STUDIES ON THE METABOLISM OF LEPTOSPIRA

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Since previous demonstrations by earlier workers have shown that some animal fluid is essential for the growth of the organisms responsible for infectious jaundice or Weil's disease, it is evident that the leptospiras require some growth factor, or combinations of factors, which they are unable to synthesize.

Preliminary experiments by Rosenfeld (unpublished) with a serum preparation so precipitated with alcohol as to give a negative biuret reaction revealed that the unknown growth factor was apparently non-protein in nature. The organisms concerned included two strains of *Leptospira icterohemorrhagiae* and one of *Leptospira canicola.*

These tests, then, suggested the use of various growth-promoting factors with *Leptospira.* The substances used were those which were known to exhibit definite effects upon the development of other organisms. These were included in the basic medium, with and without the addition of serum which represents the unknown factor. Since *Leptospira canicola* has better viability, we decided to use it as the test organism throughout this investigation.

MATERIALS AND METHODS

The culture medium

The culture medium devised by the Dutch workers and hereinafter known as the Schüffner medium was prepared according to the method of Smith and Tulloch (1937):

To 1500 ml. of tap water were added 1.5 grams of Witte's peptone. The mixture was boiled, and 300 ml. of Ringer's solution

were added. Next, 150 ml. of Sorensen's double phosphate buffer solution (72 ml. of M/15 Na_2HPO_4 to 100 ml. of M/15 KH_2PO_4) pH 7.2 were added, and the mixture boiled until precipitation was complete. The solution was cooled in the refrigerator, filtered, and the reaction checked. The medium was tubed in small, clean tubes in 3 ml. quantities and autoclaved at 15 pounds pressure for 20 minutes.

To complete this medium, 0.3 ml. of fresh, sterile rabbit serum was added to each tube, the tubes then being heated to 56°C. for 30 minutes to inactivate the serum. Inoculation was carried out by means of a pipette, the inoculum being 0.3 ml.

The inoculated medium was incubated at 30 to 32°C., and the cultures were transferred at seven-day intervals.

Growth factors

The following substances, singly and in combination, were tested for their ability to support leptospiral growth in Schüffner's medium in the absence of serum and for their effect on growth when serum was present: thiamin hydrochloride, riboflavin, nicotinic acid, nicotinic acid amide, vitamin B_6 hydrochloride, ascorbic acid, catalase, milk peroxidase. All these products, with the exception of the last two, were obtained commercially. Catalase was obtained in crystalline form from beef liver by treatment with dioxane in accordance with the method of Sumner and Dounce (1937). Peroxidase was extracted from milk by the method of Elliott (1932).

Leptospiral counts

The Petroff-Hausser counting chamber was used, since it permitted the combination of darkfield with the oil immersion objective. This chamber was equipped with a Neubauer ruling and had a depth of 0.02 mm. The leptospira could be identified easily in the counting chamber with 10× oculars and a 1.8 mm. oil immersion objective.

In the counting chamber each small square had an area of 2.5×10^{-3} mm.^{2} and a depth of 2×10^{-2} mm. This gave a total volume of 5×10^{-5} cu. mm. or 5×10^{-8} ml. per square.

Each organism seen in one square therefore represented $\frac{1}{5 \times 10^{-8}}$ or 2×10^7 organisms per ml. in the material used. The factor 2×10^7 was multiplied by the average number of organisms per square based on the total of 96 squares counted. The culture was not diluted.

