117. ON THE SYNTHESIS OF COCARBOXYLASE

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WEIJLARD & TAUBER [1938] have described a method for the synthesis of cocarboxylase from aneurin which is important not only because it renders this substance easily accessible, but also because of the general interest attached to the synthesis of a primary ester of pyrophosphoric acid in a pure state, a problem which has never been satisfactorily solved before.

Weijlard & Tauber only described their method briefly without studying or discussing the mechanism of the synthesis or the progress of the purification. Because a proper understanding of the reactions involved was lacking, great difficulties were encountered by the writer when he first tried to repeat Weijlard & Tauber's experiments with smaller quantities of aneurin than those used by them. The product finally isolated, although catalytically active, had a low P content; it could be shown to contain an admixture of 50–70% of aneurin monophosphate from which it could not be separated by repeated recrystallizations. Similar difficulties were apparently met with in the Oxford Laboratory (personal communication from Mr L. A. Stocken).

These failures led to a detailed investigation of the synthesis of cocarboxylase. As a result an alternative method of synthesis has been worked out which is probably capable of a wider application to the synthesis of primary esters of pyrophosphoric acid. In addition, a number of observations have been made which, it is hoped, will contribute to the understanding of the mechanism of this synthesis in particular and of the synthesis of pyrophosphoric esters in general.

After it had been found that by the method of Weijlard & Tauber a large proportion of the monophosphate was obtained; it was assumed that this was the result of the hydrolysis of a primary pyrophosphate originally formed and that the pyrophosphate present in the end product had by chance escaped this hydrolysis. Since the isolation and purification was carried out with the greatest possible speed and under the mildest possible conditions of temperature and pH, the hydrolysis could only have occurred during the actual phosphorylation. The esterification of aneurin involves the liberation of 1 mol. of water and it was thought possible that this might lead to an immediate hydrolysis of a part of the pyrophosphate. This view was confirmed by the opinion expressed by Wagner-Jauregg & Griesshaber [1937, 2] that the method of preparing organic pyrophosphoric acids by the action of P_2O_5 is liable to fail in all those cases in which water can be formed.

Consequently a method was devised where the liberation of water was entirely eliminated. This was attained by the interaction of the Br-derivative of aneurin (3-(2-methyl-4-aminopyrimidyl-5-methyl)-4-methyl-5-bromoethyl-

The aqueous extract was now freed from Ag^+ and inorganic pyrophosphate was removed with $\text{Ba}(\text{OH})_2$ until no further precipitate was formed. If the solution was now analysed it was repeatedly found that it contained acid-hydrolysable P and total organic P in a ratio of exactly 1 : 4. This result was obtained with such regularity that it could not be a coincidence, but must have been due to the mechanism of the reaction. It was concluded that in the reaction between bromo-aneurin and silver pyrophosphate for each molecule of primary pyrophosphoric ester 1 mol. of the secondary symmetrical pyrophosphoric ester was formed.

If such a mechanism was assumed the amount of acid-hydrolysable P and total organic P accounted for almost 100 % phosphorylation of bromo-aneurin. Estimation of the catalytic activity showed that the whole of the primary pyrophosphate present was cocarboxylase.

Naturally these findings were correlated with the peculiar composition of the acid silver pyrophosphate $\text{Ag}_3\text{H}_5\text{P}_4\text{O}_{14}$ which can be regarded as a mixture of equal amounts of the primary and secondary pyrophosphates. It was thought that it might be possible to increase the yield of the primary ester if the presence of the secondary salt could be eliminated. The only monobasic salt of pyrophosphoric acid known is the monosodium pyrophosphate, $\text{NaH}_3\text{P}_2\text{O}_7$, described by Giran [1903]. Since Giran's method is cumbersome and requires special equipment, a similar procedure was adopted as in the case of the acid silver pyrophosphate. The product isolated had indeed a ratio of Na : P of 1 : 2, as required for $\text{NaH}_3\text{P}_2\text{O}_7$.

