to leave a phenol-insoluble mucoid. This contains a substance with the characteristics of a mucopolysaccharide-protein complex.

5. The water-soluble mucoids contain a complex which is formed by interaction between the phenol-insoluble mucoids and a water-soluble protein of the cornea.

6. The acid mucopolysaccharide can be prepared free from protein by incubation with trypsin. Galactose, glucosamine and galactosamine have been identified as constituents of the mucopolysaccharide, by chromatography and ionophoresis on filter paper.

7. The corneal mucopolysaccharide is not a substrate for hyaluronidase.

8. Gel-forming proteins have been isolated from the insoluble mucoid and found to contain the same amino-acids as gelatin, but to differ from it in their electrophoretic behaviour.

9. It is suggested that *in situ* the acid mucopolysaccharide is combined with proteins of the collagen group.

I wish to thank Dr A. S. McFarlane for allowing me to make the electrophoretic measurements in his laboratory and Mrs A. Dovey for much advice on the use of the instrument.

REFERENCES

- Bergman, M. & Stein, W. H. (1939). *J. biol. Chem.* **128**, 217.
 Bowes, J. H. & Kenten, R. H. (1949). *Biochem. J.* **45**, 281.
 Bowes, J. H. & Kenten, R. H. (1950). *Biochem. J.* **46**, 524.
 Dempsey, E. W., Bunting, H., Singer, M. & Wislocki, G. B. (1947). *Anat. Rec.* **98**, 417.
 Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
 Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
 Kaye, M. A. G. & Stacey, M. (1951). *Biochem. J.* **48**, 249.
 Lugg, J. W. H. (1938). *Biochem. J.* **32**, 2114.
 Meyer, K. & Chaffee, E. (1940). *Amer. J. Ophthal.* **23**, 1320.
 Meyer, K. & Smyth, E. M. (1937). *J. biol. Chem.* **119**, 507.
 Morgan, W. T. J. & Partridge, S. M. (1941). *Biochem. J.* **35**, 1140.
 Morner, C. T. (1894). *Hoppe-Seyl. Z.* **18**, 213.
 Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 304.
 Partridge, S. M. (1948a). *Biochem. J.* **42**, 238.
 Partridge, S. M. (1948b). *Biochem. J.* **43**, 387.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
 Pirie, A. (1947). *Biochem. J.* **41**, 185.
 Pirie, A. (1949). *Brit. J. Ophthal.* **33**, 678.
 Rogers, H. J. (1945). *Biochem. J.* **39**, 435.
 Suzuki, M. (1939). *J. Biochem., Tokyo*, **30**, 185.
 Trevelyan, W. E., Procter, D. S. & Harris, S. S. (1950). *Nature, Lond.*, **104**, 443.
 Werner, I. & Odin, L. (1949). *Uppsala LäkFören. Förh.* **54**, 69.
 Wislocki, G. B., Bunting, H. & Dempsey, E. W. (1947). *Amer. J. Anat.* **81**, 1.
 Woodin, A. M. (1950a). *Brit. J. Ophthal.* **34**, 375.
 Woodin, A. M. (1950b). *Biochem. J.* **47**, xxxvii.

The Synthesis of Cozymase from Nicotinic Acid and its Derivatives by *Lactobacillus arabinosus* 17-5

BY D. E. HUGHES AND D. H. WILLIAMSON

*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry,
The University, Sheffield 10*

(Received 7 November 1951)

As shown in previous papers from this laboratory (McIlwain, 1949; McIlwain, Stanley & Hughes, 1949), glycolysis by washed suspensions of *Lactobacillus arabinosus* 17-5, which have been grown on a nicotinic acid-deficient medium, is stimulated by the addition of nicotinic acid. The stimulation is due to the synthesis of cozymase and, since this organism does not rapidly decompose the coenzyme (McIlwain & Hughes, 1948), it seemed to be suitable material in which to study its synthesis. In the present studies, the effect of limiting amounts of nicotinic acid, nicotinamide and nicotinamide nucleotides on the stimulation of glycolysis and on the synthesis of

cozymase has been investigated. Young deficient cells glycolysing in an optimal concentration of nicotinic acid rapidly synthesized cozymase until the cells became saturated with the coenzyme; synthesis of cozymase and metabolism of nicotinic acid then ceased. Glucose alone of the constituents of the growth medium was essential for the synthesis of cozymase by washed suspensions. The uptake of nicotinic acid ran parallel to the synthesis of coenzyme and no detectable amounts of any possible intermediate in the synthesis were detected in cells or the suspending fluid. Unexpectedly, nicotinamide was found to be rapidly deamidated by the

washed suspensions; deamidation was independent of the glucose and of the cozymase content of the cells.

EXPERIMENTAL

Growth conditions for obtaining cozymase-deficient cells

Lactobacillus arabinosus 17-5 was grown as described by McIlwain & Hughes (1948) on the semi-synthetic medium consisting of casein hydrolysate, salts and vitamin supple-

washed out twice with 1.0 ml. 0.9% NaCl, the washings were added to the centrifuged cells and recentrifuged. The washings were discarded and the cells washed once again with 2.0 ml. of 0.9% saline, and then resuspended in 1.0 ml. of 0.065M-phosphate buffer, pH 6.2, and heated at 80° for 15 min. As shown previously (McIlwain & Hughes, 1948) this treatment releases the cozymase from the cells with a 5-8% loss; no corrections for this loss were made. After heating, the tubes were cooled in running tap water and if not used immediately the contents were frozen solid and stored at -14°.

