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Factors Influencing the Polysaccharide Content of *Escherichia coli*

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It is known that bacteria build up reserves of polysaccharides within the cell, but little work has been done on the factors which influence their accumulation. Our object was to investigate factors influencing polysaccharide storage and the role which storage plays in the metabolism of the bacterial cell. Dawson & Happold (1943) reported that D-phenylalanine caused increased storage of polysaccharide when *Escherichia coli* was grown on a glucose, but not on a mannose, medium. Gale (1947) has stated that polysaccharide formation occurs within the cell when *Esch. coli* is allowed to metabolize glucose in excess, but this material is itself metabolized as soon as the external glucose is exhausted. To investigate this, storage was examined where onset of the stationary phase was determined by both glucose and nitrogen exhaustion in the medium.

Additional interest in variations of polysaccharide content was occasioned by frequent observations that a fall in bacterial mass, as indicated by turbidity measurements, occurred in the early stationary phase. Monod (1942) also observed decrease in turbidity, attributed to a decrease in cell size, in this phase, and it was of interest to see whether this was related to polysaccharide content.

EXPERIMENTAL

The organism used throughout this work, unless otherwise stated, was no. 5928 of the National Collection of Type Cultures.

Growth cycles were followed turbidimetrically, using a Spekker photoelectric absorptiometer with neutral filters, H 508, and microcells. Since we have shown that values for the polysaccharide content are dependent on the stage reached in the stationary phase we have been careful to ensure that samples were taken at the same phase of growth. In every experiment the inoculum has been taken from a medium containing the same constituents as the medium to be used in the experiment, and from the early stationary phase, under which conditions minimum lag is obtained (Hinshelwood & Lodge, 1943).

Flasks were incubated in a thermostat at 38°. Determinations of pH were made with a Marconi type TF 717A pH meter. Dry-weight determinations were made on 1 ml. bacterial suspension placed on a watch glass and heated overnight in an electric oven (80–90°) to constant weight.

For polysaccharide determinations portions of at least 250 ml. culture were centrifuged and the supernatant reserved for pH and sugar determinations. The cells were twice suspended in distilled water and again centrifuged, taken up in 10 ml. distilled water, filtered through glass wool and used for polysaccharide and dry-weight determinations. No sugar was detected in the supernatant after final centrifugation.

Media

Medium A, a simple salt medium containing: 5.4 g. KH_2PO_4 , 1.2 g. $(\text{NH}_4)_2\text{SO}_4$, 6.6 g. glucose and 0.66 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{l.}$; pH adjusted to 7.12 with NaOH.

Medium B, a complex amino-acid medium containing: 0.1 g. L-cystine, 0.5 g. glycine, 1 g. DL-valine, 5 g. sodium glutamate, 15 g. Na lactate, 5 g. NaCl, 2.5 g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.35 g. KH_2PO_4 , 0.30 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.5 mg. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l.}$; pH adjusted to 7.5 with NaOH.

Addition of sugar was made separately. The medium and sugar were sterilized separately by boiling on successive days.

Determination of bacterial polysaccharide

The polysaccharide content of *Esch. coli* has been determined by Dawson & Happold (1943), using the Sahyun (1931) method for the determination of tissue glycogen. Since this involves treatment with 10 N-KOH in a boiling water bath the method appeared open to objection on the grounds that this would result in loss of such constituents as amino sugars. Furthermore, adsorption on charcoal is also employed and this may result in low recoveries. The determination of reducing sugar by the Hagedorn & Jensen (1923) technique, which is not specific for reducing sugar, is also open to objection. These possibilities were, therefore, investigated.

To determine reducing sugar the method of Somogyi (1937) was adopted. This reagent was found to keep well, and periodic restandardization revealed no significant change in reduction equivalent. The hydrolysis of bacterial polysaccharide was effected with H_2SO_4 and optimum conditions for this, and the effect of alkali treatment, were investigated.

Fig. 1. Polysaccharide content of *Esch. coli* determined by the Somogyi (1937) method, after varying periods of hydrolysis of washed cell suspensions with 2N- H_2SO_4 , and after 16 hr. hydrolysis of washed cell suspensions with varying concentrations of H_2SO_4 .

