The process of distillation was slow, taking about 30 min. for the complete operation. Recoveries adequate for exploratory and comparative work may be obtained by a much more rapid steam distillation procedure in which the flow of vapour is supplemented by the passage of steam through the tube *B* (broken line, Fig. 4). By this procedure about 75 % of the nicotine may be recovered in 5 min. in 15 ml. of distillate.

SUMMARY

A method is described for the recovery of nicotine from tissues and its estimation in quantities from 1 μg.

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Decomposition and Synthesis of Cozymase by Bacteria

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Investigations concerning the metabolism of nicotinic acid and its derivatives have been surveyed recently by Schlenk (1945), and, in the case of micro-organisms, by McIlwain (1947). In the latter account, attention was directed to the synthesis, inactivation and interconversion of coenzymes I and II by micro-organisms, and particular efforts were made to obtain values for the rates at which these changes occur. Although many papers bearing on this subject were assessed, few if any were found to contain all the data required for calculating unequivocally the rates at which these important processes take place. On the other hand, such estimates of the rates as could be made by supplying likely values for missing data suggested the existence of a group of reactions with velocities of a few mμmol./mg. dry wt. of organism/hr. The possible significance in bacteria of reactions of this magnitude has been discussed elsewhere (McIlwain, 1946*b*).

Studies have now been made of the behaviour of bacteria towards nicotinic acid derivatives, especially cozymase. This behaviour has been found to be dominated in many organisms by reactions which are potentially much more rapid than those inferred previously. Before studying the slower reactions (for which, also, evidence has been obtained) the course and products of the more rapid reactions have been examined, and are reported in this paper.

EXPERIMENTAL

Organisms. A β-haemolytic streptococcus (R, the Richards strain; National Collection of Type Cultures no. 5631), two strains of *Streptococcus faecalis* (F 4208, N.C.T.C. no. 4208; FL, a laboratory strain), a *Staphylococcus aureus* (laboratory strain) and *Proteus morganii* (N.C.T.C. no. 2818) were grown in the casein-yeast medium of McIlwain (1946*a*), modified as follows: in place of NaOH, 0·5 M-$NaHCO_3$ (additional 6·8 ml./100 ml.) was added; the initial pantothenate content was 10^{-6} M; the quantity of yeast preparation was 2·5ml./100 ml.; and of the group A addenda only riboflavin, aneurin, KH_2PO_4, tryptophan, methionine, $MgSO_4$, $Fe(NH_4)_2(SO_4)_2.6H_2O$ and cystine were included. *Escherichia coli* (N.C.T.C. no. 4074) was grown in the mixture of inorganic salts and glucose of Dorfmann & Koser (1942).

Lactobacillus arabinosus 17–5 was grown in a medium elaborated for the use of the organism in assaying nicotinic acid (see below), and containing the quantities of nicotinic acid specified in the individual experiments described. Its pH fell from 6·8 to 5 during growth. *Haemophilus parainfluenzae* was also grown in the corresponding assay medium, with defined quantities of cozymase as V-factor.

Reaction with cozymase. Bacteria were normally harvested by centrifuging, washed twice with 0·9 % NaCl, suspended in that solution and a small sample taken for dry weight estimation. The suspensions were distributed between experimental vessels, which, for anaerobic experiments, were Warburg flasks with yellow P in a centre well. Saline was that of Krebs & Henseleit (1932).

Microbiological assays. Nicotinic acid was determined using *Lactobacillus arabinosus* 17–5 in very nearly the way

described by Barton-Wright (1946) (see McIlwain & Stanley, 1948). V-factor was estimated by growth of *Haemophilus parainfluenzae* (N.C.T.C. no. 4101) in a medium similar to that of Hoagland & Ward (1942); growth, however, was measured photoelectrically. Cozymase in concentrations of $0{\cdot}3–2 \times 10^{-8}$ M was used as source of V-factor in obtaining reference curves, and results are expressed in terms of cozymase. The inoculum was 0·05 ml. of a culture grown for 24 hr. in the assay medium with 10^{-7} M-cozymase. Inoculated cultures of 10 ml. final volume were incubated in 50 ml. conical flasks at 37° for 30–48 hr.

Determination of cozymase using apozymase. Though the determination of cozymase using apozymase (Euler, 1936; Myrbäck, 1933) requires care, the method appears much more reliable than was suggested by Jandorf, Klemperer & Hastings (1941). By adopting precautions specified by Axelrod & Elvehjem (1939) and others noted below, we have consistently obtained reliable results. Our experience and practice have been in some cases similar to, and, in others, different from that of Schlenk & Schlenk (1947), whose paper appeared while the present one was in preparation.

Preparation of apozymase. Fresh brewer's yeast was dried in air and then over $CaCl_2$; it can be kept in this form for some months. The washing, drying, rewashing and redrying (all these stages were found necessary) to yield apozymase were carried out according to Axelrod & Elvehjem (1939) and in one day. The product was kept in a stoppered bottle over $CaCl_2$ in a refrigerator for periods up to a fortnight.

It was found necessary to carry out the preparation of apozymase at room temperature (15–20°). When, in trying to obtain a more stable preparation, washing was performed with ice water, the apozymase obtained was deficient in cozymase, but its response to certain solutions containing cozymase increased with time. Such behaviour has been observed by Euler & Adler (1938), Euler, Adler & Eriksen (1937) and by Lennerstrand (1941), and interpreted as due to synthesis of cozymase. We have not found such behaviour in apozymase prepared as described in the preceding paragraph, and have therefore not found it necessary to adopt a method such as that of Lennerstrand's (1941) 'pyocyanin-system', which is much less sensitive to cozymase than is the normal apozymase system.

