55. Adenosine Deaminase

By T. Brady, *Biochemical Department, University College, Dublin*

(Received 15 April 1942)

In this paper a more detailed study of the preparation and properties of adenosine deaminase in tissue extracts is described as well as stages in the purification of the enzyme. The rate of enzymic deamination of 'desoxyadenosine'* (adenine-desoxyriboside) is compared with that of adenosine and the question of a separate deaminase for the desoxy compound examined.

Unlike the deaminase of muscle adenylic acid, that of adenosine is not affected to any appreciable extent by tissue inhibitors or by phosphate, bicarbonate, veronal etc., and above a very low limit the deamination is independent of the substrate concentration [Conway & Cooke, 1939]. Its high specificity was first shown by Schmidt [1928; 1932]. The tissues which Schmidt used for his experiments, the skeletal muscle and liver of rabbits, have been found to be very poor sources of the enzyme compared with the duodenum; jejunum and appendix, but even in the most active tissue of the rabbit, it is present in much lower concentration than adenylic acid deaminase in skeletal muscle [Conway & Cooke, 1939]. For the further investigation of adenosine deaminase a richer source was sought. The small intestines (jejunum and duodenum) of the cow, bullock, heifer and calf were tested, and found to be considerably more potent than the same tissues of the rabbit. The calf's intestine was the most active, giving an average concentration of 0.8 enzyme units (mg. $\text{NH}_3\text{-N/min.}$) per g. which may be compared with about 0.06 unit per g. of the jejunum or duodenum in the rabbit [Conway & Cooke, 1939]. The concentration of adenosine deaminase in the calf's intestine was found to be about four times greater in the light mucoid layer than in the deep mucosa. Further advantages in the use of the calf's mucosa as enzyme source is the ease with which it can be prepared in a dry state, and the fact that once dry it retains its activity for a very long time (at least one year) when kept in the cold.

Crude enzyme preparations

(a) *Aqueous extracts* were made by grinding the tissue with pure quartz sand and diluting to the required volume. The supernatant liquid which contained the enzyme was then freed from undissolved tissue and sand by centrifuging. The disadvantage of these extracts is that even when they are kept in the refrigerator, the activity falls off very rapidly after about 2 days.

(b) *Acetone precipitate from aqueous extracts.* An aqueous extract made as described above was treated with 4 vol. acetone. The precipitate, after standing for about half an hour, was centrifuged, washed with acetone, and dried in a vacuum desiccator overnight. Preparations made in this way contained most of the deaminase present in the original extract, but were deep brown in colour.

(c) *Acetone-dried mucosa from calf's intestine.* This was found to be the most convenient stock source of the enzyme. It may be noted that in samples of the intestinal mucosa of the cow, calf, bullock and heifer, the concentration of the enzyme fluctuates within wide limits. For the dried mucosa of the calf the range was from 0.1 to 15 enzyme units per g. (one enzyme unit = 1 mg. $\text{NH}_3\text{-N/min.}$). Further, the concentration of this enzyme is invariably highest in that portion of the intestine nearest the stomach, falling steeply away until at from 5-6 m. away from the stomach it is less than 1/10th that of the first metre.

* It seems desirable to shorten the rather cumbersome name adenine-desoxyriboside to desoxyadenosine.

In the preparation of the *stock enzyme powder* the first three metres of the intestine, cooled in ice immediately after removal from the animal, were cut into two equal lengths and treated separately. The intestine, after washing in a slow stream of cold water, was cut down the centre and the top mucosa scraped off with a spatula. The viscous mass was shaken with four times its weight of acetone. After exactly 10 min., the supernatant fluid was removed on a suction pump. The rather pasty material was broken up with a glass rod and the process repeated. The second extraction gave a yellow powdery material which was freed from acetone by draining on the suction pump for about 20 min. It was dried in a vacuum desiccator over P_2O_5 for about 8 hr. and then ground to a fine powder in a mortar. After a further drying for 12 hr. it was stored in the refrigerator, retaining its original activity over a very long period.

