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The Vitamin B-complex Requirements of Several Insects

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It has been shown by us [Fraenkel & Blewett, 1943b] that six insects, which normally feed on flour and other dried food, grow satisfactorily on a diet of casein 50, glucose 50, yeast 5, cholesterol 1, McCollum's salt mixture 1, and water 15 parts, but entirely fail to grow when yeast is omitted. With the yeast replaced by yeast extract, the growth rate is considerably slower. No growth takes place if the diet contains a water-insoluble fraction alone in place of whole yeast. Previous work has indicated that insects require several vitamins of the B-group; the position up to 1940 has been reviewed by Craig & Hoskins [1940] and Trager [1941]. The present paper deals with the growth factors required by insects which are contained in the water-soluble and in the water-insoluble fractions of yeast. Short accounts of some of the results have already appeared elsewhere [Fraenkel & Blewett, 1942 a, b; 1943a, d].

EXPERIMENTAL

The following beetles were used: *Tribolium confusum* Duv., Tenebrionidae; *Silvanus (Oryzaephilus) surinamensis* L., Cucujidae; *Sitodrepa panicea* L., Anobiidae; *Lasioderma serricornis* Fab., Anobiidae; *Ptinus tectus* Boield., Ptinidae.

The following preparations and substances were used to compound diets:

Casein A/E, vitamin and fat free (Glaxo Laboratories Ltd.). Dried debittered brewer's yeast (Glaxo Laboratories Ltd.). Yeast extract, prepared from fresh brewer's yeast according to Chick & Roscoe [1930]. 'Insoluble yeast': residue from preparation of yeast extract, after 10 successive extractions with large volumes of boiling water.

Aneurin hydrochloride (B.) (Glaxo Laboratories Ltd.), riboflavin (Glaxo Laboratories Ltd.), nicotinic acid (British Drug Houses Ltd.), pyridoxin (Glaxo Laboratories Ltd.), Capantothenate (Glaxo Laboratories Ltd. and Roche Products Ltd.), choline chloride (British Drug Houses Ltd.), *p*-aminobenzoic acid (British Drug Houses Ltd.), *i*-inositol (British Drug Houses Ltd.), biotin methyl ester (Research Laboratories, S.M.A. Corporation), and biotin (Prof. du Vigneaud).

All the tests were performed in 2 × 1 in. specimen tubes. Each tube contained 2 g. of the dry diet and each diet was

prepared in duplicate. Each test was performed with 20 larvae, 10 in each tube. The tubes were kept in a room of which the temperature was automatically maintained at 25° and the relative humidity at 70%. The criteria for assessing results are rate of growth from the newly hatched larva until pupation (*Tribolium*) or emergence of adult (other insects), and mortality. The methods of experimenting and assessing results are fully described elsewhere [Fraenkel & Blewett, 1943 b, c].

Preparation of a diet deficient in riboflavin. Yeast extract was treated with norit charcoal (Hopkin and Williams, Ltd.) according to the method of El Sadr, Macrae & Work [1940]. The yeast extract was not previously autoclaved, but as a precaution aneurin HCl was added to the diet, which then consisted of casein 50, glucose 50, cholesterol 1, yeast charcoal filtrate 15, insoluble yeast 5, McCollum's salt mixture 1 parts, aneurin HCl 50 µg./g. Riboflavin was added to the diet in graded doses and the results showing a graded response (Table 1)

Table 1. *Pupation of Tribolium on a diet deficient in riboflavin, with the addition of graded doses of riboflavin*

Riboflavin (µg./g. of diet)	No. of pupae	Spread in the pupation period (days)
None	11	60–113
0.625	16	50–74
1.25	18	32–43
2.5	16	29–39
5.0	18	30–39
10.0	16	30–39
50.0	20	28–39
Wholemeal flour (control)	19	27–30

confirmed those reported by Fröbrich [1939], who grew *Tribolium* on a diet containing yeast extract which had been heated in an alkaline medium in order to destroy the riboflavin.

