CLXXIX. THE ACCESSORY FACTOR
NECESSARY FOR THE GROWTH
OF *NEMATOSPORA GOSSYPHII*.

I. THE CHEMICAL NATURE OF THE ACCESSORY
FACTOR.

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(Received August 20th, 1931.)

SINCE the earliest attempts were made to cultivate fungi on synthetic nutrient media, cases have been met with in which certain fungi were unable to grow in such media in the absence of small amounts of extracts prepared from natural products. The best known instance of this difficulty of culture is that of ordinary yeast, *S. cerevisiae*, which has been shown to need the presence of a small quantity of "bios" to enable healthy growth to take place on salt-sugar media. The nature of "bios" remains undetermined, although the problem has engaged the attention of many workers for some 30 years.

In his studies on *Fusarium*, Brown [1925] devised a synthetic medium on which the growth of *F. fructigenum* was quite comparable with that on potato decoctions. This medium had the composition: glucose, 2 g.; asparagine, 2 g.; potassium phosphate, 1.25 g.; magnesium sulphate, 0.75 g.; agar, 15 g.; water, to 1000 cc. The same medium was found suitable for the cultivation of a large number of fungi, but cases were met with in which no growth could be obtained. Among the fungi unable to utilise the medium were certain varieties of *Nematospora*, including that investigated in the present instance, *N. gossypii*. The study of the nutritional needs of this organism was continued by Farries and Bell [1930], who were able to show that Brown's medium could be made entirely suitable if the asparagine were replaced by peptone or by the mixed hydrolysis products of certain proteins. They found further that the hydrolysis mixtures prepared from different proteins were not all equally suitable, those obtained from edestin and gelatin, for instance, being quite useless. Nor could the fungus utilise mixtures of all types of pure amino-acids, in the proportions in which they occur in a typical protein.

Farries and Bell concluded that, in general, the mixed hydrolysis products of purified proteins were valueless as a source of nitrogen for this fungus, whereas the hydrolysis mixtures from the crude proteins were entirely satisfactory, and gave good growth. They separated from egg-white, and also from

crude caseinogen, an "accessory factor," itself probably non-nitrogenous, the presence of which was essential for the utilisation of simple nitrogenous substances by *N. gossypii*. This "accessory factor" was readily obtained from egg-white by precipitation of the albumin with alcohol, the accessory being soluble in 80 % alcohol. Even the hydrolysis products of gelatin could readily be assimilated by the fungus in the presence of small amounts of this accessory.

The chemical nature of the accessory factor was not determined by Farries and Bell, although they made a number of experiments in an attempt to characterise the substance. They concluded that it was an organic compound, but not a polysaccharide, sugar, sugar alcohol or sugar acid, fat, ester or lipid. They considered that it had acid properties, since it was precipitated by copper hydroxide and lead hydroxide. Whether the substance was nitrogenous could not be established; that it was not a basic amino-acid was shown by its non-precipitability by phosphotungstic acid or mercuric chloride. The most active concentrate prepared from egg-white by Farries and Bell had a very low nitrogen content.

It was in an attempt to gain more information as to the chemical nature of the "accessory factor" that the present work was undertaken.

The strain of fungus with which the experiments were carried out was that used by Farries and Bell, and the technique adopted was that which had been carefully standardised by these workers, and which is described briefly below. The use of egg-white as a source of the accessory factor was abandoned early in the work, for reasons given below, and, after a brief survey of other possible sources of the factor, it was decided to prepare the concentrates from lentils. Subsidiary tests were made with concentrates prepared from another plant source (lupin seedling extract) and from an animal source (ox-heart) and the results were found to be comparable. There was therefore no reason to suppose that egg-white would have proved any exception in respect of the nature of the accessory factor which it contained.

EXPERIMENTAL.

Method.

The organism used throughout the work was a strain of *N. gossypii* which had been isolated by Brown from diseased cotton bolls (East African). This strain gave vigorous growth, and when cultivated on solid media formed a tough mat of mycelium on the surface of the medium. The mat could be peeled off completely without disturbing the medium, and it was shown by Farries and Bell [1930] that the amount of mycelium remaining within the medium was only 3 % of the total mycelium. The "dry weight" method of estimating the growth of the fungus could therefore be adopted, the percentage error due to incomplete removal of the fungus from the medium being constant.

