A STUDY OF CARBONIC ANHYDRASE IN PLANTS

By

Eugene Hans Lucas

A THESIS

Submitted to the School of Graduate Studies of Michigan
State College of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

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AN ABSTRACT

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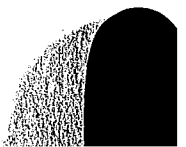
Approved G. P. Steinbauer



Soon after the discovery of the enzyme carbonic anhydrase in red blood cells in 1932 a search for a similar enzyme in plants was undertaken by a number of investigators. Until 1946 several publications appeared which disagreed among each other concerning the evidence of presence of such an enzyme in plants. Early in 1947 independent work at Oxford University and at Michigan State College led to the establishment of evidence of the presence in most green plants tested of an enzyme with carbonic anhydrase characteristics. The Oxford findings were published in 1947, the work at East Lansing was first reported at the annual meeting of the Michigan Academy of Science, Arts, and Letters in 1948.

During the following years the distribution of this enzyme in green plants and its properties were studied extensively. It was found that the enzyme is a regular constituent of the leaf tissues of green plants where it occurs in varying amounts. Some plants contain a high concentration of the enzyme in leaf extracts, others less. Only five out of 47 species investigated did not give any evidence of the presence of the enzyme. It is considered likely that shortcomings of technique were responsible for the failure to demonstrate the enzyme in these species. Leaves of the plants tested contained the enzyme in varying amounts according to their position on the plants; the greatest amounts were found in fully developed leaves. Meristematic tissues always showed evidence of increased

enzyme activity. Green parts other than leaf tissues contained varying, but always minute, amounts of the enzyme. Some flower extracts produced an activity which indicated the presence of the enzyme but roots were always devoid of it.

In vitro studies of the kinetics of the enzyme showed that it catalyzed the dehydration of carbonic acid as well as the hydration of carbon dioxide. It is this latter reaction which had led to speculations about the participation of the enzyme in a phase of the photosynthetic process. The kinetic studies which demonstrated dependence of the enzyme action on hydrogen ion concentration, temperature and substrate concentration produced a similarity with photosynthetic requirements insofar as both reactions take place within the same pH ranges (6.0 to 8.0); their temperature coefficients vary between 1.0 and 2.0 depending on the temperature range used for the calculation; and they cease to take place at temperatures above 50° C.

The enzyme is inhibited by cyanide which also inhibits CO_2 fixation in photosynthesis. Other inhibitors which have been shown to affect the animal enzyme, as sulfanilamide, did not have marked effect on the enzyme activity in vitro. No interference with the enzyme activity by 2,4-dichlorophenoxyacetic acid was observed.

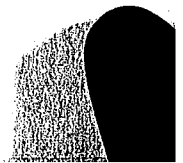
It was possible to purify the enzyme although its sensitivity to exposure to air and temperatures above 15° C is considerable and increases with its state of purification.

Chromatographic adsorption of an aqueous extract digested with papain produced a fraction of high enzyme activity having considerable stability when stored at low temperature.

ACKNOWLEDGMENT

The writer wishes to express his indebtedness to the members of his guidance committee, Drs. W. B. Drew and G. P. Steinbauer of the Department of Botany and Plant Pathology, Dr. R. U. Byerrum of the Department of Chemistry, Dr. C. L. Hamner of the Department of Horticulture, and Dr. H. J. Stafseth, representative of the Graduate Council.

Special appreciation is due to Dr. Steinbauer, Chairman of the Guidance Committee, and Dr. Byerrum who collaborated with the author in several phases of the investigation and permitted the inclusion in this thesis of results published in a joint paper under his senior authorship. The author is grateful to Mrs. Ardeth Frisbey for her assistance in assembling the material.



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INTRODUCTION AND LITERATURE REVIEW

When Meldrum and Roughton (34) first reported the discovery of a new enzyme in the red blood cells, which they called carbonic anhydrase, they stated in a short paragraph that the enzyme had not been found in plant material. Today this statement appears rather striking in view of the facility with which the presence of plant carbonic anhydrase can be established. Yet, Burr (6) failed to find the enzyme in plants which he examined by a method similar to the one employed by Meldrum and Roughton. Only a few years later, however, Neish (39) had no difficulty finding the enzyme in three plants which he selected at random. Such a discrepancy of results obtained by experienced workers who knew how to overcome difficulties when they arose, highlights one of the most interesting characteristics of this enzyme, its unequal distribution in plant species. This must at once raise the question why, if there be a uniform function of the enzyme, there should be present a larger quantity of it in one plant than in another. Indeed, there are plant species, where the enzyme, if present at all, occurs only in traces. This at least, is the result of the conventional extraction by disintegrating the tissues either by means of a hydraulic press, where the fluid is expressed undiluted after the

tissues have been broken, or by maceration in an apparatus of the blender type where the solid matter is broken up in a surrounding solvent. It is obvious that by preparing the enzyme-containing liquid in either of the mentioned ways cell contents as well as intercellular fluids become mixed and that in vitro reactions take place which may inactivate an enzyme. If this be so in the case of carbonic anhydrase it would be necessary to devise a method which insures independence from such interference. As will be seen later in this paper the possibility of such an interference can be minimized. It must therefore be assumed that there is a quantitative difference among the plant species as far as their carbonic anhydrase content is concerned. This raises a number of questions in connection with the possible function of the enzyme as catalyst of a photosynthetic phase. These will be considered in the course of the paper. At this point the mere fact is mentioned in order to illustrate the historical development of our knowledge about the enzyme.

