

STUDIES RELATING TO THE SYMBIOSIS OF SEEDS AND BACTERIA.

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GENERAL INTRODUCTION.

The subject of symbiosis is one of great and increasing importance. Symbiosis may be defined as the living together of two kinds of organisms to mutual advantage. The phenomenon is frequently met with both in the animal and vegetable kingdom. There is good evidence that the luminescence exhibited by certain insects, by the cuttlefish, and particularly by a species of fish found off the Banda Islands of the East Indian Archipelago is due to a symbiosis between the luminescent organ of the insect or fish and certain specific bacteria. The observations of Huxley, Plerantoni and others¹ on the yeasts living within various insects have been extended and confirmed by the recent work in the Department of Bio-Chemistry by Messrs. Mahdihassan and Sreenivasaya who have clearly demonstrated the presence, in the tissues of the healthy lac-insect, of yeast-like bodies which exercise a symbiotic function useful to the insect.

The outstanding example of symbiosis in plants is seen in the existence of lichens. These are compound organisms, composed of a fungus and an alga. The combination is so perfect that it produces well characterised plants, which were classified and studied separately, without the least suspicion of their dual nature until this was discovered by Schwendener in 1867. In the formation of the consortium of the lichens, the algal cells become enveloped by the mycelium of the fungus. The fungus derives its nourishment saprophitically from the organic matter produced by the assimilating alga, which, in its turn, derives a definite advantage from its consortium with the fungus, receiving from it inorganic substances and water and possibly also organic substances.

The intervention of a symbiotic fungus has been found by Bernard to be absolutely essential to the germination of the seeds of a certain species of orchid.

¹ *La symbiose et le parasitisme*, by M. Caullerie.

The symbiosis of plant and bacteria is familiar in the association of leguminous plants with bacteria in their root-nodules, resulting in the fixation of atmospheric nitrogen. Small nodules, containing nitrogen-fixing bacteria are also found in the leaves of certain plants, and hence such leaves become of prime importance as fertilisers.

The present paper deals with symbiosis between seeds and bacteria. Such a relationship has been observed in the case of the bacteria occurring on the seeds of *Cassia tora* and on paddy. Bacteria have also been found in the husk of coconut, which evidently assist in the softening and retting of the coir fibre. In each case the bacteria were specific to the seed, and the presence of other bacteria appeared to be eliminated by an antiseptic characteristic of the seed, in the cases of *Cassia tora* and paddy by substances of a basic or glucosidic nature and in the case of coconut by tannins.

Besides the simple determination of the presence of specific bacteria in, or on, seeds and their characterisation, it is obviously of special interest to determine, if possible, what part the bacteria play in relation to the life of the seeds, and, on the other hand, what relation, if any, exists between substances of an antiseptic or inhibitory character occurring in the plant and the presence or absence of certain bacteria.

The subject offers a very wide field of inquiry and although it cannot be claimed that the present research has resulted in any far-reaching conclusions, yet a number of interesting facts have been established, which, it is hoped, may find their place later on in a general view of the phenomenon of symbiosis.

The work is dealt with under the following heads :—

- I. Chemical and bacteriological investigation of certain typical seeds.
- II. Examination of the function of seed bacteria.
- III. Relation of bacteria to the seed 'extractives.'
- IV. Conclusions.

It has been found convenient to publish in an appendix some incidental experiments on the permeability of the seeds to certain antiseptics.

I. CHEMICAL AND BACTERIOLOGICAL INVESTIGATION OF CERTAIN TYPICAL SEEDS.

The seeds used for the main investigation were those of indigo and poppy, both being of economic importance, and obtainable of known quality. Reference is made also to other seeds examined less completely.

INDIGO-SEED.

Chemical examination.—The seed was obtained through the Agricultural Demonstrator to the Government of Madras at Nandyal and was a sound specimen of *Sumatrana*. The seeds were hard and brown, consisting of three main parts, viz., the outer brown testa, the middle semi-transparent endosperm, which swells and gives a mucilaginous liquid on soaking in water, and the yellow inner embryo.

Qualitative micro-chemical examination of sections of the seeds showed the presence of tannins, lignin, sugars and a glucosidic substance. Starch was not found nor was any pectin reaction given by the mucilage.

The following percentages were obtained on a quantitative proximate analysis:—moisture 9.0, nitrogen 4.4 (giving proteins 28), benzene extract (oil) 6.0, alcohol (94 per cent.) extract 8.2, crude fibre 7.9, ash 3.7.

The seeds on soaking in cold water gave a dark brown solution, containing mainly tannin, which is also the chief constituent of the alcoholic extract. Traces of a glucosidic substance were also found both in the aqueous and alcoholic extracts.

A basic substance, giving the reactions for alkaloids was obtained by crushing the seeds with a little lime-water, drying and extracting with chloroform.

To obtain a clear mucilage for separate examination the seeds were allowed to soften in water, and the endosperms separated from the rest of the seed by gentle pressure; the remaining testa and embryo were separated and kept for further experiment. The mucilaginous endosperms were extracted by boiling water, and allowed to stand over-night, when the mucilage tended to thicken. Next morning the material was strained through cloth, and to the thick slimy liquid alcohol was added till all the mucilage was precipitated as a thick white ropy mass. This was removed and dried.

The crude mucilaginous liquid (before treating with alcohol) was hydrolysed with sulphuric acid, neutralised with barium carbonate and filtered. The filtered liquid was examined for sugars when osazone and hydrazone tests showed the presence of glucose and mannose; pentoses and galactose were absent. The mucilage therefore probably contains gluco-mannan.