In order to ensure a maximum degree of regularity in the different cultures, the details of routine were standardised. The basal medium used had the following composition: glucose, 2 %; potassium phosphate, 0.5 %; magnesium

sulphate, 0.25 %; agar, 2 %. The nitrogenous constituent, from whatever source, was supplied in a concentration equivalent to 0.3 % N (see below).

The Petri dishes used were 3 in. in diameter, and the amount of medium added to each was 25 cc. A mycelium inoculation of the fungus (of which stock cultures were maintained on glucose-peptone agar) was placed at the centre of each plate, and the cultures were incubated at 30° for 6–7 days. At the end of this period the superficial mat of mycelium was peeled off the agar, dried on a tared filter-paper in a steam-oven for 48 hours and weighed. In general, the plates were treated in pairs, so that the “dry weight” figures quoted below represent the combined weight of mycelium from 2 plates.

As stated above, the nitrogenous constituent in the medium was supplied in a concentration equivalent to 0.3 % N. The main supply of nitrogen was provided by adding the appropriate amount of an amino-acid mixture prepared from gelatin by hydrolysing with 22 % sulphuric acid, removing excess of acid by baryta, and concentrating the filtrate to a known strength. Since the amounts of lentil and other concentrates tested themselves contained nitrogen, this was estimated and allowed for in adding the amino-acid mixture. In the tables given below, the term “standard basal medium” is intended to represent the salt-sugar mixture plus the 0.3 % N (from the combined sources, amino-acids and concentrate) referred to above.

The reaction of the medium was adjusted to p_H 6.8–7.0 before inoculation. The work of Farries and Bell had shown that the fungus possessed a comparatively wide range of growth, with an optimum at p_H 7.0.

In all cases where growth was found below normal, strict controls were set up to test for toxicity in the medium; this was particularly necessary, in view of the possibility of the presence of small traces of lead, barium, *etc.* in the media. In these cases, therefore, portions of the concentrate in question were added to a medium known to give good growth; if the resulting growth were at least as good as that on the standard medium, it was assumed that lack of accessory factor, and not toxicity in the concentrate, was responsible for lack of growth in the absence of the standard concentrate.

The effect upon growth of different amounts of concentrate was investigated, and it was found that addition of a small amount of concentrate produced a relatively large increment in growth, further small additions giving proportionate increases. A point was reached, however, after which the addition of a further large supply of concentrate produced only a small increment in growth, such as might often be accounted for by addition of actual food material present in the portion of concentrate added. If a curve were plotted showing the weight of concentrate added against the weight of mycelium produced, a fairly sharp change of gradient was observed where addition of more concentrate no longer produced great increase in growth. This point was taken as indicating the “critical amount” of the concentrate necessary and all comparisons of activity were made on the basis of this “critical amount” of concentrate. Examples of the method are given below.

Tests for activity in known substances.

Before attempting to fractionate the concentrates prepared from various sources, it was thought worth while to ascertain whether certain substances, which had from time to time been suggested as growth-promoting accessories by workers on animal nutrition and bacterial metabolism, could supply the essential nutrient for the growth of *N. gossypii*.

Nucleic acid (yeast), adenine sulphate, guanine hydrochloride, mannitol, inositol and inositol plus traces of Fe or Cu were tested, but in no case was any appreciable effect observed.

Sources of the "accessory factor."

As stated above, the material from which Farries and Bell prepared their active concentrate was egg-white, which they fractionated into "albumin" and "egg-extract" by precipitation with alcohol. They found that the albumin, whether hydrolysed or not, did not give any measurable growth of *N. gossypii*, but when supplemented with very small amounts of the "egg-extract" gave copious growth. According to their figures, the growth on albumin alone was 1.5 % of that on albumin plus "egg-extract." The first attempts to prepare concentrates of the active factor followed the methods given by Farries and Bell. It was found that the original egg-white (hydrolysed) gave good growth, and a sample of crystalline albumin prepared therefrom only negligible growth, thus confirming the results of Farries and Bell. On the other hand, the preparation of the "egg-extract" in good yield was found to be a matter of some difficulty, the active factor being retained somewhat tenaciously by the albumin when the latter was precipitated. On account of this difficulty in manipulation, and the expense involved in using egg-white as a starting material, other sources of the accessory factor were sought. At the same time, a number of substances were tested for the presence of the factor, so that a brief survey of the distribution of the factor among a variety of products might be obtained.