This seemed to indicate that in a solution of sodium pyrophosphate in excess pyrophosphoric acid only the monobasic salt is present. When bromo-aneurin was heated with such a solution, the degree of phosphorylation was very small and amounted only to about 3 % although the time of heating was extended to 2 hr. and the temperature raised to 120° . Again the ratio of acid-hydrolysable P to total organic P was 1 : 4. It is obvious therefore that the secondary symmetrical pyrophosphoric ester was formed even under conditions where the absence of a secondary salt of pyrophosphoric acid can be assumed.

At this stage it was decided to reinvestigate the method of Weijlard & Tauber from the same points of view. In the one experiment performed phosphorylation amounted to 100 % of the aneurin used and the ratio of acid-hydrolysable to total organic P was exactly 1 : 4. The enzymically estimated cocarboxylase however accounted for only 50 % of the acid-hydrolysable P. The higher temperature employed in this method (15 min. at 155°) had apparently brought about a considerable destruction of the molecule.

In this connexion it is interesting to note that ethyl metaphosphate combines with water in such a way that from 4 mol. 1 mol. of symmetrical and 1 mol. of asymmetrical diethylpyrophosphate are formed [Langheld *et al.* 1912]. Accordingly, Wagner-Jauregg & Griesshaber [1937, 1] only found 1/5-1/4 of the-total P of ethyl metaphosphate in an easily hydrolysable form. The same authors [1937, 2], when attempting to prepare the primary pyrophosphoric ester of lactic acid by the action of pyrophosphoryl chloride, found that not only the desired primary asymmetrical ester, but also the secondary symmetrical ester was formed.

From our own observations and the data in the literature it is perhaps permissible to conclude for non-enzymic reactions that, as a general rule, wherever a primary asymmetrical ester of pyrophosphoric acid is formed, a secondary symmetrical ester is formed simultaneously. The reasons are probably to be sought in the configuration of the pyrophosphoric acid molecule.

It remained to work out a rational method for the separation of the two esters. Weijlard & Taubér apparently succeeded in preparing the pure pyrophosphate, but it is not clear from their paper by which step of their purification procedure a separation is achieved. It must be attributed to this lack of knowledge that our earlier attempts to adapt their method to a smaller scale had failed.

An analysis of the various steps of the purification showed that neither the precipitation of the Ag salts nor the recrystallization from aqueous acetone or alcohol are in themselves capable of effecting a separation. The separation is based on the fact that the phosphotungstate of aneurin monophosphate has a much higher solubility in aqueous acetone than the phosphotungstate of co-carboxylase. This difference of solubilities is much less marked in the absence of phosphotungstic acid. The increase of the solubility of aneurin monophosphate in aqueous acetone brought about by phosphotungstic acid can easily be demonstrated by the following simple experiment:

100 mg. aneurin monophosphate were dissolved in 10 ml. *N*/10 HCl. On the addition of 100 ml. acetone a milky turbidity appeared which rapidly increased in density. When now 2 g. phosphotungstic acid, dissolved in a little acetone, were added the precipitate immediately dissolved again.

If the mixed phosphotungstates are dissolved in a small amount of aqueous acetone (2 vol. acetone + 1 vol. *N*/10 HCl) the addition of 10 times as much acetone as the *N*/10 HCl which is contained in the amount of aqueous acetone used will precipitate only the pyrophosphate. The monophosphate is precipitated only after the addition of a further 10 vol. acetone. A similar behaviour is shown by the reineckates.

Weijlard & Tauber use a rather tedious procedure of successive extractions with acetone and *N*/10 HCl for decomposing the phosphotungstate precipitate. In their method of decomposing the phosphotungstates there is a factor of uncertainty, i.e. the amount of water retained by the precipitate, which will depend on the bulk and depth of the precipitate and also on the efficiency of centrifuging. Our failure to repeat Weijlard & Tauber's experiments may perhaps be explained on the assumption that when the method was adapted to a smaller scale, the ensuing phosphotungstate precipitate was less bulky, shallower, and more closely packed down on centrifuging. The amount of water it contained was insufficient to retain in solution the phosphotungstate of the monophosphate after the addition of acetone, whereas this must have been the case in the experiments of Weijlard & Tauber. This element of uncertainty has been largely eliminated in the modification here proposed, where the entire precipitate is dissolved in a small amount of aqueous acetone before fractionally precipitating with acetone.

