5. Choline was more effective than inositol in reducing total lipids and total cholesterol in the liver, in both fat-free and fat-containing diets.

6. Choline exerted a stronger supplementary lipotropic effect than did inositol on both total lipids and total cholesterol.

7. No data were noted that would support the statement that inositol possesses unique lipotropic properties, either under these special conditions (following depletion of B vitamins) or under any other conditions.

8. Reasons for the existing confusion in the literature as to the real lipotropic potency of inositol have been discussed.

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The Stabilization of D-Amino-acid Oxidase by Flavin-adenine Dinucleotide, Substrates and Competitive Inhibitors

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The stabilization of an enzyme by its substrate has been little studied since the effect was described by O'Sullivan & Tomson (1890). Delory & King (1943) concluded that the stabilization of alkaline phosphatase by different substrates increases with the rate of hydrolysis. Kunitz & McDonald (1946) could not correlate the substrate stabilization of yeast hexokinase with the rates at which the sugars react in the hexokinase system.

The stabilization of D-amino-acid oxidase of sheep kidney has now been studied and compared with the kinetics at the same temperature and pH.

EXPERIMENTAL

D-Amino-acid oxidase was prepared by the method of Negelein & Bromel (1939). The precipitate obtained after removal of the flavin-adenine dinucleotide (FAD) by acidification in $(NH_4)_2SO_4$ solution at 0°, was washed in 16% $(NH_4)_2SO_4$ solution and suspended in 0·056 M-pyrophosphate buffer pH 8·3 (250 ml./100 g. acetone-dried kidney) and the pH adjusted to 6·5.

The same preparation of oxidase was used for all the experiments reported in this paper except those connected with Table 3, for which several different preparations were used. The solution of oxidase was stored at −10°. Small batches were thawed and subsequently stored at 0° for use as required. Each sample was rejected when 0·20 ml. failed to catalyse the uptake of 110 μl. O_2/20 min. in the activity test. (With no added FAD, about 10 μl. O_2 were consumed in 20 min.)

Catalase was prepared according to Keilin & Hartree (1945). After the first precipitation by $(NH_4)_2SO_4$, the catalase was dialysed until free from ammonium ions.

Flavin-adenine dinucleotide (FAD). The procedure of Warburg & Christian (1938) was followed to obtain a crude Ag salt of FAD from baker's yeast. The salt was suspended in water and decomposed by a method introduced by Dr M. Dixon: KCl was added to dissolve the suspended Ag salt and 2 N-HCl was added drop by drop until there was no further precipitate. The suspension was centrifuged and the pre-

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cipitate of AgCl washed with water at pH 4. The supernatant and washings were combined.

'Alumina for adsorption purposes' (Hopkin and Williams Ltd.) (50 g.) was mixed with 90 ml. 0·25 N-HCl and poured into a vertical glass tube 3·5 cm. in diameter, 20 cm. long. This tube had previously been fitted with a perforated plate and cotton-wool plug to hold the alumina. The column was washed with 200 ml. 0·05 N-HCl, compressed air being used to accelerate the flow. In the following operations care was taken not to disturb the upper surface of the alumina and to keep it covered with liquid.

The crude FAD solution (75 ml. from 1·6 kg. yeast) was adjusted to pH 1 by adding HCl and poured on to the column. The column was then washed with 1 l. of 0·05 M-sodium acetate-acetic acid buffer (pH 4·6) when the yellow FAD was adsorbed at the top of the column. The chromatogram was developed by an acetate-phosphate solution (0·05 M-sodium acetate, 0·05 M-acetic acid and 0·05 M-KH_2PO_4). The yellow eluate was collected in batches of about 50 ml. and neutralized. Selected batches were examined by paper chromatography (Crammer, 1946) in a collidine-water system on Whatman no. 4 paper. Traces of riboflavin phosphate, if present, appeared in the first fractions of the eluate which were then discarded or used to prepare riboflavin phosphate.

$(NH_4)_2SO_4$ (30 g./100 ml.) was dissolved in the combined FAD solutions. The FAD was extracted by three successive portions of *p*-cresol. Two volumes of ether were added to the combined cresol extracts and the FAD extracted by successive 10 ml. portions of water. The combined aqueous extracts were washed with ether and stored in the dark at −10°.

The FAD content was determined from the extinction coefficient of the solution at 450 mμ.; the molecular extinction coefficient was taken as $1·13 \times 10^4$ (Warburg & Christian, 1938). 11 mg. FAD were obtained from 1·6 kg. yeast.

The absorption spectrum of this FAD solution was similar to that described by Warburg & Christian at wavelengths greater than 310 mμ. The ratio of the extinction at 260 mμ. to that at 450 mμ. was 7·4. (Using pure FAD, Warburg & Christian (1938) obtained 3·3.)

When a solution containing 8 μg. FAD was examined by paper chromatography, no riboflavin phosphate, riboflavin or other coloured or fluorescent material was observed. The fluorescence of 0·03 μg. riboflavin or riboflavin phosphate can be detected on the chromatogram.

Sodium adenosinetriphosphate (ATP). The dibarium salt was prepared according to Needham (1942), converted to the monobarium salt and decomposed by treatment with an ion-exchange resin (Bailey, 1948). 95% of the total pyrophosphate P could be liberated by the combined action of myosin and myokinase.

D-Amino-acid oxidase activity was measured in Warburg manometers at pH 8·25 and 37·5° in the presence of 2×10^{-5} M-FAD, 0·040 M-DL-alanine, 0·07 M-ethanol, 0·03 M-pyrophosphate, and 0·10 ml. catalase in a total volume of 2·80 ml. The gas phase was air, the centre well contained 0·2 ml. 2 N-NaOH. 1 min. after immersing the vessels in the water bath, the reactants were mixed. After a further 7 min. readings were taken at 3 min. intervals for 20–30 min. The pressure changes were plotted against time. From the slope of the straight line thus obtained the rate of O_2 uptake was calculated. The activity is expressed as μl. O_2 consumed/20 min.

