Phytochemistry, 1970, Vol. 9, pp. 25 to 32. Pergamon Press. Printed in England

CARBONIC ANHYDRASE AND CO_2 FIXATION IN ISOLATED CHLOROPLASTS

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(*Received* 6 *May* 1969)

Abstract—Evidence that carbonic anhydrase is essential for full photosynthetic activity of spinach chloroplasts is given and the probable role of carbonic anhydrase in photosynthesis discussed. Distribution of the enzyme within chloroplasts is consistent with the view that both the formation of CO_2 from bicarbonate ions at the chloroplast surface and a further reaction of CO_2 or bicarbonate within the chloroplast must be catalysed by carbonic anhydrase in order to obtain maximum rates of photosynthesis. Photosynthesis is partially inhibited by 1 mM diamox (5-acetamido-1,3,4-thiadiazole-2-sulphonamide) and nitrite. Spinach chloroplast carbonic anhydrase is found to be inhibited by diamox, nitrate, nitrite, and halide ions.

INTRODUCTION

IN 1936 BURR pointed out that the rate of hydration of CO_2 in leaves would be inadequate to account for the observed rate of photosynthesis and suggested that either carbonic anhydrase must operate in photosynthesis, or CO_2 must be assimilated without first combining with water.[1] Although Burr was unable to detect carbonic anhydrase it was soon found to be present in leaves. At first thought to be a cytoplasmic enzyme,[2] carbonic anhydrase was recently shown to be a chloroplast enzyme in Calvin cycle plants.[3]

The possibility that carbonic anhydrase may regulate the rate of photosynthesis is indicated by the report that activity of this enzyme increases sharply when *Chlorella* is adapted to low levels of CO_2;[4] while the likelihood that carbonic anhydrase plays an essential role in CO_2 assimilation, as Burr suggested, has been demonstrated by the inhibition of photosynthesis in *Ulva pertusa*[5] and spinach chloroplast preparations[6] by diamox, a specific inhibitor of the carbonic anhydrase from animal sources.[7]

Photosynthesis by isolated chloroplasts in the presence of inhibitors of carbonic anhydrase and the distribution of the enzyme within chloroplasts have been further investigated and are here reported in detail.

RESULTS

Inhibition of Carbonic Anhydrase by Sulphonamides and Inorganic Anions

Carbonic anhydrase was partially purified from spinach chloroplasts and the effect of various compounds on the activity of the enzyme assayed as described. Controls lacking

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[3] R. G. EVERSON and C. R. SLACK, *Phytochem.* **7**, 581 (1968).
[4] M. L. REED and D. GRAHAM, *Plant Physiol.* **43**, S29 (1968).
[5] M. IKEMORI and K. NISHIDA, *Physiol. Plantarum* **21**, 292 (1968).
[6] R. G. EVERSON, *Nature*, in press (1969).
[7] H. A. KREBS, *Biochem. J.* **43**, 525 (1948).

carbonic anhydrase were run in all cases. The concentration required for 50% inhibition is tabulated in Table 1. No inhibition was detected with sulphanilamide (10 mM) nor with sulphate or phosphate (as sodium or potassium salts) up to 100 mM. The ranges of inhibition of diamox, nitrate, nitrite, iodide and chloride are shown in Fig. 1.

Although it was only 2% as inhibitory to the plant enzyme as it was to human erythrocyte carbonic anhydrase,[8] diamox was the most inhibitory of the sulphonamides tested and was equally effective in crude (see open circles, Fig. 1) and partially purified extracts, giving 100% inhibition at approximately 1 mM. Of the other inhibitors of erythrocyte carbonic anhydrase tested, *p*-toluenesulphonamide (reported to be of approximately equal potency to diamox[8]) and sulphanilamide (about 2% as effective) failed to inhibit the plant enzyme to any great extent. The result with sulphanilamide confirms previous observations on the resistance of

TABLE 1. INHIBITION OF CHLOROPLAST CARBONIC ANHYDRASE BY SULPHONAMIDE AND INORGANIC ANIONS

Inhibitors	Concentration for 50% inhibition of carbonic anhydrase activity (mM)
1. Sulphonamides:	
Diamox	0·02
P-Toluenesulphonamide*	8
1-Amino-3-chloro-4,6-benzenedisulphonamide*	2
2-Amino-4-chlorobenzenesulphonamide*	1
2. Inorganic anions:	
Azide	0·05
Nitrite	0·4
Nitrate	0·4
Iodide	0·5
Chloride	10