After a number of contradictory publications during the years 1936 and 1946 (6, 11, 38, 39, 53) Bradfield (3) showed in 1947 that the enzyme occurred in a variety of plant species. Lucas (32), stimulated by the publication of Day and Franklin (who had demonstrated the enzyme in elder leaves but not in other plants), but unaware of Bradfield's work, which took place simultaneously, undertook a search for the enzyme early in 1947. These two

independent investigations brought out very clearly the fact that carbonic anhydrase is a regular constituent of plant cells. The ubiquity of the enzyme thus shown made it very likely that a common function, perhaps in connection with photosynthesis, might exist. Day and Franklin, like Burr ten years before them, had speculated about this possibility but ended up in a blind alley: As Burr had to disclaim any participation of the enzyme in photosynthesis because he failed to show its presence, they had to modify any general concept about participation of carbonic anhydrase in the process of photosynthesis because elder leaves are not the only leaves that photosynthesize, but were the only ones in which they found the enzyme.

Waygood and Clendenning (62) based their work on the contradictory literature up to and including Bradfield's findings and supported the positive results by their own experimental work. It can now be accepted as an established fact that an enzyme of carbonic anhydrase character exists in plant tissues. That this enzyme be identical with animal carbonic anhydrase cannot be said at this time. It is unlikely, however, and some observations point to the fact that it may differ from the animal enzyme.

In their most recent summary of the status of research in carbonic anhydrase Roughton and Clark (53) characterize it as a zinc-containing enzyme present in high concentration in many animal tissues. According to this review, the reactions which it catalyzes are the hydration of carbon

dioxide and the dehydration of carbonic acid. It is pointed out that, unlike many enzymes, carbonic anhydrase catalyzes reactions which themselves proceed at an appreciable rate in absence of any catalyst. It is furthermore emphasized that the reaction rate is affected by a variety of basic inorganic catalysts. This statement is important for the study of the plant enzyme since environmental factors connected with the enzyme test may occasionally change the basic rate of reaction and thus create conditions which are applicable to the particular plant or the particular experimental setup.

Roughton and Clark (53) state, in essence, the classical findings of Meldrum and Roughton (35) which were expanded in subsequent papers by Roughton and Booth (52), Roughton (48, 49, 50, 51) and others (4, 18, 19, 24). The reactions

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \qquad (1)$$

and

$$CO_2 + OH^- \rightleftharpoons HCO_3^- \qquad (2)$$

were pointed out by Faurholt (14, 15). Below pH 7.5 the OH^- is so small that the rate of reaction 2 is negligible compared to that of reaction 1. Above pH 11 reaction 2 predominates while between pH 8 and 10 the rates of both reactions are significant. In presence of amines, carbamino formation also occurs, that is:

$$CO_2 + RNH_2 \rightleftharpoons RNHCOOH \rightleftharpoons RNHCOO^- + H^+ \qquad (3)$$

Reaction 1 is catalyzed by $HPO_4^=$, $H_2BO_3^-$, and by the more

electro-negative constituents of many buffer systems, and belongs to the general category of base-catalyzed reactions (53). The full kinetic equation below pH 7.5, but in the presence of an inorganic catalyst of concentration B^- is therefore (24, 52)

$$d[CO_2]/dt = (-k_u[CO_2] + k_o[H_2CO_3])\ (1 + \underline{1}[B^-])$$

where k_u is the velocity constant of $CO_2 + H_2O \longrightarrow H_2CO_3$ in absence of all catalysts, and k_o is the velocity constant of $H_2CO_3 \longrightarrow CO_2 + H_2O$ in absence of all catalysts and $\underline{1}$ is the catalytic coefficient of B^-.

Roughton and Clark (53) enumerate some values of $\underline{1}$ at 0°. They are as follows: (a) Weak acid anions: $HPO_4^=$ 8, $H_2BO_3^-$ 150, $SeO_3^=$ 2000, arsenite 10,000 and hypobromide 30,000. (b) Molecules of cyclic nitrogenous bases: glyoxaline 1.5, dimethylglyoxaline 12, and nicotine 13. (c) Straight chain nitrogenous bases, e.g. CH_3NH_2, show no detectable catalytic effect but exhibit a marked carbamino reaction (reaction 3). Bases like histidine, which contains both cyclic and straight chain nitrogen atoms, show both catalytic activity and carbamino-forming power.

It is certain that animal carbonic anhydrase catalyzes reaction 1 directly but it is doubtful whether it also affects the other reactions (53).

<u>In vivo</u> as well as <u>in vitro</u> a number of processes are limited by the rate of reaction 1. The over-all rate of such processes is therefore affected by carbonic anhydrase.

Many of the <u>in vivo</u> processes in animals are well

described and the participation of carbonic anhydrase has been elucidated (1, 9, 10, 16, 18, 19, 27, 28, 29, 30, 31).

The distribution of animal carbonic anhydrase is wide. It has been found in high concentration in the red blood cells, the gastric mucosa and renal cortex of the vertebrates. In smaller amounts it is established in most parts of the central nervous system, the pancreas, salivary glands and in the swim bladders and "chloride-secreting" cells of fishes (53). According to the same source, the enzyme is present in the respiratory organs and specialized regions of the alimentary canal of the invertebrates, but absent from body fluids; the blood of polychaete worms, however, shows considerable activity. Particularly large amounts are found in the gills of cephalopods, the tentacles of sea anemones, and in the esophageal diverticula of many annelids.

