PRELIMINARY NOTE ON INHIBITION OF BACTERIAL GROWTH BY AMINO-ACIDS[1].

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(With 2 Charts.)

A good deal of Pasteur's earlier work was carried out with various forms of bouillon prepared from meat, *i.e.* media containing appreciable percentages of amino-acids. Subsequently the percentage of amino-acid in bacteriological media was increased by reinforcements of the meat extract by soluble protein in the form of peptone.

But recently the value of amino-acids, as such, in promoting bacterial growth has been much investigated.

One of the first important investigations with regard to the value of different forms of nitrogenous food, for bacterial nutrition, was that of Bainbridge (1911). He demonstrated the incapacity of most bacteria to digest pure proteins and as a corollary emphasised the importance of products of protein disintegration. These results were confirmed and amplified by Rettger, Berman and Sturges (1916) and Berman and Rettger (1918). During the last six or seven years an increasing stress has been laid upon the importance of amino-acids in promoting bacterial growth and tryptic digests have been generally recommended and very largely used in the preparation of bacterial culture media (Douglas (1914), Cole and Onslow (1916), Cunningham (1918)). But in view of our own observations there is a surprising lack of any reference to inhibition of bacterial growth by excess of amino-acids. It is true that a short period of tryptic digestion is recommended in Douglas' method but little detail is given about the amino-acid content of such media by the author.

A careful study of the effects of tryptic digests on the growth of *B. typhosus* has been contributed by Norris (1918) and if he had not chosen for his observation one of a group of bacilli which is amongst the least sensitive to considerable concentrations of amino-acid the phenomena which we are about to describe would not have escaped him. In any case he points out that the products of prolonged hydrolysis by tryptic ferments or even of 24 hours hydrolysis at raised temperature (55° C.) are inferior in their growth promoting powers to the products of digestion for shorter periods. He also points out that using hydrolysates of different substrates he did not find any close relationship between growth promoting power and amino-acid content.

Long (1919), however, in a careful study of the chemical composition of media necessary for the growth of tubercle bacilli points out that as an addition

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to a basal medium insufficient in itself, a tryptic digest of casein (biuret free) is inferior in its growth promoting powers to ordinary meat extract peptone broth or to a peptic digest of casein. He suggests as a possible explanation that any considerable concentration of amino-acid may be inhibitory to the tubercle bacillus.

Lastly, Burrows and Neymann (1919) have shown quite definitely that amino-acids in concentrations isotonic with the tissues, and in the case of some of the more insoluble amino-acids in considerably smaller concentrations, very definitely arrest growth in tissue cultures. They also show that mixtures of amino-acids produced by digestion of egg-yolk with acid, which were subsequently carefully neutralised, did the same; but that peptone prepared from egg-yolk had no such effect. Our own observations which we shall now proceed to describe are not intended to throw any doubt on the accuracy of the general opinion that amino-acid is valuable in promoting growth of many bacteria but they are intended to emphasise the ease with which the amino-

