52. NICOTINAMIDE, COZYMASE AND TISSUE METABOLISM

By P. J. G. MANN and J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 1 April 1941*)

During the course of experiments on the respiratory activities of intact, minced and ground mammalian tissues we have found that there exists in brain and in liver a very active mechanism which accomplishes the breakdown of cozymase under both aerobic and anaerobic conditions. We have also found that this mechanism can be inhibited in a specific manner by nicotinamide. A preliminary note incorporating these results has already been published [Mann & Quastel, 1941]. The following communication is concerned with a description in detail of these and further results, together with observations on the effects of nicotinamide on the inhibitory activity of cobra venom in respiratory systems.

Technique

Measurements of respiratory activity

The Warburg apparatus has been used for the measurement of rates of O_2 uptake, or of CO_2 output when using the anaerobic technique of Quastel & Wheatley [1938]. The volume of fluid in the respiratory vessel was always maintained at 3 ml. and experiments were at 37° for periods usually not longer than 1 hr.

Preparation of biological material

Ground tissues. In our first experiments with ground brain, we used rat brain tissue (1 part brain to 10 parts 0·9 % NaCl) which had been finely ground in the wet-crushing mill of Booth & Green. This gave an excellent preparation having a high activity in breaking down cozymase. Subsequently, however, it was found unnecessary to use the wet-crushing mill, since a very active preparation could be made simply by grinding the tissue with sand in the presence of 0·9 % NaCl. The finely ground tissue was squeezed through muslin and the extract diluted with 0·9 % NaCl so that 10 ml. of the fluid contained 1 g. of the original tissue. This technique has been adopted for the preparation of ground tissues of rat brain, rat liver and rat kidney.

Muscle extract. Dialysed skeletal (rabbit) muscle extract as a source of cozymase-free lactic dehydrogenase was prepared by the method of Green *et al.* [1937].

Succinic and lactic dehydrogenases. The preparation made from washed pig's heart by the method of Keilin & Hartree [1938] sufficed as a source of both these enzymes. In some experiments extract of skeletal muscle or suspension of rat kidney was used as a lactic dehydrogenase.

Heart flavoprotein. This was prepared by a method similar to that described by Straub [1939].

Cozymase. A partially purified preparation, originally obtained from yeast, was used without further purification, as a high degree of purity was not required. Its activity was frequently tested by appropriate control experiments.

Media

All media in the aerobic experiments contained 0·02 *M* Na phosphate buffer *p*H 7·4 and all reagents were neutralized so that the *p*H of the fluid in the manometric vessel was 7·4. When tissue slices were investigated a phosphate-Locke medium was used. This contained 0·02 *M* Na phosphate *p*H 7·4, 0·004 *M* K^+ and 0·002 *M* Ca^{++}. An atmosphere of air was used in all aerobic experiments in which tissue slices were not involved; when intact slices were used air in the manometric vessels was displaced by O_2. In anaerobic experiments, using the ferricyanide technique, phosphate was omitted. The medium consisted of 0·025 *M* $NaHCO_3$ together with the other constituents under investigation. The atmosphere was 95 % N_2 + 5 % CO_2. 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was placed in the side tube and tipped into the main vessel after temperature equilibration. For further details of this technique see Quastel & Wheatley [1938].

In most experiments either 0·1 or 0·2 ml. of the cozymase solution was placed in the side tube of the manometric vessel, and after temperature equilibration had been attained this was tipped into the main vessel containing the tissue and respiratory constituents. Any variation in this procedure will be made clear in the text.

Results

The effects of addition of ground brain suspensions on the lactic dehydrogenase activities of muscle and kidney

The addition of small quantities of ground rat brain suspension to a lactic dehydrogenase system prepared from mammalian tissues results in a profound fall in the activity of the system. This is shown using systems prepared from rabbit skeletal muscle, ground pig heart muscle and ground rat kidney. With rabbit skeletal muscle a system is prepared similar to that used by Corran *et al.* [1939]. This consists of dialysed rabbit skeletal muscle extract (which is cell-free), pig heart flavoprotein, Na lactate, NaCN, methylene blue and cozymase. This system as shown by Corran *et al.* [1939] takes up O_2, pyruvate being formed and fixed by the cyanide. Hydrogen from the lactate passes eventually to O_2 through the chain of cozymase, flavoprotein and methylene blue. The addition to the system of 0·2 ml. of a rat brain suspension may reduce by 50 % the rate at which it consumes O_2. Typical results are shown in Table 1 and in Fig. 1. When a suspension of washed ground pig heart muscle is used as a source of lactic dehydrogenase there is no necessity to add flavoprotein as the suspension appears already to possess ample quantities. The feeble O_2 uptake secured by a mixture of washed ground pig heart muscle, Na lactate, NaCN and methylene blue is much increased by the addition of cozymase. The addition however of a mixture of cozymase and 0·2 ml. rat brain suspension reduces this rate of O_2 uptake to a value less than that which obtains in the absence of added cozymase (see Table 1). A ground rat kidney suspension provides a good source of a lactic dehydrogenase preparation relatively free from cozymase. Such a suspension, when used in small quantities, takes up but little O_2 in the presence of Na lactate, NaCN and methylene blue. The addition of cozymase powerfully stimulates the rate of O_2 uptake (Table 1, (3) and (4)). The further addition of a small quantity of ground rat brain suspension reduces the oxidative activity to very small values. This is illustrated by the results shown in Table 1.

Table 1. *Effects of ground brain suspension on lactic dehydrogenase activity*

Brain suspension was prepared by grinding finely 3·1 g. fresh rat brain in 31 ml. 0·9 % NaCl. Lactic dehydrogenase system consisted of lactic dehydrogenase preparation, pig heart flavoprotein (0·5 ml.), sodium lactate (0·13 *M*), sodium cyanide (0·08 *M*), phosphate buffer (0·02 *M*) *p*H 7·4, methylene blue and cozymase. Air; 37°.