Substitution of yeast extract by pure vitamins. As pure vitamins of the B-group became available, attempts were made to feed mixtures of pure vitamins instead of yeast extract. Except in some earlier tests, wheat-germ oil was added to the diets

placed by biotin (0.1 $\mu\text{g./g.}$) [Fraenkel & Blewett, 1943a, Fig. 3]. The results of these two series, one containing 'insoluble yeast' and the other biotin, were virtually identical, with three exceptions. In the biotin series absence of inositol delayed growth significantly, whereas absence of *p*-aminobenzoic acid had no effect. A more serious discrepancy applied to diets without pantothenic acid. These were totally inefficient in the 'insoluble yeast' series, but not quite so deficient in the biotin series, where 13 out of 20 larvae completed development, although at a greatly reduced rate. This discrepancy cannot at present be explained. It appears from a comparison of these two sets of tests that no significant amounts of vitamins could have been added to diets with 5% 'insoluble yeast'. Inositol is the only factor where the slight discrepancy in the results may, possibly, be due to retention of inositol in 'insoluble yeast'. An aneurin determination on our 'insoluble yeast' made by Dr Y. L. Wang (Cambridge) showed below 0.1 $\mu\text{g./g.}$, a quantity too small to have any effect.

Growth requirements of other insects

Concurrently with the tests of diet 6 performed with *Tribolium*, the diets which contained 'insoluble yeast' were also tested with four other beetles. The results are summarized in Table 3.

The complete diet was as good, if not better, than the controls which contained yeast extract as a source of B-vitamins. Omission of inositol or *p*-aminobenzoic acid produced a slight, but possibly significant delay in growth. Absence of choline had a very pronounced delaying effect (Fig. 3), much greater than with *Tribolium*. Absence of the other vitamins had an effect similar to that described for *Tribolium*.

Lasioderma serricorne gave results quite different from *Tribolium* and *Ptinus*. Absence of riboflavin, nicotinic acid, *p*-aminobenzoic acid or inositol seemed to have no effect. Absence of aneurin, pyridoxin or pantothenic acid delayed development considerably, but to a much slighter extent than was the case with *Tribolium* and *Ptinus*. Absence of choline chloride had a very pronounced delaying action (Table 3, and Fraenkel & Blewett [1943a, Fig. 2]).

Sitodrepa panicea also gave results different from *Tribolium* and *Ptinus*, and from *Lasioderma*. The growth rate on the complete diet was considerably inferior to that of the control, where yeast extract was given instead of pure B-vitamins. Absence of nicotinic acid, inositol, choline chloride or *p*-aminobenzoic acid greatly affected the efficiency of the diet, which became totally inefficient only in the absence of aneurin (Table 3, and Fraenkel & Blewett [1944, Fig. 4]).

Table 3. The effect on the growth of various *Coleoptera* larvae of the omission from the diet (Table 2, no. 6) of different members of the B-group of vitamins

Description of diet	<i>Tribolium</i>				<i>Ptinus</i>				<i>Silvanus</i>				<i>Lasioderma</i>				<i>Sitodrepa</i>			
	Growth rate	Days	Nos.		Growth rate	Days	Nos.		Growth rate	Days	Nos.		Growth rate	Days	Nos.		Growth rate	Days	Nos.	
Wholemeal flour	114	27-29	19		85	57-67	16		115	25-29	15		94	35-39	20		97	37-41	17	
Yeast extract + 'insoluble yeast'	100	29-36	18		100	47-58	18		100	27-35	16		100	34-39	19		100	37-39	15	
All vitamins (Table 2, no. 6)	100	29-37	18		104	47-60	18		94	27-36	16		92	34-41	19		90	38-45	13	
No aneurin	42	55-132	17		70	70-79	2		97	27-36	16		81	41-47	18		45	74-94	4	
No riboflavin	31	86-132	3		0	—	0		94	30-46	9		97	34-41	18		88	41-49	9	
No nicotinic acid	27	114	1		66	79	1		78	51	1		97	34-39	18		93	40-47	12	
No pyridoxin	70	37-53	16		63	69-90	13		91	31-41	16		87	36-48	17		84	43-47	14	
No pantothenic acid	0	—	0		70	69-77	2		103(?)	30	1		87	36-41	8		93	39-49	5	
No choline chloride	88	31-40	19		74	64-83	14		89	32-36	16		74	43-61	14		90	38-51	12	
No inositol	97	29-38	17		98	48-58	17		91	29-37	15		97	34-41	18		90	41-45	13	
No <i>p</i> -aminobenzoic acid	86	30-40	18		98	52-60	18		94	30-41	14		97	34-41	18		90	39-47	14	