Table 1. *The effect of nicotinic acid upon glycolysis by washed suspensions of Lactobacillus arabinosus* 17-5

(The cells were grown as indicated below, collected, washed as described and the effect of nicotinic acid on glycolysis measured manometrically. In each Warburg flask was 2.0 ml. containing: 0.05M-NaHCO₃; 0.05M-glucose; 2.0-4.0 mg. dry wt. cells. Nicotinic acid (5×10^{-5} M final) was added from the side arm.)

Growth period (hr.)	Nicotinic acid in growth medium ($\mu \times 10^{-7}$)	Yield of cells (mg. dry wt./100 ml.)	CO ₂ (μ moles/mg. dry wt./hr.)	
			Without nicotinic acid	With nicotinic acid
19	100	30	11.0	11.0
19	10	30	10.0	10.0
60	10	45	3.7	6.0
22	5.0	12	17.0	17.0
40	5.0	24	3.0	10.0
21	2.0	15	7.2	11.0
40	2.0	16	3.6	10.8
22	1.0	9	12.0	20.0
40	1.0	10	5.0	15.0
20	0.4	35	10.0	20.0

ment. Satisfactory yields (10-15 mg. dry wt./100 ml.) of deficient cells were obtained after 30-44 hr. growth on the medium containing from 1.5 to 2.0×10^{-7} M-nicotinic acid (Table 1). The addition of nicotinic acid to glycolysing suspensions of these deficient cells stimulated their rate of glycolysis from two- to three-fold. Higher yields of deficient cells (30-40 mg. dry wt./100 ml.) were obtained after 60-70 hr. growth on the medium containing more nicotinic acid (10^{-6} M), but these cells were unsatisfactory because their rate of glycolysis tended to fall on incubation and the ability to synthesize cozymase was poor. Non-deficient cells were grown on a medium made from enzymically hydrolysed casein, yeast extract and glucose (Nossal, 1951).

Measurements of the effects of nicotinic acid on glycolysis and cozymase synthesis.

In the main compartment of a conical Warburg cup was placed 0.1M-glucose, 0.5 ml.; 0.2M-NaHCO₃, 0.5 ml.; washed cell suspension, 0.5 ml. and sufficient Krebs & Henseleit saline to bring the final volume to 2.0 ml. The nicotinic acid or derivative was in the side arm in 0.2 ml. water. Yellow phosphorus was placed in the centre well. The cup was gassed with 95% N₂-5% CO₂, and shaken in a thermostat at 40.0°. Readings were taken at 5 min. intervals until the rate of evolution of CO₂ was constant (20-30 min.). The side arm was then emptied and readings continued at 3-5 min. intervals. To stop the reaction, the cup was taken from the thermostat and placed in an ice-salt freezing mixture. The contents were then allowed to thaw, transferred to a centrifuge tube and centrifuged for 10 min. at approx. 3500 g. The supernatant was poured off into a test tube. In order to collect the cells quantitatively the cup was

Determination of cozymase

Cozymase was determined manometrically in the apozymase system of Axelrod & Elvehjem (1942), essentially as described by McIlwain & Hughes (1948). The apparent loss of activity by many batches of apozymase upon storing was found to be associated with the formation of acidic material at 2°. This lowered the pH of the apozymase system from 6.2 to 5.6-5.8 and thus reduced its activity. In later experiments where the apozymase was dried over P₂O₅ and stored over a drying agent such as silica gel at -14° the apozymase lost no activity in a month and lost from 10 to 15% after 2 months. Large blank values in some experiments were found to be due to cozymase present in the calcium hexosediphosphate (obtained from Schwartz Laboratories Inc., New York 17, U.S.A.). To remove the cozymase, the calcium hexosediphosphate was dissolved in 1.1 equivalents of HCl and stirred with one-tenth of its weight in norite for 0.5 hr. at 2°. The norite was removed by filtration and 2 equivalents of Ba(OH)₂ added. The pH was adjusted to 8.5 and the precipitate of dibarium salt of hexosediphosphate was filtered off, washed with water and ethanol and dried. Barium was removed from small samples immediately before use by the addition of an equivalent of 0.5N-Na₂SO₄ to an ice-cold solution of the barium salt in 2N-HCl.

Determination of nicotinic acid, nicotinamide and nicotinamide derivatives

In most of the experiments, nicotinic acid was determined by the microbiological assay method of Barton-Wright (1946). When it was not desired to distinguish between nicotinic acid and amide, samples for assay were

heated in N -HCl as described in a previous paper (McIlwain & Hughes, 1948). To distinguish between the acid and amide, a portion of the sample was treated with sodium hypobromite as described by Atkin, Schultz, Williams & Freys (1943), to destroy the nicotinamide, and another portion was heated with HCl as before. The nicotinic acid in both portions was estimated by microbiological assay and the nicotinamide content of the sample calculated by difference. In some of the later work, nicotinic acid and nicotinamide were estimated by the chemical methods of Chaudhuri & Kodicek (1948) and Hughes (1949). The acetone condensation method of Levitis, Robinson, Rosen, Huff & Perlzweig (1945) was used to determine the total concentration of N^1 -substituted nicotinamide derivatives. Fluorescence was estimated in a fluorimeter employing a photomultiplier tube and similar to that described by Lowry (1948). Solutions of quinine sulphate were used as permanent standards.

