CCCV. THE NUTRITION OF *STAPHYLOCOCCUS AUREUS*. NECESSITY FOR URACIL IN ANAEROBIC GROWTH.

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AEROBICALLY, *Staphylococcus aureus* has been grown [Fildes *et al.* 1936] on a mixture of nutrients of which only one component was unknown [Knight, 1935]. This medium would not however permit anaerobic growth. For growth anaerobically, it was necessary to add pyruvic acid and a second unknown substance (Factor III, Fildes *et al.* [1936]). By a process of fractionation, confirmed by synthesis, this substance is now identified as uracil.

EXPERIMENTAL.

The general bacteriological technique is described elsewhere [Fildes *et al.* 1936]. Any details omitted here may be found in that paper.

Conditions of test.

The object of early work was the isolation of the unknown substance from crude marmite. In consequence the bacterial test was designed, at first, to show the distribution of activity after a given chemical fractionation. The fractions obtained were tested by addition to the standard medium in serial five-fold dilutions; dilutions giving comparable growths in 20–24 hours were considered to have similar contents of active material. In this way the result of any fractionation could be estimated. For later work on the inherent activity of purines, pyrimidines and other cyclic nitrogenous compounds, however, experiments had to be of longer duration. It soon became obvious that traces of growth might occur after a delay of 3 days without addition of any active substance. The reason for this has not yet been fully determined, but it was possibly due in part to a failure in anaerobic technique.

Anaerobic technique. With *Staph. aureus* growing anaerobically, oxygen is an impurity of which the merest trace may vitiate an experiment. The organism, without uracil, is under a restriction which it may escape if air is present. There is, in consequence, an extreme sensitiveness to traces of oxygen. To ensure uniformity of atmosphere in any one experiment, use was made of the McIntosh and Fildes anaerobic jar. For each filling, however, it was thrice exhausted on a Geryk pump and refilled with hydrogen + 5 % CO₂ [Gladstone *et al.* 1935] before warming the platinized asbestos capsule. Occasionally, separate platinized asbestos mats were also added. If an experiment was to continue after the first reading, opacities were estimated and the tubes returned to anaerobic conditions as quickly as possible; any subsequent growth was always regarded with some suspicion. For the more exacting work, an experiment in its early stages was observed through the jar without opening it.

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Nevertheless, these procedures could not be wholly relied on to eliminate traces of visible growth from the control. Since the atmosphere of the jar seemed free from oxygen according to indicators, it was concluded that oxygen might be dissolved in (or combined with) the medium itself. A small amount of O/R dye was therefore added to the medium; since the latter already contained a ferrous salt, the reduction of dye which was apparent in a few hours was considered to indicate the removal of oxygen. This procedure resulted in controls which were more nearly satisfactory. Nevertheless, the slight, delayed growths without uracil did not seem wholly attributable to the presence of oxygen. This matter will be referred to again.

Medium. The medium is described elsewhere [Fildes *et al.* 1936]. Its organic components were: amino-acids, glucose, pyruvic acid, an organic sulphur compound and the factor described by Knight [1935]. The organic sulphur compound was specified as dithiodiglycolic acid but in most of this work thiolacetic acid was used. As stock solution, a *M*/10 solution in *M* HCl was autoclaved and was stable for weeks. 0.2 ml. was added per tube together with 0.25 ml. *M* NaOH, making the concentration of —SH in the experiment *M*/500. Comparative tests have not shown any difference in the effect of uracil whether in the presence of —SH or —SS—. *M*/250,000 indigotetrasulphonate was used as indicator regulating the oxygen effect, on account of its lack of toxicity in immersion experiments [Chambers *et al.* 1931].

Preparation of test samples. Fractions for test were freed from any toxic materials used in fractionation, adjusted roughly to pH 7.6, brought to a dilution equivalent to a 1% solution of the original crude marmite, autoclaved and tested in fivefold serial dilutions.

Distribution of accessory substance.