PURPOSE OF WORK AND OUTLINE

The purpose of the work presented has been manifold. The following provides an outline:

(1) The question of the existence of an enzyme in plant tissues similar to animal carbonic anhydrase was to be answered unequivocally.

(2) After ascertaining affirmative evidence the properties of this enzyme were to be investigated, particularly in the light of what is known about the animal enzyme.

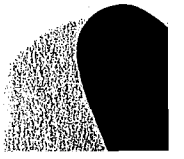
(3) The purification of the enzyme was to be attempted.

Only the first of these problems could be attacked without preliminary experimentation. The work existing in the field of animal carbonic anhydrase afforded sufficient background for such an attack of the core of the problem, i.e. whether or not such an enzyme exists in plants.

The introduction of the present paper necessarily had to be anticipative by stating, on page 3, that "it can be accepted as an established fact that an enzyme of carbonic anhydrase character exists in plant tissues".

Part of the work described in the following dates back as far as 1946. Consequently certain phases of it have been overlapping with developments published subsequently and cited in the review of literature.

It has been pointed out (53) that even in the case



of the investigation of the animal enzyme much confusion still exists about certain vital problems. This is true in spite of the fact that the classical work by Meldrum and Roughton (34) is now nearly two decades old.

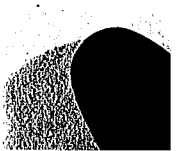
Since the three areas outlined represent more or less independent research problems they will be treated as separate entities.

EXPERIMENTAL PART

Search for Presence of the Enzyme

Introductory considerations

One of the most important requirements for obtaining unassailable results in the determination of plant constituents is the choice of a proper method of extraction. It has been said in the introduction that the demonstration of the enzyme depends on its preparation without too much interference from other substances. There are many possibilities of inter-reaction between constituents extracted from different locations within the plant. In the natural state any reaction between constituents, which are to be maintained in their active state, is prevented by spatial and temporal arrangements designated to safeguard the biologically active constituents. These barriers are broken down in an extraction method which does not attempt to reduce such a source of error to a minimum. Since it is obvious that this minimum cannot be zero unless a specific method of extraction of a specific constituent can be devised, the lowest possible level of error must be sought.



Methods and materials

The conventional procedure of disintegrating plant material is maceration in a Waring Blendor or a similar device in the presence of an extractant, in this case of water. This method was employed in many of the initial experiments, and a considerable amount of data obtained is based on it. The method consisted of blending a weighed amount of plant material with a small quantity of water, usually three times the weight of the plant tissue. Blending - in a Waring Blendor - took place for 3 minutes. Afterwards, the macerate was filtered by suction through Whatman paper No. 1. In many instances, particularly when plant material was used which contained viscous constituents, which made filtration difficult, the macerate was squeezed by hand through cheesecloth or nylon onto the suction funnel. This procedure provided a uniform preparation with a dry matter content of 10 to 30 mg per ml.

The various plant extracts differed from each other remarkably in their chlorophyll content. Presumably this was caused by the varying facility with which chloroplasts were broken in the process of blending. Some of the extracts therefore appeared turbid and green while others were clear after filtration. The filtrates thus prepared were ready for testing and were tested immediately whenever this was feasible. If refrigeration was needed, the sample was shaken vigorously before testing since sedimentation of particles was observed to occur in the cold in many of the

extracts. All samples were brought to room temperature before testing but standing at room temperature was avoided meticulously.

Another procedure of disintegration of plant material followed the ideas previously expounded. This method consisted of breaking up the plant tissues by means of immediate freezing and pounding the frozen tissue to a fine powder. The plant sample was weighed and cut into small pieces. It was then placed in a stainless steel tube which formed the core of a device which was especially built for this purpose*. It was constructed as a modification of an apparatus described by Poel (47) and used for preparation of acellular homogenates from muscle samples. Although the requirements of plant tissues differ from those of animal tissues the apparatus gave satisfactory service. It is shown in Figure I together with the homogenizer which completes the preparation of the sample. In many instances, however, the use of the homogenizer was found to be superfluous. The samples were, in those cases, removed from the stainless steel tube after disintegration and quickly transferred to a Waring Blendor equipped with a small, homemade jar, and blended while cold.

The apparatus used for pulverization of the plant sample is essentially a wooden box, made from several layers

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*The generous assistance rendered by Professor Paul J. DeKoning of the Department of Mechanical Engineering of Michigan State College in designing and building this device is gratefully acknowledged.

