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

III. THE PREPARATION OF CONCENTRATES OF THE SECOND ACCESSORY FACTOR.

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IN an earlier communication [Buston and Pramanik, 1931, 1] it was shown that the "accessory factor" found by Farries and Bell [1930] to be necessary for the growth of *Nematospora gossypii*, the fungus responsible for the "internal boll disease" of cotton, in synthetic media consisted of at least two distinct constituents, one of which was found to be *i*-inositol. The discovery of the necessity for the presence of this compound found a parallel in the observation of Eastcott [1928] concerning "bios" [see also Buston and Pramanik, 1931, 2]. The chemical nature of the second constituent forming the accessory factor was not determined by Buston and Pramanik, who recorded only that it appeared to be precipitable by Neuberg and Kerb's mercuric acetate reagent and might therefore "be associated with the mono-amino-acids."

Since the publication of this earlier work, further attempts have been made to characterise the second factor. Much more active concentrates thereof have been prepared, and although there is as yet no indication that the substance concerned has been obtained in a state approaching purity, it is thought that the results so far obtained may be of interest.

One difficulty has been met with repeatedly; with the very active concentrates, almost any treatment seemed to result in partial destruction of activity, or at any rate in the introduction of some substance giving rise to a "staling" type of growth in the cultures. Even the most extreme care in the use of purified reagents *etc.* has not always succeeded in overcoming this difficulty. Despite this, it has been possible, from a consideration of the reactions given by the active concentrates, to draw some tentative conclusions as to the possible chemical nature of the second factor, although these must be accepted with reserve in view of the admitted impurity of the preparations.

EXPERIMENTAL.

General technique.

The experimental method adopted was that described by Buston and Pramanik; the same strain of the organism was used, and the methods of culture were identical. The standard basal medium was of the following composition: glucose, 2 %; K_2HPO_4 , 0.5 %; $MgSO_4$, $7H_2O$, 0.25 %; nitrogen, supplied from

a standard gelatin hydrolysate, 0.3 %; agar, 2 %. To this was added, where necessary, *D*-inositol (B.D.H. or Kahlbaum), 0.02 %; and the appropriate amount of concentrate of the second factor.

Using their "critical dose" technique, Buston and Pramanik showed that such a dose of concentrate (*i.e.* the amount of concentrate beyond which further addition did not result in a proportionate further increase of growth) gave a growth of 270–290 mg. of mycelium (dry weight from two plates). In general, therefore, in fixing the amount of any concentrate of the second factor required, the "unit dose" was taken as the amount of concentrate, immediately after removal of inositol, necessary to give a growth of 270 mg., the corresponding amounts of the more active concentrates being calculated from the dry-weight yields of the various fractions, due allowance being made for losses at different stages.

Source of the second accessory factor.

The original experiments of Farries and Bell showed that the accessory factor (not then recognised as a mixture) was present in a number of "crude" proteins, and absent from others. The discovery of the dual nature of the accessory factor made it necessary to examine again the latter group of proteins, since it was evidently possible that lack of activity in these was due to lack of inositol only. Further, it was thought that, should any protein be found rich in the second factor, it might serve better as a starting material for its isolation than an indefinite and complex mixture such as the lentil extract of Buston and Pramanik.

With this in view, media were prepared in which the nitrogen (0.3 % N in the medium) was supplied solely in the form of hydrolysates of different proteins; adequate amounts of inositol were added to the media, since this substance was only present as an impurity in some of the crude proteins. The results of these tests are shown in Table I. In the case of every protein except

Table I.

Protein	Growth of mycelium (mg. dry weight)
Gliadin	76
Legumin	104
Edestin	20
Excelsin	65
Zein	124
Oat glutelin	145
Caseinogen	20
Ovalbumin	20
Fibrin	45
Gelatin	0

gelatin there was evidence of the presence of the second accessory factor, although often in very small concentrations only; even fibrin and edestin, stated by Farries and Bell to be completely inactive, contained small amounts of the substance. On the whole, the proteins prepared from seeds apparently contained the greatest amounts of the accessory factor, zein being one of the richest as well as the most easily obtainable. Further samples of zein were prepared, by slightly different methods, and their activities tested; it was found that there was no connection between the activity of a sample of zein and its purity, as measured by nitrogen content (Table II). Further, experiments showed that the most active sample of zein obtained showed, weight for weight,

only two-fifths of the activity of the crude lentil concentrate, so that in spite of objections it seemed best to continue the use of lentils as the source of the accessory factor.

