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Breakdown of the Oxidized Forms of Coenzymes I and II by an Enzyme from the Central Nervous System

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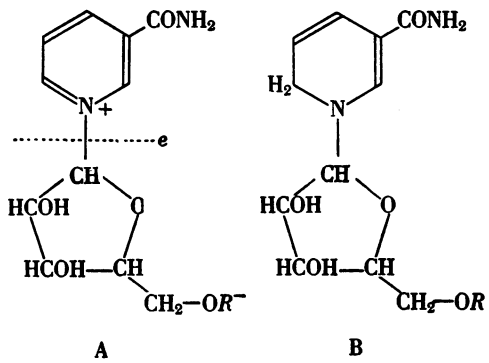
The enzymic breakdown of cozymase in various cells, and especially in tissues from the central nervous system, attracted our attention as being an unexpectedly rapid reaction (McIlwain & Hughes, 1948; McIlwain & Rodnight, 1949). It appeared that the breakdown, if unimpeded, would rapidly deplete tissues of this very important metabolite. Certain factors which would partly restrain the degradation under physiological conditions were observed, but the problem of the normal function of such a potent system remained unanswered. In general terms this appeared likely to be one of participation in, or control of, reactions in which cozymase functioned as coenzyme, and because of the importance of these the degrading enzyme has been studied further.



A B

Fig. 1. A, coenzymes I or II; *e* indicates point of fission by brain preparations; B, or an isomeride with differently placed double bonds, dihydrocoenzymes I or II.

It is fairly well established that the breakdown of cozymase in nervous tissues and in certain micro-organisms takes place at the pyridinium-N (Fig. 1; Handler & Klein, 1942; McIlwain & Hughes, 1948). In some other tissues different processes preponderate. A link between nicotinamide and a substituted ribose is at present known to exist naturally only in cozymase (Co I), coenzyme II (Co II) and nicotinamide mononucleotide, although other natural and synthetic products approach these structures in various ways. The possible action of the enzyme on a number of such substances has now been examined.

EXPERIMENTAL

Materials

Cozymase. The specimens described previously (McIlwain & Rodnight, 1949) were used.

Dihydrocozymase. The preparation followed Schlenk (1941). Reaction with a specimen of Co I (120 mg., containing 68 mg. Co I) was complete in 30 min. and differential ethanol precipitation yielded a first fraction of 44 mg. dihydrocozymase. Titration with $K_3Fe(CN)_6$: 1·2 mg. required 0·45 ml. of 0·005 M-solution; content, 0·94 μmol./mg. Manometric reaction with $K_3Fe(CN)_6$: 4·3 mg. gave 4·65 μmol. CO_2; yield, 1·08 μmol./mg. Optical density at 340 mμ. (c = 0·094 mg./ml.), 0·501; content, 0·86 μmol./mg. The pure material as disodium salt contains 1·41 μmol./mg.

Two other specimens, of 45 and 55% purity, were also examined.

Coenzyme II was prepared from liver (in different batches, sheep and horse livers were used) following in part Altman's (1948) adaptation of the procedure of Warburg, Christian & Griese (1935). After precipitation as Hg and Ba salts, materials up to 10% pure were obtained. We are indebted to Dr B. C. J. G. Knight (Wellcome Physiological Research Laboratories) for a supply of horse liver immediately after slaughter.

Dihydrocoenzyme II. Attempted preparation from a coenzyme specimen of 10% purity, by the method used for

dihydrocozymase, was unsuccessful. Solutions of the substance were prepared enzymically, as described in the text below.

Nicotinamide glycosides. We are greatly indebted to Prof. A. R. Todd and Dr L. J. Haynes (University Chemical Laboratory, Cambridge) for specimens of these compounds in their dihydro forms. They were synthetic materials of which full details will be published elsewhere. Their oxidized forms were prepared by reaction with $K_3Fe(CN)_6$ in a manometric apparatus (see below).

Analytical methods

Ferricyanide and iodine titrations of dihydronicotinamide derivatives. With I_2, a 0·005 M-solution in KI was used and titrations performed in dilute acetic acid containing starch. With $K_3Fe(CN)_6$, a 0·005 M-solution was used, and the dihydro derivative was titrated in dilute acetic acid with KI and starch as external indicator.