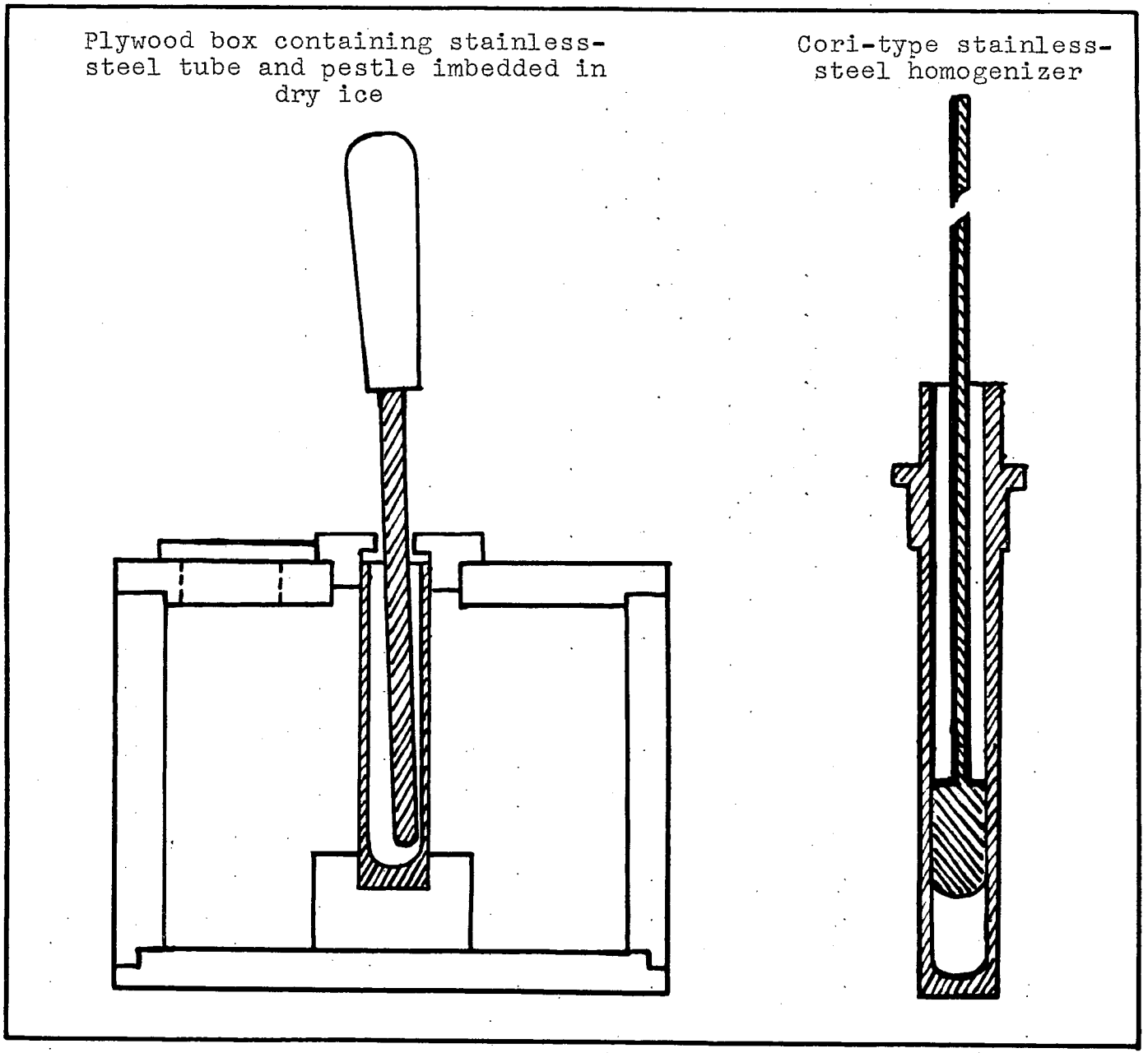
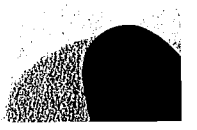


Figure I. Apparatus for disintegration of frozen plant tissues

of plywood with a stainless steel tube in the center, supported by a steel anvil. A stainless steel pestle with wooden handle is used for pulverizing the plant sample. The bottom part of the center tube is made as a separate piece and threaded on the inside so that the tube can be screwed in and later unscrewed before the plant powder is removed. This arrangement allows quantitative removal of the plant material. The space around the center tube is packed with dry ice; the cover provides two holes, one for the pestle and another one for the insertion of a plant sample. The plant sample was held in a jar or wrapped in cloth directly on or between the pieces of dry ice. After temperature equilibrium was reached it was transferred to the steel tube and pulverized. While the temperature within the dry ice areas was approximately -70° C, a temperature of -25° C to -35° C was measured inside the tube, the variation being due to larger or smaller quantities of dry ice used. It was found that for the dimensions of the box used (15 x 15 x 16 cm) 2 to 2.5 kg of dry ice sufficed to keep the temperature of the steel tube at the level mentioned for 9 to 12 hours. In other words, when the box was filled with dry ice in the morning it provided suitable working conditions for the entire day without refilling.

After pulverization for approximately one minute a sample is ready for blending. The alternative is its further disintegration by homogenizing. For this purpose a Cori-type (60) stainless steel homogenizing tube (Figure I)



was used which, for the purpose of centrifugation, has been machined with a collar 5 cm below the upper rim. This tube was also equipped with a removable bottom. The tube is preferably placed in a cup containing cracked ice or ice water. The plunger is attached to a 1/30 hp motor. The contents are homogenized in the customary manner by moving the tube up and down to force the water and tissue suspension past the wall of the rotating plunger. After this has been performed 10 to 15 times the homogenate can be decanted and filtered or centrifuged if this is preferable. In both cases satisfactory results have been obtained.

The test for carbonic anhydrase essentially followed the outline for the manometric method devised by Meldrum and Roughton (35). Some modifications were employed. Instead of the boat-shaped glass trough used by Meldrum and Roughton a 125 ml Erlenmeyer flask with divided bottom was used. The flask was fitted with a rubber stopper through which a glass tube extended, which was connected by 60 cm of rubber tubing with a standard Warburg manometer. This manometer was attached to a Precision constant temperature water bath above which was built a shaking device consisting of a 1/150 hp, 1800 rpm motor. This motor was equipped with a 10:1 ratio worm gear reducer giving drive shaft output of 180 rpm and 1.6 inch-pounds torque. By a suitable arrangement the number of oscillations of the flask was fixed at 120 per minute. Absolutely uniform speed during the operation of the shaker is essential in all enzyme

determinations but particularly in cases where measurements are taken at very short intervals and where the total period of operation is brief. The Erlenmeyer flask, in which the required quantities of reagent had been placed, was attached to a steel spring clamp that held it firmly while shaking took place. A rubber band slipped around the clamp further secured the position of the flask. The temperature of the water bath was held at 30° C. In order to reach temperature equilibrium the flask remained in the water bath 5 minutes prior to shaking.

