Studies on the Purification and Properties of Aldehyde Oxidase from Horse Liver

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The detection and partial purification of an enzyme in *pig* liver which catalyzed the oxidation of aldehydes but not xanthine has been reported by Gordon, Green and Subrahmanyam¹. The present report contains studies on a similar enzyme found in *horse* livers. The enzyme was partially purified by fractional precipitations with ammonium sulfate, and potassium phosphate buffer at about pH 7.8 followed by electrophoresis. The procedure resulted in a product which was purified about twentyfold with a recovery of about 20 per cent of the total activity of the starting material.

The most highly purified preparations of the horse liver enzyme possessed an activity on a weight basis that was about equivalent to onethird of the activity obtained by Gordon *et al.*¹ on their best preparation of enzyme obtained from pig liver. However, as judged by the lack of an absorption peak at 405 m μ (see Fig. 1), the horse liver preparation appeared to be quite free of catalase. Catalase was apparently still present in the preparation made by Gordon *et al.*¹ Despite the fact that the purified horse liver enzyme possessed an orange color, it did not possess any pronounced absorption peak from 200 to 500 m μ , except the one at 275 m μ which is attributable to aromatic amino acids (Fig. 1). Absorption bands due to flavineadenine dinucleotide, expected at 380 and 450 m μ ¹, were not detected.

Stimulation by ammonium ions

It became apparent quite early in these studies that ammonia or ammonium ions in quite high concentration produced a marked stimulation of the enzyme. This observation arose from experiments designed to test the stability of the

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Fig. 1. Absorption spectra of electrophoretically separated fractions from a preparation of horse liver aldehyde oxidase: fraction containing the enzyme activity —; and fraction containing no enzyme activity - - - -■

enzyme to dialysis. Gordon *et al.*¹ reported the pig liver enzyme to be completely inactivated by dialysis for 24 hours against distilled water at 0°. Acting on the supposition that this inactivation of the pig liver enzyme could have been due to a drop in pH or to removal of salt during the dialysis, a fraction of horse liver enzyme which had been prepared by fractional precipitation with ammoniacal ammonium sulfate¹ was subjected to dialysis at 0° against 0.01 *M* potassium phosphate buffer at pH 7.4. It was noted that the activity of the preparation dropped rapidly during the first 6 hours of dialysis and then remained fairly constant at a level of about 20 per cent of the activity of the original material during dialysis for another 24 hours. However, upon the addition of various amounts of ammoniacal ammonium sulfate to the dialyzed enzyme, the resulting mixtures possessed activities that were equal to or even greater than that of the original undialyzed preparation.

Since the addition of sodium sulfate, sodium chloride or potassium phosphate at the proper pH to dialysed preparations of the enzyme did not bring about an increase in activity, while additions of ammonium sulfate, ammonium chloride and ammonium phosphate at the proper pH did bring about an in-

Fig. 2. Effect of ammonia concentration on the activity of horse liver aldehyde oxidase at pH 8.0 with salicylaldehyde as substrate when the ammonia was added as a salt of: chloride —; sulfate — — —; and phosphate — · — · —. The experiment was performed in Thunberg tubes containing 0.1 ml of 0.0113 M methylene blue, 0.1 ml of 0.062 M salicylaldehyde, 0.1 ml of enzyme, 0.9 ml of borate (0.2 M)-phosphate (0.2 M) buffer at pH 8 and 1 ml various concentrations of ammonium salts so as to give the concentrations indicated in the figure.

crease in the activity of dialysed preparations, it was apparent that the stimulation was due to ammonia or ammonium ions. Cognizance was taken of this fact in modifying the methylene blue method of assay of Gordon *et al.*¹ so that an excess amount of ammonia was present in all assays.