Table II.

Zein, precipitated from dilute alcohol by	Nitrogen in zein %	Growth of mycelium mg. dry weight
Water	16.1	93
Acetone	13.8	365

Fractionation of the lentil concentrate by chemical methods.

(a) *Removal of inositol.* So little evidence was available as to the chemical nature of the second accessory factor that it was hardly possible to devise a logical method of fractionation based upon such evidence. It became necessary therefore to work on somewhat empirical lines, using methods which have found wide application in allied fields of research.

In the first place, some 70 g. of lentil concentrate were prepared, following the method of Buston and Pramanik; from this, the inositol was removed by precipitation with barium hydroxide and alcohol at 0°. Whereas Buston and Pramanik found that one such precipitation sufficed to separate the inositol completely from the second factor, in this case it was found that no sharp separation was obtained at the first precipitation, both the precipitate (inositol) and filtrate fractions needing to be re-fractionated. Even so, a certain amount of the second factor found its way into the inositol fraction, which thus retained some of its activity when tested alone. On the other hand, the filtrate at this stage was free from inositol, and was quite inactive alone, its activity being restored on the addition of pure inositol. A possible reason for the failure to separate the two factors sharply is put forward below (p. 1866). The distribution of activity between the fractions is shown by the results of the plating tests, given in Table III.

Table III.

	Growth of mycelium, per unit dose (mg. dry weight)		
	Precipitated fraction	Filtrate fraction	Filtrate <i>plus</i> inositol
First fractionation	312	245	332
Filtrate refractionated	185	0	224

From 50 g. of crude lentil concentrate, 6.3 g. of solid remained in the filtrate fraction after the second precipitation and was inactive in the absence of inositol. A series of platings using a range of doses of this concentrate showed that the standard growth of 270 mg. was produced in the presence of 25 mg. dry matter per 100 cc. medium (together with 20 mg. inositol).

(b) *Precipitation with mercuric acetate and with phosphotungstic acid.* Buston and Pramanik had reported that the second accessory factor was completely precipitable by the mercuric acetate-sodium carbonate reagent of Neuberg and Kerb. Accordingly the inositol-free fraction of the concentrate was treated with mercuric acetate in the presence of barium hydroxide, this latter substance being used in preference to sodium carbonate in view of the objections to the presence of large amounts of sodium in the concentrates. A 1 % solution of the concentrate was used, and 30 % of the dry matter passed into the mercuric acetate precipitate fraction; the whole of the activity appeared in this fraction.

Platings with a portion of this fraction (after removal of mercury *etc.*) corresponding to 20 mg. of the inositol-free filtrate (*i.e.* 7 mg. of the ppt. fraction at this stage) per 100 cc. medium gave a growth of 269 mg. dry mycelium.

The possibility has been pointed out above, that the second factor might be of the nature of an amino-acid; accordingly, determinations of nitrogen were made on the various fractions of the concentrate. The mercuric acetate precipitate fraction was found to contain 18.1 % N on dry matter, a figure so high as to suggest that basic substances might be present. Accordingly the precipitation of the substance with phosphotungstic acid was studied. A series of precipitations was performed, the concentration of the mercuric acetate precipitate fraction and of the phosphotungstic acid being varied. In all cases precipitation was carried out in 5 % sulphuric acid solution, and a sufficient excess of the reagent was used. The results of growth tests carried out in the usual manner on the several fractions are given in Table IV. From these results,

Table IV.

Solution of con- centrate %	Phosphotungstic acid reagent									
	25 % solution						10 % solution			
	Dry matter		Growth			Dry matter		Growth		
	ppt.	filtr.	ppt.	filtr.	p. + f.	ppt.	filtr.	ppt.	filtr.	p. + f.
10	2010	600	251	Trace	286	1355	940	218	Trace	277
4	375	350	168	177	228	425	500	169	160	270
1	377	325	163	157	300	625	525	155	152	286

(Growth figures—mg. dry mycelium.)

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it appeared that no sharp separation could be obtained by the use of phosphotungstic acid, although, as shown by the growths given by the recombined fractions, there was little loss of activity. Maximum precipitation, both of active substance and of total dry matter, took place in the stronger solutions both of concentrate and reagent; in the weaker solutions, however, precipitation of total substance and of active material were not always parallel. It seemed therefore that true precipitation, and not a mere adsorption, was concerned. It was concluded that the best method of treating the concentrates, after removal of inositol, would be to precipitate first with the Neuberg-Kerb reagent and then from a solution containing 1 % dry matter with 10 % phosphotungstic acid reagent. By this means, the greater part of the active substance passed into the phosphotungstic acid precipitate, although some 25 % escaped precipitation.

