INFLUENCE OF VITAMIN-E ON LIVERESTERASE AND CHOLINESTERASE

by

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INTRODUCTION

In the course of experiments on the relation between vitamin E and esterase activity it was found that the *in vitro* addition of *α-dl*-tocopheryl phosphate (TPh)* to rat liver-esterase preparations invariably led to a marked inhibition of this enzyme whether it was prepared from normal or E-deficient rats. This inhibition of liveresterase was investigated further in order to find out whether this phenomenon could have a specific significance with regard to the physiological action of vitamin E or should rather be considered as an unspecific "detergent" action on a protein molecule.

The latter possibility has recently been stressed in the literature[1,2,3] and was tested by studying the effect of TPh next to that of two well known anionic detergents (Na-lauryl sulphate and Na-cetyl sulphate)** on liveresterase as well as on pseudocholin-esterase and true cholinesterase. It was assumed that if the action of TPh were distinct from that of the anionic detergents (*e.g.*, in being more marked and selective), the inhibition of liveresterase by TPh might have a physiological significance. If however, the actions of the three compounds studied were comparable in degree and specificity for all enzymes tested, this would indicate that unspecific detergent actions were operating and little or no physiological significance could be attributed to the observed inhibition of liveresterase by TPh.

Indeed the first series of experiments described below indicates that TPh exerts such an unspecific detergent action *in vitro*. In a second series of experiments *α-dl*-tocopherol* was added *in vitro* to liveresterase preparations from normal and E-deficient rats. This compound was used because it cannot be expected to have an anionic detergent action lacking the ionogenic group in its molecule and should therefore enable to distinguish between detergent and other perhaps more specific effects inherent to the *α-dl*-tocopherol structure in *α-dl*-tocopherylphosphate.

MATERIALS AND METHODS

Two liveresterase preparations were used *viz.*, a rat-liver homogenate prepared as described previously[4] and a partially purified preparation of acetone dried horse-liver.

Pseudocholinesterase (ChE) was prepared from horse serum by the first two steps described by STRELITZ[5]. After the first ammoniumsulphate precipitation in the 2nd step, the cake was dissolved

* *α-dl*-tocopherylphosphate and *α-dl*-tocopherol were kindly supplied by Hoffmann-La Roche.

** We are indebted to Dr H. L. BOOY, Laboratory of Medical Chemistry, Leyden, for these compounds.

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in distilled water, neutralized and dialysed for two days against distilled water in the icebox. This solution keeps for many months.

True ChE was prepared from ox nucleus caudatus in the same way as described by COHEN, KALSBEEK, AND WARRINGA[6] for rat brain.

Enzyme activity was determined at 24° C by the continuous titration method[6] either with bromthymolblue as an indicator or with a direct reading p_H-meter. Unless otherwise stated the final concentrations used were ethyl butyrate 0.015 *M* and acetylcholine chloride 0.01 *M* in a volume of 10 ml. E-deficiency in rats was obtained as described in a previous paper[4].

RESULTS

1. *Experiments with α-dl-tocopherylphosphate (TPh)*

a. *Liveresterase*

TPh was incubated with rat liveresterase in a volume of 1.5 ml at room temperature during 20 minutes before the activity determination. No significant difference in percentage inhibition was found however, in experiments in which this incubation period was omitted.

The inhibition appeared to be non-competitive with the substrate (ethyl butyrate) as will be seen from Table I where a constant concentration of TPh produces about equal percentages inhibition at a number of different substrate concentrations.

TABLE I

INHIBITION OF LIVERESTERASE BY TPh AT VARIOUS ETHYL BUTYRATE CONCENTRATIONS

Substrate final conc.	% activity
0.015 *M*	55
0.0075 *M*	60
0.0030 *M*	61
0.0015 *M*	59

0.5 ml of a liversuspension diluted 1/50 was used
Final concentration of TPh 0.1 mg/ml

Inhibition depended largely on the amount of enzyme preparation present. In Table II two experiments are given with different amounts of liver homogenate. The amount of TPh needed for a certain inhibition runs roughly parallel with the enzyme concentration.