Preparation of hexosediphosphate. Neuberg's (1942) method was followed, using fresh baker's yeast; fermentation was complete in 5 hr. and the yield of calcium hexosediphosphate from 200 g. of sucrose was *c.* 16–20 g. A solution of sodium hexosediphosphate was prepared freshly each day; the Ca salt (208 mg.) was dissolved in 0·49 ml. of ice-cold N-HCl and the theoretical quantity of sodium oxalate, in 3·5 ml. water, added gradually. The pH was brought to 7 by N-NaOH (0·4 ml.), the mixture left cold for 10 min. and centrifuged. The solution was taken to pH 6·2 with N-HCl (*c.* 0·15 ml.) for use in the apozymase system; it contained *c.* 4 mg. organic P/ml.

Cozymase determination. This followed Axelrod & Elvehjem's (1939) description, but was performed in Warburg vessels with one side arm and of total volume *c.* 18 ml. With the apozymase used, response of the system at 30° to cozymase was found to be maximal with phosphate concentrations between 0·055 and 0·07 M, and the quantity of phosphate buffer added for cozymase determination was such as to give a final concentration of 0·065 M. This concentration is higher than that adopted by Axelrod & Elvehjem (1939), but other reagents were used in concentrations described as typical by those authors. If the solutions whose cozymase content was being determined were significantly buffered, they were brought to pH 6·2, and if their phosphate content was more than 0·02 M, that of the remainder of the system was reduced accordingly. With 0·2 g. of apozymase/vessel, the system was used for determination of up to 15 or 20 mμmol. of cozymase, and standard solutions of 7·5 and 15 mμmol. of cozymase were included with each batch of unknown solutions. It is necessary to avoid citrate buffers in solutions whose cozymase is being determined by apozymase.

Other determinations of cozymase were made by dismutation of hexosediphosphate (method and muscle preparation of Jandorf *et al.* 1941) and reaction with hyposulphite, following Warburg & Christian's (1936) description.

Cozymase specimens. Pure cozymase has not been obtained, and unless otherwise stated, specimen A of Table 1 has been used, but its behaviour has been compared with two independent specimens B and C of Table 1. The different methods of estimating the cozymase content of specimen A, quoted in Table 1, gave values differing by not more than 6%, and their mean value of 40% purity has been adopted in interpreting results with this specimen. The principles underlying methods I and II are entirely different from each other and from that underlying methods III and IV. The approximate agreement between methods I and II suggests that specimen A contains practically all its nicotinic acid as a quaternary salt such as cozymase. The approximate

Table 1. *Analytical data concerning cozymase specimens*

	Determination and standard value	Cozymase specimen A	B	C
I	Acid formation in reaction with $Na_2S_2O_4$ (μmol./mg.)	1·75	0·50	1·80
	Purity by comparison with theoretical value of 4·5 μmol./mg. ($C_{21}H_{27}O_{14}N_7P_2$, mol.wt. 663) (%)	37	11	40
II	Nicotinic acid content (%)	8·0	—	—
	Purity (theoretical value 18·6%) (%)	43	—	—
III	Catalytic activity in apozymase system (μmol. CO_2/μg./hr.) (S.D. of 15 results, with specimen A, 0·18)	1·47	0·44	1·75
	Purity (by comparison with value of 4·0 derived from Axelrod & Elvehjem, 1939) (%)	37	11	44
IV	Catalytic activity in Jandorf *et al.* (1941) system (μmol. CO_2/μg./hr.)	0·89	—	—
	Purity (by comparison with value of 2·15, derived from Jandorf *et al.* 1941) (%)	41	—	—

agreement between methods II, III and IV implies that the specimen contains relatively little material containing nicotinic acid which is not cozymase. The agreement between method III and the other methods justifies our dependence on method III in most of the subsequent work. We have also shown (Table 7) that the nicotinic acid grouping of specimen A exists as its amide, without detectable free acid (cf. Schlenk, 1945).

Extraction of bacterial cozymase. Extractions were performed aerobically in phosphate buffer, pH 6·2, as this is the medium in which cozymase is estimated in the apozymase system, and pH 6·2 is close to that of optimal stability of cozymase. Previous investigators have heated for short periods at temperatures of 70–100° in order to obtain cozymase from natural materials, and we have examined the yield of cozymase from streptococci under such conditions. Results quoted in Table 2 show that heat treatment for 8–30 min. at these temperatures yields extracts containing about the same quantity of cozymase, but that 15 min. at 70° is optimal. The quantity of cozymase so obtained was found to be nearly the same as that extracted from the cells by grinding with glass. Heating at 70° for 15 min. has therefore been employed throughout the studies reported in this paper for extracting cozymase; Table 2 shows that 5% of the substance may be lost under these conditions, but values quoted have not been corrected for such loss.

RESULTS

Inactivation of cozymase

Behaviour of various bacterial species. Washed suspensions of bacteria were incubated in buffered salt solutions containing 10^{-5}–10^{-4}M-cozymase. The majority of species inactivated cozymase (Table 3). Reaction at the rate of 5–30 mμmol./mg./hr. was found in β-haemolytic streptococci, *Haemophilus parainfluenzae*, *Staphylococcus aureus* and *Proteus morganii*. *Escherichia coli* and *Streptococcus faecalis* were less active; *Lactobacillus arabinosus* 17–5 was the only organism of those examined with activity $<0{\cdot}5$ mμmol./mg./hr.

