methyl iodide or methyl bromide, the free carboxyl groups are esterified, but peptide methylation only occurs slowly, if at all. Neither the peptide methylation nor the esterification of the free carboxyl groups of wool is prevented by treating it before methylation with dilute alkali, nitrous acid or formaldehyde. On the other hand, removal of amide groups by acid hydrolysis leads to increased esterification.

2. When wool is acetylated with acetic anhydride, the subsequent methylation of free carboxyl groups by either methyl sulphate or methyl halides is restricted. Treatment of wool and silk fibroin with acetic anhydride in methanol leads to *N*- and *O*-acetylation, simultaneously with the methylation of free carboxyl groups.

Thanks are due to the Council of the Wool Industries Research Association for permission to publish this paper and to Mr A. B. Gibson for assistance in some of the experimental work. The authors are also indebted to Dr M. P. Balfe of the British Leather Manufacturers' Research Association for the acetone-dehydrated collagen used in the experiments.

REFERENCES

Ashley, J. N. & Harington, C. R. (1929). *Biochem. J.* **23**, 1178.
Baernstein, H. D. (1932). *J. biol. Chem.* **97**, 663.
—— (1936). *J. biol. Chem.* **115**, 25.
Bailey, K. (1937). *Biochem. J.* **31**, 1406.
Barritt, J. & King, A. T. (1926). *J. Text. Inst. Manchr,* **17**, 386.
—— Bowen, H. H., Goodall, F. L. & Whitehead, A. (1938). *Analyst,* **63**, 782.
Bergmann, M. & Koster, H. (1926). *Hoppe-Seyl. Z.* **159**, 179.
—— —— (1927). *Hoppe-Seyl. Z.* **167**, 91.
Blackburn, S., Carter, E. G. H. & Phillips, H. (1941). *Biochem. J.* **35**, 627.
—— Middlebrook, W. R. & Phillips, H. (1942). *Nature, Lond.*, **150**, 57.
Boon, W. R. & Robson, W. (1935). *Biochem. J.* **29**, 2684.
Bowes, J. H. & Pleass, W. B. (1939*a*). *J. int. Soc. Leath. Chem.* **23**, 453.
—— —— (1939*b*). *J. int. Soc. Leath. Chem.* **23**, 365.
Dakin, H. D. (1920). *J. biol. Chem.* **44**, 499.
Dauphinee, J. A. & Hunter, A. (1930). *Biochem. J.* **24**, 1128.
Dirr, K. & Felix, K. (1932). *Hoppe-Seyl. Z.* **205**, 83.
Elsworth, F. F. & Phillips, H. (1938). *Biochem. J.* **32**, 837.
Felix, K. & Dirr, K. (1928). *Hoppe-Seyl. Z.* **176**, 29.
Gleysteen, L. F. & Harris, M. (1941). *Text. Res.* **11**, 117.
Harris, M. & Rutherford, H. A. (1939). *Bur. Stand. J. Res., Wash.*, **22**, 535.
Horn, M. J., Jones, D. B. & Ringel, S. J. (1941). *J. biol. Chem.* **138**, 141.
—— —— —— (1942*a*). *J. biol. Chem.* **144**, 87.
—— —— —— (1942*b*). *J. biol. Chem.* **144**, 93.
Karrer, P. & Ehrenstein, M. (1926). *Helv. chim. Acta,* **9**, 323.
Lugg, J. W. H. (1938). *Biochem. J.* **32**, 2123.
Mease, R. T. (1934). *Bur. Stand. J. Res., Wash.*, **13**, 617.
Middlebrook, W. R. & Phillips, H. (1942). *Biochem. J.* **36**, 294.
Mizell, L. R. & Harris, M. (1943). *Bur. Stand. J. Res., Wash.*, **30**, 47.
Nicolet, B. H. & Shinn, L. A. (1941). *J. biol. Chem.* **139**, 687.
—— Shinn, L. A. & Saidel, L. J. (1942). *J. biol. Chem.* **142**, 609.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry,* **2**, 835. London: Baillière, Tindall & Cox.
Pratt, A. E. (1926). *J. biol. Chem.* **67**, 351.
Rutherford, H. A., Harris, M. & Smith, A. L. (1937). *Bur. Stand. J. Res., Wash.*, **19**, 467.
—— Patterson, W. I. & Harris, M. (1940). *Amer. Dyest. Rep.* **29**, 583.
Shinn, L. A. & Nicolet, B. H. (1941). *J. biol. Chem.* **138**, 91.
Speakman, J. B. & Townend, F. (1937). *Nature, Lond.*, **139**, 411.
Steinhardt, J. & Harris, M. (1940). *Bur. Stand. J. Res., Wash.*, **24**, 335.
Synge, R. L. M. (1939). *Biochem. J.* **33**, 1913.
Theis, E. R. & Jacoby, T. F. (1942). *J. biol. Chem.* **146**, 163.
Vickery, H. B. (1940). *J. biol. Chem.* **132**, 325.
—— (1941). *Ann. N.Y. Acad. Sci.* **41**, 87.
—— & Block, R. J. (1931). *J. biol. Chem.* **93**, 105.
Wolfrom, M. L., Konigsberg, M. & Soltzberg, S. (1936). *J. Amer. chem. Soc.* **58**, 490.