Fig. 2 shows the effect of ammonia concentration at pH 8.0 on the activity of a dialysed preparation of horse liver aldehyde oxidase. The results show that the activity of the preparation increased in an almost identical manner regardless of whether the ammonia was added as a salt of chloride, sulfate or phosphate until a concentration of about 0.5 M ammonium ion in the reaction mixture was reached. Increasing the concentration of ammonium ions above

Fig. 3. Effect of substrate concentration on the activity of horse liver aldehyde oxidase: acetaldehyde plus equivalent amounts of ammonia ●—●; and the ammonia derivative of acetaldehyde ○— — —○. Experiment performed in Thunberg tubes containing 1 ml of enzyme, 1 ml of 0.2 M borate buffer at pH 8.2, 0.1 ml of 0.0113 M methylene blue and 0.1 ml of solutions of either the ammonia derivative of acetaldehyde or of acetaldehyde plus an equivalent amount of ammonia as $(\text{NH}_4)_2\text{HPO}_4$.

0.5 M in the reaction mixture resulted in an inhibition of activity. This inhibition varied somewhat with the type of salt used.

Because of the effect of ammonia in stimulating the activity of the enzyme, it was thought that the ammonia might take an active part in the reaction, possibly through a mechanism such as that illustrated in the following equation, where M. B. indicates methylene blue:

Fig. 4. Effect of substrate concentration on the activity of horse liver aldehyde oxidase in the presence of an excess of ammonium ions: acetaldehyde plus an equivalent amount of ammonia $\circ$ — $\circ$; ammonia derivative of acetaldehyde $\bullet$ — $\bullet$. Conditions the same as for Fig. 3 except that 1 ml of 1 M $(\text{NH}_4)_2\text{HPO}_4$ was used instead of 1 ml of borate buffer.

In order to test this assumption, the ammonia derivative (II) of acetaldehyde was prepared² and compared as a substrate at various concentrations with acetaldehyde plus an equivalent amount of ammonia added as diammonium hydrogen phosphate. The results, which are illustrated in Fig. 3, indicated that at least at low substrate concentrations the two substrates were practically equivalent. Essentially the same results were obtained (see Fig. 4) when the ammonia derivative (II) and acetaldehyde were compared as substrates in a medium containing large amounts of ammonium ions. However, at high substrate concentrations, the ammonia derivative (II) was a slightly better substrate than the free aldehyde. Furthermore, the ammonia derivative, unlike the free aldehyde, did not inhibit the reaction when present in an

amount several times that necessary to produce optimum activity. Since the ammonia derivative of acetaldehyde is quite unstable in aqueous solution, one can not conclude that it is being used directly as a substrate. At any rate the derivative (II) does not appear to be greatly superior to the free aldehyde as a substrate.

Since the rather large amounts of ammonia used in these tests precluded detecting the use of ammonia in the reaction by measuring the disappearance of ammonia during the experiment, attempts were made to demonstrate the presence of acetamide (III) in the reaction products. The procedure used by Speck³ for the determination of glutamine in the presence of ammonia was adapted to the problem. No evidence was obtained for the formation of appreciable amounts of acetamide during the reaction. Thus the results do not support the proposed theory for the stimulatory affect of ammonia on the oxidation of aldehydes by horse liver aldehyde oxidase. They do not, however, eliminate the possibility that ammonia is utilized in some other fashion in the oxidative reaction.

Optimum pH

In Fig. 5 the effect of pH at constant ammonia concentration on the activity of the enzyme is shown when furfural was used as a substrate. Essentially the same results were obtained when acetaldehyde or salicylaldehyde were used as substrates. These results indicate that in the presence of 0.91 *M* ammonium ion in the reaction medium, the optimum pH occurs at 8.1 ± 0.3 . The lower value of pH 7 was reported by Gordon *et al.*¹ for the pig liver enzyme.

Substrates

A number of aldehydes and aldehyde derivatives were tested to see if they would serve as substrates for the action of the partially purified enzyme in methylene blue reduction tests. Acetaldehyde, *formaldehyde*, heptaldehyde, furfural, salicylaldehyde, paraldehyde, benzaldehyde, crotonaldehyde, chloroacetal and pyridoxal possessed some activity as substrates. Quinine⁴ and xanthine possessed a very slight but still detectable activity as substrates. Acetone would not serve as a substrate.