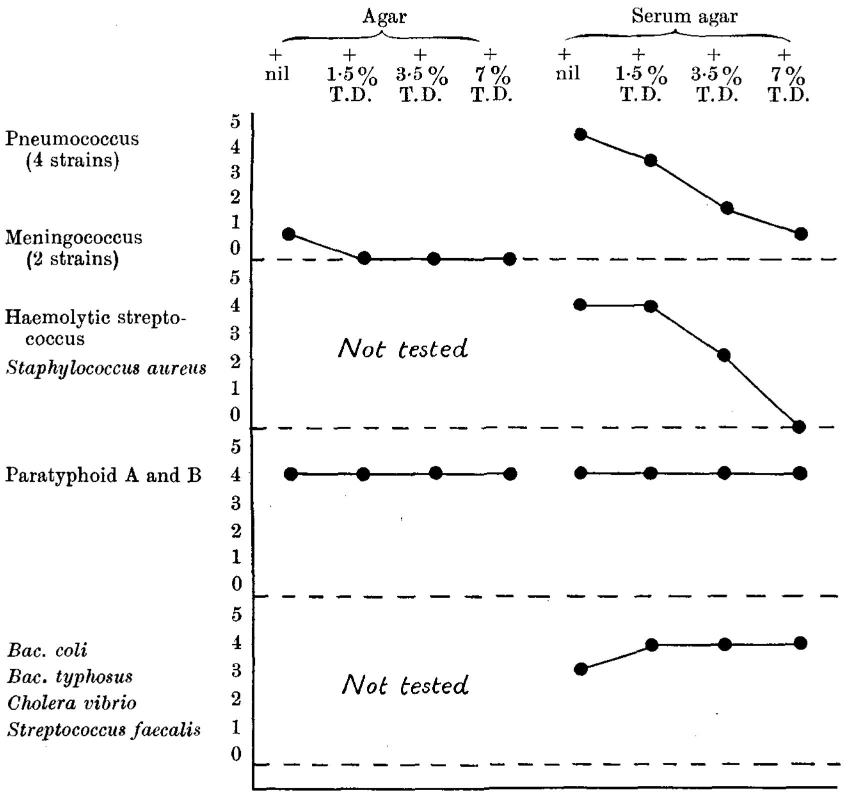

Chart 1. Represents effects on the growth of various bacteria of additions of amino-acid in the form of a tryptic digest of coagulated horse serum:

(*a*) to plain agar;

(*b*) to plain agar + 10 % of horse serum previously heated at 56° C.

(The trypsin was obtained aseptically from the pancreatic duct of a dog, and activated by a sterile filtrate of an aqueous extract of intestinal mucous membrane.)

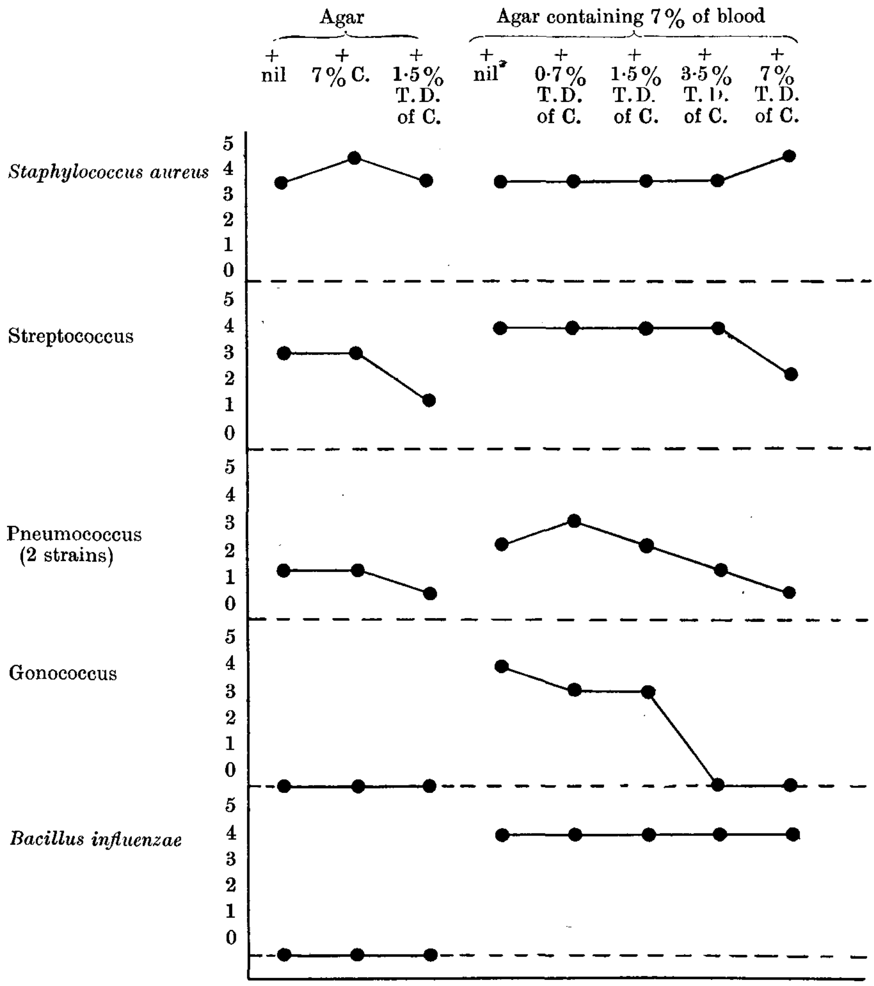

Chart 2. Represents the effect of the addition of a tryptic digest of casein:

(*a*) to agar;

(*b*) to agar + 7 % of unheated sterile blood.