Manometric reaction with ferricyanide (cf. Haas, 1937). The dihydronicotinamide derivatives were dissolved in 1 ml. of 0·02 M-$NaHCO_3$ saturated with 5% (v/v) CO_2 in N_2 in the main compartment of conical Warburg vessels of approx. 15 ml. The side arms of experimental and control vessels contained 0·3 ml. of 0·05 M-$K_3Fe(CN)_6$ which was tipped into the main compartment after equilibration with the above gas mixture; reaction was usually complete in 1 hr. This method was used also for reoxidizing dihydrocozymase to cozymase for use as substrate; the resulting solution, containing also the ferri- and ferro-cyanides, was employed as it had been established that such concentrations of the reagents had no effect on cozymase breakdown.

Nicotinamide and acid formation during reactions with the coenzymes were determined as described previously (McIlwain & Rodnight, 1949).

Spectrophotometric methods. The Beckman DU quartz spectrophotometer was used for determining dihydronicotinamide derivatives at concentrations of about 10^{-4} M and in 3 ml. of aqueous solution. Horecker & Kornberg's (1948) value of $6{\cdot}22 \times 10^6$ sq.cm./mol. was used for calculating concentrations of coenzymes I and II from the observed optical densities at 340 mμ. In demonstrating the stability of dihydrocozymase to the enzyme, reaction mixtures were examined between 320 and 400 mμ. before and after an incubation which inactivated 1·5 μmol. cozymase in a control experiment. No change in the absorption of 1·6 μmol. dihydrocozymase was found.

Determination of coenzyme II. This followed Adler, Euler, Günther & Plass (1939) and Ochoa & Weisz-Tabori's (1948) use of the *iso*citric dehydrogenase of mammalian heart. Sheep heart was treated according to Straub's (1942) procedure, and the acetone powder kept dry in a refrigerator. Portions of 0·5 g. of this were extracted weekly with 10 ml. of phosphate buffer (pH 7·3), the extract dialysed, kept cold, and used as *iso*citric dehydrogenase. DL-*iso*Citric acid was prepared according to Fittig & Miller (1898) and Nelson (1930).

In the determination, the following solutions were made to 2·95 ml. in spectrophotometer cells: 0·1 M-veronal buffer (pH 7·2) 1·0 ml.; 0·01 M-$MnCl_2$, 0·2 ml.; the Co II-containing solution and the dehydrogenase (0·05 ml.). The optical density at 340 mμ. was measured at room temperature (approx. 20°), readings being taken until values were steady, and 0·05 ml. M-*iso*citrate at pH 7·2 was then added. Readings were again taken, and from the change the volumes and the known extinction coefficient of dihydrocoenzyme II, its concentration was calculated. The course of an experiment of this type is illustrated in Fig. 2.

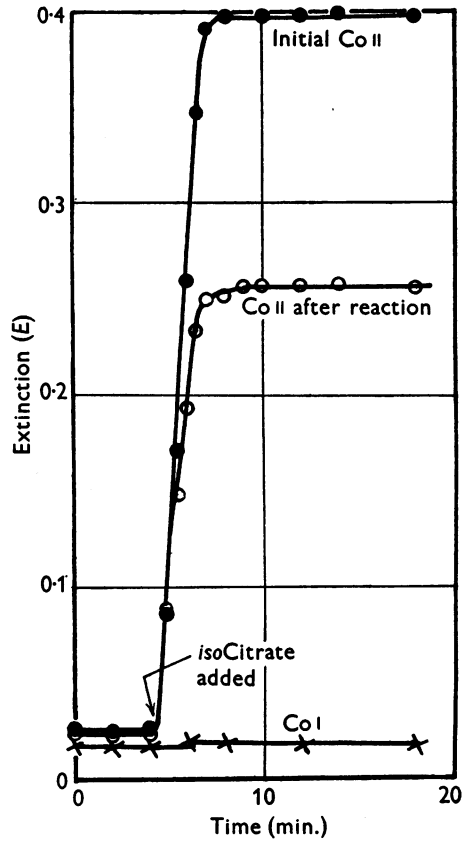


Fig. 2. Use of *iso*citrate system to show degradation of coenzyme II by a brain preparation. The initial and final Co II solutions (one-sixth of reaction mixture) are shown by the graph to contain respectively 0·86 and 0·56 μmol. of the coenzyme. The result with cozymase was from a vessel containing 1 μmol. cozymase, similarly diluted, and illustrates the specificity of the *iso*citric system and the purity of the cozymase.