From an original lentil concentrate giving a growth of 275 mg. dry matter in doses of 200 mg. per 100 cc. medium, it was possible to prepare from this phosphotungstic acid precipitate concentrates of the second accessory factor, 4 mg. of which, in the presence of 20 mg. of inositol per 100 cc. medium, gave the same growth. The material recovered from the phosphotungstic filtrates, though showing less activity per unit weight, still accounted for 25 to 30 % of the original active substance. The total recovery of factor II at this stage amounted to roughly 70 %, most of the remainder being lost with the inositol fraction.

About 300 g. of lentil concentrate were treated according to the above scheme in batches of 100 g. giving some 4 g. of the phosphotungstic precipitate fraction and 5 g. of the filtrate fraction. (In the following pages, it is to be understood that "phosphotungstic precipitate" and "phosphotungstic filtrate" refer to the fractions at this stage.)

(c) *Other chemical methods.* The further concentration of the active substance proved a matter of considerable difficulty. Attempts were made to prepare and fractionate crystalline picrates, but, although picrates of varying solubility were readily obtained, a considerable loss of activity was always found; this was not due, however, to the introduction of toxic substances into the fractions. Evidence was obtained that this inactivation was due to the mild oxidising action of the picric acid. In view of the reported stability of the substance towards oxidising agents, this seemed remarkable; however, the earlier experiments concerning oxidation were all made on the crude concentrates, and an apparent loss of stability with purer materials is not unknown in allied fields of research.

In another series of experiments, fractionation by means of copper salts was attempted [cf. Town, 1928]. From a small portion of the concentrate containing 250 unit doses of the second factor, the copper salts were prepared, and the following fractions separated: (i) insoluble in cold water, (ii) soluble in water, insoluble in methyl alcohol, (iii) soluble in water and in methyl alcohol, insoluble in absolute alcohol, (iv) soluble in absolute alcohol. In the growth tests on the fractions, after removal of Cu, fractions (i), (ii) and (iv) were found to be entirely inactive; fraction (i) was slightly toxic, possibly owing to the presence of traces of copper, but fractions (ii) and (iv) were free from toxicity. On the other hand, fraction (iii) was found to possess activity, but only to the extent of one-third that of the original concentrate fractionated. Thus there had evidently been a considerable loss of activity during the process, possibly owing to adsorption of the active substance on the copper sulphide precipitates. It may be recalled that Buston and Pramanik were unable to recover completely the activity of a concentrate which had been in contact with copper sulphide.

Fractionation of the lentil concentrate by physical methods.

(a) *Fractional electrolysis.* In view of the above recorded failures of chemical methods of fractionation, resort was had to methods of more physical nature. In the first place experiments were made using the method of fractional electrolysis, and evidence was obtained that the active substance tended to migrate towards the anode, while the greater part of the solids present moved towards the cathode. There was however a very considerable destruction of activity in the series of cells, and the method did not offer any prospect of giving more active concentrates.

(b) *Fractionation by solvents.* In a series of recent communications Block and Cowgill [1932] have studied the possibility of extracting vitamins of the B group from crude preparations by means of organic solvents and have claimed that they have obtained very considerable concentration under certain conditions. From the basic properties of the substances with which they were dealing Block and Cowgill argued that these substances should be soluble in organic solvents as bases and insoluble as salts.

Since in the case of the *Nematospora* factor it was impossible to state whether it was of acidic or basic nature, extractions with ether were carried out with both acid and alkaline solutions of the substance, solutions of the crude concentrate, without previous removal of the inositol, being used in the first place. An apparatus similar to that described by Wilson [1932] was employed; the ether used was previously purified carefully to remove traces of peroxides [see Garbarini, 1909]. The extracts and residues were in all cases freed from ether, acid *etc.*, and growth tests were made, such amounts of each fraction being used as would correspond to 0.2 g. of the lentil concentrate, on a basal medium con-

taining 20 mg./100 cc. of inositol, *i.e.* an adequate amount of inositol, irrespective of the amount present in the individual extracts. The results (Table V) showed that no extraction had taken place from the alkaline medium, indicating that it was unlikely that the substance was of basic nature. On the other hand, a certain amount of activity was removed by ether extraction from acid solution, although even here the greater part remained unextracted, and more prolonged treatment did not increase the proportion extracted.