Mucilage-content of germinating seeds.—The following experiments were made to determine whether the colloidal mucilage was rendered soluble by the germinating seed, to furnish food for the seedling plant. 200 indigo-seeds of one and two days germination and 200 ungerminated seeds were boiled separately with 50 cc. water for four or five hours, then left over-night to cool and to allow the mucilage to thicken. Next day the contents of the flasks were decanted, the seeds washed with small quantities of water and the extracts made up to 40 cc.; 5 cc. from each extract were treated with equal volumes of alcohol and the precipitated mucilage collected on weighed filter papers. The alcoholic filtrate in each case was tested for reducing sugars by Fehling solution. The following results were obtained:—

| | Mucilage content in grams. | Fehling reaction. |
|----------------------------|-------------------------------|----------------------|
| A. Ungerminated seeds | 0.0248 | Trace. |
| B. Germinated seeds, 1 day | 0.0192 | „ |
| C. Do. 2 days | 0.0160 | Red ppt. |

It is thus clear that the mucilage is saccharified during germination and used as food by the growing plant.

Fermentation of the seeds.—The seeds on soaking in water quickly began to ferment with evolution of gas. Preliminary examination showed that hydrogen, methane and carbon dioxide were produced, together with acetic and butyric acids; quantitative analysis of the gas showed the approximate proportions of the constituents to be: hydrogen 7, methane 1, carbon dioxide 2.5. No fermentation of the seeds took place when the seeds were treated with 2 per cent. copper sulphate solution, and immersed in sterile water, nor when thymol was added to a mass of crushed seeds and water. The fermentation therefore is due to bacteria.

Bacteriological examination.—After a number of preliminary tests (described in a separate note) the bacteria which may be termed incidental were removed, and the bacteria persisting on the seed were developed in the following manner. The seeds were treated with mercuric chloride solution (1:1000) for ten minutes, washed with sterile tap-water, both intact and crushed being then placed on nutrient

PLATE 1.



INDIGO-SEED BACTERIA.

agar plates. No bacterial growth was found round the crushed particles, showing that no bacteria were present inside the seed. Occasionally, however, bacterial growth appeared round the uncrushed seeds. On examination these bacteria were always found to be the same morphologically. Identical forms were obtained in a mash of seeds which had been heated in the steam steriliser for ten minutes on three successive days. Cultures of these resistant bacteria were made and termed 'indigo-seed' bacteria.' They showed the following cultural characteristics. Under the microscope they appeared as short motile rods,¹ were spore-forming and gram positive. They gave good growth on slopes of seed-mash agar and of ordinary nutrient agar, on potato slope only a slight growth appeared. No growth was obtained with stab cultures on nutrient agar. In nutrient broth a scum formed but no gas was evolved. No appreciable fermentation took place in any of a number of broth tubes to each of which one of a number of the following carbohydrates had been added, viz., starch, cane-sugar, glucose, dextrin, arabinose, xylose and galactose. The bacteria are therefore aerobic and do not ferment carbohydrates, but thrive on indigo-seed mash and nutrient agar.

Fermentation by seed-bacteria.—Having found that a mash of the seeds containing all the seed-constituents was readily fermented by the seed-bacteria, it was of interest to ascertain whether the fermentation was concerned with all or only some of these constituents. Accordingly, small quantities of the following portions of the seeds were placed in small flasks filled with water, sterilised and inoculated with indigo-seed bacteria, arrangements being made to collect any gases evolved: (a) pure mucilage, precipitated by alcohol, (b) crude mucilage, untreated with alcohol, (c) embryos. In no case was fermentation observed. Even when peptone in increasing quantities was added to the mucilage, the former only underwent fermentation, leaving the mucilage intact. The same experiments were repeated with negative results, using Durham's tubes, and every possible permutation and combination of the seed-constituents. It would appear therefore that no part of the seed by itself is capable of fermentation, nor even three of the constituents together; only when a mash is made of the whole crushed seed will the bacteria thrive. Similar behaviour has been observed in the case of the acetone bacillus.

POPPY-SEEDS.

Bacteriological examination.—The poppy-heads under investigation were kindly sent by Dr. Annett, Agricultural Chemist to the Government of Bengal. They had not been lanced for the exudation

¹ See Plate 1

of their latex, and therefore their interior had not come in contact with the outside atmosphere. A capsule was taken out carefully from the wrapping of cotton wool in which it had been sent, and after flaming it on the outside and slightly cutting it open with a sterilised knife some seeds were scattered on a nutrient agar plate and some were crushed in a sterilised mortar with a few drops of sterile water, the paste being transferred to a nutrient agar-poured plate. The next day it was found that the plate on which the uncrushed seeds had been scattered showed no sign of the presence of bacteria, while on the plate with the crushed seed there was the appearance of copious growth round the crushed particles. To confirm this observation, streak-cultures were made on agar slopes and undoubted bacterial growth was obtained. Later the method of experiment was slightly modified so that all the tests concerned with one capsule could be carried out on one plate, giving readily comparable results, thus the following streaks were made on one plate:—

(1) First blank-washings of sterilised pestle and mortar with sterile tap-water. (2) Row of aseptically handled, uncrushed seeds. (3) Crushed seeds without water. (4) Ditto with water, as used in (1). (5) Second blank-washings of pestle and mortar after washing away the paste.¹

It was noteworthy that in almost all cases the seeds crushed without water gave very little bacterial growth, whilst if water was added to the crushed mass, or the seeds crushed in water, the growth was abundant.