The thermal inactivation of the oxidase was studied using thin-walled 15 ml. centrifuge tubes of uniform shape. Each tube contained 0·20 ml. enzyme, 1·0 ml. 0·10 M-pyrophosphate and any other addition in a total volume of 2·2 ml. at pH 8·25. After incubation the tubes were cooled in melting ice. The oxidase activity of 2·0 ml. of the solution was then determined, the concentrations of FAD and alanine being adjusted to 2×10^{-5} and 0·04 M, respectively, during the activity determination. When examining the stabilization by DL-methionine or by DL- or D-valine these substrates were used for the activity determination at a final concentration of 0·022 M of the D-isomer. When the stabilizing effects of different concentrations of benzoate were studied the concentration of benzoate during the activity determination was adjusted to be the same throughout each series; similar precautions were taken in experiments with adenosine-5′-phosphate. Incubations were performed in air since the rate of inactivation is the same in air as in N_2.

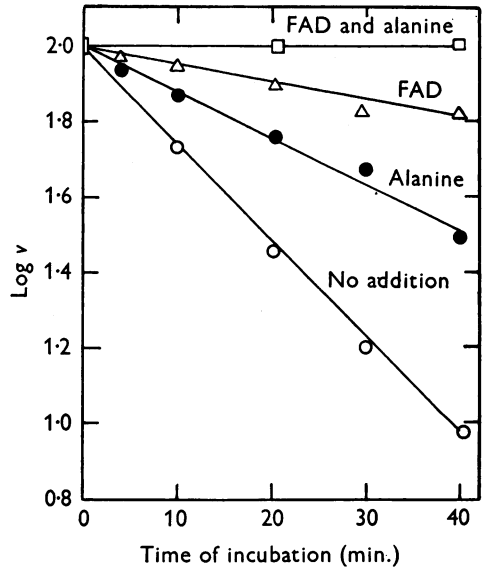

Fig. 1. Stabilization by DL-alanine and/or FAD. v = μl. O_2 consumed/20 min. in the activity test after incubation at 37·5° for the times shown. 4×10^{-6} M-FAD and/or 0·051 M-DL-alanine present as indicated.

The fluorescence of FAD was examined using exciting light of 400–500 mμ. obtained by filtering light from a 12 V., 36 W. car headlamp bulb. The fluorescent light was passed through a yellow filter and the intensities compared using a photo-multiplier and galvanometer. Under these conditions no fluorescence was recorded from aqueous solutions of quinine sulphate.

RESULTS

Stabilization by DL-alanine and/or FAD. At 37·5° and pH 8·25 the spontaneous inactivation of D-amino-acid oxidase is a first order reaction: when FAD or DL-alanine are present the rate of inactivation is reduced. In the presence of both FAD and DL-alanine the oxidase is further stabilized (Fig. 1).

Table 1. *Effect of reducing the rate of cooling on the inactivation of* D-*amino-acid oxidase*

(Immersed in water at 53° for 2 min. The figures are the D-amino-acid oxidase activities of 2·0 ml. of the cooled solution expressed as μl. O_2 consumed/20 min.)

Addition before heating ...	None	0·051 M-DL-Alanine	2×10^{-5} M-Flavin-adenine dinucleotide
Method of cooling:			
(*a*) Melting ice	50	68	90
(*b*) Kept 1 min. in water at 38° before cooling by melting ice	48	66	90
(*c*) Not heated	115	—	—

Table 2. *Effect of the presence of flavin-adenine dinucleotide (FAD) during cooling on the inactivation of* D-*amino-acid oxidase*

(Heated 2 min. in water at temperature shown, FAD added as indicated, immerse in water at 38° for 1 min., cool 5 min. in melting ice. The figures shown are the D-amino-acid oxidase activities of 2·0 ml. of the cooled solution expressed as μl. O_2 consumed/20 min.)

Temperature (°)	0·20 ml. 5×10^{-4} M-FAD added immediately before cooling	0·20 ml. 5×10^{-4} M-FAD added after cooling to 0°
0	129	129
38	119	114
52	64	60
56	47	44
58	31	28

Table 3. *Specificity of stabilization*

(2×10^{-5} M-FAD or 0·050 M-DL-alanine added, as indicated, to each series of four tubes. Only tubes x'_0, x' contain the substance shown in the first column. x, x' incubated at 37·5° for 30 min.; x_0, x'_0 kept at 0°. The figures in the last column indicate the effect of the substance in the first column: >1·0, stabilization; 1·0, no effect; <1·0, inactivating effect.)

