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 & Syngé, 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 anti-bacterial 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.

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Studies on Tannin Compounds

1. CHANGES DURING AUTOLYSIS OF MINCED CACAO BEAN

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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 ferment-

tation a peak temperature of about 50° is reached after approximately 72 hr. A full account of cacao fermentation may be found in Knapp (1937). The constituents of the bean undergo certain changes which are in part due to the natural enzymes and in part to various yeasts, moulds and bacteria. The fermentation results in reduction of the amount of tannin, besides other changes which are said to improve the flavour. In an attempt to separate the changes due to the enzymes of the bean and the enzymes of the micro-organisms, experiments were performed in which fresh cacao beans were finely minced and allowed to autolyse and the changes in tannin components followed at successive intervals of time.

EXPERIMENTAL

A. Autolysis in air

The testas were removed from about 500 g. fresh cacao beans and minced as rapidly as possible (about 2 min.). Zero time was taken as half-way through mincing, and at intervals a 25 g. portion of this material was added to 100 ml. of boiling water in a beaker. The contents were rapidly brought to the boil in order to inactivate the enzymes, and then left to cool. A representative sample of the intact, unkilld bean was prepared by plunging fresh beans with their testas into boiling water for 2 min., removing the testas, mincing and treating in the manner of the other samples. Acetone was added to each beaker to make the concentration 40% (by vol.) and the whole was left to stand overnight at room temperature (*c.* 26°) to extract the tannins. The boiling, in addition to the extraction with 40% acetone in the cold, was calculated to give maximum tannin extraction (Huber, 1929). Next day each preparation was filtered, washed with 40% acetone and made up to volume with 40% acetone. Measured fractions of these solutions were used for the following determinations based on the procedure proposed by Duthie (1938). (1) Tannin precipitated by saturated cinchonine sulphate solution; (2) a Stiasny determination on the filtrate from the cinchonine tannate ('residual Stiasny'); (3) a Stiasny determination on the original solution ('total Stiasny'). In addition, the amount of tannin was determined by a modified Lowenthal procedure (Association of Official Agricultural Chemists, 1940). The cinchonine tannate value was multiplied by 0.534 to convert to tannin (Jensen, 1928). The values obtained by the permanganate oxidation were expressed in terms of gallotannin for the sake of a basis of comparison.

In all experiments with this procedure the behaviour was closely similar, and an example is shown in Fig. 1. There was a rapid decrease with time in the amounts of cinchonine-sulphate precipitate, residual Stiasny precipitate, total Stiasny precipitate and substances oxidized by permanganate. The values from each method of measurement reached constancy after approximately 50 min. and in no case was a zero value attained.

Duthie (1938) suggested that the residual Stiasny is a measure of 'catechin and similar phenolic

compounds'. It will be noticed from Fig. 1 that the residual Stiasny fraction closely follows the downward trend of tannin precipitated by cinchonine sulphate and the total Stiasny fraction. It seems unlikely, therefore, that residual Stiasny

Fig. 1. The decrease with time of tannin fractions in minced cacao bean. ●—● Total Stiasny precipitate. ○—○ Cinchonine sulphate precipitate. ×—× Residual Stiasny precipitate. □····□ Tannin oxidized by permanganate.

measures catechin, which would be expected to remain constant during autolysis. Moreover, the catechin of the cacao bean appears not to be readily soluble. Adam (1928) found that up to 16 days' extraction with ether was necessary to remove all traces of catechin from dry cacao material free from fat and xanthine bases, and using the colorimetric method of Mitchell (1923), he estimated that unfermented beans contained about 0.8% of catechin, expressed on a dry weight basis, or approximately 0.4% on the fresh weight. It is unlikely that any catechin was extracted by the procedure adopted above, although Nierenstein (quoted in Jensen, 1928) mentions the possibility that catechin is converted to tannin during boiling. The residual Stiasny values of 2–3% of the fresh weight of the bean are much higher than would be expected if measuring catechin. No trace of catechin could be detected in the 40% acetone extract and it is concluded that catechin need not be taken into account in discussing the results obtained. The residual Stiasny

precipitate appears to be a measure of an integral part of the tannin molecule.