The growth rate (col. 1, under appropriate insect heading) is calculated as a reciprocal of the period in which half the number of insects completed their development and is expressed as a percentage of the controls. The period of development (col. 2) records the spread in the larval period (*Tribolium*) or larval plus pupal period (other insects). Col. 3 records the number, out of 20, completing development. The 'all vitamins' diet was that recorded in Table 2, no. 6. The relative growth rates of *Tribolium* and the other insects are not strictly comparable because they are based with *Tribolium* on the larval period only and with the other insects on the larval plus pupal period. Length of the pupal period of the insects in question is approximately 10 days.

Ptinus tectus [Fraenkel & Blewett, 1943a, Fig. 1] in the absence of single B-factors gave results almost identical with those described for *Tribolium*.

Silvanus surinamensis sometimes gave erratic results which could not be attributed to dietary factors. In the absence of aneurin, pyridoxin,

choline chloride, inositol or *p*-aminobenzoic acid, growth was not impaired. Absence of riboflavin caused high mortality and considerable delay in growth, and without nicotinic acid or pantothenic acid hardly any growth took place.

Effects of choline, inositol and *p*-aminobenzoic acid

To obtain a clear indication as to the nutritional importance of choline, inositol and *p*-aminobenzoic acid, a series of tests was devised for *Tribolium* and *Ptinus*, in which the diets contained these substances in various combinations. Diet no. 6, Table 2, was used. With *Tribolium* no difference was found in the efficiency of diets containing all the vitamins compared with diets lacking either inositol, or *p*-aminobenzoic acid, or both. Without choline chloride, the diet became considerably inferior, much more so than in the corresponding tests of Fig. 2. Pupation started after 37 days, as compared with 30 days on the 'all vitamins' diet. Omission of inositol + choline, or *p*-aminobenzoic acid + choline or of all three substances had no effect beyond that shown by the absence of choline.

With *Ptinus* the result was somewhat different (Fig. 3). Omission of inositol, or *p*-aminobenzoic

Fig. 3. *Ptinus tectus*. Explanations as for Fig. 2.

acid singly had a slight, but possibly not significant delaying effect, while a cumulative effect of the absence of both these substances became manifest. Without choline chloride, the diet became very inefficient. Omission of *p*-aminobenzoic acid + choline had no further effect, but without choline + inositol the diet was inferior to the 'no choline' diet. From these tests it appears that inositol is of some nutritional value for *Ptinus*.

Biotin as an insect growth factor

In the experiments so far described, with one exception (p. 688), the diets contained the insoluble fraction of yeast. Earlier experiments [Fraenkel & Blewett, 1943b] had shown that the water-soluble fraction of yeast was not fully equivalent to whole yeast, but had to be supplemented

by the insoluble fraction, which was eventually found to be effective because it supplied biotin. The initial steps in the analysis which led to the recognition of biotin as the missing factor are described elsewhere [Fraenkel & Blewett, 1942a]. The result of a test with crystalline biotin methyl ester (25 μ g. dissolved in 1 ml. of ethanol) is given in Fig. 4. The solution was taken up in a 1 ml.

