179. TANNINS AS HYDROGEN CARRIERS IN BIOLOGICAL OXIDATION

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(Received 10 October 1940)

EVIDENCE has been presented [Roberts, 1939; 1940] which indicates that the effect of extensive bruising on tea leaf is to decrease the activity of the H-carrying coenzymes (cozymase). In the so-called tea fermentation which follows this bruising, it was established that carbohydrate oxidation, as measured by the rate of CO₂ production, parallels tannin oxidation. Further, if excess ascorbic acid be added to the system the primary oxidation product of tea tanpin is reduced again immediately on its formation, and until the whole of the ascorbic acid has been oxidized no carbohydrate oxidation is apparent. These observations led to the conclusion that in tea fermentation, the tannin acted as one of the H-carriers in the oxidative stages of carbohydrate breakdown.

The possibility that cozymase was completely or almost completely inactivated by mechanical damage to the leaf was not considered, as it was believed that an *o*-quinone could not function as a H-acceptor in the absence of cozymase (Co). The later stages in the reactions in dilute suspensions of minced tea leaf were therefore considered to be

Owing to the decrease in activity of cozymase as a result of the mechanical damage it was believed that the *o*-quinone of tea tannin was not so readily reduced and that there was in consequence a much greater tendency for it to be removed from the sphere of the reaction by undergoing irreversible condensations.

Since the publication of this work, fresh evidence has been brought forward by Dixon & Zervas [1940] which conclusively shows that *o*-quinone requires the presence of cozymase for it to function as H-acceptor for the alcohol- and malic-dehydrogenases of yeast.

However, as a result of work from this laboratory [Deb & Roberts, 1940] on the measurement of anaerobic fermentation in mechanically damaged tea leaf, it has been shown that in all probability there is an even greater destruction of cozymase activity in bruised tea leaf than was formerly visualized. It would appear that the residual cozymase activity in finely minced tea leaf is too low to account for more than a part of the carbohydrate oxidation that actually takes place, and consequently the possibility must be envisaged that the *o*-quinone of tea tannin can function directly as H-acceptor without the aid of cozymase.

A difference of this type between *o*-quinone itself and the *o*-quinone of tea

tannin is perhaps not surprising, as the chroman ring undoubtedly has a great effect on the reactivity of the catechol group in the tea tannin molecule, witness the much greater rate of oxidation by tea oxidase of tea tannin in comparison with catechol and pyrogallol [Harrison & Roberts, 1939].

In tea fermentation therefore the most likely path of H-transfer is in accordance with the following scheme:

The dotted arrows represent alternative paths for H transfer, but these will be of secondary importance in comparison with the main path, which utilizes only tannin and cytochrome as carriers between substrate and O_2 . Not all of the tannin *o*-quinone will be effective as a H-acceptor, as this substance has a strong tendency to undergo irreversible condensation to form the deeply coloured tannin products, characteristic of fermented tea. Eventually the greater part of the tannin will have been converted into such complex condensation products, and oxidation of carbohydrate will come practically to a standstill. The R.Q. of the reaction will be a measure of the proportion of the tannin oxidation product functioning as a H-acceptor.

The *o*-quinone of a tannin is in all probability produced by direct oxidation by the cytochrome system. The actual oxidation of the tannin is believed to be non-enzymic and hence the tannin concentration may determine the rate of oxidation to a very large extent.

