# THE CHEMICAL NATURE OF GROWTH FACTORS REQUIRED BY MOSQUITO LARVÆ

## I. RIBOFLAVIN AND THIAMIN

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Previous work (1–4) has shown that the larvæ of the yellow fever mosquito (*Ædes ægypti*) require certain accessory growth factors which they normally obtain from living microörganisms. A proper quantity of living yeast suspension in distilled water supports good growth of the larvæ, but the same quantity of heat-killed yeast suspension under sterile conditions supports only a very slight growth. If, however, it is supplemented with an adequate amount of autoclaved liver extract, growth proceeds at a maximum rate. Liver extract without yeast gives no growth. Very large amounts of heat-killed yeast suspension (30 to 40 times as much as is required in the presence of liver extract) support fair growth even in the absence of liver extract. Thus at least two substances or groups of substances are required by the larvæ. Only one of these (designated as growth factor A) is present in liver extract. Both are present in heat-killed yeast, but much more yeast is needed to furnish adequate amounts of the one present in liver extract than of the other (4).

The sources and some of the properties of both growth factors have been described in previous publications (2, 4). This paper will show that riboflavin is one of the essential substances supplied by growth factor A and that thiamin (vitamin $B_1$) is an essential substance supplied by both of the crude growth factor preparations.

### METHODS

#### *The Larval Growth Test*

All the tests were conducted in 18 × 160 mm. test tubes holding 6 ml. of the medium to be tested. The various constituents of the medium were prepared as separate stock solutions adjusted to a pH of 5.8–6.0 and autoclaved ½ hour at 120° C. The required amounts

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of each solution were then pipetted into each experimental tube and made up with sterile distilled water to a total volume of 6 ml. When the growth factor A activity was being measured, each tube received 0.3 ml. of a washed, killed yeast suspension in distilled water (4).

In some experiments concerned with the factor A activity, and in all the experiments concerned with the other factor and with thiamin, the killed yeast was replaced by yeast extract (yeast vitamin Harris) and casein. Since the yeast extract used contains factor A (4) it was necessary first to treat it for ½ hour with 10 grams of fuller's earth to 100 ml. of 5 per cent solution of yeast vitamin Harris. The fuller's earth was then removed by centrifugation. This procedure removes much of the factor A (4). Amounts of treated yeast extract which supply enough of the second factor when A is supplied in some other form, do not contain enough factor A to support good growth in the absence of added A. In experiments in which whole yeast was replaced by yeast extract and casein, it was also necessary to add $Ca^{++}$ (3), and this was provided as $CaCl_2$ at a concentration of 0.01 M.

Each experimental tube was inoculated with 3 newly-hatched bacteria-free *Ædes ægypti* larvæ secured by the methods previously described (4). Duplicate tubes were prepared for each medium in each experiment. The tubes were incubated at 28 ± 1° C. and were observed daily for the first 10 days and every other day thereafter, the number of larvæ in each stage of development being noted. From the data thus obtained, the rate of development could be expressed as a number, $N \times 1/T$, in which $N$ is the percentage of larvæ reaching the 4th (last) larval instar within 10 days, and $T$ is the average time in days required by these larvæ to reach the 4th instar. The adequacy of this growth index in the measurement of growth factor A has already been shown (4). Under optimum conditions, at 28° C., $N \times 1/T = 100 \times 1/4 = 25$.

It cannot be too strongly emphasized that significant results with the growth factors here concerned can be obtained only if the larvæ are reared in the complete absence of living microörganisms. To this end, the ordinary bacteriological methods to insure sterility were used and the sterility of each tube was tested by inoculation of a loopful of material to dextrose digest agar. With doubtful or particularly important tubes, broth and cooked meat under vaseline were also inoculated. Contaminated tubes, which occurred but rarely, were discarded.

