SUMMARY

1. Methods are described for the synthesis of ε-carbobenzoxy lysine, α-N-acetyl lysine and ε-N-acetyl lysine from the copper complex of lysine.

2. ε-N-acetyl-*l*-lysine is available for the growth of rats on a lysine deficient diet, whereas α-N-acetyl-*l*-lysine is not so available.

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The Thermal Decomposition of Aneurin and Co-carboxylase at Varying Hydrogen Ion Concentrations

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Molitor & Sampson [1936] have stated that pure aneurin (vitamin B_1 hydrochloride) in aqueous solution at *p*H 3·5 may be heated to 120° without undergoing decomposition. Foodstuffs containing aneurin are rarely if ever prepared, cooked and eaten in such an acid condition, and this observation, therefore, is nutritionally of little practical importance. Farrer [1941] has made an extensive study of the effect of *p*H's ranging from 3 to 9 upon the destruction of aneurin in solution at 100° and finds that even at *p*H 3 there is 16 % loss after 1 hr. and 29 % after 3 hr. It may therefore be that assay methods were not sufficiently advanced at the time of publication of Molitor & Sampson's work to indicate with certainty the small loss which it would appear probable had taken place. On the other hand, as will be discussed more fully later, the buffer used by Farrer may have contained impurities which catalyzed the destruction which was noted. Beadle, Greenwood & Kraybill [1943] have verbally presented details of a study similar to that of Farrer, but a full account of their work has only just come to hand.

Apart from these references to rate of destruction under specific and carefully controlled conditions and certain early studies [Chick & Hume, 1917; Sherman & Grose, 1923; Sherman & Burton, 1926; Guha & Drummond, 1929] there is little factual information in the literature on the subject apart from statements concerning losses on baking bread and in cooking a few other foodstuffs. In view of the fact that the fortification of foodstuffs with vitamins and other dietary essentials is being widely carried out (in respect of vitamin B_1 and other factors) it was considered desirable to determine such losses over a fairly wide range of conditions (but in the absence of oxidizing or reducing agents) which would serve to indicate the magnitude of loss to be expected in any non-pressure cooking or preparatory process (e.g. blanching) applied to foodstuffs.

While wheat and some other cereals contain vitamin B_1 as hydrochloride only, in other vegetable (e.g. soya) and most animal products B_1 activity resides in the pyro-phosphoric ester (co-carboxylase) and/or monophosphate of the vitamin in addition

to the free non-esterified form. Also, when a fermentation process is applied to a foodstuff (e.g. panary fermentation), phosphorylation of its vitamin B_1 takes place. In view of the uncertainty as to the relative stabilities of free aneurin and co-carboxylase it was decided to compare them under identical conditions. Clearly, if there is any foundation for the suggestion of Rosenberg [1942] that the relatively high stability of vitamin B_1 in foods as measured by activity is due to the vitamin being present largely in this combined form, then, other things being equal, fortification of foods should preferably be carried out with co-carboxylase rather than with aneurin.

The effect of metallic ions (especially Cu^{++}) in catalyzing oxidation was also considered as a possible factor in the destruction of aneurin. There is no doubt that many of the more labile constituents of foodstuffs may be profoundly affected during their cooking by the nature of the vessel in which the cooking is carried out. Therefore the effects of a number of metals of which cooking vessels are usually constructed or with which they are lined were compared at concentrations low enough to be of the same order as those which might be expected to occur in practice. Metals used in this connexion were Al, Zn, Sn, Fe, and Cu.

EXPERIMENTAL

Aneurin destruction. Glass distilled water was used throughout. Buffer solutions were made from Analar reagents as follows: 0·2*M* acid potassium phthalate; 0·2*M* acid potassium phosphate; and a solution 0·2*M* in respect of both of the above salts. This latter mixed buffer was accurately calibrated for addition of *N*/5 acid and alkali using an electrometric *p*H meter (quinhydrone electrode) accurate to 0·02 *p*H, and was used to cover a range of *p*H 5·4–6·8. The accuracy of buffers made from phthalate and phosphate separately (after the method of Clark & Lubs) was also regularly checked on the same meter.

A solution of pure aneurin was prepared in glass distilled H_2O (previously acidified to *N*/25 with HCl) to a final strength of 40 μg./ml. Such solutions have been found to be quite stable over periods of many months.