In the method of purification finally adopted, the phosphotungstate precipitation is carried out twice. The silver precipitation is retained as a precaution though it could probably be omitted. After recrystallization from aqueous acetone and alcohol a product was obtained which crystallized readily and gave the correct analytical figures. The yield was 50–60% of the cocarboxylase estimated in the original solution of the phosphorylation products. The yield from bromo-aneurin is about 17% and the overall yield from aneurin about 10%. This is about the same yield as in Weijlard & Tauber's method (8–9%). Whereas in this method the losses chiefly occur during the actual phosphorylation owing to thermal destruction, this is counterbalanced in the method here described by the losses occurring during the preparation of the bromo-aneurin. If it were possible to prepare this substance in a better yield either from aneurin itself or by a method similar to one of those used for the synthesis of aneurin,

it might be possible to improve the overall yield considerably. Until then most workers will prefer to use the method of Weijlard & Tauber because of its greater simplicity, with certain modifications in the procedure of purification as described in the experimental part.

The P figures tend to be a little too high in the product prepared by the method of Weijlard & Tauber, probably owing to a slight contamination with thiazole pyrophosphate. Through the courtesy of Dr Weijlard I have been supplied with a sample of Merck's cocarboxylase and I have thus been able to compare this preparation with those I had prepared myself. Table 1 shows the

Table 1. *Comparison of P contents and catalytic activities of various cocarboxylase preparations*

Substance	A	B	C	D	Calculated for cocarboxylase
Total organic P (%)	13.72	13.70	13.18	12.92	12.93
Acid-hydrolysable P (%)	6.64	6.26	6.38	6.50	6.46
μ l. CO ₂ developed after 15 min. with					
1 μ g.	56.5	64	—	63.5	—
3 μ g.	185.5	178	—	184	—
10 μ g.	420	466	—	420	—

result of this comparison with regard to P analyses and catalytic activity. Substance A is Merck's cocarboxylase, substance B is a preparation made by the method of Weijlard & Tauber using the modified purification procedure, substance C was prepared from bromo-aneurin and silver pyrophosphate and analysed at the same stage of purification as substance B, substance D was prepared from substance C by two further recrystallizations from aqueous alcohol. The acid-hydrolysable P was estimated after 20 min. heating with *N* acid in a boiling water bath. The catalytic activity was estimated with the soluble enzyme preparation recently described [Weil-Malherbe, 1939]. Each manometer cup contained 1 mg. protein, *M*/30 phosphate buffer, *pH* 6.2, *M*/30 pyruvate, 0.1 mg. Mg⁺⁺, 0.1 mg. Mn⁺⁺ in a total volume of 3 ml..

It appears that the catalytic activities of the three preparations are practically identical. Mr L. A. Stocken kindly carried out a comparison of the catalytic activity of substance D with that of pure natural cocarboxylase isolated from yeast by Prof. Lohmann and he reported, rather surprisingly, that the synthetic cocarboxylase had only 60 % of the activity of natural cocarboxylase. There is no doubt that substance D is a reasonably pure product as shown not only by the P analyses, but also by the figures for C, H and N (see Experimental Part). The low catalytic activity might suggest that the synthetic product exists in two isomeric modifications though it is hard to see what kind of isomerism there could be.