Source of lactic dehydrogenase	Methylene blue %	Cozymase mg.	Rat brain suspension ml.	μl. O_2 uptake
				In 1 hr.
(1) 0·3 ml. rabbit skeletal muscle extract	0·017	1	0	205·2
		1	0·2	108·9
		1	0·4	64·0
(2) 0·3 ml. washed ground pig heart muscle	0·04	0	0	45·9
		2	0	136·2
		2	0·2*	28·5
(3) 0·2 ml. ground rat kidney suspension	0·04	0	0	33·5
		2	0	179·5
				In 30 min.
(4) 0·2 ml. ground rat kidney suspension	0·04	0	0	30·1
		1	0	66·0
		1	0·2	10·5

* In this experiment the cozymase and rat brain suspension were incubated together for 15 min. at 37° before adding to the main system.

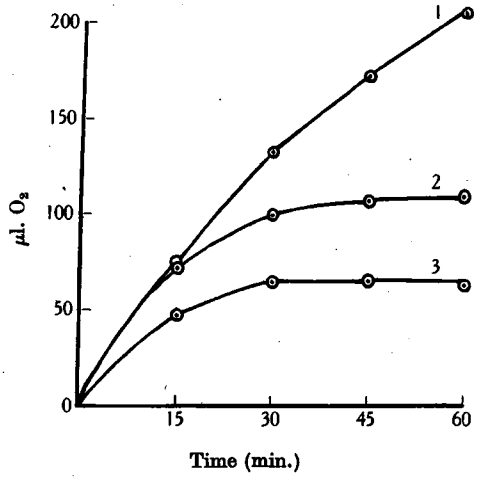

Fig. 1. Effects of ground brain suspensions on oxygen uptake by lactic dehydrogenase system (muscle extract, flavoprotein, lactate, cyanide, cozymase and methylene blue).

1. Lactic dehydrogenase system.
2. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension.
3. Lactic dehydrogenase system + 0·4 ml. ground rat brain suspension.

Destruction of cozymase by suspensions of ground mammalian tissues

The powerful inhibitory effects of ground brain suspensions on lactic dehydrogenase systems are due to their destruction of cozymase. This is proved by the following facts:

(1) When cozymase is added to a system containing lactic dehydrogenase, a ground brain suspension, lactate, cyanide and methylene blue, the inhibition of

the rate of O_2 uptake due to the brain develops slowly, the rate of development of the inhibition depending on the amount of brain used. Thus, as seen in Table 1 (1) and in Fig. 1, the presence of 0·4 ml. rat brain suspension brings about a much larger inhibition of the rate of O_2 uptake than occurs in the presence of 0·2 ml. brain suspension. If, however, the cozymase is incubated together with 0·2 ml. brain suspension for 15 min. at 37°, before addition to the mixture of lactic dehydrogenase, lactate, cyanide and methylene blue, a large inhibition of the rate of O_2 uptake takes place at once—the mixture behaving in fact as though no cozymase had been added. This is shown by the results given in Table 2. It is clear from these results that both rat brain and rat liver are able

Table 2. *Destruction of cozymase by tissues*

Lactic dehydrogenase system and brain suspension as in Table 1. Liver and kidney suspensions made by grinding tissue with sand and saline (1 g. tissue to 10 ml. saline) and squeezing through muslin. Air; 37°.

Source of lactic dehydrogenase	Methylene blue %	Treatment of cozymase	μl. O_2 uptake in 1 hr.
(1) 0·3 ml. rabbit skeletal muscle extract	0·017	1 mg. added. Untreated	262·8
		1 mg. incubated with 0·2 ml. brain suspension for 15 min. at 37° before addition to lactic dehydrogenase system	44·3
		1 mg. incubated in absence of brain suspension for 15 min. at 37° before addition to lactic dehydrogenase system	272·0
(2) 0·3 ml. washed ground pig heart muscle	0·04	1 mg. added. Untreated	136·2
		1 mg. incubated with 0·2 ml. rat kidney suspension for 15 min. at 37° before addition to lactic dehydrogenase system	243·6
(3) 0·3 ml. rat kidney suspension	0·04	No cozymase added	59·9
		2 mg. added. Untreated	189·4
		2 mg. incubated with 0·2 ml. liver suspension for 15 min. at 37° before addition to lactic dehydrogenase system	57·8

to destroy cozymase. However, after the addition of an incubated mixture of rat kidney suspension and cozymase to the lactic dehydrogenase of pig heart, an acceleration instead of an inhibition of O_2 uptake takes place. This is due to the fact that rat kidney has but little ability to destroy cozymase and also provides an extra source of lactic dehydrogenase.

(2) The inhibition of the lactic dehydrogenase-cozymase system by a brain suspension takes place anaerobically, using ferricyanide as oxidant of reduced cozymase instead of flavoprotein and methylene blue (Table 3). This result shows that the inhibition cannot be attributed to a destruction of flavoprotein by the brain suspension.

(3) The inhibitory effect of cozymase on succinic dehydrogenase activity [Keilin & Hartree, 1940] is entirely neutralized by the addition to the system of a small quantity of brain suspension. This result is shown in Table 12 and will be referred to in more detail later.