Preparations of the cozymase-degrading enzyme

Tissue suspensions were made by grinding with sand, or by homogenizing, and were washed with saline. Aqueous extracts of washed tissue were made by repeated extraction of such suspensions with water. These have been described by McIlwain & Rodnight (1949). Preparations *SsDC* were made by extracting suspensions with strong salt solutions, dialysing and centrifuging. Data concerning them are given in the descriptions of the experiments in which they were used.

RESULTS

Dihydrocozymase

No change has been observed to be caused in dihydrocozymase by various brain preparations, and its much greater stability than cozymase in

nervous tissue is therefore noteworthy. The analytical methods used to detect possible change would in several cases have detected a reaction with a velocity less than 1 % of that observed with cozymase. Table 1 shows that no reaction leading to liberation of nicotinamide was detectable, nor one leading to the formation (as with cozymase) or absorption of acid. Moreover, dihydrocozymase itself was determined in certain reaction mixtures, before and after incubation, by its absorption band at 340 mμ.; this showed less than 3 % change. The dihydrocozymase was not pure, but three independently prepared specimens from three specimens of cozymase were examined. The initial cozymase specimens were degraded by brain preparations. Also, in one case the dihydrocozymase which had failed to react was reoxidized by ferricyanide and the resulting cozymase found to act as substrate. The dihydrocozymase specimen which failed to react did not inhibit the reaction with cozymase. Lack of reaction with dihydrocozymase thus cannot be attributed to effects of associated materials.

of whole brain tissue which had been ground with sand and thoroughly washed with 0·9 % sodium chloride. It was, however, unstable in the presence of homogenized whole brain or blended heart muscle, aerobically, when oxidation to cozymase itself could be expected.

Nicotinamide glycosides

The most interesting of these compounds was the naturally occurring nicotinamide ribofuranoside. This contains the nicotinamide-ribose link which in cozymase is split during the present reaction. Nicotinamide riboside itself was, however, stable to the brain preparations used. Any reaction with three different enzyme preparations was of less than 1 % of the speed with cozymase (Table 1). As criteria of reaction, appearance of nicotinamide (Koenig reaction) and acid formation were used.

In view of the stability of the riboside, it was not surprising that compounds less closely related to cozymase did not act as substrates. The mannoside, galactoside and glucoside were examined by the

Table 1. *Possible liberation of nicotinamide from its derivatives by brain preparations*

(All enzyme preparations were of ground tissues, washed with and diluted in 0·9 % NaCl. Rates were determined from manometric observation of the course of evolution of CO_2 from bicarbonate containing solutions, and checked by nicotinamide estimation at the end of the reactions. The possible reaction with dihydrocozymase was also examined, with similar results, with two other preparations of grey and white matter from sheep brain, one from guinea-pig brain, and one from ox spinal cord. The riboside was examined also with preparations from white matter (sheep) and spinal cord (ox), and the mannoside, glucoside and galactoside with another preparation in addition to that quoted below; results were the same.)

Potential substrate (M × 10^{-4})	Source of enzyme preparation	Rate of reaction (μmol./mg./hr.)
Cozymase (5)	Whole sheep brain	0·95
Dihydrocozymase (5)	Whole sheep brain	<0·03
Cozymase (5)	White matter from centrum ovale of sheep brain (preparation A)	0·92
Dihydrocozymase (5)	White matter from centrum ovale of sheep brain (preparation A)	<0·008
Dihydrocozymase (5) with ferricyanide (11)	White matter from centrum ovale of sheep brain (preparation A)	0·84
Cozymase (3·8)	White matter from centrum ovale of sheep brain (preparation B)	0·96
Dihydrocozymase (4)	White matter from centrum ovale of sheep brain (preparation B)	<0·007
Dihydrocozymase (4) with cozymase (3·8)	White matter from centrum ovale of sheep brain (preparation B)	0·94
Cozymase (5)	Whole sheep brain	0·68
Nicotinamide ribofuranoside (16)	Whole sheep brain	<0·007
Nicotinamide mannoside (10)	Whole sheep brain	<0·007
Cozymase (5)	Sheep brain, white matter	1·13
Nicotinamide glucoside (10)	Sheep brain, white matter	0·01
Nicotinamide galactoside (10)	Sheep brain, white matter	<0·01

Dihydrocozymase added to brain preparations was found to be stable not only anaerobically, under which conditions most of the present reactions were carried out, but also aerobically when in the presence

methods of Table 1, and any reaction found to be less than 1 % of that with cozymase. Dihydro derivatives of all four compounds were also examined, with a similar result.