The effect of an addition of undigested casein is also shown.

T.D. = Tryptic Digest of serum. C. = 10 % suspension of Casein. T.D. of C. = Tryptic digest of 10 % Casein. The curves represent the amount of growth of various bacteria as modified by changes in the media. The bacteria whose growth was similarly affected are taken together. The dotted line represents no growth in each of the horizontal compartments, and heights above the base line represent degrees of growth, the five degrees indicating: 1. A few colonies. 2. Numerous colonies, easily counted. 3. Colonies too numerous to be easily counted. 4. Colonies coalescing. 5. Very heavy growth.

acid content of a medium can be raised beyond the point at which it is favourable to the growth of some bacteria.

Our attention was drawn to the matter by observations made in the course of some work on the factors underlying the growth promoting property of serum for pneumococcus, streptococcus, etc. (McLeod and Wyon, 1921).

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It was found that when serum was subjected to tryptic digestion three phases in its value for promoting the growth of these delicate bacteria could be observed to succeed one another: (1) its value was slightly increased or unaffected, (2) it lost its growth promoting power, (3) it became inhibitory.

Examples of phase (3) are set out in Charts I and II.

The general indications from these results are that a digest of serum seems to be more inhibitory than one of casein, but that in the concentrations employed there is a definite inhibition of streptococcus, gonococcus, pneumococcus and meningococcus but not of the intestinal bacteria, or of *B. influenzae*.

INVESTIGATIONS OF SEPARATE AMINO-ACIDS, ETC.

In view of the above results an attempt was made to investigate the phenomenon more fully.

As a result it was found that several of the hardier organisms were inhibited by moderate concentrations of amino-acid, viz. *Staphylococcus albus* and *aureus*, *B. subtilis*, *B. dendroides*, *B. dysenteriae* (Flexner), *B. diphtheriae*.

Tyrosine in a single experiment in a concentration of 1 % inhibited *B. pyocyaneus* slightly and *B. proteus* completely. And in another experiment *B. pyocyaneus* was inhibited by a high concentration of glycine (2·5 %).

B. typhosus, *B. paratyphosus* and *B. prodigiosus*, however, were never inhibited by any of the concentrations of amino-acid used.

METHOD.

Several different media were used in these experiments, and those for which results are recorded were:

Medium 2. Peptone 1 %, NaCl 0·5 %, meat extract agar.

Medium 3. Peptone 0·5 %, agar.

Medium 7. Peptone 0·2 %, KH_2PO_4 0·3 % in water.

The steps in preparing the completed media were as follows: (1) one of the above basal media in concentrated form was adjusted colorimetrically to P_H 7·6 and autoclaved; (2) a 5 % (or less) solution of the amino-acid under examination was adjusted when possible, to P_H 7·6 and sterilised by boiling; (3) a constant amount of (1) was mixed with varying amounts of (2) and sterile distilled water was added so as to make the total quantity equal throughout a series of media of different amino-acid content.

The mixtures were made with sterile pipettes in order to avoid the need of further sterilisation.

With some amino-acids, such as tyrosine, acid had to be used to attain solution. In these cases the required alkali was added to the final mixture.

Where several organisms were to be tested simultaneously, plates of the media were poured, the plates marked out into sections and each bacterium inoculated on one of these. Inoculations were made with a fine platinum wire from emulsions in sterile peptone water of 24 hour agar or blood agar cultures.

In the case of the fluid medium (7) growth was judged by daily subcultures to agar. Controls of the basal medium alone made up to standard volume were always employed.

The organisms used were:

(1) *Staphylococcus aureus* throughout.

(2) In some experiments the following in addition: *Staphylococcus albus*, *B. subtilis*, *B. dysenteriae* (Flexner), *B. diphtheriae*, *Pneumococcus*, *Streptococcus haemolyticus*, *Streptococcus faecalis*.

All but the last suffered inhibition at one time or another, the staphylococci appearing to be on the whole the most sensitive, and *S. albus* particularly so.

In some observations made with a glucose, nucleic acid, phosphate agar medium the following were usually inoculated and never showed inhibition: *B. typhosus*, *B. paratyphosus*, *B. prodigiosus*.

The number of different concentrations of amino-acid employed was kept rather small on account of the immense labour involved in preparing a large number. Hence some of the gaps recorded between the inhibitory and non-inhibitory concentrations are wider than is desirable.

