LI. ANEURIN (VITAMIN B_1) AND PYRUVATE METABOLISM BY *STAPHYLOCOCCUS AUREUS*

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KNIGHT has shown [1937, 1, 2] that nicotinic acid or its amide and aneurin (vitamin B_1) are both necessary for the growth of *Staphylococcus aureus* in a medium which contains only definite chemical compounds, mainly certain amino-acids and glucose [Fildes *et al.* 1936; Fildes & Richardson, 1937]. Furthermore, pyruvate plays an important part in growth under anaerobic conditions. It was therefore of interest to investigate the part played by aneurin in the metabolism of the organism, in view of the findings of Krebs [1937] concerning pyruvate dismutation by staphylococci, and of Peters and his school [1936] on the importance of aneurin in restoring the deficient O_2 uptake of avitaminous pigeon brain, especially with pyruvate as substrate.

Krebs [1937] showed that staphylococci can effect an anaerobic dismutation of pyruvate to lactate, acetate and CO_2, according to the equation (1).

$$2CH_3CO.COOH + H_2O = CH_3CHOH.COOH + CH_3COOH + CO_2. \quad \ldots(1)$$

He maintained that the oxidation of pyruvate by staphylococci proceeded through this anaerobic phase, followed by oxidation of the lactate formed.

The present paper provides further evidence in support of this view. In the case of staphylococci grown with minimal amounts of aneurin for good growth in the presence of ample excess of all other essential nutrients, the O_2 uptake with pyruvate as substrate is very small, but it is increased immediately by the addition of aneurin chloride, while the O_2 consumption of organisms grown with ample excess of the vitamin is not affected. The failure of O_2 uptake by organisms grown in the presence of suboptimal amounts of aneurin is essentially due to inability to carry out the anaerobic phase, since the anaerobic CO_2 production in the presence of pyruvate is also small in the absence of added aneurin. Krebs' failure to show any effect of aneurin on this process is evidently due to the presence of an ample supply of the vitamin in his culture medium (broth agar), whereas in our case it has been possible by adjustment of the quantity of aneurin in the culture medium, to produce at will "aneurin-deficient" or "aneurin-sufficient" cocci.

Experimental methods

(1) *Manometric methods.* Methods employing simple Warburg manometers were used as described by Dixon [1934]. All experiments were carried out at 37·5°. In anaerobic experiments the 5% CO_2 in N_2 gas mixture was freed from oxygen by passing over red-hot copper. In aerobic experiments in which the suspension medium was buffered by phosphate the gas was air; CO_2 was removed by 20% KOH, 0·2 ml. per vessel. Metabolic rates are expressed as usual, as μl. gaseous metabolite at N.T.P. per hr. per mg. dry wt. of cocci. (1 millimol = 22,400 μl.)

(2) *Materials.* Pyruvate solutions were made up as required from a stock solution of 5N pyruvic acid, prepared as described by Clift & Cook [1932] and stored at 0°. Lactate was freshly made up from crystalline $l(+)$-lactic acid (B.D.H.). The aneurin was a synthetic specimen. 4-Amino-5-aminomethyl-2-methylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole were specimens supplied to Mr B. C. J. G. Knight by Dr A. R. Todd.

(3) *Bacteria.* The organisms were cultured aerobically for about 40 hr. in 250 ml. Erlenmeyer flasks each containing about 40 ml. of the basal medium described by Fildes & Richardson [1937]. Aneurin was used in a concentration of $10^{-7}M$ when an ample excess was required. Most of the organisms used in this work, however, were grown in a medium in which the aneurin concentration had been reduced to 3 or $4 \times 10^{-9}M$. Under these conditions the bulk of growth was not greatly reduced and the ratio of dry weight to volume of organisms was not significantly altered. Organisms grown under these conditions are described as aneurin-deficient. Further reduction of the aneurin concentration to $2 \times 10^{-9}M$ led to variable amounts of growth, and even when the growth appeared to be good the metabolism was often low, as if the number of surviving organisms was small.

The organisms were collected by centrifuging at 2500 r.p.m. for $\frac{1}{2}$ hr. They were washed once with a volume of 0·9 % NaCl equal to half that of the culture medium used. It was shown that further washing had no appreciable effect on the metabolism of the organisms. Growth tests, with the contents of the vessels at the end of a manometric experiment as a source of aneurin, failed to detect its presence except where it had been added to the suspension medium.