TABLE II

INHIBITION OF LIVERESTERASE BY TPh

Amount of enzyme added	mg of TPh added	% activity
0.5 ml liversuspension 1/10	5	74
	10	24
	15	4
0.5 ml liversuspension 1/50	1	59
	2	22
	3	0
	5	0

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Dialysis during one night against distilled water could not reverse the inhibition of liveresterase by TPh.

Essentially identical results were obtained with partially purified horse liveresterase.

b. *Pseudo ChE*

This enzyme was also inhibited by TPh. The inhibition could be decreased only slightly by the addition of $CaCl_2$ as will be seen from Table III. So it seems very unlikely that the inhibition of pseudo ChE by TPh must be ascribed to a removal of Ca^{++} ions as has been described for the inhibition of the succinoxidase system[7].

TABLE III

INFLUENCE OF $CaCl_2$ ON THE INHIBITION OF PSEUDO CHOLINESTERASE BY TPh (1 mg)

$CaCl_2$ final conc.	% activity
No $CaCl_2$	28
0.01 *N*	36
0.1 *N*	38
1 *N*	35

A number of experiments were performed in order to answer the question whether the binding of TPh to the enzyme was reversible or irreversible.

Dialysis against distilled water during 3 days of an enzyme preparation treated with a TPh concentration producing a decrease in activity to 13%, only increased the activity from 13% to 17%. In another experiment dialysis during 7 days resulted in an increase in activity from 6% to 27%.

In preliminary experiments on the lipase activity of liver homogenates it was found that the hydrolysis of olive oil emulsion was also inhibited by TPh, whereas the hydrolysis of Tween 80 was practically not inhibited in equal TPh concentrations. It seemed possible that the lipophilic groups of TPh were attracted by the Tween thus preventing a combination of TPh with the enzyme.

Tween 80 was found to reverse also the binding of TPh to pseudo ChE. 0.4 ml pseudo ChE was incubated during 20 minutes with 0.4 mg TPh in a volume of 2 ml. Afterwards 1 ml Tween 80 250 mg/ml was added and the mixture was incubated during another 10 minutes. Compared with a control the activity was increased from 15% to 38%. Tween 80 itself slightly inhibited pseudo ChE, the activity being 83% of the control. No difference was observed between the activities of enzyme preparations incubated with TPh for periods of 20 and 30 minutes resp.

These results suggest that Tween 80 effects a true reversion of TPh inhibition. A similar observation has been made by MILLER AND DESSERT[1] regarding the influence of Tween 80 on the inhibition of hyaluronidase by TPh.

The inhibition of Pseudo ChE by TPh also depends largely on the enzyme concentration used. Experiments with two amounts of enzyme preparation and with TPh, Na-cetyl sulphate and Na-lauryl sulphate as inhibitors are shown in Table IV, experiment 1.

In experiment 2 of Table IV the action of TPh is compared with that of physostigmine sulphate.

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TABLE IV

INHIBITION OF PSEUDO CHOLINESTERASE BY TPh, Na-CETYL SULPHATE, Na-LAURYL SULPHATE AND PHYSOSTIGMINESULPHATE

	ml enzyme	Amount of inhibitor added mg	Amount of inhibitor added µmol	% activity
Exp. 1:	0.4	Tocopheryl phosphate		
		0.5	0.9	84
		1.0	1.9	53
		2.0	3.7	25
	0.8	1.0	1.9	75
		2.0	3.7	41
		4.0	7.4	14
	0.4	Na-cetyl sulphate		
		0.25	0.7	95
		0.5	1.4	41
		1.0	2.9	13
		2.0	5.8	9
	0.8	0.5	1.4	88
		0.75	2.2	62
		1.0	2.9	33
		2.0	5.8	11
	0.4	Na-lauryl sulphate		
		1.0	3.5	95
		2.0	7.0	85
		2.5	8.7	82
		3.0	10.4	28
		4.0	13.9	9
	0.8	2.5	8.7	82
		3.0	10.4	51
		4.0	13.9	18
Exp. 2:	0.3	Tocopheryl phosphate		
		0.3	0.6	71
		0.4	0.7	50
		0.5	0.9	37
		0.8	1.5	20
		1.0	1.9	7
	0.6	0.6	1.1	65
		0.8	1.5	46
		1.0	1.9	34
		1.4	2.6	17