*Preparation of the Flavin-Purine Complex*

The 60 per cent alcohol precipitate obtained in the preparation of Cohn's Fraction G (5) was extracted 3 times with 10 volumes of hot

75 per cent acetone. The filtrate was cooled and concentrated. When this was kept overnight in the cold room a heavy precipitate separated out.

This granular yellow precipitate, 20 mg. of which are derived from 100 grams of liver, has a nitrogen content of 28 to 30 per cent. Dr. Max Tishler found that it contains 80 to 85 per cent xanthine by the perchlorate precipitation of Biltz. Mr. Edward Meilman, working with one of us, found it to contain adenosine and sometimes inosine, glutathione, and traces of nicotinic acid amide. The precipitate regularly contains 1.2 per cent flavin phosphoric acid. Its further composition is being investigated.

*Preparation of the "Alcohol-Ether Filtrate" and the "Calcium Filtrate"*

The charcoal elute described by Subbarow, Jacobson and Fiske (6) was precipitated with 10 volumes of 95 per cent alcohol and 10 volumes of ether. The mixture was left in the cold room for 48 hours and the precipitate filtered off. The filtrate, after removal of the alcohol and ether, was the alcohol-ether filtrate fraction. To prepare the calcium-filtrate fraction, the filtrate was concentrated to a small volume and the concentrate neutralized with $Ca(OH)_2$ and precipitated with 5 volumes of alcohol. The calcium precipitate mostly contained glutathione and adenylic acid. The filtrate was concentrated to remove alcohol. So far, from the filtrate small amounts of ergothionine, tryptophane, tyrosine, nicotinic acid amide, guanosine and ethanol amine have been isolated.

## RESULTS

*The Resolution of Growth Factor A into Two Fractions*

Growth factor A was at first considered to be only one substance, but preliminary attempts at its purification soon indicated that the factor A activity was due to at least two substances. Treatment of a partly purified liver extract in 80 per cent alcohol with barium hydroxide gave a precipitate and a filtrate, each of which, after regeneration, had a very low activity. But combination of both fractions almost restored the original activity.

In a previous paper (2) it was shown that treatment of liver extract by charcoal removes the factor A activity. At that time no successful elution of the active material from the charcoal had been accomplished. Meanwhile, Subbarow, Jacobson and Fiske (6) had obtained a partially purified anti-pernicious anemia material prepared by adsorption on charcoal and subsequent elution with alcohol. This elute gave fairly good growth of the *Ædes ægypti* larvæ only if used at relatively high concentrations, on the basis of the weight of liver from which it was

derived. It was then found that if this elute were supplemented with the flavin-purine complex prepared from liver, high values for $N \times 1/T$ could be obtained with much lower concentrations of the elute, although the flavin-purine complex alone had no factor A activity whatever (Table I). In the presence of the high concentrations of elute needed to obtain $N \times 1/T$ values of about 20 in the absence of flavin-purine complex, metamorphosis to the adult stage required a longer time and was accomplished by far fewer insects than in the presence of both elute and flavin-purine complex. A concentration of each of the two components, such that 100 ml. of medium contained that amount of each derived from 50 grams of liver, gave $N \times 1/T$ values of 20 or more, while half this concentration gave values of about 10. These results show that the amount of elute plus flavin-purine complex derived from 50 grams of liver has a factor A activity equivalent to that amount of Lilly liver extract No. 343 derived from about 12.5 grams of liver (a 0.5 per cent solution).

TABLE I

*The effect of the flavin complex*

| Concentration as grams of liver from which was derived amount of fraction present in 100 ml. of culture medium | | |
|---|---|---|
| Charcoal elute | Flavin complex | $N \times 1/T$ |
| 600 | 0 | 18.2 |
| 400 | 0 | 20.0 |
| 200 | 0 | 13.3 |
| 100 | 0 | 2.1 |
| 200 | 200 | 23.7 |
| 100 | 100 | 25.0 |
| 50 | 50 | 20.0 |
| 27 | 27 | 10.1 |
| 13.5 | 13.5 | 1.7 |
| 6.7 | 6.7 | 0 |
| 0 | 50 | 0 |

The growth factor present in the charcoal elute has been subjected to further purification, the activity going into the alcohol-ether filtrate and the calcium-filtrate fractions prepared as already described. In the presence of suitable concentrations of these more highly purified fractions, the same amounts of flavin-purine complex gave the same growth as in the presence of the charcoal elute itself. The nature of the substances responsible for the activity of the calcium-filtrate fraction is now being investigated.

