The Thermal Destruction of Vitamin B₁

3. THE INFLUENCE OF COPPER ON THE RATE OF DESTRUCTION OF ANEURIN IN BUFFER SOLUTIONS AT 100°

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The first recorded statement concerning the influence of copper on the rate of destruction of aneurin is that of Booth (1943), who claims that aneurin is destroyed more rapidly in the presence of Cu than in its absence. Work in these laboratories on the behaviour of aneurin during the manufacture of yeast extract which contained significant amounts of Cu suggested that, perhaps, this metal was not such an accelerant of aneurin destruction as might be expected from Booth's statement. It was decided, therefore, to use the methods employed in earlier vitamin B₁ destruction studies (Farrer, 1945 *a, b*) for a more detailed investigation of the influence of Cu on the rate of destruction of aneurin.

Booth reported that aneurin in phosphate-phthalate buffer at pH 5.4 was destroyed more rapidly when 2 p.p.m. of Cu were added to the solution in the form of CuSO₄, but this is the only experiment recorded in his paper. The work herein describes the behaviour of aneurin in various buffer solutions at pH values between 3 and 7 in the presence of 2 and 20 p.p.m. of Cu, and, as in the earlier papers, the determination of the unimolecular reaction constant, *k*, in each case has permitted a quantitative comparison of the results. It has been found that the effect of Cu is variable.

EXPERIMENTAL

Reagents

Buffer salts. Because of the influence of very small amounts of heavy metals on aneurin destruction (q.v.) and the difficulty in general of obtaining satisfactory reagents for this type of work by recrystallization, the following solutions were made from A.R. reagents and distilled water and purified by the CaCO₃ adsorption method described by Stout & Arnon (1939): m-Na₂HPO₄, 0.5M-sodium potassium tartrate, 0.5M-glycine, potassium hydrogen phthalate-K₂HPO₄ (0.1M with respect to each salt). The purified solutions were filtered, and a measured volume of the filtrate was just acidified with the 6N-HCl (q.v.) and boiled for 15 min. to expel CO₂. When cool the solution was adjusted to the original volume with twice distilled water. The solutions were tested for heavy metals by means of the dithizone method and showed contamination of the order of considerably less than 0.1 p.p.m. in the concentrated solutions. This was considered to be satisfactory especially

as the reagents were diluted at least tenfold before use in experiments upon aneurin destruction.

Citric acid. An attempt to purify sodium citrate in the same way was not entirely successful. A solution (0.5M) was prepared from some citric acid which had been thrice recrystallized. Buffer solutions with Na₂HPO₄ were tested for heavy metals by evaporating a large sample (70 ml.) and ashing before proceeding with the dithizone test. The result obtained was completely negative and consequently this citric acid (0.5M solution) was used with confidence.

6N-Hydrochloric acid. HCl (A.R.) was twice distilled from all-glass apparatus.

Twice distilled water. Doubly distilled water was prepared in all-glass apparatus with a 'strip action' still. This water was used in the preparation of all the solutions.

Copper solution. Copper sulphate (0.795 g. of A.R. CuSO₄·5H₂O) was dissolved in distilled water (250 ml.). When 5 ml. of this solution were diluted to 200 ml., the resulting Cu concentration was 20 p.p.m. Concentrations of 2 p.p.m. were obtained by appropriate dilution of the CuSO₄ stock solution.

Other metal solutions. Solutions of FeNH₄(SO₄)₂·12H₂O, ZnSO₄·7H₂O and NiSO₄(NH₄)₂SO₄·6H₂O were made up with distilled water so that 5 ml. contained 200 mg. of each metal (the Fe solution was slightly acidified with 6N-HCl to remove opalescence). Hence when 5 ml. were diluted to 200 ml. the resulting solution contained 1 p.p.m. of metal.

Aneurin stock solution. Pure aneurin hydrochloride (10 mg.) was dissolved in twice distilled water, acidified with 2 or 3 drops of 6N-HCl, and the solution diluted to 250 ml.

Method

The Jansen thiochrome method was used and the apparatus and procedure were the same as previously described (Farrer, 1941, 1945*a*).