Effect of length of hydrolysis on polysaccharide determination.

Portions (5 ml.) of a bacterial suspension were hydrolysed in sealed ampoules with 2N- H_2SO_4 for varying periods in a boiling water bath, the contents transferred quantitatively, neutralized and made to 50 ml. Portions (5 ml.) were used for reducing sugar determinations (triplicate). The polysaccharide content of the organism is expressed in terms of glucose equivalents as a percentage of the dry bacterial weight. Fig. 1 shows that hydrolysis is complete at approximately 10 hr. Accordingly, for convenience, all hydrolyses were carried out overnight.

Effect of acid strength on polysaccharide determination.

Fig. 1 shows the variation in polysaccharide content obtained when hydrolysis of the bacterial suspension was carried out with various acid strengths. It will be seen that there is little difference over the range 1-6N, maximum effect being obtained with 2-4N-acid. Accordingly, we decided upon 2N-acid for hydrolysis in all subsequent work.

Effect of pre-treatment with alkali. The effect of treatment with 2N-NaOH prior to acid hydrolysis was investigated. Portions (5 ml.) of bacterial suspensions were made 2N with respect to NaOH, and the unsealed ampoules covered with glass bulbs and placed in the boiling water bath for given periods. They were then removed, made 2N with respect to H_2SO_4 , sealed and returned to the water bath. Treatment with 2N-NaOH for 2 hr. prior to 10 hr. hydrolysis with 2N- H_2SO_4 gave a polysaccharide content of 7.1% dry bacterial weight as compared with 15.5% with the control. Pre-treatment with NaOH therefore results in a much lower figure for polysaccharide content.

Comparison of polysaccharide determinations by the Somogyi and Hagedorn & Jensen methods. For convenience, hydrolysis in this experiment was conducted for 2 hr. only, and then the reducing sugar liberated determined by both the Somogyi (1937) and Hagedorn & Jensen (1923) methods. In three different experiments the latter method gave much higher figures, in some cases double the Somogyi value: viz. Somogyi 3.5, 2.7, 3.7 and Hagedorn & Jensen 7.3, 5.3 and 5.6% dry bacterial weight respectively. Clearly, the Hagedorn & Jensen method is determining reducing substances other than sugars. The findings that alkali causes destruction of approximately 50% bacterial polysaccharide, and that the Hagedorn & Jensen method of reducing sugar determination gives very high results with bacterial hydrolysates, was fully borne out when a direct comparison of Dawson & Happold's (1943) method was made with our own (see p. 333).

Comparison of polysaccharide determinations by the Dawson & Happold technique and by acid hydrolysis. Seeking to explain the difference in observations recorded by Dawson & Happold (1943) using the Sahyun (1931) method, and ourselves, employing acid hydrolysis, on the effect of D-phenylalanine, we undertook a direct comparison of both methods and also carried out the reducing sugar determinations by the Hagedorn & Jensen and the Somogyi techniques. Thus a comparison was afforded of both important stages in the procedure: first hydrolysis and then reducing sugar determination.

Two flasks were set up each containing 1 l. of medium B to which 1% (w/v) of glucose and DL-tryptophan was added. D-Phenylalanine (0.13 g.) was added to one of these flasks and both inoculated with two loops of bacterial suspension. Polysaccharide determinations were carried out, when the stationary phase had been reached, by both methods. The results (Table 1) confirm our previous findings, namely, that destruction of 40-50% of bacterial polysaccharide is brought about by the KOH treatment in the Sahyun method, and also that the Hagedorn & Jensen method of sugar determination gives results which are about 50% too high, presumably due to the presence of reducing substances other than sugars. Quite clearly the Sahyun method for glycogen is not suitable for total bacterial polysaccharide determinations, and neither is the Hagedorn & Jensen method suitable for the determination of reducing sugar liberated from such material. All our work therefore has, with justification, been based on acid hydrolysis of the cells followed by determination of reducing sugar by the Somogyi method.

It will be noted that, using the Sahyun and Hagedorn & Jensen methods, the polysaccharide content is slightly higher in the presence of D-phenylalanine. However, using the method of Dawson & Happold, the polysaccharide content obtained is very much greater than that recorded by them (about 5%) and this is apparently due to a strain difference. To investigate this possibility the polysaccharide content of various strains has been examined (see p. 335).

