corresponded to ethyl-3-indoleacetate (R_f 0.81) and 3-indoleacetonitrile (R_f 0.76). However, the zone neither developed typical colors with Salkowski and Ehrlich spray reagents nor possessed an ultraviolet absorption spectrum typical of the indole moiety (see above). The eluate from the growth stimulating zone of R_f 0.00 to 0.16 in figure 4 had an ultraviolet absorption spectrum identical with the eluate from the zone of R_f 0.70 to 0.90 in figure 3 (major max. at 272 mμ, minor max. at 279 mμ, minima at 255 and 277 mμ shoulder at 265 mμ). This indicates a conjugated system and is very similar to the absorption spectrum of benzoic acid. A "neutral" growth substance with similar properties has been extracted from immature corn kernels in this laboratory (1).

The results of this investigation add evidence to the concept that IAA and other indole derivatives are not the only group of naturally occurring plant growth substances (2, 4).

Summary

In the acid fraction of corn pollen, 3-indoleacetic acid and two other growth promoting substances were observed. The growth regulator in the "neutral" fraction did not possess an indole moiety.

The authors are indebted to Mrs. JoAnne DeVries and Miss Nancy Carson for performing the biological assays.

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UTILIZATION OF D-GLUCURONATE BY CORN COLEOPTILES[1]

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D-Glucuronic acid and D-xylose, which may be regarded as its decarboxylation product, occur naturally in plant cell wall polysaccharides. Although considerable interest has been focused on D-glucuronate metabolism in animals in connection with its role in detoxication little has been established until very recently on its origin and metabolism in plants.

Hough and Pridham (10) have shown that D-glucuronolactone is metabolized in plum mesocarp and this is one of the compounds which Isherwood, Chen and Mapson have shown to function as a precursor for ascorbic acid synthesis in water cress (11). Altermatt and Neish (1) in a paper which appeared after the completion of the present work, have followed the utilization of D-glucuronolactone and several other compounds in the synthesis of cell wall materials in wheat. They have emphasized that D-glucose and D-glucuronolactone are superior to pentoses as precursors of the D-xylose units in xylan, and the labeling data show clearly that loss of carbon 6 is involved in the conversion of D-glucuronolactone to xylan. A similar loss of C-6 from a glucose skeleton was observed in previous work on the origin of the pentose units in wheat xylans (13) and in the pectins of boysenberry and strawberry fruits (16, 17). Some of this work has been reviewed (2).

As a result of their recent work, Altermatt and

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Neish propose that uridine diphosphoglucose (UDP-glucose) occupies a central role as the starting point for uridine diphospho-glucuronate and xylan synthesis and for the analogous uridine diphosphogalactose ⟶ araban series of reactions (1).

While investigating the fate of D-glucuronic acid in the plant as part of our studies on carbohydrate catabolism we have found it to be rapidly decarboxylated by some tissues. This paper describes an investigation into features of the decarboxylation and the fate of the 5-carbon residue in the coleoptiles of corn seedlings. It will be seen that the results (which have been described previously in a preliminary form (18) are in accord with the suggestions of Altermatt and Neish (1); indeed we had been led to speculate independently along similar lines.

Methods

In most of the experiments, the coleoptiles of 3-day-old corn seedlings, variety Wf9/38-11 were used. These were obtained by germinating the grain on moist paper at 30° C. The coleoptiles were cut into slices about 0.5 mm thick, and 1 g fresh weight of tissue was incubated with phosphate buffer (pH 5.0 or 7.0) at 25° C in the presence of the appropriate substrate. Large (100-ml) Warburg flasks containing 3 ml of liquid were used, so that the tissue was always well aerated. Oxygen consumption could be measured, and the carbon dioxide was absorbed in CO_2-free sodium hydroxide which was removed for determination of $C^{14}O_2$ and replaced as frequently as necessary during each experiment.

D-Glucuronic acid-6-C^{14} and D-glucuronolactone-6-C^{14} were supplied by the National Bureau of Standards (Dr. H. S. Isbell), and D-glucuronolactone-U-C^{14} (i.e., D-glucuronolactone uniformly labelled with C^{14} in all 6 carbon atoms) was donated by the Corn Products Refining Company. D-Glucuronic acid-U-C^{14} was prepared by hydrolysis of an aqueous solution of the lactone at 100° C for one hour; the acid was then separated from unchanged lactone by paper chromatography in ethyl acetate : acetic acid : water (3 : 3 : 1 v : v : v (5)) and eluted with water.