From the mother liquors the second phosphoric ester was isolated. It had a P content of 7.5–7.6 % and contained no acid-hydrolysable P. Although it is very probable that the substance formed during the phosphorylation is the secondary symmetrical pyrophosphate, it might have been hydrolysed during the isolation. That such had been the case could be shown by the titration of the product, before and after heating with *N* acid for 2 hr. in a boiling water bath. This treatment would have hydrolysed any persisting pyrophosphate group with the result that a new acidic group would have been formed for each aneurin radical. Actually, exactly two acidic groups were titrated against phenolphthalein both before and after heating with acid. This is the result to be expected for aneurin monophosphate, whereas only one acidic group per aneurin radical would have been titrated in the case of the secondary symmetrical

pyrophosphate. The latter therefore seems to be an unstable compound which is hydrolysed even under the mild conditions prevailing during the isolation.

Reference has been made to the fact that of the cocarboxylase formed only 50–60 % could be isolated in a pure state. Much time and labour has been spent in attempts to improve this yield, but without success. Some observations have been made, however, which it may be worth while to put on record. Our experiments were done along two lines: first the precipitation of the two esters by the salts of various heavy metals was studied in the hope of finding a more efficient separation. According to Lohmann & Schuster [1937] the only insoluble salt of cocarboxylase is the Ag salt. This is not correct since a number of other salts of heavy metals were found to precipitate cocarboxylase completely from 0.0025 *M* solution at pH 7. The following ions had this effect: Hg^{++} (mercuric acetate), Bi^{+++} (bismuth nitrate), Cu^+ (cuprous chloride), Pb^{++} (lead acetate), UO_2^{++} (uranyl acetate), V^{++} (vanadium chloride). The precipitation became complete only at a pH of 7 or slightly above. But in all cases the monophosphate was precipitated as well, at least partly. In some experiments a certain fractionation could be obtained, especially with the Hg^{++} and Bi^{+++} salts. But the result was not easily reproducible, probably owing to small differences of pH.

Secondly, attempts were made to use organic bases, such as strychnine, brucine and acridine, for the preparation of salts of cocarboxylase which it was hoped might have a greater tendency to crystallization. Acridine has been recommended by Wagner-Jauregg [1936] as specially suitable for the preparation of crystallized derivatives of phosphoric esters. In the case of the aneurin phosphates it was however not possible to prepare these salts. No crystallization occurred from the aqueous solutions and from dilute alcohol the unchanged aneurin phosphate crystallized out. Equally unsuccessful was the use of acridine methochloride.

EXPERIMENTAL PART

Phosphorus estimations. Phosphorus was estimated colorimetrically by the method of Berenblum & Chain [1938]. If ammonium molybdate is added to a solution containing aneurin phosphates a voluminous precipitate is formed. If the blue colour is developed in the presence of the precipitate it is very firmly adsorbed on its surface. With the method of Berenblum & Chain this complication does not arise since phosphomolybdic acid is extracted before the reducing agent is added. This method was found to be very reliable and satisfactory in other respects too. Its great sensitivity enabled the P-estimation to be carried out with quantities of 0.5 mg. cocarboxylase and 0.8–1.0 mg. monophosphate.

Total organic P was estimated after digestion with 60 % perchloric acid, pyro-P after heating with *N* H_2SO_4 in a boiling water bath for 20 min.

Pyrophosphoric acid. The commercial product (B.D.H.) was found still to contain a large proportion of orthophosphoric acid. It was rapidly heated to 300° and kept there for a few minutes. After cooling, a neutralized solution gave a pure white precipitate with AgNO_3 ; an acid solution did not coagulate a solution of egg albumin. P found: 35.05 %, calc. for $\text{H}_4\text{P}_2\text{O}_7$: 34.84 %.

Preparation of $\text{Ag}_2\text{H}_2\text{P}_2\text{O}_7$. 17 g. AgNO_3 were dissolved in 100 ml. water and 100 ml. *N* NaOH were added. The precipitate was filtered and well washed. 80 g. of pyrophosphoric acid were dissolved in 400 ml. water at 0°. The silver oxide precipitate was stirred into this solution where it dissolved almost completely. After filtering, 3 vol. alcohol were added. The precipitate was collected

on a Büchner funnel, washed with alcohol, acetone and ether, and dried. Analysis showed that it consisted of a mixture of 23% $\text{Ag}_4\text{P}_2\text{O}_7$ and 77% $\text{Ag}_2\text{H}_2\text{P}_2\text{O}_7$. Yield: 11 g. Found: Ag 60.2%, P 14.1%. Calc. for mixture: Ag 60.1%, P 14.1%.

