CXXVIII. THE NUTRITION OF *STAPHYLOCOCCUS AUREUS*. THE ACTIVITIES OF NICOTINAMIDE, ANEURIN (VITAMIN B_1) AND RELATED COMPOUNDS

By BERT CYRIL JAMES GABRIEL KNIGHT[1]

From the Department of Bacterial Chemistry (Medical Research Council), Bland-Sutton Institute of Pathology, Middlesex Hospital, W. 1

(*Received 29 April 1937*)

In a previous paper [Knight, 1937] the aerobic growth of a strain of *Staphylococcus aureus* in a medium of known chemical composition was described. It was shown that nicotinic acid and aneurin together could replace the "staphylococcus growth factor" preparation (high-vacuum distillate [Knight, 1935]) ordinarily used, which had to be added to the deficient basal medium, containing chiefly amino-acids and glucose [Fildes *et al.* 1936] before the growth of typical strains of the organism could take place. It was shown that the growth factor was a complex of at least two components, one being replaceable by nicotinic acid (or its amide), the other by aneurin.

It has now been found by Dr G. P. Gladstone (unpublished) that 12 other typical strains of *S. aureus*—all, in fact, that have been tested—have similar nutrient requirements satisfied by nicotinic acid plus aneurin; none of these strains is satisfied by either substance alone. These growth requirements therefore seem general for the organism. With the further addition of uracil [Richardson, 1936] the organism can be grown anaerobically in the same medium.

The present paper concerns the quantitative activities of nicotinamide and aneurin, and the ability of the organism to use certain compounds related to aneurin instead of this substance itself.

The activity of nicotinamide

A titration of nicotinamide in the presence of excess of aneurin, using the amino-acid basal medium, is recorded in Table I. The technical details of the bacteriological testing for growth activity are given by Fildes *et al.* [1936] and need not be repeated here.

It is seen that maximum growth is obtained with approximately 10^{-6} *M* nicotinamide. For routine growth purposes 10^{-5} *M* nicotinic acid or the amide provides an ample quantity of either of these substances.

Nicotinic acid in the high-vacuum distillate. Spectrophotometric examination of the high-vacuum distillate [Knight, 1935; 1937] by Mr E. R. Holiday indicated that nicotinic acid or some closely related compound was present. Estimated by comparison with a solution of nicotinic acid, not more than 2 % of this distillate consisted of nicotinic acid. Nicotinamide gives a sufficiently different absorption spectrum for it to be possible to say that the selective absorption of the distillate can be accounted for by the acid. Short of actual isolation, the evidence is clear that it is to nicotinic acid or some closely related compound[2] that the biological activity of the one component of the distillate is in fact due.

[1] Halley Stewart Research Fellow.

[2] Synthetic pyridine-2-carboxylic and pyridine-4-carboxylic acids have not yet been tested.

Table I. *Titration of nicotinamide*

Basal medium: amino-acids, glucose [Fildes *et al.* 1936].
Aneurin chloride (synthetic [Todd & Bergel, 1937]) $2{\cdot}0 \times 10^{-7}$ *M* in each tube.
Nicotinamide added in serial dilution.
Aerobic growth at 37°.

Nicotinamide		Growth		
Dose γ per 10 ml. of medium	Conc. (*M*)	22 hr.	27 hr.	46 hr.
100·0	$8{\cdot}2 \times 10^{-5}$	+ +	+ + + +	+ + + +
20·0	$1{\cdot}6 \times 10^{-5}$	+ +	+ + + +	+ + + +
4·0	$3{\cdot}3 \times 10^{-6}$	+ +	+ + + +	+ + + +
0·8	$6{\cdot}6 \times 10^{-7}$	+ +	+ + + +	+ + + +
0·16	$1{\cdot}3 \times 10^{-7}$	tr.	+ +	+ + +
0·032	$2{\cdot}6 \times 10^{-8}$	tr.	+	+

+ + + + = maximum growth; other + signs proportional to mass of growth judged by opacity.
tr. = opacity just visible to naked eye.

The activity of aneurin

The following specimens of synthetic aneurin have been tested: (1) specimen from Prof. R. A. Peters, Baeyer product; (2) specimen synthesized by Todd & Bergel [1937]; (3) Hoffmann-La Roche product.

