Studies of lignin biosynthesis using isotopic carbon: I. Long-term experiment with $C^{14}O_2$

Stone, J. E.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1139/v53-030
Canadian Journal of Chemistry, 31, 3, pp. 207-213, 1953-03-01

STUDIES OF LIGNIN BIOSYNTHESIS USING ISOTOPIC CARBON

I. LONG-TERM EXPERIMENT WITH $C^{14}O_2$[1]

By J. E. Stone

ABSTRACT

Wheat plants were fed with $C^{14}O_2$ at the stage of growth corresponding to rapid lignification. Thereafter, plants were harvested every few days until maturity. The specific and total activity of the plants, and the specific and total activity in some lignin degradation products were determined. Results indicated that (i) $C^{14}O_2$ was still being respired from the plants at maturity, (ii) all the C^{14} which gets into the lignin does so within 24 hr. from the administering of $C^{14}O_2$, (iii) the total activity originally acquired by the syringaldehyde portion of the lignin remains constant throughout the growth of the plant, that is, the lignin (as represented by syringaldehyde) is a final end product of the plant and is not a part of the respiratory system, (iv) the total activity originally acquired by the vanillin suffers an initial drop for about two weeks after activation and then becomes constant, (v) the *p*-hydroxybenzaldehyde activity drops continuously throughout the life of the plant.

INTRODUCTION

When considering the mechanism of lignin biosynthesis it is usual to postulate a precursor which might by its structure and known reactions lead to lignin. There must be many intermediates between carbon dioxide and lignin but it is very probable that a simple aromatic compound, or to be more explicit, a phenol substituted in the para position, lies somewhere along the route. Klason (2) suggested that coniferyl alcohol or aldehyde might be a lignin precursor, and Freudenberg's recent work (1) on the polymerization of coniferyl alcohol to a lignin-like substance by polyphenol oxidase seems to support this contention.

It is probable that much light can be shed on this problem by the feeding of suspected precursors, labelled with C^{14}, to plants which are actively synthesizing lignin. Activity should appear in the lignin if the added compound is a precursor. One disadvantage to this method however is that carbon dioxide is the only precursor which can be added without risking some disturbance of the normal metabolism of the plant. A more serious difficulty arises owing to the fact that lignin is not a well-defined crystalline compound which can be obtained in a pure state. Because of this it would be rather difficult to decide whether activity which appeared in the isolated lignin was due to a true incorporation of the precursor into a normal lignin molecule or whether it was due to the copolymerization of the actual precursor and the supposed one which had been added. Polyphenol oxidases are present in many plants and the mere isolation of a radioactive lignin-like substance from a plant which had been fed a radioactive phenol would need interpreting with caution.

The difficulty goes even further than this because the lignin as isolated from the majority of plants is highly contaminated with complexes of the humic acid type which are definitely not lignin-like from the chemical point of view. Regardless of the precursor used, from $C^{14}O_2$ to C^{14} labelled coniferyl alcohol, the

[1] *Manuscript received August 11, 1952.*
Contribution from the National Research Council, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as N.R.C. No. 2923.

total activity acquired by the isolated "lignin" fraction would be meaningless.

It appeared necessary, therefore, to study the activity acquired by the crystalline degradation products of lignin, i.e. vanillin, syringaldehyde, and possibly *p*-hydroxybenzaldehyde, rather than lignin itself. It was decided to use $C^{14}O_2$ for activating the plants because, although it has the disadvantage of causing the majority of plant components to become radioactive after a few seconds, continuing growth in an atmosphere of normal carbon dioxide should distinguish between those which were intermediates and those which were stable end products. The former should become depleted in activity with time while the latter should increase in total activity.

It was hoped that the present work would provide answers to the following questions. After wheat plants are given a fixed dose of $C^{14}O_2$ and their growth is continued in carbon dioxide,

(1) How long does it take for the lignin, as represented by the three phenolic aldehydes, to acquire its maximum activity?

(2) Is lignin a stable end product of plant metabolism or is it part of a dynamic system with a consequent gradual loss of activity?

(3) What percentage of the activity originally administered to the plant shows up in the phenolic aldehydes? If an appreciable amount, this could be a convenient biological method for preparing C^{14} labelled aromatic compounds.

Future papers in this series will deal with the conversion of $C^{14}O_2$ to aldehydes during the first few minutes and hours of photosynthesis, and the feeding of multicarbon plant metabolites labelled with C^{14}.

EXPERIMENTAL

Apparatus

A dual purpose chamber was constructed for growing the plants in an enclosed atmosphere and for carrying out subsequent large sheet paper chromatography.