A suspension of the organisms in a small quantity of 0·9 % NaCl was standardized in quadruplicate by means of the haematocrit. The dry weight of the organisms was determined in a number of cases by washing 0·3 ml. of the suspension free from salt with 5 ml. distilled water, and drying the centrifuged organisms to constant weight in the steam oven. A control with 0·3 ml. of saline was performed simultaneously. The ratio of dry weight to haematocrit volume was constant within the limits of experimental error—0·32 for organisms grown under the above conditions (Fujita & Kodama [1934] give 0·23 for *Staph. aureus* grown on agar). The haematocrit volumes were converted into dry weights by means of the ratio 0·32 when the dry weight was not determined directly.

The standardized suspension was diluted with 0·9 % NaCl so that 1 ml. formed a suitable quantity to add to the buffered medium in the manometer vessels. The diluted suspension was used at once since the activity of aneurin-deficient staphylococci diminished appreciably on storage, even at 0°.

The effect of aneurin on O_2 uptake in the presence of pyruvate

Varying amounts of synthetic aneurin chloride in distilled water were tipped from the side-bulbs of conical manometer vessels after a preliminary control period. The results for a crop of organisms grown with optimal concentration of aneurin are shown in Table I. The increase in the rate of O_2 uptake on adding aneurin after 30 min. is not significantly different from the rise which occurs in control vessels to which no aneurin is added. Even when the rate of respiration begins to fall after 150 min. the effect of added aneurin is small, even if significant.

In the case of aneurin-deficient organisms, on the other hand, the low falling O_2 uptake of the control period is raised to a maximum 3 to 6 times the control value by the addition of similar amounts of aneurin, as shown by Table II.

I to III. *The effect of added aneurin on O_2 uptake*

Pyruvate, 24m*M*. Phosphate, *p*H 7·6, 32m*M*., 3·5 ml. fluid

Table I. Staph. aureus *grown with 10^{-7} M aneurin*

Crop A, 3·76 mg. cocci per vessel.

Added aneurin $\times 10^{-8}$ *M* at		Q_{O_2} at (min.)				
30 min.	160 min.	0–30	50–110	120–150	160–190	170–200
0	—	40·9	41·4	36·9	36·9	—
0	173	44·8	47·5	44·2	—	47·4
9	—	42·1	45·9	—	—	—
43	—	41·6	44·7	—	—	—
173	—	37·9	45·3	38·4	40·5	—

Table II. "*Aneurin-deficient*" Staph. aureus

Crop	Wt. cocci per vessel mg.	Conc. of aneurin during growth	Aneurin $\times 10^{-8}$ *M* added at 30 min.	Q_{O_2} at (min.) 0–30	50–100
B	3·23	3×10^{-9} *M*	0	6·8	6·2
			3	7·4	14·1
			9	8·1	16·6
			21	7·9	18·6
			43	8·3	18·7
C	4·76	3×10^{-9} *M*	0	6·7	4·2
			7	12·7	18·9
			19	7·7	19·4
			43	4·4	22·1
			173	8·6	21·0
D	4·32	2×10^{-9} *M*	0	6·4	2·7
			7	6·2	12·6
			19	6·3	12·9
			43	6·3	16·3
			173	7·1	18·7

Table III. *Viable count and maximum O_2 uptake*

Crop of organisms	Viable count Organisms per mg. dry wt. Initial	Final	Mean (M)	Q_{O_2} 50–110 min. B_1 at 30 min. $=43 \times 10^{-8}$ *M*	$\frac{Q_{O_2} \times 10^8}{M}$
A	1·05	1·04	$1{\cdot}05 \times 10^9$	44·7	4·25
B	0·57	0·47	$0{\cdot}52 \times 10^9$	18·7	3·6

Fig. 1 contrasts the effect of increasing concentrations of added aneurin on the O_2 uptake of organisms grown in the presence of the minimal concentration of aneurin (3×10^{-9} *M*; crop B) with the lack of effect of added aneurin on the O_2 uptake in the case of organisms grown simultaneously, but in the presence of excess of aneurin (100×10^{-9} *M*; crop A).