* Prior incubation 10 min at 20° before assaying at 0°.

carbonic anhydrase from higher plants (cf. *Ulva*[5]) to sulphonamides,[9, 10] but the chloroplast enzyme is approximately five times as sensitive to inhibition by chloride and iodide and fifty times more sensitive to inhibition by nitrate than are the enzymes from human erythrocytes.[11] The inhibitory effect of chloride is noteworthy in view of the use of high concentrations of NaCl in common procedures for isolating chloroplasts from leaf homogenates.[12]

Inhibition of carbonic anhydrase by nitrite does not appear to have been reported previously.

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[9] J. R. G. BRADFIELD, *Nature* **159**, 467 (1947).
[10] P. M. SIBLY and J. G. WOOD, *Australian J. Sci. Res.* **B4**, 500 (1951).
[11] J. A. VERPOORTE, S. MEHTA and J. P. EDSALL, *J. Biol. Chem.* **242**, 4221 (1967).
[12] M. GIBBS, E. LATZKO, R. G. EVERSON and W. COCKBURN, in *Harvesting the Sun, Photosynthesis in Plant Life* (edited by A. SAN PIETRO, F. A. GREER and T. J. ARMY), p. 111, Academic Press, New York and London (1967).

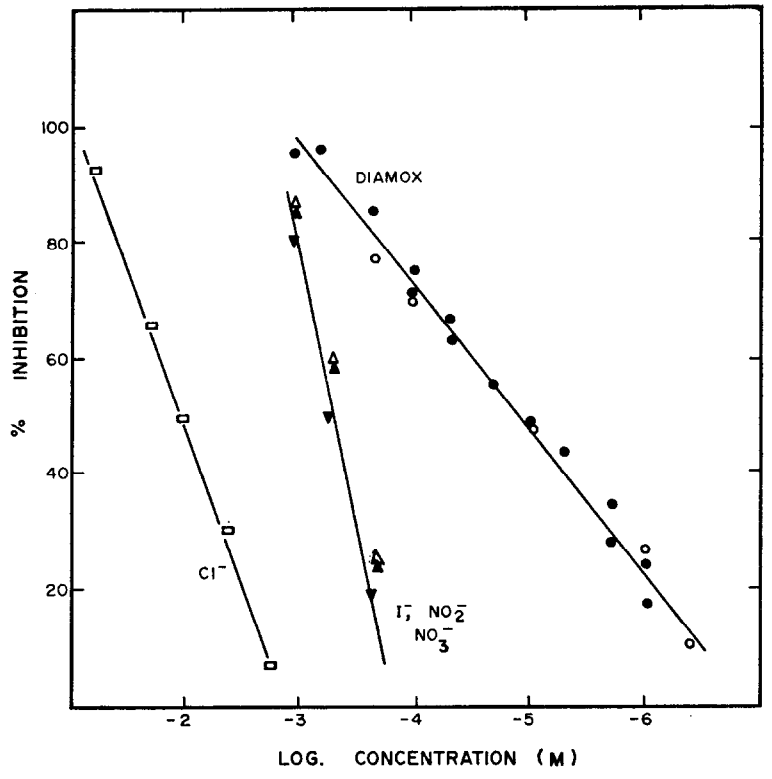

FIG. 1. INHIBITION OF CARBONIC ANHYDRASE BY DIAMOX (●,○), NITRITE (▲), NITRATE (△), IODIDE (▼) AND CHLORIDE (□).

The enzyme preparation was partially purified as described in the Experimental section, except in a few diamox assays shown in the graph as (○) where crude supernatant was used.