Coenzyme II

This was found to be acted on by the enzyme only slightly less rapidly than cozymase. The reaction was first followed in bicarbonate-buffered mixtures at pH 7 by liberation of nicotinamide from a preparation containing about 4% of the coenzyme, and the rate of reaction found to be approx. 80% of that with cozymase. Purer coenzyme specimens, and various preparations of the enzyme, gave values between 70 and 85% of the rate with cozymase (Table 2; Fig. 3).

The rate of reaction was also followed by determination of the coenzyme II at different times during the reaction. Fig. 2 shows the determination of coenzyme II in an experiment in which this was done. Warburg vessels contained in their main compartments 0·9% NaCl and 0·01 M-$NaHCO_3$ 1 ml.) with a brain preparation *WS* (a sodium sulphate precipitate of a water extract of sheep brain washed with 0·9% NaCl, with activity of 25μ mol. cozymase degraded/hr./mg. N). The coenzyme II (1 μmol./0·2 ml., as material of 9% purity) was

Table 2. *Breakdown of coenzyme* II *by brain preparations*

(Reaction mixtures contained the coenzymes (1 μmol.), $NaHCO_3$ (22·5 μmol.), NaCl (155 μmol.) and brain preparations in 2·5 ml., with 5% (v/v) CO_2 in air at 37°. At intervals portions were removed, the reaction stopped with Zn salts, and nicotinamide determined as described by McIlwain & Rodnight (1949). The extract *SsDC* contained 1·2 mg. protein/ml. and 0·5 ml. was used in each experiment.)

Co II content of preparation (μmol./mg.)	Brain preparation	Rate of breakdown of coenzyme II (μmol./mg./hr.)	Rate of breakdown of coenzyme II (% of rate of cozymase in same experiment)
0·056	Guinea pig, mixed tissue ground and washed with NaCl	0·34	89
0·056	Ox spinal cord, as above	0·51 (μmol./vessel/hr.)	71
0·087	Sheep brain extract *SsDC*	1·40	77
0·087	Sheep brain extract *SsDC*	1·62	85

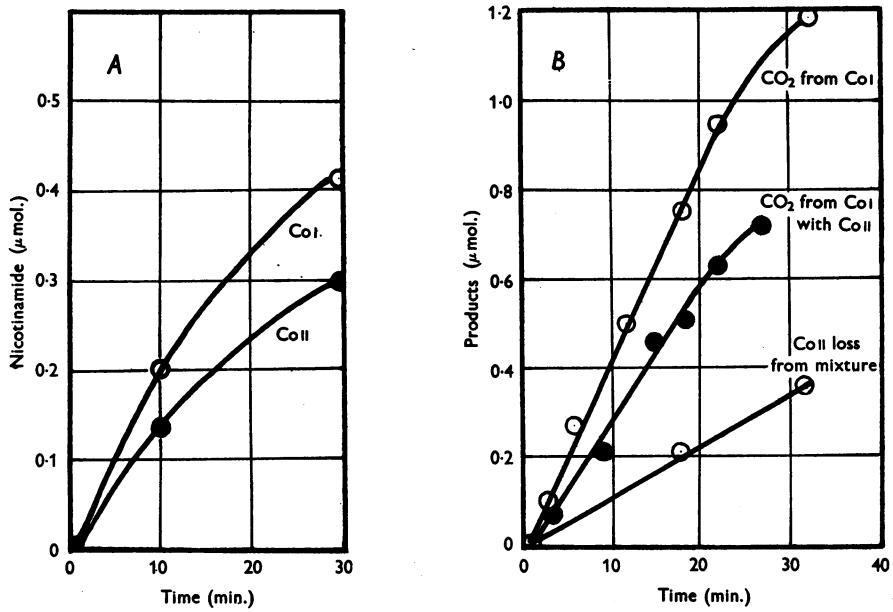


Fig. 3. Kinetics of coenzyme breakdown by brain preparations. A, nicotinamide liberated from coenzymes I and II, each 1 μmol. in 2·5 ml. NaCl-$NaHCO_3$-N_2/CO_2 (pH 7), by ground washed sheep spinal cord (equivalent to 8·3 mg. dry wt.) at 37°. B, CO_2 evolved during breakdown of cozymase (1·3 μmol.) in 1·4 ml. NaCl-$NaHCO_3$-N_2/CO_2 (pH 7) and of a mixture of cozymase and coenzyme II (total 1·3 μmol., in ratio 3/4) by a similar preparation of the degrading enzyme from ox spinal cord. Loss of coenzyme II from the same mixture is also shown.