The whole seeds showed no growth, except in the case of one or two, which under the microscope were found to have a slightly ruptured skin.

Bacteria were also found in the seeds of ordinary garden poppies and in seeds brought from the local market. Their occurrence appears therefore to be normal.

General cultural characteristics of poppy-bacteria.—The poppy-seed bacteria when examined under the microscope, appeared to be pure cultures of very short motile rods,² spore-forming and gram positive. They grew freely on slopes of nutrient agar, and poppy-seed mash agar, but showed practically no signs of growth from stab-cultures in nutrient agar, or in the following media:—nutrient broth, broth tubes containing either glucose, lactose, dextrin or starch; glucose bile-salt, nitrate peptone, milk or potato-slope. It is evident that the bacteria are strictly aerobic and do not ferment

¹ See Plate 2.

² See Plate 3.

PLATE 2.



BACTERIAL GROWTH FROM CRUSHED POPPY-SEED.

PLATE 3.



POPPY-SEED BACTERIA.

carbohydrates, thus bearing a close resemblance to the bacteria of indigo-seeds. Experiments to determine whether these bacteria attack cellulose, in the form of filter paper, either under aerobic or anaerobic conditions gave negative results. The bacteria have a slight lypolytic action on poppy-seed oil.

MISCELLANEOUS OBSERVATIONS.

Tomato seeds.—During the course of the work, occasion was taken, on the occurrence of wilt in some growing tomato plants to examine them for bacteria, to see whether disease may begin where symbiosis ends, or whether symbiosis may be the result of a gradual toleration of an alien organism, at first appearing as an enemy. The healthy seeds of the fruit did not, however, show indications of the presence of bacteria either within or without. At the same time it may be mentioned that the bacteria found in the wilted leaves and in some of the seeds, gave the same cultural phenomena as those of the organism described by E. F. Smith in his *Bacterial Diseases of Plants*; in some cases a fungus, *Macrospora solani*, pathogenic to solanaceous plants, was also found (*cf.* Duggar, *Fungus Diseases of Plants*).

No bacteria were found within linseed, castor or annatto seeds after these were treated for ten minutes with 1-1000 mercuric chloride, crushed and transferred to nutrient agar plates.

Mode of entry of bacteria into the seeds.—In those cases where bacteria are actually found within the endosperm, it is natural to inquire how they find entry. In the case of indigo-seed the tests made to determine the permeability or otherwise of the wall of the endosperm to antiseptics revealed the fact that although for the most part impermeable, the endosperm was open to attack at one point, which was shown as a tiny red spot when the de-husked seed had been dipped into potassium iodide solution, subsequent to the treatment of the unhusked seed with mercuric chloride. The penetration of the liquid had taken place through the micropyle where the endosperm is practically absent or very thin, and had continued right up to the spot where the embryonic cotyledons abutted on the endosperm.

In the case of the bacteria in poppy-seeds it was possible to make observations on the growing plant. An average-sized poppy-head was flamed and portions of a section of it were placed on a nutrient agar plate, brought into the garden; but for some reason no growth was obtained. Some poppy-heads were then cut with stems about 3' long and the cut ends of the stem dipped quickly into melted paraffin, the whole being brought over to the laboratory for examination.

Portions of the stem, seeds from the middle and upper portions of the head were placed aseptically on a nutrient agar plate, both with and without moistening with water. In the absence of water no growth took place in any case; in the presence of water, bacterial growth was evident from the piece of cut stem and from the seeds in the middle portion of the head, but none from seeds derived from the upper portion.

Smaller poppy-heads and their stems as well as young buds were similarly examined to see at what stage the bacteria appeared. They were found in the seeds of poppy-heads from 0.5 to 1 cm. diameter, and in the stem of the former, but not in the buds, nor in poppy-heads of less than 0.5 cm. diameter, nor in the corresponding stems. Although it must not be overlooked that the tissues of the immature plant may be too delicate to stand the sterilisation of their external surface by flaming, so that any internal bacteria may thus be destroyed, the more probable conclusion would seem to be that the bacteria are not present in very young buds or stems, but ascend the stem to the capsule as the size of the fruit increases.

II. EXAMINATION INTO POSSIBLE FUNCTION OF SYMBIOTIC BACTERIA.

The fact that certain bacteria appear specific to certain seeds would suggest that their presence is due to their exercising some function beneficial to the seed. It was of interest therefore to determine, if possible:—

- (1) Whether the bacteria helped in the actual germination of the seed.
- (2) Whether they promoted the growth of seedlings.
- (3) Whether they fixed atmospheric nitrogen for the benefit of the plant.
- (4) Whether they assisted the nutrition of the plant by breaking down the complex seed-proteins.

(1) *Seed-bacteria and germination.*—According to Nilson,¹ the agent which opens the series of changes constituting germination in barley is the lactic acid bacterium, which is always present in the barley grain under normal conditions, and not the enzymes, the presence of which in the barley grain is doubtful, according to recent researches, and which are formed as a consequence of germi-

¹ *J. Amer. Chem. Soc.*, 1904, 26, 289.

PLATE 4.



Showing germination of *Cassia Tora* seeds under perfectly sterile conditions. The seedling is quite healthy as seen from the photograph and there is no bacterial growth on the nutrient agar poured in afterwards.

PLATE 5.