Diplococcin, an Anti-bacterial Protein Elaborated by Certain Milk Streptococci

By A. E. OXFORD, *The Biochemical Laboratory, London School of Hygiene and Tropical Medicine, University of London*

(*Received* 6 *March* 1944)

Whitehead (1933) first showed that milk sometimes contains lactic acid-producing streptococci which inhibit the action of the very similar organisms used industrially as cheese starters. He further proved that the inhibition was due to a protein-like substance produced by the inhibiting organisms. It was not however made clear whether this substance was a constituent of the medium altered by bacterial

action, an excreted metabolic product, or a part of the bacterial cell which had been dissolved by the medium. The problem has recently been investigated on the practical side by Meanwell (1943), and the present author has been able to examine two strains of inhibitory cocci isolated by him. The anti-bacterial effect is in fact due to a protein-like substance of small molecular weight which is normally a constituent of the bacterial cell. It is proposed to call this substance '*Diplococcin*' since the bacteria which produce it exhibit a diplococcal arrangement in stained preparations. The present paper is mainly concerned with the isolation of diplococcin from the bacterial cell.

EXPERIMENTAL

Cultures. Two pure cultures of anti-bacterial streptococci, labelled F 7 and F 8, were received from Mr L. J. Meanwell. These were used for the isolation of diplococcin. The test-organism used was obtained from a cheese starter labelled 303. It was immediately plated out and an isolate made from a single colony. This culture, called 303 S, was used throughout in the routine testing of bacteriostatic material isolated from F 7 and F 8 cultures.

Method of assay for anti-bacterial potency

Stock cultures of 303 S, viz. 2-day growths on heart agar slopes, were kept in the cold room and used after a week. The cultures used in the assay were always 1-day cultures in 2% glucose-broth, derived from a similar culture. A 0·05 or 0·1% solution of the material to be assayed, in 0·4% acetic acid, was sterilized at 100° for 30 min. When a liquid was to be assayed it was first brought to pH 3–4 before sterilization. The reason for this treatment is that diplococcin is not stable at 100° at a pH above 4 (see below).

Two mixtures containing 0·5 and 0·25 ml. of the solution and 4·5 and 4·75 ml. of glucose-broth, respectively, were made aseptically, and further dilutions were made from them, each tube to contain 5 ml., of which at least 4·75 ml. were glucose-broth. Usually a series of eight dilutions was employed to cover the range 10,000–1,000,000. The controls (*A* and *B*) were made correspondingly, the former containing 0·5 ml. and the latter 0·25 ml. of sterile 0·4% acetic acid respectively. All ten tubes were inoculated with one loopful (0·004 ml., containing about 20,000 viable bacteria) of a culture of 303 S. This was made by transferring one loopful of a well-shaken 24-hr. culture in glucose-broth, which had remained 45 min. at room temperature after being shaken, into 4 ml. of sterile glucose-broth and mixing well. After thorough shaking, the inoculated tubes were incubated at 24° and read after 18 and 24 hr. After 18 hr. control *A* should show a faint turbidity and control *B* a good turbidity, whilst after 24 hr. *A* should show a good turbidity and *B* maximum growth with deposit.