All these specimens of aneurin were active at the same concentrations, within the limits of the test, when tested in presence of excess of nicotinic acid. Two typical titrations of aneurin are recorded in Table II, from which it is seen that a detectable effect with aneurin, namely an amount of growth in 10 ml. of medium giving an opalescence just visible to the naked eye, can be obtained at approximately $5{\cdot}0 \times 10^{-10}$ *M* (i.e. 0·00015γ/ml.). Heavy growth is obtainable at $1{\cdot}0 \times 10^{-8}$ *M* without noticeable increase in opacity when larger doses are added. Taking this figure, equivalent to 0·3γ/100 ml. of medium, as approximating to

Table II. *Titration of aneurin*

Basal medium: amino-acids, glucose [Fildes *et al.* 1936].
Nicotinamide added to each tube.
Aerobic growth at 37°.
Aneurin added in serial dilution.

Nicotinamide $4{\cdot}0 \times 10^{-5}$ *M*			Nicotinamide $1{\cdot}0 \times 10^{-5}$ *M*		
Aneurin* *M*	Growth 20 hr.	Growth 40 hr.	Aneurin† *M*	Growth 22 hr.	Growth 46 hr.
$1{\cdot}0 \times 10^{-6}$	+	+ + + +	—	—	—
$2{\cdot}0 \times 10^{-7}$	+ +	+ + + +	$2{\cdot}5 \times 10^{-7}$	+ + + +	+ + + +
$4{\cdot}0 \times 10^{-8}$	+ +	+ + + +	$5{\cdot}0 \times 10^{-8}$	+ + + +	+ + + +
$8{\cdot}0 \times 10^{-9}$	+ +	+ + + +	$1{\cdot}0 \times 10^{-8}$	+ + + +	+ + + +
$1{\cdot}6 \times 10^{9}$	+ +	+ + +	$2{\cdot}0 \times 10^{-9}$	+ +	+ + +
$3{\cdot}2 \times 10^{-10}$	tr.	tr.	$4{\cdot}0 \times 10^{-10}$	?	+ +
—	—	—	$8{\cdot}0 \times 10^{-11}$	?	?

* Aneurin synthesized by Todd & Bergel [1937].
† Hoffmann-La Roche synthetic product.
+ + + + = maximum growth; number of + signs proportional to mass of growth judged by opacity.
tr. = trace of growth. ? = doubtful trace of growth.

that required for optimum growth, it may be compared with the optimum concentration found by Tatum *et al.* [1936] to be required for *Propionibacterium pentosaceum 11*, namely 0·5γ/100 ml. of medium. The requirements of the two organisms for this substance are therefore closely similar.

For routine growth purposes a concentration of $1{\cdot}0 \times 10^{-7}$ *M* provides an ample quantity of aneurin for *S. aureus*.

Aneurin derivatives

A specimen of an "aneurin" without the 5-β-hydroxyethyl group which is attached at the 5-position in the thiazole ring of aneurin proper (see Fig. 1) was tested. This substance had been synthesized by Dr A. R. Todd (unpublished). At a concentration of 10^{-5} *M* it was inactive, but tested in the presence of 4-methyl-5-β-hydroxyethylthiazole ($2{\cdot}0 \times 10^{-7}$ *M*) this derived aneurin gave good growth (see Table III).

Thiochrome. Two different synthetic specimens [Todd, Bergel, Fraenkel-Conrat & Jacob, 1936] of this substance were tested. Alone at concentrations of the order of $1{\cdot}0 \times 10^{-5}$ *M* a slight growth was obtained, with much enhanced activity in presence of 4-methyl-5-β-hydroxyethylthiazole (see Table III). Thiochrome is the only aneurin derivative of the two tested which shows any activity alone (at least up to 10^{-5} *M*).

The "aneurin-component" of the high-vacuum distillate

From the mode of preparation of the growth factor concentrate from autolysed yeast extract, involving a high-vacuum distillation above 105°, and from the stability of the growth factor to autoclaving at acid and alkaline reactions [Knight, 1935], it was impossible that aneurin itself could be responsible for the activity of that component of the distillate which it could replace. It was therefore likely that thermal or other degradation products of aneurin were present in the distillate, which could be used by the organism as well as the complete aneurin molecule.