*Comparison of the Effects of Riboflavin and the Flavin-Purine Complex*

Repeated experiments have shown that, in the presence of the alcohol-ether filtrate or the calcium filtrate, pure crystalline riboflavin will support excellent growth of the larvæ (Table II). Very much

TABLE II

*Comparison of the effects of flavin complex and riboflavin.* Each of the tubes contained for lines 1–12, alcohol-ether filtrate, for lines 13–24, calcium filtrate, each at a concentration such that 100 ml. of solution contained the material derived from 53 grams of liver.

| Concentration mg. per 100 ml. | | $N \times 1/T$ | Percentage of larvæ transforming to adults | Percentage of larvæ transforming to normal adults |
|---|---|---|---|---|
| Flavin complex | Riboflavin | | | |
| 0 | 0 | 0 | 0 | 0 |
| 20.0 | 0 | 23.1 | 100 | 100 |
| 10.0 | 0 | 23.1 | 67 | 67 |
| 5.4 | 0 | 6.5 | 33 | 0 |
| 0 | 1.0 | 16.6 | 50 | 0 |
| 0 | 0.5 | 15.8 | 33 | 0 |
| 0 | 0.33 | 10.7 | 0 | 0 |
| 0 | 0.17 | 10.7 | 17 | 0 |
| 0 | 0.08 | 13.4 | 33 | 0 |
| 0 | 0.04 | 14.1 | 33 | 0 |
| 0 | 0.025 | 14.1 | 33 | 17 |
| 0 | 0.008 | 6.0 | 33 | 17 |
| 0 | 0 | 0 | 0 | 0 |
| 13.4 | 0 | 24.0 | 100 | 100 |
| 10.0 | 0 | 18.9 | 67 | 67 |
| 6.6 | 0 | 23.1 | 100 | 100 |
| 3.3 | 0 | 14.8 | 50 | 50 |
| 0 | 0.25 | 19.8 | 67 | 0 |
| 0 | 0.17 | 21.4 | 83 | 0 |
| 0 | 0.125 | 18.0 | 67 | 0 |
| 0 | 0.080 | 19.8 | 67 | 50 |
| 0 | 0.040 | 22.2 | 67 | 33 |
| 0 | 0.017 | 14.8 | 67 | 33 |
| 0 | 0.008 | 6.2 | 17 | 17 |

lower concentrations of riboflavin are required than of the flavin-purine complex. In the presence of alcohol-ether filtrate, as little as 0.025 mg. of riboflavin per 100 ml. gave growth almost as good as did 1 mg. of riboflavin, but somewhat less than that obtained with 10 mg. of flavin-purine complex; 0.008 mg. of riboflavin per 100 ml. gave growth as good as 5.4 mg. of flavin-purine complex. In the presence of the calcium filtrate, 0.04 mg. of riboflavin per 100 ml. gave a value for $N \times 1/T$ as high as that obtained with 6.6 mg. of flavin-purine