Cozymase, nicotinamide ribotide and riboside can act as 'V' factor for bacteria (Gingrich & Schlenk, 1944). Activity as 'V' factor was estimated by measuring the growth response of *Haemophilus parainfluenzae* as described by McIlwain & Hughes (1948). National Type Culture Collection, strains no. 4101, no. 4625 and eight locally isolated strains were tested. Where the strain was found to reduce nitrate, tests for nitrite were made according to Hoagland & Ward (1942). It was found that although nitrite was often detected where no growth could be seen, the response of various strains towards nitrate was too unreliable to be used as a quantitative method for estimating 'V' factor.

Chemical determinations

Ammonia was distilled in a modified Parnas apparatus or in Conway units and determined by the method of Russell (1945) or with Nessler's reagent. A modification of the method of Van Slyke was used for the estimation of glucose (see Wheatley, 1947). The ribose and phosphate contents of the nicotinamide nucleotides were determined as described by Schlenk (1943). Lactic acid was estimated by the method of Friedman & Graeser (1933).

Materials

The first of the two specimens of cozymase used as a standard was that described as 'C' by McIlwain & Hughes (1948); it contained 44% coenzyme I (CoI) and no detectable free nicotinic acid or nicotinamide. The second specimen was prepared from brewer's yeast according to LePage (1949), and contained 50–52% CoI when compared with the first specimen in the apozymase system, and approximately 2.0% free nicotinamide, as judged by microbiological assay. Specimens of dihydronicotinamide ribofuranoside (nicotinamide riboside) were made available through the courtesy of Dr L. J. Haynes, Chemistry Department, University of Cambridge. Tests for 'V' factor activity showed that it had one-quarter to one-tenth of the activity of CoI on a molar basis as 'V' factor (Table 2) (see also Haynes & Todd, 1950). In most freshly isolated strains the amounts of riboside needed for visible growth were higher than those needed in the older strains (see Schlenk & Gingrich, 1944). Specimens of nicotinamide glucoside, galactoside and arabinoside were also synthesized by Dr Haynes; they were without 'V'-factor activity for all strains tested (Table 2). Nicotinamide ribose phosphate (nicotinamide ribotide) was

prepared from cozymase by the action of potato pyrophosphatase according to Kornberg & Pricer (1949). Nicotinic acid and nicotinamide were commercial specimens which had been recrystallized several times from water-ethanol mixtures.

Table 2. 'V'-factor activity of nicotinamide nucleosides

(*Haemophilus parainfluenzae* Strain 4101 was grown as described by McIlwain & Hughes (1948) and growth measured photometrically. Nitrite was detected by the method of Hoagland & Ward (1942); + indicating just detectable amounts and + + + maximum amounts, and 0 no test for nitrite.)

Growth factor added	Concn. of nicotinamide derivative ($M \times 10^{-7}$)	Photo-meter reading at 22 hr.	Nitrite at 22 hr.
None	—	5.2	0
Cozymase	1.3	25	+ + + +
	0.75	20	+ + +
	0.35	13	.
	0.17	6	0
Nicotinamide riboside	20	20	+ + + +
	4	9	+ +
	1	5	+
	0.2	5	+
Nicotinamide galactoside	100	5	0
	10	5	0
Nicotinamide galactoside with cozymase ($0.75 \times 10^{-7} M$)	100	20	+ +
	10	20	+ +
Nicotinamide arabinoside	100	5	0
	10	5	0
Nicotinamide arabinoside with cozymase ($0.75 \times 10^{-7} M$)	100	20	+ + + +
	10	20	+ +
Nicotinamide glucoside	100	5	0
	10	5	0
Nicotinamide glucoside with cozymase ($0.75 \times 10^{-7} M$)	100	20	+ + +
	10	20	+ +

RESULTS

Stimulation of glycolysis by varying concentrations of nicotinic acid

The addition of nicotinic acid in concentrations from 3×10^{-8} to $10^{-4} M$ to glycolysing washed suspensions of deficient cells caused a gradual increase in the rate of glycolysis (Fig. 1). The magnitude of the increase was greatest at and above 1.0 – $5.0 \times 10^{-6} M$; below this concentration the effect decreased and generally could not be detected in concentrations below $3 \times 10^{-8} M$ (Table 3). The effect reached its maximum after a lag period of 10–50 min. This lag period was shortest at nicotinic acid concentrations above $5 \times 10^{-6} M$, and increased as the concentration was reduced to $10^{-7} M$ (Fig. 1). This stimulating effect of nicotinic acid is due to the formation of cozymase which in the deficient cells limits glycolysis (McIlwain *et al.* 1949). Thus the lag period may

be taken as indicating the time taken for the cells to synthesize sufficient cozymase to saturate the glycolytic system and hence indirectly indicates the rate of cozymase synthesis. It would therefore appear that below $5 \times 10^{-5} M$ the concentration of nicotinic acid is a limiting factor in cozymase synthesis. When limiting amounts of nicotinic acid

Fig. 1. The effect of varying concentrations of nicotinic acid on glycolysis by deficient cells. Cells were grown for 41 hr. on medium containing $1.57 \times 10^{-7} M$ -nicotinic acid and collected and washed as described. Glycolysis was followed manometrically. In each Warburg cup were 2 ml. containing 0.05 M-glucose, 0.05 M- NaHCO_3 , 3.2 mg. dry wt. of cells. Nicotinic acid added from the side arm as indicated by the arrow.

