STUDIES ON CULTURAL REQUIREMENTS OF BACTERIA

VII. AMINO ACID REQUIREMENTS FOR THE PARK-WILLIAMS NO. 8 STRAIN OF DIPHTHERIA

J. HOWARD MUELLER AND ISRAEL KAPNICK

Department of Bacteriology and Immunology, Harvard University Medical School, Boston, Massachusetts[1]

Received for publication, September 25, 1935

It is a well-recognized fact that the nutritional requirements of an individual strain of any organism may be materially altered by prolonged cultivation on artificial media. Examples are so numerous and so well known that they need not be reviewed in detail. In many instances such changes are accompanied by alterations in the nature of the organism itself as in smooth to rough variation, while in other cases no such changes have been noted. The need of a particular organism for such an amino acid as tryptophane has been found to disappear after repeated subcultures on media with gradually diminishing concentrations of this amino acid (Fildes, Gladstone and Knight, 1933). The ability of organisms to grow on an exceedingly simple synthetic medium has been enhanced by the gradual substitution of such a solution for much more complex mixtures. Many other examples naturally suggest themselves.

The diphtheria group has been widely studied in connection with its growth requirements, perhaps more so than other varieties of bacteria because of the interest attached to the process of toxin formation. It is now well known that great differences

[1] The writer is indebted to Professor N. B. Dreyer of the Department of Pharmacology and Professor R. P. Smith of the Department of Pathology, of Dalhousie University, Halifax, for their courtesy in extending him the hospitality of their laboratories, where a part of this work was carried out during the summer of 1935.

exist between various strains of this organism. In the literature are to be found reports of strains which grow and even produce toxin on synthetic media of the type suggested by Braun (1928) or by Wadsworth and Wheeler (1934), of others which grow on the so called "semi-synthetic" media containing small amounts of peptones or tissue extractives, and of still others which do well only on the usual infusion peptone media. To what extent these variations were present originally in the particular strain of organism and to what extent they have been produced artificially by prolonged adaptation is a matter which remains still to be cleared up.

The writer has studied in some detail the requirements of a single strain of the diphtheria bacillus isolated in 1928 by Dr. Ho Yu (1930) and grown since that time on a peptone meat-infusion broth with short intervening periods on Loeffler's medium. This organism may conveniently be called the HY strain. The results of this study have been presented in earlier publications (Mueller, 1935). It seems now desirable to extend such studies to other strains of diphtheria bacilli and the most logical one to begin with would seem to be that which is used in most laboratories for the production of a toxin of high titer, namely the Park-Williams No. 8 strain. With the probability in mind that differences will be found to exist among cultures of this strain obtainable from various sources because of variations in conditions to which the organism has been subjected over a period of years, this study has been carried out with a single strain of the Park-Williams No. 8 obtained from the Massachusetts State Antitoxin Laboratory through the courtesy of Dr. E. S. A. Robinson early in 1934. It will naturally be extended to strains of the Park-Williams organism from other sources and if differences are found to exist they will be presented in due time. That an extension of the study will necessarily have to be made when further information is available concerning the nature of the substances involved in the tissue extractive fraction, goes without saying. However, since the amino acid requirements for the HY strain which has already been studied are complex, there

would appear to be sufficient basis at the present time for a comparison with other strains on the basis of these compounds.

METHODS EMPLOYED

The same procedure has been followed in studying this strain as that used heretofore and described in detail in earlier publications. The substances used have been dissolved in water, added in the required proportion to special centrifuge tubes, the pH adjusted to 7.4 to 7.6 and the volumes brought to exactly 10 cc. After sterilization for ten minutes at 10 pounds pressure, inoculation with a loopful of pellicle from a 24-hour broth culture, and incubation in a sloped position at 35°C. for 60 to 72 hours, the bacterial growth has been killed by heat, centrifuged and washed and the nitrogen determined by the Pregl micro-Kjeldahl method. Results are given in terms of such bacterial nitrogen and for purposes of orientation it may be stated that 0.8 to 1.0 mgm. nitrogen is produced under the same conditions by the same strain on 10 cc. of the usual unenriched meat infusion peptone broth, this representing satisfactory growth.