complex, while 0.017 mg. of riboflavin was about equivalent to 3.3 mg. of flavin complex. Since the flavin complex contains 1.2 per cent flavin-phosphate, it is readily apparent that the flavin as contained in the complex has about the same activity, so far as larval growth is concerned, as free riboflavin. But it was soon noted that many of the larvæ growing in media containing riboflavin in place of flavin complex had difficulty in metamorphosing into the adult stage. This fact is well shown in Table II. In the presence of alcohol-ether filtrate, those concentrations of flavin complex which gave the highest $N \times 1/T$ values permitted 67 to 100 per cent of the larvæ to emerge as adults and all the adults which emerged were normal and vigorous. Adults were considered normal if they were able to emerge completely from the pupal sheath and to fly up from the surface of the liquid. Abnormal adults were those which either failed to emerge completely from the pupal sheath, or, having done so, were too weak to get free from the surface of the liquid. In Table II it may be seen that in media containing alcohol-ether filtrate and concentrations of riboflavin giving high $N \times 1/T$ values, very few adults emerged and most of these were abnormal. Larvæ which failed to transform into adults usually died, either in the late larval stage or in the pupal stage. In the presence of calcium filtrate, the same differences are apparent between flavin complex and riboflavin. Here again, riboflavin in the most favorable case permitted only 50 per cent of the larvæ to transform into normal adults. With flavin complex, concentrations which gave the fastest larval growth also gave the highest percentage of normal adults, but with riboflavin the highest percentage of normal adults was in general obtained with concentrations which gave submaximal larval growth rates.

Riboflavin is thus an essential substance for the growth of mosquito larvæ. Flavin-purine complex, however, contains some other substance which is possibly of little or no importance for larval growth but is very important for metamorphosis. The nature of this material is unknown. Perhaps the difference between the effects of the flavin complex and the riboflavin may be accounted for by the fact that the flavin in the complex exists as flavin phosphate.

### *The Need for Thiamin*

It has already been shown (2) that if larvæ of *Ædes ægypti* are grown with casein, $CaCl_2$, liver extract, and yeast extract previously treated with fuller's earth, both the factors present in liver extract and those present in the yeast extract withstand 5 hours of autoclaving at 120° C., if each extract is tested in the presence of the other extract

autoclaved for only ½ hour. But if both the extracts are autoclaved for 5 hours, the amount of growth obtained is greatly reduced (Table III). When such autoclaved liver and yeast extracts are supplemented with crystalline thiamin in low concentration, growth is restored almost to that obtained with extracts autoclaved for only ½ hour (Table III). This is true whether the casein be free from water-soluble vitamins only, or from both fat- and water-soluble vitamins, although with the latter type of casein, growth is never as good as with the former, possibly because of a deficiency in a fat-soluble growth factor. Table III also shows the interesting fact that a thiamin solution autoclaved for 5 hours is fully as effective as one sterilized by Berkefeld filtration. This can only mean that certain decomposition products of thiamin are as effective as the intact compound. These decompo-

TABLE III

*The effect of thiamin.* Each tube contained 0.01 M $CaCl_2$.

| Type of casein | Treatment of liver extract (0.5% Lilly 343) and yeast vitamin Harris 0.33% after fuller's earth adsorption | Concentration of added thiamin mg./100 ml. | $N \times 1/T$ | |
|---|---|---|---|---|
| | | | Exp. A | Exp. B |
| Harris. Free from water-soluble vitamins | Autoclaved ½ hour | 0 | 23.1 | 25.0 |
| " " " " " | Autoclaved 5 hours | 0 | 6.2 | 7.5 |
| " " " " " | " " " | 0.80 | 20.7 | 16.7 |
| " " " " " | " " " | 0.33 | 17.6 | 15.4 |
| " " " " " | " " " | 0.17 | 15.4 | 16.7 |
| " " " " " | " " " | 0.08 | — | 18.2 |
| " " " " " | " " " | 0.33 —autoclaved 5 hours | 18.8 | 20.0 |
| Harris. Free from water- and fat-soluble vitamins | Autoclaved ½ hour | 0 | 18.8 | 14.3 |
| " " " " " " " | Autoclaved 5 hours | 0 | 0 | 0 |
| " " " " " " " | " " " | 0.80 | 12.8 | 9.6 |

sition products apparently are not formed when liver extract or yeast extract is autoclaved for 5 hours. Presumably, under these conditions, other less easily utilized products are formed. This accords with the fact that growth does go on even to the adult stage in the medium autoclaved for 5 hours and not supplemented with thiamin. The slower rate and greater difficulty with which it proceeds are perhaps the result of a slow synthesis of an essential substance which is readily synthesized from thiamin itself or certain of its decomposition products. The utilization of certain decomposition products of thiamin has been shown for *Phycomyces* by Schopfer and Jung (7), for excised tomato roots by Robbins and Kavanagh (8) and for *Staphylococcus aureus* by Knight (9).