Preparation of an acid silver pyrophosphate of the approximate formula $\text{Ag}_3\text{H}_5\text{P}_4\text{O}_{14}$. 10 g. of silver pyrophosphate ($\text{Ag}_4\text{P}_2\text{O}_7$; prepared by precipitation of $\text{Na}_4\text{P}_2\text{O}_7$ with AgNO_3) were mixed with 60 g. pyrophosphoric acid and heated in an oil bath of 150° for 1 hr. After cooling the mass was dissolved in about 200 ml. acetone. An insoluble residue was filtered off. The acetone extract was poured into 2000 ml. acetone, the white precipitate was allowed to settle and was finally centrifuged, washed with acetone and ether and dried. Found: Ag 47.98%, P 18.12%. Calc. for $\text{Ag}_3\text{H}_5\text{P}_4\text{O}_{14}$: Ag 47.90%, P 18.35%.

Preparation of monosodium pyrophosphate. 2 g. $\text{Na}_4\text{P}_2\text{O}_7$, anhydrous, were mixed with 8 ml. pyrophosphoric acid and gently heated in a test tube over a Bunsen flame until dissolved. After cooling, the mass was dissolved in 600 ml. acetone and 200 ml. ether were added. An oily precipitate formed which was allowed to settle and was hardened by treatment with fresh acetone. The precipitate was centrifuged and dried *in vacuo* on a porous plate. It had a glassy appearance and was very hygroscopic. Found: Na (by uranyl zinc precipitation): 10.11%, P 27.28%. Na : P = 1.0 : 2.0. Calc. for $\text{NaH}_3\text{P}_2\text{O}_7$: Na 11.49%, P 31.01%.

Preparation of bromo-aneurin. 1 g. aneurin hydrochloride was dissolved in 60 ml. anhydrous acetic acid at about 80° . The solution was saturated with dry gaseous HBr with careful exclusion of moisture. The temperature was first kept at about 50° to prevent the aneurin from crystallizing out; it was gradually lowered to 0° and the saturation was completed at this temperature. Sometimes a turbidity appeared owing to the precipitation of aneurin hydrobromide, but this is of no importance. The solution was now heated in sealed tubes for 3 hr. at 130° . After cooling, the combined liquids were precipitated with 300 ml. ether; the precipitate was centrifuged, washed with ether and dried *in vacuo* over moist KOH until all HBr had been absorbed. It was now dissolved in 20–25 ml. of hot methyl alcohol. On cooling in ice the solution usually remained clear; if any unchanged aneurin was present, it crystallized out at this stage. The solution was warmed again and 5 ml. ether were added. On cooling, the bulk of the bromo-aneurin crystallized out in long felted needles. The substance is sufficiently pure for the preparation of cocarboxylase. For analysis it was twice more crystallized from methanol-ether. Yield: mean of 7 preparations: 754 mg. Highest yield: 1.00 g., lowest yield: 565 mg. M.P. 234° (decomposition). Calc. for $\text{C}_{12}\text{H}_{17}\text{N}_4\text{SBr}_3$: ionizable Br 32.69%, total Br 49.03%, C 29.45%, H 3.51%, N 11.46%. Found: ionizable Br 32.60%; 32.78%. Total Br (method of Robertson [1915]) 49.13%. Analysis of C, H and N (Weiler): C 29.13%, H 3.71%, N 11.0%.

