Biochemistry. — *„Concerning the Synthetic Action of Bacteria in the Paunch of the Cow".* By Prof. B. SJOLLEMA and J. E. VAN DER ZANDE. (Communicated by Prof. H. ZWAARDEMAKER).

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The question whether bacterial processes occurring in the paunch of ruminants are significant for the metabolism of these amimals [1]), should be given more attention to than here to fore, since, by way of trial, ruminants are fed with urea, made from the nitrogen in the air. For the significance of the substitution of urea for protein in the animal's diet depends to a great extent on the capacity of the bacteria of the paunch to synthesize from urea, in the presence of non-nitrogenous substances, the amino-acids which the higher animals are not able to build up.

Tryptophane is one of the amino-acids indispensable to man and to the higher animals. It is highly improbable that mammals can synthesize tyrosine from non-aromatic substances.

We have tried to ascertain whether these two substances can be built up by the bacteria occurring in the cow's paunch, when, beyond ammonia no other source of nitrogen is present than urea, asparagin or aspartic acid.

Our procedure was as follows: [2])

Directly when the animal was killed, part of the contents of the paunch was brought to our Laboratory in a sterile bottle, fitted with a glass stopper [3]).

With the help of a sterile wire a little of the paunch contents (i.e. of the turbid fluid after removal of the coarser particles) was transmitted to sterile nutrient solutions, contained in Erlenmeyer-flasks plugged with cotton-cool, and which were of a depth of 1

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[1]) Here we refer to the development of volatile acids in the paunch from sugar, as demonstrated before by one of us (B. S.). See Bericht III 5th International. Congres für "angewandte Chemie" Berlin 1903, p. 825.

[2]) It was adopted because bacterial growth could not easily be recognized directly in the turbid juice of the paunch (even when much diluted), and also because we wanted quantitative data regarding tryptophane-formation.

[3]) We would here gratefully acknowledge our thanks to Mr. HOEFNAGEL and to Mr. DE GRAAF, respectively director and sub-director of the Utrecht abattoir, for their kind assistance in obtaining the material required for these experiments.

to $1^1/_2$ cm. The flasks were then left standing in an incubator at 36° C. Duplicate cultures were made for each experiment.

When the bacteria were fairly developed (which was the case after two days) one of the cultures was examined for the presence of the amino-acids, alluded to above; the other remained in the incubator. Moreover a new culture-medium was inoculated with it. We used USCHINSKY's solution, unmodified or modified as indicated below.[1])

Since the p_H of the paunch contents was about 7,4, we took care to let the p_H of our culture media be the same.

In order to demonstrate the presence of tryptophane we applied the reactions of VOISENET (with HCl, formaldehyde and nitrite) and of HOPKINS-COLE (with H_2SO_4 and glyoxylic acid). MILLON's reagent was used for ascertaining the presence of tyrosine. VOISENET's reagent stains differently with indole and with tryptophane. Indole after shaking out with ether was reacted on with dimethylpara amidobenzaldehyde.

USCHINSKI's solution, whether modified or not, but invariably without an aromatic or heterocyclic compound, inoculated with a small quantum of the paunch-contents, always gave in the sediment (obtained by centrifugation after the addition of alcohol) after a sojourn at 36° C. in an incubator, a very distinct tryptophane, and tyrosine-reaction, whereas initially the reactions were negative.

A better growth and more powerful reactions were obtained by mixing 10 c.c. of the fresh paunch fluid with 25 c.c. of USCHINSKI's solution.

Whereas the reactions in the sediment were invariably positive, the supernatant fluid displayed negative reactions.

In order to make sure that the tryptophane and the tyrosine reactions were not due to other indole or phenol-derivatives, the sediment was, in a few cases, centrifuged anew with diluted alcohol and once more with ether (indole). The reactions of the sediment were as distinct as before. The cultures themselves were also shaken out with ether some times. With the above-named aromatic aldehyde the ether gave a negative indole-reaction. It was evident, therefore, that neither free tryptophane, nor other free indole-derivatives, nor free phenol-like bodies were present. The positive reactions may, therefore, be attributed to the body-protein of the bacteria.

On inoculation of new USCHINSKY solutions with the cultures an

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[1]) The ordinary USCHINSKY-solution contains K, Na, Ca, Mg, PO_4, Cl and SO_4; besides glycerol, ammonium-lactate and sodium aspartate.

excellent growth could be noted, and after a couple of days positive tryptophane, and tyrosine-reactions of the sediment.

The present investigation, therefore, shows clearly that there are bacteria in the paunch of the cow, capable of building up tryptophane and tyrosine with an aliphatic nitrogen-compound and with ammonia. With every one of the six paunches we succeeded in obtaining this result.

We consider the presence of tyrosine to be established when bacterial bodies show a phenol-reaction (MILLON's) The non-specificity of the tryptophane reactions is of no importance in our experiments. They are only needed to show the presence of an indole-derivative so long as tryptophane is considered as sole indole-derivative in the protein-molecule [1]).