Where tannin is the principal substance oxidized by the cytochrome system, i.e. when the normal system of H-acceptors has been inactivated by the disorganization that follows extensive damage to plant tissue, we may expect a correlation between tannin content and the rate of O_2 uptake by the damaged tissue, so long as the tannin concentration remains a limiting factor. If the tannin content is such that the rate of reduction of oxidized cytochrome is equal to the maximum rate of oxidation of cytochrome by its oxidase, any increase in tannin concentration will have no further accelerating effect on the rate of O_2 uptake. The fact, previously established by Harrison & Roberts [1939], that addition of tea tannin to a suspension of 200 mg. finely minced tea leaf in 3 ml. water produces no acceleration in O_2 uptake, shows that in this particular case the limiting concentration has been reached. On the other hand the relatively short period for which minced tea-leaf suspensions maintain their original rate of O_2 uptake ($-Q_{\text{O}_2}$ for 0–10 min. is almost invariably greater than $-Q_{\text{O}_2}$ for 10–20 min.) suggests that this limiting concentration is not greatly exceeded when 200 mg. minced tea leaf are suspended in 3 ml. water.

The tannin contents of different samples of tea leaf do not differ sufficiently to establish a correlation between tannin content and rate of O_2 uptake in a fine mince. The less satisfactory method of varying the tannin content by using leaf from different plants was therefore employed. This has the compensation of extending the results obtained for tea to other plant tissues, many of which show similar effects to tea on mincing or otherwise damaging the tissue.

EXPERIMENTAL

Leaf tissues. Tannin contents of leaf samples were determined by the Lowenthal process in a 1 hr. infusion. Each tannin probably has a characteristic factor of its own but to avoid determining a variety of such factors, which in

itself would be a major research, it was assumed that 1 ml. of N $KMnO_4$ would be equal to 0.0416 g. of tannin (the tea tannin factor). The figures recorded for tannin percentages are therefore purely arbitrary, but probably represent the relative amounts of tannin in the various tissues with fair accuracy.

Rates of O_2 uptake in a very fine mince were determined manometrically. Minced leaf equivalent to 45–50 mg. dry wt. was suspended in 3 ml. water. The temperature of the bath was 36° , this being the lowest practical in Assam during the hot months. Where CO_2 was determined, the direct method of Warburg was used: in four vessels O_2 uptakes were measured with a CO_2 absorbent (KOH on filter paper) and in four other vessels containing comparable samples of tissue the CO_2 absorbent was omitted. This method, although shown to be inaccurate for mammalian tissue slices, gives quite good results for plant tissues. Retention of CO_2 by tissues seems to be low, and the buffer retention is negligible, as the tissues are suspended in distilled water. Turner [1938] has shown this method to give values for the R.Q. of carrot slices almost identical with those obtained by the more complicated method of Dickens & Simer.

The rate of O_2 uptake ($-Q_{O_2}$) is defined as the initial rate in μ l. O_2 /hr. for 1 mg. dry wt. of tissue. The figures quoted in Tables 2–6 represent uptakes in μ l. O_2 /100 mg. tissue (fresh weight).

The results obtained for young leaves from many different species are summarized in Table 1.

Table 1

Family	Species	$-Q_{O_2}$	% tannin (dry wt.)	R.Q.
MONOCOTYLEDONS:				
Cannaceae	<i>Canna</i> (cultivated variety)	11.0	5.8	—
Liliaceae	<i>Hemerocallis fulva</i>	3.0	0.0	—
Amaryllidaceae	<i>Eucharis grandiflora</i>	1.0	0.0	—
	<i>Hippeastrum equestre</i>	1.8	0.0	—
Gramineae	<i>Zea mays</i>	2.9	0.0	—
DICOTYLEDONS:				
Tropaeolaceae	<i>Tropaeolum majus</i>	2.2	0.0	0.9
Theaceae	<i>Eurya acuminate</i>	4.4	3.3	0.31
	<i>Pyrenaria barringtoniaefolia</i>	18.5	13.8	0.07
	<i>Schima wallichii</i>	6.1	10.7	0.29
	<i>Gordonia excelsa</i>	2.0	14.9	0.44
	<i>Camellia thea</i>	20.0	22.0	0.36
	<i>C. drupifera</i>	4.5	27.7	0.37
	<i>Eugenia balsamea</i>	18.2	18.9	0.56
	<i>E. jambolanea</i>	13.0	16.6	0.32
Myrtaceae	<i>Hibiscus rosasinensis</i>	1.6	0.0	1.0
Malvaceae	<i>Daucus carota</i>	13.8	1.7	—
Umbelliferae	<i>Allamanda cathartica</i>	5.1	1.6	0.36
Apocynaceae	<i>Gardenia florida</i>	2.3	2.2	1.1
Rubiaceae	<i>Coreopsis</i> (annual spp.)	14.8	3.8	—
	<i>Dahlia</i>	18.0	1.2	—
	<i>Eclipta alba</i>	22.5	6.8	0.54
Compositae	<i>Bignonia venusta</i>	4.8	0.9	0.30
	<i>B. aequinoxialis</i>	5.0	1.0	0.39