The phosphate buffer of Meldrum and Roughton (equal volumes of 0.2M Na_2HPO_4 and 0.2M KH_2PO_4) was used. Instead of 0.2M $NaHCO_3$ in 0.02M HaOH, however, 0.1M Na_2CO_3 in 0.01M NaOH was employed since more uniform blank readings were obtained and the value of the blank reading was at a more convenient level. Originally, readings had been taken after 10, 20, 30 and 60 seconds, as had been suggested by Meldrum and Roughton. Many tests showed, however, that it was unnecessary to take more than one reading. This was subsequently done after 30 seconds. The amount of CO_2 evolved during this time of shaking, at the indicated speed and at the uniform temperature of 30° C, was utilized to express enzyme activity. It was calculated as micromoles. The formula used to indicate the activity in comparable terms was $\frac{R - R_0}{g}.C$, where R = rate of catalyzed reaction, R_0 = rate of uncatalyzed reaction, C = correction factor for the flask, and g = weight in grams of dry matter. The

activity resulting from this formula, expressed as micromoles of CO_2, was assumed to be the enzyme activity (E). In order to specify environmental conditions accompanying the test which could be modified whenever needed an exponent may be used to denote the temperature at which the measurements are taken: E^{30} would then indicate enzyme activities determined at the temperature of 30° C. A subindex, for instance 30, can also be used to denote the number of seconds during which the evolution of CO_2 is measured, as E_{30}. Thus, E^{30}_{30} indicates the enzyme activity measured at the temperature of 30° C during a period of 30 seconds. This system resembles the specification of environmental conditions given in measurements of optical rotation.

The plant materials used were freshly gathered leaves of species that became available. Although occasionally other parts of the plants were examined the overwhelming majority of the samples consisted of leaf blades. The samples were made uniform by collecting an excess, cutting or tearing apart the leaf blades - under exclusion of heavy midribs - and sampling from the well-mixed supply. Since fresh plant material was the paramount source of the enzyme, description of occasional deviations can be omitted here; they will be mentioned whenever work with other kinds of material will be reported.

The main microorganism used in these studies is Euglena gracilis, var. bacillaris. Preliminary work employed liquid cultures of Euglena deses in which measured amounts

of the centrifuged and resuspended organisms were tested. (33). Surface cultures of Euglena gracilis, var. bacillaris* were prepared in Petri dishes on agar of the following composition (23)(Tables 1 and 2).

TABLE 1

COMPOSITION OF BASAL MEDIUM

(per liter of final medium, pH 6.5)

	g	mg
$NH_4H_2PO_4$	0.8	
Potassium citrate, monohydrate	0.2	
$MgSO_4 \cdot 7\ H_2O$	0.2	
Sodium butyrate	2.0	
Monosodium glutamate	1.0	
$CaCl_2$	0.1	
$FeSO_4 \cdot 7\ H_2O$		20.0
$MnSO_4 \cdot H_2O$		6.0
$CoSO_4 \cdot 7\ H_2O$		5.0
$ZnCl_2$		0.8
$Na_2MoO_4 \cdot 2\ H_2O$		1.0
$CuSO_4 \cdot 5\ H_2O$		0.08
Thiamine chloride		0.1

To each liter of this solution 10 ml each of a 1000 ppm vitamin B_{12} solution and of the following vitamin mixture was added:

*Obtained through courtesy of Dr. W. J. Robbins, Director, The New York Botanical Garden, New York City.

TABLE 2

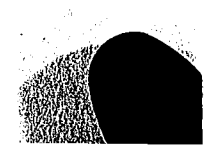
COMPOSITION OF VITAMIN SOLUTION

(per 250 ml of stock solution)

	mg
Thiamine chloride	25.0
Riboflavin	12.5
Pyridoxine hydrochloride	12.5
Calcium pantothenate	50.0
Para-aminobenzoic acid	12.5
Nicotinic acid amide	50.0
i-Inositol	100.0
Pimelic acid	100.0
Choline chloride	50.0
Nucleic acid	12.5
Folic acid	0.004
d-Biotin	0.125

Per liter of final solution 10 g of agar was added.

After extracts had been prepared from either plant materials or microorganisms they were added to the reagents in amounts ranging from 0.25 to 2 ml depending on the enzyme concentration. Two ml of each of the reagents was pipetted into the separate compartments of the Erlenmeyer flask. Bacteriological pipettes which allowed rapid transfer were used. The liquids were allowed to flow into the compartments by gravity since blowing would have caused splashing and therefore could have resulted in accidental mixture of the reagents.



Results and discussion

Enzyme activity in plant species. A number of preliminary tests were unsuccessful because of the nature of the plant material used. It was soon established, however, that many plant extracts catalyzed the reaction employed in the test. This work was done early in 1947 and the results assembled in Table 3 indicate activity as obtained on the basis of the formula developed by Meldrum and Roughton, namely $\frac{R-R_o}{R_o}$, where R and R_o equal the rates of the catalyzed and uncatalyzed reactions respectively. In order to have an expression for enzyme activity which resembles the enzyme units used later in the course of this work the resulting figures were multiplied by 100.