To determine the radioactivity of the substrates, samples were wet-ashed by the method of Stutz and Burris (20), and the CO_2 absorbed in barium hydroxide. CO_2 released by the tissues was recovered from the alkali by precipitation as barium carbonate, which was then spread as a thin film (4 to 16 mg/sq cm) on planchets, dried, and its activity measured in a windowless gas flow counter. All activity measurements are expressed in counts per minute (cpm) at infinite thinness, i.e., corrected for background and self-absorption.

Results

Ability of Different Tissues to Decarboxylate D-Glucuronic Acid: Table I shows the O_2-consumption and $C^{14}O_2$-production of a number of tissues, sliced thinly and incubated for four hours at 25° C with 50 micromoles (20,600 cpm) of D-glucuronic acid-6-C^{14} at pH 5.0. In general, those tissues with the higher rates of respiration were most efficient in converting the radioactive D-glucuronate to $C^{14}O_2$. The highest activity was shown by young tissues of the three cereals, and the best of these, corn, was used for all subsequent experiments.

Table I

Decarboxylation of D-Glucuronic Acid-6-C^{14} by Various Plant Tissues

	CO_2, cpm *	O_2-uptake **
Corn, coleoptile	2040	1940
Corn, root	1880	1350
Barley, coleoptile	734	930
Wheat, coleoptile,	630	950
Castor bean, hypocotyl	217	1040
Castor bean, endosperm	129	1500
Pea, epicotyl	242	600
Soy bean, epicotyl	224	1000
Lima bean, epicotyl	168	700
Kidney bean, immature seed	314	890
Kidney bean, pericarp	193	320
Tomato, petiole	153	226
Kalanchoe, peduncle	22	295
Bryophyllum, leaf	420	490
Coleus, stem	110	135

* Per g fresh weight in 4 hours.
** Microliters per g fresh weight in 4 hours.
50 micromoles of D-glucuronic acid-6-C^{14} containing 20,600 cpm was supplied as substrate in each case.

Progress of Decarboxylation by Corn Coleoptiles: The course of $C^{14}O_2$ production from 20 micromoles and 50 micromoles of D-glucuronic acid-6-C^{14} is shown in figure 1. With the smaller quantity, half of the radioactivity had been recovered in 16 hours, and 75 % in 21 hours. Respiratory activity declined steadily during the experiment.

Aerobic Nature of the Process: In the absence of air (replaced by nitrogen), production of $C^{14}O_2$ was almost completely inhibited (fig 2).

Origin of CO_2 Produced from D-Glucuronic Acid: In order to determine whether C-6 alone ap-

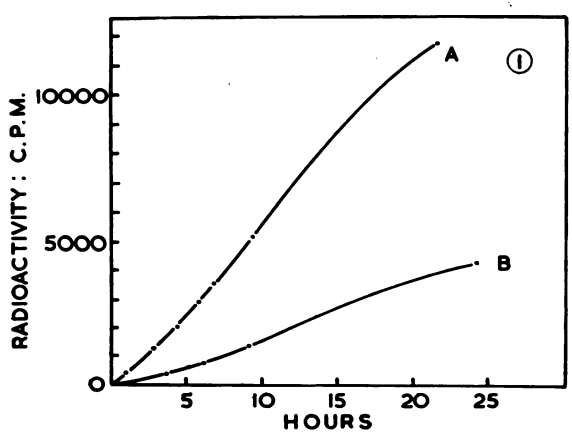

Fig. 1. $C^{14}O_2$ production from D-glucuronic acid-6-C^{14} (A. 50 micromoles; 20,600 cpm: B. 20 micromoles; 5650 cpm) by 1.0 g sliced coleoptiles; pH 7.0.

peared as CO_2, equivalent molar and radiochemical amounts of the specifically (6-C^{14}) and the uniformly labeled acids were used. The specifically labeled acid therefore contained 6 times as much activity in the 6-carbon position as did the uniformly labeled acid, so that if this were the only carbon atom released, a ratio of 6 : 1 in the radioactivity of the CO_2 from the 6-C^{14} and U-C^{14} acids would be expected. This was the value actually observed experimentally, as figure 3 (curves A and B) shows. The values for radioactivity recovered from U-C^{14} (curve A) have all been multiplied by 6 to demonstrate the close agreement of the experimental to the theoretical ratio during the first 12 hours; after that time curve A diverges from B, indicating the release, as CO_2, of carbon atoms other than C-6.