(a) *Cotton plant*. As the natural habitat of the fungus is in the bolls of the cotton plant, it was thought worth while to see whether other parts of the plant would also yield the accessory factor. Cotton leaves (about 50 g.) were ground to a pulp, and treated with 3 volumes of 95 % alcohol; the filtrate was evaporated, taken up in water and made up to 50 cc. Sufficient of this solution to correspond with 0.5 g. of solid matter was incorporated in 50 cc. of the medium and growth tested. The results of this and other tests on various extracts are summarised in Table I.

(b) *Potato*. 300 g. potato were steamed for 3 hours and the aqueous extract was precipitated with alcohol (3 volumes). The portion soluble in 75 % alcohol was taken up in water, and varying amounts were plated with the basal medium according to the usual procedure.

(c) *Lentils*. 300 g. portions of lentils were treated in the same way as the potatoes.

(d) *Lupin seedlings*. A portion of an extract of etiolated lupin seedlings which was available was treated with alcohol, as in the case of other extracts, to remove most of the inorganic salts, *etc.* The filtrate, after removal of alcohol, was tested for activity.

(e) *Yeast extract*. A sample of zymine was prepared from brewer's yeast by the acetone method. This was boiled with water for 30 minutes, and the solid material removed on the centrifuge. The extract was concentrated, and portions were incorporated in the basal medium. Similar tests were also made with a commercial yeast preparation (marmite), this being used without further treatment.

(f) *Ox-heart*. Preparations of ox-heart were used by Reader [1928] as a source of the factor promoting growth of *Streptothrix corallinus*; accordingly, a tryptic digest of fresh ox-heart was prepared according to the method described by Reader, except that a much longer period of digestion was found necessary to produce a mixture not giving the biuret reaction. The soluble portion of the resultant mixture was made up to a known concentration, and portions were added to the basal media.

(g) *Fibrin*. A sample of commercial fibrin (B.D.H.) was digested in acid solution with pepsin for 1 month. The insoluble residue was filtered off, and the filtrate tested for activity.

Table I.

Extract tested	Weight of concentrate in 50 cc. medium	Dry weight of mycelium (2 plates)
	g.	g.
Cotton leaves	0.5	0.15
Potato	0.1	—
	0.2	0.05
	0.3	0.11
	0.4	0.14
	0.5	0.18
Lentils	0.1	0.28
	0.2	0.31
	0.3	0.33
	0.4	0.35
	0.5	0.375
Lupin	0.05*	0.06
	0.1	0.12
	0.2	0.195
	0.3	0.25
	0.4	0.295
	0.5	0.32
Yeast	0.05	0.08
	0.075	0.10
	0.1	0.13
(Marmite)	0.075	0.11
	0.15	0.185
Ox-heart digest	0.1*	0.08
	0.3	0.19
	0.5	0.25
Fibrin digest	0.1	—
	0.5	—

* Ash-free organic matter.

The relative activities of the concentrates prepared from different materials were clearly indicated by these tests. The greatest activity was shown by lentil extracts, while lupin extract, yeast and ox-heart were fairly rich in the factor. Potato was relatively poor, and fibrin deficient in the factor. (This deficiency was noted by Farries and Bell.) To the list of substances rich in the accessory must be added those recorded by Farries and Bell, *viz.* egg-white, impure caseinogen and commercial peptone.

In view of the results thus obtained, it was decided to use lentils as the starting material in attempts to prepare more active concentrates than those hitherto dealt with.

Preparation of the lentil concentrate.

About 50 kg. of lentils, dealt with in portions of 500 g., were steamed with twice their weight of water for 3 hours, the residual solid was removed, and the extract evaporated to about 300 cc. The addition of 3 volumes of alcohol gave a large precipitate, containing inorganic salts, proteins, *etc.* This was filtered off, and the filtrate again evaporated to a small bulk, and precipitated with alcohol. The filtrate at this stage was evaporated to a syrup and treated with dry acetone, thus granulating the substance. The solid was dried *in vacuo*, when it was obtained as a light brown, somewhat hygroscopic powder, containing 2.88 % N. The yield from 50 kg. of lentils amounted to about 100 g. Its activity was similar to that of the sample obtained on the small scale, as described above—0.1 g. in 50 cc. medium gave 0.28 g. dry mycelium.

When different amounts of the lentil preparation were incorporated in the medium, the corresponding growths obtained were those shown in Table II.

Table II.

