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A TRACER STUDY WITH OXYGEN-18 IN PHOTOSYNTHESIS BY ACTIVATION ANALYSIS

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ABSTRACT

A method has been devised for tracer studies with oxygen, using paper chromatography and proton activation of oxygen-18 to fluorine-18. This technique is relatively simple and can detect a fraction of a microgram of O^{18}, as well as giving some information about the chemical nature of the compound into which it is incorporated. The procedure has been applied to a preliminary study of the path of oxygen in green algae. Three short-term photosynthesis products containing O^{18} were observed.

A TRACER STUDY WITH OXYGEN-18 IN PHOTOSYNTHESIS BY ACTIVATION ANALYSIS*

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Introduction

Since oxygen is an important element in organic matter, it is of interest to study the path of oxygen in plants, from the time oxygen as water is taken up by plants to the time it is given off as gaseous oxygen. The half lives of the radioactive isotopes of oxygen are all very short, so in general it is necessary for oxygen tracer studies to use one of the two stable isotopes occurring in small percentage in ordinary oxygen. These are oxygen-17 and oxygen-18, with abundances of 0.039% and 0.204% respectively.

There are several ways to detect these isotopes, such as mass spectrometry, nuclear magnetic resonance (applicable only to O^{17}), and density measurements. Another way is by activation analysis, and the purpose of the work reported here was to investigate this oxygen-analysis method and to apply the procedure to a study of the chemical incorporation of oxygen into algae. To do this experiment by nuclear magnetic resonance or mass spectrometry would have involved large quantities of very expensive chemicals and (or) distressingly tedious procedures for reducing a complex sample to a large number of small samples derived from some separation process. In the activation analysis here reported, entire chromatograms can be bombarded and radiautographed, thus simplifying the analytical procedure and providing chemical information at the same time.

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†Present address: Royal Institute of Technology, Stockholm, Sweden. This work was partly supported by a grant from the Rockefeller Foundation to Prof. Calvin.

Selection of Isotope

Both the isotopes of oxygen that are suitable for tracer work, oxygen-17 and oxygen-18 are available in enriched form. The isotope chosen in this work was O^{18}, which was used as water in an enrichment of 20%. The main reason for this choice was that the isotope has a high capture cross section for protons (2×10^{-25} cm^2 for 4-Mev protons[1,2]), yielding fluorine-18 by the reaction $O^{18}(p,n)F^{18}$. Fluorine-18 has a half life of 1.8 hours, and emits positrons of an energy of 0.64 Mev. Calculations showed that 0.1 to 1 μg of O^{18} could easily be detected by radioactivity measurements of F^{18} if a 4-Mev proton beam of 1 to 10 μa was allowed to hit the oxygen target for a few minutes. The amount of F^{18} activity formed during a bombardment with 4-Mev protons during a few minutes is roughly $\phi\, t\, m\, (4 \times 10^{-11})$ disintegrations per unit time, where ϕ is the flux, t the bombardment time in minutes, and m the amount of O^{18} in μg. It was also found that, after 1 hour of aging a bombarded sample of organic material containing enriched O^{18}, interfering radioactivity from carbon, nitrogen, and other oxygen isotopes was negligible if protons of an energy of about 4 Mev had been used for the bombardment.

The meain reason for not using O^{17} was that it was not available in very high enrichment (1 to 1.8%). Also, the only reaction that seemed to be useful for the activation of that isotope is $O^{17}(n,\alpha)C^{14}$, with a cross section of 0.5×10^{-24} cm^2, yielding the radioactive isotope carbon-14. The amount of C^{14} activity produced with a neutron irradiation of O^{17} is roughly $\phi\, t_d\, m\, (0.5 \times 10^{-14})$ disintegrations per unit time, where ϕ is the flux, t_d the time in days, and m the amount of O^{17} in μg. This is a very small amount of activity to be detected when one considers that O^{17} is available in only 1% enrichment and that the highest neutron flux available is about 10^{14} n/sec/cm^2. Carbon-14 is also formed by a high-cross-section (1.75×10^{-24} cm^2) neutron reaction on nitrogen-14, which would give serious interference in the analysis of unknown nitrogen-containing compounds. Nitrogen would give rise to C^{14} activity of about $\phi\, t_d\, m\, (2 \times 10^{-14})$ disintegrations per unit time, where m is here the amount of N^{14} in μg.

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[1] Dubridge, Barnes, Buck, and Strain, Phys. Rev. 53, 447 (1938).

[2] Blaser, Boehm, Marmier, and Scherrer, Helv. Phys. Acta 24, 473 (1951).

