EXPERIMENTAL BIOLOGY GROUP : SUMMARIES OF SCIENTIFIC PAPERS

The following are summaries of the proceedings of the 11th meeting of the Experimental Biology Group (EBG) held on 14 February 1964:

SEPARATION OF PARTICLES BY ELECTROPHORESIS THROUGH DIFFERENT CONCENTRATIONS OF AGAROSE

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It has been shown that the pore size of agar gel diminishes as the concentration is increased.[1] By using a potential gradient as the force to drive particles through a series of agarose gels of increasing concentration, it should be possible to separate a mixture into fractions of more or less homogeneous particle size. Agarose, the neutral galactose polymer of agar, is especially useful for electrophoresis since undesired electroendosmotic effects are virtually eliminated. The preparation of agarose by precipitation with polyethylene glycol has been described.[2]

Different concentrations of agarose were dissolved in borate buffer, pH 8·6[3]; and sieving layers, 0·5 cm. thick, were formed in a convenient apparatus with the highest concentration of agarose at the bottom of the column and 0·5% agarose separating each sieve. The mixture to be separated was diluted with agarose at 38°C to give a 0·25% solution, which was allowed to gel at the top of the column and covered with 0·5% agarose. The rest of the apparatus, filled with borate buffer, pH 8·6, connected the agarose column to electrode vessels containing silver- silver chloride-electrodes covered with saturated NaCl. Electrophoresis was continued for 25 - 26 hours with a current of 15 mA (10 volts/cm.).

By means of this technique, the particles in a mixture of pantropic Rift Valley fever virus (PRVF, 70 mμ), and enteric cytopathogenic bovine orphan virus, ECBO 1 (30 mμ), were separated by using a series of agarose gels varying in concentration between 0·5 and 10%. These two viruses have almost identical electrophoretic mobilities in sucrose gradients at pH 8·6[4, 5] but PRVF was held back at the 0·5% agarose level and ECBO 1 concentrated at the 8% agarose level.

In another experiment, MEF_1 poliovirus concentrated at the 3% agarose level when allowed to migrate through a series of agarose gels ranging in concentration from 1 to 6%. MEF_1 virus has approximately half the mobility of ECBO 1 in zone electrophoresis at pH 8·6.[3] When ECBO 1, which has the same particle size as MEF_1, was tested at the same potential gradient over the same range of gel concentrations, it migrated to the 6% agarose level. Therefore, although this is a useful method for separating particles of different size, the electrophoretic mobilities of all constituents of a mixture need to be taken into consideration when using this technique.

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THE OCCURRENCE OF PHOSPHATE-DISSOLVING BACTERIA IN SOIL AND ON PLANT ROOTS

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Six hundred and seventy-three bacterial isolates have been isolated from soil and the roots of wheat (*Triticum sativum*) and lupin (*Lupinus angustifolius* var. S.E. Blue) using the method of Louw and Webley.[2] The plants were grown in Mitscherlich pots[4] using a mixture of one part of sand to two parts of soil by volume. All the pots received the same basal dressing of macronutrients,[2] except that nitrogen was omitted in the case of the legume. The seed of the latter was inoculated immediately before planting with inoculant. The following phosphate treatments were applied: No phosphate, monocalcium phosphate, and tricalcium phosphate. The phosphate was added as 1·2 G. P_2O_5 per pot. A few pots from each phosphate treatment were kept as uncropped controls.

The bacterial floras in the uncropped soil and on the roots of the plants were counted by the plate-dilution method at different stages of plant growth. The isolates were isolated from the pots treated with monocalcium phosphate and tricalcium phosphate when the plants were 14 weeks old. Their ability to dissolve insoluble calcium carbonate, dicalcium phosphate, tricalcium phosphate and hydroxyapatite was studied under pure culture conditions by means of the techniques developed by Louw and Webley.[1, 3]

The plate count of the general bacterial flora on the roots of both plants increased markedly during the growing period of the plants, lupin roots showing the greatest increase. No increase was observed in the uncropped soil. The proportion of calcium carbonate and phosphate-dissolving bacteria was much greater on the plant roots than in the soil away from the roots. This particularly applied to lupin roots. The groups of bacteria mainly responsible for dissolving the phosphates were Gram negative short rods and pleomorphic types. Although the proportion of short rods was greater on plant roots, especially on lupin roots, the pleomorphic types seemed to be better dissolvers of insoluble phosphate.