Procedure

In the case of the phosphate buffer, 20 ml. of the purified, acidified solution and 4 ml. of the aneurin stock solution were pipetted into a 200 ml. volumetric flask. In appropriate cases 5 ml. of Cu solution of suitable concentration were also added. (In the confirmatory experiments 5 ml. of either Fe, Zn or Ni solutions were substituted for the Cu solution.) After dilution to about 170 ml. with twice distilled water, the pH was adjusted to the desired value by the addition of the 6N-HCl, and the solution was made up to volume. The concentration of the final solution was thus 0.1M with respect to phosphate. The final pH was checked; pH determinations were performed with the glass electrode.

The other buffer solutions were prepared in a similar manner. 40 ml. of mixed phosphate-phthalate solution were used to give a final concentration of 0.02M with respect to each salt, and 20 ml. of the Na₂HPO₄ formed the basis for the remaining three solutions which, by adding 8 ml. of tartrate, citrate or glycine solution in addition to the phosphate, were thus 0.1M with respect to phosphate and 0.02M with respect to the other buffer constituents when diluted to the final volume.

The prepared solutions were then boiled in a flask fitted to a reflux condenser by means of a ground-glass joint. At regular intervals portions (about 20 ml.) of the boiling solution were pipetted into 50 ml. conical flasks and cooled rapidly under the tap. Samples (5 ml.) were then taken for analysis by the thiochrome method. At the conclusion of the experiment the pH was again checked. Both glass and quinhydrone electrodes were used in this work.

Fig. 1. pH/log k curves for aneurin destruction in phosphate solutions with and without the addition of Cu (k =velocity coefficient for the thermal destruction of aneurin).

RESULTS

As in the earlier work, the galvanometer readings are equivalent to $(a-x)$ in the monomolecular reaction equation:

$$k = \frac{2.303}{t} \log_{10} \frac{a}{a-x}$$

and $\log_{10} (a-x)$ was plotted against time. From the resulting straight lines, k has been calculated for each experiment and graphs have been drawn of $\log k$ against pH (see Figs. 1-8).

In the phosphate (Fig. 1) and the phosphate-phthalate (Fig. 2) buffer solutions Cu at a concentration of 2 p.p.m. or 20 p.p.m. accelerates aneurin destruction at all acidities. It should be stated that the effective concentration of Cu in the solutions to

which 20 p.p.m. of Cu were added was a good deal less than this figure as, on boiling, precipitation of Cu₃(PO₄)₂ was brought about by the large excess of phosphate ions.

In phosphate solutions to which tartrate, citrate or glycine were added both acceleration and deceleration of aneurin destruction have been encountered (Figs. 3, 4 and 5). With all three substances the same results were generally obtained with 20 p.p.m. of Cu as with 2 p.p.m. although, at the higher pH values for phosphate-glycine solutions the effect of 2 p.p.m. of Cu tends to disappear.

A complicating factor in the solutions used in this work is, however, the presence of comparatively large amounts of phosphate and a precipitate identified as Cu₃(PO₄)₂, was encountered in most of the tartrate solutions to which 20 p.p.m. of Cu were added.

Fig. 2. pH/log k curves for aneurin destruction in phosphate-phthalate solutions with and without the addition of Cu.

Experiments were therefore carried out to investigate this precipitation. Solutions of acidity between pH 4 and 6 were made up to contain Na₂HPO₄, sodium potassium tartrate and CuSO₄ in the concentrations of 0.1, 0.02 and 0.005M respectively and were adjusted to the desired pH with HCl. The extinction (derived from Beer's law) which is indicative of complex formation (q.v.) was measured and the solutions were then boiled for 1 hr. No precipitate was obtained at pH 4.40 but a precipitate did appear in the solutions at pH 5.15, 5.50 and 5.85 and was identified as Cu₃(PO₄)₂ precipitated by the common ion effect of a large excess of phosphate ions as in the phosphate and phosphate-phthalate solutions described above. The extinction of the solution at pH 4.40 was unchanged, that of the solution at pH 5.15 was reduced by about two-thirds and those of the solutions of pH 5.50 and 5.58 were virtually nil.

There were no precipitates with the citrate phosphate buffer (Fig. 4) and aneurin destruction is retarded by Cu from pH 3-6.5. There is a hint of

acceleration above pH 6.5 but the rate of aneurin destruction generally is so rapid even slightly above pH 7 that the matter was not investigated further.