Anaerobic destruction of cozymase by brain

Cozymase is broken down by a brain suspension under strictly anaerobic conditions. The experiment demonstrating this is carried out by using a Warburg manometric vessel equipped with two side arms. In one side arm is placed the brain suspension, in the other ferricyanide solution. The main vessel contains a lactic dehydrogenase preparation, cozymase, Na lactate, NaCN and $NaHCO_3$ (0·025*M*). The air in the apparatus is displaced by 95 % N_2 + 5 % CO_2, and only after strictly anaerobic conditions are obtained is the brain suspension tipped into the main vessel containing the cozymase. Finally, after temperature equilibration, the ferricyanide is tipped into the main vessel. A control experiment, with no brain suspension present, is also carried out. As the results given in Table 3 show, the effect of the brain suspension under strictly anaerobic conditions is to reduce the activity of the lactic dehydrogenase system.

Table 3. *Anaerobic destruction of cozymase by brain*

Rat kidney suspension was used as source of lactic dehydrogenase. Brain suspension was made up as in Table 1.

Medium. Bicarbonate (0·025*M*)-saline, Na lactate 0·13*M*; NaCN 0·08*M*; 1 mg. cozymase. 95 % N_2 + 5 % CO_2; 37°. Vessels with two side arms were used:

In 1st vessel: 0·2 ml. brain suspension placed in one side arm.
0·2 ml. 8·3 % $Na_3Fe(CN)_6$ placed in other side arm.
Lactic dehydrogenase system in main vessel.

In 2nd vessel: As above, but no brain suspension present.

After anaerobic conditions were secured, the brain suspension was added to the lactic dehydrogenase system in first vessel. After equilibration, the ferricyanide was added to both vessels.

	μl. CO_2 output in 1 hr.*
Lactic dehydrogenase system; no brain suspension added	378·8
Lactic dehydrogenase system; 0·2 ml. brain suspension added	204·9

* Retention of CO_2 due to addition of small volume of brain suspension is here neglected.

This result shows that the destruction of cozymase by the brain suspension is not due to an oxidative breakdown and we conclude, for the time being, that the destruction of the coenzyme is accomplished by a hydrolytic enzyme. We will refer to this as cozymase nucleotidase.

Table 4. *Thermolability of cozymase nucleotidase*

Lactic dehydrogenase system: Washed ground pig heart suspension (0·5 ml.), 0·13*M* Na lactate; 0·02*M* phosphate buffer *p*H 7·4; 0·08*M* NaCN; 0·04 % methylene blue. 1 mg. cozymase. Ground brain suspension used as cozymase nucleotidase. Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system, no cozymase added	34·1
Lactic dehydrogenase system, with cozymase	183·0
Lactic dehydrogenase system + cozymase previously incubated with 0·2 ml. brain suspension for 15 min. at 37°	38·9
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. brain, which had been heated for 5 min. at 70°	161·3
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. brain which had been heated for 5 min. at 100°	173·4

Thermolability of cozymase nucleotidase

Results given in Table 4 show that the enzyme in brain responsible for the breakdown of cozymase is thermolabile. It is completely destroyed by exposure

to 100° for 5 min. and almost completely destroyed by exposure to 70° for the same period.

Can the inactivation of cozymase be due to preferential adsorption on brain proteins?

The possibility arises [cf. Lipton & Elvehjem, 1940] that the inactivation of cozymase by a brain extract may not be due to hydrolysis of the coenzyme but to its preferential adsorption on, or combination with, brain proteins. According to this suggestion the coenzyme is not destroyed, but removed from its sphere of action by adsorption on proteins having a high affinity for it.

This problem has been attacked in the following way. Cozymase is incubated together with a brain suspension at 37° for 15 min., when the inactivation of cozymase is complete. The mixture is divided into lots *A*, *B* and *C*. Mixture *A* is left undisturbed. Mixture *B* is heated for 5 min. at 70°. This treatment is known to destroy the inactivating mechanism and presumably, therefore, the proteins responsible for adsorption of the cozymase. Since the cozymase, by hypothesis, is not broken down by adsorption on brain proteins, it should be liberated from the heat-treated proteins. Hence mixture *B*, in contrast to mixture *A*, should contain free cozymase. Mixture *C* is heated for 5 min. at 70° and to this, after cooling, is added free cozymase. If the brain proteins, alleged to be responsible for the adsorption of cozymase, are destroyed by the heat treatment, they will now be unable to inactivate the added cozymase. Hence it should follow if the adsorption theory be correct, that mixture *A* will contain no free cozymase, and that mixtures *B* and *C* will contain free cozymase, *C* being richer in this respect than *B*. On the other hand if the adsorption theory be incorrect, both mixtures *A* and *B* will contain no free cozymase whilst mixture *C* will contain the free cozymase which was subsequently added to it.

Experiment (see Table 5) shows that no free cozymase is liberated from the incubated cozymase-brain mixture after heat treatment and that the heated mixture is unable to inactivate any added cozymase. These facts are in accordance with the view that cozymase is inactivated by breakdown by an enzyme in the brain and not by adsorption on special proteins.

Table 5. *Effect of heating cozymase-brain extract mixture after incubation*

The following mixture was prepared: Ground rat brain suspension (0·6 ml.), cozymase (3 mg.) in 2·4 ml. phosphate buffer (0·02*M*) *p*H 7·4. This was incubated at 37° for 15 min. and divided into three equal lots (*A*, *B*, *C*).

A was not treated further.

B was heated for 5 min. at 70° and cooled.

C was heated for 5 min. at 70°, cooled and to it was added 1 mg. cozymase.