Behaviour of D-Glucuronolactone: D-Glucuronic acid in aqueous solution comes into equilibrium with its corresponding D-glucuronolactone (6). The reaction appears to be a slow one at normal temperatures, although Rabinowitz, in his work on decarboxylation by kidney preparations found that the lactone and acid were equally effective as substrates and considered them to be readily interconvertible (4).

The four curves in figure 3 show the decarboxylation of the free acid and lactone, in both the specifically and uniformly labeled forms, by corn coleoptile tissue. It is clear that the lactone is attacked,

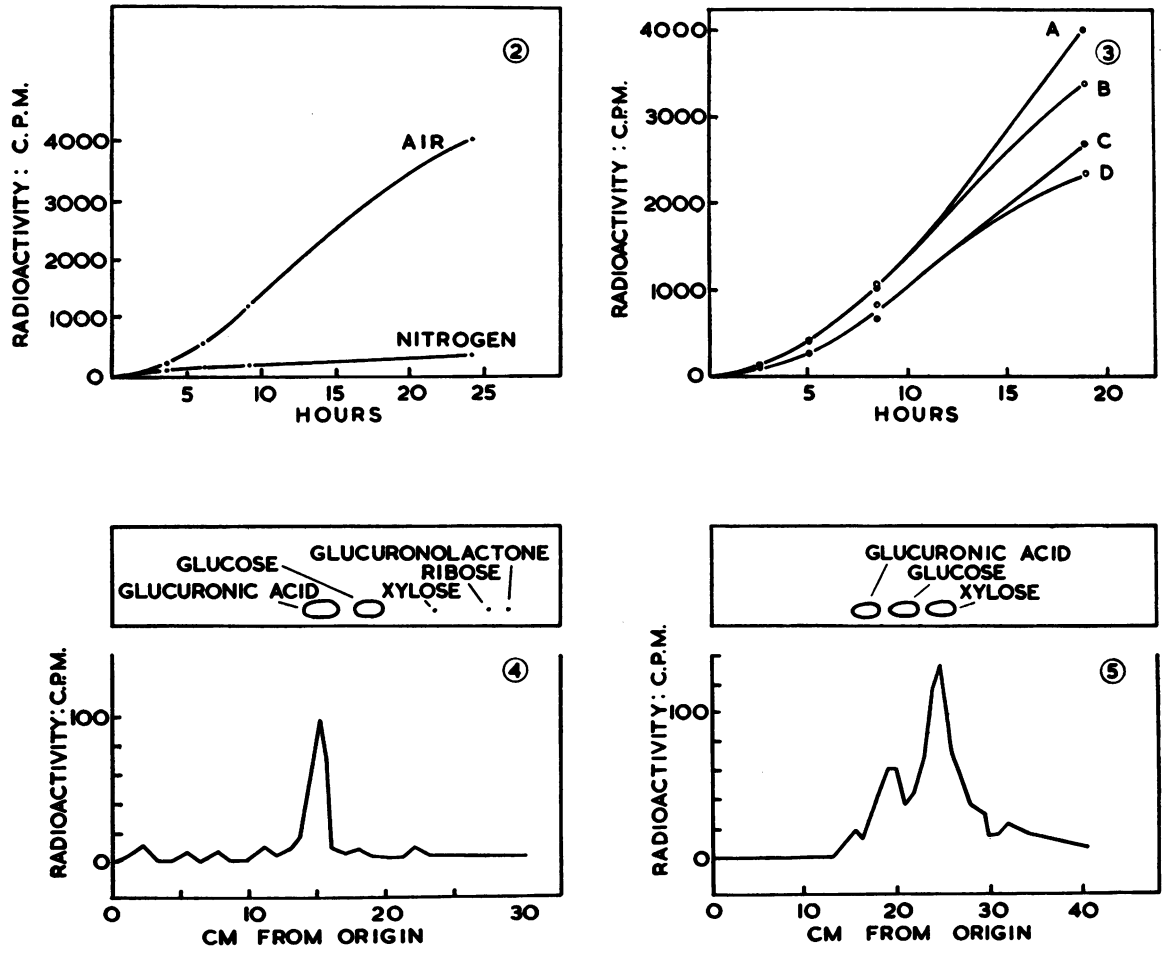

Fig. 2. $C^{14}O_2$ production from D-glucuronic acid-6-C^{14} (20 micromoles; 5650 cpm) in air and in nitrogen by 1.0 g sliced coleoptiles; pH 7.0.