Table 1. Comparison of polysaccharide determinations by the Sahyun and acid-hydrolysis methods with reducing sugar determinations by the Hagedorn & Jensen and Somogyi techniques

Method of hydrolysis	Polysaccharide content (% dry bacterial wt.)			
	Sahyun		2 N-H ₂ SO ₄	
	Hagedorn & Jensen	Somogyi	Hagedorn & Jensen	Somogyi
Control	15.5	10.8	28.2	19.5
0.013% (w/v) D-phenylalanine	17.5	12.8	26.8	18.7

Accuracy of the method. Two sets of duplicate determinations were carried out, with different dry weights; hydrolyses and reducing sugar determinations being all carried out independently, all possible experimental variables were introduced. For two different samples of *Esch. coli* grown under different conditions the polysaccharide contents (% dry bacterial weight) were: sample 1—13.5, 13.3; sample 2—17.5, 17.4.

Final method for bacterial polysaccharide determinations. Portions (5 ml.) of bacterial suspension (washed free from sugar present in medium as previously described) were placed in ampoules, 0.3 ml. conc. H₂SO₄ added to each to make 2 N with respect to acid, and the ampoules sealed and placed in a boiling water bath overnight. They were then opened and the contents transferred quantitatively, with filtering to remove debris, to 50 ml. graduated flasks, neutralized and made to the mark with water. Portions (5 ml.) were used in triplicate for the determinations with 5 ml. Somogyi reagent. Liberated I₂ was titrated with 0.005 N-Na₂S₂O₃, which was made from 0.1 N-stock solution immediately before use.

RESULTS

The variation of polysaccharide content of Escherichia coli at various phases of the growth cycle

Variations in polysaccharide content with time. Growth was followed by turbidity measurements in a medium in which glucose was known to be in excess (simple medium A). The fluctuation of polysaccharide content with time is shown in Fig. 2. It is seen that between 20 and 38 hr. a fall in polysaccharide occurs. In another experiment the polysaccharide content during this phase of growth was followed more fully over a period of 46 hr. The polysaccharide content 1.75 hr. before onset of the stationary phase was 16.9%; 22 hr. after onset it

was 12.2% and 42 hr. after, 9.0%. A fall in polysaccharide content over long periods of the stationary phase has been confirmed in several subsequent experiments concerned with the influence of other factors.

Fig. 2. Fluctuation of polysaccharide content of *Esch. coli* with time. Growth cycle followed turbidimetrically (○—○), and polysaccharide content (△—△) determined at various stages of growth and stationary phase.

Influence of factors determining onset of the stationary phase. Using the simple medium A, glucose concentration was maintained at 20 g./l. and the concentration of (NH₄)₂SO₄ varied. A linear relationship was obtained between stationary population and (NH₄)₂SO₄ concentration up to 1.2 g./l., the highest concentration used, at which the stationary population was approximately 10⁹ bacteria/ml. Similarly, with excess (NH₄)₂SO₄ present at a concentration of 4 g./l., a linear relationship existed up to the highest concentration of glucose used (0.9 g./l.) and the stationary population was approximately 3.7 × 10⁸ bacteria/ml. These findings are in accordance with those of Dagley & Hinshelwood (1938) and Hinshelwood & Lodge (1939) for *Aerobacter aerogenes*, and indicate that over these ranges of foodstuff concentration the onset of stationary phase is determined by exhaustion of foodstuff and not by adverse conditions or toxic products. This was confirmed by following the consumption of glucose in the case of a medium for which glucose exhaustion during growth was anticipated and one for which glucose was in excess of growth requirements (Fig. 3).

In Fig. 3 the fall in turbidity in the stationary phase is clearly shown. From this information it was possible to follow the storage of polysaccharide during the growth of *Esch. coli* when, on one hand, glucose exhaustion determined onset of the stationary phase and, on the other hand, when the glucose was in excess of requirements. Results of these experiments are given in Table 2. It is evident that

the fluctuations of polysaccharide content in the stationary phase are not determined by glucose exhaustion.