Table V.

Fraction	Growth of mycelium (mg. dry weight)	
	Alkaline extraction	Acid extraction
Ether extract: 1 unit	Nil	41
2 units	Nil	91
Water phase: 1 unit	300	223
2 units	323	—
Toxicity test on ether extract	293	—

In other experiments, ether was replaced by butyl or amyl alcohol, the extractions being carried out in a faintly acid medium, using a continuous extraction apparatus modelled on that of Dakin [1920]; the extractions were usually carried out at reduced pressure, the temperature of the liquid being kept below 80°. Experiments with the crude concentrates again showed partial extraction of the active substance from acid solution, but further experiments using the more active concentrates obtained from the precipitate and filtrate fractions after the phosphotungstic acid treatment (p. 1862) showed that the active substance in these two fractions behaved somewhat differently towards the solvent.

(i) A 250 cc. portion of the filtrate fraction, containing 280 unit doses, was extracted in all for 18 hours with butyl alcohol (this solvent being preferred to amyl alcohol on account of its lower B.P.). On testing, it was found that the aqueous phase was completely inactive, showing that the active factor had passed completely into the butyl alcohol. The butyl alcohol itself, however, seemed to contain traces of some substance which checked the normal growth of the fungus, causing growth of the "staling" type, and a similar type of growth was observed even when the extract and residue were recombined. The mycelium, after growing normally at first, later gave irregular and sparse growth, so that the dry weights of mycelium obtained showed a distinct falling off of activity¹. The results of typical growth tests are given in Table VI.

Table VI.

Fraction	Growth of mycelium (mg. dry weight)
Aqueous phase after 12 hours	Nil
Alcoholic extract: 1 unit	146 (S)
2 units	242
Recombined	124 (S)

(S indicates staling growth.)

¹ Not all the samples of butyl alcohol used seemed to contain the substance producing "staling," for in at least one set of extractions normal growth was obtained (see Table VII, col. 3). It is understood that butyl alcohol is produced by a fermentation process and may contain traces of β -hydroxybutyric acid; this acid has been shown [Fuller, 1933] to possess bactericidal properties; it seems therefore not impossible that it may also affect the growth of other micro-organisms.

(ii) A portion of the concentrate from the phosphotungstic acid precipitate fraction was similarly extracted with butyl alcohol. After 18 hours' extraction, a growth test with 1 unit dose of the aqueous phase still gave 237 mg. (dry weight) of mycelium—roughly 85 % of the full growth, showing that but little of the activity had passed into the alcoholic solution. A further portion of the same concentrate was hydrolysed for 3 hours with 1 % sulphuric acid prior to extraction, and in this case the whole of the active substance passed rapidly into the alcoholic extract, the aqueous phase after 12 hours possessing no activity whatever. A further test was made on the filtrate fraction, after hydrolysing this in the same manner, but the treatment had no effect on the

Table VII.

Fraction	Growth of mycelium (mg. dry weight)		
	Filtrate hydrolysed	Precipitate hydrolysed	
		1	2
Aqueous phase after 6 hours	140	—	—
Aqueous phase after 12 hours	Nil	Nil	Nil
Alcoholic extract: 1 unit	253	204 (S)	280
2 units	255	—	—
Recombined	244	—	—

(S indicates staling growth.)

subsequent extraction (Table VII). The only explanation of the difference in behaviour of the active substance present in the phosphotungstic filtrate and precipitate fractions respectively seems to be that, in the latter, it forms part

of some more complex molecule, being probably combined with a basic substance, whereby it is rendered precipitable, whereas in the former it is present in an uncombined form, without basic properties. In either form, however, the substance is capable of exercising its growth-promoting function.

With regard to the activity of the extracts obtained after hydrolysis, that from the filtrate gave a growth of 255 mg. when supplied in a dose of 1.8 mg. per 100 cc. medium; for the precipitate fraction the dose was 1.2 mg. per 100 cc. Possibly the somewhat lower activity of the filtrate fraction was due to the presence therein of certain inactive substances, not present in the precipitate fraction, which were capable of extraction with butyl alcohol.

Further concentration of the active factor has not at the moment proceeded beyond this stage, except that some preliminary tests have shown that, from the precipitate fraction after hydrolysis, the whole of the bases are precipitated by phosphotungstic acid at p_H 2, and are quite inactive.

The steps in the concentration of the lentil extract are summarised in the accompanying diagram (see p. 1865); the figures quoted under each fraction represent the amount necessary, in 100 cc. of the medium, to produce the standard growth (270 mg.).