Inhibition of Photosynthesis in Spinach Chloroplasts

Diamox partially inhibits photosynthesis by isolated chloroplasts. Figure 2 shows that CO_2 fixation from 1 mM $NaHCO_3$ is inhibited by both diamox and azide but not by sulphanilamide. Azide at the concentration used inhibits carbonic anhydrase,[2,9] but it also inhibits other photosynthetic reactions.[13] The fact that sulphanilamide did not inhibit photosynthesis confirms that, apart from a possible effect on carbonic anhydrase, sulphonamides are not necessarily toxic to chloroplasts, whatever their effects may be on growth.[14]

Another inhibitor of carbonic anhydrase, nitrite, was subsequently shown to inhibit photosynthesis when present at a concentration of 1 mM. As in the case of diamox, inhibition by nitrite could be relieved by raising the bicarbonate concentration. In Fig. 3 diamox and nitrite are seen to inhibit photosynthesis in the presence of 0·5 mM $NaHCO_3$ but diamox is not inhibitory, and nitrite inhibition (after a lag) is much reduced when the concentration of $NaHCO_3$ is tenfold higher. Such a reversal of inhibition by high substrate levels suggests that either the inhibition of carbonic anhydrase is competitive, or that its uncatalysed reaction is no longer rate-limiting for photosynthesis, when the substrate level is 5 mM. Although

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inhibition in the case of nitrite may be partly due to competition between nitrite reductase and ferridoxin NADP reductase for reducing power[15] the similarity between the effects of nitrite and diamox suggests that both inhibitors probably act via the same enzyme, carbonic anhydrase, for which the inhibition by diamox is known to be highly specific. The possibility arises that interaction occurs between photosynthetic carbon fixation and nitrate metabolism at this point.

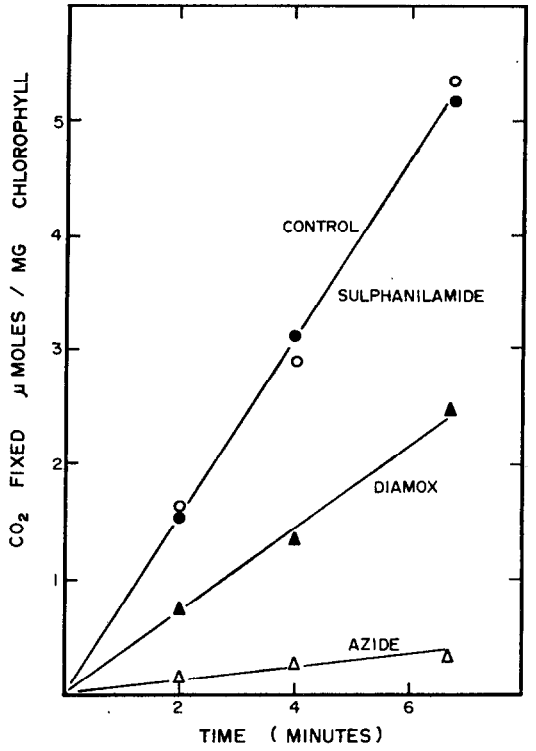

FIG. 2. INHIBITION OF PHOTOSYNTHESIS IN ISOLATED CHLOROPLASTS BY DIAMOX AND AZIDE BUT NOT BY SULPHANILAMIDE (ALL 1 mM).

Spinach chloroplasts were exposed to the inhibitors for 2 min in the light before completing the reaction mixture with 1 mM $NaHCO_3$ (containing C^{14}).

In twelve assays using 1 mM bicarbonate, inhibition by diamox was found to be independent of the rate of photosynthesis between 20–100 μmoles CO_2 fixed/mg chlorophyll (inhibition = 49 ± 8 per cent). At lower rates of photosynthesis little or no inhibition was found. While these results may reflect a varying percentage of active chloroplasts from preparation to preparation,[12] because there is a constant fraction of photosynthesis inhibited it is possible that part of the uptake of CO_2 or bicarbonate is not controlled by the diamox-sensitive carbonic anhydrase. Although diamox-insensitive carbonic anhydrase[16] has been reported in parsley, such activity could not be detected in spinach leaf extracts.

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[16] K. S. FELLNER, *Biochem. Biophys. Acta* **77**, 155 (1963).