Circumstances affecting the reaction. Most of the following investigations were with streptococci. The reaction reached its greatest velocity at a pH near 6 or 7 (Table 4; see also Table 8). We have usually studied it at pH 7–7·5 in order to assess its relation to growth. With high concentrations of cozymase, very rapid rates of inactivation were reached by all three species examined. In Table 5, cozymase at an initial concentration of $1{\cdot}2 \times 10^{-3}$M is seen to be inactivated at over 440 mμmol./mg./hr. The system does not approach saturation even at that concentration; values for $(\log a/b)/t$ (a = initial, b = concentration at time t) are not falling at the higher concentrations of Table 5A. In Table 5B, also, the velocity is proportional to the initial concentration of cozymase, except with the lower concentration

Table 2. *Extraction and stability of cozymase from streptococci*

(Streptococci throughout were the Richards strain, grown in the casein yeast medium for (in different batches) 20–40 hr.)

Material extracted or treated	Conditions of extraction or treatment	Cozymase		
Streptococci, batch A	At 70°, 15 min.; 2·4 mg. cells/ml.	6·3		mμmol./mg. cells
	At 70°, 30 min.; 2·4 mg. cells/ml.	5·9		
	At 85°, 15 min.; 2·4 mg. cells/ml.	5·8		
		(Batch B)	(Batch C)	
Streptococci, batches B and C	At 70°, 15 min.	9·8	4·9	
	At 85°, 15 min.	9·0	4·3	
	At 100°, 8 min.	8·5	3·8	
Streptococci, batch B	Ground with glass	9·5		
	Ground with glass and extract heated at 70°, 15 min.	9·2		
Streptococcal extract	None	(*a*)		
	Heated at 70°, 15 min.	94% of (*a*)		
Cozymase	None	(*b*)		
	Heated at 70°, 15 min.	94–97% of (*b*)		
Streptococci with added cozymase:				
Cells (4·8 mg.)	At 70°, 15 min.	48		mμmol.
Cozymase	At 70°, 15 min.	82		
Cozymase with cells (4·8 mg.)	At 70°, 15 min.	126		

The rapid inactivation of cozymase by streptococci makes it possible that losses may occur by enzymic activity during the heating to 70°, but we have not found this to be the case. The extraction has been carried out in small volumes of liquid (usually <2 ml.) placed in tubes in a water bath at 70°. Temperatures approaching 70° are quickly attained in the tube, and cozymase added to the system is recovered satisfactorily (Table 2).

Table 3. *Bacterial inactivation of cozymase*

(Reactions were at 37°, with shaking, in tubes or Warburg vessels. The latter, with yellow P in a centre well and N_2 in the gas space, were employed for anaerobic reactions. Changes in cozymase refer to the whole reaction mixture including the cozymase of the cells.)

Reaction mixture			Reaction performed aerobically (A) or anaerobically (AN)	Rate of change in cozymase (mμmol./mg./hr.)
Organism and dry wt. of cells/ml.	Cozymase (M × 10^{-5})	Other constituents		
β-Haemolytic streptococcus (R); 3·9 mg./ml.	2·4	0·02M-Na and K phosphates, pH 7·6	A	−29
Streptococcus faecalis (FL); 3 mg./ml.	1·2	0·03M-Na and K phosphates, pH 7·6, with saline	AN	−1·8
	1·2	Do.	A	−1·7
Staphylococcus aureus (E); 3·2 mg./ml.	1·2	Do.	AN	−7·0
	1·2	Do.	A	−5·2
Proteus morganii (2818); 1·3 mg./ml.	1·2	Do.	A	−4·2
	1·2	Do.	AN	−4·5
Lactobacillus arabinosus 17–5; 21·5 and 45 hr. old; 2·3 mg./ml.	6	As above; also with glucose (0·05M)	A	<0·5
Lactobacillus arabinosus 17–5; 40 and 44 hr. old; 3·6 mg./ml.	6	As above; pH 5 and 7	A	<0·5
Haemophilus parainfluenzae, N.C.T.C. 4101; *c.* 2 mg./ml.	6	0·03M-Na and K phosphates, with saline; pH 6 and 7	A	−6·7 to 8·4
Escherichia coli; 7 mg./ml.	2·4	As above at pH 7·5	A	−0·6
	2·4	Do.	AN	−3·5
	2·4	As above with glucose (0·02M)	AN	−1·1

Table 4. *Cozymase inactivation by streptococci: different specimens*, pH, *concentrations and temperatures*

(Reactions were aerobic and in phosphate saline, except * which was in acetate buffer. Cozymase specimens: see Table 1.)

Cozymase					
Specimen	Initial concentration (M)	Organism (batch)	Temp.	pH	Rate of inactivation (mμmol./mg./hr.)
A	4×10^{-4}	R (*a*)	10	7·5	172
A	4×10^{-4}	R (*a*)	20	7·5	267
A	4×10^{-4}	R (*a*)	37	7·5	390
A	4×10^{-4}	R (*b*)	37	7·5	295
C	$3·6 \times 10^{-4}$	R (*c*)	37	7·5	160
C	$3·6 \times 10^{-4}$	R (*d*)	37	7·5	266
B	$3·6 \times 10^{-4}$	R (*d*)	37	7·5	250
A	$1·2 \times 10^{-3}$	FL	37	7·5	288
A	$3·6 \times 10^{-4}$	F 4208	37	7·5	144
A	4×10^{-4}	R (*e*)	37	5*	37
A	4×10^{-4}	R (*e*)	37	6	183
A	4×10^{-4}	R (*e*)	37	7	159
A	4×10^{-4}	R (*e*)	37	8	84

when measurements were less accurate. Different cozymase specimens and different streptococcal strains behaved similarly, and the reaction was still rapid at 10° (Table 4).

The course of change in cozymase in one reaction mixture was followed both by apozymase and by the muscle preparation of Jandorf *et al.* (1941). Results obtained by the two methods did not differ by more than 6%.