contained in a side arm, yellow phosphorus in a centre well, and 5% (v/v) CO_2 in N_2 in the gas space. Other vessels contained cozymase, and others no substrate. After equilibration at 37°, reaction was started by tipping. Carbon dioxide evolution was followed and vessels removed at different times and put in ice water. To the portions taken for coenzyme II determination, ⅓ vol. of 0·5 M-nicotinamide was added and specimens taken for test with the *iso*-citric system. From determinations which included that of Fig. 2, the rate of breakdown of coenzyme II was found to be 76% of that of cozymase.

The simultaneous degradation of the two coenzymes in a mixture containing them both in similar concentrations is shown in Fig. 3B. The results indicate that the two coenzymes compete for the degrading system.

Dihydrocoenzyme II

Demonstration of the stability of dihydrocoenzyme II in the presence of the enzyme was carried out as follows.

To 9·85 ml. of solution containing 1·16 μmol. Co II and the $MnCl_2$ and veronal buffer (0·1 M) of the *iso*citric system in a glass-stoppered tube, was added 0·15 ml. of the heart *iso*-citric dehydrogenase. Sodium DL-*iso*citrate (0·1 M, 0·025 ml.) was added, and the optical density of a portion of the solution followed in a closed cell at 340 mμ. Another cell containing all reagents but no Co II was used as control. In 16 min. at room temperature the optical density became almost steady at a value corresponding to 1·06 μmol. of dihydrocompound; a further 0·005 ml. of the *iso*citrate was added, and the reading rose to correspond to 1·11 μmol. The whole solution was then heated quickly to 100° for 1 min., cooled in running water and centrifuged. The optical density of the solution compared with that of the control similarly treated fell by 10%, presumably due to the known instability of the dihydrocompound. Samples of this solution were used as source of dihydrocoenzyme II in the following experiment.

Spectrophotometer cells were prepared of which three contained the foregoing reaction mixture (2 ml.). To one of these was added 1 ml. of water; to two others a preparation *SsDC* of the cozymase-splitting enzyme from ox cord, as a clear solution. Another cell which served as control in the spectrophotometer contained the control solution of the previous paragraph (2 ml.) with the enzyme. Other cells contained the same control solution, enzyme and cozymase (0·27 μmol.). Change in the latter mixtures was determined by estimating nicotinamide; change in the former by spectrophotometric estimations at 340 mμ.

The results (Fig. 4) showed that the system rapidly inactivated cozymase (rate greater than 0·55 μmol./hr.), but caused very little change in dihydrocoenzyme II. The latter change in both control and experimental vessels was of about 0·02 μmol./hr.; any difference between these two due to an action of the enzyme itself on dihydrocoenzyme II was of less than 0·005 μmol./hr., i.e. less than 1% of the rate of reaction with cozymase. The small change observed in all vessels containing the dihydrocompound was presumably due to its instability in the reaction mixture in air at pH 7·2; no attempt had been made to remove dissolved oxygen initially present. In a vessel containing the enzyme in 3 ml. with both dihydrocoenzyme II (0·28 μmol.) and cozymase (0·18 μmol.), the dihydrocompound underwent no change other than that of the control, while cozymase was decomposed rapidly (Fig. 4). The experiment

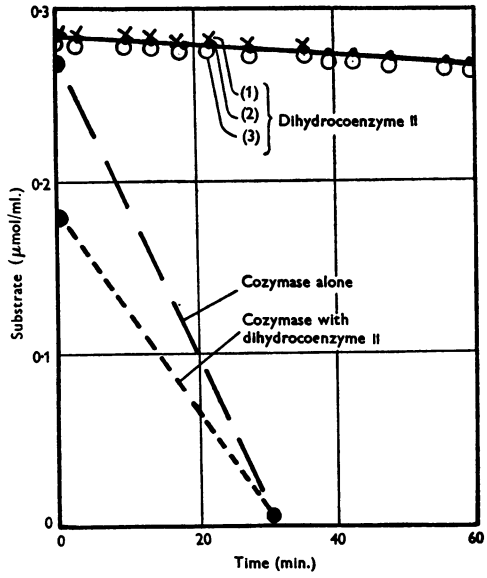


Fig. 4. Stability of dihydrocoenzyme II in the presence of the enzyme degrading cozymase. Above: dihydrocoenzyme II determined spectrophotometrically in three solutions (indicated by different points) which contained (1) no further additions, (2) and (3) the ox spinal cord preparation *SsDC* (1·5 mg. dry weight). Below: change in cozymase with the same quantity of cord preparation, with and without dihydrocoenzyme II.