TISSUE EXTRACTIVE FRACTION

In place of a crude meat infusion or of Liebig's Meat Extract we have used in this study a preparation from beef liver which we shall call a "liver elute." The procedure followed in obtaining this material has already been described (Mueller, 1935). It consists briefly in precipitating a concentrated aqueous liver extract with alcohol up to 95 per cent, removing the alcohol from the filtrate, adsorbing with charcoal and eluting with acid alcohol. After concentration to remove alcohol, 1.0 cc. of this material is equivalent to 80 grams of liver, and contains 0.0475 gram solids dried at 110°. The solution itself will serve as a starting point for a further study of the substances involved. Undoubtedly many compounds are present in this fraction but it represents a considerable degree of purification over a crude infusion, by a method which should be susceptible to duplication.

AMINO ACID REQUIREMENTS

Preliminary experiments which need not be given in full indicated that our culture of the Park 8 strain grew badly on the same medium which permitted heavy growth of the HY strain of the diphtheria bacillus. Further experiments indicated that the fault lay, not with the tissue extractive fraction (liver elute) nor with the inorganic constituents, but with the amino acids. Certain of these substances required by the HY strain were evidently unessential, at any rate for first generation growth, and moreover it appeared that one or more amino acids were essential to the Park 8 strain for which the other strain had no requirements. By the usual process of separation of the amino acids into three groups by the Dakin butyl-alcohol extraction method, it was found that this new requirement was localized in the mono-amino monocarboxylic fraction. This fraction in turn, upon further examination, appeared to owe its additional activity entirely to its content of leucine, provided the amino acids used for the earlier strain were also present. It was now possible in a series of experiments to determine which of the other amino acids could be omitted without causing diminution in growth and the amino acids essential to heavy growth were in this way limited to valine, leucine, glutamic acid and cystine. The addition of methionine, while not essential for fairly heavy growth in the first generation, produced a marked increase in the quantity of nitrogen obtained, either with or without the concomitant presence of histidine. The latter substance had been found to play an apparently important rôle together with methionine in the study of the HY strain, but with this strain of the Park 8 it is evidently not essential.

The results may be most clearly expressed in a series of curves. For the first six of these the following composition was determined as giving satisfactorily heavy growth and controls were made in such a way that the indicated quantities of all but one substance were present in each tube. The material which was omitted was then added in increasing amounts with the idea of learning in this way the smallest amount necessary for maximal growth.

dl-Valine	10.0 mgm.
dl-Leucine	10.0 mgm.
d-Glutamic acid HCl	50.0 mgm.
l-Cystine	1.0 mgm.
Glycerol	0.05 cc.
Liver elute	0.25 cc.
Salt mixture*	

* The salt mixture had been determined upon in a preliminary series of experiments using both the Park 8 and the HY strain as test organisms. It is added in constant amounts, as indicated below, to each tube.

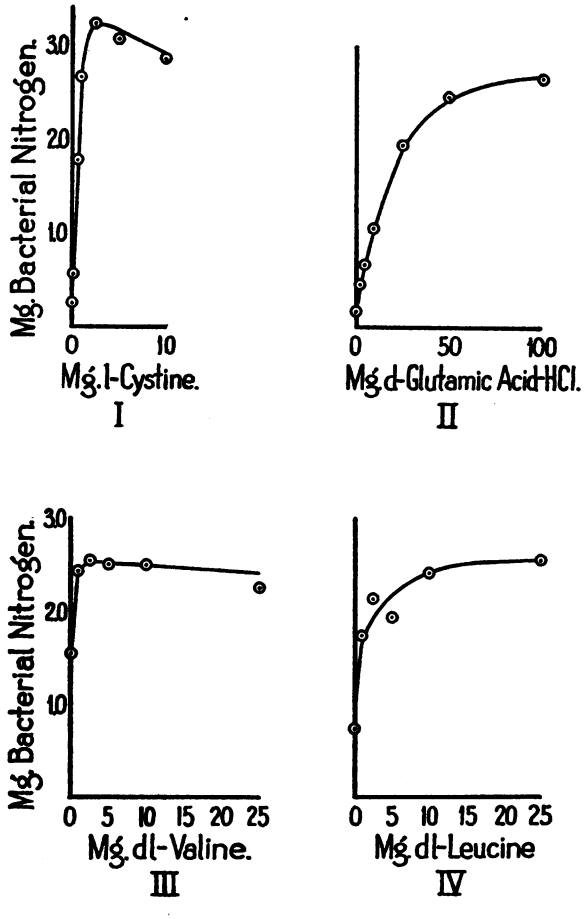
NaCl	50 mgm.
KH_2PO_4	3.5 mgm.
$MgCl_2 \cdot 6H_2O$	3 mgm.