An examination of this table shows that, broadly speaking, leaves with a low tannin content show a very slow rate of O_2 uptake in a fine mince, whereas leaves of high tannin content show a comparatively rapid rate. In all six cases where tannin is not present in the tissues, the rate of O_2 uptake in the finely minced tissue is very low. The correlation coefficient unfortunately falls short of significance, $r = 0.356$ (0.396 required for significance when $P = 0.05$).

This is perhaps not surprising, as the correlation may be obscured by several

factors. Thus the effect of fine mincing on the rate of O_2 uptake varies markedly with the type of tissue. In the case of young shoots from tea (*Camellia thea* Link), the finely minced petiole shows a much higher rate of O_2 uptake than the blade, despite the former's lower tannin content. Some of the inconsistencies in the above table may be explained in terms of a greater proportion of petiole to blade tissue in some of the more rapidly respiring cases. In addition to lack of uniformity in the tissue resulting in different reactions of respiratory activity to damage, another factor, the toxic effect of the tannins, must be considered.

In the two cases *Gordonia excelsa* and *Camellia drupifera*, where tissues of high tannin content show a low $-Q_{O_2}$ in a mince, the strength of the tannins is of a high order. Unlike tea tannin they can precipitate gelatin directly, without the assistance of acid and salt, and solutions of these tannins are sufficiently toxic to depress the rate of O_2 uptake in other minced tissues, particularly tea.

Such strong tannins may fairly be considered to be toxic even to their own oxidase systems, and we consider it legitimate to exclude these two results when calculating the correlation coefficient between $-Q_{O_2}$ and tannin content. The correlation coefficient now becomes 0.6160 which is easily significant for $P=0.01$.

The majority of the tanniferous leaves show O_2 uptake and CO_2 output curves similar to those of tea [cf. Roberts, 1939]. The initial uptake is rapid, slowing down as tannin oxidation becomes complete. The R.Q. usually varies from 0.3 to 0.5 indicating that condensation of the *o*-quinone molecule is rather more likely than its functioning as a H-acceptor. When the greater part of the tannin molecules have been transformed into oxidized and condensed tannin products, both O_2 uptakes and CO_2 outputs sink to low levels.

The non-tanniferous leaves not only show very low initial rates of uptake, but this rate is not maintained for long. It would seem that the reaction comes to a standstill after all the carriers are in their oxidized form. The R.Q. in such cases is found to be close to unity. Typical results for two representative species are summarized in Table 2.

Table 2

		Min.				
		15	30	45	60	120
<i>Tropaeolum majus</i>	O_2 (μ l.)	8	13	14	16	19
	CO_2 (μ l.)	7	10	11	12	15

		Min.						
		15	30	45	60	75	90	105
<i>Eugenia jambolanea</i>	O_2 (μ l.)	49	95	133	171	192	217	237
	CO_2 (μ l.)	8	18	34	48	57	67	76

Not all species fall into these two clearly differentiated types. Thus *Gardenia florida* (Table 3) shows a continuous uptake of O_2 and evolution of CO_2 for the finely minced leaf tissue.

