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THE ENZYMIC HYDROLYSIS OF TRIACETIN (GLYCERYL TRIACETATE)*

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The ability of cobra venom to hydrolyse acetylcholine was first described by Jynegar, Sehra, Mukerji, and Chopra (1938). The esterase responsible for this reaction was further studied by Bovet and Bovet (1943) and Bovet-Nitti (1947); they established that the hydrolysis was brought about by an enzyme distinct from the lecithinase present. Bovet-Nitti (1947) showed that the venom was able to hydrolyse a number of aliphatic esters, in particular acetyl esters including triacetin, and she therefore considered that the enzyme responsible was not a cholinesterase but an "acetylase" which would hydrolyse esters of acetic acid including acetylcholine.

Zeller (1947) found that the cobra venom esterase would hydrolyse acetyl-β-methylcholine but not benzoylcholine, and he classified it as a "true" cholinesterase according to the classification of Mendel and Rudney (1943). In later publications Zeller (1948a and b), in agreement with Bovet-Nitti (1947), sees in the ability to hydrolyse aliphatic esters a property which distinguishes the cobra venom enzyme from the mammalian true cholinesterase.

At the same time, however, Bodansky (1946) had found that triacetin was hydrolysed by mammalian tissues. The hydrolysis by mouse brain was inhibited by DFP (di*iso*propylfluorophosphonate). He obtained preparations of cholinesterase from red cells which hydrolysed acetylcholine and triacetin but not methyl butyrate and tributyrin.

These findings suggested the possibility that the pattern of substrate specificity of the cobra venom enzyme was in fact similar to that of the true cholinesterase of mammalian tissues, and in this paper we have therefore investigated whether triacetin and choline esters are hydrolysed by the same enzyme. Three preparations were studied: cobra venom, horse serum, and a homogenate of dog's caudate nucleus.

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Methods and Materials

1. *Substrates*

Acetylcholine bromide and acetyl-β-methylcholine chloride were commercial samples. Benzoylcholine chloride was prepared by Dr. H. R. Ing. Triacetin was a commercial sample redistilled, suspended in Ringer's solution, and brought to *p*H 7.4 before use.

2. *Enzyme preparations*

Cobra venom was given to us by Dr. C. H. Kellaway, F.R.S. It was dissolved in Ringer's solution. Horse serum was sent by Dr. E. S. Duthie from the Lister Institute, Elstree; a purified preparation of horse serum cholinesterase, free of ali-esterase, prepared according to Strelitz (1944) was kindly sent by Dr. J. W. Legge. A homogenate of dog's caudate nucleus was prepared and stored as a suspension in Ringer's solution (100 mg. fresh weight of tissue per ml.).

For the measurement of enzymic activity manometric methods were used. In all experiments the temperature was 37.5° C. and the reaction was carried out in an atmosphere of 95 per cent N_2 and 5 per cent CO_2 in Krebs's bicarbonate Ringer. Unless otherwise stated the reaction volume was 3.0 ml. The small blank due to non-enzymic hydrolysis is subtracted in the results given below.

Results

1. *Horse serum* (Table I)

It is known that the cholinesterase of horse serum will hydrolyse benzoylcholine (Mendel, Mundell, and Rudney, 1943). Fig. 1*b* shows the rate of hydrolysis of 0.006*M* benzoylcholine and of 0.006*M* triacetin and also of a mixture of the two esters. At a concentration of 0.006*M* benzoylcholine the horse serum cholinesterase is known to be saturated with substrate (Mendel, Mundell, and Rudney, 1943). It will also be seen that the rate of liberation of CO_2 with the mixture is equal to the sum of the rates with the esters separately. This indicates that triacetin is hydrolysed by an enzyme other than cholinesterase.

This result was confirmed by an experiment with the purified preparation of horse serum cholinesterase of which 0.5 ml. was used per flask. The result given in Table I shows that both acetylcholine and benzoylcholine were actively hydrolysed but that the hydrolysis of triacetin was negligible compared with its hydrolysis by untreated horse serum.

TABLE I

HYDROLYSIS OF CHOLINE ESTERS AND OF TRIACETIN IN HORSE SERUM

Substrate concentrations 0.006*M*

Substrates	μl. CO_2 liberated in 15 min. with	
	0.2 ml. horse serum	purified horse serum cholinesterase
Acetylcholine (ACh) ..	—	29
Benzoylcholine (BzCh)	62	20
Triacetin (TA) ..	46	1
BzCh + TA	108	—

The cholinesterase of horse serum is known to be inhibited by Nu 683 [(2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide dimethylcarbamate] (Hawkins and Gunter, 1946). Table II shows that the hydrolysis of triacetin was unaffected by concentrations of Nu 683 which completely inhibited the hydrolysis of benzoylcholine.