Solutions of the following salts were prepared containing 80 parts/million of the metal in each instance: $CuSO_4$, $ZnSO_4$, $FeCl_3$, $FeSO_4$, $SnCl_2$, $Al_2(SO_4)_3$.

Rate of destruction of aneurin was studied at *p*H's ranging from 2·4 to 7·4, particular emphasis being laid on the *p*H 5–7 range in view of the relative rarity of foods falling outside this range.

50 ml. of the appropriate buffer solution was taken in a 250 ml. Erlenmeyer flask and was adjusted to the desired *p*H with *N*/5 HCl or NaOH. In all instances insufficient *N*/5 HCl was used by exactly 1 ml. (or excess *N*/5 NaOH by 1 ml.) in order to allow for the subsequent addition of 5 ml. of the *N*/25 acid aneurin solution. In appropriate cases, 5 ml. of the metal solution were added, and then in all instances the volume of solution was made up to 195 ml. with H_2O. One or two minute fragments of porous pot were added and the solution was quickly brought to boiling point, 5 ml. of the aneurin solution (containing 200 μg., i.e. giving a final concentration of 1 μg./ml.) were added and boiling of the solution was started immediately under reflux, an all-glass system being used.

The time at which the aneurin addition was completed was noted and the contents of the flasks were sampled at the times indicated on the graphs of rate of destruction (Figs. 1–3)—usually after ½, 1, 1½, 2 and 3 hr. Sampling was carried out by momentarily removing the source of heat from the flask, and then rapidly pipetting 20 ml. of the solution into a tube containing 1 ml. of conc. HCl immersed in cold H_2O. By this means, destruction of the aneurin was immediately stopped. The final solution for assay even in the absence of destruction, would contain slightly less than 1 μg./ml., and this amount was determined both theoretically and experimentally to serve as a 'control' value and basis for assessing the percentage destruction which had occurred. Experimentally it was determined by rapidly bringing an acid solution of aneurin (*p*H 3, conc. 1 μg./ml.) to the boil and immediately pipetting 20 ml. into 1 ml. of conc. HCl. Theoretical and experimental values agreed within the limits of experimental error. At the conclusion of the 3 hr. boiling, all solutions after cooling were checked for *p*H. In certain instances, buffers of double the above concentration were used. Rates of destruction of aneurin in these cases were identical with those obtaining with the lesser concentration and are therefore neither graphed nor considered separately.

Co-carboxylase destruction. Co-carboxylase (aneurin pyrophosphate) cannot be estimated by the thiochrome method until it has been split up into phosphoric acid and aneurin. The plan of the experimental work had therefore to be substantially altered to make possible the complete hydrolysis of the ester before assay was carried out.

All estimations of co-carboxylase destruction were made using the mixed buffer. The co-carboxylase used was supplied by Merck and Co., Inc. (U.S.A.) and a solution containing 800 μg./5 ml. in *N*/25 HCl was prepared. The stability of this solution at room temperature was equal to that of aneurin solution under similar conditions—there was no loss after several months. In view of the fact that the co-carboxylase in this solution would become converted to monophosphate within a short time, experiments were carried out on old and freshly prepared solutions, i.e. on monophosphate and co-carboxylase, but no difference was found in their behaviour. It was therefore not considered necessary to use fresh co-carboxylase solutions for the work.

The setting up of the flasks was substantially the same as for aneurin destruction experiments—40 ml. of buffer solution + the necessary amount of *N*/5 NaOH to give the desired *p*H (after the addition of the 5 ml. of *N*/25 acid co-carboxylase solution) + H_2O to bring the total volume to 155 ml. The 5 ml. of co-carboxylase solution were added in a similar way to that described for aneurin, and 20 ml. portions were removed from the total volume of 160 ml., also in the same way as for aneurin. (Each 20 ml. of solution, it should be noted, originally contained 100 μg. of co-carboxylase.) Each portion was run into a 100 ml. flask containing sufficient *N*/5 HCl to bring the *p*H of the contents to 4 ± 0·1, was diluted to approx. 50 ml. and as soon as its temperature had fallen to about 40°, 1 ml. of a solution of taka-diastase in 15% ethanol (representing 50 mg. of the Parke Davis standard dry substance) was added. Incubation at 37° overnight (16 hr.) brought about complete hydrolysis of the co-carboxylase; after coming to room temperature the contents of each flask were made up to 100 ml. and was assayed.