Fig. 3. $C^{14}O_2$ production from 20 micromoles (5650 cpm) of: A. D-glucuronic acid-U-C^{14} (cpm in $CO_2 \times 6$); B. D-glucuronic acid-6-C^{14} (cpm in CO_2); C. D-glucuronolactone-U-C^{14} (cpm in $CO_2 \times 12$); D. D-glucuronolactone-6-C^{14} (cpm in CO_2); by 1.0 g sliced coleoptiles; pH 7.0.

Fig. 4. Chromatogram of alcohol-soluble contents of coleoptiles and medium after incubation with D-glucuronolactone-U-C^{14}. Only D-glucose and D-glucuronic acid were detected in color tests for sugars, as shown in the upper part of the figure.

Fig. 5. Chromatogram of products of hydrolysis from alcohol-insoluble residue obtained from coleoptile sections after incubation with D-glucuronolactone-U-C^{14}.

although $C^{14}O_2$ release (curves C and D) lags somewhat behind that from the corresponding acids (curves A and B). However, the ratio of radioactivity in the CO_2 from C-6-labeled lactone to that from uniformly labeled lactone was 12 : 1 rather than 6 : 1. The values for $C^{14}O_2$ formation from the D-glucuronolactone-U-C^{14} (C) have been multiplied by 12 to show this relationship. No satisfactory explanation can be offered for the fact that in each experiment in which the two lactones were compared, a ratio of 12 : 1 (rather than 6 : 1 anticipated from the acid results) was obtained. It appears that only half of the added uniformly labeled lactone can be decarboxylated in vivo; in a chemical decarboxylation of the two lactones, achieved by boiling in 1 : 1 hydrochloric acid for 1.5 hours (12a) the radioactivity recovered in the CO_2 from glucuronolactone-6-C^{14} was 6.02 times that from an equivalent amount glucuronolactone-U-C^{14}.

PRODUCTS OF THE DECARBOXYLATION: The product expected after the removal of CO_2 at the 6-carbon atom from D-glucuronic acid is D-xylose. Presumably, this would then be liable to oxidation (3), but the fact that the two curves in figure 3 do not begin to diverge until after some 12 to 15 hours suggests that the residue of five carbon atoms is not, in fact, broken down further during this time. Accordingly, coleoptiles and medium were extracted in boiling 80 % ethanol, after an 18 hour incubation with 100 micromoles of D-glucuronolactone-U-C^{14}, and the extract analyzed for soluble products, by paper chromatography in ethyl acetate : acetic acid : water (3 : 3 : 1 v : v : v). A radioactive scanning of the paper was made by cutting it into 1-cm strips and counting these individually without elution; the results of this analysis are shown in figure 4, together with the positions of marker substances on the chromatogram.

In the absence of any trace of pentose, or other derivative of the decarboxylation, other treatments were tried. For example, when the extract was fractionated into acidic, basic, and neutral components on ion-exchange resins, all the activity remained in the acid fraction, and this was found to be entirely due to unchanged D-glucuronic acid itself. Different conditions of extraction, and treatment with phosphatase before extraction (to break down any insoluble phosphorylated derivatives, e.g., of sugars) also proved unfruitful.

Attention was therefore turned to the alcohol-insoluble material. This was strongly radioactive, and a quantitative comparison of the C^{14} in the CO_2 released with that in the residue yielded the results shown in table II in two separate experiments. These figures establish the fact that any 5-carbon product of the decarboxylation is converted rapidly into some insoluble form. An attempt was made to determine which components of the residue were labeled, by hydrolysis and chromatography, but the large excess of inactive cellulose made a quantitative analysis impracticable. Figure 5 illustrates a chromatogram obtained by hydrolyzing the dried alcohol-insoluble residue with 1 % sulphuric acid under reflux for 12 hours (12), and developing the concentrated and neutralized hydrolyzate in ethyl acetate : acetic acid : water. The most strongly radioactive component was D-xylose, with some activity in D-glucose, and a little in uronic acid.