Sterilised *Cassia Tora* seeds set to germinate on nutrient agar plate.

nation. His views, however, are contradicted by Windisch and K. Schoenewald,¹ who have proved that barley does not lose its germinating power even when sterilised with an alcoholic solution of mercuric chloride which would kill all bacteria. S. U. Pickering² has effected germination in agar-agar with perfectly sterilised seeds of *Lilium italicum*, *L. perenne*, clover and spinach, and in one case mustard, and therefore comes to the conclusion that bacteria are not necessary for germination.

In the present investigation the conditions of germination were observed in the case of the following seeds, viz., indigo, *Cassia tora*, linseed and tomato.

The seeds were soaked in 1/1000 mercuric chloride solution for 10-15 minutes, then thoroughly washed with sterile tap-water, a few placed on agar plates to test their sterility, and others set to germinate on sterile sand, contained in a sterile flask or test-tube. No sign of bacterial growth was noticed in any case where vigorous germination took place, but in a few cases where the seeds failed to germinate, bacterial growth was observed.

The accompanying photographs³ illustrate the germination of antiseptically treated *Cassia tora* seeds, after they had been soaked in sterile tap-water in a plugged sterile tube, and the excess of soaking liquid had been decanted. After about three days' vigorous growth of seedlings, some melted and sterilised nutrient agar was poured into the tube in such a way that any bacteria attached to the rootlets would readily grow; no development of bacteria took place.

In the case of poppy-seeds, the conditions were rather different from the instances just described, inasmuch as the poppy-seed bacteria exist within the actual seed; nevertheless when a few seeds were scattered on a nutrient agar slope with a few drops of sterile water, and allowed to germinate, it was noticed invariably that wherever germination took place there was no bacterial growth round the seedlings, but this developed in absence of germination.

It may of course be argued that where healthy germination takes place, the bacteria, whether useful or not in the initial stages, are afterwards digested along with other food material by the enzymes of germination. In none of the cases investigated, however, is there actual proof that the presence of bacteria is essential to germination.

¹ *Woch. Brau.*, 1905, 22, 200.

² *J. Agri. Sc.*, 1907-8, Vol. II. 411.

³ See Plates 4 and 5.

(2) *Bacteria in relation to the growth of seedlings.*—Although the presence of bacteria may not be essential to the actual process of germination, some hitherto unpublished experiments by one of us with D. L. Sen, using wheat-grains treated with dilute copper sulphate, gave clear indication that the presence of bacteria was helpful to the growth of seedlings. In these experiments three sets of wheat-grains were placed in moist sterile sand in three plugged sterile flasks. The first were unsterilised, the second treated with copper sulphate and washings of the untreated seeds added, while the third set consisted of treated seeds. The results of growth showed clearly that in this case at any rate the bacteria had a beneficial effect.

The same type of experiment was carried out with indigo-seeds, treated in one case with 1/1000 mercuric chloride for 15 minutes with subsequent washing with sterile water, and in another treated similarly with a saturated solution of boric acid. The results were similar to those obtained with the wheat-grains and lead to the conclusion that seed-bacteria are helpful in promoting the growth of seedlings after germination is complete.

(3) *Seed-bacteria and nitrogen-fixation.*—Experiments with poppy-seed bacteria, grown in Ashby's medium gave no evidence that they possessed any power of nitrogen-fixation.

(4) *Seed-bacteria and the disintegration of proteins.*—It has been seen that the bacteria of indigo and poppy-seeds showed no tendency to ferment carbohydrate material, and also that apparently they do not help to fix nitrogen, so that more detailed study of their special function, viz., the disintegration of protein, becomes of special interest. For this purpose a medium was used described by Aibel,¹ having the following composition: asparagine 6 gms., potassium phosphate 1 gm., manganese sulphate 1 gm., water 1000 c.c.

Tubes of this medium were inoculated with fresh cultures of indigo and poppy-seed bacteria, and after four days incubation at 37°, part of the contents of each tube was tested for ammonia by Nessler; an intense reaction was obtained which was not developed by blanks consisting of the bacterial culture and the broth separately. The ammonifying power of these bacteria was confirmed by the examination of the water into which some poppy-seeds had been shaken direct from a poppy-head about a year before. The tube containing the seeds and water had been plugged, fitted with a rubber cap and left in the culture room to develop. No change excepting a slight turbidity was seen. After observing the production of ammonia in the Aibel medium, the

¹ 'Influence de la nature de l'aliment carbone sur l'utilisation de l'azote par le *Bacillus subtilis*.' E. Aibel, *Compt. rend.*, 1920, 171, 479.

liquid in this tube was examined by removing 1 c.c., diluting to 50 c.c. and applying the Nessler test, an intense reaction occurring. It was evident that the seed-proteins had been broken down to ammonia, either by the bacteria present in the seeds, or by the enzymes, or by both. That ammonia was not present in the original seeds was shown by shaking some seeds direct from a poppy-head with water, and testing the liquid for ammonia with a negative result.

It was found that a mash of poppy or indigo-seed, autoclaved at 115° for fifteen minutes, developed ammonia on inoculation with specific seed-bacteria, and further that indigo-seed mash could be fermented by poppy-seed bacteria and *vice versa*. In neither case was the ammonia transformed to nitrite or nitrate even after long standing, showing that the only bacteria present were those capable of ammonifying proteins.