The effect of substrate concentration on the activity of the enzyme was investigated for salicylaldehyde, *n*-heptaldehyde, furfural and acetaldehyde. The results of these investigations for acetaldehyde and furfural are shown in Figs. 4 and 6. The results show that there is a rather narrow range of optimum concentration of substrate and that the activity of the enzyme falls off quite rapidly when the optimum concentration is exceeded. The concentration of the aldehyde in the reaction mixture which gave the highest activity is shown in column two of Table 1.

Fig. 5. Effect of pH on the activity of horse liver aldehyde oxidase at an ammonia concentration of 0.91 M in the reaction mixture and with furfural as the substrate. The Thunberg tubes contained 1 ml of enzyme, 0.1 ml of 0.1 M furfural, 0.1 ml of 0.0113 M methylene blue and 1 ml of various ammonium phosphate buffers prepared by mixing various amounts of 1 M $(\text{NH}_4)_2\text{HPO}_4$ with either 2 M $\text{NH}_4\text{H}_2\text{PO}_4$ or 0.66 M $(\text{NH}_4)_3\text{PO}_4$. The pH noted is that found on the reaction mixture after reduction of the dye.

It was obvious from these results that estimates of the relative activities of substrates as determined by Gordon *et al.*¹ in which all aldehydes were tested at the same concentration were likely to be misleading. Therefore, acetaldehyde, furfural, salicylaldehyde and *n*-heptaldehyde were compared as substrates at their optimum concentrations against the same enzyme preparation. The results showed that both salicylaldehyde and furfural were much better substrates than acetaldehyde when each was compared at its optimum concentration. *n*-Heptaldehyde on the other hand was a very inferior substrate.

In connection with the studies on the ammonia stimulation effect, it was thought advisable to investigate the use of certain aldehyde derivatives as

Table 1. Relative effectiveness of various aldehydes as substrates for aldehyde oxidase.

Tests were performed in Thunberg tubes in which the main bulb contained 1 ml of enzyme and 1 ml of 1 *M* $(\text{NH}_4)_2\text{HPO}_4$; and the side bulb contained 0.1 ml of 0.0113 *M* methylene blue and 0.1 ml of a solution of the various aldehydes so as to give, after mixing the contents of the Thunberg tube, the molarity indicated in the table.

Aldehyde	Molarity of aldehyde in test	Relative activity (acetaldehyde = 100 %)
Acetaldehyde	0.046	100 %
Furfural	0.0046	175 »
Salicylaldehyde	0.0028	175 »
<i>n</i> -Heptaldehyde	0.0014	1.8 %

substrates for the enzyme. Of particular interest to the author was the class of compounds called thiazolidines which have recently been reported to occur in nature as an integral part of the penicillin molecule⁵. Several aldehydes were condensed with L-cysteine to give 4-thiazolidine carboxylic acids of the general formula (IV).

The compounds from formaldehyde (IV, R = H), furfural (IV, R = $\text{C}_4\text{H}_3\text{O}$) and benzaldehyde (IV, R = C_6H_5) were prepared according to the directions of Schubert⁶. The compound from acetaldehyde (IV, R = CH_3) was made according to the directions found in the monograph on *The chemistry of penicillin*⁷. All of these 4-thiazolidinecarboxylic acids except the one prepared from formaldehyde served as substrates for the action of the enzyme. However, none of the active thiazolidines were as effective as substrates as the corresponding aldehydes. The thiazolidines needed to be present in much higher concentrations than the free aldehydes in order to give maximum activity. A comparison of furfural and 2-furfuryl-4-thiazolidinecarboxylic acid as substrates is shown in Fig. 6. It is possible that the substrate activity of the thiazolidines is due to a partial reversion of the compounds in solution to aldehydes and cysteine^{6,8}.