Reactions given by the active concentrates.

The observation that after hydrolysis the active factor was no longer precipitable by phosphotungstic acid, as well as the fact that in the uncombined form it escaped precipitation with this reagent in the first place (thus giving rise to the unexplained loss of 25–30 % of the activity in the phosphotungstic acid filtrate fraction) made it clear that the substance concerned was not a base. The most active concentrates still contained approximately 5 % N, on dry matter; the product obtained by butyl alcohol extraction of the filtrate fraction was richer in nitrogen (14.8 % N), although somewhat less active. Although this figure showed a considerable fall from the 18 % N recorded for the original phosphotungstic precipitate fraction, the possibility that activity was due to some nitrogenous substance was not excluded. In the combined form there seemed no doubt that the molecule contained a basic nitrogenous unit. The tests with the crude lentil concentrate (Table V) seemed to show that in the combined form the substance was insoluble in ether.

Some evidence seems to exist that the active substance is of acidic nature. Its extraction from acid solution only by ether; its behaviour under the influence of the electric current; its apparent ability to form copper salts and its non-precipitability in the free state by phosphotungstic acid point in this direction. It may be also that the persistent appearance of a portion of factor II in the inositol fraction was due to the formation of a barium salt by a part of the substance which was present in the lentil extract in the free state.

The most active concentrates were found to give a strong Molisch reaction, so that carbohydrates were still present. Pentoses were detected in the concentrates immediately before the butyl alcohol extraction, but the orcinol reaction was not given by the final concentrates. On the other hand, these concentrates gave a distinct naphthoresorcinol test for uronic acid, the test being indefinite in the case of the less active concentrates. It is interesting to recall that in the original paper of Farries and Bell [1930] it was suggested that the accessory factor was of the nature of a sugar acid; one class of sugar acids—the uronic acids—seem to be possessed of certain peculiar properties giving them a special influence on the course of metabolism. The stability of the substance here described towards heat and strong acids seems to preclude any possibility

of its being of the nature of a uronic acid, although the presence of a substance giving the naphthoresorcinol test is interesting. In any event, there is no suggestion that a state of purity has been approached; if it is possible to make any comparisons on the basis of the activity of the purest vitamin B preparations, at least 90 % of even the most active concentrate must be impurity, and there is no justification in attempting to assign the active factor to any chemical class.

Inorganic constituents of the concentrates.

Although Farries and Bell had already shown that the ash of their active "egg extract" was inactive in promoting growth of *N. gossypii*, the recent observations of Richards [1932] on the ability of certain metals, particularly thallium, to act as growth stimulants for yeasts, made it desirable to re-examine the question of the possibility of inorganic constituents of the lentil extracts acting as growth-promoting substances. Samples of the concentrate were examined spectrographically by Mr Hugh Ramage, to whom the authors' thanks are due. Apart from K, Na, Ca and Mg, the only element present in more than infinitesimal amount in the concentrate was nickel, which was present to an amount estimated at between 0.002 and 0.003 % of the crude concentrate. A series of platings was therefore made in order to discover whether nickel, in amounts of the order found in the concentrates, had any effect on the growth of the organism, but the results were entirely negative.

Tests with isomeric inositols.

In order to ascertain whether any of the naturally occurring isomerides of *i*-inositol, or related substances, could replace this body in the media, certain of these substances were prepared and their activity tested, in conjunction with factor II. Those used were: quercitol; quebrachitol; *l*-inositol, from quebrachitol; sennitol; *d*-inositol, from sennitol. In no case was any activity found.

SUMMARY.

1. The second factor necessary for the growth of *Nematospora gossypii* occurs in lentils in combination with an inactive nitrogenous base. It is readily liberated by gentle hydrolysis and exerts its growth-promoting influence both in the free and combined forms.

2. The second factor is also found associated with proteins, especially those of seeds, but probably does not form part of the protein molecule.

3. The active substance is precipitable by Neuberg and Kerb's mercuric acetate reagent, but (in the free form) not by phosphotungstic acid. In the combined form, the presence of a basic unit renders it precipitable by the latter reagent.

4. In the free form, the substance is extracted from acid solutions with organic solvents and seems to possess certain weakly acidic properties.

5. The most active concentrates prepared promote full growth when present to the extent of 1.2 mg. per 100 cc. medium, as compared with a dose of 200 mg. of the original lentil extract. Some reactions given by the concentrates are recorded and their possible significance is discussed.

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