	Concentration (mM)	Other additions	μl. O_2 consumed/20 min. in activity test				$\frac{x'}{x'_0} \cdot \frac{x_0}{x}$
			x_0	x	x'_0	x'	
Substrates							
DL-Alanine	50	None	100	29	98	57	2·00
DL-Methionine	26	None	98	30	98	52	1·73
DL-Valine	60	None	114	42	113	85	2·00
D-Valine	30	None	114	42	114	80	1·91
Inhibitors							
(*a*) Substrate antagonists							
L-Leucine	34	None	94	24	76	36	1·85
L-Leucine	34	FAD	94	75	76	72	1·18
L-Leucine	34	Alanine	94	52	76	43	1·01
Sodium benzoate	0·13	None	119	58	97	62	1·32
Sodium benzoate	0·13	FAD	119	103	97	96	1·14
Sodium benzoate	0·13	Alanine	119	74	97	63	1·04
(*b*) FAD antagonists							
Adenosine-5′-phosphate	10	None	110	36	99	71	2·15
Adenosine-5′-phosphate	10	FAD	110	95	100	85	0·98
Adenosine-5′-phosphate	10	Alanine	110	58	99	76	1·46
Adenine	10	FAD	110	95	109	94	0·99
Adenine	10	Alanine	110	67	109	60	0·89
Adenosine	10	FAD	110	95	110	95	1·00
Adenosine	10	Alanine	110	67	110	62	0·93
Caffeine	10	FAD	110	95	110	94	0·99
Caffeine	10	Alanine	110	67	110	57	0·85
Mepacrine (atabrine)	20	FAD	129	108	127	108	1·02
Mepacrine	20	Alanine	129	77	127	33	0·44
Quinine	4	FAD	129	108	127	107	0·99
Quinine	4	Alanine	129	77	127	78	1·03
Affecting rate of oxidase action by less than 5% at the concentrations used							
L-Alanine	10	None	114	28	113	30	1·06
L-Valine	30	None	114	42	112	45	1·09
Glycine	90	None	115	44	114	48	1·08
β-Alanine	45	None	138	40	140	43	1·06
Sodium chloride	90	None	115	44	114	46	1·05
Guanosine-3′-phosphate	10	None	110	40	110	37	0·93
Inosine-3′-phosphate	2	None	115	44	115	44	1·00
Sucrose	100	None	94	24	95	25	0·97
Sodium DL-lactate	80	None	98	30	98	30	1·00
Riboflavin	0·28	None	119	71	115	26	0·38
Riboflavin phosphate	0·07	None	119	71	117	20·5	0·30

Variations of the conditions of cooling. The results of the experiments summarized in Tables 1 and 2 indicate that there is no reversal of the inactivation either when the rate of cooling is reduced or when FAD is added immediately before cooling.

Specificity of the stabilization. In addition to DL-alanine, the substrates DL-methionine, DL-valine and D-valine stabilize D-amino-acid oxidase (Table 3). Several compounds, not being substrates, were examined for any effects on the inactivation of the oxidase and on the rate of the enzyme-catalysed reaction. The results are presented in Table 3. The effects on the enzymic catalysis were examined at a series of concentrations of DL-alanine and of FAD, namely $4{\cdot}4\times10^{-2}$M-DL-alanine and 5×10^{-6}M-, 5×10^{-7}M- or 2×10^{-7}M-FAD and also 5×10^{-6}M-FAD and $2{\cdot}2\times10^{-2}$M- or $4{\cdot}4\times10^{-3}$M-DL-alanine.

L-Leucine and sodium benzoate, which are competitive inhibitors of the oxidase (Edlbacher & Wiss, 1944; Klein & Kamin, 1941), also stabilize the enzyme. The protective effects of these compounds are reduced in the presence of 0·05M-DL-alanine.

Quinine and mepacrine (atabrine) inhibit D-amino-acid oxidase by competing with FAD (Hellerman, Lindsay & Bovarnick, 1945). The inhibition produced by quinine is completely reversible by FAD, but that produced by mepacrine is only partially reversible. Quinine does not affect the inactivation of the oxidase, but mepacrine increases the rate of inactivation. This effect of mepacrine is prevented by 2×10^{-5}M-FAD. Of the FAD antagonists examined quinine, adenine, adenosine and caffeine do not protect D-amino-acid oxidase, but adenosine-5′-phosphate (AMP) protects the oxidase. The protection by AMP is prevented by 2×10^{-5}M-FAD.

Formation of complexes between FAD and some inhibitors. Weber (1948, 1950) has shown that the quenching of the fluorescence of riboflavin by purines in aqueous solution is due to the formation of complexes between the riboflavin and the quencher. It is feasible that some inhibitors of D-amino-acid oxidase act by forming similar complexes with FAD.

Suppose the fluorescent FAD is in equilibrium with a quencher (Q) to form the non-fluorescent complex (FAD) Q

$$\text{FAD}+Q=(\text{FAD})\,Q.$$

If the total concentration of FAD is negligibly small compared with the total concentration of quencher, quenching by complex formation is governed by (Weber, 1950)

$$\frac{(C_o)}{K}=\frac{I_o}{I}-1, \qquad (1)$$

where (C_o) is the concentration of quencher, K is the dissociation constant of the complex and I_o/I is the ratio of the fluorescent intensities in the absence and in the presence of the quencher respectively.

The quenching of the fluorescence of FAD by several inhibitors of D-amino-acid oxidase is represented graphically in Fig. 2, where I_o/I has been

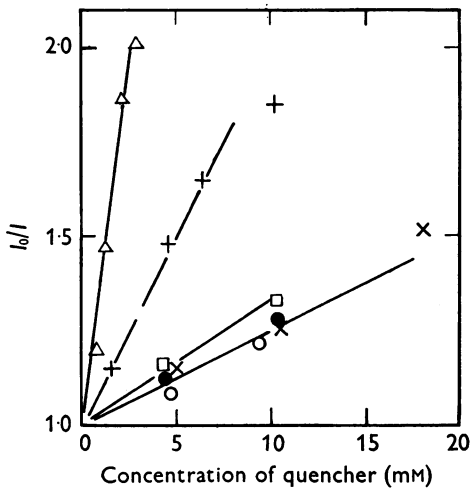

Fig. 2. Quenching of fluorescence of FAD by several inhibitors of D-amino-acid oxidase. I_o is the fluorescent intensity of 5×10^{-6}M-FAD in 0·040M-pyrophosphate pH 8·25 at 18°. I is the fluorescent intensity when the quencher is also present: quinine, △—△; caffeine, +—+; adenosine, □—□; ATP, ×—×; ADP, ●—●; AMP, ○—○.

plotted against the concentration of quencher. From Fig. 2 and equation 1 the corresponding values of K have been evaluated (Table 4).

Table 4. *Quenching of fluorescence of FAD by FAD antagonists*

(pH 8·3; 17°; 5×10^{-6}M-FAD; 0·040M-pyrophosphate buffer; K = dissociation constant of complex obtained from Fig. 2 by using equation 1.)