The table indicates most striking differences in enzyme activity of tissue extracts of even closely related plants. In spite of this fact, there are certain consistencies which cannot be overlooked. The legumes, looked upon as a family, obviously contain the enzyme in considerable quantities. The only exceptions were the leaf extracts of peanut, which showed low activity, and those of broom which not only had no activity but evidently retarded the evolution of CO_2. Considerable variations were noted in solanaceous plants: While pepper leaves had rather high activity, tomato leaves were extremely low; the leaves of Jerusalem cherry ranked about halfway between them. The brassicas tested were very high in activity.

TABLE 3

CARBONIC ANHYDRASE ACTIVITIES OF PLANT EXTRACTS AND *EUGLENA* SPECIES

Family, species and part	Apparent enzyme activity ($E_A = \frac{R - R_o}{R_o} \times 100$)
Apocynaceae	
Vinca minor, leaves	0
Chenopodiaceae	
Chenopodium album, leaves	105 ± 16.0
Cruciferae	
Brassica oleracea, var. capitata, leaves	105 ± 11.4
Raphanus sativus, leaves	114 ± 11.1
Gramineae	
Hordeum vulgare, leaves	28 ± 3.4
Triticum vulgare, leaves	39 ± 2.6
Leguminosae	
Arachis hypogaea, leaves	12 ± 1.3
Cytisus genista, leaves	0 (reaction retarded)
Lathyrus odoratus, leaves	75 ± 7.3
Lupinus angustifolius, cotyledons	55 ± 4.6
" " , leaves	72 ± 4.1
Medicago sativa, leaves	64 ± 6.2
" " , stems	18 ± 3.2
Phaseolus lunatus, leaves	80 ± 6.4
Pisum sativum, leaves	96 ± 12.8
Liliaceae	
Allium cepa, leaves	0
Polygonaceae	
Polygonum persicaria, leaves	126 ± 13.5
Primulaceae	
Cyclamen persicum, leaves	0
Rubiaceae	
Rubus strigosus, leaves	119 ± 4.8
Solanaceae	
Capsicum annuum, leaves	80 ± 6.2
Lycopersicon esculentum, leaves	7 ± 3.1
Solanum pseudocapsicum, leaves	36 ± 3.9
Umbelliferae	
Daucus Carota, leaves	23 ± 2.0
Urticaceae	
Urtica urens, leaves	0
Euglenaceae	
Euglena deses	1.9 ± 0.6
Euglena gracilis, var. bacillaris	2.3 ± 0.8

Two weeds, lady's-thumb and lamb's-quarters, although members of different families, showed the highest activity recorded in the early tests. The grasses tested ranked low in activity. The distribution pattern is typical of plant enzymes whose functions, although of a general biological nature, seem to be specialized to fit into the scheme of metabolism of the individual species.

The discovery of plant carbonic anhydrase in about two dozen species in 1947 apparently disproved earlier statements (6, 11, 38) about the absence of an enzyme having the properties of carbonic anhydrase, including the report by Day and Franklin (11) who established it in only one out of 16 species. Since this part of the work described here was done within six months after the paper by Day and Franklin appeared it seemed necessary to ascertain the reasons for such a striking discrepancy. It was considered possible that the plants Day and Franklin found negative (they identified, in their publication, only one of the 15) might be low in enzyme content or devoid of the substance; this possibility was conceded in view of the fact that the early records (see Table 3) contained five plants completely devoid of enzymatic activity and one of so low an activity that it could have been overlooked. It was therefore attempted to explain the discrepancy on the basis of a difference in the approach. This proved to be correct although the final proof was furnished by Waygood and Clendenning (62) three years later. Accurate information as to

the procedures used in the work that resulted in negative findings (6, 11, 38) is not available but it is likely that they may have been negative for the same reason.

Extraction experiments. Sweet pea leaves (Lathyrus odoratus) were used for these experiments. The plants had been grown in the greenhouse since early spring 1947 and had developed to approximately seven feet long vines which were trellised in the customary way. Leaves were selected at random using at least six plants at the same time to insure uniformity. Only healthy leaves were picked. The plants were in full bloom at the time of the tests.

Thirty g of leaves was blended with 60 ml of ice water in a Waring Blendor for 2 minutes and filtered through cheesecloth. Ten ml of plant extract was shaken with 6 ml of ice water, 4 ml of absolute ethanol and 5 ml of chloroform in a centrifuge tube for 1 minute. This procedure was essentially the one used by Meldrum and Roughton (35) which in turn was similar to the method used by Tsuchihashi (59) for preparing catalase. The mixture was centrifuged for 10 minutes at 2500 rpm. Three layers were formed: (1) Aqueous supernatant, (2) mat consisting of chloroplastic material, and (3) chloroform layer in which much of the chlorophyll was dissolved.

The same experiment was repeated with different plant materials and varied amounts of tissue and solvents but the ratio between them remained the same as in the first

experiment described. In all instances the enzyme activity was more or less evenly divided between the fractions 1 and 2 (Table 4). The reasons for this distribution were not investigated at that time. It was noted, however, that the activity of fraction 1 was more stable than that observed in fraction 2 (Figure II).

TABLE 4

CARBONIC ANHYDRASE ACTIVITY IN LEAVES OF LATHYRUS ODORATUS*

Experiment number	Percentage of enzyme activity based on E_A of original material		
	Aqueous fraction	Mat of solids	Chloroform fraction
1	46	48	0
2	45	45	0
3	50	45	0
4	58	46	0

*Fractions obtained by centrifuging a water-ethanol-chloroform system containing the source of enzyme.