Fig. 3. pH/log k curves for aneurin destruction in phosphate-tartrate solutions with and without the addition of Cu.

Fig. 4. pH/log k curves for aneurin destruction in phosphate-citrate solutions with and without the addition of Cu.

An attempt made to use unbuffered solutions for studying the effect of Cu before the part played by the Cu complexes was realized gave unsatisfactory results. The interchangeability of Na_2HPO_4 and K_2HPO_4 has been demonstrated from time to time and CuCl_2 gave exactly the same results as CuSO_4 .

DISCUSSION

From the results, Cu can both accelerate and retard the destruction of aneurin, according to the conditions obtaining in the solution. While acceleration only has been observed in phosphate and phosphate-phthalate buffer solutions, both acceleration and deceleration occur in solutions containing tartrate, citrate or glycine. These compounds are among those with which Cu forms complexes. It is suggested that the retarding effect with Cu found in the solutions containing them is due to the presence of these complexes.

Fig. 5. pH/log k curves for aneurin destruction in phosphate-glycine solutions with and without the addition of Cu.

Wark (1923, 1924, 1927) studied the complexes formed by Cu with hydroxy-acids and describes acids with anions containing Cu. Speaking of Cu malate he states that, although the normal salt is said to preponderate in solution, there is always a sufficient concentration of hydrogen and cupri-malate ions to give the characteristic properties of this anion. By analogy, the same author postulated the existence of cupri-salicylic, -glycollic and -lactic acids in equilibrium with the Cu salts in solutions of which the pH values are 3.5, 4.7 and 4.7 respectively. Bobtelsky & Jordan (1945) support this by reporting that Cu (and Ni) complexes with citric and tartaric acids behave as monobasic acids, so there is good reason for assuming the presence of cupri-citrate and cupri-tartrate anions in the solutions studied in this work. It is also likely that the large excess of citrate and tartrate over Cu in these solutions will favour the formation of the complex anion.

From the behaviour of boiling phosphate-tartrate- CuSO_4 solutions recorded above it would seem that at pH values between 5 and 6 the solubility product of $\text{Cu}_3(\text{PO}_4)_2$ is small compared with the dissociation constant of the complex and hence the tendency is for the latter not to be formed. Nevertheless, there would appear to be sufficient Cu left in solution to give the same results as are obtained with solutions containing 2 p.p.m. of Cu in which there is no precipitation. It is difficult to imagine free Cu^{++} in solutions containing such an excess of tartrate and when solutions 0.02 and 0.005M with respect to sodium potassium tartrate and CuSO_4 respectively were boiled at pH 4.54, 5.11 and 5.86 for 1.5 hr. there was no precipitation of the normal salt and no apparent change in extinction so that the cupri-tartrate seems to be relatively stable at 100° under conditions more nearly approaching those obtaining in the aneurin studies. The acceleration of aneurin destruction by Cu over most of the range studied and the deceleration below pH 5 are therefore likely to be due to the complex cupri-tartrate anion, the variation in behaviour being a pH effect conditioned by the phosphate which seems to determine the general shape of the pH/log k curves.

The case of the citrate-phosphate buffer (Fig. 4) is more straightforward. The absence of precipitation suggests that the cupri-citrate complex is more stable than the cupri-tartrate and it is submitted that the deceleration of aneurin destruction (see Fig. 4) may be ascribed to the presence of the copper-citrate complex anion.

Borsook & Thimann (1932) present evidence for the existence of several complexes of the amino-acids glycine and alanine with Cu in dilute solution at pH values from about 2 upwards. In the pH range studied in the present work, particularly since the glycine concentration greatly exceeds that of the Cu, it seems that two complexes are encountered: the so-called 'second acid complex', which, according to Borsook & Thimann, can exist at pH 3-6, and the 'neutral copper-glycine', which is said to predominate in the range pH 5-8. In the pH/log k curve for the phosphate-glycine solutions (Fig. 5) the similarity to the curve for phosphate solutions is clearly seen, but, with the addition of Cu, it is apparent that the reaction velocity is modified by the Cu complexes to show a deceleration of aneurin destruction below pH 5.7 and an acceleration above this pH. No precipitates were encountered in these solutions.