The dimensions of the chamber were 27 in. × 16 in. and 30 in. high, constructed of plate glass cemented into an angle iron frame and provided with casters for easy moving. The cover, also made of plate glass, was ground to fit the top of the chamber. This cover contained three $\frac{1}{2}$ in. holes for (*a*) the carbon dioxide generator, (*b*) a thermometer, (*c*) a tube for watering the plants. When the chamber was used for chromatography these holes were used for adding the solvent to the three stainless steel troughs after equilibration of the paper. Air was drawn through a 2 in. hole in the bottom of the chamber and exhausted outside the greenhouse by means of a blower connected to a side hole. Illumination was provided by daylight supplemented by artificial light to give a total of 17 hr. of light and 7 hr. of darkness per day.

Procedure

Thatcher wheat was grown (three plants per 5 in. pot) under normal conditions until alkaline nitrobenzene oxidations indicated that the plants were reaching the stage when rapid lignification was about to set in (4). Nine pots containing 27 plants of similar size were selected and placed in the growing chamber. The latter was sealed and 1 millicurie of $C^{14}O_2$ added. Illumination was maintained for 24 hr. and then the blower started in order to sweep out any

remaining $C^{14}O_2$. After air had been drawn through the chamber and exhausted outside the greenhouse for one hour, a single plant was removed for analysis. Thereafter, a plant was removed at intervals of a few days up to the cessation of growth. As much as possible of the root was retained by carefully washing away the earth clinging to it.

The plants were dried in a rapid current of air at 35°C. and ground to pass a 40-mesh screen. Samples of about 10 mgm. weight were burnt to carbon dioxide in a normal combustion train and the carbon dioxide trapped as sodium carbonate and converted to barium carbonate. The barium carbonate was filtered and its "specific activity" at infinite thickness determined using a Simpson gas flow counting chamber and an RCL scaler. The residue remaining in the crucible after the combustion gives the percentage ash in the plant material and all results were calculated on an ash-free basis.

The nitrobenzene oxidations and determination of *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde were carried out by the micromethod of Stone and Blundell (3). About 60-80 mgm. samples were used and after the oxidation, one half of the liquor (0.5 ml.) was placed on a paper chromatogram sheet 18 in. × 22 in. and developed with the organic layer of petroleum ether – *n*-butyl ether – water 6:1:1. The pure phenolic aldehydes which separated on the paper were extracted with ethanol, the solutions evaporated to small volume (about 5 ml.), made alkaline with alcoholic potassium hydroxide, and aliquots removed for the determination of (*a*) percentage aldehyde, (*b*) specific activity of aldehyde. The latter was carried out by evaporating 1.0 ml. on to an aluminum disk and measuring the counts per minute obtained. The very small weight of material on each disk gave an "infinitely thin" sample so that the specific activity of the aldehydes recorded as counts per minute per mgm. should be interpreted on this basis.

RESULTS AND DISCUSSION

The results are shown graphically in Figs. 1 to 5 and are almost self-explanatory. Fig. 1 demonstrates the fall in specific activity of the total plant carbon

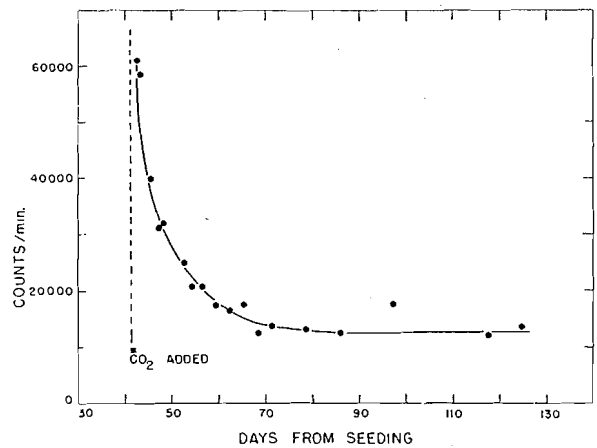


FIG. 1. Specific activity of total plant carbon (measured as barium carbonate, infinitely thick). $C^{14}O_2$ added on 41st day from seeding. First active plant removed for analysis 25 hr. later.

with time, this fall being due to two causes. One is the continuing growth of the plant in inactive carbon dioxide with a consequent dilution of activity. The other cause is the loss of $C^{14}O_2$ through respiration. This latter is demonstrated in Fig. 2

