Separation of Pigment Cells of Cacao

THE purple pigment in the cotyledons of unfermented Forastero cacao is distributed in separate cells which are readily seen in cut sections. These separate cells can also be seen in sections from the Pale Criollo cacaos, though they are not coloured purple.

During the fermentation process, the contents of these cells dissolve, and diffuse throughout the cotyledons and into the testa of the bean. Some constituents of the substance of the cells undergo oxidative and other changes, and some of the material is lost into the 'sweatings' which drain from the fermenting beans.

When the freshly harvested beans are dried rapidly, there is no diffusion of the substance of the pigment cells, which can be seen apparently intact in sections of the cotyledons of beans dried in this way. These dried pigment cells have a higher density than the other cells of the tissue, and a separation of them has been effected as follows.

The dried beans are freed from testa and radicle, the cotyledons crushed to a suitable degree—a household coffee mill with the finest setting has been found suitable—and the ground mass suspended in petrol. The fat is dissolved, and of the mixture of insoluble suspended matter, the protein and starch are almost completely removed by sedimentation and decantation. The heavier mixture of free pigment cells and coarse tissue, mainly cellulose, is then sifted through a 240-mesh sieve suspended in petrol. The coarse, less dense, tissue is held on the sieve and is rejected; the free pigment cells pass through the sieve. Further careful sedimentation and decantation, preferably from a shallow dish, give a final product of separated pigment cells with about 5 per cent of adherent extraneous matter which is not removed by further washing with petrol.

The whole amount of the pigment cells in the cotyledons is not recovered in this way; some cells are too firmly attached to the lighter tissue and do not pass through the fine sieve. When carefully dried beans are used, about one-half of the total can be recovered as separated cells.

These dried pigment cells are dark purple, almost black, in the case of West African Amelonado cacao, somewhat lighter and redder purple in the case of most West Indian and South American cacaos, and a light puce colour in the case of East Indian and other 'white' Criollo cacaos.

The pigment cells are rather larger than the other cells of the dried cotyledons, the maximum lengths being about 50 μ and 35–40 μ respectively.

It has been found that the whole of the purin substances, and probably the whole of the polyphenolic substances, in the cotyledons are concentrated in these pigment cells. They dissolve almost completely in water, giving a solution of *p*H 6·5–6·7, the same as that recorded for an aqueous extract of the fresh whole cotyledons[1].

The proportion of pigment cells in the total tissue of the cotyledons has been determined from a comparison of theobromine contents, and found to vary from 11 to 13 per cent by weight in the air-dry cotyledons. This compares well with the figure of about 10 per cent by count quoted by Knapp[2].

This separation of the polyphenolic and purin constituents from the other components of the bean provides a new and better approach to the study of these constituents, particularly the polyphenols. Solutions can be prepared which are free from extractives from other components of the bean, and are less labile than solutions from extracts of the whole cotyledon.

This is being applied, and analyses obtained giving comparisons between cacaos from different sources.

This work was carried out in the laboratories of Messrs. Rowntree and Co., Ltd., York, and is communicated by permission of the directors.

H. B. BROWN

Laboratories,
Messrs. Rowntree and Co., Ltd.,
York.

[1] Whymper, *Chimie et Industrie*, **30**, 507 (1933).

[2] Knapp, "Cacao Fermentation", 58 (John Bale, Sons and Curnow, 1937).

Intra-arterial Injection of Methylene Blue for Staining Nerve Endings in Striated Muscles

THE difficulty of staining nerve endings with methylene blue is well known. I have found that intra-arterial injection of methylene blue in an anæsthetized animal consistently stained the nerve endings of striated muscles with greater uniformity than was possible by intravenous injection or the local infiltration of methylene blue. The method used is as follows.

Under ether anæsthesia, a convenient artery supplying the muscles the nerve endings of which are to be stained (for example, the femoral artery for staining the endings in the muscles of the hind-legs) is exposed and dissected free. The staining fluid consists of 0·25 per cent zinc-free methylene blue in a solution of 0·8 per cent sodium chloride, 0·2 per cent glucose, 0·02 per cent magnesium bromide, buffered to a *p*H of 6 with phosphate buffer[1], with 1 mgm. of hyaluronidase[2] added to 20 ml. of the solution immediately before use. The fluid is warmed to the body temperature and slowly injected into the exposed artery by means of a syringe and a needle of suitable size until the skin over the injected part turns deep blue in colour. The amount required for this depends, of course, on the dimension of the area to be injected, hence preliminary trials are usually necessary to find the right dose. In adult rats and chickens, injections of about 4 and 40 ml. respectively into the femoral artery stained the nerve endings in the hind-legs. The tendency at the beginning is to inject too little, but an overdose results in the undesirable staining of the blood cell and muscle nuclei or in the death of the animal.

After injection, arterial bleeding from the withdrawal of the needle is stopped by clamping the artery with artery forceps, and the animal is kept alive under anæsthesia for 10 min. before killing it by bleeding. The muscles are removed, the large muscles are sliced to about 3 mm. in thickness, and they are suspended on hooks in an oxygen chamber kept damp by a piece of wet cotton-wool. After about half an hour the 'blued' muscles are examined under the microscope and pieces that contain stained nerves and endings are selected for fixation overnight in chilled 8 per cent ammonium molybdate solution. Serial frozen sections are cut, rapidly dehydrated in chilled *n*-butyl alcohol, cleared in xylene and mounted in canada balsam.

This method had been successfully used in staining the nerve endings of the leg muscles of rat, rabbit,

©1954 Nature Publishing Group