Table 3

		Min.					
		15	30	45	60	90	120
<i>Gardenia florida</i>	O_2 (μ l.)	15	25	36	45	56	72
	CO_2 (μ l.)	14	25	36	46	63	80

This is obviously a case where mincing has not seriously inactivated the reducing powers of the tissue. The tannin in *Gardenia* may not condense readily on oxidation, coenzyme inactivation may be less apparent, or alternatively

dehydrogenase activity may be high enough to reduce any *o*-quinone immediately on formation. It is impossible to decide between these three alternatives at the moment.

At the other extreme, we have *Pyrenaria barringtoniaefolia* where the R.Q. is 0.07. Here very little carbohydrate oxidation is apparent and most of the *o*-quinone must condense before it has had a chance of functioning as H-acceptor.

Minced leaf from the carrot (*Daucus carota*) and from the three Compositae investigated, show much higher initial rates of O₂ uptake than would be expected from their tannin contents. This high rate is only transient and soon falls to a low value. With *Eclipta alba* the rate of uptake is initially so high that oxidation may be nearly complete before the minced tissue is weighed out into the Warburg vessels. Samples of this plant contain a large proportion of succulent stalk, and as was found for tea, such tissue undergoes very rapid oxidation. *Eclipta alba* is also of interest as the tannin condensation product is dark blue in colour unlike the more usual brown of these substances. This blue colour is made use of locally as an ink, and also for tattooing and hair dyeing.

Fruit tissues. The effect of fine mincing on fruit tissues is similar to that on leaves. Thus the R.Q. of apple as measured for thin slices, is reduced to 0.60 on fine mincing, and the mince rapidly darkens in colour. The fruit of *Phyllanthus emblica* behaves similarly. Here the R.Q. is 0.44 after mincing, and the rate of O₂ uptake in the mince is 82 μ l./hr./100 mg. tissue, as against 14 μ l. for the apple. This higher rate is paralleled by the relatively high tannin content (10% of dry wt.) of the latter fruit. The tannin condensation products of this fruit, like those of *Eclipta alba*, are blue.

Specificity of tannins as carriers. It might be expected that addition of a tannin to finely minced non-tanniferous leaves such as *Tropaeolum*, *Hibiscus* etc. would stimulate the rate of O₂ uptake. Neither of the above species reacts significantly in this way as shown by the results in Table IV for *Tropaeolum*.

Table 4

	Min.				
	15	30	45	60	90
	O ₂ uptake (μ l.)				
<i>Tropaeolum</i>	10	11	12	13	14
<i>Tropaeolum</i> + tea leaf infusion	13	14	15	16	17

The failure of added tea tannin to stimulate O₂ uptake was further investigated and it was found that the oxidase system of a given plant, while generally capable of promoting oxidation of tannins from its own species, has comparatively little action on foreign tannins.

This is shown quite clearly in the following experiment. Finely minced portions of leaf, from tea and from *Eugenia balsamea*, were suspended in 2 ml. of water in Warburg vessels and shaken for 50 min., at the end of which time O₂ uptake had slowed down considerably. Fresh substrate in the form of green leaf infusions from either of the two species was then added and the uptakes recorded over a further period. Tea tannin stimulated the O₂ uptake of tea leaf to a much greater extent than that of *E. balsamea*, while the *E. balsamea* tannin had a greater effect on the O₂ uptake catalysed by its own enzymes (Table 5).

For tea the oxidation of tea tannin is not directly an enzymic process but is brought about by oxidized cytochrome [Roberts, 1940; Deb & Roberts, 1940]. Enzymic specificity therefore is unlikely to account for these differences, and it is suggested that plant tissues possess some sort of "immunity" to the toxic effect

Table 5. *O₂ uptake after addition of fresh substrate*

Minced tissue	Infusion added	Min.				
		15	30	45	75	105
Tea	Tea	68	108	125	146	159 μ l.
Tea	<i>E. balsamea</i>	25	42	50	63	73 μ l.
<i>E. balsamea</i>	Tea	23	44	61	86	102 μ l.
<i>E. balsamea</i>	<i>E. balsamea</i>	86	115	134	147	158 μ l.

of their own specific tannins, and that foreign tannins in consequence have a greater inhibitory effect on oxidase activity. The general toxic effect of tannins on enzyme activity is well known.