Working Conditions

The initial purpose of the work was to analyze qualitatively and, if possible, quantitatively for the oxygen that is incorporated in different compounds in algae grown in O^{18}-enriched water for a short period of time. In order to separate the different compounds in an alcohol extract of the algae, the extract was concentrated and chromatographed in one dimension with butanol—propionic acid—water solvent.[3] The compounds are then found as spots on the paper. The amount of the solution from the algae to be chromatographed is chosen in such a way that every spot on the chromatogram contains 10 to 100 μg of substance, of which 20% to 50% is usually oxygen. Since we were using water containing 20% O^{18}, every spot would thus contain 0.4 to 10 μg O^{18}, mostly spread over an area of 1 cm^2. Chromatograph paper weighs about 10 mg/cm^2 and is about 50% oxygen, of which 0.20% is O^{18}. This gives an O^{18} content of 10 $\mu g/cm^2$ in the paper which is comparable to that in the spots of the material to be analyzed. It is thus not feasible to perform an O^{18} analysis directly on ordinary paper. A synthetic oxygen-free paper would be useful, but here other isotopes can interfere. Thus a polyvinylchloride paper looks interesting until one considers that a 4-Mev proton bombardment would give rise to an argon-37 activity from Cl^{37} of about $\phi t \times 10^{-11}$ disintegrations per unit time per cm^2 of paper, where ϕ is the proton flux and t the bombardment time in minutes.

Transfer of a Paper Chromatogram to a Metal Sheet

For these reasons it was necessary to devise a method to transfer the compounds of a developed one-dimensional paper chromatogram to a metal sheet. This had to be done without changing the pattern of the compounds on the developed paper, since this pattern is used to identify the compounds in the different spots. After trials of different ways of serrating the edge of the paper and of eluting the material onto the sheet, including transfer by cloth and transfer by ascending and descending elution, it was found that good results are achieved with the apparatus shown in Fig. 1. The chromatogram was divided into 3- to 4-inch pieces and the segments were eluted one at a time.

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[3] A. A. Benson et al., J. Am. Chem. Soc. 72, 1710 (1950).