Preparation of cocarboxylase from bromo-aneurin. 1.2 g. $\text{Ag}_2\text{H}_2\text{P}_2\text{O}_7$ were mixed with 5 ml. pyrophosphoric acid and heated in an oil bath at 150° until dissolved. The mixture was allowed to cool to about 80° . 600 mg. bromo-aneurin were now added and heated in an oil bath at 100° for 40 min. with constant stirring. After cooling, the mass was ground up with 90 ml. acetone and centrifuged. The precipitate was washed with 2 more lots of 90 ml. acetone. It was now treated with 50 ml. ice-cold water, the insoluble residue was centrifuged and washed once with 20 ml. water and once with 10 ml. $N/10$ HNO_3 . The combined extracts were brought to pH about 6 with cold saturated $\text{Ba}(\text{OH})_2$ (about 35 ml.) and Ag^+ was removed with H_2S . The precipitate was centrifuged

off and well washed with water 3 times. The solution was concentrated *in vacuo* at 30° to about 60 ml. After cooling in ice, cold saturated $\text{Ba}(\text{OH})_2$ (about 10 ml.) was added until the reaction was alkaline to thymolphthalein. The precipitate was quickly centrifuged off and the supernatant fluid was tested for the appearance of a further precipitate on addition of $\text{Ba}(\text{OH})_2$. If it remained clear it was immediately neutralized with $N \text{ H}_2\text{SO}_4$ (1.5–2 ml.) and again centrifuged. Both precipitates were well washed.

At this stage the solution was usually made up to 100 ml. and aliquots were analysed for total organic and acid-hydrolysable P and for catalytic activity. Also the insoluble residue of AgBr was reprecipitated and weighed. The following is the result of a typical experiment: AgBr after dissolving in conc. NH_3 and reprecipitation with HNO_3 : 688.4 mg. Calc. for 600 mg. bromo-aneurin: 690 mg. Found: total organic P 47.5 mg., acid-hydrolysable P 11.8 mg. Acid-hydrolysable P: total P = 1:4.03. 11.8 mg. acid-hydrolysable P indicate 183 mg. cocarboxylase which account for 187 mg. bromo-aneurin. 47.5 mg. $- 2 \times 11.8 \text{ mg.} = 23.9 \text{ mg.}$ P indicate 321 mg. monophosphate which account for 376 mg. bromo-aneurin. Amount of bromo-aneurin phosphorylated: 563 mg. = 94 %. Amount of cocarboxylase present calculated from the assay of catalytic activity by comparison with a pure sample of synthetic cocarboxylase: between 180 and 190 mg.

The isolation was continued as follows: the solution which had been made up to 100 ml. was acidified by the addition of 8 ml. $10N \text{ H}_2\text{SO}_4$. After removal of the precipitated BaSO_4 the solution was precipitated with 10 ml. 25 % phosphotungstic acid. The precipitate was centrifuged and washed with $0.8N \text{ HCl}$. It was now dissolved in 40 ml. of a mixture of 1 part $N/10 \text{ HCl}$ and 2 parts acetone, an insoluble residue was centrifuged off and 130 ml. acetone were added. After standing overnight the supernatant solution was set aside for the preparation of the monophosphate and the gummy precipitate was washed with acetone, dissolved in 2 ml. $N/10 \text{ HCl}$ and precipitated with a mixture of 4 ml. alcohol and 16 ml. acetone. The following day the precipitate was dissolved in 30 ml. water and 5 ml. $N \text{ AgNO}_3$ were added. The precipitate of AgCl was removed and washed with $N/100 \text{ HNO}_3$. The solution was adjusted to pH 7 with $N/10 \text{ NH}_3$. The precipitate of the silver salt was decomposed with H_2S and the solution freed from H_2S *in vacuo*. $5N \text{ HCl}$ was added to give a concentration of $0.8N$. The phosphotungstic acid precipitation was repeated with 10 ml. of a 25 % solution and the precipitate was dissolved in 9 ml. of the HCl -acetone mixture. The clear solution was precipitated by the addition of 30 ml. acetone. The crystalline precipitate was once more recrystallized from alcohol-acetone, as above, and finally dissolved in 1 ml. $N/10 \text{ HCl}$ and precipitated with 4 ml. alcohol. Yield: 107 mg. After two more recrystallizations from dilute alcohol the following analyses were obtained: Total organic P: 12.92 %; acid-hydrolysable P 6.50 %. Analyses of C, H and N (Weiler): C 30.42 %, H 4.52 %, N 11.5 %. Calc. for cocarboxylase: C 30.08, H 4.42, N 11.71, total P 12.93, acid-hydrolysable P 6.45 %.