TABLE II

RELATIONSHIP BETWEEN C^{14} IN CO_2 AND IN THE "INSOLUBLE" FRACTION AFTER METABOLISM OF D-GLUCURONOLACTONE-U-C^{14} BY CORN COLEOPTILES

EXTRACTING SOLVENT	CPM IN INSOLUBLE RESIDUE	CPM IN CO_2	RATIO
Hot 80 % ethanol	50,000	9,600	5.2 : 1
Cold 10 % trichloracetic acid	25,200	5,100	4.9 : 1

DISCUSSION

The close adherence to a 6 : 1 ratio in figure 3 (curves A and B) shows that, as in the rat kidney preparations of Rabinowitz (14, 15) it is the carbon atom in position 6 which is lost during decarboxylation of D-glucuronate by corn coleoptile tissue. However, a much greater proportion of the supplied D-glucuronate was decarboxylated in the present experiments than in those of Rabinowitz and Sall (15); whereas the animal preparations effected only 10 to 12 % decarboxylation, the decarboxylation went virtually to completion when 10 micromoles of substrate were supplied to 1 g of corn coleoptiles. Rabinowitz (14, 15) was unable to decide whether the acid or the lactone was the immediate substrate for decarboxylation, because both were equally reactive in his system. In corn, on the other hand, the curve of $C^{14}O_2$ release from the lactone lags slightly behind that for the acid, which might suggest that the lactone is first hydrolyzed. Experiments in which D-glucuronolactone-U-C^{14} was used and the alcohol-soluble products separated chromatographically, showed that corn coleoptile tissue was in fact able to bring about a thermolabile hydrolysis to D-glucuronic acid. At pH 5, where only a trace of acid was produced in the absence of the tissue, hydrolysis was complete within 24 hours when slices were added. At pH 7, where the spontaneous hydrolysis of the lactone occurs at a noticeable rate (about 50 % hydrolysis in 24 hr) no lactone remained after this time in the presence of the tissue, and the radioactive area coincided with D-glucuronic acid (fig 4). No cell-free preparation capable of hydrolyzing the lactone has yet been obtained from corn coleoptiles; Eisenberg and Field have recently described such an enzyme in liver homogenates (7).

The absence of any trace of pentose in the experiments such as that illustrated in figure 4 indicates that the products of decarboxylation are not liberated in the free state, and the high level of radioactivity in the residue supports this suggestion. The products are apparently rapidly converted to insolu-

ble material, of which D-xylose, combined in polysaccharide, is the main radioactive component (fig 5). The presence of D-xylose in a hydrolysis under such drastic conditions as these must be accepted with caution, but there are two reasons for accepting the D-xylose as a valid component of the residue. First, the chromatograms of the digest revealed large amounts of uronic acid which had withstood the acid hydrolysis, but which had very low activity (fig 5). Second, the ratios of (C^{14} in residue) : (C^{14} in CO_2) are so close to theoretical (i.e., 5 : 1) that only traces of radioactive substances, other than the products of decarboxylation, could be present in the insoluble residue (table II).

Existence of an active process such as this in the plant leads to consideration of its significance in vivo. Although free D-glucuronic acid is not known to occur naturally in plants the present experiments show that it is decarboxylated at a rate at least as great as that of D-gluconic acid (3), which as 6-phosphogluconic acid, is an intermediate in the pentose phosphate cycle. It is now suggested that this reaction may throw additional light on the question of pentosan synthesis in plants since it demonstrates convincingly the occurrence in vivo of the split between carbons 5- and 6- of a 6-carbon chain. In addition, it seems clear that the decarboxylation leads quantitatively to xylan synthesis by a process not involving free D-xylose as an intermediate.

These facts are in accord with the following mechanism of xylan synthesis proposed independently by Altermatt and Neish (1) from their extensive work on labeling patterns in the xylan units of wheat polysaccharides.

The facts that the decarboxylation of supplied D-glucuronate has an O_2 requirement and that UDP derivatives of pentose have now been isolated in small amounts from wheat by Ginsburg, Stumpf and Hassid (8) are in harmony with the proposed involvement of UDP derivatives as xylan precursors. The concept derives further support from the work on glucuronyl transfer in animal tissues (19) and from the finding that in the cell-free decarboxylation of D-glucuronate in rat liver, UTP is an effective co-factor (14).