In the seventh curve, increasing amounts of methionine are added to the above complete medium to show the further stimulating effect of this substance. In the eighth experiment the quantities of valine and cystine have been changed to 2.5 mgm. of each, since Charts I and III indicate such quantities to be optimal, 2.0 mgm. of dl-methionine have been added, and the effect of leucine again studied with this control medium, the results shown in curve IV for this amino acid being not entirely satisfactory.

With the exception of curve VII, in which duplicate tubes were not run, each point plotted is the average of two tubes.

The charts show clearly the growth-stimulative effect of each of the constituents of the medium except the inorganic portion. There is some evidence that excessive quantities of any of the materials become inhibitory, as, indeed one should expect, and that for optimal growth one must select suitable concentrations, which can be done from an inspection of the curves. The only substance calling for particular comment is glutamic acid. This amino acid must be supplied in considerably greater quantities than any of the others. It is unfortunately still impossible to determine whether its effect may not be due to an impurity rather than to glutamic acid itself. Neither sodium nor ammonium glutamate can replace it. Aspartic acid is without effect, and a specimen of dl-hydroxy glutamic acid supplied us through the

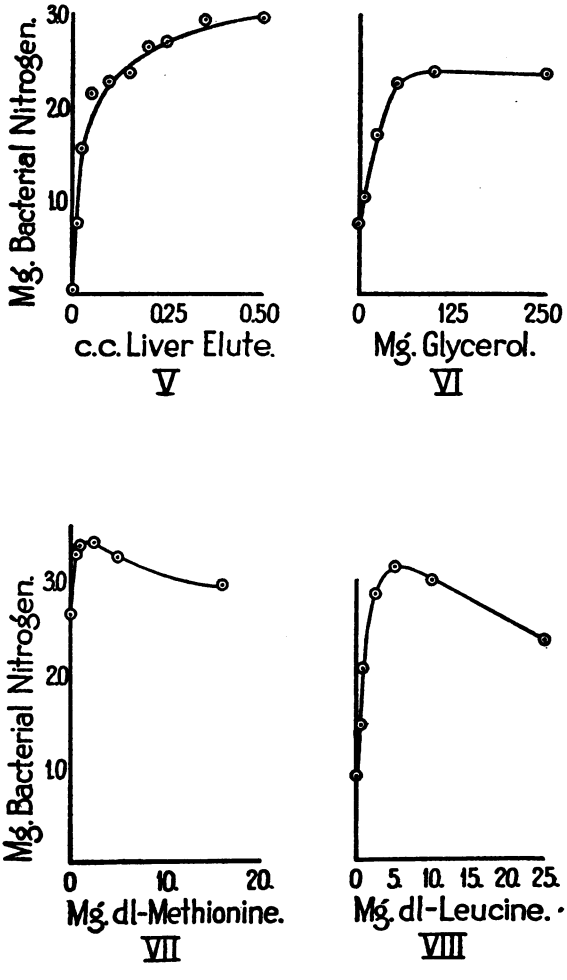
courtesy of Dr. Hans T. Clarke of the College of Physicians & Surgeons in New York was equally ineffective. Synthetic glutamic acid has not been available to us, and a definite answer to the problem will be possible only when this material can be



substituted for the natural product. We hope to be able to carry out such experiments in the near future. Indirect evidence that glutamic acid itself is involved is found in the experiments of Abt. (Abt, 1925). From a quantitative study of the ratio of CO_2 to NH_3 formed in growing cultures of the diphtheria bacillus,

this author concludes that glutamic acid is the amino acid metabolized in greatest quantity.

In all of the work described in this, as in our earlier reports dealing with the diphtheria bacillus, only first generation growth



resulting from a relatively heavy inoculum, has been dealt with. It seemed desirable to determine whether or not this organism could be passed through repeated subculture on such simplified media. A series of tubes were therefore prepared, each con-

taining 10 cc. of a medium of the following composition, the quantities being chosen from an examination of their several curves:

l-Cystine	2.5 mgm.
dl-Valine	2.5 mgm.
dl-Leucine	5.0 mgm.
d-Glutamic acid HCl	50.0 mgm.
dl-Methionine	2.0 mgm.
Liver elute	0.25 cc.
Glycerol	0.05 cc.
Salt mixture	

After sterilization in the autoclave, one tube was inoculated from the pellicle of a broth culture, incubated for 18 hours in a vertical position, and a loopful of the pellicle transferred to a second tube. The first tube was now slanted as usual in order to increase the surface, and incubation continued for a further period of 60 to 72 hours, at which time the bacterial nitrogen was determined. In this way, the organism was passed through seven consecutive daily transplantations on the above medium. The nitrogen in milligrams resulting in these tubes day by day was as follows: 1.93, 2.37, 2.68, 2.87, 2.93, 3.01 and 3.12.