Effects of other substances on the reaction. Inactivation of cozymase in the presence and absence of oxygen is compared with several organisms in Tables 3 and 6. Differences between the two conditions were in general not striking and often within experimental error. Any difference was in the sense of greater loss anaerobically; the greatest contrast was found with a *Streptococcus faecalis* (Table 6) and *Escherichia coli* (Table 3).

Table 5. *Course of the streptococcal inactivation of cozymase*

(In Exp. A, organisms of strain R were grown in 500 ml. of standard medium, collected, washed twice in 0·5% NaCl and the bulk, of dry wt. 190 mg., suspended in 10 ml. of 0·5% NaCl and mixed with cozymase (33 μmol.) in 10 ml. of 0·04M-Na and K phosphates, pH 7·6. The mixture was shaken at 37° in air, and specimens (0·2 ml.) were taken at the intervals indicated, diluted in cold water, and their cozymase determined in the apozymase system.

For Exp. B, a smaller quantity of a similar reaction mixture was serially diluted to give the four different concentrations of cozymase (and of organisms) which are indicated.)

Reaction period (min.)	Cozymase content: μmol. in whole	Cozymase content: M × 10^{-4} (*c*)	Mean $Q_{Coz.}$ (mμmol./mg./hr.)	Log *a*/*b* / Time (hr.)
		Exp. A		
0	33·3 (*a*)	16·7	—	—
20	13·5 (b_1)	6·75	−155	1·18
40	6·04 (b_2)	3·02	−117	1·11
60	2·28 (b_3)	1·24	− 59·4	1·16
90	1·22 (b_4)	0·61	− 16·7	0·96
		Exp. B		$Q_{Coz.}/c$
15	—	12	−444	−37
15	—	4	−140	−35
30	—	1·33	− 48	−36
60	—	0·44	− 11	−25

Table 6. *Distribution of cozymase between bacterial cells and suspending fluid during its inactivation*

(Reactions were carried out in 2·5 ml. of phosphate saline, pH 7·5.)

Reaction mixture: Organisms (mg. dry wt.)	Reaction mixture: Other reagents	Reaction mixture: Added cozymase (mμmol.)	Reaction: Aerobic (A) or anaerobic (AN)	Reaction: Time (hr.)	Cozymase (μmol.): In cells	Cozymase (μmol.): In solution	Cozymase (μmol.): Total	Cozymase (μmol.): Loss
			Streptococcus faecalis					
8·4	—	—	A	2	43	4	47	—
8·4	—	62	A	2	60	35	95	14
8·4	—	62	AN	2	49	33	82	27
8·4	—	62	AN	0·5	54	48	102	7
8·4	Glucose; 0·01M	62	AN	0·5	61	38	99	10
			Staphylococcus aureus					
9·5	—	—	AN	2	53	< 1	53	—
9·5	—	30	AN	0·5	54	< 1	54	29
9·5	Glucose; 0·01M	30	AN	0·5	48	< 1	48	35
9·5	—	30	A	2	53	7	60	23
			Proteus morganii					
3·7	—	—	AN	2	41	3	44	—
3·7	—	34	AN	2	37	3	40	38
3·7	—	34	A	2	38	2	40	38
			Escherichia coli 4074					
7·4	—	—	—	—	26	—	26	—
7·4	—	93	AN	0·5	42	13	55	64
7·4	—	93	AN	2·0	40	< 1	40	79
7·4	Glucose; 0·01M	93	AN	0·5	58	14	72	47
7·4	—	93	A	2·0	56	1	57	62

Glucose did not have a large effect on the reaction; instances of greater loss in its presence are shown in Table 6 with streptococci and staphylococci. During these experiments both organisms caused a rapid breakdown of the added glucose, largely to lactic acid. Glucose, on the other hand, decreased the inactivation of cozymase brought about by *Esch. coli*. Sodium pyruvate, α-ketoglutarate, and glutamine (all at 7×10^{-3}M) were not found to alter the rate of change in cozymase added anaerobically to suspensions of the R streptococci. Variation in the phosphate concentration of the medium between 0·008 and 0·08M caused no change in the reaction with cozymase.

Distribution of cozymase between cells and solution. The experiments of Tables 3–5 concerned reaction mixtures in which the added cozymase was in large excess over that of the bacteria, or in which the cozymase of the complete reaction mixture had been determined after liberating the substance from the cells. The experiments of Table 6 were performed with relatively little added cozymase, and the distribution of cozymase between the bacteria and the solution was examined. Added cozymase was found first to enter the cells and then to be lost without reappearing in solution. Much added cozymase could be lost with relatively little change in the cellular cozymase. Almost all the nicotinic acid derivatives resulting from the breakdown of cozymase in Exp. A, Table 7, were found in solution and not in the cells.

Products of cozymase inactivation by streptococci

Nicotinamide moiety. Although the specimens of cozymase which were employed in this investigation were not pure, they contained little nicotinic acid which was not in the form of cozymase (Table 1). The bulk of the nicotinic acid of streptococci also appeared to exist as cozymase (Table 7C). When streptococci were caused to react with added cozymase, the cozymase content of the system fell without a corresponding decrease in its content of nicotinic acid derivatives. This is shown in each experiment of Table 7.