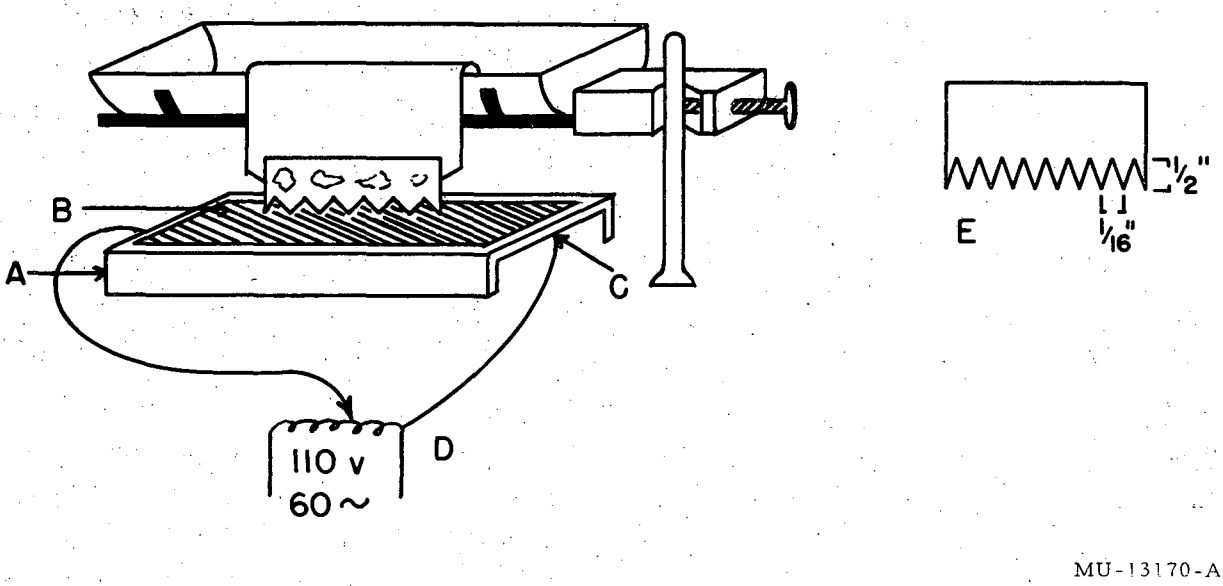

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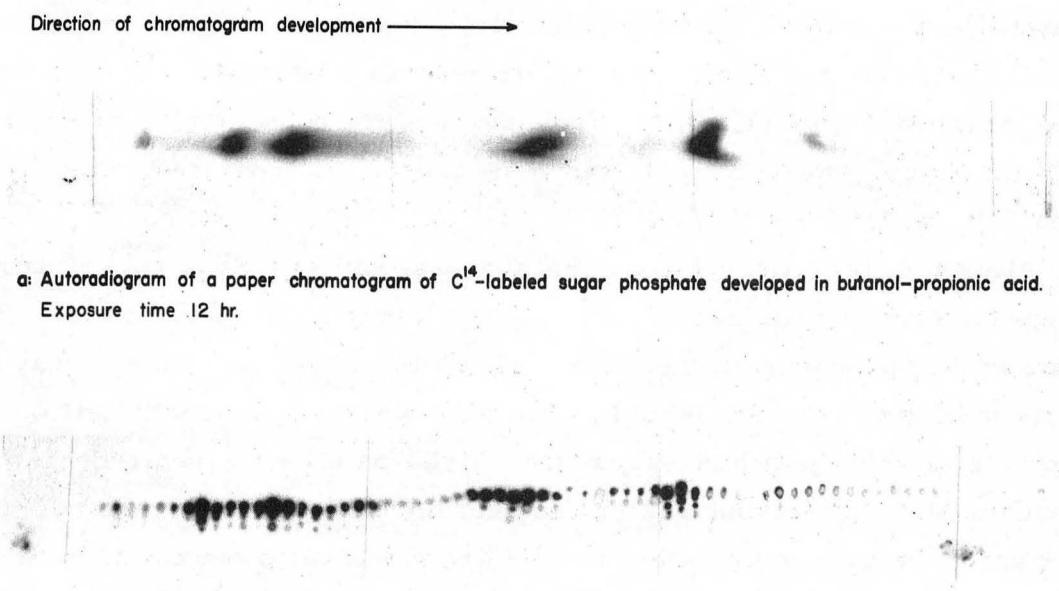
Fig. 1. Apparatus for transferring a developed one-dimensional paper chromatogram to a metal sheet. A, metal supporting plate; B, tantalum sheet; C, heating tape under sheet; D, variable voltage control; E, sheet of brass used to serrate edges of chromatogram.

One edge of the chromatogram was cut with a scalpel, using as a pattern the edge of a piece of brass that has the desired form (see Fig. 1E). The cut paper edge was allowed to just touch a metal sheet B, which rested on the metal plate A, which was heated by means of a resistance strip. The elution system was covered with a glass vessel. A row of drops was allowed to form while the plate was heated slightly. The heat was then regulated in such a way that the beginning of a row of drops was continually forming where the paper touched the metal sheet. The system was kept in that condition for about one hour, causing the eluted material to concentrate at the edge of the paper. In addition to the heat, removal of the glass hood can be used as a regulating factor. After 1 hour the heat was shut off and a row of drops was allowed to form on the sheet. At no time were the drops allowed to coalesce, either on the paper or on the sheet. Then the cover was taken away and the paper was lifted from the sheet. The heat was turned on and left on until the drops on the sheet had evaporated. The paper was lowered again so that it touched the sheet about 1/2 cm beyond the first row of drops. The system was again covered and another row of drops was allowed to form, but without heating. The evaporation was repeated. A third row of drops was then allowed to form 1/2 cm further from the center.

In order to test the reliability of the transfer by this technique, a paper chromatogram of C^{14}-labeled sugar phosphates was developed in a system of butanol—propionic acid, autoradiographed, and eluted by the above method. After the elution the metal sheet and the paper were autoradiographed. The X-ray film showed that the pattern of the chromatogram had been reproduced on the sheet (Figs. 2a, 2b). A small amount of radioactivity was still left at the edge of the paper, but most of the active material had been eluted onto the plate.

Irradiation Development and Identification of Radioisotopes

If one is to be able to trace spots containing O^{18} on a metal sheet by proton bombardment and autoradiography, the metal itself must not become radioactive, or the radioactivity induced in the metal must have such a short half life that it disappears 1 to 2 hours after the bombardment, the time that the sheet can be aged. Two materials that fulfill these conditions are aluminum and tantalum. However, there are impurities in most materials,



a: Autoradiogram of a paper chromatogram of C^{14}-labeled sugar phosphate developed in butanol–propionic acid. Exposure time 12 hr.

b: Autoradiogram of the metal sheet to which the paper chromatogram in "a" had been eluted. Exposure time 12 hr.

ZN-1715

Fig. 2. Films showing transfer of paper chromatogram pattern to metal sheet.

and, as activation analysis is a very sensitive analytical method, even a hundredth of a percent or less of an element could give rise to interfering radiactovity. Even pure aluminum usually contains some 0.01 to 0.001% copper, which has a very high capture cross section for protons. By calculation it was found that the copper content had to be lower than 0.005% to not interfere with the oxygen analysis. Very small impurities of zinc would also cause interfering radioactivity. The best aluminum available was said to have a purity of 99.999% though spectral analysis showed that the copper content was larger than 0.001%. The oxide layer on aluminum does not interfere with the oxygen analysis as, on a rough aluminum surface, it is about 90Å thick, having a specific gravity of 3, and thus contains only 3×10^{-3} μg of O^{18} per cm^2.

Pure aluminum and reactor-grade tantalum were tested in a preliminary bombardment. In addition O^{18}-containing spots were applied to the metal sheets. The reason for testing the aluminum in spite of its impurities was that it is an inexpensive and easily available material, compared to tantalum.