If the explanation for the variable effect of Cu on aneurin destruction is the occurrence of complex anions containing Cu, complex anions containing other metals might also be expected to modify the rate of aneurin destruction. Booth mentions that Fe, Sn, Zn and Al were without effect on aneurin destruction but he seems to have used phosphate-phthalate buffer solutions.

Reference to Fig. 6 shows that in phosphate solutions Fe, Zn and Ni have no effect on the rate of destruction of aneurin. However, in phosphate-citrate solutions these metals, for the most part, are

Fig. 6. pH/log k curve for aneurin destruction in metal-free phosphate solutions (with points omitted for convenience: see Fig. 1) on which are superimposed points for similar solutions containing Fe, Zn and Ni.

Fig. 7. pH/log k curve for aneurin destruction in phosphate-citrate solutions without added metals compared with similar curves obtained in the presence of Fe, Zn, Ni and a Cu-Fe mixture.

accelerants (Fig. 7) although the pH/log k curves are nearly horizontal. Bobtelsky & Simchen (1939) refer to an iron-citrate complex anion $(\text{Fe}_2\text{Cit}_3)^{-}$, which is formed in the presence of excess citrate and is said to be very stable, and reference to a zinc citrate complex has been made by Bobtelsky & Jordan (1945). Ni has not been studied as closely as Fe in

the present work but has shown acceleration of the same order as the latter at pH 5.5. Bobtelsky & Jordan (1945) found Ni complexes with citrate analogous to those with Cu. These results are thus an extension of the earlier work in that it seems that, with the exception of the special case of acceleration by Cu^{++} , it is the presence of various anions which cause the variations in the behaviour of aneurin.

This work affords an explanation of some results obtained when this investigation was first begun. Since there was no reason for suspecting that Fe or any heavy metals other than Cu, for that matter, catalyzed aneurin destruction, and, in fact, Booth had reported specifically that several had no effect, recrystallized reagents were used and tests for Cu only were carried out on the solutions by the addition of sodium diethyldithiocarbamate. With such solutions a pH/log k curve was obtained for aneurin destruction in phosphate-citrate solutions in the presence of 2 p.p.m. of Cu. In Fig. 8 it is compared

Fig. 8. pH/log k curves for aneurin destruction in phosphate-citrate solutions, with and without added Cu, made from recrystallized but insufficiently purified reagents.

with a similar curve without added Cu. The rate of aneurin destruction has been considerably accelerated. With the specially purified reagents described herein a series of experiments was carried out in which 2 p.p.m. of Cu and 1 p.p.m. of Fe were added to the aneurin containing phosphate-citrate solution (Fig. 7). The results obtained were close to the earlier curve for the solution containing Cu, i.e. aneurin destruction was accelerated but not to the same extent as with Fe alone. It seems therefore that the earlier solutions were contaminated with traces of Fe and that the behaviour of the aneurin is the result of a compromise between the two metal complexes present.

Further confirmation of the ideas put forward herein is found in the fact that Cu retarded the rate of aneurin destruction in solutions of yeast extract which contain amino-acids with which it is capable of forming complexes.

The method of preparing the buffer solutions used in this work inevitably leads to their contamination with Ca. However, the pH/log k curves for phos-

phate and phosphate-phthalate solutions purified by CaCO_3 adsorption are exactly the same as curves obtained with solutions of thrice recrystallized reagents which must be very low in Ca, used in the preliminary part of this work and in other work (Farrer, 1947). Moreover, when CaCl_2 (Ca = 20 p.p.m.) was added to the latter solutions, there was no change in the rate of destruction of the aneurin. However, Bobtelsky & Jordan (1945) report that Ca can form a complex with citrate although the equilibrium is usually in favour of the free ions and precipitation of calcium citrate. In solutions so dilute with respect to Ca it is hard to say what is the position and the results obtained with the specially purified phosphate-citrate solutions show small variations from the results obtained with the recrystallized reagents. These may possibly be due to a Ca effect but are more likely to be due to traces of heavy metals, which give more stable complexes, in the latter, and the presence of Ca has therefore been ignored in interpreting the results obtained.

From time to time abnormally fast rates of destruction have been encountered. Booth has already mentioned this point and there appears to be no obvious explanation of it. Similarly, in the absence of any indication of an oxidation of aneurin, there appears to be no simple explanation for the mechanism of the accelerating effect of Cu.