Compound	K (mmol./l.)
Adenosinetriphosphate	39
Adenosinediphosphate	37
Adenosine-5′-phosphate	40
Adenosine	30
Caffeine	10
Quinine	2·7

The relation between D-amino-acid oxidase activity and FAD concentration is

$$v=\frac{Vf}{K_F+f}, \qquad (2)$$

where v is the reaction velocity in the presence of a concentration f of FAD, V is the maximum velocity obtained by increasing the concentration of FAD, and K_F is independent of f and v.

If an inhibitor acts by forming a complex with the FAD it can readily be shown from equation 2 that if $K \gg K_F$

$$v = \frac{Vf}{K_F(1+i/K)+f}, \quad (3)$$

where i is the concentration of inhibitor.

If an inhibitor acts by combining with the oxidase protein in competition with the FAD a similar equation may be obtained

$$v = \frac{Vf}{K_F(1+i/K_I)+f}, \quad (4)$$

where K_I is the dissociation constant of the inhibitor from its complex with the oxidase protein.

If v_o is the reaction velocity in the absence of inhibitor, from equation 4

$$\frac{v_o}{v} = 1 + \left\{1 - \frac{v_o}{V}\right\}\frac{i}{K_I}. \quad (5)$$

K_I may be evaluated (method I) by plotting $1/v$ against $1/f$ in the presence of a constant concentration of inhibitor. The straight line thus obtained can be produced to intercept the axis $1/v = 0$, where $\frac{1}{f} = \frac{1}{K_F(1+i/K_I)}$. In the absence of inhibitor the similar intercept is $1/K_F$. K_I can be obtained from the ratio of these two intercepts.

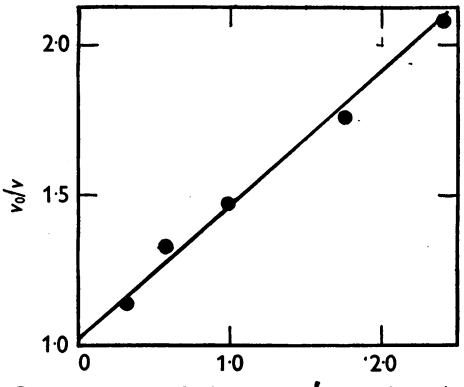

Fig. 3. Inhibition of D-amino-acid oxidase by adenosine-5′-phosphate (AMP). v_o is the reaction velocity in the absence of AMP (44 µl. O_2/20 min.) v is the reaction velocity in the presence of AMP. Concentration of FAD about 2×10^{-7} M.

K_I may also be evaluated (method II) from equation 5: v_o/v is plotted against i with a constant concentration of FAD such that v_o is about half V. K_I is calculated from the slope of the straight line thus obtained. For example, in Fig. 3 with AMP as an inhibitor, the slope of the straight line is 450 l./mole, $v_o = 44$ µl./20 min. and V (in the presence of 2×10^{-5} M-FAD) = 79·5 µl./20 min. From equation 5

$$K_I \text{ for AMP} = K_{AMP} = \frac{1 - 44/79{\cdot}5}{450} = 1{\cdot}0 \times 10^{-3} \text{ M}.$$

Table 5. *Inhibition of oxidase by substances antagonizing flavin-adenine dinucleotide*

(pH 8·25; 37·5°; $2{\cdot}2 \times 10^{-2}$ M-DL-alanine.)

Compound	Method I: Concentration tested (mM.)	Method I: K_I (mM.)	Method II K_I (mM.)
Adenosinetriphosphate	8·5	11	—
	5·4	11	—
Adenosinediphosphate	7·7	1·3	1·4
Adenosine-5′-phosphate	7·4	1·1	1·0
	0·74	1·0	—
Adenosine	19	45	—
Adenine	7·3	22	—
Caffeine	20	11	11
Hypoxanthine	17	24	
Quinine	2·0	2·8	3·0
Adenosine-3′-phosphate	5·3	No inhibition	—

Effect of concentration of protectors on the stabilization. The inactivation of D-amino-acid oxidase in the presence of 5×10^{-6} M-FAD has been examined at various concentrations of sodium benzoate. The rate constant of inactivation (k) has been plotted against the concentration of protector (Fig. 4)

$$k = \frac{1}{t} \ln (x_0/x'),$$

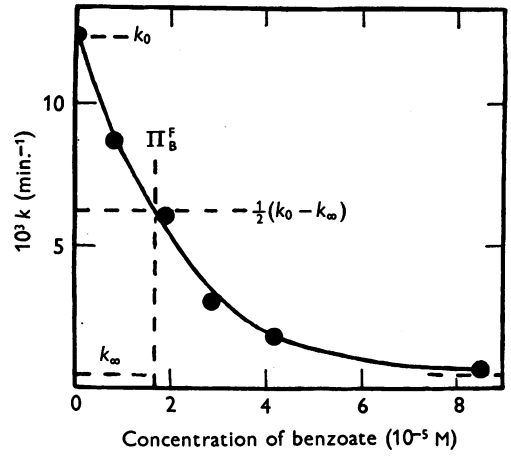

Fig. 4. Stabilization of D-amino-acid oxidase by sodium benzoate in the presence of 5×10^{-6} M-FAD. k is the rate constant of inactivation of the oxidase, assuming that this process is a first-order reaction. The dotted lines indicate how Π_B^F has been evaluated.

where x' is the oxidase activity remaining after incubating at 37·5° with the appropriate concentration of benzoate; x_0 is the corresponding activity obtained with a control tube kept at 0°; t is the time of incubation at 37·5° (= 30 min.).

The curve obtained in Fig. 4 resembled the effect of substrate concentration on the rate of enzyme action. By analogy with the Michaelis constant, the protection constant, Π_B^F, has been evaluated.