The amino-acids were obtained from various sources, commercial and private. There was no reason to doubt their purity, except perhaps in the case of tryptophane. Estimations of amino-nitrogen by Van Slyke's nitrous acid method were carried out for 4 of the 11, alanine, tyrosine, aspartic acid and glutamic acid with the following results:

	Amino-nitrogen observed	Per cent. calculated
Alanine	14·67	15·73
Tyrosine	7·43	7·73
Aspartic acid (di-sodium salt)	8·38	7·91
Glutamic acid hydrochloride	8·09	7·65

The nitrogen content of histidine was indirectly verified by a Van Slyke estimation of a mixture containing tyrosine 0·1 %, glutamic 0·5 % and aspartic acids 0·5 % and histidine hydrochloride 0·5 %.

The figures for the acid digest and for autolysed yeast are calculated from Van Slyke estimations on the assumption that all the amino-acid is monamino. This of course introduces an error, but the order of magnitude of the figures so obtained is almost certainly correct.

For these last the figures quoted in columns 5 and 6 of the Table are calculated as follows:

If V is the number of milligrams of amino-nitrogen (Van Slyke) found per cubic centimetre of digest, then the concentration in millimols per litre of amino-acid is $\frac{1000V}{14}$.

If there is X per cent. digest in the medium, the concentration of amino-acid in the medium is $\frac{1000V}{14} \times \frac{X}{100}$, which $= \frac{VX}{1\cdot4}$ millimols per litre.

Column 7 represents the mean of the inhibitory and non-inhibitory concentrations and represents approximately the limiting concentration beyond which growth is unlikely to occur.

1	2	3	4	5	6	7	8
				Millimols per litre			
Bacteria inhibited	Medium 2. Peptone 1 % Meat extract agar	Medium 3. Peptone 0·5 % agar	Medium 7. Peptone 0·2 % KH_2PO_4 0·3 %	Inhibitory	Not inhibitory	Mean of columns 5 and 6	Per cent. amino-acid corresponding to column 7
I							
S2	—	Histidine	—	14	9	11·5	0·18
S2, Sub., Fl.	Tyrosine	—	—	—	9	11·5	0·21
S2	—	—	Tyrosine	14			
S2	—	Tryptophane	—	30	20	25	0·51
S2	Cystine	—	—	40	20	30	0·96
S1, S2, Sub., K	—	Cystine	—	20	—	—	—
S2	Leucine	—	—	—	50	65	0·85
S1, S2, Fl., K	—	Leucine	—	80	—		
S1, S2, Sub., K	—	Alanine	—	150	80	115	1
S2, Sub., Fl., K; Str. H.	Glycine	—	—	150	110	130	0·96
Pneumo.	Glycine	—	—	210	—	—	—
S2	Glutamic acid	—	—	—	120	130	1·9
S2, Pneumo.	—	—	Glutamic acid	140	—		
S2	HCl-hydrolysate of casein	—	—	100	50	75	—
S2	Autolysed yeast	—	—	36	18	27	—
II							
S2	Asparagine	—	—	170	—	—	(2·25)
S2	Phenylalanine	—	—	—	30	30	0·5
S2	—	Phenylalanine	—	—	30		
Pneumo.	—	—	Phenylalanine	30	—	—	—
III							
S2	Aspartic acid	—	—	—	130	—	(1·73)
S2	Urea	—	—	—	740	—	(4·4)
S2	Sod. acetate	—	—	—	740	—	(4·4)

Abbreviations in column 1: S1 = *Staphylococcus albus*. S2 = *Staphylococcus aureus*. Sub. = *B. subtilis*. Fl. = *B. dysenteriae* (Flexner). K = *B. diphtheriae*. Str. H. = *Streptococcus haemolyticus*. Pneumo. = Pneumococcus. Section II gives cases where only incomplete determinations were made. Section III gives figures where no inhibition was observed.

RESULTS.

The amino-acids in section 1 of the Table, being those in which fairly complete determinations were made, are arranged in the order of potency as judged by the molecular concentration at the inhibitory threshold (column 7).

It will be noted that the most potent are the cyclic compounds, and that the potency of the monamino-monocarboxylic acids increases with molecular weight.

Of the two dicarboxylic acids, glutamic acid is of low potency, while aspartic acid showed no inhibition at a relatively high concentration.