SUMMARY

1. Methods already described have been used to study the effect of two different concentrations of Cu (2 p.p.m. and 20 p.p.m.) on the rate of destruction of aneurin in the presence of five different buffer solutions at pH values from 3 to 6.8.

2. In phosphate and phosphate-phthalate solutions Cu accelerates the rate of destruction of aneurin at all pH values studied, greater acceleration being obtained with the greater concentration of Cu.

3. In phosphate solutions containing tartrate, citrate or glycine both acceleration and deceleration of aneurin destruction have been brought about by Cu and the variations in reaction velocity have been attributed to the presence in the solutions of complex anions containing Cu which act as positive or negative catalysts of aneurin destruction.

4. Confirmation of this explanation has been found in the fact that, while Fe, Zn and Ni are without effect on aneurin destruction in phosphate solutions, all three metals can modify the reaction velocity in phosphate solutions containing citrate with which all three can form complex anions.

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The Thermal Destruction of Vitamin B₁4. THE INFLUENCE OF THE CONCENTRATION OF BUFFER SALTS
ON THE RATE OF DESTRUCTION OF ANEURIN AT 100°

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In the course of the work on the influence of copper (Farrer, 1947), it was noticed that aneurin was destroyed more slowly in buffer solutions considerably more dilute than those used in earlier experiments (Farrer, 1941, 1945 a, b). The influence of the concentration of buffer salts on the thermal destruction of aneurin has therefore been investigated further, with the methods already developed. It has been found that the concentration of buffer salts can, under certain conditions, greatly influence the rate of destruction of aneurin but that the effect can vary in different buffer solutions.

EXPERIMENTAL

Reagents

The work reported herein was carried out before the effect of copper upon the thermal destruction of aneurin had been completely investigated (Farrer, 1947). Consequently reagents purified by recrystallization and not CaCO₃ adsorption were used. However, this work has been allowed to stand as the solutions were copper-free and reaction velocity determinations with 0.1M-phosphate and 0.02M-phosphate-phthalate solutions purified by CaCO₃ adsorption gave results exactly comparable with those obtained with the recrystallized reagents. Moreover, the results of Farrer (1947) show that other heavy metals (Fe, Zn and Ni) have no effect on aneurin destruction in the absence of complex-forming anions, and Booth (1943) could find no effect on aneurin destruction by adding Fe, Sn, Al or Zn to phosphate-phthalate solutions.

Phosphate buffer solutions. Na₂HPO₄·12H₂O was thrice recrystallized from glass-distilled water. A 0.2M solution was prepared with glass-distilled water and diluted as required for the various concentrations. The pH was adjusted with glass-distilled 6N-HCl.

Phosphate-phthalate buffer solutions. Samples of potassium hydrogen phthalate and KH₂PO₄ were twice recrystallized from glass-distilled water and a stock solution 0.2M with respect to each salt was made up. A slight flocculent precipitate, identified as an Al salt was always obtained no matter how carefully the salts were recrystallized (cf. Clark, 1928). Repeated determinations of *k* carried out in the presence of 5 p.p.m. of Al gave the same results as were obtained in its absence (as indicated by the alizarin spot test) which confirmed Booth's result. Accordingly, the precipitate was removed by filtration. The pH was adjusted to the desired value with KOH (copper-free).

Phosphate-citrate buffer solutions. A 0.1M solution of the citric acid used by Farrer (1947) was prepared with glass-distilled water. The 0.2M-Na₂HPO₄·12H₂O solution described above was used for these solutions too.

The aneurin stock solution and the method of assay were as described by Farrer (1947).

Procedure. In the case of the phosphate-citrate buffer, 20 ml. of solution of the desired pH were prepared by pipetting the appropriate volumes of the citric acid and phosphate solutions into a 200 ml. volumetric flask; 4 ml. of aneurin solution were added, and the solution was made up to volume giving a tenfold dilution of the McIlvaine buffer used in the earlier work (Farrer, 1945a). The final pH was measured.

The preparation of the phosphate and phosphate-phthalate solutions and the subsequent procedure were the same as described by Farrer (1947).

RESULTS

The method of interpretation is the same as was used in the earlier work. For the phosphate (Fig. 1) and phosphate-phthalate (Fig. 2) solutions pH/log *k* curves are given and in Figs. 3 and 4, *k* has been plotted against the salt concentration for solutions of