In some experiments the total material soluble in 40% (v/v) acetone at successive intervals was also measured. Two examples of the results obtained are shown in Table 1. There was a progressive decrease in

Table 1. *The amount of total Stiasny precipitate and the amount of material soluble in 40% (v/v) acetone in two samples of cacao beans at various times during autolysis*

Time (min.)	Total Stiasny preci- pitate (% fresh weight)	Material soluble in 40% (v/v) acetone (% fresh weight)	Total Stiasny preci- pitate (% fresh weight)	Material soluble in 40% (v/v) acetone (% fresh weight)
0	6.00	9.83	8.39	12.71
4	4.70	10.26	5.00	10.05
8	4.23	9.16	—	—
12	3.51	9.44	—	—
16	3.10	9.39	3.28	8.53
20	2.63	7.98	—	—
24	2.41	8.00	—	—
54	1.72	7.48	2.32	7.64
110	1.78	7.19	—	—

$r = +0.876$ (significant at $P = 0.01$); regression of total Stiasny on acetone-water soluble = 1.133
 $r = +0.999$ (significant at $P = 0.01$); regression of total Stiasny on acetone-water soluble = 1.201

the total amount of acetone-water soluble material and there was a high, fully significant, positive correlation between the amount of soluble material and the corresponding total Stiasny value. The linear regression coefficients of total Stiasny value (percentage fresh weight) on acetone-water soluble material (percentage fresh weight) ranged from 1.13 to 1.22 in different experiments, indicating that the decrease in total acetone-water soluble material was less than the decrease in total Stiasny value, from which it is inferred that there is a progressive, linear accumulation of some substance or substances during autolysis, soluble in 40% acetone. The nature of this was not determined. The amount of tannin, as measured by the cinchonine sulphate precipitate, the residual Stiasny precipitate and the total Stiasny precipitate, reached a constant value after the same length of time (approximately 54 min.). It appears that when minced cacao bean material is allowed to autolyse in the manner described above, a certain tannin fraction, subsequently referred to as 'oxidizable tannin', is rapidly transformed. The remaining fraction ('resistant tannin'), which also reacts with cinchonine sulphate and the Stiasny reagent, and has a positive value even after 110 min. hydrolysis (Fig. 1), is apparently not acted upon by the enzyme system responsible for the transformation of the oxidizable tannin. If

C , T and R are the total amounts of tannin as represented by the cinchonine sulphate, total Stiasny and residual Stiasny determinations at any given time, and c , t and r the corresponding amounts after 54 min. (resistant tannin), then $C - c$, $T - t$ and $R - r$ will represent the amounts of oxidizable tannin at any given time. In Fig. 2, data for the oxidizable fractions alone have been plotted, viz. the residual Stiasny values ($R - r$), the difference between the total Stiasny and the residual Stiasny values, i.e. $[(T - t) - (R - r)]$, at successive times. Results are presented for five experiments with different samples of beans. It is seen that these two fractions bear a fairly close similarity to one another both in magnitude and trend. In all experiments with pigmented beans, consideration of the oxidizable tannin alone yielded results similar to those shown in Fig. 2, although the amounts of resistant tannin differed considerably. It thus appears that the tannin molecule is partitioned into two approximately equal portions by the action of cinchonine sulphate. The portion of the oxidizable tannin precipitated by cinchonine sulphate ($C - c$) does not always follow the total Stiasny minus residual Stiasny curve $[(T - t) - (R - r)]$ very closely (Fig. 2), and it is suggested that this is because the amount of cinchonine sulphate precipitate is dependent upon the pH (Ware & Smith, 1933), but the partition of the tannin molecule by the cinchonine sulphate is not so dependent on pH. Hence it is likely that the difference between total Stiasny and residual Stiasny is a better estimate of the tannin precipitated by cinchonine sulphate than the value calculated from the weight of cinchonine sulphate precipitate. In these experiments no attempt was made to control the pH before precipitation by cinchonine sulphate, although 150 ml. of saturated cinchonine sulphate, added to only 25 ml. of tannin solution, maintains the pH of precipitation within certain limits. If, as suggested, precipitation by cinchonine sulphate measures only approximately one-half of the tannin, the values obtained by the cinchonine sulphate method hitherto reported in the literature seriously underestimate the amount of tannin present in the cacao bean.