III. RELATION OF BACTERIA TO SEED-EXTRACTIVES.

It was frequently noted in the course of the foregoing experiments that the bacteria present in the seeds did not begin to function until sufficient water was added to cause appreciable attenuation of the bacterial environment. This was especially the case with poppy-seeds which were scattered direct from the poppy-head on to the agar plate without any preliminary washing of the actual seeds with antiseptic. Moreover the bacteria remaining on other seeds after removing 'incidental' external organisms by rapid antiseptic washing, were of one kind specific to the seed. It would therefore appear that the seed contained some agent actually antiseptic to all but certain specific organisms, and inhibiting even to these until the seed was moistened. There was direct evidence of this in the case of *Cassia tora*,¹ where the agent in question appeared to be a 'bitter principle,' in paddy,² where an alkaloid of similar character to Funk's anti-beri-beri vitamine was isolated, and in coconut,³ where tannin was shown to be the inhibitory agent. The presence of a sparse but specific bacterial population has been noted in the inner coat of the peel of the banana,⁴ which is held in check during normal ripening processes. It would be interesting to ascertain the relationship between what may be termed the 'active principles' or 'extractives' of the seeds and the accompanying bacteria, inasmuch as the matter bears on the subject of plant-vitamines, concerning which many diverse opinions are held. Thus Funk considers the anti-beri-beri vitamine of rice to be an organic base; according to which view the effect of this vitamine would be

¹ This *Journal*, 1921, 4, 205.

² Fowler and Sen, This *Journal*, 1921, 4, 119.

³ Fowler and Marsden, This *Journal*, 1924, 7, 39.

⁴ E. M. Bailey, *J. Amer. Chem. Soc.*, 1912, 34, 1706.

simply antiseptic, as it has been shown by Fowler and Sen¹ that the bacterial population of polished rice is far more numerous and varied than that of unpolished rice, containing presumably, Funk's base.

On the other hand Ciamician and Ravenna,² from experiments with young haricot-beans, consider that alkaloids may exist as vegetable hormones, exciting chlorophyllous activity. Annett³ regards them simply as waste-products of nitrogen metabolism.

Again Bottomley⁴ contends that organic manures supply small quantities of necessary 'auximones' or plant vitamins stimulating to plant growth. Similarly, such small amounts of plant decoctions have been found stimulating to bacteria and to moulds.

Thus Willaman⁵ found that *Sclerotinia cinerea*, a brown rot fungus of peaches and plums cannot grow on media made up of sucrose, salts and asparagin, unless very small quantities of plant decoctions, especially of plums and peaches are added to the media. Linossier⁶ grew various moulds on attenuated broth and found that the growth of some was improved by adding small quantities of infusion of dry grapes, chiefly where the vitality of the mould was below normal. This growth-promoting property is attributed by the author to the presence in the extract of certain substances of unknown composition, capable of promoting growth, and which according to him are indispensable to the growth of the plant. Itano⁷ claims that nucleic acid and vitamine B exercise a markedly stimulating effect on a certain type of azotobacter. The question is rendered still more complex by the contention of Bierry and Portier⁸ that bacteria themselves, especially those present in seeds, act as vitamins.

On the other hand Lumiere⁹ is of opinion that these organic extracts are not essential to the growth of plants and maintains that the addition of these organic extracts to attenuated media may be advantageously replaced by addition of mineral salts of definite composition, at least in the case of moulds which were utilised in the experiments he describes. Mazé¹⁰ was successful in growing maize in a solution containing only mineral constituents; fluorine was found to be indispensable, in the proportion of only 1-3000 of the saline content of the culture solution on which the maize was grown.

¹ *loc. cit.*

² *Compt. rend.*, 1920, 171, 830.

³ *Mem. Dept. Agric, India*, 1921, 2, 154.

⁴ *Proc. Roy. Soc.*, 1915 B, 88, 237.

⁵ *J. Amer. Chem. Soc.*, 1920, 42, 549.

⁶ *C. R. Soc. de Biol.*, 1919, 381 ; 1920, 346.

⁷ *Jour. Bact.*, viii, 5, 483.

⁸ *Compt. rend.*, 1918, 166, 963.

⁹ *Ann. Inst. Pasteur*, 1921, 35 102.

¹⁰ *Ibid.*, 1919, 33, 139.

The question to be determined in the present research was whether the 'extractives' of the plant are definitely inhibitory or stimulating agents, or does their effect depend on circumstances or environment. The following experiments, though by no means final, have revealed some interesting facts.

In the first place the preparation of the various extracts may be briefly described:—

INDIGO-SEEDS.

Aqueous extract.—150 grams of clean indigo-seeds were soaked in distilled water for an hour, the liquid being decanted and reserved; a further extraction was made at 50–55° for fifteen minutes, the residue being freed from liquid by pressing through a cloth. The two extracts were mixed, and evaporated to a syrup on the water-bath. Qualitative chemical examination showed the syrup to consist mainly of tannins, sugars and mucilage.

Alcoholic extract.—275 grams of crushed seeds were extracted with petrol in a shaking-machine to remove oil; the extraction was repeated three times in ten hours, and the last traces of oil removed by percolation with petroleum ether. The oil-free material was then extracted in a shaking-machine with 70 per cent. alcohol for about twelve hours with four changes of liquid; the combined alcoholic extracts were filtered and distilled under reduced pressure. The almost solid residue was dissolved in hot water, the solution neutralised, and treated with lead acetate and basic lead acetate till no more precipitate formed. Tannins, albuminoids, gums, etc., are thus precipitated, and after filtration, the dissolved lead was removed by hydrogen sulphide. The filtrate was gently heated to remove hydrogen sulphide, neutralised with sodium carbonate and evaporated, the residue being extracted with alcohol. This extract was used in the experiments to be described. On acidification, the extract gave a precipitate with phosphotungstic acid, platinum chloride, Mayer's solution, picric acid and iodine in potassium iodide, pointing to the presence of alkaloids or other organic bases.