Weight of lentil concentrate per 50 cc. medium	Dry weight of mycelium
g.	g.
0.01	—
0.025	0.06
0.05	0.13
0.075	0.195
0.10	0.28
0.20	0.31
0.30	0.33
0.40	0.35
0.50	0.375

It was evident from these results that the "critical dose" of the lentil concentrate lay in the region of 0.1 g. per 50 cc. of medium. Accordingly, all tests were made on a basis of 0.1 g. original concentrate per 50 cc. medium. Thus it was necessary at each stage of the fractionation to determine the weight of substance in each fraction, corresponding to 0.1 g. of the original substance; since there was a small loss of total substance at every stage of the fractionation process, it was assumed that the fractions (*e.g.* filtrate and ppt.) suffered the same proportionate loss.

DEY

Fractionation of the lentil concentrate.

In the course of their attempts to fractionate the "egg-extract," Farries and Bell found that the active factor was precipitated by copper hydroxide or lead hydroxide; accordingly, the first attempts to fractionate the lentil concentrate were made on these lines. It was found, however, that although the active substance was apparently precipitated by these reagents, it was impossible to recover it from the precipitates, probably on account of the fact that nearly all the active substance was adsorbed on the copper (or lead) sulphide produced when the precipitates were decomposed. For this reason these experiments were not proceeded with. A number of preliminary tests indicated that a considerable degree of concentration could be obtained by treatment of the lentil extract in the following manner.

- (i) Removal of basic substances (inactive) by phosphotungstic acid.
- (ii) Removal of the majority of organic acids as barium salts, by precipitation with baryta and alcohol.
- (iii) Precipitation of the active factor as a mercury compound by Neuberg and Kerb's reagent (mercuric acetate and sodium carbonate).

Accordingly, 80 g. of the lentil preparation were submitted to the above process. The precipitation with phosphotungstic acid was carried out in the ordinary way, in the presence of 5 % sulphuric acid, which was subsequently removed from the filtrate by baryta. The activity was found to be confined to the filtrate, the basic substances being entirely inactive, even in high concentrations. From the 80 g. of original substance, 60.8 g. remained in the filtrate. The usual growth tests gave the following results:

Table III.

Medium	Dry weight of mycelium g.
Basal + 0.075 g. substance from phosphotungstic acid filtrate	0.245
Basal + 0.1 g. substance from phosphotungstic acid filtrate	0.315
Basal + 0.1 g. substance from phosphotungstic acid ppt.	—

A portion (30 g.) of the substance not precipitated by phosphotungstic acid was dissolved in 200 cc. of water, and the liquid was saturated with pure barium hydroxide; 3 volumes of alcohol were then added, and the mixture was kept at 0° overnight. The precipitate was washed with 75 % alcohol, and the precipitate and filtrate fractions were decomposed separately by means of sulphuric acid. The material recovered from the precipitate amounted to 21.6 g., and from the filtrate 5.6 g. The loss of material thus amounted to 2.8 g., and the proportion of precipitate fraction to filtrate fraction was almost exactly 4 : 1. Media were prepared containing the appropriate amounts of the two fractions and growth tested (Table IV). Since, as shown in the table, the activity had apparently been almost entirely lost, the usual toxicity tests were applied, but no evidence of the presence of any toxic substance could be obtained. Portions of the two fractions were then recombined, to reconstitute the original substance, when full activity was found to have been restored.

Table IV.

Medium	Dry weight of mycelium g.
Basal + 0.08 g. Ba ppt. fraction	0.02
Basal + 0.02 g. Ba filtrate fraction	0.018
Basal + 0.08 g. ppt. + 0.02 g. filtrate	0.285
Basal + 0.07 g. ppt. + 0.03 g. filtrate	0.265

These results gave a clear indication that the active substance present in the lentil concentrate could be fractionated into two substances, either of which alone was inactive, but in combination possessing the full activity of the original concentrate. By submitting the two fractions again to the precipitation, the activity of the separate fractions was further reduced, the amount of mycelium obtained being too small to allow of its being scraped from the surface of the medium. The process was repeated with a further small batch of the lentil concentrate (6 g.) and a similar result again obtained.