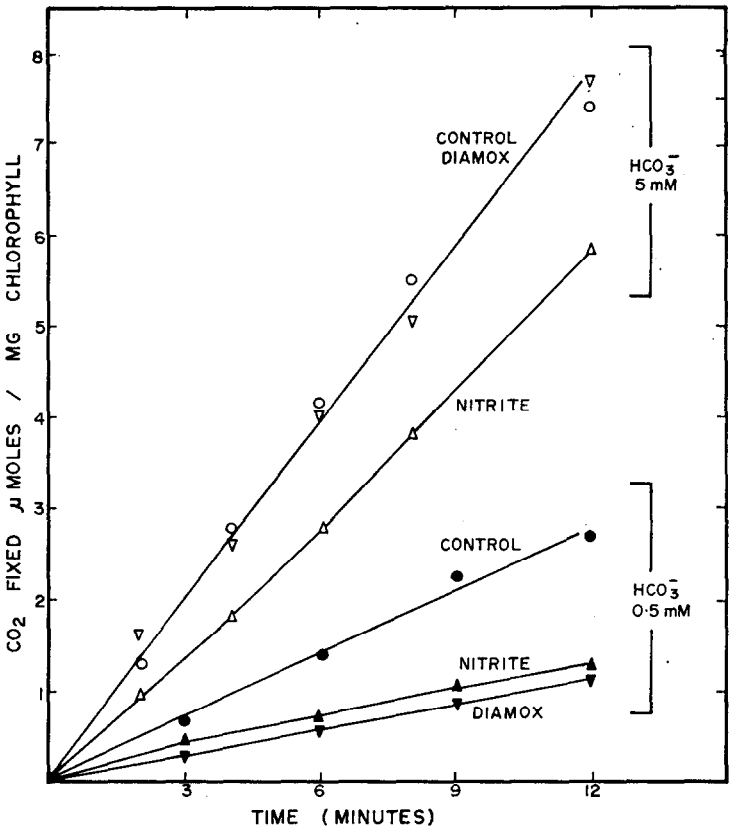

FIG. 3. BICARBONATE-DEPENDENCE OF INHIBITION BY DIAMOX AND NITRITE (BOTH 1mM) OF PHOTOSYNTHESIS IN ISOLATED CHLOROPLASTS.

Conditions as for Fig. 2.

Carbonic Anhydrase at the Chloroplast Surface

Gibbs *et al.*[12] have suggested that the chloroplast membrane may contain "the enzyme mechanism for setting the pace for CO_2 fixation". In order to detect whether carbonic anhydrase is present at the chloroplast surface where it could mediate the uptake of CO_2, a series of enzyme assays were carried out with lysed and intact chloroplasts in osmotically buffered solutions. The standard assay was modified by the inclusion of 300 mM sorbitol in both the veronal buffer and the saturated CO_2 solution. Equal samples of filtered leaf homogenate yielded equal amounts of chloroplasts after centrifuging. Prior to assay for carbonic anhydrase activity these chloroplasts were washed once in the preparation medium and resuspended in buffer or in buffer–sorbitol solutions at 0°. Assays on equivalent volumes (0·1–0·2 ml) of these suspensions, performed in the veronal–sorbitol reaction mixture showed that less than one-fifth of the total carbonic anhydrase of the chloroplasts could be detected prior to lysis (Table 2). Preparations from Swiss chard (*Beta vulgaris*) and lettuce (*Lactuca sativa*) leaves gave similar results.

Chloroplasts were also assayed in sorbitol in the presence and absence of the non-ionic detergent Triton X or chloroform. Results confirmed that the chloroplasts had to be fragmented before total carbonic anhydrase could be estimated (Table 3).

TABLE 2. DISTRIBUTION OF CARBONIC ANHYDRASE IN CHLOROPLASTS

Source of chloroplasts	Carbonic anhydrase activity	
	% on intact chloroplasts	% released by lysis
Spinacea oleracea	11·3	88·7
	10·2	89·8
	8·8	91·2
	14·4	85·6
	7·6	92·4
Beta vulgaris	15·0	85·0
Lactuca sativa	6·1	93·9

TABLE 3. RELEASE OF CARBONIC ANHYDRASE BY DISRUPTION OF SPINACH CHLOROPLASTS

Treatment	Carbonic anhydrase activity (units/mg chlorophyll)
(*a*) Control (chloroplasts intact)	850
(*b*) Sorbitol omitted from suspension (chloroplasts lysed)	7500
(*c*) 0·01% Triton X-100	5800
(*d*) 1% Chloroform	7100

Aliquots of chloroplast suspension were incubated in the assay mixture (veronal buffer+300 mM sorbitol etc.) 3 min at 0° before addition of CO_2-saturated sorbitol solution to complete the assay.