The fact that the main tannin component of the cacao bean quickly disappears during autolysis in air provides a simple means of estimating this fraction; it is only necessary to measure the decrease in tannin during 1 hr. autolysis. The results presented in Fig. 2 show that the amount of this tannin varies in different samples of beans.

B. Autolysis in distilled water

A series of experiments was performed in which fresh, minced cacao beans were allowed to autolyse in distilled water. Several 250 ml. beakers of approximately similar weight were counterpoised in turn on a balance and water

Fig. 2. The decrease with time of three fractions of the oxidizable tannin of minced cacao bean. ●—● Total Stiasny precipitate minus residual Stiasny precipitate. x—x Residual Stiasny precipitate. ○····○ Cinchonine sulphate precipitate.

Fig. 3. The changes with time of tannin fractions in minced cacao bean standing under water. △—△ Total material soluble in 40% acetone. Other symbols as in Fig. 1.

added to bring the weight to 130 g., so that each beaker contained approximately 60 ml. water. Fresh beans with testas removed were quickly minced and 5 teaspoonfuls of the mince put in each beaker, which was then weighed. Boiling water was added to each beaker after a given time and the contents brought to the boil. The subsequent procedure was similar to that for the air autolysis experiments.

In these experiments there was no rapid decrease in tannin with time, presumably because of the restricted oxygen supply, but the amount of tannin increased. There was an initial fall in some cases before the increase occurred. Fig. 3A illustrates the results obtained in an experiment where no initial fall in tannin amount was observed, except in the case of the residual Stiasny at 4 min. Apart from this anomaly the cinchonine sulphate, total Stiasny and residual Stiasny fractions show similar trends. The maximum rise in each occurred after 12 min., except the values obtained by the permanganate method, where the maximum occurred after 4 min. The results were more variable by this last method than in the other procedures, probably owing to the difficulty in judging the end-point of the titration. In Fig. 3B is shown an example of the results found in some experiments where there was an initial drop and a subsequent rise in the values obtained by all four methods of measurement. The permanganate oxidation value slightly exceeded the initial value after 54 min., but in no other case did the subsequent values exceed the initial values. As in the previous experiment the maxima of the total Stiasny and cinchonine sulphate values were attained after 12 min. The maximum of the residual Stiasny values was reached after 20 min. The acetone-water soluble material of the successive samples showed a similar trend to the tannin fractions and reached a maximum, a little less than the initial value, after 16 min. It thus appears that in these experiments where oxygen was partially excluded from the minced beans, the amount of tannin increases over and above the amount initially present. The extra amount of tannin obtained in this way varied considerably with different samples of beans. It seems fairly certain that the amount of extra tannin recorded does not necessarily measure the true increase, since ordinary autolysis proceeds at the same time, both in water and for a short period before the minced bean is put in water, although every effort was made to reduce this time to a minimum. In other words the data obtained in water autolysis experiments are resultants of two opposite processes, disappearance of tannin due to enzyme oxidation and production of additional tannin from some precursor. As the first process is considerably slowed by lack of available oxygen, the second process is more prominent, resulting in a net increase of tannin. The fact that acetone-water soluble material shows a trend similar to that of tannin in

these experiments suggests that the extra tannin is produced from a substance which is itself insoluble in 40% (v/v) acetone.

Owing to scarcity of cinchonine sulphate it was not possible to develop these experiments, but it is hoped to continue them in the future.

DISCUSSION

The main tannin component of the cacao bean appears to be split into two approximately equal portions by cinchonine sulphate. In this connexion the data of Clarke, Frey & Hyland (1939) are of interest. They determined the increase in tannin in leaves of *Lespedeza sericea* during growth. A re-examination of their data shows that the amount of tannin fixed by hide-powder (revised Wilson-Kern method) is practically equal to the difference between the formaldehyde precipitate and fixable tannin during a 2½-fold increase in tannin. Humphries (1943) pointed out that the residual Stiasny of the cacao bean probably measures the same substance as is fixed by gelatin (Lowenthal method), and it is probable that this portion is also fixed by hide-powder. Hence the tannin fraction in *Lespedeza* leaves measured by the formaldehyde precipitate minus fixable tannin in all likelihood corresponds with the fraction total Stiasny minus residual Stiasny in the cacao bean. If this is the case, the tannin of *Lespedeza* leaves appears to be of the same nature as that present in the cacao bean.