One unit of anti-bacterial substance was defined as the least amount which, dissolved in 5 ml. of glucose-broth, will prevent visible growth under the above conditions in 18 hr. or, what was almost always the same thing, prevent maximum growth from being attained in 24 hr. In the sequel, the anti-bacterial potency is expressed throughout as units/mg. in the case of solids, and units/ml. in the case of liquids. Thus, the sterilized supernatant obtained from the growth of organisms F 7 or F 8 for one day in glucose-broth usually contained 28 units/ml.

Preparation of an active extract from bacterial cells separated from an inactive medium

Organisms F 7 and F 8 grew poorly in heart broth containing no added carbohydrate, the pH remaining near neutrality. The metabolism solution so obtained was devoid of activity. Whitehead obtained similar results with caseinogen broth, but made no attempt to isolate the anti-bacterial substance from the bacterial cells.

Organism F 7 was grown for 6 days in 3 l. of heart broth; the bacterial cells were spun down and the clear supernatant decanted. The deposit was washed once with distilled water (200 ml.), then dried *in vacuo* at room temperature over H_2SO_4 to yield 0·10 g. of dried cells which, after grinding in a glass mortar, were refluxed for several hours with 20 ml. of 0·4% acetic acid (cf. Freeman, 1942). The cooled extract was centrifuged at high speed until almost clear, decanted, heat-sterilized and assayed: *found*, 10 units/ml.

Large-scale production of the anti-bacterial substance from the cells

Since it appeared probable that the anti-bacterial agent could most conveniently be obtained from the cells, it was necessary to find a medium which would allow good growth of organisms F 7 and F 8, but which would not yield any organic precipitate upon acidification, as does undiluted heart broth, to contaminate the bacterial cells. The heavily buffered medium C of Birkinshaw, Charles & Clutterbuck (1931), to which 2·5–5% of heart broth had been added, was found to be admirably suited for this purpose. Sucrose was chosen as carbohydrate source since it could be autoclaved with phosphates at pH 7–8 without appreciable darkening taking place.

The medium had the following composition: $NaNH_4HPO_4$, $4H_2O$, 490 g.; KH_2PO_4, 870 g.; $MgSO_4$, $7H_2O$, 50 g.; KCl, 50 g.; $FeSO_4$, $7H_2O$, 1 g.; sucrose, 3 kg.; heart broth, 2·5 or 5 l.; distilled water, about 90 l. The pH was adjusted to 7·8 by the cautious addition of concentrated KOH solution and the volume made up to 100 l. with distilled water. The medium was distributed in 1·5 l. lots in 2 l. conical flasks which were plugged and sterilized by autoclaving. A number of 5 ml. tubes of the above medium was also prepared, inoculated with organism F 7 or F 8, and used after a day's incubation for sowing the flasks, one tube to each flask. The temperature of incubation was 24°.

The following points have been noted: it is not necessary to shake the flasks except immediately after sowing; it is not advantageous to extend the period of incubation beyond 11 days, since further incubation gives no increase in the amount of anti-bacterial substance in the cells, and increases

the risk of mould infection; when the growth is harvested the relatively clear top three-quarters of the medium may be decanted and discarded.

The cells were collected by the use of a De Laval centrifuge. They were washed on the centrifuge by distilled water (500 ml.) and the head of the centrifuge was inverted and allowed to drain before dismantling. All parts which appeared to have bacteria on them were immediately dried *in vacuo* at room temperature over H_2SO_4. After 3 days the flakes and scales of dried bacteria were detached without difficulty. It is not necessary to kill the bacteria by heat or otherwise, nor to de-fat them by use of an organic solvent. The dried bacteria were worked up in 5 g. lots after being finely powdered in a mortar. Each lot was first refluxed with 500 ml. of distilled water containing 10 drops of glacial acetic acid for 75 min. (oil-bath at 140°), in order to remove adhering constituents of the medium. After being cooled, the suspension was spun down to yield a virtually inactive supernatant at pH 4. The solid residue was resuspended in 100 ml. of distilled water and re-centrifuged. The deposit was refluxed for 5 hr. with a solution of glacial acetic acid (2·1 ml.) in distilled water (500 ml.). After standing overnight the mixture was spun down in 100 ml. lots at high speed for several hours, and the almost clear supernatant carefully decanted. In general this contained 100 units of activity/ml. A second similar extract of the cells had less than one-fourth of the activity of the first extract.