Table 3. *The effect of varying concentrations of nicotinic acid on glycolysis by deficient cells*

(Cells were grown for 40 hr. on a medium containing $1.5 \times 10^{-7} M$ -nicotinic acid. Glycolysis was followed manometrically. In each Warburg flask was 2.0 ml. containing: 0.05 M-glucose; 0.05 M- NaHCO_3 ; 2.3 mg. dry wt. of cells: the nicotinic acid was added from the side arm. The values for CO_2 production are the maximum that occurred during 2 hr. after adding the nicotinic acid.)

Concn. of nicotinic acid ($M \times 10^{-8}$)	CO_2 ($\mu\text{moles/mg.}$ dry wt. cells/hr.)
None	7.2
3.0	7.8
10.0	9.7
30.0	17.7
100	18.0
150	19.3
500	19.0

are added to the cells, all the nicotinic acid is taken up and converted into cozymase (see p. 335). A limiting concentration of 1.0 to $5.0 \times 10^{-6} M$ would mean therefore that 1.0 – $2.0 \mu\text{moles}$ of nicotinic acid/mg. dry wt. are needed to saturate the glycolytic system of the cells.

The rate of cozymase synthesis at varying concentrations of nicotinic acid

Varying concentrations of nicotinic acid ranging from 5×10^{-4} to $3 \times 10^{-7} M$ were added to glycolysing deficient cells. The cups were placed in a freezing

Fig. 2. Cozymase synthesis in varying concentrations of nicotinic acid. Cells were grown for 21 hr. on medium containing $2 \times 10^{-7} M$ -nicotinic acid, collected and washed. Reaction with nicotinic acid, in concentrations as indicated on the graphs, and cozymase determinations were as described in the text.

Fig. 3. The initial rate of cozymase synthesis in varying concentrations of nicotinic acid. Cells were grown for 21 hr. on medium containing $2 \times 10^{-7} M$ -nicotinic acid. The reaction with nicotinic acid was for 10 min. at 40° . Cozymase estimations were carried out as described in the text.

mixture to stop the reaction at 5, 10, 15 and 30 min., the cells collected, cozymase extracted, and estimated as already described. Under these conditions no measurable amounts of cozymase appeared in the reaction fluid and it is therefore assumed that the rate of cozymase accumulation in the cells indicates

the rate of cozymase synthesis. As will be seen from Fig. 2 the cozymase content of the cells rapidly rises following the addition of nicotinic acid. At the higher concentrations of nicotinic acid, the maximum rate of cozymase synthesis lasted for approximately 20–30 min. then decreased and ceased when the cells contained 12–14 $\mu\text{m-moles/mg. dry wt.}$ (Fig. 2). At the lower concentrations of nicotinic acid the rate of cozymase synthesis continued approximately linearly until all the available nicotinic acid was converted into the coenzyme (see also p. 335). The maximum rate of cozymase synthesis (16–25 $\mu\text{m-moles/mg. dry wt./hr.}$) occurred in concentrations at and above $5 \times 10^{-5}\text{M}$ -nicotinic acid and fell off progressively as nicotinic acid concentration was reduced (Fig. 3). These results confirm the conclusions drawn from the experiments in the preceding paragraph, which suggested that the rate of cozymase synthesis was limited by the concentration of nicotinic acid.

Fig. 4. The effect of growth period on the rate of cozymase synthesis. Cells were grown in medium containing $2 \times 10^{-7}\text{M}$ -nicotinic acid and samples taken at the times indicated. The cells were collected, washed and the reaction with nicotinic acid ($5 \times 10^{-5}\text{M}$ final) and cozymase estimations carried out as described.

The effect of growth period upon the cozymase synthesis

The maximum rate of cozymase synthesis, at and above $5 \times 10^{-5}\text{M}$ -nicotinic acid, was found in washed cells grown for 16–20 hr. In three batches of cells grown for a shorter period (10 hr.) the rate of synthesis was 15–20% lower than maximum. The rate of cozymase synthesis fell progressively as growth was extended from 20 to 70 hr. (Fig. 4). At 38 hr. the initial rate was 1.5–2.0 $\mu\text{m-moles/mg. dry wt./hr.}$ and increased to 6.0–10.0 $\mu\text{m-moles/mg. dry wt./hr.}$ after 50–90 min. In cells grown for 70 hr., however, the rate of cozymase synthesis remained low (0.5–1.0 $\mu\text{m-mole/mg. dry wt./hr.}$) on incubation up to 3 hr. During this period the rate of

glycolysis increased from 1 $\mu\text{mole/mg. carbon dioxide/mg. dry wt./hr.}$ to 4 $\mu\text{moles/mg. dry wt./hr.}$ Younger cells with an initial rate of glycolysis of from 2 to 5 $\mu\text{moles carbon dioxide/mg. dry wt./hr.}$ were found to synthesize cozymase at rates of 2–4 $\mu\text{m-moles/mg. dry wt./hr.}$, that is at a rate two- to three-fold faster than in the old cells whose rate of glycolysis was the same. Although, as is shown later, glycolysis is essential for cozymase synthesis, it would appear that the synthesis in older cells is limited by processes other than glycolysis.