At this point it was realised that the results so far obtained—particularly the partition of the active factor into two inactive components—showed a close parallel with those reported by several observers on the “bios” of yeast. Eastcott [1928] claimed to have obtained two such fractions from a bios concentrate, and further stated that one of the factors was *i*-inositol (ppt. fraction). Although many subsequent workers had disputed her results [Narayanan, 1930] it was thought worth while to test the effect of inositol on the growth of *N. gossypii* in the presence of the baryta filtrate fraction. (Inositol alone had been found inactive in promoting growth—see above, p. 1659.) The results are given in Table V.

Table V.

Medium	Dry weight of mycelium g.
Basal + 0.02 g. Ba filtrate + 0.0005 g. inositol	0.13
” ” ” + 0.001 g. ”	0.15
” ” ” + 0.005 g. ”	0.19
” ” ” + 0.007 g. ”	0.245
” ” ” + 0.01 g. ”	0.315

Other media were tested in which the inositol was replaced, wholly or in part, by mannitol, but in no case was the presence of mannitol found to influence the amount of growth. The inositol itself was also carefully recrystallised several times, but again the repurified product did not differ in activity from the original sample (Kahlbaum). The evidence seemed conclusive that small amounts of inositol were able to replace the “baryta precipitate” fraction in the medium, in conjunction with the “baryta filtrate.”

For this reason, the isolation of inositol from the baryta precipitate fraction was attempted. 20 g. of the fraction were dissolved in water, and the solution was treated with cold neutral lead acetate; after removal of the small precipitate, the liquid was treated with excess of basic lead acetate and ammonia, and allowed to stand overnight. The precipitate was removed, and decomposed with hydrogen sulphide in the presence of sulphuric acid; from the solution,

excess of sulphuric acid was quantitatively removed, and the liquid was concentrated to a small bulk. Five volumes of absolute alcohol and 1 volume of ether were added, and the whole was kept at 0° for 24 hours. The solid was removed and taken up in as little hot water (containing about 5 % nitric acid) as possible, and reprecipitated with alcohol and ether. After again taking up in hot water, the solution was treated with hot baryta solution; the filtrate was freed from barium with ammonium carbonate, evaporated to a small bulk, and precipitated with alcohol and ether. The precipitate was recrystallised from 50 % acetic acid, and after three recrystallisations was obtained as a white crystalline material, M.P. 223°. This contained¹ C, 40.4 %; H, 6.9 %. Calculated for inositol, (CHOH)₆, C, 40.0 %; H, 6.7 %. A mixed M.P. with a sample of Kahlbaum's inositol, recrystallised three times, showed no depression.

The hexa-acetyl derivative, prepared by the method of Müller [1907], had M.P. 214°; it showed no depression when mixed with a sample of hexa-acetyl-inositol prepared from Kahlbaum's inositol. It was therefore considered that the product isolated from the baryta precipitate fraction was inositol, which was known to be widely distributed among natural products. The amount isolated was 0.88 g. from 20 g. of the baryta precipitate fraction. A calculation of the amount present, based upon the relative activities of pure inositol and the precipitate fraction indicated the presence of about 2 g. of inositol in 20 g. of the fraction. The yield thus amounted to approximately 40 %, which was considered reasonable in view of the complexity of the process of separation.

Its growth-promoting activity was tested in the usual way, and was found to be the same as that of Kahlbaum inositol. 0.007 g. in conjunction with 0.02 g. of the baryta filtrate fraction in 50 cc. of medium gave 0.235 g. of mycelium (dry weight). The remainder of the baryta precipitate fraction, from which the inositol had been removed by the process described above, was carefully freed from lead, acetic acid, *etc.*, and portions tested for activity. It was found to be completely inactive and non-toxic, thus proving that the active factor had been entirely removed by precipitation with basic lead acetate. An experiment with pure inositol had shown that this substance was completely separated from a mixture with sugars by the same method. There seemed no doubt, therefore, that the substance responsible for the activity of the baryta precipitate fraction was inositol, and that no other active substance was present.

The baryta filtrate fraction.

So far, little has been done in attempts to isolate the active substance from the baryta filtrate fraction. Precipitation with the mercuric acetate reagent of Neuberg and Kerb was found to throw down the active substance, so that the possibility exists that it is associated with the amino-acids. That it is not one of the ordinarily occurring amino-acids follows from the fact that the hydrolysis mixture obtained from gelatin, supplemented with tryptophan,

¹ The micro-combustions were carried out by Dr J. A. B. Smith, to whom our thanks are due.

tyrosine and inositol was found unable to support the growth of *N. gossypii*. Investigations on this section of the work are still in progress.