For the first bombardment a stationary beam and sample at the 60-inch cyclotron was used. The beam covers an area of about 0.2 by 1 inch. Each target was bombarded 4 minutes with 4.5-Mev protons at 10.5 μa.

Aluminum Target

The O^{18} was applied to the aluminum in the form of barium aluminate prepared from O^{18}-rich water by dissolving barium to a known concentration in such water and putting a known amount of this hydroxide solution onto the target, which was then heated in a vacuum oven in order to evaporate the water without exchange with the water molecules in the air. Two spots of this barium aluminate, each containing about 10 μg of O^{18}, were applied to the plate together with a spot of barium aluminate prepared from ordinary water.

After the bombardment the target showed a very high radioactivity even after an again period of two hours (about 150 mr/hr at a distance of 1 cm). No significant difference could be found between the area with O^{18}-rich barium aluminate and the area with ordinary barium aluminate. Twenty-four hours after the bombardment about one-third of the radioactivity measured after 2 hours' aging still remained. One of the aluminate spots was washed off the target, and the radioactivity of the target was then measured. It remained

the same as before washing off the spot, which shows that the radioactivity originated from impurities in the aluminum.

Tantalum Target

In order to obtain O^{18}-enriched organic material, Chlorella pyrenoidosa was grown at 24°C, with shaking, for 2 days in O^{18}-enriched water. One ml of ordinary Myers medium* was evaporated to dryness in a 5-ml volumetric flask. One ml of 20% O^{18} water† was added to the salts and inoculated with 10% of algae suspension. Dry 4% CO_2 in air was supplied to the culture at a pressure somewhat over 1 atmosphere in a closed vessel. Twice during the growth period the gas in the flask was flushed and renewed. The algae were centrifuged and washed twice with ordinary distilled water. Seventy-two μg of the algae (dry weight), with a calculated O^{18} content of about 4 μg, were applied in a water suspension to a tantalum sheet as a drop, which was then evaporated.

After aging of the bombarded target for 2 hours, the radioactivity at a distance of 1 cm was 5 mr/hr. An autoradiogram taken of the plate after 2.5 hours showed a black spot where the algae had been applied and a slight dark shadow on the whole area where the beam had been hitting the target. Figure 3a shows the developed film. The exposure time was 12 minutes.

A γ-ray spectrum in the lower energy range was then taken of the target by means of a hundred-channel pulse-height analyzer. The result is shown in Fig. 4. From this figure it can be seen that there is a pronounced peak at 0.51 Mev, the energy of annihilation radiation for positrons. This peak could thus correspond to F^{18}, which is a β^+-emitter. There are also other peaks.

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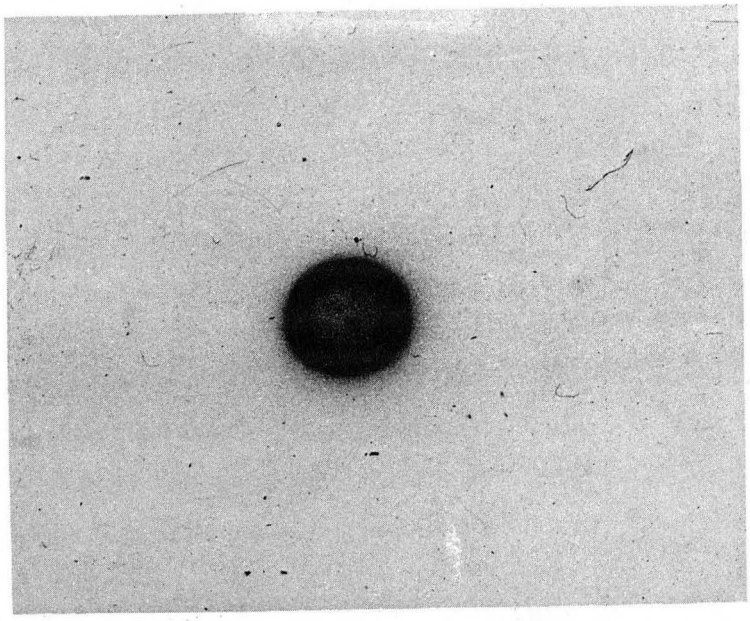
*Composition 12 mM KNO_3, 10 mM $MgSO_4 \cdot 7H_2O$, 8 mM KH_2PO_4, 0.1 mM Ca $(NO_3)_2$, 0.11 mM Fe-EDTA, 0.5 ppm B, 0.5 ppm Mn, 0.05 ppm Zn, 0.02 ppm Cu, 0.01 ppm Mo, and 0.01 ppm Co. Pyrex-redistilled water was used throughout.

†This water was analyzed by Melvin P. Klein of UCRL, Livermore, and found to contain 58% D. Experiments by Vivian Moses in our laboratory have shown that deuterium content of the water as high as 60% slows down the rate of photosynthesis somewhat and only slightly modifies the metabolic pattern as determined by incorporation studies.