Fig. 3. Relation between growth of *Esch. coli* and amount of glucose in the simple ammonium salt-glucose medium A. (a) Excess glucose present: growth (○—○) glucose content of medium during growth (△—△). (b) Limiting concentration of glucose present: growth (●—●); glucose content of medium during growth (▲—▲).

Cessation of cell division and polysaccharide storage. When the stationary phase is reached the total bacterial mass, as measured by turbidity, does not remain constant, but invariably shows a steady decline. We compared the fall in polysaccharide content during the stationary phase with this fall in bacterial mass. It is seen, however, from Table 2 that storage of polysaccharide has not in every case reached its maximum when division ceases, and from Fig. 2 it is also seen that the maximal storage does not exactly coincide with the beginning of the stationary phase. Thus decrease in bacterial mass cannot be correlated with fall in polysaccharide content.

The effect of D-phenylalanine on polysaccharide content of Escherichia coli

Results using the method of acid hydrolysis. Dawson & Happold (1943), using the Jebbs strain of *Esch. coli*, reported that 0.01% (w/v) DL-phenylalanine (but not the L-isomer) caused an increase in cell polysaccharide grown in the

Fig. 4. Polysaccharide content of *Esch. coli* grown in presence and absence of D-phenylalanine on simple ammonium salt-glucose medium A; control, ○—○; 0.013% (w/v) D-phenylalanine added before inoculation, ●—●; and 0.013% (w/v) D-phenylalanine added when stationary phase reached (7.25 hr.), △—△.

amino-acid medium B. We sought to confirm this observation using both the simple and complex media and using the present method of polysaccharide determination. Of three flasks containing 1500 ml. simple medium A, 0.013% (w/v) D-phenylalanine was added to one, and growth curves were determined. To another, 0.013% (w/v) D-phenylalanine was added when the stationary phase was reached. Samples

Table 2. *Effect of limiting concentration of glucose and ammonium sulphate on stationary population and polysaccharide content of Escherichia coli*

Glucose (g./l.)	(NH ₄) ₂ SO ₄ (g./l.)	Stationary population (10 ⁶ /ml.)	Time after reaching stationary phase (hr.)	Polysaccharide content (% dry bacterial wt.)
(a) Onset of stationary phase determined by glucose exhaustion				
1.50	5.0	725	0	9.7
			19	13.4
			24	8.8
2.25	5.0	925	0	12.3
			19	12.2
			24	10.2
(b) Onset of stationary phase determined by (NH ₄) ₂ SO ₄ exhaustion				
5.0	1.2	737	0	14.0
			19	14.7
			24	11.1
5.0	1.8	950	0	11.6
			19	16.5
			24	11.5

(350 ml.) of cultures were withdrawn at intervals; the polysaccharide content of the cells determined, and these are shown in Fig. 4. D-Phenylalanine has no effect on growth rate or polysaccharide storage in this medium. The decline in polysaccharide content previously noted is shown in each case. The experiment was repeated using an inoculum of which about twenty-five generations of the cells had been grown in the medium containing 0.013% (w/v) D-phenylalanine. Here again there was no significant difference between cultures containing D-phenylalanine and those without.

Using the amino-acid medium B, to which glucose was added to a concentration of 1% (w/v), polysaccharide contents were estimated when turbidities showed in each case that the culture had just attained its stationary phase. Besides adding D-phenylalanine to two of the four flasks, 1% (w/v) tryptophan was also added to two of the flasks since this was present in the medium used by Dawson & Happold (1943). The results obtained again show no increase in polysaccharide content due to the presence of phenylalanine, e.g. control, 22.2%; 1% (w/v) DL-tryptophan added, 21.5%; 1% (w/v) DL-tryptophan + 0.02% (w/v) D-phenylalanine, 20.3% and 0.02% D-phenylalanine, 19.5% polysaccharide dry bacterial weight.