It is clear that there is no tendency for the organism to diminish its growth on this medium. On the contrary, with adaptation, which takes place rapidly, the strain grows more and more luxuriantly. To explain the relatively poor growth in the first two generations as compared with the maxima of the curves and with the growth in the next few generations the following statement must be made: The preliminary studies and the curves were carried out with the strain of Park-Williams No. 8 obtained, as stated above, from the Massachusetts Antitoxin Laboratory more than a year ago, and carried since that time by daily transfer on a 2 per cent "proteose" peptone meat infusion broth. After completing the curves, which was done in Halifax during the summer, the work was interrupted, to be resumed in Boston. The Halifax strain was not brought back to Boston since the same original strain had been carried there on the same stock medium and under the same conditions. It appeared, however, that something had gone amiss with this strain, and it became neces-

sary to go back to the original Massachusetts Antitoxin Laboratory strain. The passage experiment was therefore carried out with this freshly acquired strain, which had been kept during the past fifteen or sixteen months by methods differing somewhat from those which we had used. Evidently the conditions which had been fixed as optimal for the strain as we carried it were not completely so for the strain when kept under different ones. That the changes involved were not deep-seated is indicated by the rapidity with which growth attained the same maximum indicated by the charts.

As a more exact explanation of what had occurred, it was found that increasing the quantity of dl-valine from 2.5 to 5 mgm. and adding 5 mgm. of glycine produced 3.30 mgm. bacterial nitrogen in the first generation. The slight change was evidently attributable to a variation in the ability of the organism to produce these two substances.

Comparison of the results obtained with the HY and the Park-Williams strains show distinct differences, as well as similarities. Fildes and Richardson (1935) have carried out similar studies with the *Clostridium sporogenes*, and find a somewhat similar list of amino acids, some of which they list as indispensable, others as necessary only for heavy growth. We have not attempted to separate the requirements in this way, but for the present group them all as necessary for heavy growth. The similarities and differences shown by the two strains of diphtheria and by the *Cl. sporogenes* may most easily be seen from the following tabulation:

Diphtheria (HY)	*Diphtheria (P-W. No. 8)*	*Cl. sporogenes*
Glycine	Valine	Leucine
Valine	Leucine	Tryptophane
Methionine	Methionine	Phenylalanine
Phenylalanine*	Cystine	Tyrosine
Tryptophane	Glutamic acid	Arginine
Cystine		Histidine
Histidine		Cystine
Glutamic acid		Methionine
		Valine

* Found in later work to have very little effect.

A general similarity is evident, but at the present stage of the study of bacterial cultural requirements one cannot predict how valuable a more complete knowledge of the facts will prove to be, either for purposes of classification and differentiation, or from the more general point of view of processes underlying the metabolism of amino acids. In the latter connection, such studies as those of Stickland (1934) on the oxidation-reduction mechanisms of amino acids in bacterial media are interesting and suggestive.

CONCLUSIONS

1. A strain of the Park 8 diphtheria bacillus was found to grow heavily on a medium containing only l-cystine, d-glutamic acid, dl-valine, dl-leucine, dl-methionine, glycerol, inorganic salts and "liver elute." The method of preparing the "liver elute" is described.

2. The medium is suitable for serial culture, the amount of growth increasing with adaptation.

3. Moderate variation in conditions under which the strain is routinely maintained may lead to differences in its amino acid requirements.

REFERENCES

ABT, G. 1925 Ann de l'Inst. Pasteur **39**, 387.
BRAUN, H. 1928 Münch. Med. Wchnschr., **75**, 457.
FILDES, P., GLADSTONE, G. P., AND KNIGHT, B. C. J. G. 1933 Brit. Jour. Exp. Path., **14**, 189.
FILDES, P., AND RICHARDSON, G. M. 1935 Brit. Jour. Exp. Path., **16**, 326.
MUELLER, J. H. 1935 Jour. Bact., **29**, 515; **30**, 513.
STICKLAND, L. H. 1934 Biochem. Jour., **28**, 1746.
STICKLAND, L. H. 1935 Ibid., **29**, 288 and 889.
WADSWORTH, A., AND WHEELER, M. W. 1934 Jour. Inf. Dis., **55**, 123.
YU, H. 1930 Jour. Bact., **20**, 107.