Another observation (with medium 7) showed that glutaminic acid in high concentration (140 millimols) favoured the growth of *B. pyocyaneus* and *B. subtilis*, whereas glycine at 330 and tyrosine at 60 inhibited them. This is in accord with the statement of Long as regards the tubercle bacillus that dicarboxylic acids are peculiarly valuable for promoting its growth.

The result with sodium acetate (740 millimols), sodium chloride (1230 milli-

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mols), ammonium chloride (890 millimols) and urea (740 millimols) show that the physical effect of crystalloids in high concentration is not responsible for the phenomenon.

It is of interest, that such complex mixtures as acid-hydrolysate of casein and autolysed yeast are inhibitory at concentrations of amino-acid of the same order of magnitude as in the case of single amino-acids. Solutions of peptone were also tried in percentages varying by units from 10 to 2. Inhibition (delay of 24 hours) of *Staphylococcus aureus* was found present at 6 % and not at 5 %. Several formaldehyde titrations had been done with this particular peptone. A mean figure was taken and increased by 30 % which had been found in several cases to be the mean amount by which formaldehyde and Van Slyke estimations differed with the same material. (Opportunity did not occur for a Van Slyke estimation at the moment.) Calculation on this basis showed that 6 % peptone corresponded to 110 millimols amino-acid per litre and 5 % to 90—mean 100, again of the same order of magnitude as the inhibitory concentrations of single amino-acids.

In the sample of autolysed yeast used, the concentration 27 in the table corresponds to 9 % of the autolysate in the medium.

Funk (1920) found the stimulation of yeast growth by yeast extract to get less as concentrations of extract increased. This result he interpreted as giving evidence of the presence of inhibitory substances. It is suggested that this is another example of inhibition by amino-acid.

A biochemical observation made incidentally and quite beside the mark was that when testing high concentrations of glycine, tyrosine and glutaminic acid in medium 7 for inhibition of *B. pyocyaneus*, tyrosine delayed growth for 24 hours, but on the third day the tyrosine tube was the only one of the four (counting the control) which showed the green pigment associated with the organism. This may possibly be a new fact for workers on bacterial pigments.

Metabolism Experiment.

In the hope of elucidating somewhat the question why amino-acids should be inhibitory, a three-weeks metabolism experiment with *Staphylococcus aureus* was undertaken, using a high percentage of glycine and a low percentage of peptone, the idea being to see whether an amino-acid below but near the inhibiting concentration led to differences in metabolism which might suggest a reason for inhibition at slightly higher concentration.

Three media were inoculated simultaneously:

(1)	0·9 % glycine,	0·2 % peptone,	0·3 % KH_2PO_4
(2)	0·2 „	0·2 „	0·3 „
(3)		0·2 „	0·3 „

Results of some significance were:

(In milligrams of nitrogen per 5 c.c. medium.)

	Amino-nitrogen	Ammonia-nitrogen
(1)	Fall from 7·78 to 5·71.	Rise from 0·07 to 1·4.
(2)	Fall from 2·1 to 0·74.	Rise from 0·04 to 1·02.
(3)	Slight rise from 0·32 to 0·40.	Slight rise from 0·02 to 0·19.

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The figures for amino-nitrogen show clearly that the amino-acid is attacked and broken down at a concentration (0·9 %) not far removed from the inhibitory concentration.

Why the organism can attack the amino-acid at a concentration of 0·9 % and not at 1·1 %, as determined in other experiments, remains a problem.

DISCUSSION.

It is interesting that the amino-acid concentrations found inhibitory are on the whole similar to, though somewhat lower, than those recorded by Burrows and Neymann as inhibitory for growth of animal tissue; these authors, however, did not attempt to determine the lowest inhibitory concentrations for most of the amino-acids which they tested. There is one notable divergence, however, Burrows and Neymann found 0·05 % cystine inhibitory to the growth of tissue cultures; whereas in our experiments cystine is one of the less inhibitory amino-acids.

In this connection it is interesting to note that of all amino-acids tested cystine was found by Davis and Ferry (1919) to be the most active in promoting growth and toxin production by *B. diphtheriae* in a medium otherwise unsuitable for toxin production; the concentration of cystine which they added was 0·05 %.