The demonstrated inability of tissues to make direct use of D-xylose in pentosan synthesis (1, 8) must, on this line of reasoning, be ascribed to an inability of the tissue to form directly a UDP derivative. As the labeling data show (1, 8) D-xylose is apparently first converted into hexose units in the pentose phosphate pathway. This hexose unit is then presumably converted to its UDP derivative before oxidation to UDP-glucuronate and decarboxylation to UDP-pentose. This would ascribe the origin of UDP-pentoses to reactions of the corresponding hexose derivatives.

It is implied by the ability of the tissues to utilize D-glucuronate that this can be converted into a UDP form, although no direct evidence for this reaction can be cited. The results from pentose utilization (1, 8) and on uronic acid formation in the cell free system (19, 21) make it appear unlikely that free D-glucuronic acid is an obligatory intermediate in the synthesis of xylans from hexose. The reaction leading to uronic acid products which has been elucidated is one which involves UDP-glucose rather than free glucose, and the enzyme involved is a DPN linked dehydrogenase which yields UDP-glucuronic acid as the product. This reaction has now been demonstrated in pea extracts (21) and the enzyme has been highly purified from this material. The activity of such an enzyme in the present connection could clearly limit the natural rate of xylan (and polyuronide) synthesis from D-glucose. However, it is also clear from the fact that the young tissues had the greater ability to deal with added D-glucuronate that the whole complex of enzymes involved may be more active in the younger, actively growing tissues. In these tissues a significant part of the glucose might be diverted towards pentosan synthesis, and such a diversion leading to an excess of C-6 in the respired CO_2 could well account for occasional values of greater than unity observed for the C-6 : C-1 ratio by Beevers and Gibbs (4).

Summary

Out of a variety of tissues tested, young corn coleoptile was superior in its ability to decarboxylate D-glucuronic acid-C^{14} under aerobic conditions. By comparing radiochemical yields in the $C^{14}O_2$ produced from D-glucuronic acid-6-C^{14} and D-glucuronic acid-U-C^{14} by 1-g coleoptiles, it was shown that, over a period of about 12 hours (during which nearly half of the 20 micromoles of substrate supplied had been utilized) the only carbon from the substrate to appear in the CO_2 was that from C-6. D-Glucuronolactone-C^{14} was also decarboxylated, and the slices were shown to bring about the hydrolysis of the lactone to the free acid.

No soluble products of the decarboxylation of D-glucuronic acid-U-C^{14} could be detected, but the insoluble residue after 80 % alcohol extraction or 10 % TCA precipitation contained five times as much radioactivity as the respired CO_2. Hydrolysis of this material revealed that the bulk of the radioactivity was in D-xylose units derived from polysaccharide. This suggests that xylan synthesis and decarboxylation of D-glucuronate are very closely linked.

Other evidence on the origin of D-glucuronate and its utilization is also summarized, and the present experiments are considered to lend support to some recent proposals of Altermatt and Neish (1) implicating UDP-glucose, UDP-glucuronate and UDP-xylose in xylan synthesis from glucose in plant tissues.

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CHANGES IN OXIDATIVE ENZYME ACTIVITY DURING THE CURING OF CONNECTICUT SHADE TOBACCO[1]

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The activities of enzymes initially present are believed to be responsible for the major chemical changes that occur during the curing of tobacco leaves (3, 10). Accordingly, the stability of specific enzymes under curing conditions could markedly influence the final composition of the cured leaf. Vickery and Meiss (10) and others (3, 4) have shown that the major changes in composition occur during the first 8 to 12 days of air curing. Respiration, which causes a loss of about 16 % of the total organic solids, also ceases by the end of this period (10). However, most previous studies of enzymes have dealt with non-oxidative reactions, and it has been observed that the activities examined persist even beyond 12 days (1, 2, 3, 8). Such results seem surprising since at least 50 % of the proteins are broken down during curing (4, 10). It would appear that proteins of widely different degrees of stability are present and it has recently been suggested (8) that many of the enzymatically active proteins are included in the stable group.

Since oxidations are fundamental to the curing process, the activities of some soluble oxidative enzymes which vary greatly in their mechanism of action have been investigated. A marked decline, and in some cases a complete loss, of enzymic activity as well as a disappearance of oxygen uptake by leaf tissue has been observed. These results correlate well with earlier investigations on the chemical changes that occur during curing.

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