Biosynthesis of Nucleic Acids in *Bacillus megaterium*

1. THE ISOLATION OF A NUCLEAR MATERIAL

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So-called 'chromatin bodies' have been observed in whole cells, protoplasts and spores of *Bacillus megaterium* by cytological and electron-microscopical techniques (Fitzjames, 1953, 1958; Robinow, 1956). In ultra-thin sections of whole cells the chromatin body appears as an area less electron-dense than the surrounding cytoplasm and made up of a network of fine fibrils with no bounding membrane (Piekarski & Giesbrecht, 1956; Giesbrecht & Piekarski, 1958; Giesbrecht, 1958). In protoplasts the morphology of the chromatin body appears to depend on the nature of the stabilizing medium. In media of low ionic strength the bodies are dispersed in an open arrangement of filaments, but in media of high ionic strength the bodies are aggregated into a tight structure with a chromatin material arranged round a non-chromatinic core (Fitzjames, 1958).

The investigations of Spiegelman, Aronson & Fitzjames (1958) and of de Lamater (1959) have strongly suggested that a discrete nuclear apparatus ('nucleoids' or 'nuclear material') can be isolated from protoplasts of *B. megaterium* and that this body can be equated with the chromatin area identifiable in electron micrographs of whole cells. The methods described for the preparation of these nuclear bodies, however, are prolonged and unsuitable for metabolic studies. Also, the procedures employed destroy some of the cellular components in order to liberate the nuclear bodies from the cytoplasm. The methods of Spiegelman *et al.* (1958) entail dissolving away the cytoplasmic membrane with lipase, and the methods of de Lamater (1959) entail standing the protoplasts in sodium glycocholate for up to 24 hr. and then disrupting the cells by ultrasonic treatment. Several other attempts to isolate a nuclear material have been reported (Ezekiel, 1960), but in most of these cases only a portion of the material has been recovered.

The purpose of the present paper is to describe a rapid and convenient method of isolating nuclear material from *B. megaterium* and fractionating the bacterial cell into three components, namely (a) a nuclear fraction containing almost all of the cellular DNA, together with some RNA and protein, (b) a cytoplasmic fraction containing all of the

cellular ribosomes and most of the cellular RNA, and (c) a membrane fraction made up of cytoplasmic membranes stripped clean of attached RNA or other material.

A preliminary account of some of this work has been given (Godson & Butler, 1962*a, b*).

METHODS

Organism. The strain of *Bacillus megaterium* (originally KM) was cultured as described by Butler, Crathorn & Hunter (1958). It was grown in a glucose-salts medium ('C-medium'; McQuillen, 1955) in aerated Roux bottles at 30°. To obtain cells in the logarithmic phase of growth, the Roux bottles were inoculated on the evening before the experiment and placed in a water bath at room temperature. During the early hours of the next morning (usually 5–6 hr. before harvesting) the air blowers and the thermostatic heater of the water bath were switched on by an automatic timing device. Growth of the culture under these conditions is reproducible and in this way cells of an accurately known age can be obtained. For most of the experiments described below the cells were harvested after 2–3 hr. of logarithmic growth.

Preparation of protoplasts. Whole cells were converted into protoplasts by resuspending them, at a concentration of approx. 10 mg. dry wt./ml., in C-medium (McQuillen, 1955) containing KH_2PO_4 (0.5M) and brought to pH 7.3 with NaOH (phosphate-salts medium), and adding 0.03% of lysozyme. The time required to convert the cells into protoplasts depended on the age of the culture: with cells harvested in the early logarithmic phase complete conversion took place within 5–10 min., but with cells harvested in the stationary phase, 45–60 min. was required, and cells from very old cultures were never completely converted into protoplasts.