ZN-1708

Fig. 3a 72 μg (dry weight) of H_2O^{18}-grown algae on a tantalum plate bombarded 4 minutes with 4-Mev protons at 10.5 μa. The autoradiogram was taken 2-1/2 hours after the bombardment. Exposure time, 12 minutes.

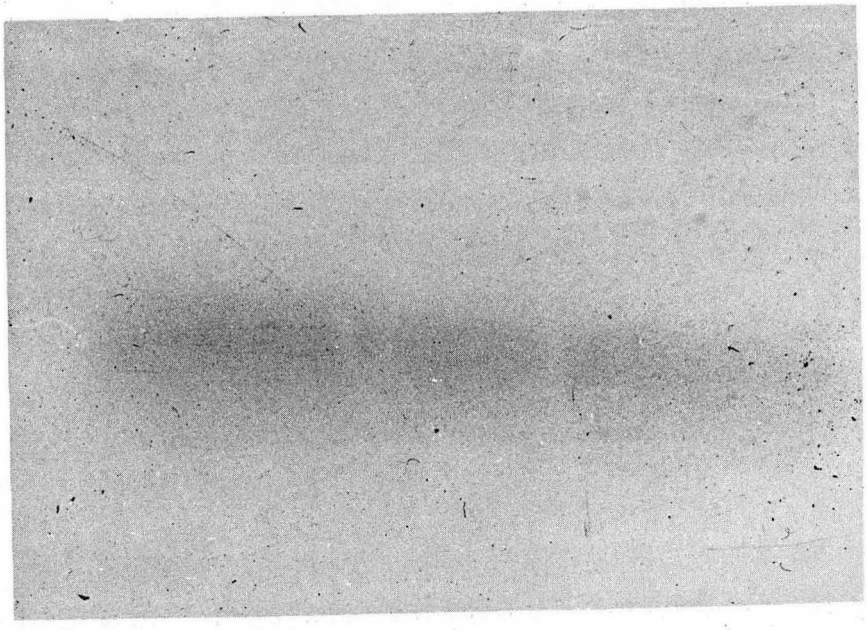


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Fig. 3b Radioautogram of target, starting 26 hours after the bombardment. Exposure time, 7 hours.

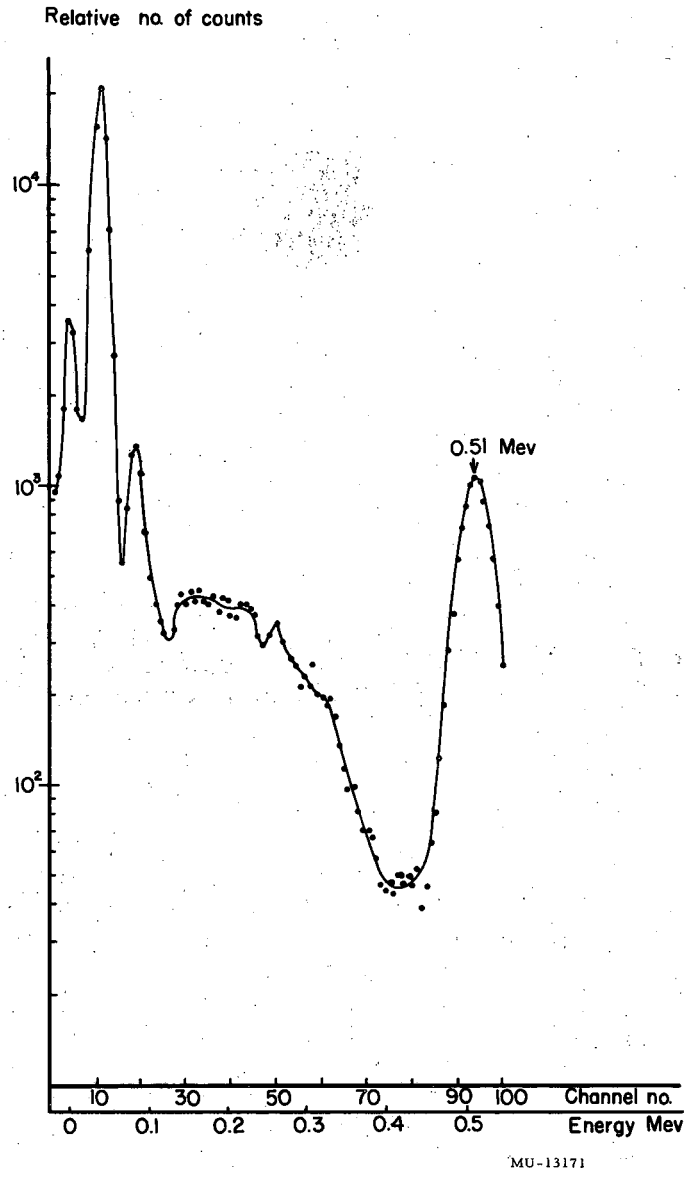

Fig. 4. Energy spectrum taken by a 100-channel X-ray pulse-height analyzer of a proton-bombarded tantalum target with a spot of O^{18}-rich green algae.