Alcoholic extract 'A'.—This extract was obtained by following the same procedure as above, except that after the removal of hydrogen sulphide, the solution was acidified with hydrogen chloride and precipitated with phosphotungstic acid. A yellowish brown precipitate was obtained. To this moist phosphotungstate, sodium carbonate was added till a paste was formed, this being shaken with strong alcohol, filtered and evaporated. The residue was called alcoholic extract indigo-seed 'A'.

Indigo-seed oil.—The petroleum ether extract of the indigo-seeds was distilled and the residue evaporated on the water-bath. The oil is greenish and has a bitter taste and strong smell.

Alcoholic extracts of poppy-seed and of tomato-seeds were obtained in a similar manner.

A decoction of poppy-heads was obtained by boiling the crushed heads with water, filtering and evaporating to a syrup.

(1) *Experiments on antiseptic properties of extractives.*—For testing the action of seed-extracts on bacteria, Prof. Delépine's thread method¹ was used, slightly modified to suit the special conditions. Silk threads, sterilised at 115° for half an hour, were immersed in an emulsion of the test organism (in this case *B. coli*), dried rapidly for an hour, and brought into contact with a concentrated syrup of the extractive for about seven minutes, washed quickly with sterile tap water and placed on a nutrient agar slope. Good growth was obtained showing that the aqueous extract of indigo-seeds had no antiseptic effect. No antiseptic effect was obtained when the procedure was reversed, and the bacterial emulsion carefully poured over the threads soaked in extract.

In case the failure of the extractives to act as antiseptics was due to dilution, occurring when the bacterial emulsion was poured over the soaked threads, these were carefully drawn along bacterial slopes, so as to impregnate them with bacteria without causing any dilution of the antiseptics. Six extracts, viz., aqueous of indigo-seed, alcoholic of indigo-seed, alcoholic 'A' of indigo-seed, alcoholic of rice-polishings, alcoholic of tomato seed and aqueous of poppy-head were used with the following organisms: (1) indigo-seed bacteria, (2) *B. coli*, (3) Tomato-disease bacteria, (4) soil bacteria, (5) Wild yeast, (6) monilia, (7) *S. thermanti*, and in every instance growth was observed.

No particular difference was noted in the appearance of growths obtained from threads soaked in extractive, or from unprepared threads similarly impregnated with bacteria, but evidence was obtained that spore-formation took place much earlier in the case of organisms treated with extractives than with those not so treated.

From all the above trials it would seem that whatever the nature of the action of extractives on bacteria may be, it is certainly not actually lethal. That under certain circumstances even strong antiseptics, such as mercuric chloride, retard development without lethal

¹ *J. Soc. Chem. Ind.*, 1910, 29, 334.

result has been shown both by Geppert and by Delépine. It would also seem probable that the employment of the extractives in the manner described does not reproduce the conditions existing in the living seed.

(2) *Experiments on stimulating properties of extractives.*—The foregoing experiments were concerned with the action on bacteria of the extractives in a concentrated condition; the effect of highly dilute solutions was now examined. Flasks containing equal quantities of Raulin's solution were seeded with a fresh culture of *Penicillium glaucum* (the size of a pin's head) and to each of the flasks was added a few drops of an extractive. The results observed were striking. In three days' time, when the blank containing Raulin's solution showed only slight growth of the moulds, there was abundant growth in the flasks containing the extractives, tomato-seed giving the best growth.

On the other hand it was observed several times during the work that whenever indigo-seeds were allowed to soak in water, and the soakings removed and replaced by fresh water, there was better germination than when the soakings were not removed. The brown liquid that is produced on soaking the seeds contains much tannin, and this seems to inhibit root-growth, possibly by impeding the activity of the seed bacteria.

As a general result of these tests it may therefore be tentatively concluded that both bacteria and extractives play their part in the economy of the seed and the growing plant. In a concentrated condition the extractives would seem to exercise an inhibitory effect on the bacteria, and possibly even a toxic effect on the seeds, when these are moistened and the extract allowed to remain unchanged in their vicinity. Upon dilution, however, these substances may serve as stimulants to the bacteria which in turn assist plant growth by breaking down the seed-proteins and possibly even by decomposing some of the toxic products in the extractives.

SUMMARY OF CONCLUSIONS.

(1) Every seed so far examined has been associated with specific bacteria, either within the seed (poppy), within the husk (rice), attached to the seed by the mucilage coat (*Cassia tora*) or residing on the testa (indigo-seed).

(2) No part of indigo-seed by itself is capable of fermentation.

(3) All poppy-seeds so far examined (field, garden and market) contain bacteria.

(4) So far poppy and barley are the only two types of seeds found to contain bacteria; others have them on the outside.

(5) Bacteria associated with the seed are not essential to its germination.

(6) They are helpful to growth of seedling.

(7) They break down seed-proteins, converting them into simpler substances assimilable by plants.

(8) This property is not restricted to particular seed-proteins but is extended to those of quite different types.

(9) Every seed so far examined has a specific extractive, removable by water or other suitable solvent and having a well-defined basic or glucosidic nature.

(10) Extracts of seeds are not always of an antiseptic nature but in very small doses act as stimulants to growth.