Fig. 6. Effect of substrate concentration on the activity of horse liver aldehyde oxidase: furfural — — — —; and 2-furfuryl-4-thiazolidinecarboxylic acid ————. The Thunberg tubes contained 1 ml of enzyme, 1 ml of 1 M $(\text{NH}_4)_2\text{HPO}_4$, 0.1 ml of 0.0113 M methylene blue and 0.1 ml of solutions of either furfural or 2-furfuryl-4-thiazolidinecarboxylic acid of various concentrations.

EXPERIMENTAL

Assay method. — In the main bulb of a Thunberg tube were placed the enzyme in a volume of 1.0 ml and 1.0 ml of 1 M $(\text{N}_4\text{H})_2\text{HPO}_4$. In the side bulb of the Thunberg tube was placed 0.1 ml of a solution that was 1 M with respect to acetaldehyde and 0.0113 M with respect to methylene blue. The tube was evacuated for at least one minute on a water pump, and then placed in a water bath at 37° for 2.0 minutes. The contents of the two bulbs were mixed and the time for complete reduction of the methylene blue was noted. The enzyme contained one unit of activity when the reduction time was sixty seconds under the above conditions. In practice it was found advantageous to use an

amount of enzyme that would cause reduction of the dye in a period of 20 to 40 seconds. This was because a plot of (1/reduction time) against dilution of the enzyme, although linear for crude enzyme, fell off rather rapidly for purified preparations of the enzyme.

Since very crude fractions of the enzyme would bring about reduction of the dye in the absence of substrate, it was necessary in these cases to determine the activity in units in the absence of substrate and subtract this value from the value obtained in the presence of substrate in order to secure a fair approximation of the activity due to the aldehyde oxidase enzyme.

Activity per unit weight. — The activity was referred to the optical density of the solution at 280 $m\mu$. The ratio of the activity in units per cubic centimeter to the optical density at 280 $m\mu$ of a 1 centimeter thick solution was used as a measure of purity. For abbreviation this is written as "units/o.d.". Turbid solutions were clarified by centrifugation at high speed in an angle head before making the optical density determinations.

The relationship of the optical density at 280 $m\mu$ of a solution to the dry weight of material was determined on a purified fraction of enzyme (0.22 units/o.d.) from which catalase had been removed by electrophoresis. From the results of dry weight and absorption spectra measurements it was found that an optical density reading of 1.88 for a 1 cm thick solution corresponded to 1 mg of material per cubic centimeter of solution. Thus a fair approximation of the activity per unit protein can be obtained by the following equation:

$$\text{units/mg} = (\text{units/o.d.}) \times 1.88$$

In many of the fractionation procedures the removal of catalase was followed by determining the optical density at 405 $m\mu$ as well as at 280 $m\mu$. The ratio of absorption at 280/405 was used as a measure of the catalase present. This ratio became progressively larger as the catalase was removed, reaching a value of about 7.4 when a peak at 405 $m\mu$ was no longer detected.

Fractionation of horse liver aldehyde oxidase

Starting material. — In the course of this work both fresh and frozen horse livers were used. The frozen horse livers had been stored at -15° for periods ranging from three to fifteen days. Experience with these two types of material showed the fresh, unfrozen livers to be a much better source of starting material than livers which had been stored for several days in the frozen state. About twice as much enzyme with about twice the specific activity was obtained from fresh livers as was obtained from the frozen livers.

During the early stages of fractionation of this enzyme turbid solutions which could not be readily clarified were encountered. Due to the turbidity of the solutions it was found impossible to fractionate with salt by visual observation of precipitates. Thus it was necessary to add the salt to certain predetermined concentrations and to collect the fractions therefrom. The salt concentrations were determined by measuring the specific gravity of the solutions with floating pycnometers and relating the specific gravity to salt concentration by use of a standard curve. As fairly dilute solutions of protein were used in the fractionations, the contribution of the protein to the density of the solution was quite small. However, in order to make the results strictly reproducible it was found advisable to adjust the protein concentrations of the solutions before fractionating with salt. This was accomplished by diluting the protein solution until the desired optical density reading at 280 $m\mu$ was obtained. For most fractionations the solutions were

Table 2. Steps II to V. Fractionation of horse liver aldehyde oxidase.