Let k_0 be the rate constant of inactivation with no sodium benzoate and k_∞ be the minimum rate constant of inactivation obtained by increasing the concentration of sodium benzoate.

Then Π_B^F is equal to the concentration of sodium benzoate at which $k=\frac{1}{2}(k_0+k_\infty)$. From Fig. 4, $\Pi_B^F=1{\cdot}7\times10^{-5}$M.

The inhibition of D-amino-acid oxidase by benzoate obeys relations analogous to equations 4 and 5. The inhibition constant (K_B) has been evaluated at the same temperature and pH as used for the determination of Π_B^F and using the same preparation of

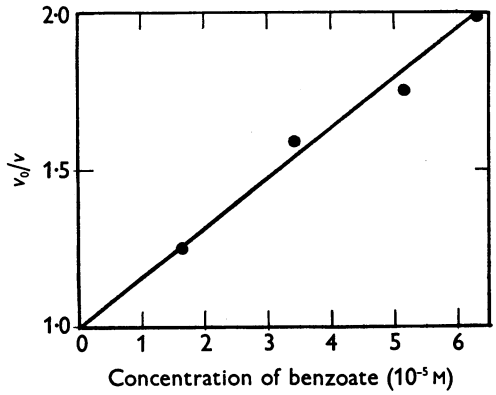

Fig. 5. Inhibition of D-amino-acid oxidase by sodium benzoate. v is the reaction velocity in the presence of benzoate. v_0 is the reaction velocity in the absence of benzoate (68 μl. O_2/20 min.). Concentration of DL-alanine = 0·0080 M.

oxidase. v_0/v was plotted against the concentration of sodium benzoate (Fig. 5) with a constant (non-limiting) concentration of DL-alanine (0·0080 M). The slope of the straight line is $1{\cdot}58\times10^4$ l.mol.$^{-1}$. With 0·080 M-alanine the reaction velocity was 99 μl. oxygen/20 min.; by extrapolation to infinite concentration of alanine, $V'=102$ μl. oxygen/20 min. Since $v_0=68$ μl. oxygen/20 min.,

$$K_B=(1-68/102).\frac{1}{1{\cdot}58\times10^4}=2{\cdot}1\times10^{-5}\text{M}.$$

The protection constants have also been evaluated for other substances: Π_X^Y has been used to represent the protection constant of substance X in the presence of substance Y at a concentration sufficiently high to give the maximum protection by Y alone; Π_X represents the protection of X in the absence of Y.

For the effects of AMP in the presence of 0·051 M-DL-alanine (Figs. 3 and 6) the protection constant $\Pi_{AMP}^{AL}=0{\cdot}95\times10^{-3}$M while the inhibition constant, $K_{AMP}=1{\cdot}0\times10^{-3}$M.

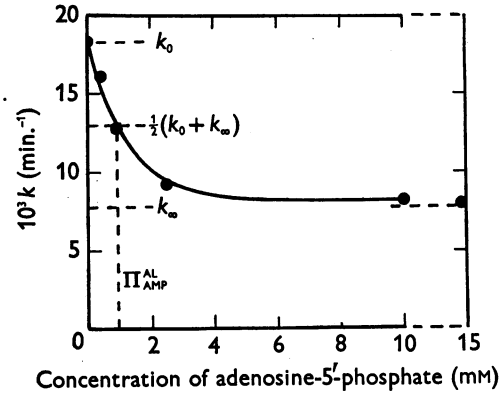

Fig. 6. Stabilization of D-amino-acid oxidase by adenosine-5′-phosphate in the presence of 0·051 M-DL-alanine. k is the rate constant of inactivation of the oxidase, assuming that this process is a first-order reaction. The dotted lines indicate how Π_{AMP}^{AL} has been evaluated.

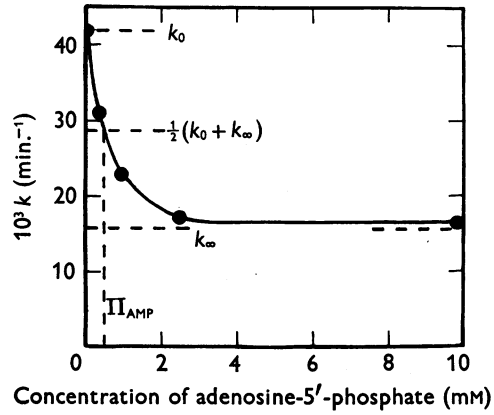

Fig. 7. Stabilization of D-amino-acid oxidase by adenosine-5′-phosphate in the absence of alanine. k is the rate constant of inactivation of the oxidase, assuming that this process is a first-order reaction. The dotted lines indicate how Π_{AMP} has been evaluated.

The protection constants Π_{AMP}, Π_F, Π_{AL}, Π_{AL}^{AMP} have also been evaluated (Figs. 7–10).

$$\Pi_{AMP}=4{\cdot}8\times10^{-4}\text{M};$$
$$\Pi_F=2{\cdot}1\times10^{-7}\text{M};$$
$$\Pi_{AL}=4{\cdot}5\times10^{-3}\text{M};$$
$$\Pi_{AL}^{AMP}=1{\cdot}0\times10^{-2}\text{M}$$

in the presence of 0·045 M-AMP. Π_{AL} and Π_{AL}^{AMP} are expressed in terms of the concentration of D-alanine.

K_{AL}, the Michaelis constant for D-alanine, has been determined in the presence of 2×10^{-5} M-FAD from the effect of the concentration of DL-alanine on the activity of the oxidase. The reciprocal of the

reaction velocity has been plotted against the reciprocal of the concentration of D-alanine (Fig. 11). The straight line thus obtained is produced to intercept the axis $1/v = 0$; K_{AL} is numerically equal to the reciprocal of the intercept on this axis. In constructing Fig. 11, the D-alanine concentration was taken as that at the middle of the period of the respective activity determination. Using a similar method, K_F (defined by equation 2) has been determined in the presence of 0·051 M-DL-alanine.