Spectrophotometric examination did not give any positive evidence on this point. This does not exclude the presence of pyrimidine or thiazole types of compound derived from aneurin, however, but only indicates that if present they must be in much smaller concentration than the nicotinic acid. The quantitative testing of aneurin and related compounds (see Tables II and III) shows that this could be so and still account for the biological activity of the distillate. Another approach was therefore made to the problem of what degradation products of aneurin might be present in the distillate which were utilizable by *S. aureus*. Through the generous co-operation of Dr A. R. Todd it has been possible to test the activity of various synthetic pyrimidines and thiazoles related to aneurin, and to see how far these could replace the "aneurin-component" of the staphylococcus growth factor.

Activities of pyrimidines and thiazoles related to aneurin

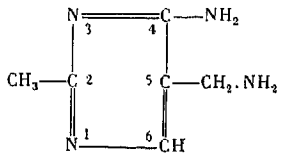
Possible thermal or other degradation products of aneurin which might be expected in the high-vacuum distillate would be derived from the thiazole or pyrimidine parts of the molecule; thiochrome was also a possibility. The blue fluorescence of the distillate in ultraviolet light [Knight, 1935] could not correspond to more than 1 % of thiochrome according to Prof. R. A. Peters's estimate (private communication). Comparing the activity of thiochrome alone with the

activity of the distillate it seems improbable that the activity of the latter could be due only to its thiochrome content.

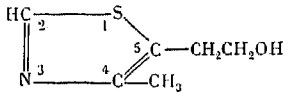
The following synthetic products were examined for activity, alone and in mixtures, as substitutes for aneurin:

Substance	Synthesized by
4-Methylthiazole	Dr A. R. Todd
4-Methyl-5-β-hydroxyethylthiazole	Todd, Bergel and Jacob [1936]
4-Hydroxy-5-hydroxymethyl-2-methylpyrimidine	Dr A. R. Todd
4-Hydroxy-5-aminomethyl-2-methylpyrimidine .	Todd, Bergel, Fraenkel-Conrat & Jacob [1936]
4-Amino-5-aminomethyl-2-methylpyrimidine	Todd & Bergel [1937]
4-Amino-5-thioformamidomethyl-2-methylpyrimidine	Todd & Bergel [1937]
4-Amino-2-hydroxypyrimidine (cytosine)	Richardson [1936]

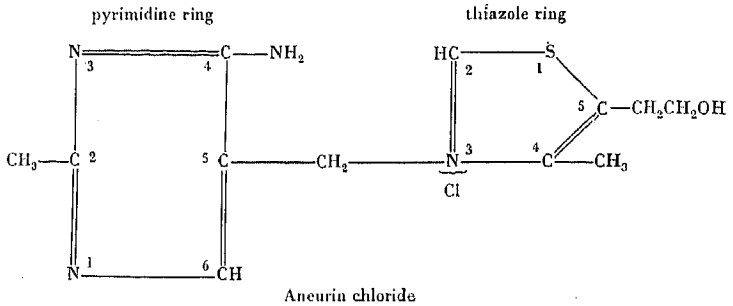
For convenience of reference the structural formulae of aneurin chloride, 4-amino-5-aminomethyl-2-methylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole are given in Fig. 1.



4-Amino-5-aminomethyl-2-methylpyrimidine



4-Methyl-5-β-hydroxyethylthiazole



Aneurin chloride

Fig. 1.

The 4-methyl-5-β-hydroxyethylthiazole is that corresponding exactly to the thiazole part of aneurin. Thiochrome can be formed by the oxidation of aneurin [Barger *et al.* 1935] whereby the nitrogen atom of the 4-amino-group becomes a bridge between the pyrimidine part of the molecule and the 2-carbon atom of the thiazole ring. The two samples of thiochrome used were synthesized directly [Todd, Bergel, Fraenkel-Conrat & Jacob, 1936].

Four of the pyrimidines had a CH_3 group in the 2-position as in aneurin. Two of these 2-methylpyrimidines had an amino-group in the 4-position, as in aneurin, the other two having hydroxy-groups in this position. In the 5-position of each 2-methylpyrimidine there was a substituted methyl group. It is the —CH_2— of this group which forms the bridge between the pyrimidine and thiazole parts of the aneurin molecule (see Fig. 1). In cytosine, the amino-group is in the same position as in aneurin, but the 2-methyl and 5-—CH_2— groups are lacking.

