in phloridzinized dogs they did not find that protein injection was followed by the increased $N_2$ and sugar excretion that followed the taking of similar quantities of protein by mouth. But since Drury[6] has demonstrated the utilization of dextrose in phloridzinized dogs it is possible to suppose that the difference may be an indication of slow digestion of protein to amino-acids, not of no digestion at all. The preliminary hypothesis on which our future experiments will be based is that the injected protein is taken up by the reticulo-endothelial cells of the body and subjected to intra-cellular digestion to amino-acids.

## 10405 P

## Utilization of Amino-Acids by *Clostridium botulinum.**

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Our knowledge of the metabolism of *Clostridium botulinum* is at present scanty. Wagner, Meyer and Dozier[1] analyzed the products of the action of *Cl. botulinum* on complex media but obtained little information as to the reactions by which the products had been formed. Knight,[2] summarizing the growth-requirements of *Cl. botulinum,* reports that the simplest medium which will support growth contains the amino-acids proline, glycine, leucine, alanine, lysine, and cystine, together probably with traces of tryptophan and of the "sporogenes growth factor." He suggests that *Cl. botulinum* may obtain its energy through the "Stickland reaction," a coupled oxido-reduction between pairs of amino-acids. Glycine and proline act as hydrogen acceptors while alanine and leucine act as hydrogen donators in the case of *Cl. sporogenes* (Stickland[3]). Therefore these amino-acids were tested to determine whether or not they are attacked directly as is true for *Cl. tetanomorphum* with various

6 Drury, D. R., Bergmann, D. C., and Greeley, P. O., *Am. J. Physiol.*, 1939, **117**, 323.

* Aided in part by a grant from the Rockefeller Fluid Research Fund.

1 Wagner, E., Meyer, K. F., and Dozier, C. C., *J. Bact.*, 1925, **10**, 321.

2 Knight, B. C. J. G., *Bacterial Nutrition*, 1936, 117-120, His Majesty's Stationery Office, London.

3 Stickland, L. H., *Biochem. J.*, 1934, **28**, 1746.

amino-acids (Woods and Clifton[4]) or by coupled reactions between pairs of amino-acids.

All the experiments reported below were carried out with washed suspensions of *Cl. botulinum,* Type B (E-44). 800 ml of plain broth plus 0.1% yeast extract were inoculated with 0.5 ml of a beef-brain culture of this organism and incubated for 20 hours at 37°C in a McIntosh and Fildes anaërobic jar. The culture was then centrifuged, the cells were washed in saline and finally suspended in phosphate buffer of pH 7.4. The suspension so prepared was immediately deaërated *in vacuo.* The usual Thunberg-tube method was employed for the detection of hydrogen donators and it was found that alanine and leucine were the only amino-acids of those essential for growth which reduced methylene blue readily. In the presence of glycine or proline, methylene blue was not reduced more rapidly than in the blank suspension alone. None of these amino-acids acted as hydrogen acceptors in the presence of reduced methylene blue, probably because the acceptor must lie on a more negative point on the O/R potential scale than methylene blue.

Direct reactions between pairs of amino acids were determined by measuring the ammonia production in the presence of glycine or proline. One ml of M/10 alanine or leucine, 1.0 ml of M/10 glycine or proline and 5 ml of the phosphate-buffer suspension of *Cl. botulinum* were placed in Thunberg tubes, which were then thoroughly evacuated. Appropriate controls were always included. After incubation for 6 hours at 37°C, ammonia was determined by distillation from 50% alcohol-borate buffer of pH 10 into standard sulfuric acid. Typical results obtained are shown in Table I.

The amounts of ammonia produced in the presence of a single amino-acid or the pair glycine plus proline are not appreciably greater than in the control suspension, but considerable amounts are produced from the pairs glycine or proline plus alanine or leucine. Apparently only one isomer of alanine is readily attacked, while proline does not appear to be deaminated.

TABLE I.

| Substrate | M/10 $NH_3$ ml | Substrate glycine | M/10 $NH_3$ ml | Substrate proline | M/10 $NH_3$ ml |
|---|---|---|---|---|---|
| Control suspension | .17 | | | | |
| Glycine | .20 | + alanine | 1.09 | + glycine | .25 |
| d-l alanine | .20 | + leucine | 1.18 | + alanine | .61 |
| l-leucine | .25 | + proline | 0.25 | + leucine | .98 |
| l-proline | .23 | | | | |

[4] Woods, D. D., and Clifton, C. E., *Biochem. J.,* 1937, **31**, 1774.

The reactions between alanine and glycine or proline have been studied in more detail and on a larger scale. Five ml of M/5 d-alanine plus 10 ml of M/5 glycine on incubation at 37°C in an atmosphere of nitrogen for 20 hours with the washed cells from 400 ml of medium gave on correction for the control 6.6 ml of M/10 $CO_2$, 12.8 ml of M/10 $NH_3$, and 10.5 ml of M/10 volatile acid (steam distillation). The volatile acid has been tentatively identified as acetic acid by micro Duclaux distillations. The results suggest that the main reaction between glycine and alanine may be represented as

$$2CH_2NH_2COOH + CH_3CHNH_2COOH + 2H_2O = 3CH_3COOH + CO_2 + 3NH_3$$

although the high value for $CO_2$ suggests that a side reaction may also be occurring.

In a similar experiment with 5 ml of M/10 d-alanine and 10 ml of M/10 l-proline, 5.0 ml of M/10 $CO_2$, 5.4 ml of M/10 $NH_3$, and 5.1 ml of M/10 volatile acid tentatively identified as acetic were recovered. Apparently proline is reduced without deamination, probably to δ-amino-n-valeric acid, as is true in the case of *Cl. sporogenes* (Stickland[5]). Experiments on a larger scale are in progress to isolate and identify by derivatives the reduction-product of l-proline and also the tentatively identified acetic acid. Preliminary results suggest that the reaction may be represented as

$$CH_3CHNH_2COOH + 2CH_2 \cdot CH_2 \cdot CH_2 \cdot \overset{|\text{———NH———}|}{CH} \cdot COOH + 2H_2O = CH_3COOH + CO_2 + NH_3 + 2CH_2NH_2 \cdot (CH_2)_3 \cdot COOH$$

These results also suggest that the oxidation of alanine by either glycine or proline may be represented as

$$CH_3CHNH_2 \cdot COOH - 4H + 2H_2O = CH_3 \cdot COOH + NH_3 + CO_2,$$

this oxidation probably taking place in 2 steps, pyruvic acid being the intermediate compound.

It has been shown in preliminary studies that *Cl. botulinum* does not obtain its energy by direct utilization of amino-acids as does *Cl. tetanomorphum,* but probably by a coupled oxido-reduction between pairs of different amino-acids in a manner similar to *Cl. sporogenes.*

[5] Stickland, L. H., *Biochem. J.*, 1934, **29**, 288.