Of raw materials tested, crude marmite was active at highest dilution in permitting growth. "Bacto peptone" and aqueous extracts of liver and malt showed moderate activity, whilst minced meat and pea extracts, and caseinogen, gelatin, egg albumin and edestin hydrolysates showed little or none. It is now seen that this distribution coincides with the probable distribution of nucleic acid and similar substances in the crude extracts.

Chemical behaviour of accessory substance from crude marmite.

As present in crude marmite, the substance was stable to autoclaving and to 3 *N* H₂SO₄ (8 vol. %) either at 130° for 3 hours or during 18 hours' boiling. Its activity, in fact, was enhanced by acid hydrolysis. It was incompletely destroyed by 18 hours' boiling with 3 *N* NaOH or Ba(OH)₂. It was stable to heating at 200° for 5 min. but was partially destroyed at 250° in 15 min. It was not distillable in a high vacuum at the latter temperature.

It was not extractable by ether from aqueous solution at any pH and only with difficulty by *n*-propyl and *n*-butyl alcohols. The latter extraction proceeded better (on hydrolysed marmite) at pH 1 or 6 than at pH 10, and six extractions under the former conditions removed about half the activity. Continuous extraction by the Dakin method proceeded rather slowly. An attempt to utilize the apparent weakly acidic properties of the substance in order to transfer it from aqueous marmite to *N* Na₂CO₃ solution by continuous rocking under a layer of butyl alcohol was only moderately successful. It was however possible, by shaking with equal volumes of aniline and alcohol, to extract 40% of the activity in one shaking into the aniline-alcohol layer. From dry "Bacto peptone", the

activity could be partially removed by continuous extraction with absolute alcohol for 12 hours. Progressive addition of alcohol to strong solutions of hydrolysed or unhydrolysed marmite produced merely a progressive precipitation of activity.

The active material was not significantly precipitated by $\text{Ba}(\text{OH})_2$, lead acetate (at $p\text{H}$ 6) or basic lead acetate (at $p\text{H}$ 7.4, Britton & Meek [1932]). It was partially precipitated by $\text{Ba}(\text{OH})_2$ followed by 75% alcohol. It was not notably adsorbed upon fuller's earth, alumina, kieselguhr or frankonite at $p\text{H}$ 6, or upon norite at any $p\text{H}$. It was partially precipitated by phosphotungstic acid in 5% H_2SO_4 . It was not precipitated by mercuric acetate or chloride in aqueous or 50% alcoholic solution, or by the sulphate in 5% H_2SO_4 . It was, however, precipitated by acid HgSO_4 in 66% alcohol, by HgCl_2 in absolute alcohol and by Neuberg's mercuric acetate-sodium carbonate reagent for amino-acids.

At this stage therefore the possibility of distinguishing between the active material and those amino-acids which comprise about 40% of the crude marmite seemed quite remote. Indeed, it seemed feasible, in view of the acknowledged importance of amino-acids in the nutrition of bacteria [Knight, 1936] that we were dealing with some uncommon amino-acid not present in proteins. In opposition to this view, the activity was not destroyed by nitrous acid; but the product of such treatment might easily have had similar biological activity.

Preparation of active concentrates from crude marmite.

There were then two methods which might give a practical separation of the active material from the bulk of the amino-acids: precipitation by acid HgSO_4 in 66% alcohol and serial extraction by aniline-alcohol. Owing to the difficulty of maintaining the right proportions in an extraction by a ternary liquid mixture, the former method was selected.

A strong solution (dry weight 50%) of marmite was precipitated with 3 vol. absolute alcohol and left overnight at 0° . The clear supernatant liquor, though of considerably diminished activity, was in a more workable condition. It was treated with 25% HgSO_4 in 10% (by vol.) H_2SO_4 , until the curdy precipitate gave place to a somewhat granular one, and filtered. The precipitate was suspended in water, freed from Hg^{++} and SO_4^{--} , and concentrated. It was then brought to $p\text{H}$ 4 and fractionated by the AgNO_3 - $\text{Ba}(\text{OH})_2$ method for imino-compounds. The active material was in the pyrimidine-glyoxaline fraction.