It will be seen from Tables I and II and especially from Fig. 1, that the maximum rate of O_2 uptake of aneurin-deficient organisms in the presence of added aneurin does not reach the value for organisms grown in the presence of excess aneurin. The discrepancy is accounted for by the difference in the number of actually living organisms as determined by the agar roll tube method by Dr G. P. Gladstone (Table III).

Table III also shows that the effect of aneurin in raising the O_2 uptake of aneurin-deficient organisms cannot be due to multiplication, since the number

surviving at the end of the experiment is significantly less than that present initially.

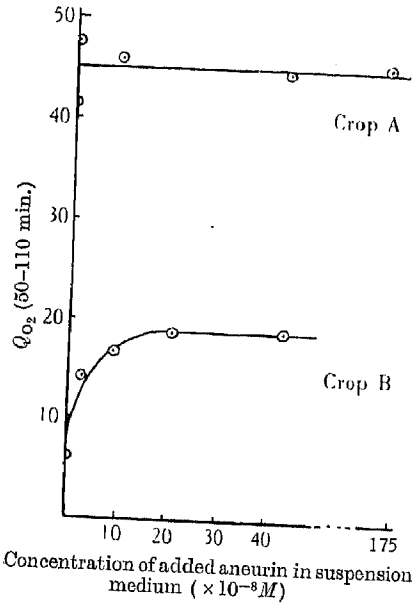

Fig. 1.

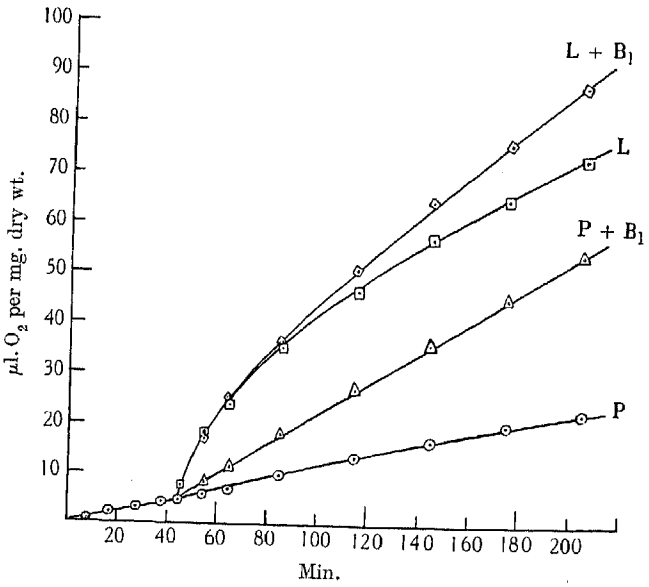

Fig. 2.

Fig. 1. O_2 uptake of *Staph. aureus*. Effect of aneurin. Phosphate, *p*H 7·6, *M*/30; pyruvate, *M*/40, 3·5 ml. fluid. Crop A, cultured in the presence of 10^{-7} *M* aneurin, 3·8 mg. cocci per vessel. Crop B, cultured in the presence of 3×10^{-9} *M* aneurin, 3·2 mg. cocci per vessel.

Fig. 2. O_2 uptake of aneurin-deficient *Staph. aureus*. Effect of aneurin with lactate and pyruvate as substrates. Phosphate, *p*H 7·6, *M*/30, 2·8 ml. fluid, 2·9 mg. cocci per vessel. B_1 = aneurin in the medium, 10^{-6} *M*. Substrates added at 40 min.: L = lactate *M*/200, P = pyruvate *M*/50.

Lactate and pyruvate as substrates

Since, according to Krebs' view, the O_2 uptake of staphylococcus in the presence of pyruvate is mainly due to the oxidation of lactate produced by a dismutation in which O_2 plays no part, the O_2 uptake of the same suspension was studied in the presence of these substrates with and without added aneurin. According to Krebs' equation (1) for the dismutation, 1 mol. of lactate is formed from 2 of pyruvate. The rate of O_2 uptake at a lactate concentration less than half the pyruvate concentration must at least equal that in the presence of pyruvate if the O_2 uptake in the latter case is to be accounted for by the oxidation of lactate. Fig. 2 shows the effect of adding after a preliminary control period lactate and pyruvate to give *M*/200 and *M*/50 solutions respectively.