The part of the plate containing the algae was then cut out from the rest of the plate and the new energy spectra were taken, which showed that the only peak derived from the algae alone was the 0.51-Mev peak. The half life of the material forming this peak was measured and found to be 1.8 ± 0.1 hours (Fig. 5), which agrees with the half life of F^{18}, 1.8 hours. One of the half lives of the radioactivity coming from the tantalum was about 6 hours.

After the energy measurements the target was put together again and on the next day autoradiographed for 7 hours. The film, which is pictured in Fig. 3b, shows that part of the radioactivity in the tantalum plate itself is still left while all activity from the algae has disappeared. This agrees with the results obtained by the half-life measurements.

Target Holder and Target Sheet

As the activation analysis method of tracing O^{18} was developed in order to analyze paper chromatograms of a length of about 10 inches, a target plate of 0.006-inch-thick tantalum sheet about 10 inches long and 2 inches wide was used; it was rotated in front of the proton beam. A holder for the target was constructed, in which the tantalum strip was held as a cylinder. On one edge of the target, strip holes were drilled, and screws held the strip to the holder which was a round lucite plate. Thus one edge of the tantalum strip was fastened to the holder, the other side was free. Inside the cylindrical strip a jet of air was directed at the target point for cooling. The proton beam was collimated into a cross-sectional area 3/4 by 1/4 inch.

In the experiments described in the following sections, the total bombardment time was 2 hours with 4.5 ± 0.2-Mev protons. The average current during this time was 2.75 μa. The target was rotated in front of the beam at 160 rpm. As the length of the target was about 10 inches and the width of the beam 1/4 inch, every spot was exposed to the beam for a total of 3 minutes. Sufficient air was blown onto the inner side of the target area to dissipate the heat.

Sensitivity

The experiment just described showed that 72 μg of O^{18}-enriched algae containing 4 μg of O^{18} could be detected on an autoradiogram of a tantalum target after bombardment with protons. The next experiment was planned to give information on how small an amount of O^{18} could be detected by activation

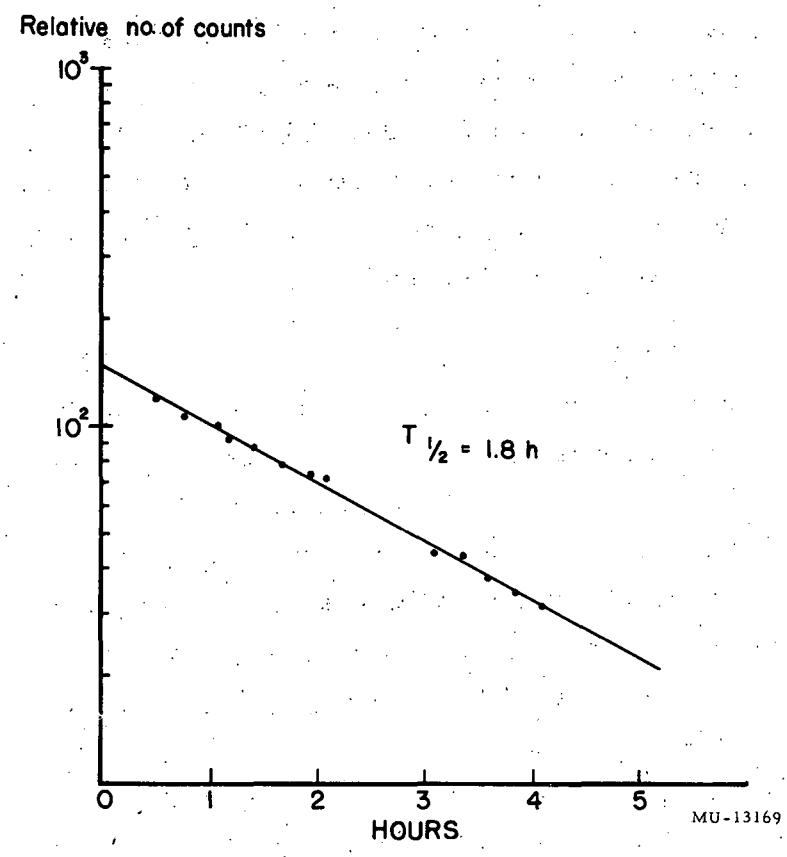

Fig. 5. Activity of the 0.51-Mev peak from green algae bombarded with protons, as a function of time.

and autoradiography. Elution and bombardment of a chromatogram of an extract from a photosynthesis experiment in O^{18}-rich water was also planned.