It was found that the amount of resistant tannin varied with different samples of beans. The ratio of the cinchonine tannin to the residual Stiasny value in the resistant tannin was by no means constant in different samples (Table 2) and this may mean

Table 2. *The amount of cinchonine tannin, total Stiasny precipitate and residual Stiasny precipitate present in the resistant tannin of different samples of cacao beans*

Sample	Cinchonine tannin (% fresh weight)	Total Stiasny precipitate (% fresh weight)	Residual Stiasny precipitate (% fresh weight)	Ratio: Cinchonine tannin/ Residual Stiasny ppt.
a	0.98	1.74	0.75	1.3
b	1.26	1.14	0.23	5.5
c	1.43	1.72	0.49	2.9
d	1.69	2.60	0.97	1.7
e	1.43	1.17	0.20	7.2
f	1.31	1.64	0.37	3.5

that at least two different tannin-like substances are present in the resistant tannin fraction. There was an indication that the amount of resistant tannin was small in unpigmented beans, but the large variety of cacao beans available have not been investigated sufficiently to be certain of the point.

Experiments were performed to see if any change takes place in the anthocyanin pigment, cyanin-3-monoside (Laurence, Price, Robinson & Robinson, 1938), during autolysis. Samples were exhaustively extracted with 90% (v/v) ethanol after various periods of autolysis, and measured portions boiled down *in vacuo* to remove ethanol, and treated with basic lead acetate. The lead precipitate was hydrolyzed for 1 hr. with *N*-HCl, and the amount of reducing sugar estimated. There was no significant change with time in the amount of reducing sugar (presumably mainly from the anthocyanin), which was taken as an indication that little change had taken place in the anthocyanin during autolysis. It is likely that, during the more drastic commercial fermentation, considerable changes in the anthocyanin do occur, which may lead to the development of tannin-like substances. Knapp & Hearne (1939) have reported the presence of leuco-anthocyanins in all parts of the cacao fruit, and these may be concerned in the residual tannin fraction.

The presence of *l*-epicatechin, closely related to cyanidin, in the cacao bean has been reported (Freudenberg, Cox & Braun, 1932). This may be the tannin-precursor found in the present investigation but so far direct evidence is lacking. The fact that a tannin-precursor is present is important, however,

as the foregoing results indicate that unless the conditions during a commercial fermentation are mainly aerobic an increase in tannin, instead of the desirable decrease, may actually occur.

SUMMARY

1. When fresh cacao beans are minced and allowed to autolyse, there is a rapid decrease in the amount of tannin as measured by (a) precipitation with cinchonine sulphate, (b) total Stiasny precipitate, (c) residual Stiasny precipitate, (d) oxidation by permanganate (Lowenthal method).

2. A constant value was attained after about 54 min. by all these methods and it is suggested that autolysis divides the tannin compounds into two fractions—oxidizable and resistant.

3. The oxidizable tannin appears to be split into two approximately equal portions by cinchonine sulphate.

4. Evidence for the existence of a tannin-precursor was obtained by allowing minced beans to autolyse in water, when an increase in tannin occurred. The tannin-precursor is not soluble in 40% (v/v) acetone.

5. The resistant tannin fraction appears to consist of at least two components.

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Biochemical Characterization of the Actions of Chemotherapeutic Agents

2. A REACTION OF HAEMOLYTIC STREPTOCOCCI, INVOLVING PANTOTHENATE-USAGE, INHIBITED BY PANTOYLTAURINE, AND ASSOCIATED WITH CARBOHYDRATE METABOLISM

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The basis upon which pantoyletaurine was designed as an antibiotic agent (Snell, 1941; Kuhn, Wieland & Möller, 1941; McIlwain, 1942a; cf. Fildes, 1940), and subsequent investigations (McIlwain, 1944),

suggested that the compound acted upon bacteria by affecting metabolic processes involving pantothenate. It is also suspected that other chemotherapeutic agents have their primary action upon