The addition of pyruvate in the absence of aneurin has little effect on the O_2 uptake, but in the presence of the vitamin a large increase occurs which is maintained for nearly 3 hr. The addition of lactate, however, produces a very much larger increase in respiration, at first independent of the presence of aneurin. In the absence of aneurin the rate of O_2 uptake subsequently falls to a fairly steady level almost identical with that obtained when pyruvate is the substrate in the presence of aneurin. The fall of the rate of O_2 uptake in

the presence of lactate is partially prevented by the presence of aneurin, the almost steady level reached in this case being significantly higher than when aneurin is absent. These facts may be explained if the lactic dehydrogenase system of staphylococcus behaves similarly to that of animal tissues for which Green & Brosteaux [1936] have shown that the oxidation of lactate is strongly inhibited by pyruvate. There is evidence for the oxidation of lactate to pyruvate by *Staphylococcus* [Sevag & Neuenschwander-Lemmer, 1936]. Since in the absence of added aneurin pyruvate promotes little O_2 uptake of aneurin-deficient staphylococci, it is concluded that any pyruvate formed from lactate cannot be removed to any great extent by further oxidation under these conditions. Moreover, with lactate in the absence of appreciable amounts of its oxidation products, aneurin appears to have no direct effect on the O_2 uptake of aneurin-deficient staphylococci as Sherman & Elvehjem have suggested [1936] in the case of the tissues of polyneuritic chicks [cf. Meiklejohn, 1933]. We are forced to the conclusion that its effect on the oxidation of pyruvate must occur in the anaerobic dismutation to lactate, in spite of Krebs' failure to detect any effect with organisms grown on agar. As already described, it is necessary to have aneurin-deficient organisms before it is possible to show any effect of added aneurin on the O_2 uptake. Krebs' organisms correspond with the aneurin-sufficient crop A of the present work.

Anaerobic production of CO_2 in the presence of pyruvate

The anaerobic production of CO_2 in the presence of a bicarbonate buffer, in which no retention of CO_2 occurs, can be used as a measure of the anaerobic dismutation of pyruvate when this compound is used as a substrate [Krebs, 1937]. Table IV shows that aneurin has a definite effect on the anaerobic production of CO_2 in the presence of pyruvate.

Table IV. *Anaerobic CO_2 production with pyruvate*

Bicarbonate, 5% CO_2 in N_2. *p*H 7·2–7·3, 2·7 ml. fluid

Crop	Wt. cocci per vessel mg.	Conc. of aneurin during growth	Suspension	Time min.	$Q_{CO_2}^{N_2}$ No B_1	$Q_{CO_2}^{N_2}$ $+B_1$
E	2·9	$4 \times 10^{-9} M$	Bicarbonate 15 m*M*. Pyruvate, 20 m*M* to give 210 μl. CO_2 per mg. cocci*. $+B_1 = 10^{-6} M$ aneurin	0– 30 45– 75 105–195	3·5 7·8 6·1	12·6 15·8 18·0
F	5·4	$4 \times 10^{-9} M$	Bicarbonate 19 m*M*. Pyruvate, 24 m*M* to give 125 μl. CO_2 per mg. cocci*. $+B_1 = 1{\cdot}2 \times 10^{-6} M$ aneurin	0– 30 30– 40 45– 60 90–120	19·0 23·2 23·7	59·3 66·3 57·5 14·9
G	6·6	$3 \times 10^{-9} M$	Bicarbonate 15 m*M*. Pyruvate, 20 m*M* to give 93 μl. CO_2 per mg. cocci*. $+B_1 = 10^{-6} M$ aneurin	0– 30 30– 90 120–180	8·9 10·1 12·2	27·5 30·3 2·5

* Calculated according to equation (1).

The autocatalytic behaviour of the reaction observed by Krebs is confirmed. This is probably due to lactate, which Krebs has shown to catalyse the reaction. From the variation of the activity of different cultures it appears that aneurin is not the sole factor concerned. The viability of the organisms may be responsible

to some extent for these differences (cf. Table III); the phosphopyridine-nucleotide coenzymes may also be concerned since Krebs [1937] has shown that they catalyse the reaction and Knight [1937, 1, 2] that nicotinic acid or its amide is necessary for the growth of staphylococcus. With the more active cultures the rate of the reaction shows a marked falling off towards the end of the experiment in the presence of added aneurin when more than half the substrate appears to have been consumed.