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THE PURIFICATION OF THE BITTER PRINCIPLE OF OLIVES

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The bitter principle (oleuropein) present in olives was isolated and partly purified by Cruess and Alsberg,[1] who obtained a creamy-white amorphous substance which yielded glucose on acid hydrolysis. Though caffeic acid was claimed to have been obtained by treatment with alkali, the structure of the compound was not established. In the present study oleuropein was prepared by means of the method of Cruess and Alsberg. When examined by paper chromatography the presence of impurities was revealed. Since the compound was amorphous and could not be purified further by crystallization, a chromatographic method was developed for obtaining chemically pure oleuropein.

Green Mission olives (2 kg.) were blanched by dipping in boiling water for 5 minutes before removing the seeds by hand. The flesh was macerated in a Waring blender after adding approximately 2 l. of acetone. The macerate was transferred to a Buchner funnel and the liquid drawn off. The material on the filter was washed with several portions of acetone, and the combined acetone extracts (ca. 5 l.) were evaporated under reduced pressure to remove the excess of acetone. The aqueous concentrate (ca. 600 ml.) was subjected to liquid-liquid extraction with chloroform to remove the oil and some of the pigments. The pH of the aqueous phase was then adjusted to 8·5 by carefully adding NaOH solution (10%) before liquid-liquid extraction with ethyl acetate for 24 hours. The ethyl acetate was evaporated under reduced pressure to yield a yellow sticky residue (46·7 G.) which did not puff readily when vacuum and heat were applied.

The isolated material was further purified by passing through a magnesol column as described by Ice and Wender,[2] except that the magnesol was mixed with an equal volume of dicalite filter aid to improve the percolation rate. Columns of 6 cm. in diameter and 8 cm. high were found suitable for this purpose. The crude material (2 G.) was dissolved in acetone (5 ml.) and applied on the column. It was washed into the column with additional acetone. The column was developed with ethyl acetate saturated with water. Oleuropein was preceded by a narrow band of an impurity visible under ultraviolet light. After the emergence of the last traces of this impurity from the column the oleuropein was collected in several fractions. Each fraction was examined separately for purity by paper chromatography. The pure fractions were combined and the solvent evaporated under reduced pressure to yield a white amorphous powder. At least 0·8 G. of purified material could be recovered with each preparation as well as an additional 0·6 G. of material containing small quantities of impurities.

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THE ANTAGONISTIC AND STIMULATORY EFFECTS OF SOIL MICRO-ORGANISMS ON CLOVER RHIZOBIA

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The antagonistic and stimulatory effects of 246 bacterial and actinomycete isolates obtained from the Outeniqua Experimental Farm at George, were tested against five isolates of *Rhizobium trifolii*. The isolates were obtained from clovers which were inoculated at planting with a commercial inoculant, as well as from self-sown uninoculated plants. However, the one rhizobial isolate was isolated from a commercial inoculant ('AB' culture for alfalfa and clover produced by the Nitragin Company, Inc., Milwaukee, Wisconsin, USA). The non-rhizobial isolates were obtained from the root surface (rhizoplane) and rhizosphere of the clovers, as well as from the adjacent soil, using the method of Louw and Webley.[3] However, the medium of El-Nakeeb and Lechevalier[2] was used for these isolations. This medium allowed favourable proportions of bacteria and actinomycetes to appear on the dilution plates.

The antagonistic and stimulatory effects of the bacteria and actinomycetes on the rhizobia were determined by one of Waksman's[4] methods. This was done by streaking the culture to be tested on the surface of an agar plate previously seeded with the rhizobial isolate. A suitable medium, recommended by Abdel-Ghaffar and Allen,[1] allowing good growth of both the rhizobia and potential antagonist (or stimulant), was used for this purpose.

The sensitivity of the rhizobia varied considerably. Twenty-four per cent of the isolates inhibited one or more of the rhizobia to a greater or lesser extent. Only 8·1% of the isolates (14 bacteria and 6 actinomycetes) inhibited all the rhizobial isolates. The rhizobia, with one exception, were inhibited by a greater percentage of isolates from the soil adjacent to the roots than by the isolates from the root surface and rhizosphere. It appears as if the root surface population was less antagonistic towards the rhizobia. The rhizobial isolate from commercial inoculant was, however, inhibited by a higher percentage of root surface isolates than the other 4 rhizobia.