A, *B* and *C* were transferred to Warburg vessels containing a lactic dehydrogenase system (washed ground pig's heart suspension 0·5 ml., Na lactate 0·13*M*, NaCN 0·08*M*, methylene blue 0·04 % and 0·02*M* phosphate buffer *p*H 7·4). Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system containing *A*	36·2
Lactic dehydrogenase system containing *B*	32·2
Lactic dehydrogenase system containing *C*	193·2

Effects of addition of nicotinamide

The addition of nicotinamide to a ground brain suspension has the effect of greatly inhibiting the cozymase nucleotidase. This is shown most clearly by incubating a mixture of brain suspension, cozymase and nicotinamide for

15 min. at 37° and adding the mixture, after incubation, to a lactic dehydrogenase preparation in presence of lactate, cyanide and methylene blue. The effect of the nicotinamide is to prevent almost completely the breakdown of the cozymase.

Typical results with (*a*) the lactic dehydrogenase of a dialysed rabbit skeletal muscle extract and with (*b*) the lactic dehydrogenase of a ground rat kidney suspension are shown in Tables 6 and 7 respectively.

Table 6. *Effects of addition of nicotinamide aerobically*

Lactic dehydrogenase system: Rabbit skeletal muscle extract (0·5 ml.), pig heart flavoprotein (0·5 ml.), Na phosphate buffer 0·02*M* *p*H 7·4, Na lactate 0·13*M*, NaCN 0·08*M*, methylene blue 0·017 %. Cozymase (1 mg.) was incubated at 37° for 15 min. with 0·2 ml. ground rat brain suspension in phosphate buffer *p*H 7·4 in the presence and absence of nicotinamide. The mixture was then added to the lactic dehydrogenase system, and O_2 consumption measured. Air; 37°.

	μl. O_2 uptake in 1 hr.
1. Lactic dehydrogenase system with untreated cozymase	215·4
2. Lactic dehydrogenase system with cozymase previously incubated for 15 min. with 0·2 ml. brain suspension	54·1
3. Lactic dehydrogenase system with cozymase previously incubated for 15 min. with 0·2 ml. brain suspension in presence of 0·3 ml. 1 % nicotinamide	167·2
4. As in 3 but using 0·1 ml. 10 % nicotinamide	199·4
5. As in 3 but using 0·3 ml. 10 % nicotinamide	171·3
6. Lactic dehydrogenase system with untreated cozymase and with 0·3 ml. 1 % nicotinamide present (i.e. final concentration of nicotinamide =0·1 %)	195·9

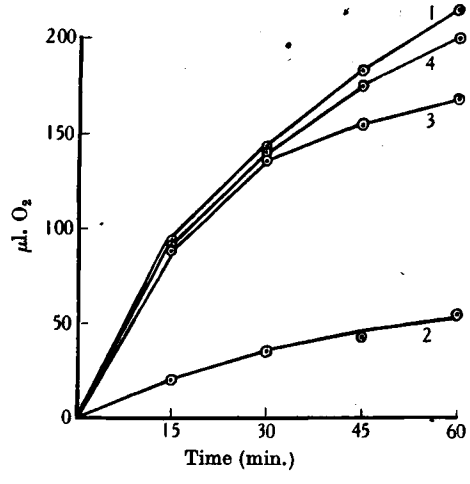

Fig. 2. Inhibitory action of nicotinamide on cozymase nucleotidase.

1. Lactic dehydrogenase system (muscle extract, etc.).
2. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension.
3. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension previously incubated with 0·1 % nicotinamide.
4. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension previously incubated with 0·3 % nicotinamide.

Examination of the results given in Table 6 and in Fig. 2 shows that the protective effect of nicotinamide increases with its concentration, but that above an

optimal concentration the nicotinamide inhibits the O_2 uptake. Probably this small inhibitory effect is that already described by Baker *et al.* [1938]. This effect has not yet been explained but it seems likely that it is due to competition of nicotinamide with cozymase for lactic dehydrogenase.

The presence of nicotinamide increases the O_2 uptake of the kidney lactic dehydrogenase system, containing added cozymase even in the absence of brain suspension. This points to the presence in the ground kidney suspension of a small quantity of cozymase nucleotidase. The very powerful inhibitory effect of a brain suspension on the kidney system is almost completely neutralized by the presence of 0·1 % nicotinamide (Table 7).

Table 7. *Effects of addition of nicotinamide aerobically*

Lactic dehydrogenase system: Rat kidney suspension (prepared by grinding fresh kidney in saline, 1 g. to 10 ml., with sand and squeezing through muslin); phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %. 0·2 ml. of the kidney suspension was placed in side tube of Warburg vessel and tipped into main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 30 min.
Kidney lactic dehydrogenase system	30·1
Kidney lactic dehydrogenase system + cozymase (1 mg.)	66·0
Kidney lactic dehydrogenase system + cozymase + 0·1 % nicotinamide	89·8
Kidney lactic dehydrogenase system + cozymase (1 mg.) + 0·2 ml. ground brain suspension	10·5
Kidney lactic dehydrogenase system + cozymase (1 mg.) + 0·2 ml. ground brain suspension + 0·1 % nicotinamide	77·1

Cozymase is not only broken down by ground brain suspensions but by intact brain slices, this effect being inhibited by the presence of nicotinamide. These results are shown in Table 8. The destruction of cozymase by the intact brain slices is only partially due to cozymase nucleotidase diffusing from the tissue

Table 8. *Effect of nicotinamide on cozymase in brain slices*

Equal weights (100 mg. wet wt.) of rat brain slices were placed in Warburg vessels containing the following media:

A 0·02*M* Na phosphate *p*H 7·4, Locke solution, 2 mg. cozymase preparation.

B As *A* but containing also 0·3 % nicotinamide.

C 0·02*M* Na phosphate *p*H 7·4, Locke solution.

D As *C* but containing also 0·3 % nicotinamide.

In another vessel was placed medium *E*: 0·02*M* Na phosphate *p*H 7·4, Locke solution and 2 mg. cozymase preparation (no tissue slices present). These were incubated at 37° in O_2 for 30 min., after which slices of tissue were removed from all vessels. To *C* and *D* were then added 2 mg. cozymase preparation and these vessels were incubated for a further 30 min. in O_2 at 37°.