It has now been shown that both the O_2 uptake and the anaerobic CO_2 production of aneurin-deficient staphylococcus in the presence of pyruvate are defective. We conclude that both the anaerobic and oxidative removals of pyruvate are impaired and are now in a position to explain some of the observed features of Fig. 2. The identity of the final rates of O_2 uptake for pyruvate in the presence of added aneurin and for lactate in the absence of added aneurin are evidently due to the occurrence of one and the same main oxidative process in both systems, i.e. the oxidation of lactate to pyruvate in the presence of the latter as inhibitor. In the presence of added aneurin pyruvate may be removed by dismutation. The inhibition of lactate oxidation by pyruvate accumulation is therefore not so pronounced in the presence of added aneurin. In an experiment with a smaller amount of lactate, which was prolonged until O_2 uptake had fallen almost to that of a control without substrate, in the presence of added

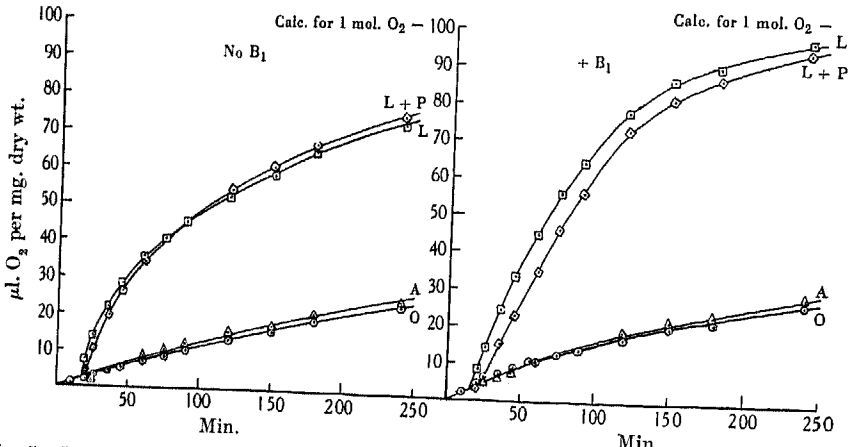

Fig. 3. Oxygen uptake of aneurin-deficient *Staph. aureus*. Phosphate, *p*H 7·3, *M*/30, 3·5 ml. fluid, 3·0 mg. cocci per vessel. No B_1 = no aneurin in suspension medium. + B_1 = aneurin in suspension medium $1{\cdot}1 \times 10^{-6}$ *M*. Substrates added at 15 min. to give 2·85 m*M* final concentration: O = control, A = acetate, L = lactate, P = pyruvate.

aneurin no pyruvate was detected by the Simon & Piaux nitroprusside test [1924]. The additional O_2 uptake with lactate above that of the control corresponded with the consumption of very nearly 1 mol. O_2 per mol. lactate (Fig. 3), in accordance with the equations:

$$2CH_3CHOH.COOH + O_2 = 2CH_3CO.COOH + 2H_2O, \quad \text{.....(2)}$$

$$2CH_3CO.COOH + H_2O = CH_3CHOH.COOH + CH_3COOH + CO_2, \quad \text{.....(1)}$$

$$CH_3CHOH.COOH + O_2 = CH_3COOH + H_2O + CO_2. \quad \text{.....(3)}$$

In the absence of added aneurin reaction (1) occurs very incompletely. The O_2 uptake is less and pyruvate accumulates (nitroprusside test). Fig. 3 also shows directly that added pyruvate has a definite inhibitory effect on the

O_2 uptake with lactate as substrate in the presence of aneurin; under these conditions the pyruvate formed by reaction (2) does not accumulate; in the absence of aneurin the accumulation of pyruvate is almost sufficient to mask the effect of added pyruvate. Acetate does not significantly increase O_2 uptake, either with or without added aneurin. It is therefore clear that the oxidation of the lactate formed by dismutation is mainly responsible for the O_2 uptake with pyruvate as substrate.