Fractionation of concentrates from other sources.

The fractionation of the active factor into two components by means of baryta and alcohol in the case of lentils made it desirable to ascertain whether a similar result could be obtained when using concentrates prepared from other sources. Consequently, the following substances were investigated: peptone, tryptic digest of ox-heart, lupin extract. The method used was precisely the same as in the case of the lentil preparation, and the results obtained are given in Table VI.

Table VI.

Medium	Dry weight of mycelium g.
Basal + 0.12 g. Ba ppt. from peptone	—
„ + 0.27 g. Ba filtrate from peptone	—
„ + 0.12 g. ppt. + 0.27 g. filtrate from peptone ...	0.185
„ + 0.06 g. Ba ppt. from ox-heart	—
„ + 0.24 g. Ba filtrate from ox-heart	0.055
„ + 0.06 g. ppt. + 0.24 g. filtrate from ox-heart ...	0.195
„ + 0.07 g. Ba ppt. from lupin extract	—
„ + 0.03 g. Ba filtrate from lupin extract	—
„ + 0.07 g. ppt. + 0.03 g. filtrate from lupin extract	0.155

The results clearly indicated that the active factor present in each of these substances was split into two components, neither of which alone was active, but which in conjunction gave the normal degree of activity.

In the case of the lupin extract, inositol was actually isolated from the baryta precipitate fraction, the method being the same as that used in the experiments with lentils. The inositol-free residue was again shown to be inactive in combination with the baryta filtrate fraction.

Various experiments on the baryta precipitate fraction (inositol).

(a) *Hydrolysis with 25 % sulphuric acid.* Eastcott [1928] found that the “bios I” (inositol) content of her preparation, as judged by its “bios” activity, was trebled on boiling with 22 % hydrochloric acid for 24 hours. This she ascribed to the presence of a precursor of inositol, from which the latter was liberated by hydrolysis with acid. That no similar effect could be observed in the present case was proved by the following experiment. 25 g. of the lentil concentrate, after treatment with phosphotungstic acid, were hydrolysed with 25 % sulphuric acid for 24 hours. A large amount (3.2 g.) of insoluble matter separated. The solution after hydrolysis was freed from sulphuric acid in the usual manner, and the filtrates were evaporated. 15.8 g. of solid material were recovered, so that there was a loss of 6 g.; the loss was probably partly due to adsorption on the barium sulphate precipitates, and partly to destruction of sugars in the concentrate by the action of the strong acid. The activity of the product after hydrolysis was tested as usual (Table VII).

Table VII.

Medium	Dry weight of mycelium g.
Basal + 0.025 g. concentrate after hydrolysis	0.11
" + 0.05 g. " " "	0.165
" + 0.075 g. " " "	0.255
" + 0.075 g. original concentrate	0.245

(b) *Recovery of the active factor from the mycelium.* In her work on "bios," Eastcott was able to recover from the yeast-crop grown on a medium containing a known amount of inositol the whole of the inositol supplied. Further, many workers have apparently shown that yeast manufactures "bios" during growth. It seemed therefore of interest to determine whether any similar effect could be traced in the case of *N. gossypii*. Extracts were prepared from the combined mycelia from 2, 4, 6, and 8 plates, and from the residual media after removal of the mycelia. Fresh media were prepared incorporating these extracts, allowance being made for both the nitrogen and the phosphate carried over in the extracts. In no case was the medium found able to support more than slight growth. If a large amount of extract of mycelium were used (1 g. in 50 cc. medium), the growth amounted only to 0.185 g. The evidence was, therefore, that far from producing the active factors during growth, the fungus largely used up the amount initially supplied in the medium. These results were thus in direct contrast to those noted by workers in the case of yeast and "bios."

(c) *Proportion of inositol to the baryta filtrate fraction giving optimum growth.* Since it had been proved that the accessory factor in the case of *N. gossypii* consisted actually of two substances, it seemed probable that the activity of an extract would depend not only on the total amount of the accessory factor present, but on the relative amounts of the two components, and that the high activity of such products as the lentil concentrate might be due to the presence of the two components in an optimum ratio. Experiments were made to see

Table VIII.