## DISCUSSION

Early in the study of the nutritional requirements of insects, it became apparent that yeast and other microörganisms were the

natural source of certain necessary foodstuffs (10–14). Several workers, as for example Bacot and Harden (15), working with *Drosophila*, and Richardson (16) with *Ephestia kuehniella*, soon found that yeast extract would supply substances essential for the growth of insect larvæ. Since yeast extract was at that time synonymous with vitamin B, these authors concluded that insects require vitamin B. Similar evidence for the vitamin B requirement of insects has been recently supplied by McCay (17) for the roach *Blatella germanica*, by Crowell and McCay (18) for larvæ of the clothes moth *Tineola bisseliella* and by Frost, Herms and Hoskins (19) for larvæ of the mosquito *Theobaldia incidens*. Hobson (20), and Street and Palmer (21) were among the first to show that the yeast extract supplied several different essential substances, and it was Hobson who found that pure vitamin $B_1$ is one of these substances. Van't Hoog (22) has gone still further and shown that both crystalline riboflavin and thiamin are substances essential for the growth of larvæ of *Drosophila melanogaster*. Growth does not occur, however, unless still a third substance or group of substances, also present in yeast extract, is supplied. Thus the water-soluble accessory growth factors required by *Drosophila melanogaster* and *Ædes ægypti* are identical so far as they are known at present. Both insects require for larval development riboflavin, thiamin, and a third factor present in yeast extract, liver extract, rice-polishings concentrate, etc. It is interesting that these requirements form a perfect parallel to the vitamin B complex requirements of vertebrates (23–25).

The nature of the third factor required by vertebrates and present in liver extract is now under investigation in several laboratories. The indications are that nicotinic acid amide or cozymase is one of the essential substances present in the third factor, but not the only one (26–29). Lepkovsky (30) has recently isolated one of these substances in crystalline form, but he gives no information as to its nature or method of preparation. So far as *Ædes ægypti* larvæ are concerned, nicotinic acid or its amide cannot replace the "calcium-filtrate" fraction of liver extract. But evidence is now accumulating that nicotinic acid amide plus certain other substances can at least partially replace the impure liver fraction.

It is worth noting that while rice-polishings concentrates have been generally considered to be free from riboflavin (as in the work of Cook, Clarke and Light (28)), they must actually contain appreciable amounts, since a 0.4 per cent solution of Ryzamin B has been found (4) to give growth about as good as a 0.3 per cent solution of Lilly liver extract No. 343. On the basis of this result, a 0.4 per cent

solution of Ryzamin B should contain around 0.02 mg. of riboflavin per 100 ml. It is not surprising that the clay adsorbate (the International Standard vitamin $B_1$) prepared from rice-polishings concentrate also can replace liver extract (4), since the flavin and other necessary factors would be adsorbed as readily as the thiamin.

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## SUMMARY

Riboflavin, and thiamin or certain of its degradation products, are necessary for the normal development of larvæ of the yellow fever mosquito *Ædes ægypti*. In the presence of all the other necessary growth factors, riboflavin permits normal larval growth at concentrations as low as 0.0004 mg. per ml. and has a definite effect at a concentration of 0.00008 mg. per ml., while thiamin permits normal growth at concentrations at least as low as 0.0008 mg. per ml. Although larval growth is normal in the presence of suitable amounts of heat-killed yeast, riboflavin, and the other liver extract factors supplied in partially purified form, metamorphosis to the adult stage is successfully accomplished by a relatively small percentage of the insects. If, however, the flavin is supplied as a flavin-purine complex containing 1.2 per cent flavin-phosphate, almost all the larvæ transform into vigorous adults. The nature of the additional factor responsible for this difference is not known.