(11) Washed indigo-seeds germinate better than unwashed ones owing to presence of toxic substances in the latter.

APPENDIX.

NOTE ON THE PERMEABILITY OF VARIOUS SEEDS TO ANTISEPTICS.

In order to examine the bacteria which occur within the external coating of a seed, and which may be considered specific to the seed under examination, it is necessary to eliminate, by simple washing or by treating with antiseptic solution, bacteria which are merely incidentally present in the outside of the husk, or of the seed-coat. Special observations were therefore made to determine how far, if at all, the external membrane of the seed was permeable to the antiseptics used, and whether such antiseptics prejudicially affected the germinating power of the seeds.

For this purpose, 7 grams each of indigo-seeds and paddy-grains (carefully examined for soundness), were soaked in 50 c.c. of 2 per cent. solutions of mercuric chloride and of copper sulphate and in a saturated solution of boric acid, and left over-night. Next morning seeds from each solution were removed, washed with sterilised tap water and,

- (1) Plated on nutrient agar plates.
- (2) Sown in sterile flasks containing sterile sand.

The following results were obtained from the agar plates :—

SEEDS PLATED FROM :

| | 2 per cent. mercuric chloride | 2 per cent. copper sulphate | Saturated boric acid solution |
|-------------|---------------------------------------|---------------------------------------|--|
| Paddy | No bacterial growth. | No bacterial growth, no germination. | Bacterial growth on nearly every seed. |
| Indigo | No bacterial growth. Seed germinates. | No bacterial growth. Seed germinates. | Bacterial growth on nearly every seed. |

It is evident that boric acid in saturated solution does not inhibit the growth of all bacteria occurring incidentally in the external surface of the seeds.

As the mercuric chloride and copper sulphate under these conditions inhibited the external bacteria, but not the germinating

power of the seed, it was of interest to discover whether the antiseptic actually penetrated the seed coat.

Mercuric chloride within the endosperm was sought by potassium iodide, potassium ferrocyanide being used for the detection of copper. Neither mercury nor copper was found, even when the seeds were placed for some hours in the antiseptic solutions, after removal of the outer husk.

It thus appears that the external membrane of the endosperm itself is impermeable to the antiseptics, if the exposure is not too prolonged. It was found, however, on careful examination, that when de-husked seeds were placed in the antiseptic and then transferred to the precipitating reagent, red spots appeared on rice grains, where the pericarp had been injured during removal of the husk, while on indigo-seeds a minute red spot occurred at the micropyle, showing that the reagent had penetrated the micropyle where the endosperm is practically absent or very thin. In the case of indigo-seeds, penetration by the antiseptic could be observed right up to the spot where the embryonic cotyledons abutted on the endosperm.

This phenomenon revealed two possibilities either harmful or beneficial to the seed, according to conditions. In the first place the simple passage of the antiseptic through the micropyle will destroy all extraneous bacteria which might obtain access to the seed at this point, while the prolonged immersion in the antiseptic might destroy the 'specific' bacteria inside the seed, and affect germination adversely. The following experiments show this probably to be the case.

The effect of antiseptics on germination was more marked in the case of the seeds referred to on page 269 which were sown on sterile sand. In this case germination was inhibited in the case of all the seeds soaked in 2 per cent. mercuric chloride, in the case of boric acid germination took place, but the indigo-seeds were evidently more affected than the paddy-grains. Seeds treated with copper sulphate solution germinated fairly well.

A second sowing was made with seeds which had been immersed in the antiseptic solutions for five days. In this case only the paddy-grains which had been immersed in copper sulphate solution showed any sign of germination in six days. In all the other cases growth was arrested entirely.

Some of the treated seeds were placed on nutrient agar plates at the time of the second sowing, and it was found that bacterial growth took place round seeds treated with copper sulphate or boric acid, but none round those treated with mercuric chloride.

Further examination of the seeds which had been exposed to mercuric chloride solution for five days showed that no bacterial growth could be obtained from the crushed seed, i.e., the growth of the bacteria, if any, within the seed had been inhibited. Treatment of both seeds, indigo and paddy, with ammonium sulphide solution (which was found more suitable than potassium iodide for the detection of small quantities of mercury in seeds), showed that, after prolonged contact, the antiseptic had penetrated throughout the seeds, including the embryos. In the case of copper sulphate, similar tests showed that the internal tissues had not taken up any appreciable quantity of the antiseptic.

This was confirmed by immersing the seeds in a known quantity of the solutions and testing the supernatant liquid for copper or mercury. In both cases it was evident that the seed had absorbed water and that the supernatant liquid had become more concentrated. Actual gravimetric determinations were made of the amount of reagent present in the supernatant liquid, with the following results:—

| MERCURIC CHLORIDE 2 per cent. | WEIGHT OF SUBSTANCE DISSOLVED IN 5 C. C. SUPERNAT- ANT LIQUID |
|---|---|
| Blank | 0.0470 |
| Paddy | 0.0880 |
| Indigo-seeds | 0.0634 |
| COPPER SULPHATE 2 per cent. | |
| Blank | 0.0900 |
| Paddy | 0.1168 |
| Indigo-seeds | 0.6764 |
| BORIC ACID (Saturated solution) | |
| Blank | 0.2625 |
| Paddy | 0.2781 |
| Indigo-seeds | 0.4275 |

In all cases there is a tendency for the seed to be permeable to water rather than to the dissolved substance. Indigo-seed behaves much more markedly in this respect than paddy, except when the reagent used is mercuric chloride, which probably has a specific action on the tissues.