Vitamin B_1 assay. Vitamin B_1 assay was carried out by a photoelectric procedure substantially the same as has been detailed elsewhere [Booth, Nicholls, Kent-Jones & Ward, 1942]. This method has been shown [Coward, 1943] to have limits of error for pure solutions of aneurin of the concentrations encountered here as follows:

No. of estimations	$P=0{\cdot}95$	$P=0{\cdot}99$
In duplicate	1·98%	2·60%
In triplicate	1·62%	2·12%
In quadruplicate	1·40%	1·84%

In a number of instances the boiled buffer + aneurin solutions containing aneurin decomposition products were exposed to ultra-violet light in order to ascertain whether thiochrome was formed during the boiling, but in no instance was this the case nor was any fluorescence observed. The same was true of isobutanol extracts from these solutions. It was therefore considered unnecessary to carry out unoxidized 'control' estimations to any considerable extent, as the 'control' values were both very low and uniform.

Each point on the curves represents the average of at least six separate determinations. Replicates agreed very well; only occasionally a phenomenally high rate of destruction was noted. This so far unexplained circumstance has been observed also by Dawson [1943]. Despite all reasonable precautions being taken, an occasional flask showed marked acceleration of destruction, this not uncommonly being at double the rate normally observed. It was considered that this might possibly be due to dissolved O_2, but bubbling N_2 through the solution for 20 min. before carrying out an experiment did not prevent the occurrence of occasional freak destruction rates. Once brisk ebullition has commenced with consequent rapid loss of dissolved gases the risk of oxidation due to dissolved O_2 rapidly diminishes [Mapson, 1941]. Therefore as a further check the buffer solution was boiled under reflux for ½ hr. before adding a solution of aneurin made up in previously boiled $N/25$ acid, the experiment being otherwise continued in the usual manner. Again, the absence of O_2 from the solution had no effect upon destruction rate.

RESULTS

In addition to the data presented graphically (Figs. 1–4), further figures were obtained which are not inserted on the graphs in order to avoid confusion.

Within the limits of experimental error, no effect was observed upon the rate of destruction of aneurin at *p*H 5·4 in mixed $M/20$ phosphate-phthalate buffer by the addition of 2 parts/million of Zn, Fe^{++}, Fe^{+++}, Sn and Al, but Cu caused it to be markedly accelerated. In view of the greater rate of destruction of co-carboxylase as compared with aneurin under identical conditions, it was considered possible that the co-carboxylase contained an impurity which catalyzed destruction. Addition of co-carboxylase to aneurin solution at *p*H 5·4 at the rate of 1 γ of co-carboxylase/1 γ of aneurin/1 ml., however, failed to accelerate the destruction of the aneurin. The removal of O_2 from the buffer solutions (prior to addition of aneurin solution), either by bubbling of N_2 for 20 min. or by boiling under reflux for ½ hr., affected neither the rate of the destruction at *p*H 5·4 during ½ hr. (when under the particular experimental conditions the maximum effect would be expected), nor the total amount of destruction during 3 hr. boiling.

DISCUSSION

It is clear both from the shape of the graphs and also from the evidence in respect of dissolved gases that primarily the destruction of aneurin at 100° is thermal and is affected by *p*H; it is not an oxidative effect. The stability of aneurin in solution at room temperature at low *p*H's where no protection is given against atmospheric oxidation also supports this view. Nevertheless, as Cu is able to catalyze destruction, it would appear that oxidation can be involved. From the point of view of food processing, therefore, minimal loss of aneurin is achieved by the employment of as low a *p*H as is practicable coupled with the avoidance of contact with Cu and with the minimum exposure to high temperature during cooking.

It will be noted that where their respective *p*H ranges overlap, there is a considerable difference between rate of destruction in phosphate and phthalate buffer (compare Figs. 1 and 2). When Fig. 3 (mixed buffer) enters into the comparison it becomes clear that phosphate exerts a protective effect of some kind, as destruction in the presence of phthalate is lessened by the simultaneous presence of phosphate.