Lysis of protoplasts with detergents. To study the lytic effect of detergents, suspensions of protoplasts stabilized in 0.5M-phosphate-salts medium were placed in a series of 250 ml. conical flasks containing the detergent, and either gently shaken at 30°, or left to stand at room temperature or immersed in an ice bath. Samples of the suspension were taken after various times and the extinctions measured at 420 m μ with a Unicam SP. 600 spectrophotometer. The samples were also examined with an interference microscope ($\times 1000$) for the presence of intact protoplasts.

The conditions finally adopted for the lysis of protoplasts with Lubrol W (I.C.I. Ltd.) (Cohn & Butler, 1958) were as follows. An equal volume of ice-cold 0.5M-phosphate-salts medium or 0.3M-sucrose containing MgCl_2

(0.01M) and tris (0.01M) adjusted to pH 7.3 with HCl (sucrose-MgCl₂-tris medium), containing 0.01 or 0.02% of Lubrol W, was added to a suspension of protoplasts (5–10 mg./ml.) stabilized in the same medium. Under these conditions, protoplasts from logarithmic-phase cells are lysed completely within 2–5 min.; the older the culture, the longer the time required for lysis of the protoplasts, and protoplasts from cells harvested 3–4 hr. after active growth had ceased took at least 1 hr. to burst.

Preparation of a nuclear material and other cell fractions. Two conditions were used for the preparation of nuclear material:

Method A. In preliminary experiments (Godson & Butler, 1962*a, b*) the protoplasts were suspended in 0.5M-phosphate-salts medium and lysed as described above. The lysate was centrifuged in the cold at 78 000*g*_{av} for 30 min. The tight gelatinous pad that sedimented under these conditions contained nearly all of the cellular DNA, some RNA and protein, and all of the cytoplasmic membranes of the cells. There was very little contamination of the pellet with the supernatant material. When the lysate was centrifuged at a low speed, nearly all of the DNA sedimented, but the pellet was very loose and seriously contaminated with the supernatant. The cytoplasmic membranes could be separated from the nuclear material by homogenizing the pellet for a very short time in a Potter homogenizer in 1–2 ml. of 0.01M-tris-HCl buffer, pH 7.3, containing MgCl₂ (0.01M), layering the homogenate on 0.9M-sucrose and then centrifuging for 30 min. in a Spinco no. SW25 rotor at 40 000*g*_{av}. The homogenization broke up the lumps of cytoplasmic membrane and reduced the viscosity of the pellet sufficiently to allow the membranes to separate from the nuclear material. The membranous material collected on the bottom of the tube and the viscous transparent nuclear material remained layered on the top of the sucrose. This could be removed with almost no contaminating sucrose by sucking it off with a pipette held a few millimetres above the surface. The nuclear material was normally centrifuged briefly at top speed in a Spinco no. 40 rotor to remove any fine membrane fragments. In this preparation the ribosomes present in the cytoplasmic fraction were degraded by the high ionic strength of the stabilizing medium to give particles with a sedimentation coefficient of less than 14S (Godson, Hunter & Butler, 1961). Hence the nuclear material prepared by this method is free from contamination with ribosomes.

Method B. To recover the ribosomes from the protoplasts, sucrose-MgCl₂-tris medium (see above) was used for stabilization. The lysis of the protoplasts with Lubrol W and the sedimentation of the nuclear material and cytoplasmic membranes was carried out as described above. The ribosomes present in the supernatant were recovered by centrifuging at 78 000*g*_{av} for several hours. The main difficulty with this method was that the nuclear material was contaminated with ribosomes that sedimented at the same time. Approx. 20% of the ribosomes were sedimented when the lysate was centrifuged at 78 000*g*_{av} for 30 min. (see Table 4). The nuclear material, even after homogenization, was too viscous for the ribosomes to be removed by centrifuging. Two methods were adopted to overcome this difficulty. Either the lysate was centrifuged at a low *g*, when not quite all of the nuclear material was recovered but with very little ribosomal contamination (method B₁), or the nuclear material was sedimented at a