Isolation of an active picrate. The active extract (500 ml.) was treated with Esbach's reagent (150 ml.), with shaking. After some hours the yellow flocculent precipitate was collected on a small Buchner funnel, washed once with a little water, dried *in vacuo* (weight, about 0·3 g.) and assayed: *found*, usually 200 units/mg. Picric acid itself has no activity at a concentration of 1 part in 5000 of glucose-broth. Tannic acid also precipitates the anti-bacterial substance but no satisfactory method for recovery has yet been found.

Table 1 summarizes the large-scale experiments so far carried out.

Table 1. *Production of anti-bacterial substance by organisms* F 7 *and* F 8

Exp.	Organism and vol. of medium (l.)	Incubation (days)	Yield of dried cells (g.)	Picrate from 5 g. of dried cells (g.)	Activity of picrate (units/mg.)
A	F 7, 94·5	10–11	23·5	0·35–0·39	100–200
B	F 7, 43·5	14–20	7·6	0·33	200
C	F 8, 43·5	18–24	8·8	0·28	200
D	F 7, 45	11–13	3·7	0·16 (from 3·7 g.)	200

All the media contained 5% heart broth except D, which contained 2·5%.

Decomposition of the picrate to yield an active protein-like substance. The picrate (0·7 g. very finely powdered) was suspended in absolute ethanol (1050 ml.), and 0·4% acetic acid (70 ml.) added with shaking. The mixture was kept for 3 days in the cold room, with frequent shaking; any visible lumps were broken up from time to time with a glass rod. The deposit was collected by centrifuging and washed on the centrifuge with absolute ethanol until the washings were colourless. The cream-coloured residue (crude diplococcin) weighed 0·4 g. when dry and contained 100 units/mg. This showed that no marked loss of activity had occurred. The residual colour could not wholly be removed by a second ethanol-acetic acid treatment, but there was no appreciable further loss in weight and the activity remained unimpaired. It may be concluded that diplococcin is insoluble in ethanol and that it is not appreciably inactivated by ethanol when it is out of solution at the beginning.

Properties of crude diplococcin. Crude diplococcin after two ethanol-acetic acid treatments had: N, 13·9%; S, nil (Weiler, Oxford). It gave almost all the usual protein tests with the exception of Heller's nitric acid and the $HgSO_4$-H_2SO_4 precipitation tests and hence is probably less complex than a meta-protein; it is laevo-rotatory, is precipitated by half saturation with ammonium sulphate, gives no Molisch reaction, and diffuses slowly through a collodion membrane. It thus appears to be a protein-like material of relatively small molecular size and may possibly be a proteose. It contains no sulphur or phosphorus and gives reactions for arginine, tyrosine and tryptophan.

Fractionation of crude diplococcin. Crude diplococcin can be freed from the remaining undecomposed picrate by treatment with cold distilled water. The almost colourless filtrate so obtained, after removal of coloured insoluble material, yields precipitates with increasing amounts of ethanol, without serious loss of activity if the experiment is conducted at 4°. There is, however, no marked concentration of activity in any one fraction.

Crude diplococcin (100 mg.) was triturated with distilled water (25 ml.) for 30 min. and filtered. The filtrate, cooled to 4°, was treated with ice-cold ethanol (100 ml.) and left in the cold room for 2 hr. The precipitate, collected by centrifuging, weighed 40 mg. and contained 100 units of activity/ml. *Found* (Weiler, Oxford) on this fraction dried at 60° *in vacuo*: C, 50·5; H, 7·3; N, 13·1%; ash, nil. The aqueous-ethanol supernatant from the first precipitate was treated with a further quantity of ice-cold ethanol (550 ml.) and left in the cold room overnight, when a further 0·8 mg. of about the same activity was precipitated. The final clear supernatant was evaporated to 30 ml. *in vacuo* below 40° to yield a further 9 mg. of insoluble material which also contained about 100 units of activity/mg. The liquid residue yielded only 6 mg. of almost inactive material when evaporated to dryness at room temperature over H_2SO_4.

Considerable loss of activity occurred when a similar ethanol fractionation was carried out at room temperature in the first stage.

Extraction of diplococcin from the bacterial cells by cold 0·4% acetic acid

A possibility exists that extensive degradation of the true anti-bacterial substance of the cells may occur, without loss of activity, during the process of extraction with boiling 0·4% acetic acid. The following experiment definitely shows that this is improbable.