On comparing the relative rates of destruction of co-carboxylase and aneurin under identical conditions, it is evident that the former is very much less stable than the latter. The reason for this is obscure, but it is quite definite and unmistakable and disposes of the hypothesis of Rosenberg [1942] previously mentioned. It furthermore rules out the desirability on other grounds of using co-carboxylase in lieu of aneurin for fortifying foodstuffs.

It will be noticed that Farrer [1941] obtains destruction rates (using citrate, phosphate and biborate buffers) in most cases roughly double those reported here. He also mentions that his figures 'show that aneurin is considerably more vulnerable to heat than results obtained by means of animal experiments would suggest'. While the figures reported here are still somewhat in excess of those reported by Sherman & Burton [1926], concordance is very much greater. It must also be borne in mind that Sherman & Burton's figures were obtained at a time when biological assays were not susceptible of the accuracy obtainable at present and in fact at a time when differentiation of the B group of vitamins had not gone very far.

The shape of the curves in Fig. 2 is worthy of comment—so far no reasonable explanation has

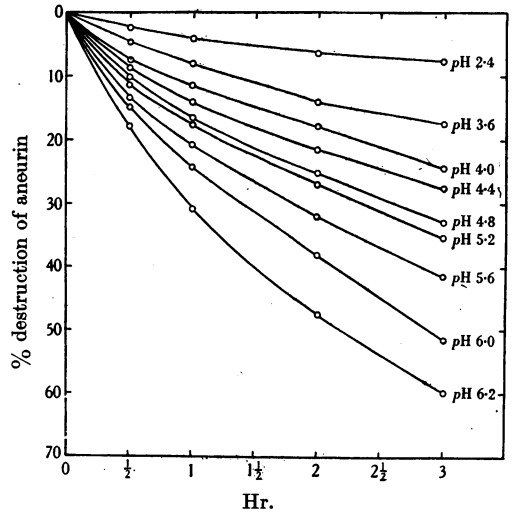

Fig. 1. Destruction of aneurin in boiling phthalate buffer.

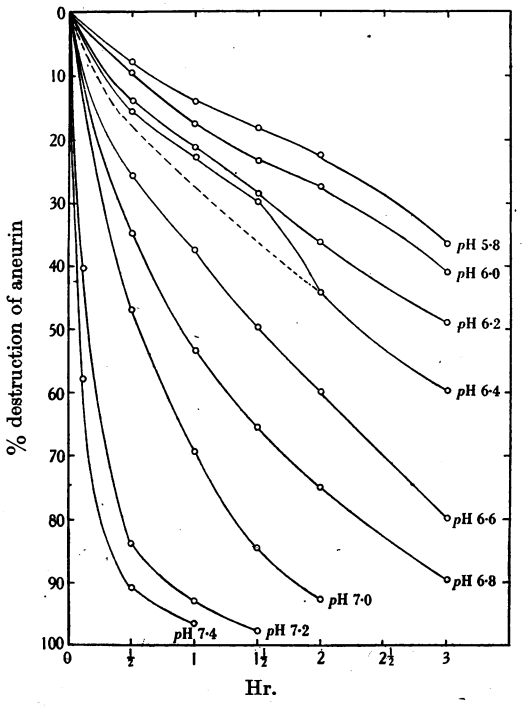

Fig. 2. Destruction of aneurin in boiling phosphate buffer.

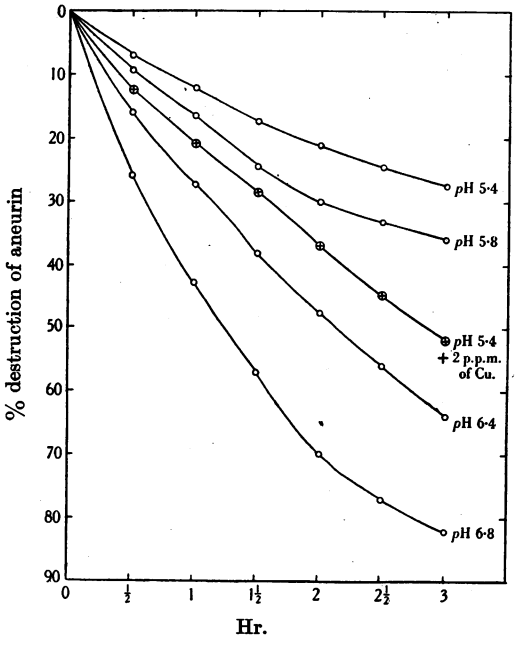

Fig. 3. Destruction of aneurin in boiling mixed (phosphate + phthalate) buffer, with and without 2 p.p.m. Cu.