high *g* and the pellet of nuclear material plus cytoplasmic membranes (in 2–3 ml. in an ice bath) subjected to ultrasonic oscillations for 1–2 min. [MSE-Mullard machine, power 60W, with a titanium probe (diam. 1.0 cm.) in a Spinco polypropylene tube (5 cm. × 1.25 cm.)] and then centrifuged for 1 hr. at 120 000*g*_{av} to remove the ribosomes and fragments of membrane (method B₂). Alternatively the nuclear material could be separated from the membranes by homogenization and then subjected to ultrasonic treatment, and the ribosomes removed by centrifuging. The ultrasonic treatment reduced the viscosity of the nuclear material sufficiently to allow the ribosomes to sediment easily. These procedures are summarized in Scheme 1.

Extraction of acid-soluble proteins. The acid-soluble proteins of the nuclear material were extracted by gently rocking in the cold for 2–24 hr. with HCl or H₂SO₄ at pH 1.7–1.8. Any precipitate was removed by centrifuging and the supernatant was dialysed in the cold against 0.2N-HCl for 18 hr. to remove non-protein material. The dialysed extract was then neutralized with ammonia to pH 8–9 and the acid-soluble proteins were precipitated by leaving the extract overnight in the cold with 7 vol. of ethanol or with acetone or with 10–15% (w/v) trichloroacetic acid.

Determination of ribonucleic acid. The RNA was determined by the orcinol method (Ceriotti, 1955). The results were checked by extracting the RNA by the Schmidt & Thannhauser (1945) method and measuring the extinction at 260 mμ in a Unicam SP. 600 spectrophotometer, and using an extinction coefficient of 32.4 mg./ml./cm. (Scott, Fraccastoro & Taft, 1956). When RNA was determined in fractions containing Lubrol W, the minute amounts of detergent present did not interfere with either method.

Determination of deoxyribonucleic acid. DNA was determined either by the indole method of Ceriotti (1952) or by the diphenylamine method of Burton (1956). Lubrol W did not interfere with these reactions.

Determination of protein. In all fractions [except the membrane fraction, which contains large amounts of poly-(β-hydroxybutyric acid)], the weight of the material insoluble in hot 5% (w/v) trichloroacetic acid after extraction of the lipids was taken as the weight of protein. This was checked by dissolving the samples in N-NaOH and determining the protein by the Lowry, Rosebrough, Farr & Randall (1951) method. Standard curves, which did not differ appreciably, were prepared from lysozyme and ribonuclease after they had been dissolved in water, precipitated with trichloroacetic acid and taken through the same procedure as the samples of cellular protein.

RESULTS

Effect of detergents on protoplasts. The effect of three detergents on protoplasts of *B. megaterium* was investigated. These were an anionic detergent, perfluoro-octanoate, a cationic detergent, deoxycholate and a neutral detergent, Lubrol W. The concentration of the detergents ranged from 0.1 to 0.0001%. All three detergents lysed the protoplasts, when present in a high enough concentration. Perfluoro-octanoate had very little effect at concentrations less than 0.05%, but at higher con-

Scheme 1. Preparation of the nuclear material, ribosome, 'soluble' and membrane fractions of *Bacillus megaterium* by method B (see the Methods section).

centrations lysed the protoplasts instantaneously. Deoxycholate gave very variable results and concentrations higher than 0.01% were necessary to induce lysis. The most promising results were obtained with Lubrol W, which was active at much lower concentrations than the other two detergents. The rate of lysis by Lubrol W was easily controlled and reproducible from experiment to experiment. A typical family of lytic curves is shown in Fig. 1. The rate of lysis by Lubrol W was dependent on the ratio of the dry weight of protoplasts to the concentration of the detergent, was slower at 0–4° than at 20° or 30° and was considerably increased when the suspension was shaken. The medium used to stabilize the protoplasts had no significant effect on the rate of lysis.