TABLE IV (continued)

	ml enzyme	Amount of inhibitor added mg	Amount of inhibitor added μmol	% activity
Exp. 2:		Physostigmine sulphate		
	0.3	$0.32 \cdot 10^{-4}$	$0.5 \cdot 10^{-4}$	72
		$0.52 \cdot 10^{-4}$	$0.8 \cdot 10^{-4}$	48
		$0.58 \cdot 10^{-4}$	$0.9 \cdot 10^{-4}$	36
		$0.65 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	31
	0.6	$0.52 \cdot 10^{-4}$	$0.8 \cdot 10^{-4}$	70
		$0.65 \cdot 10^{-4}$	$1.0 \cdot 10^{-4}$	45
		$0.78 \cdot 10^{-4}$	$1.2 \cdot 10^{-4}$	35
		$1.04 \cdot 10^{-4}$	$1.6 \cdot 10^{-4}$	27

The enzyme preparations ot experiment 1 and experiment 2 were different but of equal activity in the amounts given.

Only two enzyme concentrations were used because for lower concentrations the percentage inhibition could not be determined accurately while when higher concentrations were used the reaction velocity was so great that the titration could only be perfomed by adding large amounts of NaOH which caused unwanted shifts in p_H.

From the data of experiment 2, Table IV, it appears that the concentration of TPh giving 50% inhibition is very much higher (about 10,000 times) than that of physostigmine. This great difference in concentration is in accordance with the assumption that the mechanism of action of TPh is quite different from that of physostigmine. Physostigmine is probably bound mainly to negative groups of the enzyme and possibly to enzyme molecules only. On the other hand TPh is likely to be bound to positive groups not only of the enzyme but also of other indifferent proteins. A tentative calculation of the amounts of inhibitors bound to the enzyme preparation is in accordance with this notion.

For physostigmine this amount can be calculated using the equation of GOLDSTEIN[8] for competitive inhibition with the enzyme operating in Zone A with respect to substrate (practically all substrate free) which is nearly always the case, and in Zone B with respect to inhibitor (a considerable amount of inhibitor bound).

$$I = K_i \left[\frac{S}{k_s} \cdot \frac{1-a}{a} - 1 \right] + \left[1 - a \left(1 + \frac{k_s}{S} \right) \right] E \qquad (1)$$

where I = concentration of inhibitor, K_i = dissociation constant of enzyme-inhibitor complex, S = substrate concentration, K_s = dissociation constant of enzyme-substrate complex, E = concentration of enzyme centers, a = fractional activity. According to GOLDSTEIN the first term on the right gives the concentration of free inhibitor, the second term that of bound inhibitor. As K_i and K_s are constants and when S is constant and $a = \frac{1}{2}$ (as in our experiments) a straight line is obtained on plotting I (ordinate) against E (abscissae) which at $E = 0$ intersects the ordinate at a value giving the concentration of free inhibitor.

The concentration of bound inhibitor can be calculated for every value of E by subtracting the concentration of free inhibitor from the total inhibitor concentration giving 50% inhibition at the enzyme concentration used.

The fact that the exact value of E is not known makes no difference in the calculation as the value at which the line intersects the ordinate is independent of the scale used in plotting E.

For experiment 2 of Table IV the amount of physostigmine bound to 0.3 ml enzyme preparation was calculated from the concentration of bound inhibitor and was found to be $2.0 \cdot 10^{-5}$ μmol whereas the amount of free physostigmine was $5.8 \cdot 10^{-5}$ μmol.

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In determining these values for TPh the GOLDSTEIN equation for non-competitive inhibition was used

$$I = K_i \cdot \frac{1-a}{a} + (1-a)\,E \qquad (2)$$

which in our experiment ($a = \frac{1}{2}$) becomes

$$I = K_i + \tfrac{1}{2}E \qquad (3)$$

Here K_i stands for the concentration of free inhibitor and $\frac{1}{2}E$ for that of bound inhibitor.

It was found that 0.3 ml of enzyme preparation bound 0.6 μmol of TPh, while 0.1 μmol was free.