1 ml. of each of the above media, i.e. *A*, *B*, *C*, *D*, *E*, was then transferred to fresh Warburg vessels, containing 0·02*M* Na phosphate buffer *p*H 7·4, 0·13*M* Na lactate, 0·08*M* NaCN, 0·017 % methylene blue, and 0·16 % nicotinamide. In the side tube of each vessel was placed 0·3 ml. suspension of ground rat kidney (1 g. to 10 ml. saline). After temperature equilibration (37°) in air, the kidney suspension was tipped in the main vessels and rates of O_2 uptakes were measured.

	μl. O_2 uptake in 30 min.
Kidney lactic dehydrogenase system + 1 ml. *A*	36·4
Kidney lactic dehydrogenase system + 1 ml. *B*	112·5
Kidney lactic dehydrogenase system + 1 ml. *C*	88·7
Kidney lactic dehydrogenase system + 1 ml. *D*	131·4
Kidney lactic dehydrogenase system + 1 ml. *E*	140·4

slices in the outer medium. Much of the cozymase must be destroyed by cozymase nucleotidase within the intact tissue. The intact brain slices must, clearly, be permeable to cozymase, a conclusion already arrived at by Quastel & Wheatley [1938].

The protective effect of nicotinamide on cozymase breakdown may be demonstrated also by experiments carried out under anaerobic conditions.

If a brain suspension is, itself, used as a source of lactic dehydrogenase it may be shown that the addition of cozymase to it has but little effect on its power of reducing ferricyanide under anaerobic conditions. The addition of nicotinamide also (in the absence of added cozymase) has little or no effect on the reduction of ferricyanide. If, however, a mixture of cozymase and nicotinamide be added to the brain suspension, a powerful reduction of ferricyanide ensues. These results are shown in Tables 9 and 10. The results are easily explained by the fact that the very active cozymase nucleotidase of brain quickly

Table 9. *Effects of nicotinamide anaerobically*

One rat brain (1·6 g.) was finely chopped and suspended in 16 ml. saline. 1 ml. brain suspension was placed in each Warburg vessel containing the following medium: $NaHCO_3$ 0·025*M*, NaCl 0·13*M*, Na lactate and 0·05*M* NaCN. Nicotinamide (0·3 %) or cozymase (1 mg.) or both were present in certain vessels. After removal of air by 95 % N_2 + 5 % CO_2, and equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped from side tube into main vessel.

	μl. CO_2 output in 1 hr.
Brain + lactate system	124·4
Brain + lactate system + nicotinamide	129·5
Brain + lactate system + cozymase	136·6
Brain + lactate system + cozymase + nicotinamide	341·0

Table 10. *Effects of addition of nicotinamide anaerobically*

One half (0·6 g.) of a fresh rat brain was ground in 6 ml. Locke solution. The other half of the rat brain was ground in 6 ml. Locke solution containing 3 % nicotinamide. 1 ml. of each of these suspensions was placed in a $NaHCO_3$ (0·025*M*)-Locke medium containing 0·13*M* Na lactate, 0·05*M* NaCN, with and without the addition of cozymase (1 mg.). After removal of air by 95 % N_2 + 5 % CO_2 and equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into each vessel.

	μl. CO_2 output in 1 hr.
Brain ground in Locke solution only. No cozymase added	168·9
Brain ground in Locke solution only. Cozymase added	185·7
Brain ground in Locke solution containing nicotinamide. No cozymase added	149·6
Brain ground in Locke solution containing nicotinamide. Cozymase added	387·4

destroys all free cozymase,[1] and not until the enzyme is inhibited by nicotinamide can the addition of free cozymase show its normal catalytic effect on lactic dehydrogenase activity. The results given in Table 10 show the stimulating effect of cozymase on the reducing power of brain ground in a nicotinamide Locke solution as compared with that ground in Locke solution free from nicotinamide.

Can the apparent protective effect of nicotinamide be due to synthesis to cozymase?

A possible explanation of the effect of nicotinamide in stimulating the O_2 uptake under the given experimental conditions is that the nicotinamide is built up into free cozymase.

[1] We have evidence to show that brain tissue contains a small quantity of bound cozymase which is broken down to produce free cozymase on heating the tissue.

If this view is true the apparent protective effect of nicotinamide on cozymase breakdown by a brain suspension is not in fact due to a competition of nicotinamide with cozymase for a cozymase nucleotidase, but to the synthesis of cozymase by the brain suspension from nicotinamide and other constituents present in the brain suspension. It may be shown, however, that 0·3 % nicotinamide which will apparently prevent the breakdown of cozymase by a brain suspension gives rise to little or no free cozymase when incubated alone with the brain suspension. These results are shown in Table 11 and indicate that a synthesis of cozymase from nicotinamide under the experimental conditions

Table 11. *Test for synthesis of cozymase*

Lactic dehydrogenase system: Washed ground pig's heart suspension (0·3 ml.); Na phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %.

Cozymase (1 mg.) or rat brain suspension or both, in the presence or absence of 0·1 ml. 10 % nicotinamide, were incubated aerobically in Warburg vessels at 37° for 15 min. The lactic dehydrogenase system was then added to each vessel and the O_2 consumption was measured. Air; 37°.