The nicotinic acid estimations of Table 7 were performed after heating with acid under conditions (see experimental section) which other workers have found to liberate nicotinic acid from the majority of its naturally occurring derivatives. The preceding observations, therefore, do not imply that nicotinic acid itself is formed from cozymase. As nicotinic acid exists in cozymase as its amide, we have sought information on the fate of the amide grouping. Atkin, Schultz, Williams & Frey's (1943) method for distinguishing between nicotinamide and nicotinic acid was found to be an excellent one, and applicable when nicotinic acid was assayed with *Lactobacillus arabinosus*. Solutions were assayed before and after treatment with hypobromite. This converted the amide but not the acid to the amine, which was without growth-promoting activity. Table 7B shows the product from cozymase to behave similarly to nicotinamide in this respect. It also shows the specimen of cozymase which we have employed to contain $< 0{\cdot}5\,\%$ of free nicotinic acid, or of derivatives of nicotinic acid which failed to be inactivated by hypobromite (cf. Schlenk, 1945).

The stability of nicotinamide, when added as such to streptococci, was investigated to see whether it was comparable to that of the product from cozymase. Reaction mixtures similar to that of Exp. A, Table 5, but containing nicotinamide in place of cozymase, were kept at 37° for 90 min. Ammonia was determined by Nessler's reagent after distillation under reduced pressure. Ammonia formation occurred in the absence of nicotinamide at the rate of about 40 mμmol./mg./hr., and no difference was detected in this rate when 4 or 40 μmol. of nicotinamide were added. A difference of 10 mμmol./mg./hr. could have been detected. Corresponding quantities of cozymase would have been inactivated at rates of at least 400 mμmol./mg./hr. under the conditions of the reaction. Any hydrolysis of nicotinamide thus appears to be at a rate not exceeding 10 % of that of the inactivation of cozymase; in reality, it may be much less.

Loss of V-factor activity. The term V-factor has been retained as a collective name for a group of

Table 7. *Stability of nicotinamide moiety of cozymase in the presence of streptococci*

(In mixtures A, varying quantities of streptococci R, prepared as described in Table 5, were incubated aerobically with cozymase in 0·017 M-Na and K phosphates of pH 7·5. At the times indicated, portions of the reaction mixtures were cooled, centrifuged and the cozymase (by apozymase) and nicotinic acid in solution were determined. Mixtures B were similar but determinations were carried out on the whole reaction mixture, including bacteria. Mixtures C were buffered with veronal (0·05 M, pH 7·5) and determinations included bacteria.)

	Mixture	Cozymase content (mμmol.)	Nicotinic acid content (mμmol.)	Nicotinic acid remaining after treatment with hypobromite (mμmol.)
A.	Before reaction	57·3	55	—
	With 11·4 mg. organisms, 10 min.	3·9	57	—
	With 11·4 mg. organisms, 40 min.	3·6	60	—
	With 3·8 mg. organisms, 10 min.	23·7	59	—
	With 3·8 mg. organisms, 40 min.	11·7	61	—
B.	Organisms and cozymase before reaction	24	26	0·1
	Organisms and cozymase after reaction	0·4	27	0·15
	Nicotinic acid	—	406	420
	Nicotinamide	—	406	1·2
C.	Organisms and buffer	60	68	—
	Cozymase and buffer	102	115	—
	Above quantities of organisms, cozymase and buffer after 1 hr. at 37°	96	172	—

substances, any one of which will support the growth of *Haemophilus parainfluenzae* under certain conditions described in the experimental section. These substances are known to include coenzymes I and II, a 'desaminocozymase' (Schlenk, Hellström & Euler, 1938) and nicotinamide riboside. The simplest of these is the riboside, and the others are derivatives of it.

When cozymase had been inactivated with respect to the apozymase or muscle systems, we found that it had lost also most of its V-factor activity. Moreover, when part only of its activity in the apozymase system had been lost, a similar part of its V-factor activity had disappeared (Table 8).

Isolation of nicotinamide picrolonate. The simplest conclusion from the preceding paragraphs is that the streptococcal reaction with cozymase produces a molecule smaller than nicotinamide riboside, but still containing the nicotinamide part of the molecule. Following a method similar to that of Handler & Klein (1942), who were concerned with the breakdown of cozymase by animal tissues, we have attempted to isolate nicotinamide itself from the products of streptococcal inactivation of cozymase.

Table 7 shows that the product from added cozymase which carries the nicotinic acid portion of the molecule remains in solution and not associated with the streptococci. The reaction mixture of Table 5A was accordingly kept at 37° until samples showed all but 3% of its cozymase to be inactivated. It was then centrifuged and the organisms washed twice with 8 ml. of water. The combined solutions were acidified to pH 3 with 5 N-H_2SO_4, and Ag_2SO_4 (1·7 mmol). added until no more precipitate formed; this was separated. The solution was freed from Ag^+ by H_2S, and after removing the latter by N_2, it was taken to pH 9 with $Ba(OH)_2$, N_2 again passed, then adjusted to pH 7–7·5 with H_2SO_4, $BaSO_4$ removed and washed, the combined solutions evaporated to dryness under reduced pressure and the residue left *in vacuo* with $CaCl_2$ overnight. It was extracted by refluxing 3 times successively with 8 ml. of absolute ethanol, the ethanol solutions evaporated to dryness and the residue re-extracted 3 times with 2 ml. of absolute ethanol. Determination of nicotinic acid showed that all the acid had not been extracted by these procedures; the residues were accordingly re-extracted in the same way, when >90% of the materials of

Table 8. *Loss of V-factor activity with inactivation of cozymase*

(Smaller quantities of streptococci R were prepared as described in Table 5. In Exp. A, determinations were carried out on reaction solutions after centrifuging free from streptococci. In B, the separations indicated were carried out. Reactions were anaerobic.)