Fractions obtained after lysing protoplasts in 0.5M-phosphate-salts medium. When the lysate obtained by treating the protoplasts with Lubrol W in 0.5M-phosphate-salts medium was centrifuged at 15 000g for 5 min., 85–90% of the total DNA was sedimented. At higher centrifugal forces, 90–97% of the total DNA was sedimented (Table 1). When the lysis was carried out at room temperature or at 30°, less of the DNA sedimented than when the preparation was kept at 0–4°. Thus, at temperatures less than 20°, 92–94% of the DNA sedimented; at 34° only 86% sedimented.

The pellet that sedimented at 20 000g_{av.} for 20 min. was gelatinous and contained areas of yellow membranous material. Centrifuging of this pellet at 105 000g_{av.} for 30 min. squeezed out very little supernatant material and the pellet could therefore be regarded as relatively uncontaminated with cytoplasm. When the material was viewed under an interference microscope ($\times 1000$) small spherical bodies could be seen, but it was not certain if these were the 'nuclear bodies' described by Spiegelman *et al.* (1958) or fragments of the cytoplasmic membrane or both. The nuclear material could be separated from the cytoplasmic membranes by the procedures described in the Methods section. The distribution of DNA, RNA and protein between the various fractions is shown in Table 2. The distribution of the DNA and RNA between the fractions obtained by osmotic lysis of the protoplasts (Godson *et al.* 1961) is given for comparison.

Composition of the fractions obtained by lysing protoplasts in 0.5M-phosphate-salts medium (method A). The amount of DNA, RNA, protein and other material present in the nuclear material, the cytoplasmic fraction and membrane fraction, is shown in Table 3. In each case the pellet of nuclear material and membranes sedimented from the lysate was centrifuged at 105 000g_{av.} for 30 min. to

Fig. 1. Protoplasts of *Bacillus megaterium* were suspended at a concentration of 5 mg. dry wt./ml. in 0.3M-sucrose containing $MgCl_2$ (0.01M) and tris (0.01M) brought to pH 7.3 with HCl. The Lubrol W was added to a portion of the suspension and the extinctions of samples were measured every 2 min. at 420 m μ . The final concentrations of Lubrol W were: A, 0%; B, 0.0001%; C, 0.0005%; D, 0.001%; E, 0.003%; F, 0.005%; G, 0.01%; H, 0.05%; I, 0.1%.

squeeze out any remaining supernatant. The nuclear material was also centrifuged at 105 000 g_{av} for 20 min. to remove any minute fragments of membranous material that had remained suspended.

The RNA:DNA:protein proportions by wt. in the nuclear material are 1:7:10. These proportions were constant over a large series of experiments, even when details of the preparation had been altered somewhat. The 'other material' present in the membrane fraction is mainly the storage polymer poly-(β -hydroxybutyric acid). The RNA of the cytoplasmic fraction was not present in ribosome form, as the high ionic strength of the phosphate-salts medium degraded the ribosomes to give particles with a sedimentation constant of less than 14s (Godson *et al.* 1961). Hence the nuclear material prepared by this method was not contaminated with ribosomes.

Fractions obtained after lysing protoplasts in hyperosmotic sucrose-magnesium chloride-tris medium (method B). The distribution of RNA and DNA between the fractions obtained by this method is shown in Table 4. The fractions were prepared by centrifuging the lysate at 78 000 g_{av} for 39 min. The nuclear material was separated from the membranes by homogenization and then treated with ultrasonic vibrations and centrifuged to remove the contaminating ribosomes as described above.

Table 1. *Sedimentation at various speeds of the deoxyribonucleic acid of the protoplasts of Bacillus megaterium*

The protoplasts, suspended in 0.5M-phosphate-salts medium at a concentration of 5 mg. dry wt./ml., were lysed at 2° with 0.01% of Lubrol W. The lysate was centrifuged in a Spinco no. 40 rotor at 0°. Experimental details are given in the text.