The medium already contained histidine, and it was soon demonstrated that thiolhistidine also had no effect. The naturally occurring pyrimidines and purines were therefore submitted to test.

Preparation from yeast nucleic acid.

Commercial preparations of yeast nucleic acid and of cytidylic acid both contained much active material, the activity being enhanced on acid hydrolysis in the usual way. By fractionation of the acid-hydrolysed nucleic acid with AgNO_3 - $\text{Ba}(\text{OH})_2$, the active material was again obtained in the pyrimidine fraction. Treatment of the latter with picric acid left the bulk of the activity in the uracil fraction. On the other hand, both picrates and flavianates from the pyrimidine fraction (which presumably contained traces of guanine, adenine and hydrolytic products therefrom, in addition to cytosine), showed quite appreciable activities. Likewise, a commercial guanine preparation showed some

activity, though an adenine preparation had none. It was clear that a final decision on the relative importance of various cyclic compounds of this type could only be reached by using synthetic materials.

Uracil as the active substance.

In Table I are shown the effects of selected pyrimidines, purines and other cyclic nitrogenous compounds, mostly synthetic materials, on the strictly anaerobic growth of *Staph. aureus*. Clearly, only uracil is fully active in permitting anaerobic growth. If the other compounds can be utilized (and it is

Table I. *Effect of uracil and related substances on the anaerobic growth of Staph. aureus on the basal medium.*

Compound	Constitution (enolic tautomeride)	Growth after		
		24 hr.	44 hr.	68 hr.
Control	—	0†	0	0
Control	—	0	0	0
Control	—	0	0	0
Uracil,* <i>M</i> /20,000	2:6-Dihydroxypyrimidine	++	+++	+++
<i>M</i> /100,000	"	tr.	++	++
<i>M</i> /500,000	"	?	++	++
<i>M</i> /2,500,000	"	0	+	+
Thymine,* <i>M</i> /20,000	5-Methyluracil	0	0	tr.
<i>M</i> /100,000	"	0	0	?
4-Methyluracil,* <i>M</i> /20,000	4-Methyluracil	0	0	+
1:3-Dimethyluracil,* <i>M</i> /20,000	1:3-Dimethyluracil	0	0	tr.
1:3:4-Trimethyluracil,* <i>M</i> /20,000	1:3:4-Trimethyluracil	0	0	tr.
Thiothymine,* <i>M</i> /20,000	2-Thio-5-methyluracil	0	0	tr.
Barbituric acid, <i>M</i> /20,000	4-Hydroxyuracil	0	0	tr.
Barbitone, <i>M</i> /20,000	(5:5-Diethylbarbituric acid)	0	0	—
Xanthine, <i>M</i> /20,000	2:6-Dihydroxypurine	0	0	+
Theobromine, <i>M</i> /20,000	3:7-Dimethylxanthine	0	0	tr.
Uric acid, <i>M</i> /20,000	2:6:8-Trihydroxypurine	0	0	tr.
Guanidine HCl, <i>M</i> /20,000		0	0	+
Urea, <i>M</i> /20,000		0	0	tr.
Control		0	0	?
Pyrimidine and purine bases.				
Cytosine,* <i>M</i> /20,000	2-Hydroxy-6-aminopyrimidine	0	0	tr.
isoCytosine,* <i>M</i> /20,000	6-Hydroxy-2-aminopyrimidine	0	0	0
Guanine,* <i>M</i> /20,000	6-Hydroxy-2-aminopurine	0	0	tr.
Adenine, <i>M</i> /20,000	6-Aminopurine	0	0	0
4 Bases as above, <i>M</i> /20,000 (each)		0	+	++
4 Bases with uracil, <i>M</i> /20,000 (each)		++	+++	+++
Vitamin B ₁ , <i>M</i> /17,000	Derivative of 6-aminopyrimidine	0	0	—
Other cyclic nitrogenous compounds.				
Thiolhistidine,* <i>M</i> /2000		0	—	—
Hydantoin,* <i>M</i> /20,000		0	0	?
Allantoin, <i>M</i> /20,000		0	0	?
Creatinine, <i>M</i> /20,000		0	0	?