In air, $K_F = 1{\cdot}3 \times 10^{-7}$ M; $K_{AL} = 2{\cdot}0 \times 10^{-3}$ M.

In oxygen, $K_F = 1{\cdot}3 \times 10^{-7}$ M; $K_{AL} = 2{\cdot}4 \times 10^{-3}$ M.

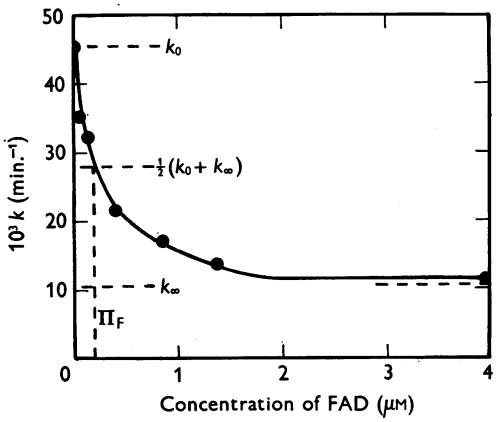

Fig. 8. Stabilization of D-amino-acid oxidase by FAD. k is the rate constant of inactivation of the oxidase, assuming that this process is a first-order reaction. The dotted lines indicate how Π_F has been evaluated.

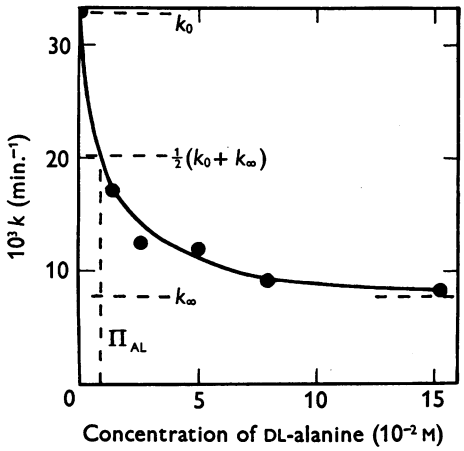

Fig. 9. Stabilization of D-amino-acid oxidase by DL-alanine. k is the rate constant of inactivation of the oxidase, assuming that this process is a first-order reaction. The dotted lines indicate how Π_{AL} has been evaluated.

DISCUSSION

The effect of the concentration of coenzymes, substrates and inhibitors on the rate of enzyme-catalysed reaction is often discussed in terms of a combination between the enzyme and the coenzyme, substrate or inhibitor. If the stabilization of D-amino-acid oxidase by FAD, substrates or competitive inhibitors were also connected with a similar combination, at the same site on the enzyme the protection constants should be related to the Michaelis constants determined at the same temperature and pH.

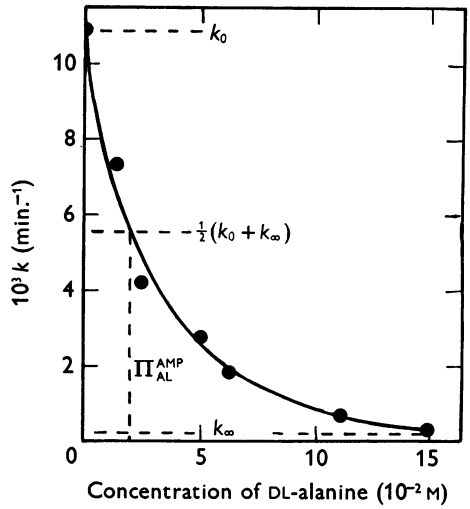

Fig. 10. Stabilization of D-amino-acid oxidase by DL-alanine in the presence of 0·045 M-adenosine-5′-phosphate. k is the rate constant of inactivation of the oxidase, assuming that this process is a first-order reaction. The dotted lines indicate how Π_{AL}^{AMP} has been evaluated.

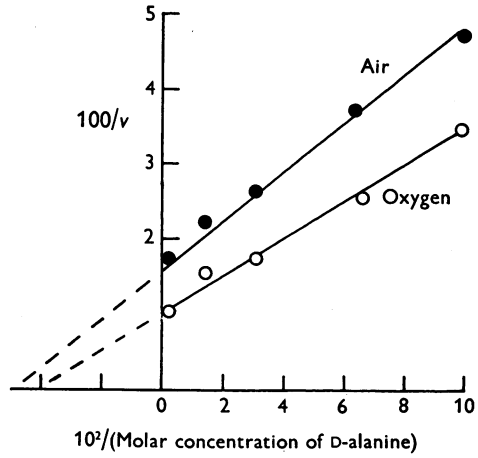

Fig. 11. Effect of alanine concentration on the activity of D-amino-acid oxidase. v = μl. O_2 consumed/20 min. Concentration of FAD = 2×10^{-5} M.

The relation between the rate of inactivation and the concentration of protector can be explained if the apo-enzyme P combines with the protector X to form a complex PX, which is less readily denatured than the apo-enzyme.

Let α_X be the dissociation constant of the PX complex, $\alpha_X = \frac{(P)\,(X)}{(PX)}$ if (P), (X), and (PX) are the equilibrium concentrations.

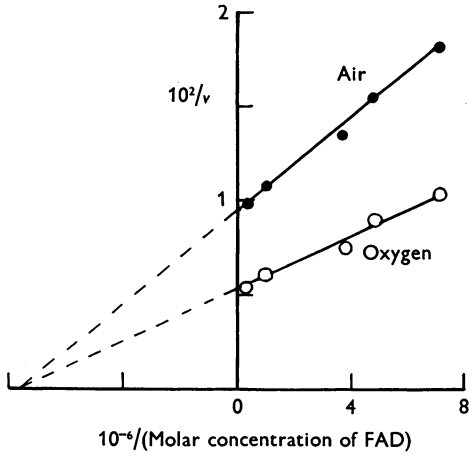

Fig. 12. Effect of FAD concentration on the activity of D-amino-acid oxidase. $v = \mu$l. O_2 consumed/20 min. Concentration of DL-alanine = 0·051 M.