The effect of replacing nicotinic acid by nicotinamide and its derivatives

In young deficient cells (19–22 hr. growth) there was no significant difference in the effects on glycolysis or on the rate of cozymase synthesis when nicotinic acid was replaced by nicotinamide, nicotinamide riboside or nicotinamide ribotide. In older cells (30–48 hr. growth) glycolysis was stimulated more rapidly by the nicotinamide and its

Fig. 5. Stimulation of glycolysis in deficient cells grown for 50 hr., by nicotinic acid and nicotinamide. Glycolysis and reaction with nicotinic acid and nicotinamide ($5 \times 10^{-5}\text{M}$ final) were measured manometrically as described in the text.

derivatives than by nicotinic acid, although the final rate of glycolysis was the same (Fig. 5). This suggests that in older cells cozymase is synthesized more rapidly from nicotinamide and its derivatives than from nicotinic acid. Experiments in which the cozymase content of the cells was measured showed no differences in the rate of cozymase synthesis. As shown previously the differences in the amount of cozymase which changed the rate of glycolysis were of the order of 1.0–2.0 $\mu\text{m-moles/mg. dry wt.}$ Differences in the rate of formation of such small amounts of cozymase were too small to be measured by the present methods. No cozymase was synthesized from nicotinamide arabinoside, nicotinamide glucoside or nicotinamide galactoside. This

result agrees with the finding that these compounds are inactive as 'V' factor for *Haem. parainfluenzae*.

Under a variety of conditions the rate of cozymase accumulation in deficient cells was, in the presence of cozymase, 10–15 % faster than the accumulation in the presence of nicotinic acid or nicotinamide. Cozymase was not degraded by the organism at rates faster than 0.01 $\mu\text{m-mole/mg. dry wt./hr.}$ (see also McIlwain & Hughes, 1948). The intact cozymase molecule thus appears to be readily adsorbed.

and no nicotinamide or other possible intermediates in the synthesis of cozymase accumulated once the saturation level was attained (Table 5). When experiments were continued for longer periods, i.e. up to 4 hr. instead of the usual 1–2 hr., a small amount of an N^1 -substituted nicotinamide compound was detected in the medium. This material had 'V'-factor activity for *Haem. parainfluenzae*, but the amounts as judged by the fluorimetric method of Levitis *et al.* (1945) were equivalent to 0.02 $\mu\text{m-mole}$

Table 4. Cellular concentration of nicotinic acid, nicotinamide or cozymase during synthesis by deficient cells

(Cells were grown on medium as described below, collected and washed. The reaction with nicotinic acid ($5 \times 10^{-5} \text{M}$) and estimation of the products was carried out as described in the text.)

	Time after adding nicotinic acid (min.)	Total nicotinic acid ($\mu\text{moles/mg.}$ dry wt.)	Nicotinamide ($\mu\text{moles/mg.}$ dry wt.)	Cozymase ($\mu\text{moles/mg.}$ dry wt.)
Exp. A. Cells were grown for 28 hr. on medium containing $2 \times 10^{-7} \text{M}$ -nicotinic acid	0	0.5	—	0.46
	10	3.6	—	3.8
	20	6.3	—	6.3
	40	11.1	—	10.8
	80	15.6	—	14.0
Exp. B. Cells were grown for 44 hr. on medium containing $1.57 \times 10^{-7} \text{M}$ -nicotinic acid	0	1.2	1.0	1.0
	30	1.8	1.2	1.4
	60	5.0	3.0	3.2

Cozymase was not taken up in the absence of glucose; some source of energy is therefore needed for cozymase adsorption as well as for its synthesis. The highest concentration of cozymase found in cells was the same (10–12 $\mu\text{m-moles/mg. dry wt.}$) whether nicotinic acid, nicotinamide or cozymase were added to glycolysing deficient cells. Cells grown with adequate nicotinic acid (10^{-4}M) on the semi-synthetic medium or on the yeast-supplemented pronutrin medium also contained the same amount of cozymase and did not adsorb further cozymase. This amount of cozymase (10–12 $\mu\text{m-moles/mg. dry wt.}$) therefore appears to be a saturation level for this organism and once it is attained the organism ceases either to adsorb further cozymase from the medium or to destroy it as in the case of other micro-organisms (McIlwain & Hughes, 1948).

The metabolism of nicotinic acid

In the absence of glucose, nicotinic acid was not removed from the medium by washed suspensions of cells which had been grown under a variety of conditions. In the presence of glucose, however, nicotinic acid was taken up by deficient cells at approximately the same rate as cozymase was synthesized (Table 4). During synthesis, small amounts of free nicotinic acid sometimes appeared in the cells, but when the cozymase content of the cells had reached 10–12 $\mu\text{m-moles/mg. dry wt.}$ cozymase synthesis and nicotinic acid metabolism ceased. When the cells were provided with up to tenfold excess of nicotinic acid the excess acid was recovered from the medium

of cozymase/2.0 ml. and were too small to be estimated in the apozymase system. It is uncertain, therefore, whether the substance leaking from the cells is cozymase or some other N^1 -substituted nicotinamide derivative.

Table 5. Recovery of nicotinic acid from medium and cells after glycolysis

(Nicotinic acid was added to glycolysing cells (3.0 mg./2 ml.) under the conditions described in Tables 2 and 3. After reacting for 90 min. the cells were collected by centrifugation, washed twice with 0.9 % NaCl, and nicotinic acid was estimated in cells and medium by microbiological assay.)