Polysaccharide content of various strains of Escherichia coli. Five different strains of *Esch. coli* were obtained through the courtesy of Dr D. E. Dolby of the Bacteriology Department of this University. They were grown in medium B containing 1% (w/v) glucose, and their polysaccharide contents determined in the usual way. It was found that there is a quite wide variation with strain, but none is as high as the strain used in this work. The polysaccharide contents were as follows: N.C.T.C. no. 5928, 21.2%; coli I, 10%; coli B, 6.9%; coli D, 9.5%; coli 4, 13.9% and coli 7148, 12.7%. The lowest storer was the 'B' strain which was a non-motile organism. It was decided, therefore, to see whether this organism (with a polysaccharide content similar to that of the Jebbs strain used by Dawson & Happold) showed an increased storage in the presence of D-phenylalanine, both in the presence and absence of glucose.

To four flasks containing 250 ml. medium B additions were made as follows: (1) 0.33% (w/v) glucose; (2) 0.33% (w/v) glucose + 0.013% (w/v) D-phenylalanine; (3) none; (4) 0.01% (w/v) D-phenylalanine. The polysaccharide content (% dry bacterial weight) of the 'B' strain grown on these four media was found to be (1) 8.2, (2) 8.1, (3) 3.3, (4) 3.3. Hence it was concluded that D-phenylalanine has no effect on the polysaccharide storage of *Esch. coli*. It is of interest to note that with this low-storing strain, as with the highest one, the addition of glucose to the medium causes a relatively large increase in stored polysaccharide. That low storage was not due to non-utilization of glucose was demonstrated by turbidity determinations during growth. The 'B' strain grew to approximately the same turbidity as the high-storing N.C.T.C. strain no. 5928.

Effect of various sugars on polysaccharide storage in the amino-acid medium

Effect of sugar concentration. Dawson & Happold (1943) reported that mannose had little effect on the polysaccharide storage of *Esch. coli* on the amino-acid medium B using the Jebbs strain of organism.

We have repeated their experiments and, in addition, investigated the effect of the sugar concentration with both mannose and several other sugars.

To flasks containing 250 ml. amino-acid medium B (pH 7.5) increasing amounts of a sterile sugar solution were added. Each flask was inoculated with two loops of a culture, which had grown on the amino-acid medium containing the sugar in question, at a time when it had just attained its stationary phase. Growth was followed turbidimetrically and the cultures were centrifuged and washed when the stationary phase was reached. The effect of sugar concentration is given in Fig. 5. It will be seen that

Fig. 5. Relation between polysaccharide content of *Esch. coli* and concentration of sugar present in amino-acid medium B; glucose, ○—○; lactose, ●—●; mannose, □—□; and galactose, ■—■.

a certain minimum storage of polysaccharide is obtained in the absence of sugar, but on the addition of it the stored polysaccharide increases to a maximum value, above which further additions of sugar produce no increase. It is also seen that, although some increase in storage was obtained when mannose and fructose (see Fig. 6) were present, the increase was markedly less than in the case of glucose, galactose and lactose.

Effect of pH. Hinshelwood & Lodge (1939), working with *Bact. lactis aerogenes*, have shown that stationary population is determined by the pH of the medium as well as by foodstuff concentration. At adverse pH values on the acid and alkaline sides of neutrality the total growth, and hence the foodstuff consumption, is greatly reduced. Accordingly, we have investigated the effect of pH on polysaccharide storage since this appears to depend on the amount of sugar metabolized. For each sugar at a concentration of 3.3 g./l. a range of media of pH between 6 and 8.5 was inoculated as before, the cells centrifuged when the stationary phase had just been reached and their polysaccharide content determined.

The dependence of polysaccharide content on pH for various sugars is shown in Fig. 6, where the

dependence of storage on pH is seen to be greatest in the case of the sugars for which the storage is highest.

Fig. 6. Effect of pH on polysaccharide content of *Esch. coli* with various sugars at a concentration of 3.3 g./l. in amino-acid medium B; glucose, ○—○; galactose, ●—●; mannose, □—□; and fructose, ■—■.

DISCUSSION

It is sometimes assumed that the function of storage of polysaccharide by the cell is to serve as a store of carbohydrate for use when the other sugars in the medium are exhausted. Although we have found that there is a decline in storage during the stationary phase this is not directly related to the exhaustion of the normal supply of carbohydrate in the medium. Whilst it is possible that the polysaccharide may serve as a reserve source of energy, an alternative explanation for the fall in polysaccharide content may be found in the fact that increasing numbers of cells are dying in the stationary phase, and the decrease is reflected in autolytic activity. We cannot conclude from our results that the process of storage ceases exactly at the same time as cell division, whether the latter is due to exhaustion of glucose in the medium or not. On these grounds it is difficult to ascribe the fall in bacterial mass as reflected in the turbidity of the culture, which Monod (1942) first reported, and which we have repeatedly confirmed, entirely to dissimilation of stored carbohydrate.