Retention of Carbonic Anhydrase by Isolated Chloroplasts

In order to assess how tightly carbonic anhydrase was bound to chloroplasts, enzyme activity diffusing from washed chloroplasts incubated at 20° was compared with the total

TABLE 4. RETENTION OF CARBONIC ANHYDRASE BY ISOLATED SPINACH CHLOROPLASTS INCUBATED AT 20°

Time incubated (min)	Carbonic anhydrase activity in supernatant (units/mg chlorophyll)	% Carbonic anhydrase activity retained in chloroplast
0	465	94·1
2	650	91·8
4	720	91·0
8	800	90·0
16	890	88·8
32	1005	86·9

Chloroplasts were prepared, washed and resuspended as described in the Experimental section except that all operations were carried out at pH 6·5 and 1 mM dithiothreitol was included in the final suspension. 1 ml replicate samples of the suspension were transferred to centrifuge tubes for incubation at 20°. After incubation samples were centrifuged at 3000 *g* for 3 min at 2° and the clear supernatant assayed for carbonic anhydrase activity as described. The zero time samples were not transferred to 20°. Pellets lysed in 1 ml 1 mM DTT, contained 8000 units of activity before and after incubation.

activity in replicate samples (Table 4). Carbonic anhydrase proved to be a structurally secure component and little of the enzyme dissociated from the chloroplasts, perhaps only that fraction normally assayed in intact chloroplasts being lost. Indeed, carbonic anhydrase retention could be assayed for an indication of chloroplast integrity.

DISCUSSION

Carbonic anhydrase catalyses the reaction of CO_2 with water, which at pH values above 6[17] can be represented as follows:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

Seeing that the substrate for the diamox-sensitive fraction of carboxylation must have passed through at least one step involving this reaction, the following possibilities arise: (i) If CO_2 is the immediate substrate for ribulosediphosphate carboxylase the rate of carboxylation may be limited by the rate of formation of CO_2 from HCO_3^-, (ii) If HCO_3^- rather than CO_2 is the substrate of ribulosediphosphate carboxylase there would appear to be no requirement for the participation of the above reaction, unless chloroplasts are relatively impermeable to HCO_3^-. If CO_2 forming outside the chloroplasts diffuses in and is converted back to bicarbonate inside the chloroplasts,[6] the rate of carboxylation could be limited by the rate of the reaction in either direction.

The maximum uncatalysed rates of both the formation of CO_2 in the reaction mixture and the formation of HCO_3^- from CO_2 within the chloroplasts can be calculated from the following equation, for which Magid and Turbeck[18] have evaluated the constants for a range of pH and temperature values:

$$-d(HCO_3^-)/dt = k_d'(HCO_3^-) - k_h'(CO_2)$$

If it is assumed that all CO_2 formed is used in photosynthesis directly, the maximum rate of CO_2 formation in 1 ml reaction mixture under the experimental conditions (1 mM HCO_3^-, 20°) is 2·03 μmoles/hr. (With 0·1 mg chlorophyll present, a limit on the possible rate of CO_2 fixation is thus set at 20·3 μmoles/mg chlorophyll/hr).

On the other hand, if it is assumed that HCO_3^- and CO_2 are at equilibrium in the reaction mixture, the CO_2 concentration under the conditions stated can be evaluated from the above equation. At equilibrium

$$\begin{aligned}(CO_2) &= (HCO_3^-)k_d'/k_h' \\ &= 17{\cdot}1\ \mu M\end{aligned}$$

and the maximum rate of bicarbonate formation in a volume of chloroplasts equivalent to 1 mg chlorophyll is 0·93 μmole/hr (0·25 ml chloroplasts are approximately equivalent to 1 mg chlorophyll[19]). Seeing that control rates of photosynthesis in the chloroplast preparations used exceeded the theoretical limits imposed by these spontaneous reactions, direct assimilation of HCO_3^- must have proceeded to some extent, or else catalysis of both CO_2 formation at the chloroplast surface and of bicarbonate formation within the chloroplast (if HCO_3^- is the carboxylation substrate) must have occurred. The experimental finding that carbonic anhydrase is present both on the surface and inside the chloroplast is consistent with the need for catalysed interconversion of CO_2 and HCO_3^- at both sites.