	μl. O_2 uptake in 30 min.
Lactic dehydrogenase system + cozymase	194·5
Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension	10·5
Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	164·5
Lactic dehydrogenase system + 0·2 ml. ground brain suspension	9·7
Lactic dehydrogenase system + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	14·7

employed does not take place. Hence the alternative explanation, that nicotinamide competes with cozymase for a cozymase nucleotidase is probably the correct one. This is supported by the result quoted in Table 10 in which it is shown that the addition of nicotinamide alone to minced brain does not increase its lactic dehydrogenase activity. Moreover, it may be shown (see Fig. 2) that the effect of nicotinamide in increasing the O_2 uptake of a lactic dehydrogenase system containing cozymase and brain suspension falls off with time, whereas if synthesis of cozymase took place, the effect of nicotinamide should increase with time.

Effects of nicotinamide on succinic dehydrogenase activity in presence and absence of cozymase

It is known [Potter, 1939; Keilin & Hartree, 1940] that the addition of cozymase to a suspension of washed ground pig's heart greatly reduces the latter's succinic dehydrogenase activity. This is attributed by Keilin & Hartree to the formation of oxaloacetate from succinate by the action of succinic dehydrogenase and of malate dehydrogenase in conjunction with cozymase. The oxaloacetate formed, even in minute quantities, inhibits succinic dehydrogenase.

It may be shown that the addition of a ground brain suspension entirely removes the inhibitory effect exercised by cozymase on the succinic dehydrogenase activity of pig's heart. If nicotinamide, however, be added at the same time as the ground brain suspension, the cozymase exercises its normal inhibitory effect. These results are shown in Table 12, and are explained satisfactorily by the presence in brain of a cozymase nucleotidase which breaks down cozymase and whose activity is inhibited by the presence of nicotinamide.

It may also be shown that cozymase inhibits the anaerobic oxidation of succinate by ferricyanide in the presence of a brain suspension acting as sole source of succinic dehydrogenase. This effect is greatly increased by the addition

Table 12. *Effects of nicotinamide on succinic dehydrogenase activity in presence and absence of cozymase*

Succinic dehydrogenase system: Washed ground pig's heart suspension (1 ml.), Na succinate 0·03 *M*; phosphate buffer (0·02 *M*) *p*H 7·4. Air; 37°.

	μl. O_2 uptake in 1 hr.
Succinic dehydrogenase system	241·2
Succinic dehydrogenase system + 0·3 % nicotinamide	253·5
Succinic dehydrogenase system + cozymase (1 mg.)	119·8
Succinic dehydrogenase system + cozymase (1 mg.) + 0·3 % nicotinamide	123·9
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension	270·0
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	272·4
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + cozymase (1 mg.)	236·5
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide + cozymase (1 mg.)	130·8

to the system of nicotinamide. These results are shown in Table 13. The phenomena are explained by the presence in the brain suspension of cozymase nucleotidase. The inhibitory action of added cozymase on the succinic dehydrogenase of brain is determined by the amount of free cozymase present, and by the speed with which it can form oxaloacetic acid as compared with the speed with which it is broken down by the cozymase nucleotidase. In the presence of nicotinamide the breakdown of cozymase by the brain is inhibited and hence more free cozymase is available to build up oxaloacetic acid.

Table 13. *Effects of nicotinamide on succinic dehydrogenase activity of brain under anaerobic conditions*

Medium: $NaHCO_3$ (0·025 *M*)-saline. 95 % N_2 + 5 % CO_2; 37°. After equilibration 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into main vessel.

	μl. CO_2 output in 30 min
1 ml. ground rat brain suspension	12·0
1 ml. ground rat brain suspension + 1·0 % nicotinamide	13·7
1 ml. ground rat brain suspension + Na succinate 0·03 *M*	337·6
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + 1·0 % nicotinamide	266·9
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + cozymase (1 mg.)	210·9
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + cozymase (1 mg.) + 1·0 % nicotinamide	128·0

Keilin & Hartree [1940] could find no inhibitory effect of cozymase on the succinic dehydrogenase of brain and attributed this apparent inactivity of cozymase to the rapid breakdown of oxaloacetic acid by brain. This explanation cannot be wholly correct as the results in Table 13 show that the presence of cozymase can inhibit succinic dehydrogenase of brain and hence that the breakdown of oxaloacetic acid by brain is not sufficiently rapid to prevent the inhibition taking place. It is more likely that the failure of Keilin & Hartree to observe an inhibitory effect of cozymase on brain succinic dehydrogenase is due to the rapid destruction of the cozymase by the brain preparation under their experimental conditions.

Effects of Indian cobra venom on lactic dehydrogenase activity in presence and absence of nicotinamide

Chain [1939] has shown that cozymase is destroyed by small quantities of cobra venom. This phenomenon we have confirmed. The inhibitory effect of the venom on the lactic dehydrogenase-cozymase system of pig heart is neutralized

by the addition of nicotinamide. 0·3 % nicotinamide neutralizes almost completely the inhibitory effect of 0·5 mg. dried cobra venom. The neutralizing effect of the nicotinamide increases with increase of concentration of nicotinamide and decreases with increase of concentration of the venom. This points to the existence of an equilibrium between nicotinamide and an enzyme in the cobra venom. Whether this enzyme is identical with the cozymase nucleotidase of brain and liver remains to be seen. Results illustrating these phenomena are shown in Table 14.

Table 14. *Effects of dried Indian cobra venom on lactic dehydrogenase activity in presence and absence of nicotinamide*

Lactic dehydrogenase system: Washed ground pig's heart suspension (1 ml.); Na phosphate buffer 0·02*M* pH 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %; cozymase 1 mg. Air; 37°.