With the present strain of *Esch. coli*, and using the method of estimation which we have developed, we have not observed any effect of small quantities of D-phenylalanine in increasing polysaccharide storage as reported by Dawson & Happold (1943). It would appear that the effect ascribed by Dawson & Happold to D-phenylalanine was, in fact, due to the method of estimation used. We conclude that the Sahyun (1931) method for glycogen determinations, coupled with reducing sugar determinations by the Hagedorn & Jensen (1923) technique, is unsuitable for bacterial polysaccharide determinations.

It is seen that although a low level of storage is obtained when the cells are provided with lactic

acid and various amino-acids a greatly increased level is obtained when sugars are provided, particularly when pH is favourable to growth and storage. On the other hand, all sugars are not as effective as glucose or galactose; lower storage is obtained with mannose (which Dawson & Happold have previously commented upon) and fructose.

SUMMARY

1. The polysaccharide content of *Escherichia coli*, grown under various conditions, has been determined by acid hydrolysis of washed cell suspensions, followed by determination of reducing sugars by the method of Somogyi (1937).

2. The polysaccharide content falls during the stationary phase, but the onset of this fall does not exactly coincide with the cessation of cell division.

3. In a glucose-ammonium salt medium this trend is obtained whether exhaustion of nitrogen or exhaustion of glucose determines the onset of the stationary phase.

4. For these reasons the fall is not attributed to the utilization of stored polysaccharide by the cells when the sugar in the medium is used up.

5. For lactose, galactose and glucose, grown at pH 7.5 in an amino-acid medium which will support some growth in the absence of a sugar, the stored polysaccharide increases markedly with sugar concentration to a maximum, beyond which further additions cause no increase. With mannose and fructose little increase is produced.

6. At a fixed sugar concentration, variation of pH affected the storage of cells grown in glucose and galactose media. In these cases there are pH optima, which are not shown with the low-storing sugars, mannose and fructose.

7. There is a wide variation of stored polysaccharide content for different strains of *Esch. coli* grown under identical conditions.

8. The Sahyun (1931) method of glycogen determination, coupled with the Hagedorn & Jensen (1923) method for reducing sugars, is not satisfactory for the estimation of total bacterial polysaccharide.

9. The increased storage of polysaccharide, reported when this method of estimation was used and the cells were grown in the presence of D-phenylalanine, is not confirmed when the method described in paragraph 1 is employed.

We wish to thank Prof. F. C. Happold, at whose suggestion the research was undertaken and from whose advice we have benefited at various stages in its progress. We are indebted to Dr H. J. Rogers who drew our attention to the advantages of the method of estimation for bacterial polysaccharide which we adopted, and indicated possible sources of error in the Sahyun (1931) method. We have also benefited from discussions with Mr E. S. Holdsworth, who is undertaking similar investigations with *Corynebacterium diphtheriae*.

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

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

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

EXPERIMENTAL

Materials

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

Dihydrocozymase. The preparation followed Schlenk (1941). Reaction with a specimen of Co I (120 mg., containing 68 mg. Co I) was complete in 30 min. and differential ethanol precipitation yielded a first fraction of 44 mg. dihydrocozymase. Titration with $K_3Fe(CN)_6$: 1.2 mg. required 0.45 ml. of 0.005 M-solution; content, 0.94 μ mol./mg. Manometric reaction with $K_3Fe(CN)_6$: 4.3 mg. gave 4.65 μ mol. CO_2 ; yield, 1.08 μ mol./mg. Optical density at 340 m μ . ($c = 0.094$ mg./ml.), 0.501; content, 0.86 μ mol./mg. The pure material as disodium salt contains 1.41 μ mol./mg.

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

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

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

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

It is fairly well established that the breakdown of cozymase in nervous tissues and in certain micro-