TABLE 1

Growth of L. canicola in normal Schüffner medium with the addition of thiamin hydrochloride

TRANSFER	CONTROL	CONCENTRATION OF FACTOR (GAMMA PER ML.)				
		1	3	5	8	10
First						
Orgs. per ml. ($\times 10^7$)	4.4	5.0		5.8		5.8
Percentage dev. from control		+14		+32		+32
Second						
Orgs. per ml. ($\times 10^7$)	1.3	2.2	2.5	2.6	2.5	1.3
Percentage dev. from control		+69	+92	+100	+92	0
Third						
Orgs. per ml. ($\times 10^7$)	3.0	4.1	3.3	2.8	3.3	2.9
Percentage dev. from control		+37	+10	−7	+10	−3
Fourth						
Orgs. per ml. ($\times 10^7$)	0.9	1.1	1.6	1.2	1.4	1.4
Percentage dev. from control		+22	+77	+33	+56	+56
Fifth						
Orgs. per ml. ($\times 10^7$)	2.1	2.3	2.4	2.8	2.3	2.0
Percentage dev. from control		+9	+14	+33	+9	−5

Before counting the organisms, the chamber was thoroughly cleansed. The suspensions were heated at 56°C. for 30 minutes to kill the organisms before enumerating them. This method of sterilization was employed because it gave a minimum of lysis. Before making each succeeding count the chamber was rinsed, first in acid alcohol and then in distilled water, and was carefully dried.

All counts were made on the seventh day of incubation, transfers being made at that same date.

The method of computation of results is illustrated by table 1. The percentage deviations from the control count were averaged over the five transfer periods. Thus, at a concentration of one

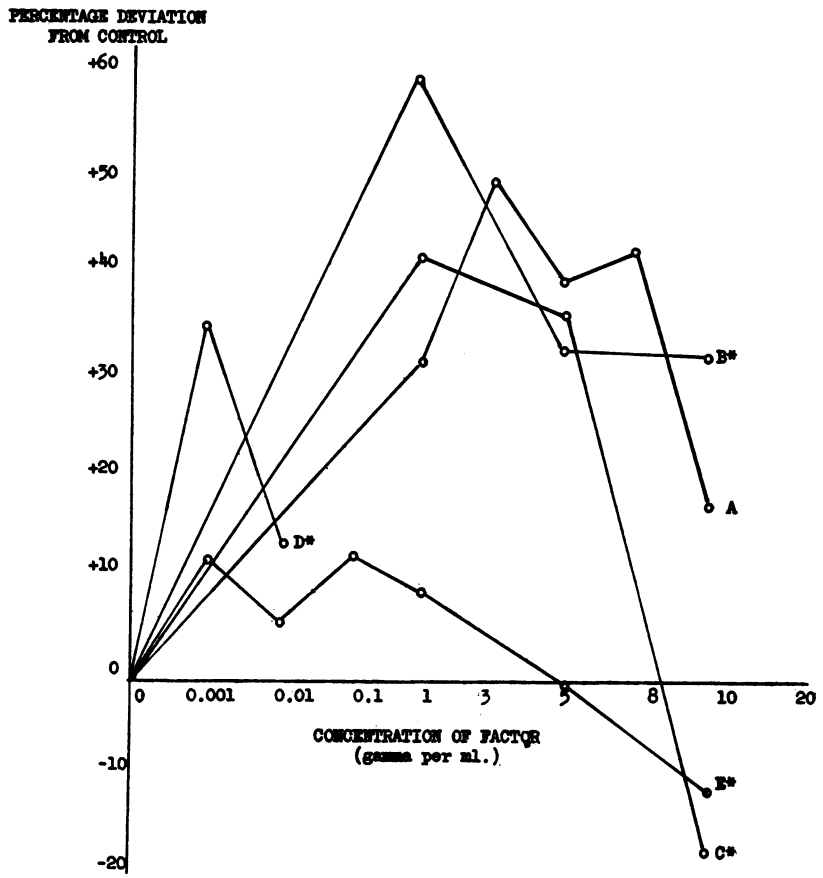

FIG. 1. GROWTH OF L. CANICOLA IN NORMAL SCHÜFFNER MEDIUM WITH THE ADDITION OF VARIOUS GROWTH FACTORS

A, thiamin hydrochloride; *B*, nicotinic acid; *C*, nicotinic acid amide; *D*, riboflavin; *E*, vitamin B_6 hydrochloride.