Nicotinic acid added to the medium (μmoles)	Nicotinic acid recovered (μmoles)	
	Cells	Medium
None	2.0	None
6.0	8.0	None
20	16	1.5
50	19	3.1
100	23	80
200	25	180

The metabolism of nicotinamide

Nicotinamide added to glycolysing cells disappeared rapidly from the medium as judged by the fluorescence after reaction with CNBr (Chaudhuri & Kodicek, 1948) (Table 6). Some of the nicotinamide (20–40 μmoles) which disappeared from the medium was accounted for by the appearance in the cells of cozymase, but the rest could not be accounted

for by the accumulation of other derivatives of nicotinic acid or nicotinamide (Table 6). After reaction with nicotinamide the mother liquor was examined by the method of Hughes (1949) (reaction with CNBr at pH 5.5, followed by sodium hydroxide), and the absorption spectrum was found to

since blank values on the cells themselves were sometimes as large as that expected to be formed from the nicotinamide (100 μ m-moles). However, ammonia formation at the rate of approximately 2 μ m-moles/mg. dry wt./hr. was found when cells were incubated with larger amounts of nicotinamide than

Table 6. *Deamination of nicotinamide during cozymase synthesis*

(The reaction was carried out as described in the text. In each Warburg flask were 2.0 ml. containing: 0.05M-glucose; 0.05M-NaHCO₃; 2.3 mg. dry wt. cells. Nicotinamide (100 μ m-moles) was added from the side arm.)

Time after adding nicotinamide (min.)	Relative fluorescence	Nicotinic acid in mother liquor (μ m-moles/2.0 ml.)	NH ₃ content of mother liquor (μ m-moles/2.0 ml.)	Cozymase content of cells (μ m-moles)
0	100	0	57	1.2
8	2.7	71	75	8.2
15	*Negative reading	84	87	12.0
30	*Negative reading	85	54	19.0
60	*Negative reading	80	50	19.3

* Negative reading indicates some absorption of the activating light probably by nicotinic acid (cf. Chaudhuri & Kodicek, 1948).

resemble that of nicotinic acid (Fig. 6). The formation of nicotinic acid from nicotinamide was confirmed by microbiological assay (Table 6). It was also found that there was some temporary increase

usual (2.0×10^{-3} M). A similar formation of ammonia occurred in the absence of glucose (Table 7). It is clear, therefore, that these organisms deamidate nicotinamide and that this reaction, unlike the uptake of nicotinic acid and the synthesis of cozymase, is independent of the presence of glucose.

Fig. 6. The change in the absorption curve of nicotinamide brought about by *Lb. arabinosus*. Nicotinamide (100 μ m-moles/2 ml.) was added to glycolysing cells under the usual conditions and the reaction stopped at 0 and 10 min. The cells were removed by centrifugation, 1.0 ml. of the mother liquors reacted with CNBr and NaOH and the absorption spectra measured in a Beckman spectrophotometer. ○—○, solution at 0 min. The absorption spectra is typical of nicotinamide ($E_{\max}$ at 352 $m\mu$). □—□, solution at 10 min. ($E_{\max}$ at 372 $m\mu$). x—x, control solution of nicotinic acid ($E_{\max}$ at 373 $m\mu$).

in the ammonium content of the medium during reaction with nicotinamide by glycolysing cells under these conditions (Table 6). The amount of ammonia expected to be formed under the usual conditions was too small to be accurately estimated

Table 7. *Nicotinic acid and NH₃ formation from nicotinamide in the absence of glucose*

(*Lb. arabinosus* was grown on the Pronutrin medium for 16 hr., collected by centrifugation and washed in 0.9% NaCl. Reaction was carried out in double side-arm test tubes (Hughes & Williamson, 1952). In each was 10.0 ml. 0.2M-phosphate buffer, pH 6.5; 0.002M-nicotinamide; and 5 mg. dry wt. cells. The tubes were incubated at 40° and the reaction stopped by the addition of 1 ml. 2N-H₂SO₄. Ammonia was estimated after distillation and nesslerization. Nicotinic acid was estimated by microbiological assay after destruction of the remaining nicotinamide.)

Time after mixing (min.)	NH ₃ formed (μ m-moles)	Nicotinic acid formed (μ m-moles)
0	0.91	1.2
15	2.1	2.3
45	3.8	4.3
90	7.6	8.2
180	13.2	13.8

The maximum rate of deamidation of nicotinamide (1–2 μ m-moles/mg. dry wt./hr.) was some 200 times faster than the maximum rate of cozymase synthesis and appeared to be independent of the time for which the cells had been grown (up to 4 days). Cells grown on the semi-synthetic medium with adequate nicotinic acid and cells grown on the 'pronutrin' medium also deamidated nicotinamide at the same rate as the deficient cells. The rate of deamidation is therefore independent of the

cozymase content of the cells. In cells grown for 40–50 hr. the initial rate of cozymase synthesis from nicotinamide was 1.5–4.0 $\mu\text{m-moles/mg. dry wt./hr.}$ Under these conditions all added nicotinamide (100 $\mu\text{m-moles}$) was converted to nicotinic acid in the first 5–8 min. of the reaction. This means that the bulk of the cozymase synthesized by the cells after 2 hr. (8–10 $\mu\text{m-moles/mg. dry wt.}$) could not have been directly derived from the added nicotinamide.