The effect of components of aneurin on metabolism

Knight has shown [1937, 2] that an equimolecular mixture of 4-amino-5-aminomethyl-2-methylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole is practically as effective as aneurin chloride itself for the growth of staphylococcus. These compounds have similar substituents to the corresponding parts of the aneurin molecule; the C atom attached to position-5 of the pyrimidine compound forms the bridge between this and the thiazole nitrogen in aneurin.

Fig. 4 shows the effect of adding these compounds singly, together and consecutively on the O_2 uptake of aneurin-deficient staphylococcus in the

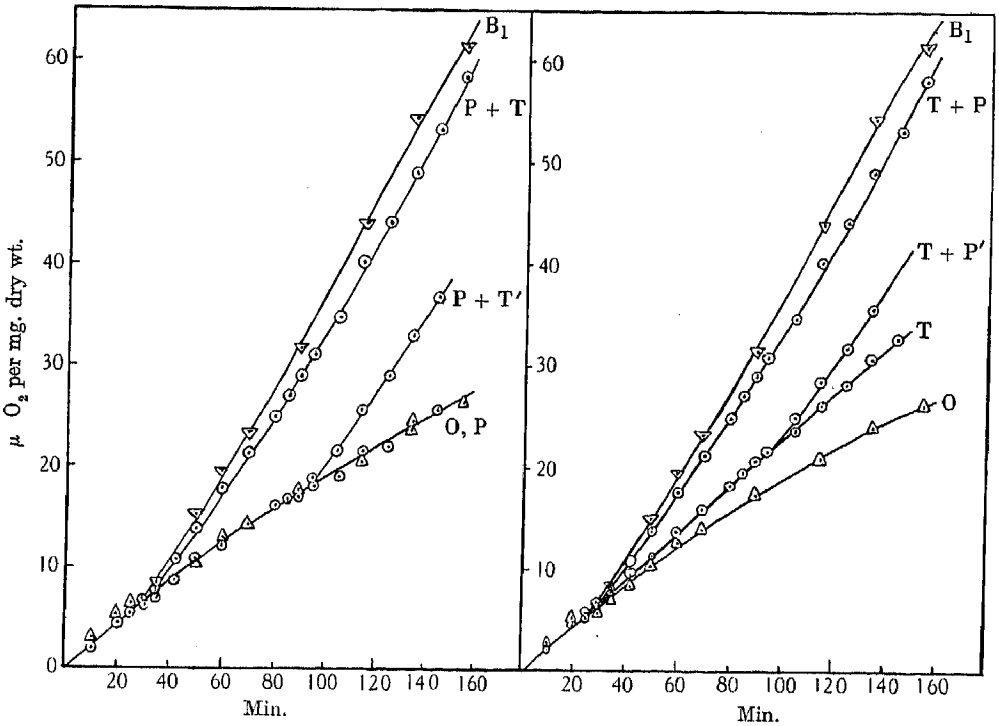

Fig. 4. O_2 uptake of aneurin-deficient *Staph. aureus*. Effect of aneurin and its components. Phosphate, *p*H 7·6, *M*/30. Pyruvate, *M*/40. Substances added to give final concentration $1{\cdot}2 \times 10^{-6}$ *M*: 4-amino-5-aminomethyl-2-methylpyrimidine at 28 min. = P, at 88 min. = P′; 4-methyl-5-β-hydroxyethylthiazole at 28 min. = T, at 88 min. = T′; aneurin at 28 min. = B_1; control = O.

presence of pyruvate. The effect of aneurin is shown for comparison and control vessels to which only distilled water was added are included.

The thiazole and pyrimidine separately have no significant effect in raising the O_2 uptake. In the experiment quoted the thiazole appears to prevent a

tendency of the respiration to fall, perhaps because this component is destroyed more rapidly in the washed cells than the pyrimidine. This small effect however has not been investigated further. As soon as both components are present together a large increase in O_2 uptake occurs, but there appears to be a slight delay before the effect becomes quite as great as that due to aneurin itself.

Table V shows that both components must be present before the anaerobic CO_2 production of aneurin-deficient staphylococci reaches a value similar to that in the presence of added aneurin.