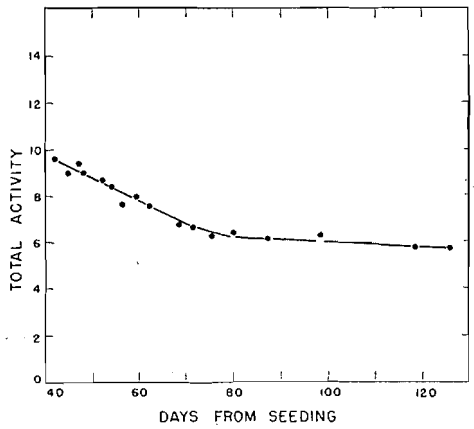


FIG. 2. Total activity of wheat plants. Weight (ash free) × specific activity.

where the total activity of the plant (specific activity × weight of plant) is seen to fall with time.

Figs. 3, 4, and 5 refer to the results of the alkaline nitrobenzene oxidation of the plants. Fig. 3 shows the change in the percentage of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde and indicates the age of the plants at the time of activation and at subsequent harvests. (In the other figures a number of points have been omitted for the sake of clarity.) It will be noted that the vanillin and syringaldehyde percentages are increasing quite rapidly during the course of the experiment, whereas *p*-hydroxybenzaldehyde rises only slightly.

The specific activities of the three aldehydes fall as the plant matures, this being demonstrated in Fig. 4. This fall is only to be expected since inactive lignin

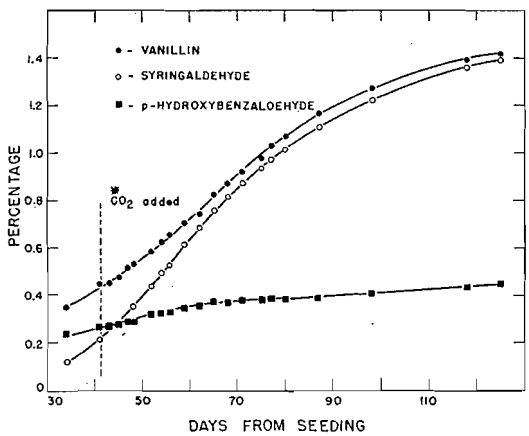


FIG. 3. Percentage of aldehydes based on whole plant.

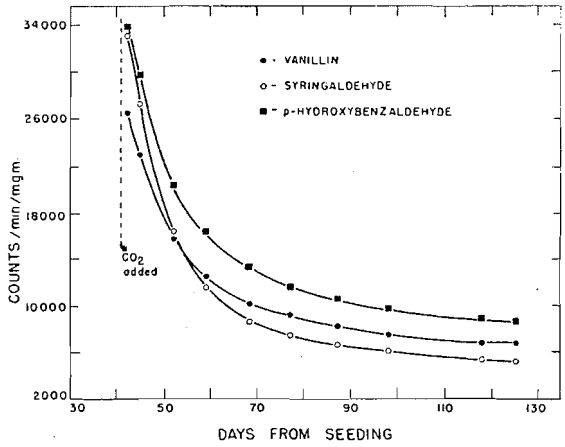


FIG. 4. Specific activity of aldehydes.

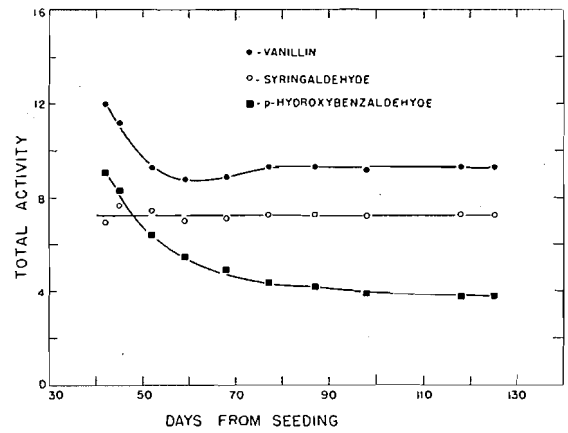


FIG. 5. Total activity of aldehydes (percentage × specific activity).

is being synthesized by the plant the whole time and is diluting the activity of the material formed immediately after the addition of $C^{14}O_2$.

Of more interest are the curves shown in Fig. 5 where the total activity of each aldehyde is given. The total activity is the specific activity (see Fig. 4) in counts per min. per mgm. multiplied by the percentage of aldehyde (see Fig. 3) and represents the total amount of activity acquired by that portion of the lignin molecule. The units of total activity are arbitrary ones and have not been calculated as microcuries since it is unnecessary for comparative purposes.