Centrifugal force (g_{av})	Time (min.)	DNA sedimented (% of the total)
14 000	5	85-90
25 000	20	91-93
100 000	10	94-96
100 000	60	94-96

Table 2. *Distribution of deoxyribonucleic acid, ribonucleic acid and protein between the various cell fractions obtained by lysing the protoplasts of Bacillus megaterium with Lubrol W*

The fractions were prepared (by method A) and their chemical compositions determined by the methods described in the text. The results are expressed as percentages of the total dry wt. of each component in the protoplasts.

Cell fraction	Amount present (% of the total)		
	DNA	RNA	Protein
Fractions obtained after lysing protoplasts with Lubrol			
Cytoplasmic fraction	4-5	96	76
Nuclear material	90	3-4	12
Membrane fraction	5	1	12
Fractions obtained after osmotic lysis of protoplasts			
Cytoplasmic fraction	65	60	70
Membrane fraction	35	40	30

In protoplasts from cells in logarithmic phase, approx. 85% of the total RNA was in ribosomal form, 12% was low-molecular-weight RNA free in the cytoplasmic fraction, and 2-4% was attached to the nuclear material. Under the conditions used, over 90% of the total DNA sedimented as the nuclear material, but after further treatment to remove membranes a little of this was lost. The RNA:DNA:protein proportions by wt. in the nuclear material prepared by this method are about the same as in the nuclear material prepared by method A.

Presence of basic proteins in the nuclear material. All attempts to prepare basic proteins from purified nuclear material were unsuccessful. Usually 100-400 mg. dry wt. of nuclear material (containing 55-220 mg. of protein) was extracted in dilute hydrochloric acid or dilute sulphuric acid for various times and the acid-soluble proteins were

Table 3. *Composition of the principal cell fractions obtained from the protoplasts of Bacillus megaterium by method A*

The fractions were prepared and their chemical compositions determined by the methods described in the text. The results are expressed as percentages of the total dry wt. of each component in the protoplasts, and each value is the average of four or more different preparations, the limits of which are given.

Cell fraction	Amount present (% of the total)			
	DNA	RNA	Protein	Other material
Nuclear material	39±4	6±1	50±4	5±2
Membrane fraction	7±1	1-2	31±4	60±8
Cytoplasmic fraction	<1	12±4	77±3	10±3

Table 4. *Distribution of deoxyribonucleic acid and ribonucleic acid between various cell fractions obtained from the protoplasts of Bacillus megaterium by method B*

The fractions were prepared and their chemical compositions determined by the methods described in the text. The results are expressed as percentages of the total dry wt. of each component in the protoplasts.

Cell fraction	Amount present (% of the total)	
	RNA	DNA
Cytoplasmic fraction		
Ribosomes	70	4-7
Soluble fraction	12	—
Membrane fraction	1-2	2-4
Nuclear material before ultrasonic treatment	12-15	92-97
Nuclear material after ultrasonic treatment		
Nuclear material	2-4	86-90
Ribosome pellet	10-15	4-5

recovered by one of the methods described in the Methods section. Table 5 shows three typical experiments in which the protein extracted by acid was less than 0.5 % of the total amount of protein in the nuclear material. These proteins were found, on amino acid analysis, to be predominantly acidic.

DISCUSSION

The ideal method of isolating nuclear bodies from protoplasts of *B. megaterium* (if the nuclear material does exist in the form of discrete bodies) should satisfy three criteria: (a) lysis should be carried out under conditions that do not break up the nuclear bodies; (b) lysis should be rapid so that short-time metabolic studies can be performed; (c) lysis and cell fractionation should be easily controlled and give exactly reproducible results. The use of a surface-active agent to lyse the protoplasts immediately suggests itself.