It was assumed that the activity found in fraction 2 greatly depended on the amount of enzyme adsorbed on its surface. By washing this mat the enzyme activity could be gradually diminished and shown in the washings (Table 5).

The more rapid loss of activity in the mat suspension which is shown in Figure II can possibly be explained by the fact that in this case the enzyme seems to be adsorbed on the surface but not very firmly attached since washing removed it with comparative ease. These enzyme particles

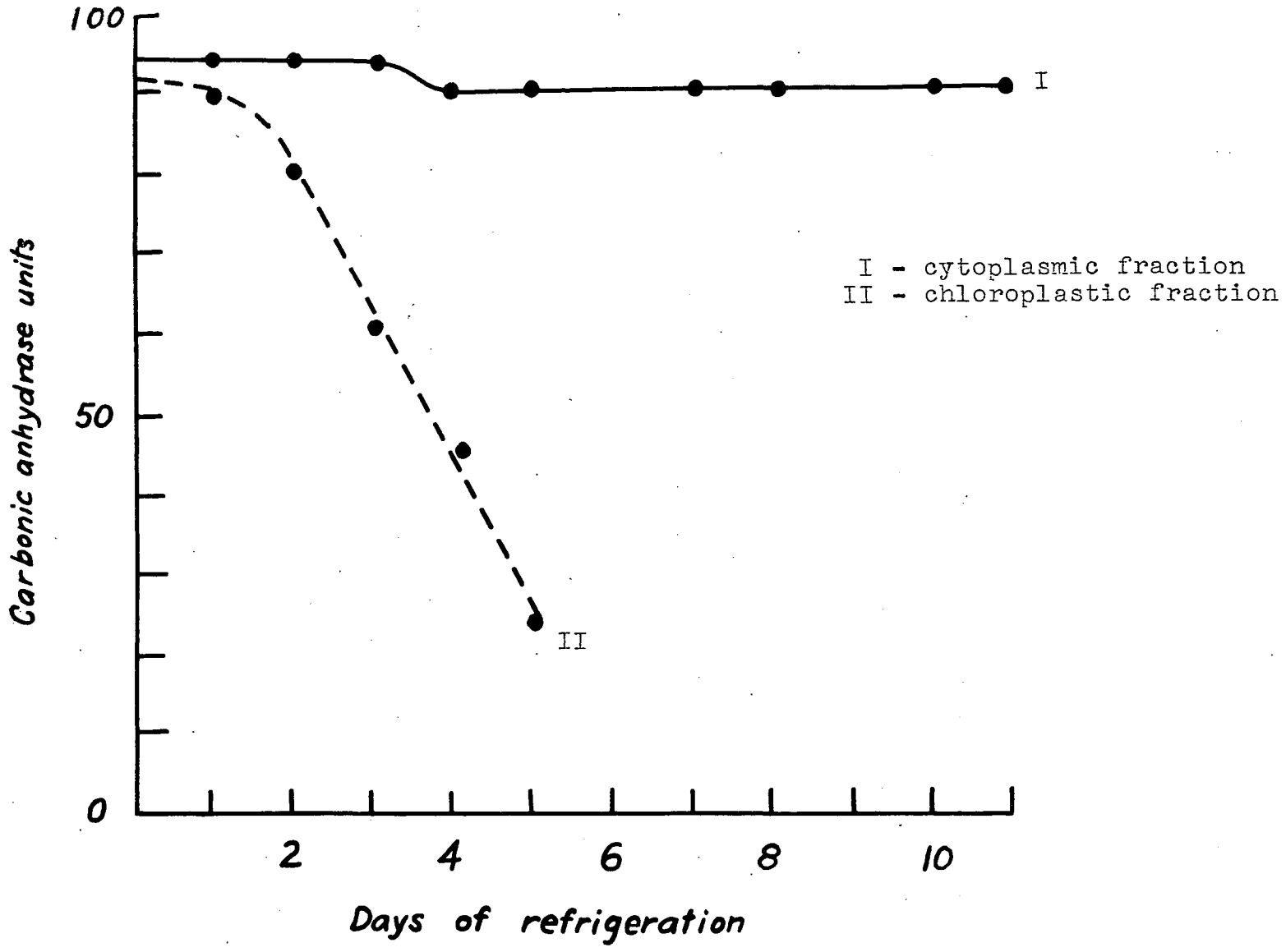


Figure II. Differential stability of carbonic anhydrase in cytoplasmic and chloroplastic fractions of a leaf extract

TABLE 5

CARBONIC ANHYDRASE ACTIVITY IN SUCCESSIVE WASHINGS OF MATS OF TABLE 4

Experiment number	Percentage of enzyme activity removed in		
	First washing	Second washing	Third washing
1	31	17	3
2	33	15	2
3	30	17	6

evidently lack the protective action of their natural surroundings; such protection is obviously provided more effectively in the cytoplasm which is completely transferred into the water phase of the system.