To test the sensitivity of the O^{18} analysis, Chlorella pyrenoidosa was grown in O^{18}-rich water for 2-1/2 days. The algae were centrifuged and washed twice with distilled water. Five samples from a suspension of the algae in distilled water were evaporated as spots on the area of the target sheet. The amounts of algae in the different spots were 85, 15, 7.5, 4, and 2 μg, and the amounts of O^{18} were 5, 1, 0.5, 0.2, and 0.1 μg, respectively.

A control sample of algae (actually a sample from the inoculating suspension) was also centrifuged and washed twice with distilled water. Three samples from a suspension of this algae were evaporated as spots on the target sheet. The amounts of algae in the three control spots were 40, 4, and 2 μg.

Figure 6 shows the autoradiograms from the several spots. The spot containing 2 μg of algae and 0.1 μg of O^{18} can still be detected against the background. A shorter exposure time would probably favor the contrast between the spot and the background. The autoradiograms of the spots of ordinary algae show that 40 μg of algae gives rise to a very slight black spot. The autoradiograms of the two smaller amounts of ordinary algae show no black spot at all. The slight spot from 40 μg of algae might be due to the F^{18} formed from the 0.2% of O^{18} in the ordinary oxygen of the algae, which would be $\sim$0.04 μg of O^{18}. Spots 6f and 6g have comparable contrast.

Preliminary Photosynthetic Experiment

In the photosynthesis experiment 250 λ of packed cells of Chlorella pyrenoidosa was suspended in 4 ml of O^{18}-rich water (20% O^{18}). The suspension was allowed to photosynthesize for 23 minutes and then centrifuged. The supernatant liquid was withdrawn and the algae were killed with 5 ml of hot 80% ethanol in water. The suspension was extracted for about 1 hour, after which time it was centrifuged and the liquid phase withdrawn. The algae were also extracted two times with 20% ethanol in water at 60°C for 20 minutes. The different extracts were combined and concentrated to almost dryness under vacuum in a Craig apparatus. The residue was taken up in 2 ml of 40% ethanol and centrifuged. The centrifugation gave a very small amount of solid substance on the botton of the

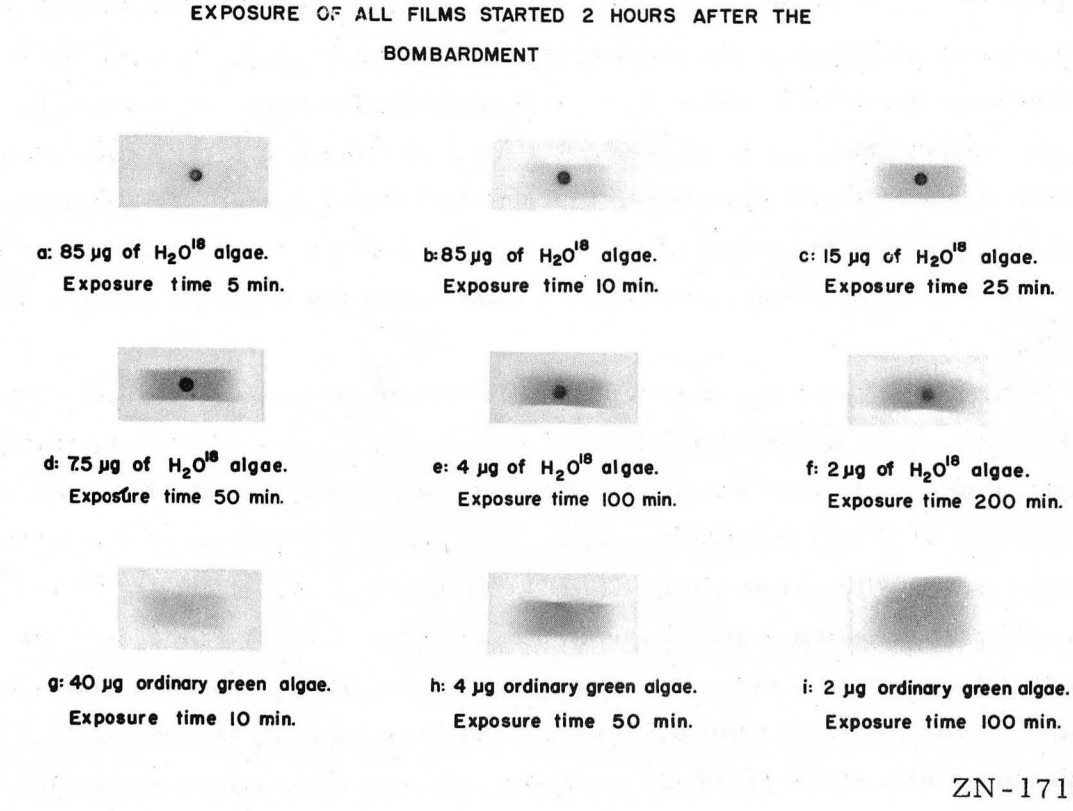

Fig. 6. Autoradiograms after proton bombardment of different amounts of O^{18}-enriched and ordinary green algae. Exposure of all films started 2 hours after the bombardment.

centrifuge tube. An aliquot of 0.4 ml of the solution was applied to a paper which was then developed in butanol–propionic acid for about 6 hours. The chromatogram, which was 13-1/2 inches long, was dried and the first 6 inches of it eluted onto a tantalum target sheet by the method described earlier in this report.