*Growth ceased at higher concentrations of factor.

gamma of thiamin hydrochloride per ml. the average percentage deviation from the control would be given by:

$$\frac{(+14) + (+69) + (+37) + (+22) + (+9)}{5} \text{ or } +30.2 \text{ per cent}$$

The same procedure was carried out on all succeeding concentrations of the factor, the average percentage deviation at a concentration of three gamma per ml. being 48.3, etc.

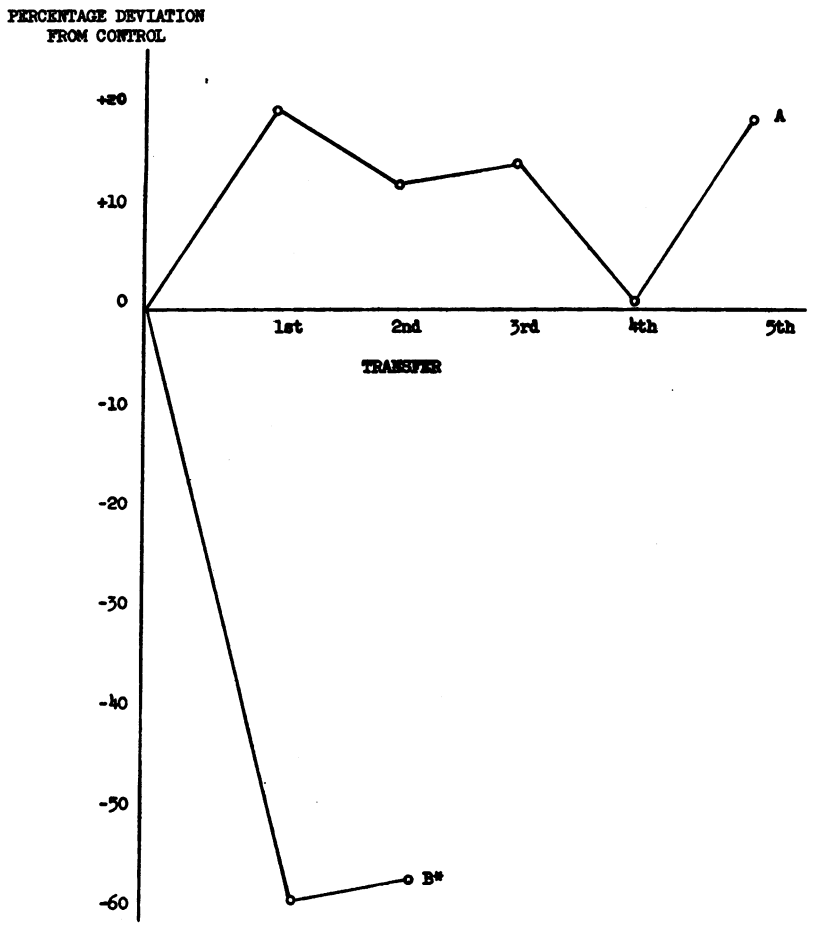

FIG. 2. GROWTH OF L. CANICOLA IN NORMAL SCHÜFFNER MEDIUM WITH THE ADDITION OF VARIOUS GROWTH FACTORS

A, riboflavin (1 gamma/ml.) plus catalase (1:1,000,000); *B*, riboflavin (1 gamma/ml.) plus peryoxidase (1:1,000,000).

*Organisms died out upon a further transfer.

RESULTS

No factor or combination of the factors tested was capable of maintaining leptospiral growth in the absence of serum. How-

ever, noticeable effects, as shown in figure 1, were evident when the various products were added to serum-containing media.