Solutions of the enzyme can be made by extracting this powder with water. The activity of these extracts unlike that of the fresh tissue remains unaltered for at least a week, even when kept at room temperature. The stability is probably due to the destruction of proteolytic enzymes by the acetone treatment.

Estimation of enzyme activity. 0.5 ml. of a solution containing 0.5 mg. adenosine (or desoxyadenosine) was added to 3.5 ml. of a $M/14$ phosphate/citrate buffer with pH of 6.2. To this was added 1 ml. of the enzyme extract, whose dilution in water had been arranged so that after 3 min. action on the substrate NH_3 in amount convenient for estimation was formed. Determination of NH_3 was by the microdiffusion technique [Conway, 1939] the central chamber of the Conway units containing 1 ml. $N/5000$ acid with indicator, which was titrated with $N/2500$ $Ba(OH)_2$ from the microburette. Deamination was immediately suspended by pipetting the solution into the saturated carbonate contained in the outer chamber of the microdiffusion apparatus.

Enzyme units (e.u.) are expressed for convenience as mg. NH_3 -N/min. at a temperature of 18° , and referred to 1 g. of the enzyme source, and are 1000 times as great as those used by Conway & Cooke [1939].

Some properties of adenosine deaminase

(1) *Influence of H and OH ions.* After 30 min. at *ca.* pH 3, and on bringing to pH 6.4, practically no deaminase activity remained. The enzyme was less sensitive to OH ions; at pH 11.0 there was only slight inactivation.

(2) *Influence of commonly used inhibitors.* The inhibitor, usually $M/100$, was allowed to act on the enzyme for about 30 min. 1 ml. of the mixture was then added to the substrate giving a final inhibitor concentration of $M/500$. The substances used may be roughly divided into the following groups:

(a) *Metals.* Cu^{++} , Ag^+ and Cr^{+++} gave total inhibition. Zn^{++} , Co^{++} and Ni^{++} reduced the deaminase activity by about 20%. Fe^{++} and Mn^{++} (also Mg^{++}) were without action on the enzyme.

(b) *Oxidizing agents* had little effect, with the exception of iodine which inhibited completely. H_2O_2 and quinone, $M/500$, were found to have no effect. $KMnO_4$, however, reduced the activity by about 80%. After 24 hr. the activity of the quinone/enzyme solution was reduced to 65% of that of the original, and $KMnO_4$ /enzyme solution was completely inactivated. $M/5$ H_2O_2 reduced the enzyme action by only 20%.

(c) *Reducing substances.* $Na_2S_2O_3$ and $Na_2S_2O_4$ were without action.

(d) *Narcotics.* Veronal, urethane and chloral hydrate were also without effect.

(e) *Inhibitors of metal-containing enzymes.* $NaCN$ and NaN_3 were without action. $Na_4P_2O_7$, on the other hand, caused a 20% inhibition.

(f) *SH-reactants.* Iodoacetic acid [Bersin, 1935; Hopkins & Morgan, 1938] and maleic acid [Morgan & Friedman, 1938] were without influence on the enzyme at pH 6.2.

(g) *Other substances* tested, including pyruvic acid, semicarbazide, cysteine, succinic and lactic acids, sodium fluoride, arsenate and quinol, were without effect on the enzyme.

(3) *Dialysis and attempted coenzyme separation.* Celloidin sacs were prepared from an 8% solution of celloidin (Schering) dissolved in a 50% ether/alcohol mixture [cf. Brown, 1915].

In order to prepare membranes of graded permeability from these sacs the procedure adopted by Brown [1915] was followed in detail. His nomenclature is also used and a '95%' sac is one which after drying has been immersed in 95% alcohol for 24 hr. and subsequently washed with distilled water. Before using these sacs for dialysis it is essential that they should be tested for leaks.

Dialysis of enzyme with celloidin membranes of different permeability. From Table 1 it will be seen that there is a very marked divergence in the rate of inactivation of adenosine deaminase with membranes of different permeability.