Fig. 4. Growth of *Tribolium confusum* on the yeast extract diet with the addition of graded quantities of biotin methyl ester.

hypodermic syringe and the quantities required were pipetted through the needle into the diets. The yeast extract diet was greatly improved by adding biotin methyl ester in quantities corresponding to 0.25 and 0.0625 μ g./g. dry diet. In larger quantities biotin had a toxic effect. Quantities lower than 0.0625 μ g./g. were not tested in this series. This very small dose produced an optimal effect which at first suggested that the optimal dose might be lower still. The toxic effects of larger quantities were not due to insufficient removal of the alcohol.

The experiment recorded in Fig. 4 was subsequently repeated with pure biotin in place of the methyl ester (Fig. 5). Growth was appreciably

Fig. 5. Growth of *Tribolium confusum* on the yeast extract diet with the addition of graded quantities of biotin.

faster on the yeast extract diet without biotin than it had been on the former occasion. This is considered to be due to traces of biotin in the yeast extract of the Fig. 5 series. The optimum concentration of biotin was reached with 0.1 μ g./g. dry food, 0.05 μ g./g. being almost as efficient. Larger

quantities again appeared to be toxic, while lower quantities show a graded decreasing response with the threshold at the very low quantity of between 0.003 and 0.006 $\mu\text{g./g.}$ This does not exclude the possibility that an optimal effect would have been shown with 0.1 $\mu\text{g.}$ biotin methyl ester.

The difficulty of standardizing the yeast extract and getting consistent results made it desirable to construct a biotin-free diet which contained the vitamins of the B-complex as pure substances, instead of in yeast extract. The results of this series are given on p. 688 [cf. also Fraenkel & Blewett, 1943a, Fig. 3]. This diet, containing no yeast fractions, but 0.1 $\mu\text{g.}$ biotin/g. of dry diet, is clearly as efficient as a diet which contains insoluble yeast as a source of biotin. Leaving out wheat-germ oil did not alter the efficiency of the diet, but without biotin growth became much slower and the efficiency of the biotin-free diet was about that of a typical yeast extract diet without biotin. The results of these tests showed that for *Tribolium* biotin is the only factor missing in a diet which contains yeast extract and no 'insoluble yeast'.

exception of *Silvanus*, omission of 'insoluble yeast' causes the diet to become deficient, and that the deficiency is made good by adding biotin.

A few tests were performed with the diamino-carboxylic acid which results from hydrolysis of the urea ring of biotin [du Vigneaud, 1942] and is capable of stimulating the growth of yeast in a biotin-free medium, exhibiting about 10% of the activity of biotin [du Vigneaud, Dittmer, Hoffmann & Melville, 1942]. For *Tribolium*, a biotin-free diet was considerably improved by the addition of 2.5 or 5.25 $\mu\text{g./g.}$ of this diaminocarboxylic acid, but still remained considerably inferior to the same diet with biotin (0.1 $\mu\text{g./g.}$) added. 1 $\mu\text{g./g.}$ or less of the diaminocarboxylic acid in the diet had no effect whatsoever (Fig. 6). These tests show clearly that biotin diaminocarboxylic acid has some effect in replacing biotin for *Tribolium*, but that, as with yeast, its activity is considerably less than that of biotin. This lesser activity cannot be compensated by using larger doses, because of a toxic effect.

All the tests so far described used diets containing glucose. On a yeast extract diet containing

Table 4. Medium growth rate, period of development and number of pupae (*Tribolium*) or adults (other insects) formed, in the presence or absence of insoluble yeast or biotin

(Explanation as for Table 3.)

Diet includes	<i>Tribolium</i>			<i>Ptinus</i>			<i>Silvanus</i>			<i>Lasioderma</i>			<i>Sitodrepa</i>		
	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.
Yeast extract + 'insoluble yeast'	100	29-36	18	100	47-58	18	100	27-35	16	100	34-39	19	100	37-41	17
Yeast extract + biotin	107	28-32	19	92	54-70	16	95	28-35	16	92	26-39	17	100	37-39	15
Yeast extract alone	80	32-46	19	83	56-70	15	95	29-36	14	69	41-64	14	91	39-51	13

Concurrently with these tests with *Tribolium*, corresponding tests were performed with four other beetles. The results are summarized in Table 4. It