Let k_0 be the rate constant of inactivation of the free apo-enzyme P and let k_∞ be the rate constant of inactivation of the complex PX.

The resultant rate constant of inactivation of an equilibrium mixture of P, X and PX is

$$k = \frac{(P)\,k_0 + (PX)\,k_\infty}{(P) + (PX)} = \frac{\alpha_X k_0 + (X)\,k_\infty}{\alpha_X + (X)},$$

when $(X) = \alpha_X$, $k = \frac{k_0 + k_\infty}{2}$.

Therefore $\Pi_X = \alpha_X$.

When sodium benzoate protects the oxidase in the presence of excess FAD, Π_B^F should be equal to the expression $\frac{(PF)\,(benzoate)}{(PFB)}$. If the inhibition by benzoate depends on the inhibitor combining with the holo-enzyme (PF) in competition with the substrate, K_B is also equal to the expression

$$\frac{(PF)\,(benzoate)}{(PFB)}.$$

The values of Π_B^F and K_B determined under similar conditions agree within experimental error.

This agreement and also that between Π_{AMP}^{AL} and K_{AMP} supports the hypothesis that the sites of inhibition and protection are identical; this hypothesis is also supported by the reduced protection by benzoate in the presence of alanine; similarly, FAD reduces the protection given by AMP (Table 3). These effects are comparable to the reversals of benzoate inhibition by alanine and of AMP inhibition by FAD respectively.

The constants Π_{AL}, Π_{AMP}, Π_{AL}^{AMP}, Π_{AMP}^{AL}, if identified with appropriate equilibrium constants, should be given by the expressions

$$\Pi_{AL} = \frac{(P)\,(alanine)}{(P\text{-}alanine)},$$

$$\Pi_{AMP} = \frac{(P)\,(AMP)}{(P\text{-}AMP)},$$

$$\Pi_{AL}^{AMP} = \frac{(P\text{-}AMP)\,(alanine)}{(alanine\text{-}P\text{-}AMP)},$$

$$\Pi_{AMP}^{AL} = \frac{(P\text{-}alanine)\,(AMP)}{(alanine\text{-}P\text{-}AMP)}.$$

So the products, $\Pi_{AL}.\Pi_{AMP}^{AL}$ and $\Pi_{AMP}.\Pi_{AL}^{AMP}$ should both equal the expression

$$\frac{(alanine)\,(P)\,(AMP)}{(alanine\text{-}P\text{-}AMP)}.$$

From the experimental values of the protection constants:

$$\Pi_{AL}.\Pi_{AMP}^{AL} = 4{\cdot}5 \times 10^{-3} \times 0{\cdot}95 \times 10^{-3} = 4{\cdot}3 \times 10^{-6}\,M^2,$$

$$\Pi_{AMP}.\Pi_{AL}^{AMP} = 4{\cdot}8 \times 10^{-4} \times 1{\cdot}0 \times 10^{-2} = 4{\cdot}8 \times 10^{-6}\,M^2.$$

The hypothesis identifying protection constants with equilibrium constants is supported by the studies of Boyer, Lum, Ballou, Luck & Rice (1946) and of Boyer, Ballou & Luck (1947). Caprylate was found to stabilize serum albumin. The concentration of caprylate producing half the maximum stabilization (about 0·0034 M) is approximately equal to the dissociation constant of the caprylate-serum albumin complex (0·0041 M) as determined by ultrafiltration studies.

Warburg & Christian (1938) considered that the effect of FAD concentration on the activity of D-amino-acid oxidase was a result of an equilibrium between the protein and FAD. Stadie & Zapp (1943) concluded that the kinetics of the oxidase agree with a theory based on equilibria assumed to exist between the protein and the substrate, FAD and hydrogen ions.

The stabilization of D-amino-acid oxidase by FAD or by substrates seems to enable the more detailed study of the equilibria which may be

involved in the action of the enzyme. These equilibria are:

$$P+A=PA, \quad (i)$$

$$P+F=PF, \quad (ii)$$

$$PA+F=PFA, \quad (iii)$$

$$PF+A=PFA, \quad (iv)$$

where A represent the substrate.

The mechanism can then be completed by the reaction:

$$PFA+O_2 \rightarrow \begin{matrix} P+F \\ \text{or} \\ PF \end{matrix} + \text{products}. \quad (v)$$

Stadie & Zapp (1943) considered a similar mechanism, but omitted reaction (i). It was assumed that the reaction velocity v is given by

$$v=k'\,(PFA),$$

where k' is independent of f, a and the total concentration of enzyme. It was also assumed that in the steady state the proportion of the total protein present as PFA is the same as if the protein was in equilibrium with FAD and substrate according to the postulated reactions. They derived a theoretical relation between v and the concentration of the reactants involving equilibrium constants.

If the same treatment is applied to the mechanism as postulated above, it is found that when $a \gg K_{AL}$, the constant K_F is the equilibrium constant of reaction (iii):

$$K_F=\frac{(PA)\,(F)}{(PFA)}. \quad (9)$$

Similarly, when $f \gg K_F$,

$$K_{AL}=\frac{(PF)\,(A)}{(PFA)}. \quad (10)$$

The product $\Pi_{AL}.K_F$ should be equal to $\Pi_F.K_{AL}$ for both are theoretically equal to the expression

$$\frac{(P)\,(F)\,(A)}{(PFA)}.$$

From the experimental values for K_F, K_{AL}, Π_F and Π_{AL}:

$$K_F.\Pi_{AL}=1{\cdot}3\times10^{-7}\times4{\cdot}5\times10^{-3}$$
$$=5{\cdot}8\times10^{-10}\,M^2;$$

$$\Pi_F.K_{AL}\ (\text{in air})=2{\cdot}1\times10^{-7}\times2{\cdot}0\times10^{-3}$$
$$=4{\cdot}2\times10^{-10}\,M^2.$$

It should be noted that the expressions for K_{AL} and K_F are independent of k'. Increasing k' by increasing the oxygen tension has little effect on K_F, but there is an increase in K_{AL}; this indicates that the assumptions made in deriving equation 10 are not fully justified.