The cells from an experiment similar to D of Table 1, but with an 8-day period of incubation, were collected, washed with distilled water (500 ml.) and resuspended in 0·4% acetic acid (1550 ml.) for 7 days at 24°. After spinning, the clear supernatant (containing 10 units/ml.) was evaporated to one-thirtieth of its volume *in vacuo* below 55°. The residual liquid, which was at pH 4, contained 100 units/ml., did not give the Heller nitric acid reaction for proteins, and appeared in all essential respects to be identical with the previously described extract obtained at 100°. Similar experiments showed that the washed cells would part with a detectable amount of their anti-bacterial substance to a phosphate buffer at pH 4 but not to distilled water at 24°.

The anti-bacterial substance in metabolism solutions of organisms F 7 *and* F 8

The above results suggested that a part of the anti-bacterial activity in metabolism solutions had been derived from the cells merely by a process of chemical extraction by a dilute solution of lactic acid buffered at pH 4–5; in other words, that the active substance in the metabolism solution is the same as that extracted from the cells by dilute acetic acid. It is significant that anti-bacterial activity is strongly in evidence only in acidic metabolism solutions.

A portion of the clear supernatant from experiment D of Table 1 (500 ml. containing 40 units/ml.) was treated with Esbach's reagent (140 ml.) and after 24 hr. the liquid was agitated slightly to separate the flocculent picrate from some massive yellow crystals, decanted and centrifuged. The deposit was washed once with water (25 ml.), dried (weight, 10 mg.) and assayed: *found*, 300 units/mg. The picrate had all the properties of diplococcin picrate. The unsown medium, which contained 2·5% of heart broth, gave no flocculent picrate under similar conditions.

Heat inactivation of diplococcin

A dilute solution of diplococcin in 0·4% acetic acid loses no activity in 2 hr. at 100°, and very little under the same conditions at pH 4, but is rapidly inactivated by heat on the alkaline side. It is also slowly and irreversibly inactivated at pH 6–7, i.e. when its solution in distilled water or saline is kept in a boiling water-bath.

Crude diplococcin (4·5 mg.) was dissolved in distilled water (9 ml.) by heating at 95° for a few minutes. The cooled and filtered solution (at pH 7) was divided into two equal parts: to one was added an equal volume of distilled water and to the other an equal volume of 0·4% acetic acid. Each was heated at 100° for 30 min. *Found:* water solution, 5 units/ml.; 0·2% acetic acid solution, 20 units/ml. The heated 1 : 4000 solution in water was then divided into two equal parts, which were diluted with 0·5 vol. of water and 0·4% acetic acid respectively. Both were heated at 100° for 90 min. *Found:* water solution, less than 1 unit/ml.; 0·13% acetic acid solution, 5 units/ml.

This shows that a solution, partially inactivated at pH 7, cannot be reactivated by bringing to pH 3 and then heating at 100° (cf. Harington & Scott, 1929, for comparable results with the heat inactivation of solutions of crystalline insulin). Solutions of diplococcin inactivated by heat at pH 7 still give the protein reactions. Diplococcin is stable in glucose-broth medium at pH 7 for at least a week at 37°.

Range of anti-bacterial effect of diplococcin

The best preparation of diplococcin had a marked but transient effect on the growth of an authentic strain of *Streptococcus cremoris* at 24° when introduced into 2% glucose-broth at a concentration of 1 part in 2 millions, and a permanent bactericidal effect at 1 part in 200,000 when a small inoculum was used, e.g. 5000 viable bacteria/ml. Almost the full effect could be obtained in heart broth containing no added carbohydrate, and since growth here was poor in the absence of diplococcin, no dilution of the culture for sowing was necessary. The effect on a distinctly more vigorous strain of *Str. lactis* was only one-twentieth of the above. Diplococcin had also a definite transient inhibitory effect on *Staphylococcus aureus* (strains N.C.T.C. nos. 3095 and 3750) in 2% glucose-broth, at 37°, at a concentration of 1 part in 50,000, and a permanent bactericidal effect at 1 in 10,000, but only if an inoculum as small as one-hundredth of a loopful of a 1000-fold diluted culture was used per 5 ml. tube. The substance had a marked inhibiting effect, at a concentration of 1 in 20,000, upon a haemolytic streptococcus which grew vigorously in blood broth at 37°. It had no action on *Esch. coli* (as Whitehead found) no matter how highly diluted the culture for sowing might be.