Although 16·2% of the isolates showed stimulatory effects towards the clover rhizobia, most appeared to be very weak. Two of the rhizobial isolates were never stimulated. Eight of the stimulants stimulated some rhizobia but inhibited others, indicating the complexity of the problem.

Further investigations along these lines are in progress.

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RESOLUTION OF A PLANT VIRUS COMPLEX BY ZONE ELECTROPHORESIS

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It is often difficult and time-consuming to identify and separate the different viruses associated in a disease complex by using differential host reactions. These reactions vary according to the relative amounts of the virus components in the mixture and are also greatly influenced by the particular virus strains used and by environmental conditions.

Potato plants showing symptoms of the calico disease were used as the source material. Inoculation tests on standard host plants gave erratic results, different symptoms being obtained in successive experiments. *Nicotiana tabacum* plants, showing symptoms of mosaic, yellowing, vein-clearing, ringspots and necrosis, were used to multiply the mixture of viruses. Leaves (20 G.) were homogenized in the presence of citrate buffer and chloroform, and the viruses concentrated by differential ultracentrifugation. Zone electrophoresis in borate buffer, pH 8·6, was performed as described previously.[1] After 24 hours of electrophoresis, 3 strongly opalescent bands, situated 2, 11 and 16 cm. from the origin, were visible in the column. Inoculation tests with the material in the bottom band (1 cm. wide) revealed the presence of potato virus X (specific reactions in *Nicotiana, Chenopodium, Datura* and *Gomphrena*). The middle band (4 cm. wide) consisted of an unresolved mixture of plant proteins (fraction 1 protein[2, 3]) and alfalfa mosaic virus as shown by serological tests and specific reactions in

Nicotiana, Chenopodium, Datura, Gomphrena, Phaseolus and *Vigna*. The top band (2 cm. wide) was shown to consist of tobacco mosaic virus by characteristic reactions in *Nicotiana, Chenopodium, Datura* and *Lycopersicon*. The three viruses were then multiplied separately, again purified, and shown in successive electrophoresis experiments to be free of the original contaminating viruses. The very low and high mobilities of potato virus X and tobacco mosaic virus, respectively, recorded in this work, agree with previous observations on these viruses.[4, 5] Both viruses are common contaminants in virus-infected plants of the *Solaneaceae* family, and since many viruses have an electrophoretic mobility intermediate between that of potato virus X and tobacco mosaic virus, zone electrophoresis is a useful method of separating viruses from such a mixture.

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IN VITRO FORMATION OF BILE PIGMENTS

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Nakajima and his coworkers[1] have carried out detailed studies of an enzyme, present in mammalian liver, which acts upon a haemoglobin-haptoglobin complex but not upon free haemoglobin. The enzyme was found to be absent from recognized reticuloendothelial tissues such as spleen and bone marrow. Enzyme preparations were assayed by the breakdown of pyridine haemochromogen, a completely artificial substrate.

In many trials we have failed to substantiate the work of the Japanese workers. However, some of our experimental results appear worthy of comment at this stage.

1. We have succeeded in separating free haemoglobin from haemoglobin-haptoglobin complex on a column of dextran gel (Sephadex G 75).

2. The products of the coupled oxidation of ascorbic acid and haemoglobin have been shown to include small molecules with visible light absorption.

3. Myoglobin has been prepared from human psoas muscle and studied as a source of bile pigments *in vitro*. In earlier *in vitro* studies[2] myoglobin gave the highest yield of biliverdin. Crude muscle extracts have been brought to 40% saturation with ammonium sulphate to precipitate contractile proteins, leaving myoglobin, haemoglobin and other colourless proteins in solution. The solution was brought to 70% saturation with $(NH_4)_2SO_4$ and the precipitate, thus produced, dissolved in 0·01 M phosphate buffer, pH 7·0, dialysed against 0·05 M NaCl, and saturated with CO in the dark. The solution was then separated from one another: passed through a dextran gel column (Sephadex G 75) when 3 main zones HbCO running first, then a zone of colourless proteins which, together with the 0 - 40% $(NH_4)_2SO_4$ fraction, we are exploring for enzymatic activity in degradation of myoglobin, and finally MbCO (actually mainly metmyoglobin: myoglobin has much greater relative affinity for oxygen and is readily autoxidized in air).

Myoglobin is a single polypeptide chain with one haem only attached and it is hoped this haemoprotein will give a less complex mixture of breakdown intermediates than haemoglobin does.

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