If it is accepted that certain bacteria are readily inhibited in their growth by low concentrations of amino-acid, as our results indicate, a difficulty arises in bringing that finding into harmony with those of other observers (Lloyd and Cole, 1916 and others).

This difficulty consists in explaining the reputed value of tryptic digests or of impure peptones for promoting the growth of such bacteria.

Since the observations of Bainbridge and others exclude the unaltered proteins as sources of nitrogenous food and since our own observations show that the presence of considerable amounts of free amino-acid will inhibit the growth of such bacteria, it seems probable that some other factor is responsible for the value of these preparations.

It appears to us that this factor is the richness of these preparations in polypeptide molecules produced in the earlier stages of protein disintegration.

Some of our experiments in which the growth promoting powers of samples of tryptic digests of tissues taken at various phases of digestion were compared showed that for bacteria not inhibited by high concentrations of amino-acids a relatively small quantity of digest taken late was as good as a larger portion of similar amino-acid content taken earlier in the digestion, whereas a bacterium like the streptococcus (haemolytic) sensitive to high amino-acid concentration was most favoured by the larger quantity of less completely digested material. In any case the conclusion suggested is rendered probable by the results of Berman and Rettger who show that when grown on various impure commercial peptone solutions many bacteria incapable of utilising peptone or proteose, as such, produce a marked fall in the biuret reacting material present in these peptones. This fall is most reasonably ascribed to utilisation by the bacteria of biuret reacting polypeptide molecules.

Lastly, Bunker (1919) in a study of toxin production by the diphtheria bacillus has shown very definitely the importance of the polypeptide fraction in promoting this activity of the bacillus.

SUMMARY.

While amino-acids in appropriate concentrations have long been known as valuable aids to bacterial growth, it does not appear to have been recognised that in relatively low concentrations they are often inhibitory.

Inhibition has been shown to occur with 10 out of 11 single amino-acids tested and with certain mixtures rich in amino-acids. The inhibitory concentration varies from 11 to 130 millimols per litre, or from 0·2 to 2 % (wt./vol.). It is of some interest that the amino-acids inhibitory in the lowest concentration were the cyclic compounds—histidine, tyrosine, tryptophane, phenylalamine. Of the chain compounds tested cystine was the most inhibitory.

Several organisms belonging to various groups of bacteria are susceptible of inhibition by amino-acids; certain intestinal organisms are not susceptible. The effect cannot be attributed to the physical effects of high concentration.

There is an apparent divergence between our results and those of workers who have recommended tryptic digests for stimulating the growth of some of the bacteria here shown to be readily inhibited by amino-acids. A suggested explanation of this divergence is that it is the products of partial protein digestion, the polypeptides, which are mainly responsible for the effect of stimulating growth, not the amino-acids. A "polypeptide medium" might prove particularly valuable for bacterial growth.

A metabolism experiment with glycine in high but sub-inhibitory concentration showed that the amino-acid is broken down by *Staphylococcus aureus*, at this concentration.

ACKNOWLEDGEMENTS.

In conclusion we have pleasure in expressing our indebtedness to Professor Stewart in whose department these investigations were carried out, and to Professors Cohen and Raper of Leeds University for some of the amino-acids used.

REFERENCES.

BAINBRIDGE (1911). *J. of Hyg.* II. 341.
BERMAN and RETTGER (1918). *J. Bact.* III. 367.
BUNKER (1919). *J. Bact.* IV. 401–406.
BURROWS and NEYMANN (1919). *Johns Hopk. Hosp. Rep.* XVIII. 341.
COLE and LLOYD (1916–17). *J. Path. and Bact.* XXI. 267.
COLE and ONSLOW (1916). *Lancet,* II. 9.
CUNNINGHAM (1918–19). *Ind. J. Med. Res.* VI. 148.
DAVIS and FERRY (1919). *J. Bact.* IV. 217.
DOUGLAS (1914). *Lancet,* II. 891.
FUNK and DUBIN (1920). *J. Biol. Chem.* XLIV. 487.
LLOYD (1916–17). *J. Path. and Bact.* XXI. 113.
LONG (1919–20). *Amer. Rev. of Tuber.* III. 86.
McLEOD and WYON (1921). *J. Path. and Bact.* XXIV. 205.
NORRIS (1918–19). *Ind. J. Med. Res.* VI. 174, 569.
RETTGER, BERMAN and STURGES (1916). *J. Bact.* I. 15.