Table 1. *Effect of dialysis*

Time in hr. ...	0	24	48	72
	Activities $\times 10^3$			
Control	4600	4500	4300	4220
'95 %'	4500	2300	870	500
'80 %'	4500	4270	3590	—
'70 %'	4500	4500	—	—

Enzyme concentration—1 g. of powder in 1000 ml. water.

Activity expressed as mg. $\text{NH}_3\text{-N/min./g.}$

Dialysed against distilled water which was changed 2-3 times every 24 hr.

The enzyme contained in the '95%' membrane was the most rapidly inactivated and this membrane was therefore chosen for subsequent experiments.

Effects of added dialysate, etc., on activity of dialysed enzyme. 90 ml. of a 1/1000 enzyme solution contained in 10 sacs were suspended in 720 ml. of distilled water which was stirred continuously. After 72 hr. the dialysed enzyme had only lost about 60% of its original activity, due to the fact that the outer liquid was not replaced by fresh water.

80 ml. of the dialysate were concentrated *in vacuo* at 35° to 10 ml. To this was added an equal volume of the dialysed enzyme, but as can be seen from Table 2 (c) there was

Table 2. *Attempted reactivation of dialysate*

Time in hr. ...	0	24	48	72	120
	Activities $\times 10^3$				
(a) Control	4400	4400	4400	4300	4300
(b) Dialysed enzyme	4200	3400	2000	1800	400
(c) Dial. enz. + conc. dialysate	—	—	—	1900	—
(d) Dial. enz. + boiled enz. extract	—	—	—	1700	—
(e) Dial. enz. + MnSO_4 , <i>M</i> /250	—	—	—	1500	—
(f) Dial. enz. + fresh enzyme	—	—	—	3500	—

Activities as mg. $\text{NH}_3\text{-N/min./g.}$ enzyme powder.

only a small increase in activity (within the experimental error). As there was a possibility that a coenzyme might have been destroyed in the course of the distillation, the dilute dialysate was added to the dialysed enzyme in a concentration of 8:1. The control (in this case the dialysed enzyme) was diluted to the same extent with distilled water, but there was no increase in activity. Similarly, additions of boiled enzyme extract, fresh enzyme extract, or Mn^{++} were not capable of reactivating the enzyme.

Deamination of desoxyadenosine

A comparison was made between the deamination rates of adenosine and desoxyadenosine [cf. Brady, 1941] by extracts from fresh tissues of different animals and from the acetone-dried calf mucosa. The results are summarized in Table 3.

Table 3. *Deamination of adenosine and desoxyadenosine*

I		II	III	IV	V
Tissue		Dilution	Adenosine deamination mg. $\text{NH}_3\text{-N/min./g.}$ tissue $\times 10^3$	Desoxyadenosine deamination mg. $\text{NH}_3\text{-N/min./g.}$ tissue $\times 10^3$	Ratio of IV to III %
Rabbit:					
Whole blood		1/50	4.4	2.18	47
Plasma		1/5	0.034	0.014	42
Ventricle		1/50	3.3	1.9	58
Auricle		1/50	6.2	3.5	56
Jejunum		1/250	63.5	33.8	54
Thymus		1/100	22.7	10.7	47
Chicken:					
Whole blood		1/50	5.6	3.55	63
Rat:					
Whole blood		1/50	0.48	0.23	48
Human:					
Whole blood	A	1/50	1.53	0.82	54
"	B	1/50	1.34	0.84	63
Acetone-dried tissues			mg. $\text{NH}_3\text{-N/min./g.}$	mg. $\text{NH}_3\text{-N/min./g.}$	
Calf:					
Light mucosa	A	1/10,000	4.80	5.00	104
Deep mucosa	B	1/5000	1.70	1.68	99
Light mucosa	B	1/30,000	15.4	17.7	116
"	C	1/50	0.121	0.118	97
Rabbit:					
Duodenum A		1/500	0.204	0.115	56
"		1/5000	0.177	0.105	59

The units in which the deamination is expressed are mg. $\text{NH}_3\text{-N/min.}$ for 1 g. of tissue (or 1 ml. blood). In the upper part of Table 3 the deamination data are, for convenience, multiplied by 10^3 . For acetone-dried tissues the activity is referred to 1 g. of the dry powder.