The pyrimidines and thiazoles were tested for activity, using the amino-acid basal medium and excess of nicotinamide, to see whether any of them could be used by the organism instead of the complete aneurin molecule. Not one of these pyrimidines or thiazoles was active when tested alone at concentrations approximately 100 to 1000 times greater than that at which aneurin is active. It is clear therefore that the organism cannot use either the 4-methyl-5-β-hydroxyethylthiazole or, for example, the 4-amino-5-aminomethyl-2-methylpyrimidine, corresponding to the two parts of the aneurin molecule, singly, to replace the complete molecule.

However, certain pairs of an appropriately substituted pyrimidine plus 4-methyl-5-β-hydroxyethylthiazole were able at M concentrations approaching those at which aneurin is effective to permit good growth of *S. aureus*. The results obtained with the various compounds alone and in presence of equimolar or greater concentrations of 4-methyl-5-β-hydroxyethylthiazole are recorded in Table III. It is seen that in presence of an equimolar concentration of this thiazole both the 2-methylpyrimidines with an amino-group in position 4 showed

Table III. *Utilization of compounds related to aneurin, in presence of 4-methyl-5-β-hydroxyethylthiazole*

Basal medium: amino-acids and glucose [Fildes *et al.* 1936].
Nicotinamide present throughout in excess (10^{-5} M).
Aerobic growth at 37°.

Substance	Conc. M	4-methyl-5-β-hydroxyethyl-thiazole M	Growth 48 hr.
—	—	$3{\cdot}5 \times 10^{-5}$	0
Aneurin	$1{\cdot}0 \times 10^{-8}$	0	+ + + +
Aneurin lacking 5-β-hydroxyethyl group	$1{\cdot}0 \times 10^{-5}$	0	0
,,	$1{\cdot}0 \times 10^{-8}$	$2{\cdot}0 \times 10^{-7}$	+ + + +
Thiochrome	$1{\cdot}9 \times 10^{-5}$	0	+(*)
,,	$1{\cdot}0 \times 10^{-5}$	0	tr.(*)
,,	$7{\cdot}6 \times 10^{-6}$	0	?
,,	$1{\cdot}0 \times 10^{-5}$	$1{\cdot}0 \times 10^{-7}$	+ + + +
,,	$1{\cdot}0 \times 10^{-6}$	$1{\cdot}0 \times 10^{-7}$	+ + +
,,	$1{\cdot}0 \times 10^{-7}$	$1{\cdot}0 \times 10^{-7}$	+ +
,,	$1{\cdot}0 \times 10^{-8}$	$1{\cdot}0 \times 10^{-7}$	0
4-Hydroxy-5-hydroxymethyl-2-methylpyrimidine	$2{\cdot}0 \times 10^{-6}$	$2{\cdot}0 \times 10^{-6}$	0
4-Hydroxy-5-aminomethyl-2-methylpyrimidine	$1{\cdot}0 \times 10^{-6}$	$2{\cdot}0 \times 10^{-6}$	0
4-Amino-2-hydroxypyrimidine (cytosine)	$2{\cdot}0 \times 10^{-5}$	$2{\cdot}0 \times 10^{-7}$	0
4-Amino-5-thioformamidomethyl-2-methylpyrimidine	$1{\cdot}0 \times 10^{-5}$ (*f.*)	0	0
,, ,,	$1{\cdot}0 \times 10^{-5}$ (*f.*)	$1{\cdot}0 \times 10^{-6}$	+ + + +
,, ,,	$1{\cdot}0 \times 10^{-6}$ (*a.*)	$1{\cdot}0 \times 10^{-6}$	+ + + +
,, ,,	$1{\cdot}0 \times 10^{-7}$ (*a.*)	$1{\cdot}0 \times 10^{-7}$	+
,, ,,	$1{\cdot}0 \times 10^{-8}$ (*a.*)	$1{\cdot}0 \times 10^{-8}$	?
4-Amino-5-aminomethyl-2-methylpyrimidine	$1{\cdot}0 \times 10^{-4}$	0	0
,, ,,	$5{\cdot}0 \times 10^{-8}$	$5{\cdot}0 \times 10^{-8}$	+ + + +
,, ,,	$1{\cdot}0 \times 10^{-8}$	$1{\cdot}0 \times 10^{-8}$	+ + + +
,, ,,	$2{\cdot}0 \times 10^{-9}$	$2{\cdot}0 \times 10^{-9}$	+ +
,, ,,	$4{\cdot}0 \times 10^{-10}$	$4{\cdot}0 \times 10^{-10}$	tr.
,, ,,	$8{\cdot}0 \times 10^{-11}$	$8{\cdot}0 \times 10^{-11}$	?