* Substance of known synthetic origin.

† ++ = maximum growth, other plus signs roughly proportional. tr. = just visible opacity.

? = dubious opacity in comparison with 0 (none). — = not observed.

‡ = jar opened. Tubes returned to anaerobic conditions before O/R indicator was visibly coloured.

unwise, even with controls, to draw conclusions from delayed growths which have been exposed to air) the organism is considerably inconvenienced in using them. It is possible that the evidence of summation in the utilization of the four bases has significance, but even there, the organism is severely limited by its lack of uracil.

Uracil was synthesized by the method of Davidson & Baudisch [1926], simplified by dissolving the malic acid and urea (1 part of each) in 2 parts warm conc. H_2SO_4 before adding 8 parts of fuming H_2SO_4 (20% SO_3). Yields were comparable (53%). (Found: N (micro-Kjeldahl with selenate catalyst and added glucose), 24.9%. $\text{C}_4\text{H}_4\text{O}_2\text{N}_2$ requires 25.0%.)

Cytosine and isocytosine were synthesized from uracil by the methods of Hilbert *et al.* [1935]. (Cytosine. Found: N, 32.4%. $\text{C}_4\text{H}_5\text{ON}_3 \cdot \text{H}_2\text{O}$ requires 32.6%. *Isocytosine* (known to be contaminated with cytosine). Found: N, 36.4%. $\text{C}_4\text{H}_5\text{ON}_3$ requires 37.8%.)

Guanine was synthesized from guanidine thiocyanate and ethyl cyanoacetate by the method of Traube & Dudley [1913]. (Guanine. Found: N, 46.0%. $\text{C}_5\text{H}_5\text{ON}_5$ requires 46.4%.)

Thymine was synthesized from thiourea and ethyl α -formylpropionate by the method of Wheeler & MacFarland [1910]. To minimize the possibility of final contamination by uracil, the dry ethyl propionate in the initial stage was fractionated and the first half of the distillate discarded. The 2-thiothymine formed in the intermediate stage was recrystallized from water. (Found: N, 21.9%. $\text{C}_5\text{H}_8\text{O}_2\text{N}_2$ requires 22.2%.)

Hydantoin, 2-thiolhistidine, and the methylated uracils were synthetic materials kindly supplied by Prof. C. R. Harington and Dr H. R. Ing.

Vitamin B₁ was a crystalline preparation for which we are indebted to Prof. R. A. Peters.

Synthesis of active substance by Staph. aureus growing aerobically.

60 ml. of a 3-day aerobic culture of *Staph. aureus* on the aerobic medium without uracil [Fildes *et al.*, 1936] were centrifuged, and the supernatant liquor decanted for test for active substance after sterilization by filtration. The bacterial matter was mildly hydrolysed by boiling for 90 min. with M HCl , neutralized, diluted to 30 ml. and also tested for active substance (Table II).

Table II. *Synthesis of active substance by Staph. aureus growing aerobically.*

	Growth in 3 days
Control	0
Control	0
Filtrate (diluted 1/10)	+
Filtrate (diluted 1/50)	0
Hydrolysed bacteria (equivalent dilution 1/5)	+ +
Hydrolysed bacteria (equivalent dilution 1/25)	+
Hydrolysed bacteria (equivalent dilution 1/125)	0
Control (M /20,000 uracil)	+ + +

Clearly, a substance having the growth-promoting properties of uracil is synthesized by *Staph. aureus* aerobically. Chemical identification of the uracil, however, must be deferred until the amino-acid nutrition of the organism has been adequately studied and simplified.