The effect of constituents of the growth medium on cozymase synthesis by washed suspensions

To the washed suspensions, the constituents of the growth medium were added as shown in Table 8 under the conditions described for measuring

Table 8. *The effect of the constituents of the growth medium on the stimulation of glycolysis by nicotinic acid in washed suspensions*

(Glycolysis was followed manometrically. In the main compartment of each Warburg vessel was 2.0 ml. containing 0.02M-glucose, 0.02M- NaHCO_3 , 2.3 mg. dry wt. cells, and the substance tested in the final concentration shown below. Nicotinic acid ($5 \times 10^{-5}\text{M}$ final) was added from the side arm.)

Substance added	CO_2 ($\mu\text{moles/mg. dry wt./hr.}$)	
	Without nicotinic acid	With nicotinic acid
None	4.0	12.0
Acid casein hydrolysate (1.0%)	3.9	10.7
Tryptophan 0.04%, cystine 0.04%	4.0	12.0
Vitamin mixture (Barton- Wright, 1946)	4.0	12.0
Adenine, uracil, xanthine (0.02 mg. of each)	4.0	12.0
Guanine (0.02 mg.)	4.8	12.8
Glutamine (0.01M)	3.8	11.4
Ribose (0.1M)	3.8	11.4
NH_4Cl 0.01M and salts B (Barton-Wright, 1946) 0.01 ml.	4.3	12.9

glycolysis. With the possible exception of guanine none of the constituents of the growth medium had any effect on the nicotinic stimulation of glycolysis. In some experiments guanine markedly decreased the length of the lag period. This effect could not be repeated at will in all batches of otherwise identical deficient cells and the effect was not investigated further. The rate of cozymase synthesis was not affected by addition of the vitamin mixture, tryptophan, glutamine, guanine, ribose, ammonium chloride, and casein hydrolysate. The omission of glucose completely stopped the synthesis of cozymase from nicotinic acid, nicotinamide and nicotinamide nucleotide. Neither pyruvic acid nor adenosinetriphosphate replaced glucose, but no other sources of energy were tested. In the presence of glucose the rate of cozymase synthesis from

nicotinic acid derivatives was the same when the reaction was carried out in air instead of in nitrogen, and when the bicarbonate was replaced by 0.05M-phosphate buffer, pH 7.8. Estimation of the glucose and lactic acid showed that the rates of glycolysis in air and in nitrogen with phosphate and bicarbonate media were identical. The rate of cozymase synthesis was not significantly altered by changes in the pH of the phosphate buffer from pH 5.8 to 8.2.

DISCUSSION

Catalytic effect of CoI. The catalytic effect of cozymase, expressed as a turnover number, i.e. mol. CoI/mol. acid formed/hr. (McIlwain, 1949) was 0.5 to 3.0×10^4 in washed suspensions of deficient cells. In cells supplemented with nicotinic acid, although the rate of glycolysis increased from two- to four-fold, the catalytic effect of cozymase remained the same. The value found for cozymase in washed suspensions agrees with the turnover number of nicotinic acid during growth under the conditions of the microbiological assay (McIlwain *et al.* 1949). This agreement indicates that deficiency in nicotinic acid does not greatly affect processes such as the synthesis of enzymes concerned in the glycolytic system as was found to be the case with biotin (Blanchard, Korkes, del Campillo & Ochoa, 1950). The catalytic effect of cozymase in cells grown upon medium containing an excess nicotinic acid, or in washed cells which had made cozymase from nicotinic acid was 3.0×10^3 , i.e. about ten times lower than in the deficient cells. The cells are therefore able to synthesize and bind about ten times more cozymase than is needed for glycolysis. The saturation level of cozymase (10–12 $\mu\text{m-moles/mg. dry wt.}$) appeared not to be limited by supplies of the other parts of the cozymase molecule, ribose, adenine or phosphate, nor by constituents of the growth medium other than glucose.

Route of cozymase synthesis. In most species of bacteria, nicotinamide is as readily or more readily available for growth as is nicotinic acid (Knight, 1945). These findings are generally believed to indicate that the acid is amidated as the first step in the synthesis of cozymase. This idea is also supported by the studies on the inhibitory effects of pyridinesulphonic acid and pyridinesulphonamide (McIlwain, 1940) and by the present studies on the stimulation of glycolysis by nicotinamide and nicotinic acid in which the amide appeared to be more readily available than the acid. Further studies on the metabolism of nicotinamide showed, however, that even in young cells, where the synthesis of cozymase was most rapid, nicotinamide was deamidated some 50 to 100 times faster than cozymase was synthesized. In fact, in the older cells very little cozymase could have been synthesized from the

added nicotinamide and was made instead from the nicotinic acid formed by deamidation of the amide. But this does not rule out the possibility that nicotinamide is an intermediate in the synthesis of cozymase since reactions causing the breakdown of essential metabolites are of common occurrence in bacteria. For instance the breakdown of glutamine has been shown to occur in *Clostridium welchii* (Krebs, 1948; Hughes & Williamson, 1952) at a much faster rate and by a different reaction than it is synthesized (Fry, 1949). In erythrocytes, while nicotinic acid readily gives rise to cozymase, no cozymase was formed from nicotinamide (Handler & Kohn, 1943; Hoagland & Ward, 1942). In certain strains of *Pasteurella* and *Leuconostoc* nicotinic acid but not nicotinamide supports growth (Johnson, 1945; Koser & Kasai, 1950). These results could be taken as indicating that nicotinamide is not an intermediate in cozymase synthesis. The evidence as to whether nicotinic acid is amidated to form nicotinamide or whether nicotinic acid riboside is formed first and is then amidated must therefore remain uncertain. *Lb. arabinosus* does not seem to be suitable material on which to study this question, since no intermediates in the synthesis of cozymase from nicotinic acid accumulate during synthesis and excess nicotinamide is decomposed by the organism.