These substances are listed below in the order of decreasing stimulation upon leptospiral growth in the presence of serum: nicotinic acid, thiamin hydrochloride, nicotinic acid amide, riboflavin, vitamin B_6 hydrochloride, ascorbic acid. The average percentage deviations for all factors except ascorbic acid are charted in figure 1. These were obtained by the method described above. Ascorbic acid, however, is not included in this chart since its effects were so slight as to be considered negligible.

Riboflavin in Schüffer's medium caused the death of the organisms in all but the lowest concentrations. It was believed that this phenomenon might possibly be a function of the respiratory activity of the vitamin. For this reason riboflavin, in a concentration ordinarily sufficient to kill the leptospira, was used in combination with catalase and with peroxidase. The effects are shown in figure 2.

DISCUSSION

Both nicotinic acid and nicotinic acid amide give optimum stimulation at a concentration of one gamma per ml. The possible function of nicotinic acid and its amide as building stones in the formation of the respiratory ferment cozymase has been suggested as an explanation of its biologic action, but complete proof of this is not yet available. It is worthy of note that concentrations of nicotinic acid and the amide at levels appreciably above the optimum exhibit marked inhibitory effects upon growth. This finding is in accordance with the general view that increased amounts of growth factors exert a negative effect.

Thiamin hydrochloride is seen to give marked stimulation as an accessory substance. At a concentration of three gamma per milliliter this effect was at a maximum. If the transformation of thiamin into co-carboxylase (the phosphoric acid ester of vitamin B_1) is the actual function of thiamin in metabolism, we then have a possible explanation of this stimulatory effect upon leptospiral growth.

Riboflavin gave noticeable stimulation at a concentration of 0.001 gamma per ml. This effect fell off rapidly at concentra-

tions about this level, and the organisms died out when the factor was used in amounts of 0.1 gamma per ml. and more. The action of riboflavin can, perhaps, be reasonably explained. This substance is converted into a flavoprotein such as the "yellow enzyme" of Warburg by combination with a protein through a phosphoric acid group. It is suggested that in this system the easily reversible oxidation-reduction reactions of the "yellow enzyme" allow it to act as an oxygen carrier between molecular oxygen and the substrate. Hewitt (1937) states that hydrogen peroxide is formed as a result of this oxygen-carrying activity. The production of hydrogen peroxide would, then, account for the inhibitory effects of riboflavin. In very low concentration the amounts of peroxide produced would presumably be small enough to be overcome successfully by the organism, probably by means of its production of catalase.

To test further this hypothesis, riboflavin was used in connection with catalase and, also, with peroxidase. The results are most striking. In the former case, riboflavin, in a concentration ordinarily sufficient to prevent growth within five transfers, maintained growth at a level constantly above that of the control. When riboflavin was used with peroxidase, however, growth ceased within a space of three transfers. A possible explanation of this latter phenomenon was the toxicity of "nascent" oxygen, as produced by the action of peroxidase on hydrogen peroxide. Apparently the action of catalase was to break hydrogen peroxide down with the production of molecular oxygen which, of course, is not detrimental to growth.

Vitamin B_6 hydrochloride was apparently without much stimulating effect upon leptospiral growth. Although some increases were observed, these seemed to lie within the area of determinative error, such as might result from the slight amount of lysis occurring during sterilization of the culture, counting, etc. This substance did, however, exhibit the characteristic inhibitory effect of growth factors used in high concentrations.

SUMMARY

1. A factor necessary for the growth of *Leptospira canicola* is known to exist in animal serum; it is unidentified, and it does not

appear to be one of the following: nicotinic acid, thiamin hydrochloride, nicotinic acid amide, riboflavin, vitamin B_6 hydrochloride, ascorbic acid.

2. The following substances function as growth-accessory factors, giving marked stimulation to leptospiral growth when used in combination with the unknown factor: nicotinic acid, thiamin hydrochloride, nicotinic acid amide, riboflavin.

3. The view that growth factors inhibit proliferation when used in large concentrations has been corroborated. A possible explanation of the mechanism responsible has been advanced in the case of riboflavin.

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