The synthesis of active substance thus demonstrated under aerobic conditions may have some bearing upon the delayed growths without uracil under anaerobic conditions which have been referred to above. It is obvious in Table I that growth of *Staph. aureus* without uracil was in no way comparable with its growth with uracil. If this need for added uracil is due to failure to synthesize it, the failure need not, of course, be absolute. In this event, traces of growth without uracil might reasonably be expected.

Synthesis of active substance is also accomplished by *Bact. typhosum* growing aerobically on ammonium lactate medium. Indeed, the general phenomenon seems comparable with that, for instance, of the metabolism of tryptophan. This amino-acid is synthesized by those bacteria which by nature or training can grow without a supply of this important nutrient [Fildes & Knight, 1933].

DISCUSSION.

It has been pointed out elsewhere [Fildes & Richardson, 1935] that the criterion of indispensability adopted for work on bacterial nutrition differs in kind from that adopted in animal nutrition. In studying the growth of our small inoculum (dry weight about 4×10^{-6} mg.) we are dealing with a successful propagation through many generations and an increase of weight of about a million-fold. Any substance which is a limiting factor in this propagation is therefore probably of deep significance to cellular nutrition in general.

Uracil is interesting, therefore, not alone in relation to the growth of *Staph. aureus* growing anaerobically. It is synthesized in the aerobic growth of both *Staph. aureus* and *Bact. typhosum* (the only other organism tested), and may be presumed to have an important function in bacterial metabolism in general. It would be premature to speculate what this may be. One may note, however, that although adenylypyrophosphate and cozymase (two adenine nucleotides) are both coenzymes active in various phases of hexose metabolism, no function has yet been assigned to other nucleotides. It is possible that a nucleotide derived from uracil exerts an effect in the metabolism of *Staph. aureus* which no derivative of adenine or like substances can exert.

The discovery that this anaerobic growth substance is actually uracil, moreover, suggests that uracil has a wide distribution in nature. This view contrasts with the older supposition that uracil is not a component of animal tissues, though characteristic of plants. It also confirms the argument of Levene [1926] that uracil is indeed a primary constituent of tissues and not a product of the deamination of cytosine by the process of isolation [Jones & Perkins, 1925]. Uracil (or its derivatives) will probably be found widely distributed in bacteria. It is present in beer yeasts and is a primary component of at least the liver of certain mammals.

Of course this argument rests on the presumed specificity of the bacterial test for uracil. As far as we have been able to show, this test is at least as specific as the more common colour reactions. It is, moreover, highly sensitive (Table I) and, we consider, is a valuable micro-test for uracil where none has been previously available.

SUMMARY.

Anaerobic growth of *Staph. aureus* upon a mixed amino-acid medium containing pyruvic acid and other necessary nutrients [Fildes *et al.* 1936] was only possible, under the conditions of test, upon addition of uracil (2:6-dihydroxypyrimidine). Of twenty-one related compounds which were tested, none had a comparable effect in permitting growth. The significance of this to the distribution and function of uracil in nature is briefly discussed.

I am greatly indebted to Dr P. Fildes for his active help and advice throughout this work, and to the other members of this department for assistance at all stages. I wish to thank the Marmite Food Extract Co., Ltd., for generous supplies of crude marmite and Dr O. Rosenheim for some specimens of natural bases.

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Note added 30 November 1936. Preliminary tests with 5 further strains of *Staph. aureus* confirm that the basal medium was adequate for aerobic, but not for anaerobic, growth. Anaerobically, addition of uracil permitted growth of 3 strains, whilst hydrolysed nucleic acid was required for the remaining 2 strains. Of the latter, the only one tested depended upon guanine and uracil for good growth. The species in general seems, therefore, to need various constituents of nucleic acid according as its synthetic powers are limited by the conditions of anaerobic growth.