It will be seen from Table 3 that desoxyadenosine is deaminated at approximately 50 % the rate of adenosine in all the fresh tissues tested. It was surprising, therefore, to find that extracts made from the acetone-dried superficial mucosa, as well as from the deep mucosa of the calf's intestine deaminated desoxyadenosine to the same extent as adenosine. As it was conceivable that the acetone treatment and subsequent drying might have changed the enzyme in some manner, a comparison was made between the acetone-dried duodenum of a rabbit (Duodenum A, Table 3) prepared by extraction of the ground tissue with water, precipitation with acetone and subsequent drying, with that of an acetone-dried sample of calf's mucosa prepared in the same manner. The results, in the case of the rabbit's duodenum are the same as when fresh tissue was used, and the calf's mucosa (a sample of exceptionally high activity) appears to deaminate desoxyadenosine at a somewhat higher rate than adenosine. The lower rate of deamination of desoxyadenosine in the aqueous extracts may be due to some inhibitor. If so, dilution of such extracts might be expected to increase their total power to deaminate desoxyadenosine, in a similar way to the marked effect produced on adenylic acid deamination [Conway & Cooke, 1939]. Dilutions of extracts from the rabbit's duodenum up to 1/5000 showed, however, no difference in the ratio of adenosine to desoxyadenosine deamination.

Deamination of the mixed substrates. If adenosine and desoxyadenosine are deaminated by two different enzymes, one would expect an additive effect with the mixed substrates. The results of a number of observations with mixed substrates are summarized in Table 4, and compared with deaminations of the single substrates, the adenosine deamination

Table 4. *Relative rates of deamination*

Deamination of	Calf's mucosa (superficial)		Calf's mucosa (deep)	Rabbit's duodenum
	I	II		
Adenosine	100	100	100	100
Desoxyadenosine	105	96	99	56
Adenosine + desoxyadenosine	110	102	95	68

rate being expressed as 100. It will be seen that for extracts from the calf mucosa or rabbit duodenum there is no appreciable increase in deamination rate with mixed substrates, and it may be concluded that there is here no separate deamination of the desoxy substance.

Stages in the purification of adenosine deaminase

Extraction. 15 g. acetone-dried calf's mucosa (14.4 e.u./g.) were extracted with 300 ml. distilled water by continuous shaking for 30 min. The residue, after centrifuging, was extracted with a further 150 ml. water. The combined extracts which were cloudy and deep yellow in colour were filtered through two layers of muslin. The pH of the extract was 6.7. Each ml. contained 0.484 e.u. and 17.8 mg. solids. The total vol. (358 ml.) contained 168 e.u.

Removal of inactive proteins with salicylic acid. Of a number of protein precipitants investigated in preliminary experiments, salicylic acid was chosen. 350 ml. of the enzyme extract were cooled, mixed with an equal volume of saturated salicylic acid and cooled in the refrigerator for 15 min. The precipitate was quickly centrifuged off, washed with a further 50 ml. saturated salicylic acid, and the washings were combined with the first supernatant, the pH of which was 4.4, volume 700 ml. The activity of this solution was not determined, but it contained a total of 155 e.u.

Precipitation with acetone. Two volumes of acetone were added as soon as possible to the above fluid. A fine precipitate containing most of the enzyme formed. After standing for approximately $\frac{3}{4}$ hr. in ice, the main bulk of this precipitate had settled out. The supernatant liquid was decanted and the precipitate centrifuged off. Two washings with cooled acetone were sufficient to free it from salicylic acid (negative FeCl_3 test in diluted acetone washings). The white precipitate, freed from coloured impurities by the salicylic acid precipitation, was dried in a vacuum desiccator over P_2O_5 . Weight 0.973 g. It contained 119 e.u. or 122 per g.