DISCUSSION

As Whitehead pointed out, it is not often that an organism militates against growth of other strains of its own species. There would seem to be more than one instance of this behaviour among the lactic streptococci, since one of Whitehead's inhibiting organisms (9S), unlike the two strains here

studied, did not ferment sucrose. It is more remarkable still that of all the Gram-positive cocci inhibited by diplococcin the most susceptible is an organism (*Str. cremoris*) that is practically indistinguishable from those which produce the inhibitor (cf. Meanwell, 1943, p. 21). It is therefore of some significance that the method used for the isolation of diplococcin from the bacterial cell is very similar to those employed by Lancefield (1928, 1933) and Stamp & Hendry (1937) for the preparation of protein antigens of haemolytic streptococci. Diplococcin may possibly be a component of the somatic antigen of the streptococci from which it is obtained, and it would be of interest to find out, by serological studies, if strains F7 and F8 contain antigenic constituents in common with *Str. cremoris*.

Nothing chemically distinctive concerning diplococcin has so far been noted, except perhaps the complete absence of sulphur. It is obviously an anti-bacterial substance in the same category as the sulphur-free polypeptides gramicidin and tyrocidine, the amino-acid composition of which has recently been worked out (Gordon, Martin & Synge, 1943 *a*, *b*). Unlike these polypeptides, diplococcin contains arginine residues, is insoluble in ethanol, shows no tendency to crystallize and is obviously of greater molecular complexity.

SUMMARY

1. Two strains of milk streptococci which inhibit cheese starters produce apparently the same antibacterial substance, tentatively called diplococcin, which powerfully inhibits the growth of *Streptococcus cremoris*, a related organism. Other Gram-positive cocci are inhibited to a lesser extent.

2. Diplococcin occurs attached to the bacterial cells and also in the medium if the latter becomes markedly acidic as the result of carbohydrate fermentation.

3. Diplococcin is insoluble in absolute ethanol, is water-soluble, and appears to be a protein-like substance of relatively small molecular size. It contains no sulphur or phosphorus.

4. Diplococcin is stable at pH 4 at 100°, but not at pH 6–7.

5. A simple method is described for the isolation of diplococcin from bacterial cells grown in a semi-synthetic medium, the yield being about 20 mg./g. of dried cells separated from 5 l. of medium.

The author is greatly indebted to Prof. H. Raistrick, F.R.S., for suggesting this problem, to Mr L. J. Meanwell of United Dairies Research Laboratories for cultures and for information concerning them, and to Mr A. Edwards, without whose aid in the preparation of media this work would hardly have been possible.

REFERENCES

Birkinshaw, J. H., Charles, J. H. V. & Clutterbuck, P. W. (1931). *Biochem. J.* **25**, 1522.

Freeman, G. G. (1942). *Biochem. J.* **36**, 340.

Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943*a*). *Biochem. J.* **37**, 86.

—— —— —— (1943*b*). *Biochem. J.* **37**, 313.

Harington, C. R. & Scott, D. A. (1929). *Biochem. J.* **23**, 384.

Lancefield, R. C. (1928). *J. exp. Med.* **47**, 91, 469, 481.

—— (1933). *J. exp. Med.* **57**, 571.

Meanwell, L. J. (1943). *Proc. Soc. agric. Bact.* p. 19.

Stamp, T. C. & Hendry, E. B. (1937). *Lancet*, **1**, 257.

Whitehead, H. R. (1933). *Biochem. J.* **27**, 1793.

Studies on Tannin Compounds

1. CHANGES DURING AUTOLYSIS OF MINCED CACAO BEAN

BY E. C. HUMPHRIES, *Department of Chemistry, Imperial College of Tropical Agriculture, Trinidad, British West Indies*

(*Received* 13 *March* 1944)

It has long been considered that tannins and tannin-like compounds have an important influence on taste and flavour of cocoa and chocolate, and many tannin analyses have been reported. Unfortunately most of the methods of tannin estimation lack specificity and the position is further complicated by the fact that several closely allied substances may be present. The present paper provides evidence that several tannin-like components occur in the cacao bean and suggests a method of differentiating them.

Freshly gathered cacao beans are subjected to a process of fermentation. This process is, briefly, as follows: the beans, still enclosed in a white mucilaginous pulp, are heaped together in a wooden box where they remain for several days, being turned over or transferred to another box during this time. The beans are then sun-dried. During the fermen-