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EXPERIMENTAL

Materials

Spinach, lettuce and Swiss chard were grown in vermiculite culture under controlled conditions. Diamox (Lederle Laboratories Division, Cyanamid Inc.) was used as the Na salt, pH 8·5. *P*-Toluenesulphonamide (Koch-Light Laboratories Ltd.), 2-amino-4-chlorobenzenesulphonamide and 1-amino-3-chloro-4,6-benzene-disulphonamide (K. & K. Laboratories Inc.) were recrystallized from ethanol and dissolved at pH 8. HEPES (*N*-2-hydroxyethylpiperazine-N^1-2-ethanosulphonic acid) was obtained from Sigma Chemical Co., and MES [2-(*N*-morphilino) ethanesulphonic acid] and Tricine [N-tris(hydroxymethyl) methyl glycine] from Calbiochem Inc. For inhibition studies solutions were prepared immediately prior to test.

Methods

Carbonic anhydrase assay. The Wilbur–Anderson veronal-indicator method described by Rickli *et al.*[20] was modified to be carried out in 2·5 ml polypropylene syringe barrels in an ice bath. 1 ml 0·025 mM veronal buffer, pH 8·2, containing 10 mg/100 ml bromothymol blue was introduced. Additions, including an appropriate volume of extract were made with a microsyringe through the tip. Following temperature equilibration, 1 ml saturated CO_2 solution at 0° was added rapidly from a coarse-needled syringe, leaving only a small bubble of air as void space in the vessel: this bubble was used for stirring. The time from addition of the CO_2 to attainment of a yellow tint was recorded from a stop-watch: a minimum of 10 sec was acceptable. Times for samples (t_e) and appropriate controls (t_b) were determined in duplicate. The uncatalysed rate of the reactions was usually about 100 sec^{-1}. Carbonic anhydrase activity was expressed as units of activity calculated from the formula $U = 10[(t_b/t_e) - 1]$.

Preparation of Chloroplasts

The method of Jensen and Basshan[21] was followed with slight modifications. Chopped leaf laminae were disintegrated at 2° in a Servall "Omnimix" for a few seconds in 3 vol. of a medium that contained 50 mM Na-MES, pH 6·5, 330 mM sorbitol, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 500 μM KH_2PO_4. Chloroplasts were obtained by centrifuging the extract after filtering through two layers of wetted Miracloth (Chicopee Mills Inc., N.Y.), and were either washed once in the same medium or re-suspended directly in a similar medium but with HEPES replacing MES. NaCl was omitted.

Preparation of Chloroplast Extract and Partially Purified Carbonic Anhydrase

Chloroplasts prepared as described were lysed at pH 8·2 in 0·01 M veronal or Tricine for 5 min at 2° and centrifuged at 4000 *g* for 15 min. This extract was quite stable without reductant. Partially purified carbonic anhydrase was obtained by passing an aliquot of chloroplast extract over Sephadex G75 equilibrated with the buffer.

Measurement of Photosynthesis

An aliquot of chloroplast suspension (50–100 μg chlorophyll) was transferred to 1 ml of a similarly constituted reaction mixture at pH 8 containing 5 mM sodium pyrophosphate. 1 mM $NaHCO_3$ containing $NaHC^{14}O_3$ was now added.

The reaction mixture was contained in 2·5 ml polypropylene syringe bodies clipped to a rotating "perspex" wheel in uniform light of 3000 ft-candles in a fumehood. Air was excluded from the syringes and stirring proceeded via the oscillation of a glass bead approx. 4 mm in dia. Additions and sampling were carried out with microsyringes. Temperature was 20 ± 1°. Samples (0·03 ml) were placed directly into drops of 4 N formic acid on polyethylene sheet in the fumehood. The drops were transferred by contact to strips of Whatman No. 1 chromatography paper dried in a forced air oven at 65° before β-counting with an end-window Geiger tube and ratemeter. The method was empirically calibrated.

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