When the above calculations are correct we must assume that one molecule of enzyme binds 15,000 times more TPh than physostigmine (1 mol of physostigmine sulphate contains 2 mols of physostigmine). This is highly improbable as one would expect the number of positive groups per molecule capable of binding detergent anions to be about 100–200 (see f.i. PUTNAM AND NEURATH[9], and NEURATH AND PUTNAM[10]) and when we assume that each enzyme molecule possesses only 1 active group capable of binding 1 physostigmine the ratio bound TPh/bound physostigmine should be 100–200 at most.

This discrepancy may be solved by assuming that not only enzyme molecules but also indifferent protein molecules present as impurities in the enzyme preparation bind TPh.

A calculation identical to the derivation of the GOLDSTEIN equation (3) shows that when apart from the enzyme a number of other proteins combine with TPh equation (3) changes into

$$I = K_i + \tfrac{1}{2}E + (1-b)\,P_1 + (1-c)\,P_2 + \ldots . \qquad (4)$$

when P_1, P_2, ... are concentrations of reacting groups of different proteins and b, c, ... the fraction of these groups being free.

On plotting I against $(E + P_1 + P_2 + \ldots .)$ again a straight line is obtained and the calculation of the amount of inhibitor bound to protein $(E + P_1 + P_2 + \ldots .)$ proceeds identically as described above.

The 0.6 μmol TPh bound to 0.3 ml of the enzyme preparation is thus assumed to be not the amount bound to enzyme alone but to enzyme + indifferent proteins.

In Fig. 1 the data of experiment 1 Table IV are given, μmol of added inhibitor giving 50% inhibition being plotted against ml of enzyme preparation.

On the assumption that equation (4) holds the two experimental points for each compound are connected by straight lines. As the lines for the different inhibitors run parallel it is concluded that identical amounts of the three compounds are bound to the enzyme + indifferent proteins, thus indicating identity of mechanism of action. The lines of TPh and Na-cetyl sulphate intersect the ordinate at comparable values, whereas the line of Na-lauryl sulphate intersects at a much higher value indicating that the former two compounds possess about equal affinity for the proteins, while the affinity of the latter compound is much smaller.

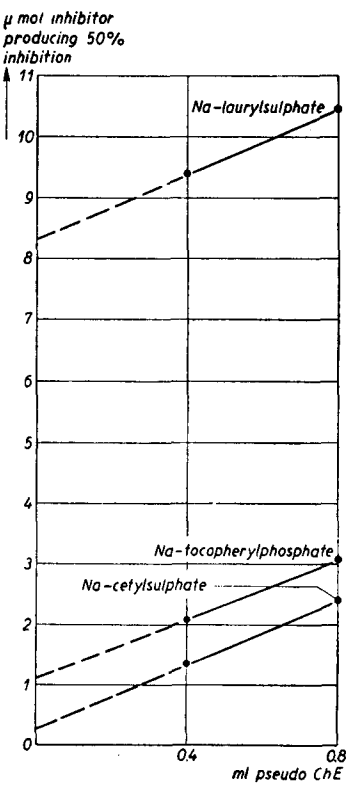

Fig. 1. Amounts of TPh, Na-lauryl sulphate and Na-cetyl sulphate (in μmol) required to produce 50% inhibition of pseudo ChE are plotted against the corresponding amounts of the pseudo ChE preparation (in ml)

c. *True ChE*

This enzyme was also inhibited by TPh. The activity of 0.4 ml enzyme preparation was reduced to 26% by 2 mg TPh and to 52% by 1 mg TPh, 0.8 ml showed 53% of control activity with 2 mg TPh added.

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$CaCl_2$ which strongly activates true ChE could only partially reduce the inhibition by TPh. An amount of 1.5 mg TPh which decreased control activity to 38% gave activities of 45% and 53% resp. in the presence of 0.1 *N* and 0.01 *N* $CaCl_2$ final concentration (compared with the activities in the presence of $CaCl_2$ alone, which for 0.1 *N* $CaCl_2$ was 252% and for 0.01 *N* $CaCl_2$ 207% of the activity determined without any addition). Thus it appears that the inhibition of true ChE by TPh is not just caused by removal of Ca^{++} ions.