From the mother liquors of the first acetone precipitation the monophosphate was precipitated in fine needles by the addition of 150 ml. acetone. The precipitate was repeatedly recrystallized by dissolving in a few ml. of $N/10 \text{ HCl}$ and adding acetone, acetone-alcohol and finally alcohol. Yield: 180 mg. m.p. 199°. Total P 7.55 % (calc. 7.45 %). No-acid hydrolysable P.

Preparation of cocarboxylase and aneurin monophosphate by the method of Weijlard & Tauber. 1 g. aneurin was used as the starting material. The procedure of Weijlard & Tauber was strictly adhered to up to the $\text{Ba}(\text{OH})_2$ pre-

cipitation and the subsequent removal of Ba^{++} with H_2SO_4 . The solution was then made up to 100 ml. and analysed. It contained 120.0 mg. of total P and 29.9 mg. of acid-hydrolysable P. Acid hydrolysable P : total P = 1 : 4.0. From these figures the formation of

460 mg. cocarboxylase, accounting for	349 mg. aneurin
and of 807 mg. monophosphate, accounting for	688 mg. aneurin
	1037 mg. aneurin

was calculated, i.e. a phosphorylation of 100 %. However, the enzymic assay of the catalytic activity revealed the presence of only 220–230 mg. cocarboxylase, i.e. about half of what was to be expected from the determination of the acid-hydrolysable P.

The isolation of the two esters proceeded on the lines described in the preceding section. Cocarboxylase: 118 mg. Total P 13.70 %, acid-hydrolysable P 6.26 %. Monophosphate: 260 mg. Total P 8.0 %, acid-hydrolysable P 0.

Attempt to prepare aneurin phosphates from bromo-aneurin and monosodium pyrophosphate. 500 mg. $\text{Na}_4\text{P}_2\text{O}_7$ (anhydrous) were dissolved in 2 ml. pyrophosphoric acid by gentle heating and 250 mg. bromo-aneurin were added. The mixture was heated in an oil bath of 120° for 2 hr. and mechanically stirred. Found in the solution after removal of inorganic P.

Acid-hydrolysable P 0.15 mg. ~ 2.32 mg. cocarboxylase ~ 2.37 mg. bromo-aneurin	
Total P 0.60 mg. ~ 4.03 mg. monophosphate	~ 4.72 mg. bromo-aneurin
	7.09 mg. bromo-aneurin

Acid-hydrolysable P : total P = 1 : 4.0. 2.84 % phosphorylation.

Titration of the second phosphoric ester of aneurin before and after heating with acid. Titrations were carried out with exclusion of atmospheric CO_2 with CO_2 free, 0.0196 *N* NaOH against phenolphthalein. 36.75 mg. aneurin hydrochloride ~ 5.28 ml. NaOH (1.00 mol.). 32.50 mg. of the product isolated from the mother liquors of the cocarboxylase fraction (7.55 % P) ~ 8.00 ml. NaOH (2.01 mol.). 37.44 mg. isolated from a solution of the above substance which had been heated with *N* H_2SO_4 for 2 hr. ~ 9.20 ml. NaOH (2.01 mol.).

Precipitation of aneurin phosphates by salts of heavy metals. (1) With mercuric acetate: 5 ml. 1.3 % mercuric acetate solution were added to 10 ml. of a solution of aneurin phosphates containing 0.75 mg. of acid-hydrolysable P and 2.94 mg. of total P. NaOH was added to pH 7. Supernatant contained 0.024 mg. of acid-hydrolysable P and 0.815 mg. of total P. Precipitate contained 0.73 mg. of acid-hydrolysable P and 2.10 mg. of total P.