The curves of Fig. 5 are interesting in that each of the three aldehydes appears to behave differently. The activity of the *p*-hydroxybenzaldehyde drops continuously throughout the life of the plant and supports the contention put forward in a previous paper (4), that this aldehyde is derived from tyrosine present in plant protein and not from the lignin fraction of the plant. Tyrosine can lose active carbon in a number of ways as it is part of a dynamic system so that loss

of activity from its oxidation product, *p*-hydroxybenzaldehyde, is not surprising.

Syringaldehyde, on the other hand, is apparently derived from an irreversible system since it loses no activity over a period of two to three months and cannot therefore be involved in respiration unless its loss of activity is exactly compensated for by a gain in activity from other active plant components. This latter is very unlikely. It will also be noted that there is no gain in total activity after the first 25 hr. from administering $C^{14}O_2$ so that evidently the path from $C^{14}O_2$ to the parent substance of syringaldehyde is complete in less than this time. This implies that it would be necessary to study the changes taking place in the first 25 hr. in order to obtain information regarding intermediates in lignin formation, and the results of such a study will be reported in later papers of this series.

Considering the total activity in the vanillin fraction (Fig. 5) it is seen to be between *p*-hydroxybenzaldehyde and syringaldehyde in its behavior. An initial drop in total activity is followed by a levelling out to constant activity. The most probable explanation for this is that the young plants contain guaiacyl-type lignans which give rise to higher percentages of vanillin than does lignin. As the plant matures these lignans are polymerized or condensed in some way to form lignin which is less susceptible to oxidation. There is evidence (4) that young wheat plants contain guaiacyl-type lignans which disappear during the early stages of lignification so this explanation is not unlikely.

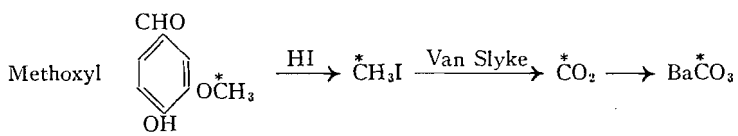
A separate experiment was conducted to find out what percentage of the activity which had been added to the plant appeared in the form of active aldehydes.

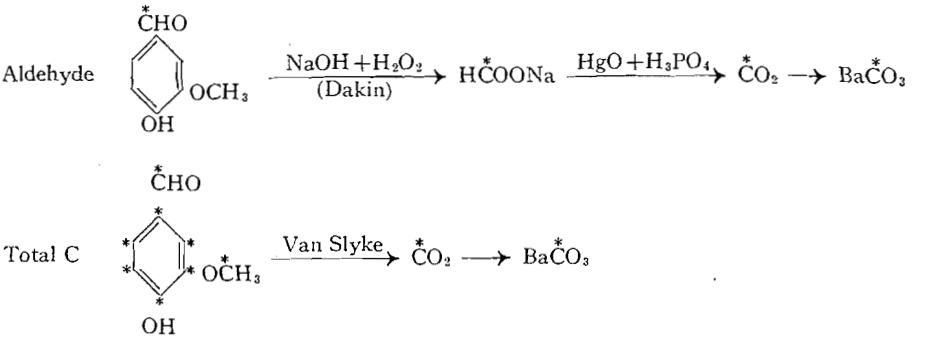
Five wheat plants (taken just prior to heading out) were kept in the dark for 18 hr. and then activated with 0.2 mc. of $C^{14}O_2$ in the light for a period of 24 hr. The plants weighed 6 gm. when dry and had absorbed 96% of the C^{14} added. The finely ground material was oxidized with alkaline nitrobenzene and the three aldehydes separated by chromatography. The results are shown in the table.

TABLE I

	Wt. of aldehyde, mgm.	Specific activity, μc. per gm. of carbon	Percentage of C^{14} added
p-Hydroxybenzaldehyde	10	60	0.3
Vanillin	49	40	1.0
Syringaldehyde	60	45	1.3

It would be expected that under the conditions of activation and growth used here, the carbon atoms in the compounds under consideration would have equal activity. This was shown to be the case. Taking vanillin as the example the reactions used for the degradations were:—





It is possible that for certain purposes this could be a convenient method for obtaining C^{14} labelled phenolic aldehydes if the uniform labelling were not a disadvantage.

REFERENCES

1. FREUDENBERG, K. Chem. Ber. 83: 519. 1950.
2. KLASON, P. Arkiv Kemi, Mineral Geol. 6: No. 15. 1917.
3. STONE, J. E. and BLUNDELL, M. J. Anal. Chem. 23: 771. 1951.
4. STONE, J. E., BLUNDELL, M. J., and TANNER, K. G. Can. J. Chem. 29: 734. 1951.