One hour and ten minutes after the bombardment, the radioautography of the target was begun. A variety of exposures were made after different decay periods. A selection of these is shown in Fig. 7. It can be seen that in the first exposure in Fig. 7 there is still a rather strong activity in the tantalum sheet. No such strong activity can be seen at the exposure taken 3 hours after the bombardment, which means that in addition to the long-lived activity there is radioactivity induced in the tantalum with a very short half life. Therefore, the tantalum target should be aged for 2 to 3 hours before autoradiograms are made of it. As can be seen from Fig. 7, the best contrast is achieved 2 to 4 hours after the proton bombardment. The eluted chromatogram shows three peaks of radioactivity which were not identified but corresponded to the mono- and diphosphate and phosphoglyceric acid areas on a paper chromatogram, as they usually appear in $C^{14}O_2$ fixation experiments.

An autoradiogram was also taken 18 hours after the bombardment as a test that very little activity remained in the spots. The exposure time was 7 hours. The developed film shows extremely slight black spots, a fact that quite agrees with the fact that the half life of F^{18} is 1.8 hours.

Summary

A method has been devised for tracer studies with oxygen using paper chromatography and proton activation of oxygen-18 to fluorine-18. This technique is relatively simple and can detect a fraction of a microgram of O^{18}, as well as giving some information about the chemical nature of the compound into which it is incorporated. The procedure has been applied to a preliminary study of the path of oxygen in green algae. Three short-term photosynthesis products containing O^{18} were observed.

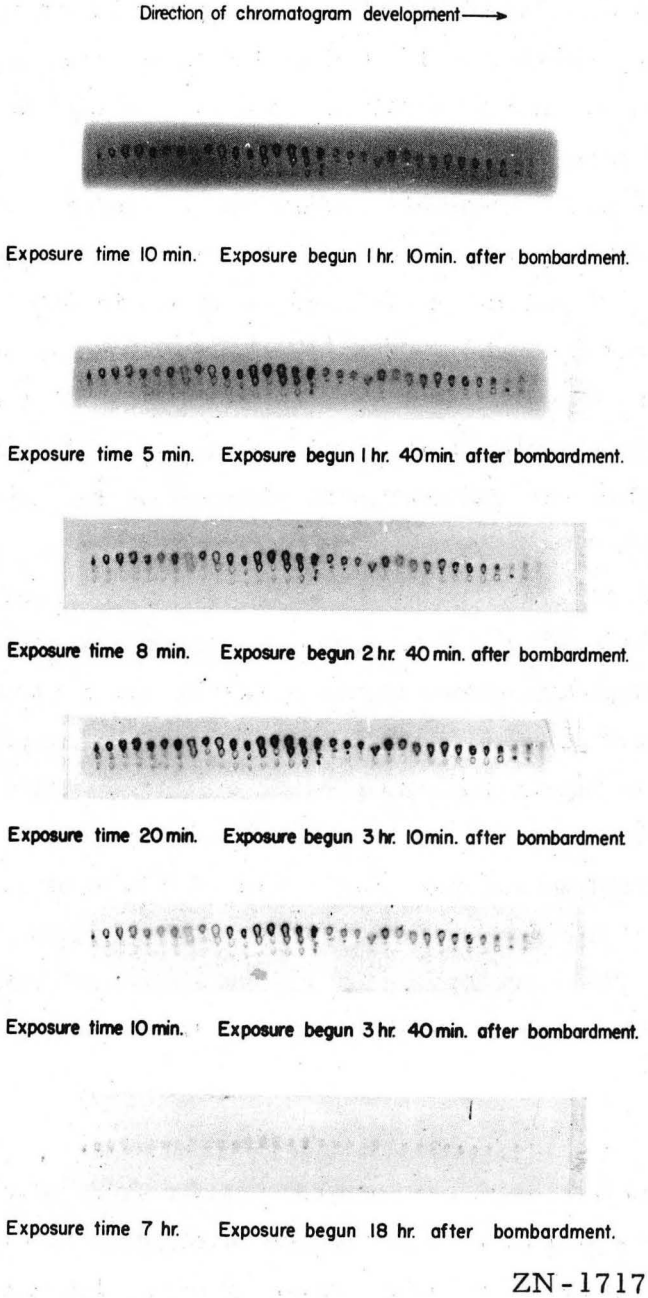

Fig. 7. Autoradiograms of a proton-bombarded tantalum target containing a transferred paper chromatogram prepared from algae grown for 20 minutes in O^{18}-enriched water.