Reaction mixture (cozymase in 2·5 ml. with)	Cozymase determined with apozymase (mμmol./ml.)	V-factor activity (as mμmol. cozymase/ml.)
Exp. A		
(1) 0·015 M-Phosphates pH 6	17·3	17·8
(2) As (1), with streptococci (6·5 mg.); 40 min. at 37°	8·2	7·8
(3) 0·015 M-Phosphates, pH 7, with streptococci (6·5 mg.); 10 min. at 37°	13·9	12·2
(4) As (3), for 40 min.	1·7	1·7
Exp. B		
(1) 0·011 M-Phosphates, pH 7·6	11·0	12·0
(2) As (1), with streptococci (8·3 mg.); 40 min. at 38°, determination in solution	0·8	1·0
(3) Cells from reaction (2)	13·0	17·4
(4) Streptococci (8·3 mg.), before reaction	16·9	17·3

Table 9. *Nicotinamide from the streptococcal breakdown of cozymase*

(Exp. B is that of Table 5A, where further details are given. The cozymase of specimens which were taken to follow the course of the reaction has been subtracted from that added. A similar reaction mixture was employed in Exp. A, but with a different batch of organisms and with nicotinamide in place of cozymase. Nicotinic acid picrolonate depresses the melting point of that of nicotinamide.)

	Experiment A	Experiment B
Added ...	Nicotinamide, 52 μmol.	Cozymase, 31·5 μmol.
Isolated as fairly pure picrolonate of nicotinamide; m.p. 214–217°, not lowered by mixing with the pure substance (μmol.)	17	16
Isolated as less pure material, m.p. 208° or greater, not lowered by mixing with nicotinamide picrolonate (μmol.)	16	5
Picrolonic acid content of purer product, by titration with methylene blue (Bolliger, 1939; theory for nicotinamide 68·5%) (%)	69	70
Yield of nicotinic acid from purer picrolonate, determined by *Lactobacillus arabinosus* (theory for nicotinamide picrolonate, 31%) (%)	—	28

nicotinic acid activity were obtained in ethanolic solution. This solution was evaporated to *c.* 0·5 and 2·5 ml. of 1% (w/v) picrolonic acid in 50% aqueous ethanol added, and the solution kept cold. Yellow crystals separated; further material was obtained from the mother liquors by evaporation and addition of more picrolonic acid. By one recrystallization from aqueous ethanol the fractions of Table 9, Exp. B, were obtained. These indicate that the bulk of the product which has nicotinic acid activity is nicotinamide, and that its yield and purity are comparable to that of nicotinamide isolated from a similar reaction mixture to which it had been added as such, in place of cozymase (Exp. A). Exp. A was carried out first to serve as a model in the isolation of the product from cozymase. The data of Table 9 afford independent evidence for the stability of nicotinamide under conditions in which cozymase is inactivated.

splitting analagous to that in the liberation of nicotinamide from cozymase would lead to the formation of nicotinic acid itself, small quantities of which can be detected. Any reaction in trigonelline brought about by streptococci, however, aerobically or anaerobically, was of <1/1000 of the rate of the organisms' reaction with cozymase.

Inactivation of cozymase by Haemophilus parainfluenzae

The change brought about by this organism appears similar to that caused by streptococci. The course of loss of cozymase activity from solutions initially about 5×10^{-5}M was paralleled closely by loss of V-factor activity (Table 10). The nicotinic acid content of a similar reaction mixture did not fall

Table 10. *Breakdown of cozymase by* Haemophilus parainfluenzae

(Strain 4101 was employed and grown in the assay medium with the addition of 3% of yeast extract; initial concentration of cozymase in the medium, 2 μg./ml. The organisms were washed twice with, and suspended in, 0·9% NaCl. The quantity of cozymase indicated was added with phosphates (to 0·03M) at pH 7. Incubation was aerobic at 37°. Determinations refer to the whole mixture, including bacteria. Cozymase itself remained unchanged in the absence of bacteria.)

Batch of organisms; time of growth; quantity used in reaction (dry wt.)	Time of reaction (min.)	Initial cozymase (mμmol./2·5 ml.)	% remaining of		
			Cozymase by apozymase	V-factor (as cozymase)	Nicotinic acid
A; 40 hr.; 4·2 mg.	0	120	(100)	(100)	—
	90	120	56	53	—
	180	120	19	19·5	—
	330	120	4	7	—
B; 46 hr.; 10·5 mg.	0	129	(100)	—	(100)
	90	129	14·4	—	97
	180	129	<3	—	100
	330	129	<3	—	104

Other changes during inactivation of cozymase; other substrates. We have found changes in the phosphate of streptococcal suspensions to be too great to obtain dependable information on the possible liberation of inorganic phosphate from cozymase during its inactivation by the organisms.

The possible production of acid during the reaction was examined by following the evolution of CO_2 from a medium containing 0·038M-bicarbonate in equilibrium with 5% CO_2 in N_2. During the inactivation of 1·7 μmol. cozymase, 2·35 μmol. CO_2 were evolved from a vessel containing streptococci, and 2·43 μmol. from one also containing the cozymase. A similar reaction mixture containing yeast nucleic acid (3 mg./ml.), instead of cozymase, yielded CO_2 in large excess of a control without substrate, and corresponding to acid formation at the rate of 300 mμmol./mg./hr. Thymus nucleic acid also reacted; any reaction with yeast adenylic acid and guanylic acid was much slower. The yeast nucleic acid was purified according to Gulland & Jackson (1938), and acid formation from it was not inhibited by 0·08M-azide, which reduced glycolysis to about 35% of its normal value. The streptococci thus appear to contain a nuclease, but their reaction with cozymase is of a different type from that with nucleic acids.

Pyridine N linked to carbon is found in other natural products. Of these, trigonelline was examined, since a

during a period at least twice as long as that which sufficed to inactivate almost all its cozymase. Breakdown therefore does not appear to lead to accumulation of nicotinamide riboside, but it does not extend beyond nicotinic acid.