Preliminary studies of the lytic effect of surface-active agents on protoplasts have been described by Salton (1958) and Gilby & Few (1958), but the products of the lysis were not investigated. Of the

three detergents, one anionic, one cationic and one neutral, chosen for this present investigation of the cell fractions obtainable after lysis of protoplasts with a surface-active agent, Lubrol W (neutral) gave the most promising results. This detergent actively lyses protoplasts at very low concentrations and in a controlled and reproducible manner. Further, almost all of the cellular DNA can be sedimented from the lysate at low centrifugal forces (10 000g_{av} for 10 min.). The pellet obtained under these conditions is fairly diffuse and heavily contaminated with the supernatant material. For this reason higher centrifugal forces are usually employed to sediment the nuclear material. The sediment also contains the cytoplasmic membranes of the cell and the storage polymer poly-(β-hydroxybutyric acid). These can be separated from the nuclear material by homogenizing the pellet to reduce its viscosity sufficiently so that the membranous material can be centrifuged out. This procedure, unfortunately, destroys any structure that the nuclear bodies might have had.

After nuclear material and the cytoplasmic membranes have been removed, the supernatant contains nearly all of the cellular ribosomes (70 % of the total RNA) and low-molecular-weight RNA (10-15 % of the total RNA, mostly 'soluble RNA'). The membrane fraction contains very little RNA and consists almost entirely of clean cytoplasmic membranes of the cell. No ribosomes can be liberated from the membranes. This is in direct contrast with the membrane fraction obtained after osmotic lysis of the protoplasts, which contains up to 50 % of the total RNA, and mainly in ribosomal form (Godson *et al.* 1961). The nuclear material contains a small amount of RNA that is tightly bound and that cannot be centrifuged out even after the structure of the DNA has been severely damaged by ultrasonic oscillation. This represents 2-4 % of the total RNA of the cell. The RNA:DNA:protein proportions of the nuclear material have remained constant throughout a large number of experiments, even when the details of the preparation have been considerably altered.

Table 5. *Protein extractable with dilute mineral acids from nuclear material of the protoplasts of Bacillus megaterium*

The methods of preparing the nuclear material, extracting the acid-soluble proteins and analysing their amino acid composition are described in the Methods section.

Preparation of nuclear material	Extraction procedure	Dry wt. of nuclear material (mg.)	Total wt. of protein in nuclear material (mg.)	Protein extracted by acid		Amount of acidic amino acids (Glu + Asp) (% of the total)	Amount of basic amino acids (His + Lys + Arg) (% of the total)
				(mg.)	(%)		
Method A	0.2N-HCl for 24 hr.	130	70	0.29	0.4	23.4	12.8
Method B ₂	0.1N-H ₂ SO ₄ for 3 hr.	135	71	0.11	0.15	24.0	10.1
Method B ₂	10% (w/v) NaCl, followed by 0.2N-HCl for 18 hr.	320	180	0.75	0.4	22.8	16

No evidence, however, can be obtained for the presence of a basic histone-like protein in the nuclear material. The presence in bacteria of a basic protein associated with the DNA is still in dispute. Zubay & Watson (1959) obtained a deoxyribonucleoprotein fraction from *Escherichia coli*, but amino acid analysis showed that the total protein of this fraction was not basic. From this and X-ray data (Wilkins & Zubay, 1959), they concluded that the *E. coli* DNA exists free and not in association with a basic protein as in higher organisms. However, Palmade, Chevallier, Knobloch & Vendrely (1958) also extracted a deoxyribonucleoprotein fraction from *E. coli* and, from the N:P ratio, suggested that the protein was histone-like. Basic proteins can be extracted from whole cells of *Staphylococcus aureus* and *Micrococcus lysodeikticus* (Cruft & Leaver, 1961), but, since proteins regarded as basic on the evidence of starch-gel electrophoresis have been extracted from the ribosomes of *E. coli* (Waller & Harris, 1961) and other organisms (Butler, Cohn & Simson, 1960), it is probable that the basic proteins extractable from whole bacterial cells are derived from the ribosome protein rather than from the protein associated with the DNA. Thus there is no satisfactory evidence of the presence of a basic histone-like protein in association with the DNA of bacteria, and this might be related to the lack of organization of the bacterial chromosome.