	μl. O_2 uptake in 1 hr.
Exp. 1.	
Lactic dehydrogenase system	164·8
Lactic dehydrogenase system + 0·5 mg. venom	95·2
Lactic dehydrogenase system + 0·5 mg. venom + 0·3 % nicotinamide	154·3
Lactic dehydrogenase system + 0·75 mg. venom	50·1
Lactic dehydrogenase system + 0·75 mg. venom + 0·3 % nicotinamide	99·1
Lactic dehydrogenase system + 1 mg. venom	36·2
Lactic dehydrogenase system + 1 mg. venom + 0·3 % nicotinamide	77·0
Lactic dehydrogenase system + 0·3 % nicotinamide	163·6
Exp. 2.	
Lactic dehydrogenase system	123·0
Lactic dehydrogenase system + 1 mg. venom	52·9
Lactic dehydrogenase system + 1 mg. venom + 0·03 % nicotinamide	53·0
Lactic dehydrogenase system + 1 mg. venom + 0·1 % nicotinamide	73·0
Lactic dehydrogenase system + 1 mg. venom + 0·3 % nicotinamide	92·4

Specificity of nicotinamide

Nicotinic acid will not replace nicotinamide as an inhibitor of the breakdown of cozymase by a rat brain suspension. Adenylic acid appears to have a feeble effect in protecting cozymase from breakdown. Propionamide and coramine are without action. These results are given in Table 15 and illustrate the specificity of action of nicotinamide.

Neither NaF nor $Na_4P_2O_7$ at the concentrations tried (see Table 16) prevents the breakdown of cozymase by a brain suspension.

Malate dehydrogenase and nicotinamide

Observations recorded in Table 17 show that substantially the same conclusions which apply to the lactic dehydrogenase system apply also to the malate dehydrogenase system. This is shown using the malate dehydrogenase of rat kidney under aerobic conditions and that of rat brain under anaerobic conditions. In the former case the addition of a suspension of rat brain effects an inhibition of malate oxidation which is neutralized by the presence of nicotinamide. In the latter case the presence of nicotinamide greatly increases the anaerobic oxidation of *dl*-malate in the presence of brain by ferricyanide.

Table 15. *Specificity of nicotinamide*

Lactic dehydrogenase system: 0·3 ml. ground rat kidney suspension; Na phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %. The rat kidney suspension was placed in the side tube of the vessel and tipped into the main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 1 hr.
Exp. 1.	
1. Lactic dehydrogenase system	31·7
2. Lactic dehydrogenase system + cozymase (2 mg.)	139·0
3. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	40·3
4. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	158·6
5. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinic acid (Na salt)	41·8
6. As in 5 but using 1 % nicotinic acid (Na salt)	38·4
7. As in 5 but using 0·1 % nicotinic acid (Na salt)	47·7
8. As in 5 but using 0·03 % nicotinic acid (Na salt)	45·1
Exp. 2.	
1. Lactic dehydrogenase system	44·2
2. Lactic dehydrogenase system + cozymase (2 mg.)	257·0
3. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	64·6
4. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	268·5
5. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 10 mg. adenylic acid (muscle)	75·7
6. As in 5 but using 10 mg. yeast adenylic acid	80·9
7. Lactic dehydrogenase system + cozymase + 10 mg. adenylic acid (muscle)	208·4
8. Lactic dehydrogenase system + cozymase + 10 mg. adenylic acid (yeast)	224·0
9. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % propionamide	57·3
10. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·1 % coramine	62·5
11. As in 10 but using 0·3 % coramine	53·4

Table 16. *Actions of fluoride and of pyrophosphate*

Lactic dehydrogenase system: Rabbit skeletal muscle extract (1 ml.), pig's heart flavoprotein, Na lactate (0·13*M*); NaCN (0·08*M*); methylene blue (0·04 %); phosphate buffer 0·02*M* *p*H 7·4 (see also Table 6). Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system + cozymase (1 mg.)	202·9
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension	44·0
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension in presence of 0·1*M* NaF	49·8
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension in presence of 0·04*M* $Na_4P_2O_7$	39·2

Table 17. *Malate dehydrogenase and nicotinamide*

Aerobic

Malate dehydrogenase system: 0·3 ml. ground rat kidney suspension, Na phosphate buffer 0·02*M* pH 7·4; Na *dl*-malate (0·017*M*); NaCN 0·08*M*; methylene blue 0·04 %. The kidney suspension was placed in the side tube and tipped into main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 1 hr.
Malate dehydrogenase system + cozymase (2 mg.)	96·4
Malate dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	26·6
Malate dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	92·5

Anaerobic

One rat brain (1·6 g.) was finely chopped and suspended in 16 ml. Locke solution. 1 ml. suspension was placed in the following medium: $NaHCO_3$ (0·025*M*)-saline, NaCN 0·05*M*, with or without Na *dl*-malate (0·017*M*), cozymase (1 mg.), and nicotinamide. After removal of air by 95 % N_2 + 5 % CO_2, and after equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into main vessel from side tube.

	μl. CO_2 output in 1 hr.
Brain system	85·6
Brain system + 1 % nicotinamide	85·8
Brain system + *dl*-malate	111·0
Brain system + *dl*-malate + 1 % nicotinamide	198·1

Discussion

It is evident that brain, both in the intact and ground condition, is able to break down cozymase at a rapid rate. The enzyme involved is present also in liver and to a small extent in kidney. It appears to be absent from a dialysed extract of rabbit skeletal muscle or from washed ground pig's heart. Possibly the same enzyme is present in cobra venom. It may also be present in tumours [cf. Boyland & Boyland, 1935] and in yeast for Leloir & Dixon [1937] have reported that crushed yeast is able to destroy cozymase.