Effect of nicotinamide on the streptococcal breakdown of cozymase: synthesis of cozymase

The effect of nicotinamide on the streptococcal breakdown of cozymase was examined since nicotinamide is a product of the reaction, and since it has been found to inhibit the analogous breakdown in animal tissues (Mann & Quastel, 1941; Handler & Klein, 1942). Retardation of the streptococcal reaction was found, but only with extremely high concentrations of nicotinamide. The retardation by 0·1M-nicotinamide (2000 times the substrate concentration) was of the order of 50% (Table 11). Nicotinic acid salts had little or no action at 0·033M.

Other products of cozymase breakdown by streptococci remain unknown, but we have attempted to obtain a synthesis of the material from nicotinamide and yeast adenylic acid. Table 11 shows that synthesis did not occur from these compounds alone,

Table 11. *Nicotinamide in the breakdown and synthesis of cozymase*

(Streptococci (strain R) were used on their first day of growth; reactions were aerobic and at 37°.)

Batch of organisms; conditions of reaction; time of growth	Reaction mixture: Substances added	Reaction mixture: Initial cozymase	Cozymase remaining (%)
A; pH 7·5; 60 min.	0	$4·8 \times 10^{-5}$ M	14
	Nicotinamide, 0·1 M	$4·8 \times 10^{-5}$ M	52
	Nicotinamide, 0·033 M	$4·8 \times 10^{-5}$ M	22
	Na nicotinate, 0·033 M	$4·8 \times 10^{-5}$ M	17
B; pH 7·5; 40 min.	0	5×10^{-4} M	26
	Nicotinamide, 0·055 M	5×10^{-4} M	47
	Nicotinamide, 0·028 M	5×10^{-4} M	29
			Change in cozymase (mμmol.)
C; pH 7·5; 40 min.	0	22 mμmol. (in cells)	—
	Adenylic acid, 0·0033 M; Nicotinic acid, 0·0033 M or both	22 mμmol. (in cells)	<1
C; pH 7·5; 10 min.	Adenylic acid, 0·0033 M; Nicotinic acid, 0·0033 M and glucose 0·033 M	22 mμmol. (in cells)	+12·4
C; pH 7·5; 40 min.	As above	22 mμmol. (in cells)	+26

but did take place when glucose also was added. In a similar reaction mixture the course of reaction was followed in more detail and the cozymase was found to increase at the rate of 20 mμmol./mg. dry wt./hr. during the first 50 min.

DISCUSSION

Streptococcal breakdown of cozymase. Inactivation of cozymase has been shown to be a very general reaction in bacteria. The reaction in streptococci reached surprisingly high velocities. With cozymase at 4×10^{-4} M, it was decomposed at rates about 400 mμmol./mg. of organisms/hr., equivalent to the decomposition during 1 hr. of a quantity of cozymase one-quarter of the bacterial dry weight. Studies of the essentials for microbial growth, and the development of microbial methods for estimating vitamin-like compounds, have tended to give the impression that bacteria normally react with only small quantities of such compounds. This is true only in one sense. Thus, the minimal concentration of nicotinic acid or its derivatives normally needed for maximal bacterial growth is less than 10^{-7} M. This value holds also for the streptococci studied here, though the quantity of cozymase in their cells was of the order of 5–10 mμmol./mg. dry wt. (Tables 2 and 6). The internal concentration of cozymase, assuming a water content of 80%, was therefore about $1–2 \times 10^{-3}$ M, or about 0·1 % of the cell weight. These are not exceptional values; cozymase contents reported for yeast (cf. Sumner, Krishnan & Sisler, 1946) correspond to internal concentrations of 7×10^{-4} M. The coenzymes of red blood cells, normally of *c.* 10^{-4} M, can be raised to 3×10^{-4} M by feeding nicotinic acid (Handler & Kohn, 1943). The latter values are for substances of V-factor activity, presumably representing the sum of coenzymes I and II. Thus, although the rapid decomposition of cozymase by streptococci was observed with concentrations greater than 10^{-4} M, cozymase frequently occurs naturally at such concentrations.

It is therefore feasible that the enzymic activity concerned may play a part in the normal economy of the cells, even though substrate saturation is not attained at a cozymase concentration of 4×10^{-4} M. It is presumably of advantage to an organism to be able to interconvert the naturally occurring nicotinic acid derivatives. An alternative possibility which we considered, but now think unlikely, was that the decomposition of cozymase might occur by the action of a nucleotidase, and although the streptococci produced acid from nucleic acids, acid from cozymase was not formed at a comparable rate. It is certain also that nicotinamide is produced as such from cozymase, and that this involves breakage of the molecule between the pyridine N and the C_1 of ribose. This linkage is known to occur naturally only in the two coenzymes. The somewhat similar link between pyridine N and $—CH_3$ in trigonelline was split, if at all, at less than 1/1000 of the rate at which cozymase was inactivated. Liberation of nicotinamide from cozymase may be preceded or followed by changes in the remainder of the cozymase molecule. Our present experiments do not give much information on this subject, except that nicotinamide riboside did not accumulate during the breakdown of cozymase. Breakdown of cozymase by acids or alkalis also liberates nicotinamide as such (Schlenk, 1943).

Synthesis of cozymase by streptococci. Streptococci were able to synthesize cozymase in spite of the rapid breakdown of added cozymase. This is implied in the finding that cells grown in presence of nicotinic acid contained cozymase, as also did non-proliferating suspensions of cells, and it seems that the cozymase initially associated with streptococcal cells was not decomposed so rapidly as the added material. An enzyme inactivating cozymase was not, however, liberated to solution by the streptococci, nor easily extracted from them, and decomposition of cozymase may always be preceded by its assimilation by the cells (cf. Table 6). The observed rate of accumulation of cozymase under conditions permitting its synthesis is therefore likely to be less than the true rate of synthesis.