Table V. *Effect of aneurin components on* $Q_{CO_2}^{N_2}$ *with pyruvate*

Bicarbonate, 18 m*M*, 5% CO_2 in N_2. *p*H 7·25. Pyruvate, 25 m*M*.

Effects of:

(*a*) 4-Amino-5-aminomethyl-2-methylpyrimidine, $1{\cdot}2 \times 10^{-6} M$ = P.
(*b*) 4-Methyl-5-β-hydroxyethylthiazole, $1{\cdot}2 \times 10^{-6} M$ = T.
(*c*) Aneurin chloride, $1{\cdot}2 \times 10^{-6} M = B_1$.

$Q_{CO_2}^{N_2}$ at time (min.)

Substance added at time (min.)	0–30 min.	30–60 min.	70–90 min.	100–120 min.
	4·15 mg cocci per vessel. Grown with $4 \times 10^{-9} M$ aneurin			
None	17	22	26	29
B_1 at 60	17·5	22	37	42·5
T at 30; P at 60	18	21	35·5	47
P at 30; T at 60	16·5	21·5	33	50
P at 30; T at 90	17·5	22	28	40
	6·25 mg. cocci per vessel. Grown with $3 \times 10^{-9} M$ aneurin			
	0–30 min.	35–50 min.	60–75 min.	80–110 min.
None	19·5	23	26	24
B_1 at 45	20	25	52	53
T at 30; P at 45	18	23	35·5	43·5
T at 30; P at 55	20	24·5	26·5	43
P at 30; T at 45	20	24	38	46
P at 30; T at 55	18	30	38	47·5

Whether it is necessary for the organism to synthesize aneurin from the two components supplied, or whether the presence together of the appropriately substituted pyrimidine and thiazole rings is a sufficient condition for the metabolism of pyruvate cannot be definitely decided from the above data. It is possible that aneurin may be broken down by the organism, and the two parts of the molecule used independently. The full effect of aneurin itself seems to appear slightly earlier than when the two components are added simultaneously from separate bulbs, suggesting that during the lag period aneurin is being formed. According to R. R. Williams [quoted by Robbins *et al.* 1937] it is to be expected theoretically that the corresponding 5-bromomethylpyrimidine would combine with the thiazole in acid solution at room temperature, although perhaps at a very slow rate unless catalysed. Such a synthesis, however, has not been demonstrated *in vitro*. It is possible that an enzyme in staphylococcus might catalyse such a reaction. At any rate, it is clear that both components of the aneurin molecule are needed for normal pyruvate metabolism.

Lipmann has shown [1936] that aneurin can be reduced by hydrosulphite in an analogous manner to the phosphopyridinium nucleotide coenzymes of Warburg & Christian [1936] and the nicotinamide methiodide model of Karrer *et al.* [1936]. Lipmann concludes that the activity of the molecule depends on

the presence of the thiazylium nitrogen analogous to the pyridinium nitrogen in the model and that in the coenzymes. None of the above observations on the activity of aneurin components is in conflict with the necessity for a thiazylium nitrogen; the suggestion that the thiazole component might be more rapidly destroyed in the aerobic experiments seems highly probable if the thiazylium nitrogen forms part of a reversible oxidation-reduction system. It is suggestive that the oxidized forms of the pyridine nucleotides are also unstable in alkaline solution, although stable in acid [Warburg & Christian, 1936].

SUMMARY

The effect of aneurin on the metabolism of *Staphylococcus aureus* occurs at very low concentrations and can only be observed in organisms which have received an inadequate supply of the vitamin during growth.

Both the pyrimidine and the thiazole rings of aneurin are necessary for the normal metabolism of pyruvate by staphylococcus whether conditions are aerobic or anaerobic.

The mechanism of pyruvate oxidation and the mode of action of aneurin are discussed.

I wish to thank Dr P. Fildes, at whose suggestion the work was first undertaken, and Mr B. C. J. G. Knight for many valuable suggestions; Dr G. P. Gladstone whose bacteriological work made possible the metabolic studies described in this paper; and Prof. E. C. Dodds for permission to carry out this work, during the tenure of a MacKenzie-MacKinnon Fellowship of the Royal Colleges of Physicians and Surgeons.

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