Fig. 6. Growth of *Tribolium confusum* on diets consisting of casein, glucose, cholesterol, salts, the eight members of the vitamin B-complex listed in Table 2, and in the presence or absence of biotin, or of graded amounts of 'biotin' diaminocarboxylic acid.

is clear [cf. also Fraenkel & Blewett, 1943b, Figs. 7-11 and Table 2] that for all the insects, with the

starch instead of glucose, *Tribolium* grew much better than in the diet containing glucose. The starch diet was almost as efficient as if it had contained 'insoluble yeast'. The effect was traced to the fact that even purified starch contained enough biotin to allow almost optimal growth of *Tribolium*.

DISCUSSION

A comparison of the effects of the absence of single B-vitamins on the different insects is given in Table 3. The most striking result is the difference in the nutritional requirements of *Tribolium* and *Ptinus* on the one hand, and *Silvanus*, *Lasioderma* and *Sitodrepa* on the other. The vitamin requirements of *Tribolium* and *Ptinus* are similar to those of the rat, dog and chick. Broadly speaking, aneurin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid are indispensable; in their absence growth is greatly affected. Choline and biotin are of somewhat lesser,

but still great importance, whereas inositol and *p*-aminobenzoic acid are of minor or perhaps no importance. With the other insects, the above-mentioned 'indispensable' vitamins seem to be less important, although their requirements proved very different in detail.

These two groups of insects differ in one important anatomical and physiological feature. *Lasioderma*, *Sitodrepa* and *Silvanus* belong to the large group of insects which contain intracellular symbionts [Buchner, 1930; Koch, 1933 *a, b*; 1936]. The latter seem to be absent with *Tribolium* and *Ptinus*. From the distribution of intracellular symbioses in insects several authors have considered their function to be provision of accessory food substances: Wigglesworth [1929], Buchner [1930], Aschner [1931], and Koch [1933 *a, b*] produced evidence in support of this suggestion for *Sitodrepa panicea*. This insect when freed of its symbionts will not grow on 'Erbswurst', an otherwise satisfactory food, but will grow on 'Erbswurst' plus 25% yeast. Our results reported in this paper strongly support the view that intracellular symbionts are sources of vitamins. In their absence, with *Tribolium* and *Ptinus*, the 'indispensable' vitamins are necessary; in their presence *Lasioderma* grows well without any of them, *Silvanus* can do without aneurin and pyridoxin and *Sitodrepa* without nicotinic acid, pyridoxin and riboflavin. More recent experiments have shown that *Sitodrepa* and *Lasioderma*, when grown in the absence of symbionts, have vitamin requirements which are much more exacting than those of the normal larvae and resemble in every respect those of *Ptinus* and *Tribolium* [Fraenkel & Blewett, 1943*d*; 1944]. But even with *Tribolium* and *Ptinus*, in the absence of some of the 'indispensable' vitamins some growth takes place, and this may be due to partial intestinal synthesis. A parallel case is the synthesis of aneurin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid and biotin by micro-organisms in the stomach of ruminants [Wegner, Booth, Elvehjem & Hart, 1941; McElroy & Goss, 1941]. Intestinal synthesis of biotin in the rat has been demonstrated by Nielsen & Elvehjem [1942] and by Daft, Ashburn & Sebrell [1942], who produced symptoms of a deficiency, curable with biotin, by feeding rats with the bactericidal drugs sulphanilylguanidine and succinyl sulphathiazole.