Step no.	Description	Starting material		Data on fractions obtained		
		Source	Protein conc. (o.d./ml)	Salt conc. ^d moles	Total activity (units)	Specific activity (units/o.d.)
II	2nd fractionation with $(\text{NH}_4)_2\text{SO}_4^a$ (a) 1st ppt. ^e (b) 2nd ppt. (c) M. L. ^f	See Text	108		21 600	0.015
				1.56	6 650	0.013
				1.92	10 500	0.023
					0	0
III	3rd fractionation with $(\text{NH}_4)_2\text{SO}_4^b$ (a) 1st ppt. (b) 2nd ppt. (c) M. L.	II-b	90		10 500	0.023
				1.35	500	
				1.86	9 000	0.031
					0	0
IV	1st fractionation with phosphate ^c (a) 1st ppt. (b) 2nd ppt. (c) M. L.	III-b	100		9 000	0.031
				1.02	1 200	
				1.20	6 250	0.076
					810	
V	2nd fractionation with phosphate ^c (a) 1st ppt. (b) 2nd ppt. (c) M. L.	IV-b	50		5 800	0.076
				0.99	500	
				1.20	4 800	0.160
					450	

^a Solid, technical grade ammonium sulfate was added.

^b Solid, C. P. grade ammonium sulfate was added.

^c A 4 M phosphate solution (10 % KH_2PO_4 and 90 % K_2HPO_4) was added.

^d Concentration of the salt in solution in moles per liter, as measured by specific gravity, when the precipitates were formed.

^e All precipitates were collected by centrifugation.

^f M. L. indicates the mother liquor from the second precipitate.

diluted so that a 1 cm thick solution would have an optical density at 280 $m\mu$ equal to 100, equivalent to 50 to 60 milligrams of protein per cubic centimeter.

Typical fractionation

Preliminary extract. — 11.3 kg of fresh horse liver, which had been freed of fat and arterial tissue, were ground in a meat grinder and extracted with 22.6 kg of tap water at 4° for 16 hours. After removal of the cell debris by centrifugation, the supernatant

liquid measured 23.1 liters and contained 24 000 aldehyde oxidase units with a purity of 0.0118 units/o.d.

Step I. First precipitation with ammonium sulfate. — Technical grade ammonium sulfate in the amount of 35 g per 100 ml of supernatant fluid was added to 23 liters of the supernatant liquid at 4°. After a period of 1.5 hours at 4°, the precipitate was collected by centrifugation. The mother liquor was either discarded or used for the preparation of horse liver alcohol dehydrogenase. The precipitate was dissolved in water to give a solution containing 21 600 units (90 %) with a purity of 0.015 units/o.d.

Steps II to V. — The active precipitate from step I was fractionated twice with ammonium sulfate and then twice with phosphate (10 % potassium dihydrogen phosphate and 90 % dipotassium hydrogen phosphate) as outlined in Table 2.

In the fractionations with ammonium sulfate the pH was purposely not adjusted (*i. e.*, solutions had a pH of about 5.7). Since the enzyme was rather unstable under these conditions, it was necessary to carry out the fractionations with ammonium sulfate as promptly as possible. Although some activity was lost by the fractionation at pH 5.7, the enzyme appeared to be more stable in later stages of purification if it were fractionated at this step without adjusting the pH to 7.5 to 8.5.

When the active material from step V (see Table 2, Fraction V-b) was dissolved in water a nearly clear solution, black-brown in color, was obtained. This solution, when stirred under a strong light, had a sheen resembling that observed during the formation of protein crystals. A similar phenomenon was observed in several preparations at this level of purity. When the solution was centrifuged for 1 hour at 18 000 rpm, a small amount of a bright yellow substance, presumably fat or lipoprotein, accumulated at the surface. This substance, which was apparently the cause for the sheen, did not contain any activity. It could not be completely removed by centrifugation but did gradually disappear on further fractionation with salt.