(*f.*) Sterilized by filtration through Seitz filter pads; actual concentration may be less than recorded.
(*a.*) Sterilized by autoclaving, 20 min. at 115°.
+ + + + = maximum growth; number of + signs proportional to amount of growth, judged by opacity.
(*) = Two different specimens of thiochrome.
tr. = opacity just visible to the naked eye.
? = doubtful trace of opalescence.

activities nearly as great as that of aneurin itself, while there was no activity with the corresponding 4-hydroxy-2-methylpyrimidines. All pairs of a pyrimidine plus 4-methylthiazole were ineffective.

Discussion

The results show that aneurin itself or a mixture of the pyrimidine plus the thiazole, both substituted exactly as in aneurin, can replace the "aneurin component" of the staphylococcus growth factor. A differently substituted pyrimidine or thiazole is inadequate. It is probable that suitably substituted compounds of these types account for the activity of the "aneurin component" of the high-vacuum distillate.

The degree of specificity is further indicated by the fact that an aneurin lacking the β-hydroxyethyl group which is attached at the 5-position of the thiazole ring of aneurin proper was inactive.

Similarly 4-amino-5-aminomethyl-2-methylpyrimidine (Fig 1) or the corresponding 5-thioformamidomethyl compound plus 4-methyl-5-β-hydroxyethylthiazole (Fig. 1) permits growth; but substitution of a 4-hydroxy-group for the 4-amino-group in the pyrimidine causes loss of activity. The organism is therefore apparently unable to substitute an amino-group for a hydroxy-group at the 4-position. The failure of 4-amino-2-hydroxypyrimidine (cytosine) plus 4-methyl-5-β-hydroxyethylthiazole to permit growth may be due to the absence of the 2-methyl group or to the absence of a 5-—CH_2— group to form the link with the thiazole, if the synthesis of aneurin is necessary (see below).

The failure of a mixture of 4-amino-5-aminomethyl-2-methylpyrimidine plus 4-methylthiazole to give growth agrees with the inactivity of the aneurin lacking the 5-β-hydroxyethyl group.

The nature of the substitution in the 5-—CH_2— group in the active 4-amino-2-methylpyrimidines appears to be of less importance, since either a 5-aminomethyl or a 5-thioformamidomethyl group permits growth, in presence of 4-methyl-5-β-hydroxyethylthiazole, provided that the remainder of the molecule is correctly substituted (i.e. 4-amino-2-methyl-). The lower potency of the 4-amino-5-thioformamidomethyl-2-methylpyrimidine as compared with the corresponding 5-aminomethyl compound, does not appear to be due to the toxicity of the former, since greater concentrations of it gave increasing mass of growth. This would not be expected if it were toxic.

From the specificity of the pyrimidine plus thiazole pair corresponding to aneurin, which permits growth, it might appear that the two compounds are used by *S. aureus* to synthesize the aneurin molecule which it really requires for use in some metabolic mechanism. There are possible objections to this view, however. For instance it might be suggested, from the inactivity alone of the aneurin lacking the 5-β-hydroxyethyl group, which, plus 4-methyl-5-β-hydroxyethylthiazole, gives growth, that the organism detaches the 4-methylthiazole of the derived aneurin and then links on the 4-methyl-5-β-hydroxyethylthiazole, giving aneurin proper. The probability of this occurring cannot be estimated however. That the β-hydroxyethyl group wandered from the uncombined to the combined thiazole is most improbable.