A comparative experiment was finally made, using a 0.25 per cent. solution of copper sulphate and wheat and barley seeds in addition to indigo and paddy. The results are shown in the accompanying photograph,¹ from which it is evident that absorption of water was greatest in the case of indigo-seeds and least in the case of paddy. This confirms the observation of Fowler and Sen² that paddy is in a degree, 'water-proof' as might be expected from its conditions of growth. The general results of the work also are in accordance with the observations of Adrian Brown,³ which indicated that dissolved copper sulphate does not penetrate to the interior of seeds, while mercuric chloride does.

Hence a fairly strong solution of this permeating antiseptic acting for a considerable length of time on the seed is liable, as we have shown, to destroy its germinating power. On the other hand, it is the most efficient agent for sterilising the outer surface of seeds. To obtain satisfactory results, therefore, a critical strength of solution and a definite duration of contact must be employed. In all cases we have used a 1:1000 concentration of mercuric chloride, and a contact of 10 to 15 minutes' duration.

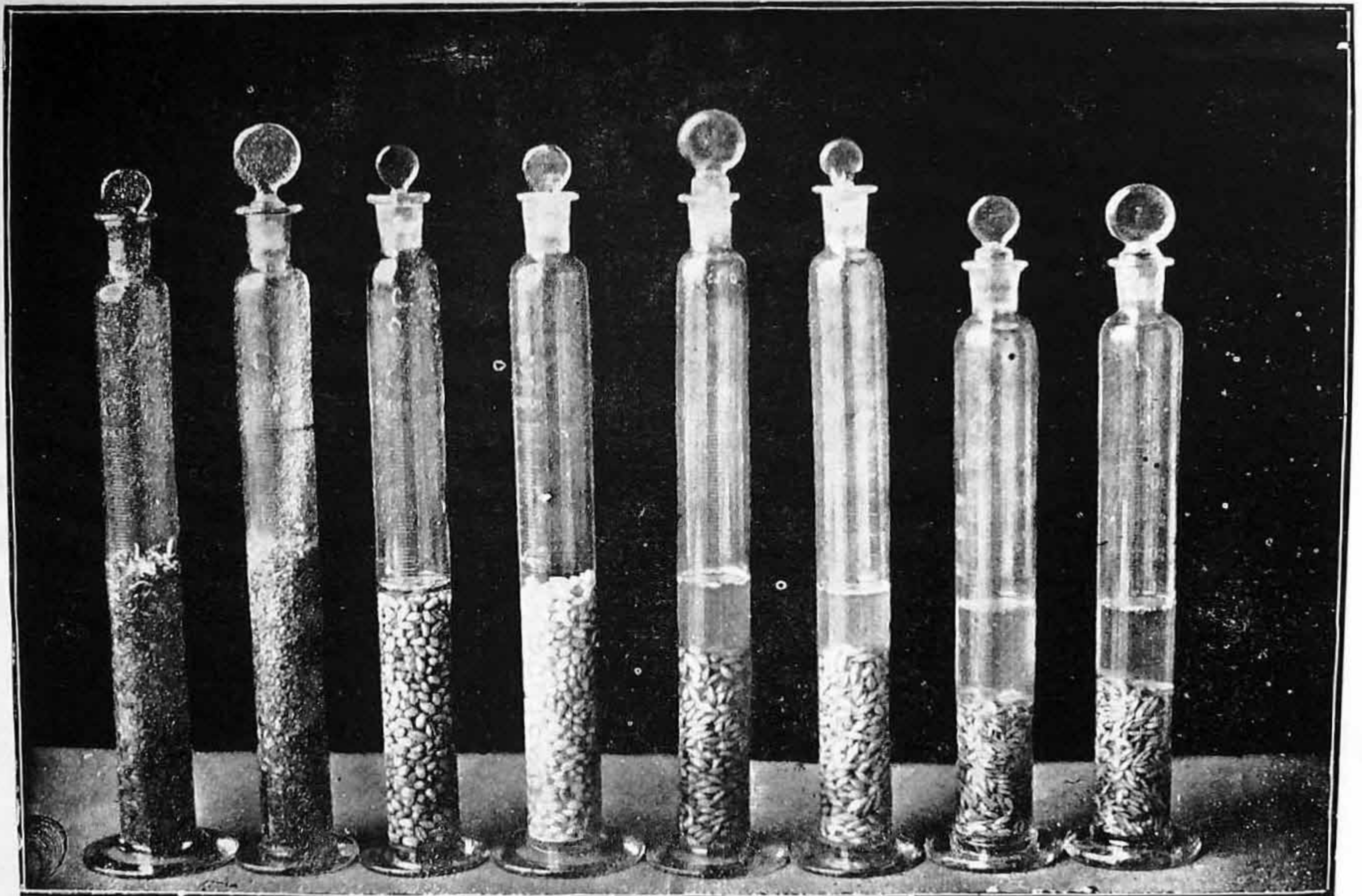
¹ See Plate 6.

² *This Journal*, 1921, 4, 144.

³ *Proc. Roy. Soc.* 1909 B, 81, 82.

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PLATE 6.



0.25%
CuSO₄ Water
Indigo-Seeds

0.25%
CuSO₄ Water
Wheat

0.25%
CuSO₄ Water
Barley

0.25%
CuSO₄ Water
Paddy

This photograph shows the comparative permeability of seed coats of different seeds. Indigo-seed, wheat, barley and paddy soaked in water and 0.25 per cent. copper sulphate solution.