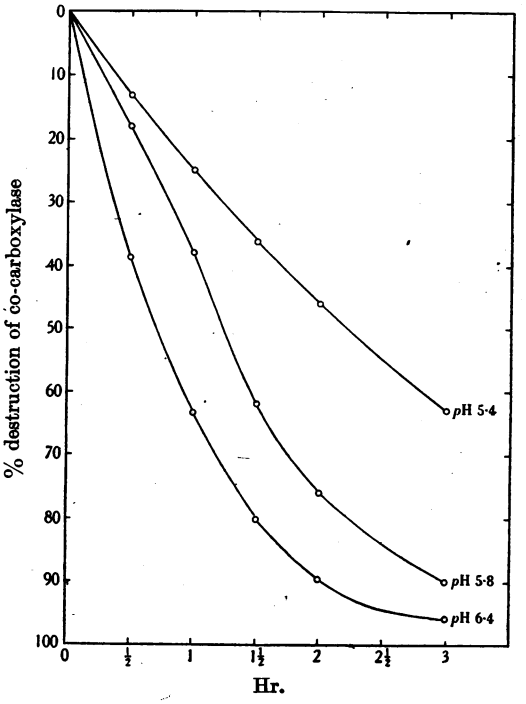

Fig. 4. Destruction of co-carboxylase in boiling mixed (phosphate + phthalate) buffer.

been found for the pronounced sigmoidal tendency at *p*H's 5·8 and 6·0, nor for the peculiar 'step' in the 6·4 curve.

While it is evident that to apply the figures presented here, obtained under rather artificial conditions, to foodstuffs in general would be unwarrantable, nevertheless there is evidence that they give a reasonable indication of the order of loss to be expected. For example, Moir [1942] finds a loss of 5–10 % of aneurin in oatmeal on boiling for 30 min., while he reports the *p*H of oatmeal to approximate closely to 6. Slater & Rial [1942] also find a small amount of destruction in domestic cooking of porridge. A rough average of the figures indicated by Figs. 1–3 gives an expected loss of about 12 %—if speculation may be allowed, the possibility of the antioxidant in oats reducing destruction, and/or the fact that porridge is not briskly boiled and may therefore fall short of 100° might be considered. Dawson & Martin [1941] find a loss of 8 % of vitamin B_1 in breadmaking, Hoffman, Schweitzer & Dalby [1940] find 5–9 %, Aughey & Daniel [1940] find 14 % loss, while Schulz, Atkin & Frey [1942] place baking loss at approximately 20 %, which at a *p*H of approximately 5·7–5·8 again agree well with the order of loss indicated here.

Aughey & Daniel [1940] also studied aneurin losses on cooking vegetables and determined *p*H's of the cooking liquors in some cases. While such *p*H's may not adequately represent the *p*H of the foodstuff cooked, significant support is given for the validity of the figures in Figs. 1–3 by *p*H and heat losses on cooking peas (9 % loss on simmering 12 min. at *p*H 6·4) and snap beans (18 % loss on simmering 40 min. at *p*H 5·8). A peculiar feature of their series of results is the rather inexplicable absence of destruction of aneurin in navy (dried) beans cooked for almost 1½ hr.

The increase in destruction of aneurin in foods occasioned by the addition of sodium bicarbonate is well known (e.g. Aughey & Daniel [1940]; Fincke [1941]; Barackman [1942]) and can be completely accounted for by the *p*H shift which it causes.

SUMMARY

1. The destruction rates of aneurin and co-carboxylase in various buffers at 100° have been followed by means of the thiochrome method of estimation.

2. The effect of traces of various metals and of atmospheric oxygen upon the destruction rate have also been compared.

3. Co-carboxylase is very much more thermolabile than aneurin at the same *p*H.

4. While Cu at the rate of 2 parts/million catalyzes the destruction of aneurin, Fe, Al, Zn, and Sn do not.

5. Destruction of aneurin appears to be for the most part non-oxidative in nature.

6. Comparison of the figures obtained in (1) with published figures by other authors of losses on cooking various foodstuffs shows reasonably good agreement.

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