Since brain contains a very active cozymase nucleotidase, it must be able to synthesize cozymase rapidly *in vitro* as well as *in vivo* in order to balance the nucleotidase activity and to account for the maintenance of oxidative activity of brain when this respires in lactate media. Alternatively it must be concluded that the cozymase nucleotidase of intact brain is ineffective under aerobic conditions—a conclusion which is incorrect as the foregoing observations have shown. There must therefore exist in intact brain an equilibrium between the mechanisms responsible for the synthesis of cozymase and those responsible for its breakdown. The existence of this equilibrium probably accounts, at least partially, for the fact that the respiration of damaged tissues is often much lower than that of intact tissues. In the former case damage to the tissues may impair synthetic mechanisms by alterations of cell permeability with loss of essential cell constituents by diffusion etc.

The high rates of breakdown of cozymase by brain or liver throw doubt on estimations of cozymase in tissue extracts where precautions have not been taken to destroy at once the cozymase nucleotidase. It is obviously essential that such destruction should be brought about immediately after dissection of the organ under investigation from the animal.

The presence of nicotinamide inhibits the breakdown of cozymase probably by competition with cozymase for the nucleotidase. The evidence shows that

the stimulating action of nicotinamide is not due to synthesis to cozymase under the given experimental conditions.

The demonstration of the action of nicotinamide in protecting cozymase from breakdown by mammalian tissues raises the question as to whether the well-known therapeutic effects of nicotinamide may not be due partly to this action. If this view is true, substances other than nicotinamide having a similar protective effect may have similar therapeutic effects. It may be noted in this connexion that Vilter & Spies [1939] have shown that quinolinic acid will cure pellagra and that Woolley *et al.* [1938] find that a variety of nicotinic acid derivatives will cure canine black tongue. Moreover, as a result of the administration of quinolinic acid, there has been found [Vilter & Spies, 1939] an increase in concentration of cozymase in the blood. It is clear that further investigation is required of the types of molecules which will inhibit cozymase breakdown by mammalian tissues.

Summary

1. The addition of a ground rat brain suspension to a lactic dehydrogenase-cozymase system or to a malic dehydrogenase-cozymase system of mammalian tissues brings about considerable diminution of the activity of the systems.

2. This is due to the destruction of cozymase by a mechanism present in brain. The evidence points to the mechanism in question being a hydrolytic enzyme which is referred to as cozymase-nucleotidase.

3. Cozymase-nucleotidase occurs in rat brain and in rat liver and to a relatively small extent in rat kidney. It is apparently absent from dialysed rabbit skeletal muscle extract and from washed ground pig's heart.

4. Cozymase nucleotidase is destroyed by heating at 70° for 5 min.

5. Evidence is given showing that cozymase is not inactivated by adsorption upon special proteins in brain.

6. The presence of nicotinamide greatly inhibits cozymase nucleotidase of brain. This may be shown by experiments carried out under aerobic and anaerobic conditions. Nicotinamide stimulates lactic and malic dehydrogenase activities of brain in presence of cozymase.

7. The stimulating effect of nicotinamide on the lactic dehydrogenase activities of tissues in presence of cozymase-nucleotidase is not due to a synthesis of nicotinamide to cozymase, but to a competition of nicotinamide with cozymase for the nucleotidase.

8. Intact brain slices break down cozymase, this effect being neutralized by the presence of nicotinamide. An equilibrium must exist in intact brain between a synthetic mechanism leading to cozymase formation and a mechanism leading to its breakdown.

9. The inhibition of succinic dehydrogenase of pig heart by cozymase is neutralized by the addition to the system of a ground brain suspension. The effect of the brain may be eliminated by the addition to the system of nicotinamide. The action of the nicotinamide here is to prevent the breakdown of the cozymase. Succinic dehydrogenase activity of a brain suspension, when examined anaerobically by the ferricyanide technique, is decreased by the addition of cozymase, the effect being enhanced by the simultaneous addition of nicotinamide. Here again the effect is due to the prevention of breakdown of cozymase by nicotinamide.

10. The action of cobra venom in inhibiting a lactic dehydrogenase-cozymase system by destruction of cozymase is retarded by nicotinamide. An equilibrium probably exists between nicotinamide and a nucleotidase in cobra venom.

11. The protective effect of nicotinamide on cozymase breakdown is not shown by nicotinic acid, coramine, propionamide, sodium fluoride or sodium pyrophosphate. Adenylic acid (of yeast or muscle) has a feeble protective action.

12. Attention is drawn to the possibility that the therapeutic effects of nicotinamide may be due partly to its protective effects on cozymase breakdown.

Our sincere thanks are due to the Rockefeller Foundation and to the Medical Research Council for their financial help, and to the Ella Sachs Plotz Foundation for a grant for the purchase of animals. We are also much indebted to Dr W. Feldberg for a supply of dried Indian cobra venom and to Dr P. Ostern for a sample of muscle adenylic acid.

REFERENCES

Baker, Fazekas & Himwich (1938). *J. biol. Chem.* **125**, 545.
Boyland & Boyland (1935). *Biochem. J.* **29**, 1097.
Chain (1939). *Biochem. J.* **33**, 407.
Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Green, Needham & Dewan (1937). *Biochem. J.* **31**, 2329.
Keilin & Hartree (1938). *Proc. roy. Soc.* B, **125**, 171.
—— —— (1940). *Proc. roy. Soc.* B, **129**, 277.
Leloir & Dixon (1937). *Enzymologia*, **2**, 81.
Lipton & Elvehjem (1940). *J. biol. Chem.* **136**, 637.
Mann & Quastel (1941). *Nature, Lond.*, **147**, 326.
Potter (1939). *Ark. Kemi Min. Geol.* **13** B, No. 7.
Quastel & Wheatley (1938). *Biochem. J.* **32**, 936.
Straub (1939). *Biochem. J.* **33**, 787.
Vilter & Spies (1939). *Lancet*, **2**, 423.
Woolley, Strong, Madden & Elvehjem (1938). *J. biol. Chem.* **124**, 715.