TABLE II

THE EFFECT OF Nu 683 ON THE ESTER HYDROLYSIS IN HORSE SERUM

Substrate	μl. CO_2 liberated in 15 min. in presence of increasing concentrations of Nu 683				
	0	$10^{-10}M$	$10^{-9}M$	$10^{-8}M$	$10^{-7}M$
0.006*M* BzCh	60	56	54	13	0
0.006*M* TA	150	140	140	146	142

From these experiments it can be concluded that the very active hydrolysis of triacetin by horse serum is due to an enzyme other than cholinesterase ; a very feeble hydrolysis by the cholinesterase cannot be excluded. There is known to be an ali-esterase in horse serum which hydrolyses tributyrin and methyl butyrate (Easson and Stedman, 1937 ; Richter and Croft, 1942), and it is possible that this enzyme is responsible for the hydrolysis of triacetin.

2. *Cobra venom*

Competition experiments were carried out using 0.25 mg. cobra venom. The result of such an experiment is shown in Fig. 1*c*. With cobra venom the rate of the reaction with the two esters is intermediate between the rates with acetylcholine and triacetin separately.

The action of inhibitors.—Two inhibitors of cholinesterases were used : eserine salicylate and

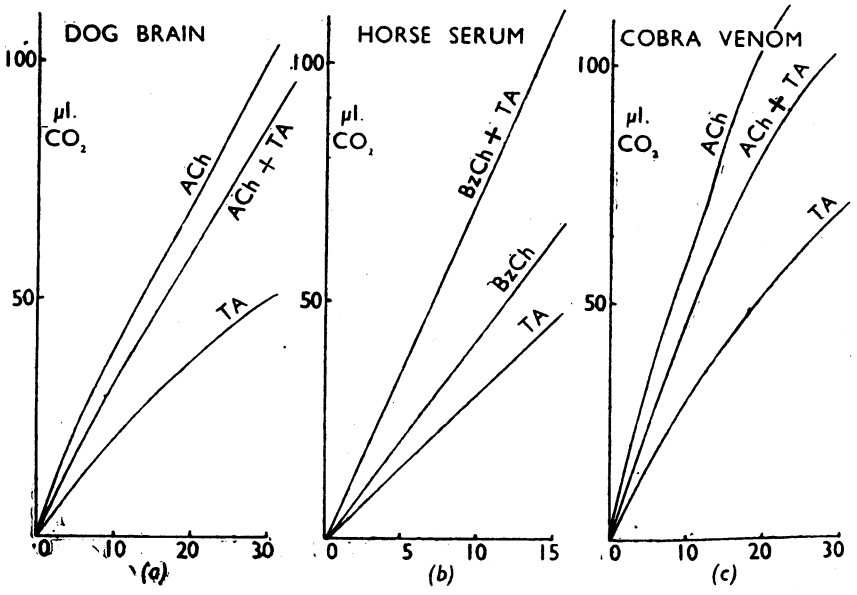

FIG. 1.—Competition experiments : (*a*) dog's caudate nucleus ; (*b*) horse serum ; (*c*) cobra venom. Abscissae : time in minutes. Ordinates : μl. CO_2 liberated.

Nu 683. The substrate concentrations were so chosen that the enzyme was approximately saturated. The results of these experiments are shown in Table III. For acetylcholine and triacetin the percentage inhibitions obtained were similar.

TABLE III

ESTER HYDROLYSIS BY COBRA VENOM IN THE PRESENCE OF INHIBITORS (Nu 683 AND ESERINE)

Inhibitor	Substrate	Percentage inhibitions during first 15 min. with inhibitor concentrations:			
		$10^{-8}M$	$10^{-7}M$	$10^{-6}M$	$10^{-5}M$
Nu 683	0.005M ACh	34	75	83	98
	0.03M TA	34	81	93	98
Eserine	0.005M ACh	26	66	80	92
	0.03M TA	20	74	92	96

These results are in agreement with the views of Bovet-Nitti (1947), according to whom the same enzyme is responsible for the hydrolysis of acetylcholine and triacetin.

3. *Dog's caudate nucleus*

The competition experiment illustrated in Fig. 1*a* shows that the hydrolysis of a mixture of 0.006M acetylcholine and 0.03M triacetin was slower than the hydrolysis of acetylcholine alone. A similar result was obtained when a mixture of triacetin and acetyl-β-methylcholine was used. The amounts of CO_2 evolved in 15 min. were:

from 0.03M acetyl-β-methylcholine	24 µl.
from 0.03M triacetin	36 µl.
from both esters	31 µl.

