluted TOCP suspension (0·5 ml.) or of extract from liver slices (0·5 ml.) with horse serum produced no inhibition of horse-serum cholinesterase.)

Time (min.)	Enzyme activity (%)
30	19
60	58
120	77
210	90

Conversion of TOCP into a more active inhibitor by rat-liver slices

It has been shown that both octamethylpyrophosphoramide (DuBois, Doull & Coon, 1950; Gardiner & Kilby, 1952) and *OO*-diethyl *O*-*p*-nitrophenyl phosphothioate (formerly called 'thiophosphate') (Gage & Payton, 1952) are converted into more active inhibitors by rat-liver slices. The results (Table 3) show that TOCP is also converted into a more active inhibitor by rat-liver slices. The concentration of inhibitor produced appears to decrease with incubation times of longer duration than 30 min. From other experiments it would appear that this is due to at least two factors. If, after

Table 4. *Effect of oral administration of tricresyl phosphates on the cholinesterase of chicken serum*

(5 ml. of a 10% (w/v) solution of the tricresyl phosphate in arachis oil was given daily by mouth to each of two chickens. Samples of blood were removed 24 hr. after such a dose for the determination of cholinesterase activity using acetylcholine (0·015 M) as substrate.)

Daily dose (mg./kg.)	No. of daily doses	Time blood sample taken (day)	Cholinesterase activity of serum (ml. CO_2/ml./min.) (1)	(2)	Remarks
Tri-*o*-cresyl phosphate					
0·2	1	0	27·6	—	Ataxia at 14 days
		1	5·8	—	Histological examination at 21 days showed extensive demyelination in the spinal chord
		4	9·7	—	
Tri-*m*-cresyl phosphate					
0·21	20	0	20·5	21·6	
		5	20·3	20·9	No paralysis or ataxia 27 days after last dose
		17	21·6	18·8	Histological examination at this time showed traces of demyelination in the spinal cord
Tri-*p*-cresyl phosphate					
0·26	18	0	—	—	
		16	20·8	—	No paralysis or ataxia 43 days after last dose
		21	20·1	19·2	Histological examination showed traces of demyelination in the spinal cord

15 min. incubation of TOCP with rat-liver slices, the slices are centrifuged off, the inhibitory activity of the supernatant decreases to 20% of the original value after 1 hr. at 37°. The inhibitor is therefore unstable under these conditions. Another factor is that the ability of the liver to produce inhibitor is not maintained under these conditions. Rat-liver slices which have been incubated at 37° for 1 hr. are unable to produce inhibitor from TOCP. Considerable variation (approx. five-fold) has also been experienced in the amount of inhibitor produced in different experiments.

The effect of tricresyl phosphates on the chicken

It has been shown that after oral administration of TOCP to chickens, there is a marked inhibition of the serum cholinesterase activity (Earl & Thompson, 1952*b*). Using a purified TOCP, a similar fall is produced after intravenous injection (Table 2). While both impure and purified TOCP produce demyelination in the chicken, the tri-*m*- and tri-*p*-cresyl phosphates are either inactive or only feebly active in this respect (Smith, Engel & Stohlman, 1932; Hunter, Perry & Evans, 1944). Experiments have been carried out to determine if the *meta* and *para* isomers produce a lowering of the cholinesterase activity of the serum. There is a danger of impurities in the *meta* isomer, which is normally received as a liquid difficult to crystallize. Both of these isomers after careful recrystallization have very little activity *in vitro* and appear to be substantially free from active impurities as judged by the solubility test. Since TOCP is obviously absorbed when administered in arachis oil, it has been presumed that both the *meta* and *para* isomers will be absorbed when given in this way. All these isomers have therefore been given orally as a 10% solution in arachis oil. The results in Table 4 show that TOCP produced a large depression of cholinesterase activity which had partially recovered in 4 days. This rate of return agrees with the results of Earl & Thompson (1952*b*). After administration of both the *meta* and *para* isomers respectively, no fall in enzyme activity was found, even though 10 g. of the compound was given. The chicken after receiving TOCP became definitely ataxic in 14 days, and the spinal cord was found upon histological examination to have extensive demyelination of the fibre tracts of the lateral and anterior columns.

DISCUSSION

Most of the *in vitro* inhibitory activity of TOCP against human serum cholinesterase and rat serum tributyrinase is due to an impurity. A saturated solution of a 'purified' specimen possessed negligible *in vitro* activity; this specimen was pure within the limits of a solubility test which has been developed. This finding re-emphasizes the warning previously given (Aldridge & Davison, 1952*b*): 'Without further evidence (of purity) statements of inhibitory power (as concentrations to produce 50% inhibition) must be regarded as only applying to the particular specimen of inhibitor being examined.' We have no information about the chemical structure of this active impurity and know only that it inhibits human pseudo cholinesterase more than it does true cholinesterase. It has also been shown (Table 2) that if this purified TOCP is injected into rabbits and chickens, their blood cholinesterases are rapidly inactivated. These results suggest that TOCP is converted into a more

inhibitory compound *in vivo* and this view is supported by the fact that rat-liver slices produce such a compound *in vitro*. *In vivo*, pseudo cholinesterase is inhibited more than true cholinesterase but again we have no information about the chemical structure of the inhibitory compound.

Although the theory involving a simple relationship between the inhibition of the pseudo cholinesterase of the central nervous system and demyelination is now untenable (Davison, 1953), it is possible that those few organophosphorus compounds which can produce demyelination (Barnes & Denz, 1953) must have the chemical properties necessary for the inhibition of esterases. If demyelination is brought about by the inhibition of an enzyme, it is important to realize that organophosphorus compounds appear to be general inhibitors of enzymes possessing carboxylic esterase activity (Aldridge, 1953). It is not always clear which enzymes possess this activity, for it has recently been shown that some proteolytic enzymes do hydrolyse carboxylic esters (Schwert, Neurath, Kaufmann & Snoke, 1948). It is possible that the products of the inhibitory reaction between the organophosphorus compounds and esterases in general may be important in the production of demyelination. There is, however, no information about the nature of these products after TOCP administration, because a metabolite is the active inhibitory agent.

SUMMARY

1. Using a solubility test, the *in vitro* inhibitory power of tri-*o*-cresyl phosphate against cholinesterase (TOCP) has been shown to be due to an active impurity. A purified specimen possessed negligible *in vitro* activity.

2. The *in vivo* inhibitory power of TOCP against cholinesterase in rabbit, rats and chickens cannot be accounted for by its *in vitro* activity.

3. Oral doses of tri-*m*- and tri-*p*-cresyl phosphates do not produce inhibition of the cholinesterase of chicken serum and do not produce more than traces of demyelination in the spinal cord.

My thanks are due to Geigy Company Ltd. for a gift of tri-*o*-cresyl phosphate and to Mr F. H. McKenzie (A. Boake, Roberts & Co. Ltd.) for the tri-*m*- and tri-*p*-cresyl phosphates. I am also grateful to Dr F. A. Denz for the histological examinations of the spinal cords of the treated chickens, and to Miss J. E. Cremer and Mr C. R. Kennedy for valuable technical assistance.

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