Medium	Dry weight of mycelium g.
Basal + 0.01 g. lentil extract	—
" " " + 0.001 g. inositol	0.065
" " " + 0.005 g. " "	0.07
" + 0.025 g. " " " "	0.06
" " " + 0.001 g. inositol	0.085
" " " + 0.005 g. " "	0.09
" + 0.05 g. " " " "	0.12
" " " + 0.001 g. inositol	0.185
" " " + 0.005 g. " "	0.195
" + 0.075 g. " " " "	0.195
" " " + 0.001 g. inositol	0.21
" " " + 0.005 g. " "	0.23
" + 0.10 g. " " " "	0.28
" " " + 0.001 g. inositol	0.296
" " " + 0.005 g. " "	0.30
" + 0.01 g. " " " "	0.08
" + 0.05 g. " " " "	0.27
" + 0.075 g. " " " "	0.29
" + 0.10 g. " " " "	0.305

whether the addition of extra inositol to the lentil preparation gave any increased activity (Table VIII).

These results indicated that the activity of the lentil extract was somewhat increased by the addition of small amounts of inositol, the effect being most noticeable when dealing with small amounts of the concentrate. Relatively much less effect was observed when the medium contained larger amounts of the lentil preparation, suggesting that the fungus was unable to utilise amounts of inositol beyond a certain concentration.

The effect of adding inositol to marmite was also tested. As shown above (p. 1660), marmite was not found to be a particularly rich source of the accessory factor, but when supplemented with inositol, its activity was considerably increased. The conclusion was drawn that marmite was rich in the second factor but deficient in inositol.

Table IX.

Medium	Dry weight of mycelium g.
Basal + 0.075 g. marmite	0.11
" " " + 0.01 g. inositol	0.24
" + 0.15 g. marmite	0.185
" " " + 0.01 g. inositol	0.31

(d) *Activity of derivatives of inositol.* In an attempt to ascertain whether the activity of inositol could be due to the presence of small amounts of active impurities, media were tested in which portions of the hexa-acetylinositol, prepared as described above, were incorporated. The derivative itself is insoluble in water, but an active impurity, unless itself converted into an insoluble acetyl compound, would be soluble and removed during acetylation. Further, the inositol obtained by hydrolysis of the acetyl compound should recover the full initial activity of the original inositol. On determining the activity of the derivative, it was found that the media containing the hexa-acetyl inositol supported a certain amount of growth (Table X). On further examining

Table X.

Medium	Dry weight of mycelium g.
Basal + 0.03 g. baryta filtrate + 0.025 g. hexacetyl inositol	0.225
Basal + 0.03 g. baryta filtrate + 0.007 g. inositol derived from hexacetyl compound... ..	0.285

this point, it was found that the acetyl compound was partly hydrolysed during the autoclaving of the medium; a loss of about 10 % occurred by autoclaving with water alone, and a hydrolysis amounting to some 80 % in the presence of small quantities of potassium phosphate. Thus the activity shown by the hexa-acetylinositol could be regarded as due to the liberation of free inositol in the medium. A test using a sample of inositol prepared from the hexa-acetyl derivative by hydrolysis gave the same amount of growth as given by the original inositol. If the activity of inositol were due actually to the presence

of an impurity, this impurity must be inseparable from inositol by acetylation, and the amount present too small to influence the ordinary chemical and physical properties of inositol and its hexa-acetate.

DISCUSSION.

The experiments described above seemed to show conclusively that the accessory factor necessary for the growth of *N. gossypii* could be fractionated into two mutually complementary substances, one of which was shown to be inositol. The nature of the second factor was not determined; it was found to be precipitated with the monoamino-acids by Neuberg and Kerb's reagent, but it was not one of the amino-acids normally occurring among protein hydrolysis mixtures. Whether it was a single substance was not determined, but there was no evidence to the contrary.

What part is played by inositol in the metabolism of the fungus is entirely unknown. It is curious that this substance has been stated repeatedly to have some special significance in the promotion of growth or activity, and its isolation as a constituent of "bios" has been reported [Eastcott, 1928]. The view that inositol is concerned in "bios" activity is, however, by no means generally accepted. Thus Narayanan [1930], criticising the work of Eastcott, stated that "the activity of inositol must have been due either to the presence of an impurity, or to some such effect as has been described by Reader in the case of mannitol." (The following communication contains a further discussion of this point.)