The maximum rate of accumulation of synthesized cozymase which was observed was 20 mμmol./mg. dry wt./hr. The streptococci used grow, when under satisfactory conditions, with generation times of 20–30 min. The rate of reaction (Q) in mμmol./mg./generation time is therefore 6·7–10. As indicated by McIlwain, Roper & Hughes (1948), this means that the cells produced by such growth could have a quantity of cellular cozymase of $q = Q \log_e 2$, or in this case of 4–6 mμmol./mg. dry wt. This is about the cell content actually observed (Table 2).

Breakdown of cozymase by other organisms. Findings with respect to the breakdown of cozymase in bacteria and in animal tissues are similar, though in neither are they very extensive. Brain suspensions, like the streptococci, liberated nicotinamide (Handler & Klein, 1942). *Haemophilus parainfluenzae* produced either nicotinic acid or nicotinamide. The reaction in animal tissues, as in bacteria, was rapid. The breakdown by rat brain, and probably by cobra venom, was inhibited by nicotinamide (Mann & Quastel, 1941), and we found a lesser breakdown by streptococci in the presence of nicotinamide, though high concentrations of this were required.

The bacteria which we found to inactivate cozymase represent many taxonomic groups: streptococci, staphylococci, *Escherichia*, *Proteus* and *Haemophilus*. They are also very varied in their reactions to nicotinic acid and its derivatives in growth: *Escherichia coli* not requiring such addition for growth, *Proteus morganii* and the cocci requiring nicotinic acid, and *Haemophilus parainfluenzae* requiring nicotinamide riboside. For this reason, breakdown of cozymase by *H. parainfluenzae* has a significance which is different from that of the breakdown by the other organisms. Reaction with cozymase by *H. parainfluenzae* was at about the same rate as by the other bacteria, but whereas these were capable of resynthesizing cozymase from the products of its breakdown, *H. parainfluenzae* could not do so. It thus inactivates rapidly a compound which is indispensable to its growth. This situation, which is presumably of ecological importance, has parallels in the behaviour of other organisms to other growth essentials (McIlwain, 1947).

One reason for our study of the breakdown of cozymase by *H. parainfluenzae* was as follows. Lwoff & Lwoff (1937) concluded from indirect evidence that added coenzymes I and II could be interconverted by this organism, though after assimilation such interconversion was not possible. Production from added cozymase of a simpler substance of V-factor activity would have provided a mechanism for the interconversion, but we were unable to demonstrate the production of such a substance.

SUMMARY

1. Cozymase at concentrations of about 10^{-4}–10^{-5}M was inactivated by streptococci, staphylococci, *Escherichia coli*, *Proteus morganii* and *Haemophilus parainfluenzae* (but not by *Lactobacillus arabinosus* 17–5) at rates of 5–30 mμmol./mg. dry wt. of cells/hr. at pH 6–7·5 and 37°.

2. Streptococcal cells have been found to be about 10^{-3}M with respect to cozymase, which was the form in which most of their nicotinic acid existed. Streptococci inactivated 4×10^{-4}M-cozymase at the rate of 400 mμmol./mg./hr., and so could inactivate their own dry weight of the substance in 4 hr.

3. Nicotinamide was produced from cozymase during its inactivation by streptococci. A nicotinamide derivative of V-factor activity, such as the riboside, did not accumulate. The breakdown by *Haemophilus parainfluenzae* was similar.

4. The breakdown in several bacteria was not greatly influenced by air (but was sometimes greater anaerobically), or glucose (this sometimes retarded it), and was still rapid at 10°.

5. Streptococci were able to synthesize cozymase from nicotinic acid and adenylic acid in the presence of glucose; rates of +20 mμmol./mg. dry wt./hr. were observed, which were about those required to account for the observed cell content of the substance.

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The Amylose Content of the Starch Present in the Growing Potato Tuber

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During the last few years it has been shown that starch consists of two components: amylose, which is a linear polymer and comparable with cellulose, and amylopectin, which is a branched chain polymer comparable with glycogen. The advances in this field have been greatly assisted by the development of the potentiometric iodine titration method for estimating the percentage of amylose in starch (Bates, French & Rundle, 1943; Hudson, Schoch & Wilson, 1943). By use of this procedure the amylose content of many starches has been determined, and starches have been examined in which the amylose content varies from less than 1% in the waxy starches to over 50% in certain varieties of pea starch (Bates *et al.* 1943; Hilbert & MacMasters, 1946). The published values of amylose contents for starches of similar botanical origin, e.g. maize, sometimes differ and this suggests that the amylose content is not necessarily a constant property of the starch from any given type of botanical species. Furthermore, by use of the newer methods for end-group determination, and with a knowledge of the amylose content of a starch, it is now possible without separation of the components to determine the proportion of glucose residues present in the amylopectin fraction of the starch as terminal groups (Brown, Halsall, Hirst & Jones, 1948). For example, the sample of rice starch which was examined by Hirst & Young (1939), who used the methylation technique, has now been shown to contain 12% of amylose, and hence we have present in the amylopectin fraction one non-reducing terminal group/26 glucose residues. In other samples of rice starch, however, an amylose content of 14–15% (Brown *et al.* 1948) has been found, and the amylopectin fraction has been shown to contain one non-reducing terminal residue/20 glucose residues. This indicates that different varieties of rice starch do not all contain the same type of amylopectin. It will be recalled that Meyer & Heinrich (1942) have claimed that the starches extracted respectively from potato tubers, leaves and shoots, contain different pro-