Material at this or a slightly greater stage of purity was used for most of the electrophoresis experiments.

Steps VI to VIII. — Results of further fractionation with phosphate are described in Tables 3, 4, and 5. In these fractionations the solution was diluted to an optical density of about 70 at 280 m μ and then 4 M phosphate was added dropwise with the collection of 4 or 5 fractions containing the activity. Each fraction was analysed for enzyme activity and optical density at 280 and 405 m μ .

The results of the last three fractionations with phosphate are typical of several experiments carried out in this fashion. Material with a specific activity of 0.27 units/o.d. could be obtained quite readily. However, in several instances further fractionation of such material with phosphate gave considerable inactivation of the enzyme. As a result the more purified material although containing less catalase, as judged by the ratio of absorption at 280/405, possessed a lower specific activity than the starting material.

Electrophoresis

Several preparations of a purity of 0.2 units/o.d. were investigated by electrophoresis at pH 7.8 to 8.8. These preparations showed the presence of two main components which could be separated by electrophoresis. The component moving the fastest toward the anode contained the enzyme. The slower moving component possessed an absorption spectrum in agreement with that of catalase. The absorption spectra of fractions separated in this fashion are reproduced in Fig. 1.

Table 3. Step VI. Third fractionation with phosphate.

Fraction	Total units %	Total extinction at 280 m μ %	Units/o.d.	280/405
Starting material	100	100	0.16	5.1
1st ppt.	2	3	0.099	4.9
2nd ppt.	61	42	0.240	5.8
3rd ppt.	26	24	0.177	5.2
M. L.	8	32	0.041	5.0
Total recovery	97	101		

Table 4. Step VII. Fourth fractionation with phosphate.

The second and third precipitates from step VI (Table 3) were combined and refractionated.

Fraction	Total units %	Total extinction at 280 m μ %	Units/o.d.	280/405
Starting material	100	100	0.214	5.5
1st ppt.	15	15	0.212	6.25
2nd ppt.	43	34	0.270	6.25
3rd ppt.	15	12	0.270	5.50
4th ppt.	10	11	0.204	5.25
M. L.	6	28	0.053	4.65
Total recovery	89	100		

Table 5. Step VIII. Fifth fractionation with phosphate.

The second and third precipitates from step VII (Table 4) were combined and refractionated with 4 M phosphate.

Fraction	Total units %	Total extinction at 280 m μ %	Units/o.d.	280/405
Starting material	100	100	0.27	5.88
1st ppt.	27	33	0.244	6.95
2nd ppt.	22	25	0.246	7.00
3rd ppt.	9	10	0.252	6.35
4th ppt.	8	10	0.220	5.77
M. L.	7	21	0.094	5.00
Total recovery	73	99		

Experiments were undertaken to determine at what pH these two components separated the most rapidly. The same initial enzyme preparation was subjected to electrophoresis at pH 7.3, 8.3 and 9.5 in buffers possessing essentially the same ionic strength as determined by conductivity measurements. Nearly the same current density was used in all three cases. An automatic compensator was used and set at such a rate as to keep the fastest moving boundary (the enzyme) practically motionless. The rate of separation of the catalase and enzyme boundaries was then determined. At the conclusion of the experiment the identity of the two reactions was checked by enzyme assay and light absorption measurements. From the results of this experiment (Table 6) it is evident that increasing the pH increases the rate of separation of the two boundaries. It also, of course, increases the rate of travel of the two boundaries toward the anode.

Table 6. Separation of enzyme and catalase by electrophoresis.

pH	Type buffer	Applied voltage	Current ma	Separation cm/hour *
7.3	Phosphate	330	17.5	0.150
8.3	»	330	17.5	0.190
9.5	Borate	300	18.5	0.214

* The separation refers to the images projected on a ground glass scale and not to the actual separation rate in the solution.