d. *Influence of TPh on egg albumin*

It was felt that the inhibitory action of TPh was a rather unspecific binding to positive groups of any protein. Therefore the reaction of TPh was assessed on a randomly chosen protein, egg albumin which has no enzymatic activity. In a micro-electrophoresis apparatus[11] the I.E.P. was determined of a 0.02% egg albumin solution with and without TPh in a concentration of 0.2 mg/ml. The addition of TPh shifted the I.E.P. from 4.8 to 2.8. An experiment with the TISELIUS apparatus of the Perkin Elmer Corporation performed in phosphate buffer of p_H 6.0 showed that with the same ratio of TPh to egg albumin, as used for the determinations of the I.E.P., besides the boundaries formed by egg albumin and TPh alone a new boundary was formed which moved faster than the egg albumin alone indicating that a complex was formed with a greater negative charge than egg albumin.

2. *Experiments with α-dl-tocopherol (Toc.)*

From the above described experiments it seems probable that the action of TPh *in vitro* is unspecific and therefore bears little or no relation to the action of vit.-E *in vivo*. Therefore a number of experiments were performed with the free alcohol (Toc.) *in vitro* using liveresterase preparations from normal and E-deficient rats.

As however Toc. is practically insoluble in water, emulsions have to be used. In accordance with the method described by AMES AND RISLEY[12], Toc. was dissolved in 0.2 ml acetone in the tube of a homogenizer of POTTER AND ELVEHJEM. The stirrer was started and 2 ml liversuspension was slowly added. The mixture was stirred during 5 minutes and left standing at room temperature during another 10 minutes. After this 1 ml was taken for the determination of enzyme activity. The acetone concentration used had no influence on enzyme activity. The effect of Toc. on liveresterase preparations from E-deficient female rats was compared with those from normal female rats.

In preliminary experiments a slight activation of liveresterase by Toc. *in vitro* was found in liverpreparations from E-deficient rats. No difference in activity was found when the Toc. concentrations were varied from 0.001–10 mg/ml.

Therefore a series of determinations was made in 10 E-deficient and 7 normal rats using a concentration of 1 mg/ml Toc.

Liversuspensions of 1/200 were prepared and from each suspension 5 determinations were made with acetone alone and 5 with Toc. added. The average of the Toc. values was expressed as percentage of the average of the blank.

For normal animals the percentages were 100, 99, 100, 116, 107, 105, 101, for E-deficient rats 122, 112, 102, 102, 107, 113, 105, 99, 101, 105.

It must be concluded that Toc. *in vitro* had no effect on liveresterase activity in our experiments.

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DISCUSSION

The fact that TPh, Na-cetyl sulphate and Na-lauryl sulphate are comparable in their action and are probably bound to the preparation of pseudo ChE in equal amounts supports the conception that TPh acts (like the other two compounds) as an anionic detergent.

TPh with its branched hydrocarbon chain of 16 carbons and a dicyclic structure attached to the phosphate group and Na-cetyl sulphate with a straight chain of 16 carbons attached to the sulphate group inhibit pseudo ChE in about equal concentrations whereas Na-lauryl sulphate with a straight chain of 12 carbons inhibits in much higher concentrations. This provides further evidence that the length of the hydrocarbon chain is an essential factor in determining the affinity for protein (reflected in K_i values of equations (3) and (4)).

More evidence for an unspecific action of TPh with proteins is provided by the fact that many different enzymes are inhibited and by the experiments in which it was shown that the enzymologically inactive egg albumin also reacts with TPh.

This unspecific effect which is moreover accentuated by the high concentrations of TPh necessary to inhibit esterases makes a physiological significance in connection with vit. E activity highly improbable.

This conclusion seems to be justified by the fact that Toc. which possesses no detergent properties (and therefore presumably does not react unspecifically with protein) was found without effect *in vitro* on liveresterase prepared from normal and E-deficient rats.

SUMMARY

1. *α-dl*-tocopheryl phosphate (TPh) added *in vitro* inhibits crude rat liveresterase, purified horse liveresterase, horse serum pseudo cholinesterase and ox nucleus caudatus true cholinesterase.

2. The percentage inhibition at a given concentration depends largely on the amount of enzyme preparation used.

3. The inhibition of pseudo cholinesterase by TPh is difficult to reverse but a certain amount of reversion can be obtained by dialysis against distilled water during 7 days and by the addition of Tween 80.