Insects require essentially the same factors of the vitamin B-complex as rats, chicks and micro-organisms. *Drosophila* requires aneurin, riboflavin [van t' Hoog, 1935, 1936], nicotinic acid, pyridoxin and pantothenic acid [Tatum, 1939, 1941]; mosquitoes require the same substances [Trager & Subbarow, 1938; Subbarow & Trager, 1940]; *Tribolium* requires aneurin, riboflavin [Fröbrich, 1939; Offhaus, 1939], and nicotinic acid [Barton-Wright,

1941]; *Galleria* requires nicotinic acid [Rubinshtein & Shekun, 1941]. In none of these cases did the insects grow satisfactorily without the further addition of yeast or yeast fractions. Insects have also been shown to require for optimal growth in addition to yeast the water-insoluble fraction of yeast (Hobson [1933] for blowfly larvae, Fröbrich [1939] for *Tribolium*, and Tatum [1939, 1941] for the *Drosophila* larva). The need of *Tribolium* for biotin [Fraenkel & Blewett, 1942*b*] has been confirmed by Rosenthal & Reichstein [1942].

The need of certain insects for choline chloride now appears clear. In tests with *Tribolium*, omission of choline definitely decreases the efficiency of the diet. The need for choline was still more marked in experiments with *Ptinus* (Fig. 3) and most strikingly demonstrated with *Lasioderma* [Fraenkel & Blewett, 1943*a*, Fig. 2], in which choline seems to be the most important of the B-vitamins. With inositol and *p*-aminobenzoic acid the position is less clear and the results are conflicting, a position which closely resembles that experienced with vertebrates [György, 1942].

For *Tribolium* a diet without any yeast, but with the water-soluble B-vitamins and biotin in pure substance, with cholesterol and without wheat-germ oil, is in every respect as good as one with yeast or yeast fractions. This would appear to show that no further factors are required. *Ptinus*, *Sitodrepa* and *Lasioderma* also grow about equally fast with either insoluble yeast or biotin. All the insects, with the exception of *Sitodrepa*, grow as well on mixtures of pure B-vitamins as on yeast extract. Taking all the evidence together, biotin and sterols seem to be the only water-insoluble factors in yeast which are required by the insects in question. Also they do not seem to require any B-vitamins in addition to those already discovered (exception, *Sitodrepa*).

SUMMARY

1. Five insect species have been grown on an artificial diet which contains all the chemically identified members of the vitamin B-complex in pure form. For *Tribolium* and *Ptinus*, aneurin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid are essential vitamins; choline chloride is certainly, and inositol and *p*-aminobenzoic acid are possibly, of importance as growth factors.

2. *Lasioderma*, *Sitodrepa* and *Silvanus* do not seem to require some of the vitamins which are indispensable for *Tribolium* and *Ptinus*. This difference in the vitamin requirements is due to the presence in *Lasioderma*, *Sitodrepa* and *Silvanus* of intracellular symbiotic micro-organisms, which synthesize some of the vitamins.

3. When bred on a diet of casein, glucose, cholesterol, salts and aqueous yeast extract, insects

require, in addition, for optimal growth a substance contained in the water-insoluble residue of yeast, which was found to be biotin.

4. The optimal requirements of biotin for *Tribolium* are approximately 0.1 µg./g. of the dry diet, and the threshold of action is between 0.003 and 0.006 µg./g.

5. The insects tested do not appear to require any water-insoluble factor present in yeast in addition to a sterol and biotin.

6. The insects used, with the exception of *Sitodrepa*, grow on a mixture of pure B-vitamins as well as on yeast extract. It is concluded that these

insects do not require B-vitamins beyond those already isolated.

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The Sterol Requirements of Several Insects

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It has been shown [Fraenkel & Blewett, 1943a] that six insects which normally feed on flour and other stored food grow satisfactorily on a diet of casein 50, glucose or starch 50, yeast 5, cholesterol 1, salts 1 and water 15 parts. Without cholesterol, growth became slightly (*Tribolium*, *Silvanus*, *Lasioderma*), appreciably (*Ptinus*) or severely (*Sitodrepa*, *Ephestia*) impaired (Table 2 (1)). If the diet con-

tains aqueous yeast extract in place of yeast together with cholesterol, all the insects develop, though at a reduced rate resulting from a deficiency of biotin. Without cholesterol the diet becomes entirely or almost entirely inefficient for all the insects tested (Table 2 (3) and Fraenkel & Blewett [1943a]). In the present work the specificity of the sterol requirements of these insects is investigated.