This powder retains 70% of the enzyme activity present in the original crude powder. It is nine times as active, is quite stable when kept in a refrigerator, and appears to be a good starting material for further purification.

As a further step in this direction, the powder was extracted with 250 parts of water and heated to 54–55° for 10 min. in a water bath. The fine cloudy suspension of denatured protein which separated was removed by centrifuging at high speed. The clear solution which resulted had only a slightly less deaminating power than the untreated solution. It contained 2.5 mg. dry weight per ml., and 185 e.u./g.

DISCUSSION

Adenosine deaminase is in general very stable towards inhibitors. It is irreversibly inactivated by 30 min. at pH about 3. Apart from this, among common inhibitors examined, and with a 30 min. action at $N/100$ strength, the enzyme is inhibited to a marked extent only by certain metals such as Cu, Ag and Cr and by iodine. Sodium pyrophosphate produced a 20% inhibition, which may possibly indicate a metal-protein as the active system, perhaps one containing copper [Massart, Dufait & van Gremberger, 1939]. On the other hand, NaCN and NaN_3 , typical inhibitors of metal-containing enzymes [Keilin & Mann, 1940], were without action.

Until recently, the hydrolases were regarded as a class of enzymes which were not separable into coenzyme and apoenzyme [Haurowitz, 1939]. However, Klein [1935] was able to inactivate the enzyme nucleosidase by dialysis, and reactivate it by addition of arsenate-ion. Edlbacher & Baur [1938] inactivated arginase by dialysis and reactivated it by addition of various metallic ions of which Mn^{++} was the most active. He suggested that Mn^{++} was the coenzyme of arginase, and Richards & Hellermann [1940], using a different method to obtain reversible inactivation, confirmed Edlbacher's observations. Shortly afterwards Albers [1939] was able to separate a highly purified phosphatase solution into coenzyme and apoenzyme, and by adding the concentrated dialysate to the inactive apoenzyme was able to restore most of the original activity. The earlier observations of Onodera [1915] on the coenzyme of urease are much disputed, and the position with this enzyme must be regarded as still in doubt [Oppenheimer, 1926]. Adenosine deaminase, like the hydrolases mentioned above, is inactivated by dialysis. So far, however, attempts to reactivate it have proved unsuccessful.

Desoxyadenosine deamination. With an adenosine deaminase concentration of 0.0012 unit/ml. (1 unit = mg. N/min.), Conway & Cooke [1939] found that the rate of deamination is independent of the substrate concentration down to 3 μ g. amino-N/ml., below which the rate fell markedly and was only 0.35 μ g. N/min. at a substrate value of 0.6 μ g. amino-N/ml. Examining the desoxyadenosine deamination with an enzyme concentration of 0.53×10^{-3} unit/ml. it was found in the present investigation that no appreciable fall in rate occurred down to a substrate concentration of 0.6 μ g./amino-N, the value being then 0.48 mg./min. If we interpret the enzyme action as a combination with substrate and subsequent breakdown of the complex, it would appear that desoxyadenosine combines more rapidly than adenosine. Comparing the deamination rates of the two substrates at higher concentrations, they are almost the same in extracts of the calf's mucosa showing that here the rate of breakdown of the complex is about the same for the two substances. With the mixed substrates there is no increase or change in rate, which is the expected result with the one enzyme operating. In extracts of the rabbit's duodenum and using substrates above minimal levels, desoxyadenosine deamination is only about half that of adenosine. If the rate at which the desoxy substance combines with the substrate is the faster it is to be expected that on using the mixed substrates the deamination should not be the mean for the two nucleosides, but should lie nearer that for the desoxy compound, and this is the result found. For example, if the adenosine deamination rate is taken as 100, that of desoxyadenosine is 56, but the deamination rate of the mixed substrates is 68 rather than the mean of the two rates which is 78.

Purification of the enzyme. The stage already reached gives an activity of 180 mg. NH_3 -N/min./g. of the dry material. This would correspond to about 210 units/g. at 20° (from some determinations made on the temperature effect). Interpreted in terms of adenosine per second per g. the figure becomes 0.07, and this may be compared with the activity of crystalline urease which deaminates approximately 0.95 mg. urea per second per g. [Sumner, 1930], so that the enzyme preparation may be expected to afford a good basis for the further stages of purification (the investigation of which the author was not in a position to pursue).