4. The inhibition of pseudo cholinesterase and true cholinesterase by TPh is not (exclusively) due to the removal of Ca^{++} ions.

5. The action of TPh is identical with that of the anionic detergents Na-lauryl sulphate and Na-cetyl sulphate.

6. TPh, Na-lauryl sulphate and Na-cetyl sulphate are probably bound to the pseudo cholinesterase preparation in equal amounts, whereas about 15,000 times less of physostigmine is bound.

7. *α-dl*-tocopherol added as an emulsion *in vitro* to liver homogenates of normal and E-deficient rats has no effect on liveresterase activity.

8. It is concluded that TPh acts as an anionic detergent and that the *in vitro* effect bears no relation to the physiological action of vitamin E.

RÉSUMÉ

1. Le phosphate de *α-dl*-tocophérol (TPh) ajouté *in vitro* inhibe l'estérase crue de foie de rat, l'estérase purifiée de foie de cheval, la pseudocholinestérase de sérum de cheval, et la cholinestérase vraie de nucleus caudatus de boeuf.

2. L'inhibition (en %) à une concentration donnée dépend en grande partie de la quantité de préparation d'enzyme employée.

3. L'inhibition de la pseudocholinestérase par le TPh est difficile à réverser; l'on obtient cependant une réversion partielle par dialyse contre de l'eau distillée pendant 7 jours et par addition de Tween 80.

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4. L'inhibition de la pseudocholinestérase et de la cholinestérase vraie par le TPh n'est pas due (exclusivement) à l'élimination des ions Ca^{++}.

5. L'action du TPh est identique à celle des détergents anioniques: laurylsulphate de sodium et cétylsulphate de sodium.

6. Le TPh, le laurylsulphate de sodium et le cétylsulphate de sodium sont probablement fixés à la préparation de pseudo-cholinestérase en quantités égales, tandis qu'une quantité 15,000 fois moindre de physostigmine est fixée.

7. L'*a-dl*-tocophérol ajouté sous forme d'émulsion *in vitro* à des homogénats de foie de rats normaux et à carence de vitamine E n'a aucun effet sur l'activité de l'estérase de foie.

8. Nous concluons que le TPh agit comme un détergent anionique et que l'effet *in vitro* n'a aucun rapport avec l'action physiologique de la vitamine E.

ZUSAMMENFASSUNG

1. *In vitro* zugefügtes *a-dl*-Tokopherylphosphat (TPh) hemmt ungereinigte Rattenleberesterase, gereinigte Pferdeleberesterase, Pferdeserum-Pseudocholinesterase und wahre Rinder-Nucleus caudatus-Cholinesterase.

2. Die Hemmung (ausgedrückt in %) bei einer bestimmten Konzentration hängt hauptsächlich von der angewendeten Menge Enzympräparat ab.

3. Die Hemmung der Pseudocholinesterase durch TPh ist schwer umzukehren; teilweise kann sie durch 7tägige Dialyse gegen destilliertes Wasser und durch Zusatz von Tween 80 rückgängig gemacht werden.

4. Die Hemmung von Pseudocholinesterase und von wahrer Cholinesterase durch TPh ist nicht (ausschliesslich) auf die Entfernung der Ca^{++}-Ionen zurückzuführen.

5. Die Wirkung von TPh ist identisch mit derjenigen der anionischen Detergentien Natriumlaurylsulphat und Natriumcetylsulphat.

6. TPh, Natriumlaurylsulphat und Natriumcetylsulphat werden wahrscheinlich in ungefähr gleichen Mengen an das Pseudocholinesterase-Präparat gebunden, während etwa 15,000 mal weniger Physostigmin gebunden wird.

7. *a-dl*-Tokopherol, das in Form einer Emulsion *in vitro* Leberhomogenaten von normalen Ratten und solchen mit Vitamin-E-Mangel zugefügt wurde, hat keinen Einfluss auf die Leberesterase-Aktivität.

8. Es wird gefolgert, dass TPh wie ein anionisches Detergenz wirkt und dass seine Wirkung *in vitro* nicht mit der physiologischen Wirkung von Vitamin E zusammen hängt.

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Received November 24th, 1950