The inactivating effect of a foreign tannin on oxidase activity may in some cases be reversible. Thus on successive additions of infusions of *E. balsamea* and tea leaf to fermented tea leaf suspensions, O_2 uptake is accelerated by the second but not by the first addition of fresh substrate. This effect is illustrated in Fig. 1.

Fig. 1.

E. balsamea infusions when added to freshly minced tea leaf suspensions have no significant effect on the initial rate of O_2 uptake, although they do actually increase the total uptake. However, at the end of 90 min. comparatively little of the total *E. balsamea* tannin has been oxidized. Uptakes are much higher after 90 min. when tea infusions are added to the system in this way.

Camellia drupifera tannin, however, significantly depresses the rate of tea fermentation, the $-Q_{O_2}$ falling from 18.9 to 15.3 as a result of the addition of

Table 6

	Min.						
	10	20	30	45	60	75	90
	O_2 uptake (μ l.)						
Tea fermentation	63	110	145	164	170	177	181
Tea fermentation + <i>E. balsamea</i> infusion	62	112	153	190	200	210	217

1 ml. of a *C. drupifera* infusion (20 g./500 ml.) to 200 mg. tea leaf suspended in 2 ml. water. With partly fermented tea leaf the inhibition by *C. drupifera* infusions exceeded 50 %. Unfortunately further experiments with *C. drupifera* could not be carried out as supplies of fresh leaf were no longer available.

DISCUSSION

The low O_2 uptakes by minced suspensions of non-tanniferous leaves, and the establishment of a correlation between the tannin content of a leaf and its rate of O_2 uptake in a fine mince confirm the belief that tannin is an essential carrier in oxidation processes taking place in minced leaf tissue. Taking into account the observations quoted elsewhere, which indicate that cozymase is almost completely inactivated by extensive damage to the tea leaf, we may conclude that, as carbohydrate oxidation is apparent in fermenting tea leaf, the *o*-quinone of tea tannin may function as a H-acceptor for dehydrogenase systems without the assistance of cozymase. This apparently distinguishes the *o*-quinone of tea tannin from *o*-quinone itself.

This conclusion however is not in accordance with all the experimental facts. Previous results from this laboratory [Harrison & Roberts, 1939] have shown that addition of catechol or pyrogallol to a suspension of finely minced tea leaf increases the output of CO_2 . We cannot be certain that this additional CO_2 comes from carbohydrate oxidation but until further evidence is available it would be premature to conclude that *o*-quinone added to tea leaf should behave in the same way as in the systems investigated by Dixon & Zervas.

Although there are many analogies between the biological oxidation systems present in yeast and animal tissues on the one hand, and in leaf-tissue on the other, we are not yet justified in assuming that the same carriers are required, and the role of flavoproteins and pyridine nucleotides in plant respiration deserves intensive study.

SUMMARY

A correlation has been found, rejecting two anomalous cases, between the tannin content of leaf tissue and its rate of O_2 uptake in a fine mince. This confirms previous ideas that tannins function as H-carriers in biological oxidation systems.

The properties of the *o*-quinone of tea tannin as H-acceptor are discussed. Apparently, it does not require the presence of cozymase.

The apparent specificity of a plant oxidase for tannins from its own species is considered to be due to an "immunity" of the enzymes of a given species to the toxic effects of its own tannins.

The authors wish to thank the Director, Mr P. H. Carpenter, for his encouragement throughout the course of this work and the Indian Tea Association for permission to publish these results.

Dr W. Wight's assistance and advice on the botanical side of this work is gratefully acknowledged, and thanks are also due to Mr Percy-Lancaster of the Royal Agri-Horticultural Society of India for identification of some of the species used.

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