(2) With uranyl acetate: 2 ml. 10 % uranyl acetate solution were added to 10 ml. of the same solution of aneurin phosphates. Neutralized with NaOH. Supernatant: acid-hydrolysable P 0.02 mg. Total P 0.068 mg. Precipitate: acid-hydrolysable P 0.73 mg. Total P 2.83 mg. Similar results were obtained with bismuth nitrate, cuprous chloride, lead acetate and vanadium chloride.

APPENDIX

The reaction of aneurin and aneurin phosphates with nitrous acid

Most authors agree that aneurin reacts only very slowly, if at all, with nitrous acid. According to Williams & Spies [1938] the reaction takes place only in the presence of mineral acid with heat.

I have studied the reaction by the method of Warburg *et al.* [1935]. The manometer vessels described by Dickens & Šimer [1930] for the measurement of the R.Q. in phosphate medium were found very suitable. 0.1 ml. of the solution

containing the amino compound was accurately delivered into the circular groove together with 0.1 ml. glacial acetic acid. The side bulb contained 0.4 ml. 35 % barium nitrite solution and the central compartment 2 ml. alkaline KMnO_4 . The gas space was filled with oxygen-free N_2 . The reaction was carried out at 20° . After a short time for temperature equilibration the taps were closed, a reading was taken and the barium nitrite solution was tipped into the circular groove. The manometers were left without shaking for the desired time. At the end of the reaction the alkaline KMnO_4 was mixed with the other solutions.

Table 2. *Reactions of aneurin, aneurin monophosphate and aneurin pyrophosphate with nitrous acid*

Addition	Time allowed for reaction hr.	N_2 evolved $\mu\text{l.}$	% of theory
4.33 mg. aneurin	3	184	68
3.61 mg. cocarboxylase	3	15	8.9
3.16 mg. aneurin	5	164	82.5
2.83 mg. aneurin	6	139	78
3.68 mg. aneurin monophosphate	6	28.5	14.4
2.90 mg. cocarboxylase	6	39	28.8

Table 2 shows that aneurin does react with nitrous acid though very slowly. It has not been possible to obtain a theoretical yield of N_2 . But it is noteworthy that the amino group is still less reactive in the phosphate esters.

A mechanism of enzymic decarboxylation has recently been proposed [Weil-Malherbe, 1940] in which the first step is the formation of a Schiff base as originally suggested by Langenbeck [1933]. Stern & Melnick [1939] have independently discussed a similar scheme, but they seem to think that the low reactivity of the NH_2 group makes it improbable that such a reaction occurs. But obviously the properties of the NH_2 group may be profoundly altered in combination with the enzyme.

SUMMARY

1. A method is described for the synthesis of cocarboxylase by the interaction of "bromo-aneurin" and silver pyrophosphate.

2. Although no water is liberated during the reaction, it is found that the ratio of acid-hydrolysable P : total P = 1 : 4. This is interpreted as meaning that for each molecule of the primary pyrophosphoric ester (cocarboxylase) another molecule of the secondary symmetrical pyrophosphoric ester is formed. The same ratio of acid-hydrolysable P : total P is found if the phosphorylation of aneurin is carried out by the method of Weijlard & Tauber, or by the interaction of "bromo-aneurin" and monosodium pyrophosphate.

3. The separation of the primary and secondary pyrophosphoric esters is possible owing to the greater solubility of the phosphotungstate (or the reineckate) of the secondary ester in aqueous acetone. A modified procedure of purification is described.

4. The secondary pyrophosphoric ester seems to be a very unstable and easily hydrolysable compound, since the substance isolated is aneurin monophosphate, as shown by titration.

5. The catalytic activities of various samples of crystalline cocarboxylase prepared either by the new method or by the method of Weijlard & Tauber are identical, but are only 60 % of the activity of natural cocarboxylase.

6. Aneurin phosphates are precipitated in neutral solution by the ions of several heavy metals.

7. The reactions of aneurin and aneurin phosphates with nitrous acid were investigated. After 5 hr. about 80 % of the theoretical amount of N_2 was evolved from aneurin, but much less from the phosphoric esters.

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