The action of FAD antagonists on the D*-amino-acid oxidase system.* Hellerman *et al.* (1945) have investigated the ability of various substances (chiefly antimalarial drugs), to antagonize FAD in the D-amino-acid oxidase system.

The present work shows that adenosinediphosphate (ADP), AMP and quinine are more effective inhibitors than ATP, adenosine, adenine and caffeine. The formation of non-fluorescent compounds between the inhibitor and FAD can account for the inhibition of D-amino-acid oxidase by quinine adenosine or caffeine, but not by ADP or AMP. It seems that ADP and AMP can combine with the oxidase-(D-alanine) complex in competition with the FAD. From Tables 4 and 5 it is concluded that the affinity of the protein-alanine complex for ADP and AMP is at least twenty times the affinity for the related compounds adenosinetriphosphate, inosine-5′-phosphate, adenosine-3′-phosphate, guanosine-3′-phosphate or adenosine. This seems reasonable for ADP and AMP are more closely related to FAD than are the other compounds.

SUMMARY

1. At pH 8·25 and at 37·5°, flavin-adenine dinucleotide (FAD), substrates and the competitive inhibitors L-leucine and sodium benzoate protect D-amino-acid oxidase apo-enzyme from thermal inactivation.

2. Of several substances which inhibit D-amino-acid oxidase by antagonizing FAD, adenosine-5′-phosphate has been found to protect the oxidase. Quinine, mepacrine, adenosine, adenine and caffeine inhibit in this way, but do not protect the oxidase.

3. The stabilization has been investigated in relation to the kinetics of the enzyme at the same temperature and pH.

4. Several substances, not being substrates or inhibitors, yet related to FAD or D-amino-acids, do not protect the oxidase under the conditions investigated.

5. The protection of the oxidase is discussed in terms of an equilibrium between the protein and protector. Evidence is presented that the site of protection by benzoate or by AMP is the same as the site of inhibition.

6. The formation of non-fluorescent complexes between the inhibitor and FAD can account for the inhibition of the oxidase by quinine, adenosine and caffeine. This process does not account for the inhibition by AMP or adenosinediphosphate.

7. It is concluded that AMP and adenosinediphosphate are able to combine with the enzyme protein in competition with the FAD. The affinity of the protein for ADP or AMP is at least twenty times the affinity for adenosinetriphosphate, inosine-5′-phosphate, adenosine-3′-phosphate, guanosine-3′-phosphate or adenosine.

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Biochemistry of Fluoroacetate Poisoning. Isolation of an Active Tricarboxylic Acid Fraction from Poisoned Kidney Homogenates

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The experiments described in this paper* were done in order to advance knowledge of the biochemistry of fluoroacetate poisoning. The proved failure of this compound to inhibit isolated enzymes made it difficult to relate the toxic action to an enzymic effect, until Bartlett & Barron (1947) made the important step of showing that in tissue slices fluoroacetate led to accumulations of acetate. Another significant point was made by Saunders (1947) and colleagues who found an alternation in the toxicity with increasing length of carbon chain in the ω-fluoroesters; compounds with an even number of carbon atoms were toxic. Then Liébecq & Peters (1948, 1949) found an instance with centrifuged kidney homogenates where fluoroacetate caused depression of oxygen uptake without an increase of acetate but with accumulation of citrate; at the same time the oxidation of added citrate was inhibited by the poison. This led them to propose the jamming hypothesis; according to this sodium fluoroacetate can be activated and condensed with oxaloacetate to form a fluorotricarboxylic acid, which either itself, or in the form of some derivative, inhibits the tricarboxylic acid cycle and so causes accumulation of citrate. A similar hypothesis was advanced independently by Martius (1949). Kalnitsky & Barron (1948) later reported accumulation of citrate, but interpreted this differently. It was an important extension from these *in vitro* tests when Buffa & Peters (1949) found that the accumulation of citrate was also induced readily *in vivo* by injection of fluoroacetate into animals. This has been recently confirmed by Potter & Busch (1950).

The best proof for the jamming hypothesis would be the isolation of a toxic fluorotricarboxylic acid either from the tissues of poisoned animals or from the products of some poisoned tissue preparation. Calculation showed that this was impracticable *in vivo*, owing to the small amount of substance present in poisoned tissues; attempts were therefore made to isolate the tricarboxylic acids formed during the oxidation of fumarate by a kidney homogenate *in vitro* in the presence of fluoroacetate. In this work, there will be described the isolation of a 'citrate' fraction from poisoned kidney tissue, a component of which has the power to inhibit citrate metabolism *in vitro*; this is not fluoroacetate, but is believed to be a tricarboxylic acid which contains fluorine in small amount.

* The main facts recorded here were given in lectures to the Physiological Society at Oslo in March 1950, also in Uppsala; see also Buffa, Lotspeich, Peters & Wakelin (1950).

EXPERIMENTAL

Determination of the inhibitory activity of tricarboxylic acid fractions using kidney homogenates

The object of the test was to determine the capacity for inhibiting the disappearance of citrate. Two methods were used.

(1) The technique developed in this laboratory by Liébecq & Peters (1949) was slightly modified. The guinea pig kidney homogenate, made in a cooled mortar, was not squeezed