SUMMARY

1. The superficial mucosa of the calf's intestine has been found to contain adenosine deaminase in high concentration.
2. A partial purification of the enzyme from this source gives an activity of 180 mg. NH_3 -N/min./g. (about 3000 times that found in the rabbit's intestine).
3. The effects of many commonly used inhibitors have been studied, and marked effects obtained only with the heavy metals, iodine and strong oxidants; but the enzyme is

sensitive to changes in hydrogen-ion concentration, and a 30 min. exposure to pH 3 produces an irreversible inhibition.

4. The enzyme is inactivated by dialysis, but so far it has not been possible to reactivate it.

5. Enzyme preparations from the calf's intestinal mucosa deaminate desoxyadenosine at approximately the same rate as adenosine, but from the blood of man, fowl, rat and rabbit and various tissues of the rabbit including intestine, at only half the rate.

A study of the attack on mixed substrates shows that there is no separate enzyme for the desoxy compound, but this substance appears to unite more rapidly with the enzyme. The complex so formed (except for calf mucosa) breaks down more slowly than the similar adenosine complex.

The author is indebted to the Irish Medical Research Council for a research grant, and to Prof. E. J. Conway for helpful discussion and advice.

REFERENCES

- Albers, D. [1939]. *Hoppe-Seyl. Z.* **261**, 43.
 Bersin, Th. [1935]. *Ergebn. Enzymforsch.* **4**, 68.
 Brady, T. G. [1941]. *Biochem. J.* **35**, 855.
 Brown, W. [1915]. *Biochem. J.* **9**, 591.
 Conway, E. J. [1939]. *Microdiffusion Analysis*, etc. London.
 Conway, E. J. & Cooke, R. [1939]. *Biochem. J.* **33**, 479.
 Edlbacher, S. & Baur, H. [1938]. *Hoppe-Seyl. Z.* **254**, 275.
 Gulland, J. M. & Story, L. F. [1938]. *J. chem. Soc.* p. 259.
 Haurowitz, F. [1938]. *Fortschr. Biochem.* Part III.
 Hopkins, F. G. & Morgan, E. J. [1938]. *Biochem. J.* **32**, 611.
 Keilin, D. & Mann, T. [1940]. *Biochem. J.* **34**, 1163.
 Klein, W. [1934]. *Hoppe-Seyl. Z.* **224**, 244.
 — [1935]. *Hoppe-Seyl. Z.* **231**, 125.
 Massart, L., Dufait, R. & van Gremberger, G. [1939]. *Hoppe-Seyl. Z.* **262**, 270.
 Morgan, E. J. & Friedman, E. [1938]. *Biochem. J.* **32**, 862.
 Onodera, N. [1915]. *Biochem. J.* **9**, 575.
 Oppenheimer, C. [1926]. *Die Fermente und ihre Wirkungen*, **2**, 790.
 Richards, M. M. & Hellermann, L. [1940]. *J. biol. Chem.* **134**, 237.
 Schmidt, G. [1928]. *Hoppe-Seyl. Z.* **179**, 243.
 — [1932]. *Hoppe-Seyl. Z.* **208**, 185.
 Sumner, J. B. [1930]. *Ber. dtsch. chem. Ges.* **63**, 582.

Note added 8 July 1942. The pure crystalline desoxyadenosine prepared as in a previous paper [Brady, 1941] showed a higher melting point from that published for Klein's preparation [1934] and the possibility arose that it might be an isomeride. The absorption spectrum has been observed meanwhile by M. G. Kelleher (Department of Physics, Trinity College, Dublin) and the maximum absorption was found at 2591 Å.U. with an accuracy of ± 5 Å.U. This is very close to that recorded for Klein's substance [Gulland & Story, 1938], so that the two preparations may be taken as chemically identical.