SUMMARY

1. Methods are described for cleanly fractionating protoplasts of *Bacillus megaterium* into a nuclear material, a cytoplasmic fraction and a membrane fraction.

2. About 90–95% of the cellular DNA is isolated with the nuclear fraction, the RNA:DNA:protein proportions of which are 1:7:10.

3. Nearly all of the cellular ribosomes and over 90% of the total RNA are present in the cytoplasmic fraction.

4. The membrane fraction is made up of clean cytoplasmic membranes with very little RNA or other material attached.

5. Basic protein cannot be extracted from the nuclear fraction.

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Biochem. J. (1963) **88**, 182

Antimetabolites in the Nutrition of *Aedes aegypti* L. Larvae

NICOTINIC ACID ANALOGUES

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Nicotinic acid antagonists have been employed in studies of insect nutrition (Shyamala & Bhat, 1958; Levinson & Bergmann, 1959), insect tissue cultures (Sanborn & Haskell, 1960), and insect development (Blaustein & Schneiderman, 1960). The effects obtained have usually been attributed to vitamin deficiencies. However, there is little direct evidence that antimetabolites affect insects in the same manner as they do higher animals. Few quantitative studies on the antivitamin-vitamin relationship, with insects as experimental animals, are available. The purpose of the present study was to investigate the effect of five better-known nicotinic acid analogues on *Aedes aegypti* L. larvae, grown on a chemically defined diet.

The specificity of the nicotinic acid requirement of various organisms has been extensively studied. Dietary nicotinic acid can be replaced by tryptophan, many nicotinamide derivatives, and even some nicotinic acid analogues (Hundley, 1954). Small doses of 3-acetylpyridine had a vitamin-like activity for the dog and the rat (McDaniel, Hundley & Sebrell, 1955; Guggenheim & Diamant, 1958). The nicotinamide-3-acetylpyridine relationship was, therefore, studied in mosquito larvae, given a basic casein diet, on which the exact nicotinamide requirements were determined.

MATERIALS AND METHODS

Methods of obtaining sterile larvae, of preparing the basal media, of testing their sterility, and of evaluating the results were described by Akov (1962). One larva was reared per tube, and its stage recorded daily until it

reached the adult stage or died. The results are expressed by an index, consisting of the sum of percentages of pupae and of adults divided by the median day of pupation. An index of about 25 was usually obtained on the basic diet (at 28°), and can be considered to be the optimum. Under these conditions up to 100% of the larvae reached the adult stage with a median larval period of 8 days. An index of zero is considered to indicate complete inhibition. The inhibition index is the ratio of moles of antimetabolite to moles of vitamin that causes 50% inhibition (Woolley, 1952).

The basal nicotinamide-free diet consisted of solid vitamin-free casein (50 mg./tube) and 5 ml. of liquid medium of the following composition: RNA (sodium salt), 1 mg./ml.; inorganic salts, 1.45 mg./ml.; cholesterol, 6 µg./ml.; biotin, 0.1 µg./ml.; folic acid, 0.6 µg./ml.; thiamine hydrochloride, 2 µg./ml.; riboflavin, 2 µg./ml.; pyridoxine hydrochloride, 4 µg./ml.; calcium pantothenate, 10 µg./mg.; choline chloride, 100 µg./ml. The appropriate amount of nicotinamide and the analogue were added before the larva was inoculated.

RESULTS

The effect of graded doses of nicotinic acid and nicotinamide is given in Table 1. Both forms of the vitamin were equally effective. The effect of various antimetabolites was tested on a diet containing the minimal amount of nicotinamide (2 µg./ml.) to give optimum results. Any possible sparing effect was tested in the presence of 0.5–2.0 µg. of nicotinamide/ml. Amounts of nicotinamide up to 2 mg./ml. had no detrimental effect.

The addition of L-tryptophan to nicotinamide-free or low-nicotinamide diets had no beneficial effect (Table 2).