Positive results were also obtained in the experiments in which asparagin (or sodium-aspartate) had been replaced by urea. The bacterial growth was, however, decidedly slower. The ammonium-lactate had been substituted in these experiments by potassium lactate, so that urea was the sole source of nitrogen.

After 2×24 hours the tryptophane-reactions were as a rule weak in the turbid culture solution and very clear in the sediment, which had been obtained through centrifugation.

A couple of times we added tryptophane to the USCHINSKY solution which resulted in the formation of indole contrary to the other experiments.

Direct addition of indole inhibited bacterial growth considerably; it was arrested completely by 50 mgms per 100 c.c.

Whether tryptophane can be developed from indole, as assumed by LOGIE, is not borne out by the present experiments, for, where addition of a small quantity of indole caused some bacterial growth, the formation of tryptophane may have resulted from the presence of ammonium-nitrogen or asparagine-nitrogen.

When substituting glucose for the glycerol and the lactic acid of the USCHINSKY-solution a tryptophane synthesis takes place which is almost equal to that in the ordinary USCHINSKY-solution.

In experiments under approximately anaërobic conditions the growth was inferior to that obtained in the manner above-described. An experiment, in which air was drawn through the fluid by suction, did not yield a larger growth than usual.

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[1]) Since gelatine does not yield VOISENET's, nor MILLON's reaction and proline and oxyproline are contained in it, it follows that these two amino-acids do not give these reactions.

The histidine reactions thus far obtained, were still somewhat doubtful.

Several microscopic preparations were made of the cultures. Sometimes different species were present, i.e. diplococci, rod-shaped bacteria; sometimes staphylococci and streptococci; in one case the predominance of one species was such as to render it difficult to find another. These almost pure cultures were not always made up of the same bacteria; sometimes they were small ovoid, at other times rod-shaped bacteria.

It being known that even various stocks of one and the same species may differ largely as to the chemical changes they engender, we did not ascertain whether the developing species were in any way concerned in the result of the reaction.

According to an approximate quantitative determination in a culture, three days old, the sediment of 100 cc. contained about 3 mgms of tryptophane, i.e. per Liter 30 mgms, or 3 grms per 100 L. (putting the paunch contents at 100 L.).

A man of 70 k.g. weight requires per day about $2^1/_2$—3 grms of tryptophane. Assuming the same ratio for a cow, this animal would require per day about $17^1/_2$—20 grms. The quantity necessary for the producton of milk has not been taken into account here.

Putting the tryptophane content of milk per L. at about 750 mgms, and putting the daily flow of milk at, say, 12 Liters, the animal would have to take in another quantum of 9 grms of tryptophane.

As far as we are aware tryptophane synthesis by bacteria (B. coli and B. FRIEDLÄNDER) from ammoniac and aliphatic nitrogen-compounds, has been demonstrated only once, viz. by LOGIE [1]).

From the publication of BRAUN and CAHN—BRONNER [2]), which came to our notice when our experiments had nearly come to an end, it may be inferred that their experiments also pointed to tryptophane synthesis, for they could grow coli, paratyphoid-, and FRIEDLÄNDER-bacteria when ammonia nitrogen was the only source of nitrogen present. Where they report, that under perfectly anaërobic conditions ammoniac-assimilation is impossible, even after the supply of more energy, the question rises (granting their theory to hold generally) whether in the rumination process an aërobic condition exists which allows any synthesis worth mentioning.

It may rationally be supposed that, wherever micro-organisms manage to live on inorganic or aliphatic nitrogen-sources, they them-

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[1]) J. of Pathol. and Bact. Bd. 23, 224 (1919/1920).

[2]) Biochem. Zeitschrift Bd. 131, 272 (1922).

selves derive the cyclic amino-acids from these sources, it being a fact that protein, containing these amino-acids, is always present in these organisms.

In how far the amino-acids, formed in the paunch, are of use to the metabolism of ruminants, will have to be made out by food-experiments, which will also have to show whether the bacterial protein, formed in the paunch, is resorbed.

Let it be observed that we have never succeeded in demonstrating tryptophane (or tyrosine) in the fresh turbid paunch-fluid (after the removal of the solid particles) and also that we were not more successful in this respect after cultivating for some days in the incubator, either under aërobic or anaërobic conditions.

Meanwhile we should not omit stating that reactions in a fluid like the paunch-fluid, are far less sensitive than those in unstained solutions. Only when 7 mgms of tryptophane per 100 cc. was added in the form of protein (bloodplasma) a perfectly distinct tryptophane-reaction was recognizable.

Still, the phenomenon, just alluded to, does not point to an abundant tryptophane formation in the paunch, which is the more striking since the paunch fluid with USCHINSKY's solution (10 : 25) yields negative results at starting, but exhibits distinct reactions after 2×24 hrs.

The above experiments show: 1°. that various bacteria present in the paunch of cows can build up the amino-acids tryptophane and tyrosine from ammonia nitrogen plus asparagine (or aspartic) nitrogen, and also from urea as nitrogen-source.

2°. that these bacteria can form quantities of tryptophane in the culture-medium of USCHINSKY, which may be of some significance for the metabolism in cows; however it is not quite certain whether this synthesis is equally intense in the paunch.

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