An alternative explanation is that, in whatever mechanism it is used by *S. aureus*, the aneurin molecule is broken down to its pyrimidine and thiazole components which are then used in the metabolic mechanism, both components still being necessary. Actual synthesis of aneurin from the two components by *S. aureus* has not yet been tested experimentally.

If synthesis of aneurin does occur, the lowered potency of 4-amino-5-thioformamidomethyl-2-methylpyrimidine as compared with the corresponding 5-aminomethyl compound, might be due to the organism having greater difficulty in eliminating the thioformamido-group before linking with the 4-methyl-5-β-hydroxyethylthiazole, which takes place through the 5-—CH_2— group. It is of interest in connexion with the possible routes of aneurin synthesis in nature that *S. aureus* should be unable to use the 5-thioformamidomethyl group to form part of the thiazole ring, as in the method of synthesis described by Todd & Bergel [1937]. But it may be able to link the correctly substituted pyrimidine and thiazole to form the aneurin molecule.

The activity of thiochrome alone may be due to the ability of *S. aureus* to open and reduce the link —C—N=C— between the pyrimidine and thiazole rings in thiochrome, giving aneurin, although with difficulty, as suggested by the relatively great concentration of thiochrome needed to show activity. The greatly enhanced activity of thiochrome plus 4-methyl-5-β-hydroxyethylthiazole suggests that the thiazole part of the thiochrome is broken off, leaving the pyrimidine; this is then active in the usual way.

From the foregoing results it is clear that aneurin, or the directly related equivalent chemical components of the molecule, are highly specific in the growth of *S. aureus*.

The "aneurin component" in gelatin

Since it was found [Knight, 1937] that nicotinic acid alone permitted some growth of *S. aureus* when added to the gelatin-hydrolysate medium formerly used [Knight, 1935], although it did not do so on the amino-acid medium [Fildes *et al.* 1936], it follows that the gelatin medium contains small amounts of compounds which the organism can use as the "aneurin component" of the complex growth factor. It was observed, however, that increasing concentrations of nicotinic acid did not result in increasing mass of growth beyond a rather poor amount. This indicated that the "aneurin component" was present in the gelatin hydrolysate only in very limited quantity. This was just sufficient for testing the "staphylococcus growth factor", as it then was, before the biological activity was found to be due to two necessary components.

In using a gelatin hydrolysate medium it must therefore be recognized that in addition to its amino-acid content this medium contains very small but often decisive traces of aneurin degradation products.

Summary

Aneurin plus nicotinic acid can completely replace the "staphylococcus growth factor", enabling the growth of 12 typical strains of *S. aureus* to take place in a medium of known chemical composition.

For routine growth purposes 10^{-5} *M* nicotinic acid (or amide) and 10^{-7} *M* aneurin provide ample quantities of these two substances.

Certain components of the aneurin molecule, namely the corresponding pyrimidine plus the corresponding thiazole, can be utilized by the organism instead of the complete molecule.

On the contrary, certain other closely related compounds (differently substituted pyrimidines, thiazoles and aneurins) cannot be utilized, indicating a high degree of specificity in the growth of *S. aureus*.

I am deeply indebted to Dr A. R. Todd of the Lister Institute for generously supplying the synthetic compounds related to aneurin, without which this work

would have been impossible; to Mr E. R. Holiday for the spectrophotometric observations; to Prof. R. A. Peters; and to Dr P. Fildes and Dr G. P. Gladstone for their continued active help in carrying out the bacteriological side of the work.

REFERENCES

Barger, Bergel & Todd (1935). *Nature, Lond.*, **136**, 259; *Ber. dtsch. chem. Ges.* **68**, 2257.
Fildes, Richardson, Knight & Gladstone (1936). *Brit. J. exp. Path.* **17**, 481.
Knight (1935). *Brit. J. exp. Path.* **16**, 315.
—— (1937). *Biochem. J.* **31**, 731.
Richardson (1936). *Biochem. J.* **30**, 2184.
Tatum, Wood and Peterson (1936). *Biochem. J.* **30**, 1898.
Todd & Bergel (1937). *J. chem. Soc.* 364.
—— —— & Jacob (1936). *J. chem. Soc.* 1555.
—— —— Fraenkel-Conrat & Jacob (1936). *J. chem. Soc.* 1601.