In her observations on the effect of mannitol on bacterial growth, Reader [1929] used a medium in which 0.5 % glucose constituted the source of carbohydrate. To this was added a small amount (1/20 "unit dose" per 20 cc.) of torulin, and 0.5 % of mannitol. The resultant growth of *S. corallinus* showed a large increase (from 300 % to 600 %) over that given in the absence of mannitol. A similar effect, though much less pronounced, was given by citrate. When the concentrations of torulin and mannitol were reduced to 1/400th of their original amount, the excess growth due to mannitol was not observed. The explanation of these "anomalous results" put forward by Reader was that mannitol served as a "specific source" of carbohydrate food-supply for her organism. Since Eastcott had found that the addition of inositol to "bios II" gave an increased growth of yeast amounting to about 300 %, Reader considered that this result could similarly be explained on the grounds that inositol served as a "specific source" of carbohydrate for yeast.

This interpretation does not seem applicable to the results here described for the following reasons. Reader, using a medium containing glucose and torulin (no mannitol), obtained a reasonably good growth of her organism, under the standard conditions adopted; similarly, Eastcott, using a medium containing "bios II" without inositol obtained a 20-fold increase of yeast cells in 24 hours. On the other hand, *N. gossypii* does not give any measurable

growth even in the presence of large amounts of the baryta filtrate fraction, which corresponds to Eastcott's "bios II"; growth is of the starvation type, the surface of the medium being faintly clouded, as in the case of media containing gelatin only. It has been found possible to remove and weigh crops of mycelium amounting to 0.008 g. (dry weight, from 2 plates), and the growth of starvation type probably does not amount to more than 2-3 mg. dry weight per 2 plates. The addition of mannitol to the medium used by Reader gave a 5- or 6-fold increase of growth of *S. corallinus*; the addition of inositol to the medium in the case of *N. gossypii* resulted in a far greater increase of growth—at least 30 times. Further, the amount of mannitol used by Reader was at least 10 % of the amount of glucose in the medium, whereas in the present case active growth was produced when the inositol present was only 0.6 % of the glucose. None of the results given by Reader refers to the use of mannitol in such extreme dilution, except when torulin (corresponding to the baryta alcohol filtrate here) was also diluted. The conditions of the experiments here described seem to correspond most nearly to those in which Reader used the diluted solutions of mannitol and torulin, and in which she did not obtain "anomalous results." The evidence here is that inositol is a true growth-promoting factor, essential for healthy growth, and not merely a "specific food factor."

In this connection, an estimation was made of the amount of glucose taken from the medium during the growth of the fungus, by determining the reducing sugar present in 50 cc. of medium before and after growing the fungus thereon. It was found that a crop of the fungus, dry weight 0.28 g., had used 370 mg. of glucose during growth, the amount of inositol supplied in the medium being 8 mg. The small amount of inositol which could have been used, in comparison with the amount of glucose actually used, gave further support to the contention that inositol is more than a "specific food factor."

If the activity of inositol is to be explained on the basis of the presence of an impurity, the amount of impurity must be too small to be detected by the ordinary tests for purity, or removed by the chemical methods usually available.

SUMMARY.

1. The statement of Farries and Bell, that *Nematospora gossypii* is unable to assimilate simple nitrogenous compounds in synthetic media, unless supplied with small amounts of an "accessory factor," is confirmed.

2. The accessory factor is shown to be present in association with crude proteins from various sources, and in extracts of lentils and etiolated lupin seedlings; cotton leaf extracts, potato, and yeast extracts show less activity.

3. The accessory factor yields two components on precipitation with barium hydroxide and alcohol; neither alone is active, the presence of both fractions being necessary.

4. The active substance in the fraction precipitated by barium hydroxide and alcohol can be replaced in the medium by small amounts of inositol.

5. The active substance present in the fraction not precipitated by barium hydroxide and alcohol is precipitated by mercuric acetate and sodium carbonate.

6. Inositol has been isolated from the baryta precipitate fraction; the fraction after removal of the inositol is inactive.

7. The activity of some relatively inactive substances (*e.g.* marmite) is greatly increased by the addition of inositol; such substances are therefore considered to contain the "baryta filtrate" fraction, and to owe their lack of activity to a deficiency of inositol.

8. Inositol is not considered to act as a "specific food factor," as is the case with mannitol in the growth of *S. corallinus* (Reader).

We wish especially to record our indebtedness to Prof. W. Brown, with whom the general research on the nutritional requirements of fungi originated, and at whose suggestion we undertook the more purely chemical investigations involved, and to Prof. A. C. Chibnall for much valuable advice and criticism.

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