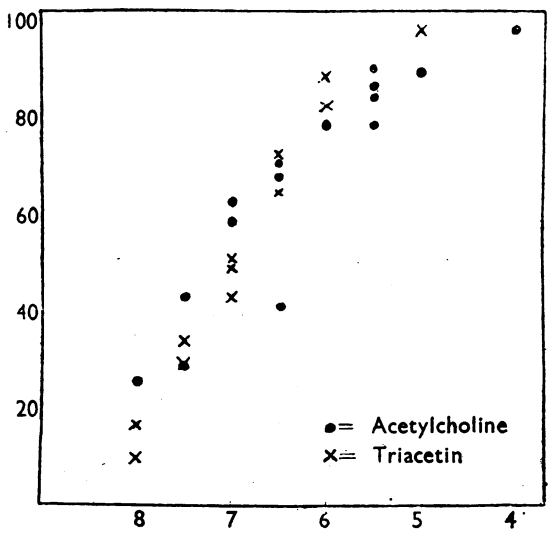

FIG. 2.—Inhibition by eserine of enzymic hydrolysis of acetylcholine (dots) and triacetin (crosses) by a suspension of dog's caudate nucleus. Each flask contained the equivalent of 4 mg. of fresh weight of tissue. Abscissa: negative logarithm of molar concentration of eserine. Ordinate: per cent inhibition.

The action of inhibitors.—In these experiments both substrates were used in concentrations which were near saturation concentration. If the esters were hydrolysed by the same enzyme it would be expected that the percentage inhibitions found under those conditions for any given inhibitor would be the same. The concentrations used were 0.006M acetylcholine and 0.03M triacetin. The results obtained with eserine salicylate are given in detail in Fig. 2, which shows that the percentage inhibitions with both substrates were not significantly different.

TABLE IV

ESTER HYDROLYSIS BY DOG'S CAUDATE NUCLEUS IN THE PRESENCE OF INHIBITORS

Inhibitor	Molar concentration	Percentage inhibition in first 15 min. with substrates	
		0.006M ACh	0.03M TA
Eserine ..	10^{-8}	13	14
,, ..	10^{-7}	62	49
,, ..	10^{-6}	80	87
,, ..	10^{-5}	90	100
Nu 683 ..	10^{-8}	4	2
,, ..	10^{-7}	40	22
,, ..	10^{-6}	78	64
,, ..	10^{-5}	88	84
Caffeine ..	10^{-3}	32	64
Benzoylcholine	10^{-2}	32	72
,,	3×10^{-2}	56	76
BQ10* ..	10^{-8}	23	14
,, ..	10^{-7}	52	46
,, ..	10^{-6}	90	72
,, ..	10^{-5}	100	76

* Decamethylenebisquinolinium dibromide

Similar results were obtained with other inhibitors in experiments summarized in Table IV. The substance BQ10 is decamethylenebisquinolinium dibromide recently prepared by Barlow and Ing (1948). Caffeine was shown to be an inhibitor of brain cholinesterase by Zeller and Bissegger (1943) and by Nachmansohn and Schneemann (1945).

These experiments suggest that the cholinesterase of dog's caudate nucleus hydrolyses triacetin.

Discussion

The experiments described in this paper are in agreement with the assumption that the cholinesterases present in cobra venom and dog's caudate nucleus are responsible for the hydrolysis of triacetin. The caudate nucleus is a tissue particularly rich in the "true" cholinesterase of Mendel and Rudney (1943). They define this enzyme as one "acting exclusively on choline esters." Our results show that this definition will have to be modified, and that the specificity towards acetylcholine is only relative and not absolute, as already postulated by Nachmansohn (1946).

Our experiments with caudate nucleus are in agreement with similar experiments of Adams and Whittaker (1948) on a purified preparation of "true" cholinesterase from human erythrocytes in which the hydrolysis of triacetin was found to be catalysed by the cholinesterase present. They found that this enzyme will split many other aliphatic esters besides acetylcholine. It thus appears that the ability of cobra venom to hydrolyse esters of acetic acid other than acetylcholine does not distinguish it from the "true" cholinesterases of mammalian tissues, and hence it seems unnecessary to classify the cobra venom enzyme as a third type of cholinesterase ("C" type of Zeller, 1948a, b).

Summary

1. The hydrolysis of triacetin by three sources of cholinesterase—horse serum, cobra venom, and dog's caudate nucleus—was investigated.

2. Triacetin hydrolysis by horse serum is mainly due to an enzyme other than cholinesterase.

3. The cholinesterases of cobra venom and dog's caudate nucleus hydrolyse triacetin.

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