was not adequate to give comparison of the rates of reaction with cozymase in the presence and absence of dihydrocoenzyme II, but these could not have differed greatly. Therefore, although nothing in the reaction mixture prevented the breakdown of cozymase, the dihydrocoenzyme II was stable.

Other N-*alkyl heterocyclic compounds*

Nicotinamide methosulphate and trigonelline (each 3×10^{-3} and 3×10^{-4} M) did not lead to detectable nicotinamide, nicotinic acid or free acid when incubated with sheep or guinea-pig brain preparations; reactions, 0·5% of the speed of that with cozymase, could have been detected. No acid was formed when the enzyme was incubated with benzimidazole methiodide or phenazine methosulphate at the same concentrations.

DISCUSSION

The major finding of the present experiments is that of the differential action of the cozymase-degrading enzyme on the oxidized, in distinction to the reduced, forms of the coenzymes. This is understandable when the structural differences between the two forms at the point of action of the enzyme are considered (Fig. 1). The enzyme acts on a bond at an ionized quaternary nitrogen atom. In dihydrocozymase this is replaced by a tertiary nitrogen atom presumably with an excess instead of a deficit of electrons. As the breakdown of cozymase is little if at all affected by the presence of either of the dihydrocompounds, these appear to have little affinity for the enzyme. The minimum structure compatible with activity of the enzyme has not been defined by the present experiments, but would appear to be at least a nicotinamide nucleotide (as distinct from nucleoside). The action of the enzyme at varying stages of purification on coenzyme II, as well as on cozymase, renders inappropriate the terms 'diphosphopyridine nucleotidase' and 'diphosphopyridine nucleosidase' which have been applied to it. These names, also, do not discriminate between the present enzyme and the cozymase pyrophosphatase of Kornberg (1948). Substrate competition in our experiments also indicates that the degrading enzyme from the central nervous system acts on both coenzymes.

The coenzyme molecules contain some fifty bonds of which eight or nine are of types which are readily split by various enzymes, for example, the bonds between ribose and phosphoric acid or between the carbon and nitrogen of the amide. It is therefore noteworthy that the present enzyme attacks the point at which the coenzymes differ most from their dihydro derivatives.

The effects of this differential attack seem likely to be far reaching (McIlwain, 1949*a*, *b*). They can immediately be expected to include changes in redox potential and in systems sensitive to such changes, and modifications of equilibria involving coenzyme-catalysed dehydrogenases. Inhibition of cozymase breakdown by low concentrations of substances (including phenosafranine) which disturb the Pasteur effect (McIlwain, 1949*b*) suggests that the system degrading cozymase does indeed play a central part in carbohydrate metabolism in normal tissues, possibly participating in the phosphorylation associated with tissue oxidations (cf. Judah & Williams-Ashman, 1949). It is noteworthy that in brain the speed of cozymase degradation was found (McIlwain & Rodnight, 1949) to be 2·2–3·3 times that of the oxygen uptake, both changes being expressed in molar quantities. The ratio appears to be similar in muscle and the enzyme exists in many animal tissues (Spaulding & Graham, 1947) although at a lower level.

SUMMARY

1. Preparations from the mammalian central nervous system, which rapidly inactivated cozymase, inactivated also coenzyme II at 70–85% of the rate of their reaction with cozymase.

2. Inactivation of coenzyme II, like that of cozymase, was accompanied by liberation of nicotinamide.

3. Competition took place between the two coenzymes for the degrading system, and presumably, therefore, a common enzyme was responsible for the two reactions.

4. Nicotinamide ribofuranoside, nicotinamide glycosides less closely related to cozymase, and several other nicotinamide derivatives were not affected by the enzyme.

5. Dihydrocoenzymes I and II were not affected by the enzyme, and did not affect the breakdown by it of coenzymes I and II.

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