SUMMARY

1. Glycolysing washed suspensions of cozymase-deficient cells of *Lactobacillus arabinosus* 17-5 synthesized cozymase from nicotinic acid, nicotinamide riboside and nicotinamide ribotide.

2. The newly formed cozymase stimulated the rate of glycolysis by the cells from two- to five-fold. Maximum stimulation occurred in the presence of 1 to 5.0×10^{-6} M-nicotinic acid.

3. As judged by their effects on glycolysis, nicotinamide, nicotinamide riboside and ribotide were in older cells more readily available for cozymase synthesis than was nicotinic acid.

4. The rate of cozymase synthesis in any batch of cells was dependent on the added nicotinic acid concentration and was maximal at and above 5×10^{-6} M-nicotinic acid.

5. The maximum rate of cozymase synthesis (18 – 24 μ m-moles/mg. dry wt./hr.) occurred in cells grown for 18 – 24 hr. and fell progressively as the cells were grown for longer periods.

6. Cozymase synthesis and the uptake of nicotinic acid ceased when the cells contained from 10 to 12 μ m-moles cozymase/mg. dry wt.

7. Cozymase uptake and synthesis and the uptake of nicotinic acid and nicotinamide did not occur in the absence of glucose. No other constituent of the growth medium had marked effect on these processes.

8. Nicotinamide was deamidated rapidly (1 – 2 μ m-moles/mg. dry wt./hr.). This reaction was independent of the presence of glucose and of the cozymase content of the cells.

The authors wish to express their gratitude to Prof. H. A. Krebs, F.R.S., for his advice and criticism. Thanks are due to Dr L. J. Haynes for gifts of synthetic nicotinamide derivatives.

REFERENCES

- Atkin, L., Schultz, S. A., Williams, W. L. & Freys, C. N. (1943). *J. Amer. chem. Soc.* **65**, 992.
- Axelrod, A. E. & Elvejehm, C. A. (1942). *J. biol. Chem.* **131**, 71.
- Barton-Wright, E. C. (1946). *Practical Methods for the Microbiological Assay of Vitamin B complex and Essential Amino-Acids*. London: Ashe Laboratories.
- Blanchard, M. L., Korkes, S., del Campillo, A. & Ochoa, S. (1950). *J. biol. Chem.* **187**, 875.
- Chaudhuri, D. K. & Kodicek, E. (1948). *Biochem. J.* **44**, 343.
- Friedman, T. E. & Graesser, J. B. (1933). *J. biol. Chem.* **100**, 291.
- Fry, B. A. (1949). *1st Int. Congr. Biochem. Abstr.* p. 465.
- Gingrich, W. & Schlenk, F. (1944). *J. Bact.* **47**, 535.
- Handler, P. & Kohn, H. I. (1943). *J. biol. Chem.* **150**, 447.
- Haynes, L. J. & Todd, H. R. (1950). *J. chem. Soc.* p. 303.
- Hoagland, C. L. & Ward, S. M. (1942). *J. biol. Chem.* **146**, 115.
- Hughes, D. E. (1949). *Biochem. J.* **45**, xxxiv.
- Hughes, D. E. & Williamson, D. H. (1952). *Biochem. J.* **51**, 45.
- Johnson, B. D. (1945). *J. biol. Chem.* **159**, 227.
- Knight, B. C. J. G. (1945). *Vitamins and Hormones*, **3**, 105.
- Kornberg, A. & Pricer, W. E. (1949). *J. biol. Chem.* **182**, 763.
- Koser, S. A. & Kasai, G. J. (1950). *J. infect. Dis.* **86**, 95.
- Krebs, H. A. (1948). *Biochem. J.* **43**, 51.
- LePage, G. A. (1949). *Biochem. Preparations*, **1**, 29.
- Levitis, N., Robinson, J., Rosen, F., Huff, J. N. & Perlzweig, W. A. (1945). *J. biol. Chem.* **167**, 169.
- Lowry, O. H. (1948). *J. biol. Chem.* **173**, 677.
- McIlwain, H. (1940). *Brit. J. exp. Path.* **21**, 136.
- McIlwain, H. (1949). *Proc. roy. Soc. B*, **136**, 12.
- McIlwain, H. & Hughes, D. E. (1948). *Biochem. J.* **43**, 60.
- McIlwain, H., Stanley, D. A. & Hughes, D. E. (1949). *Biochem. J.* **44**, 153.
- Nossal, P. M. (1951). *Biochem. J.* **49**, 407.
- Russell, J. A. (1945). *J. biol. Chem.* **156**, 457.
- Schlenk, F. (1943). *Arch. Biochem.* **3**, 93.
- Wheatley, V. R. (1947). *Laboratory J.* **8**, 191.