Stability

Stability to storage at 4°. — A variety of conflicting results were obtained in stability studies performed on various fractions stored at 4°. As a rule crude preparations (0.04 to 0.10 units/o.d.) at pH 7.5 to 9 could be kept for a period of several weeks at 4° without appreciable loss of activity. Purer preparations (0.1 to 0.2 units/o.d.) were more apt to suffer greater losses of activity during the same period. Inactivation that did eventually occur in these relatively stable solutions was probably due primarily to the development of microorganisms.

However, not all preparations of the enzyme exhibited this relatively great stability to storage. In some cases the activity would decrease as much as 5 to 10 % per day from the day that it was stored. The marked differences in the stability of various preparations could not be definitely traced to any one factor. However, it was noted that nearly all, if not all, of the preparations which had been fractionated with ammonium sulfate without pH adjustment (*i. e.*, at about pH 5.7) in the early stages of the purification exhibited a marked stability to storage.

Stability to lyophilization. — A fraction of enzyme containing 0.04 units/o.d. which had been dialysed against 0.05 M phosphate at pH 8.1 was subjected to lyophilization. The material that had been dried in the frozen state retained 66 per cent of the activity of the starting material.

Stability to dialysis. — Considerable variation in the stability of the enzyme to dialysis was noted. The stability to dialysis of various preparations seemed to be related to the stability to storage at 4° of these preparations. Preparations that were stable to storage

could, on the whole, be dialysed for several days in the pH range 7.3 to 9.5 without any large losses of activity. On the other hand preparations which lost from 20 to 50 % of their activity on dialysis also proved to be quite unstable on storage.

Successful dialysis experiments were obtained on all the buffers tried, *i. e.*, phosphate, borate, and ammonia-ammonium sulfate. Thus instability of certain preparations could not be traced to the buffer salt.

Crude preparations tended to be much more stable to dialysis than preparations having a purity of 0.2 units/o.d. or better. However, in several instances preparations of this purity were successfully dialysed.

The activity lost by some preparations on dialysis did not appear to be due to the loss of a prosthetic group or co-factor other than ammonia. Ammonia, of course, was added back to dialysed solutions before assay. For example, one catalase-free preparation of enzyme was dialysed for a week against constant changes of dilute ammonia water at pH 8.8. The enzyme lost all of its activity during this time, but lost none of its total light absorption in the range 500 to 230 $m\mu$. Thus the removal of a prosthetic group, absorbing in the range 230 to 500 $m\mu$, seemed to be eliminated. In another instance, a crude preparation was dialysed in a similar fashion against dilute ammonia water at pH 8.5. During the dialysis, the enzyme lost approximately 50 % of its activity. The dialysate was concentrated to dryness *in vacuo* at 40°. The addition of the residue to the impermeate did not increase the activity of the enzyme. In still another instance, a few cubic centimeters of the original enzyme extract were boiled for five minutes. The insoluble material separated, and the cooled supernatant was added to a preparation which had lost about one-half of its activity on dialysis. The addition of the boiled extract did not increase the activity of the dialysed preparation.

From all of these results it seems safe to conclude that the losses of activity which occur occasionally on dialysis are due to some type of inactivation of the enzyme similar to that occasionally experienced on storage of the enzyme. Certainly there is no evidence for the removal of any co-factor other than ammonia during the dialysis.

SUMMARY

1. A procedure for the partial purification of aldehyde oxidase from horse liver is described.

2. The activity of the enzyme as measured by the methylene blue reduction test is enhanced by the presence of ammonium salts. Studies on the nature of this stimulation by ammonium salts are reported. In the presence of ammonium salts, the enzyme has its optimum activity at pH 8.

3. A number of aldehydes including formaldehyde are effective as substrates for the enzyme. Furfural and salicylaldehyde are about twice as effective as substrates as is acetaldehyde.

4. At high substrate concentration, the ammonia derivative of acetaldehyde is a slightly better substrate than free acetaldehyde. Although L-cysteine derivatives of several aldehydes (thiazolidines) can be used as substrates, they are not as effective as the corresponding free aldehydes.

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