## REFERENCES

1. TRAGER, W., 1935*a*. *Am. Jour. Hygiene*, **22**: 18.
2. TRAGER, W., 1935*b*. *Am. Jour. Hygiene*, **22**: 475.
3. TRAGER, W., 1936. *Biol. Bull.*, **71**: 343.
4. TRAGER, W., 1937. *Jour. Exper. Biol.*, **14**: 240.
5. COHN, E. J., G. R. MINOT, G. A. ALLES, AND W. T. SALTER, 1928. *Jour. Biol. Chem.*, **77**: 325.
6. SUBBAROW, Y., B. M. JACOBSON, AND C. H. FISKE, 1936. *New Eng. Jour. Med.*, **214**: 194.
7. SCHOPFER, W. H., AND A. JUNG, 1937. *Compt. rend. Acad. Sci. Paris*, **204**: 1500.
8. ROBBINS, W. J., AND F. KAVANAGH, 1937. *Proc. Nat. Acad. Sci.*, **23**: 499.
9. KNIGHT, B. C. J. G., 1937. *Biochem. Jour.*, **31**: 966.
10. DELCOURT, A., AND E. GUYÉNOT, 1911. *Bull. Sci. de la Fr. et Belg.*, **45**: 249.
11. NORTHROP, J. H., 1917. *Jour. Biol. Chem.*, **30**: 181.
12. BAUMBERGER, J. P., 1919. *Jour. Exper. Zoöl.*, **28**: 1.
13. WOLLMAN, T., 1919. *Compt. rend. Soc. biol.*, **82**: 1208.
14. GLASER, R. W., 1924. *Am. Jour. Trop. Med.*, **4**: 85.
15. BACOT, A. W., AND A. HARDEN, 1922. *Biochem. Jour.*, **16**: 148.
16. RICHARDSON, C. H., 1926. *Jour. Agric. Res.*, **32**: 895.
17. MCCAY, C. M., 1933. *Jour. Biol. Chem.*, **100**: lxvii.
18. CROWELL, M. F., AND C. M. MCCAY, 1937. *Physiol. Zoöl.*, **10**: 368.

19. FROST, F. M., W. B. HERMS, AND W. M. HOSKINS, 1936. *Jour. Exper. Zoöl.*, **73**: 461.
20. HOBSON, R. P., 1933. *Biochem. Jour.*, **27**: 1899.
21. STREET, H. R., AND L. S. PALMER, 1935. *Proc. Soc. Exper. Biol. and Med.*, **32**: 1500.
22. VAN'T HOOG, E. G., 1935. *Zeitschr. f. Vitaminforsch.*, **4**: 300.
23. GYÖRGY, P., R. KUHN, AND TH. WAGNER-JAUREGG, 1933. *Naturwiss.*, **21**: 560.
24. BIRCH, T. W., P. GYÖRGY, AND L. J. HARRIS, 1935. *Biochem. Jour.*, **29**: 2830.
25. LEPKOVSKY, S., T. H. JUKES, AND M. E. KRAUSE, 1936. *J. Biol. Chem.*, **115**: 557.
26. ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG, AND D. W. WOOLLEY, 1937. *Jour. Am. Chem. Soc.*, **59**: 1767.
27. FROST, D. V., AND C. A. ELVEHJEM, 1937. *Jour. Biol. Chem.*, **121**: 255.
28. COOK, C. A., M. F. CLARKE, AND A. E. LIGHT, 1937. *Proc. Soc. Exper. Biol. and Med.*, **37**: 514.
29. EDGAR, C. E., AND T. F. MACRAE, 1937. *Biochem. Jour.*, **31**: 893.
30. LEPKOVSKY, S., 1938. *Science*, **87**: 169.