The chloroform treatment of plant material usually resulted in an increased enzyme activity, sometimes twice as high as the normal activity attained. This increased activity, however, was lost on standing. It has not been ascertained as yet whether this phenomenon is similar to the one observed by Euler and Blix (12) in their experiments with catalase in yeast cells. They reported that small amounts of substances toxic to protoplasm, as toluene or chloroform, increased the catalytic action of the cells to sixfold the original value. Table 6 illustrates the effect in a typical case of chloroform treatment. The stimulation of enzyme activity did not persist but, as Table 6 shows, the level of activity fell below that of the supernatant when tested immediately. The question why only the portion

TABLE 6

EFFECT OF CHLOROFORM TREATMENT ON CARBONIC ANHYDRASE ACTIVITY OF AN AQUEOUS EXTRACT OF *LATHYRUS ODORATUS* LEAVES

Experiment number	E_A of		
	Original preparation	Aqueous fraction	Aqueous suspension of solid mat
Tested immediately			
1	76	98	212
2	75	98	177
3	70	100	194
Tested after 24 hours of refrigeration			
1	69	91	88
2	70	90	90
3	65	94	95

of the enzyme which was found associated with the chloroplastic material was stimulated has not been answered definitely. Traces of chloroform which could have interfered with the test were evaporated before testing. Chloroform-water mixtures were tested as controls. Considering the findings of Euler and Blix (12) which referred to living cells the question must be raised as to whether the association with the chloroplasts may not be of intrinsic importance. The study of this phase will require continued attention since the question of localization of the enzyme has not been solved unequivocally although there seems to be ample evidence that it is primarily present in the cytoplasm (62, 63). According to these sources, the aquatic plants

and Sambucus racemosa were the only plants where the enzyme was apparently limited to the chloroplast sediments. They found this to be due in the case of Sambucus to the presence of a natural flocculating agent, believed to be tannin, which apparently causes the cytoplasmic proteins and chloroplasts to be deposited together when centrifuged. On resuspending the chloroplast sediment in water and adjusting the pH from 6.1 to 8-10 they observed the cytoplasmic proteins to be dispersed and hence were able to measure the major part of the carbonic anhydrase activity in the supernatant fraction. This procedure, convincing as it seems to be, still leaves open the question whether the chloroplast may not be the source of the enzyme by which it is continually produced and given off into the surrounding cytoplasm. Oparin and co-workers (40, 41, 44) have shown that enzyme activity in the living cell is often obscured by their adsorption on other protein molecules. They were the first ones to point out the participation of tannins in the inactivation of enzymes, thus creating artifacts as observed by Waygood and Clendenning. They emphasized, however, that tannins do not exert specific effects. The inactivation of enzyme, in their case amylase, was not due to the tannins present but to the protein precipitate on which the enzyme was adsorbed. Any substance causing protein precipitation could and would therefore cause a seeming loss or an inactivation of the enzyme; the use of one or the other of these terms would depend on the fraction of the preparation

to which reference is made. In order to determine whether the observations of Oparin and co-workers could be ascertained for carbonic anhydrase and thus possibly establish a link between their findings and the observations of Waygood and Clendenning, several experiments were undertaken, which are described later under the heading "Precipitation experiments".

The efficiency of a single extraction in the Waring Blendor or similar device must be seriously doubted. Even though a considerable portion of the enzyme activity seems to reside in the cytoplasm repeated extractions have revealed the interesting fact that extracts with increased enzyme activity could be obtained. It must be considered, however, that in all work dealing with so highly destructible chemical entities as enzymes, particularly when they are separated from their natural environment with all its protective mechanisms, the steady loss of enzymatic activity counteracts the purpose of increasing the activity of the preparations. Similar countereffects have been observed in purification work which will be reported later in this paper.

Frozen leaves of Chenopodium album were ground with dry ice in acetone. The mixture was frozen and allowed to stand for several hours after the acetone had been removed by expressing through nylon. The expressed acetone was evaporated and the residue taken up in water. The remaining solid material was blended with water, suction-filtered through Whatman No. 1 paper and frozen for storage. The

residue was blended with the filter paper, suction-filtered as above and frozen. Shortly before testing the preparations were thawed. The results are presented in Table 7.

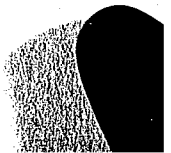
TABLE 7

REPEATED EXTRACTION OF CARBONIC ANHYDRASE UNDER CONDITIONS DESIGNED TO PREVENT LOSS OF THE ENZYME

Experiment number	Enzyme activity (E)		
	Acetone extract	Water extract I	Water extract II
1	0	135	249
2	0	147	347
3	0	150	365

Under normal laboratory conditions such a retention of the enzyme will be compensated by the continuous inactivation of it. At room temperature this inactivation will progress rather rapidly. Oxidation processes, affecting the enzyme either directly or indirectly by unbalancing protective systems contribute to this destruction; the use of disintegration devices such as the Waring Blendor play their part in the acceleration of these processes, partly by the temperature increase created within the macerate, partly by the incorporation of air into it. The use of ascorbic acid as an antioxidant has been successful to some extent.

A graphical representation of the increase in actual enzyme activity, as contrasted to apparent enzyme activity which is expressed by means of apparent enzyme units (E_A),



shows that under favorable conditions of extraction the enzyme concentration can be enhanced considerably (Figure III). In this case the frozen Chenopodium leaves were directly extracted with water without the preliminary acetone treatment described above. Otherwise, the procedures were identical.

The diagram also shows that the apparent enzyme units cannot be used to express a true estimate of the amount of carbonic anhydrase in a given plant tissue; nevertheless they convey a correct picture of the relative strengths of different plant extracts. This discrepancy merits further discussion particularly in view of the fact that all publications, which have so far appeared on the subject of plant carbonic anhydrase, have used the enzyme units introduced by Meldrum and Roughton (35) or at least units derived on the basis of the same equation ($\frac{R-R_o}{R_o}$ = enzyme units). The justification for this method of expressing enzyme activity lies in the fact that plant tissue consists of so much water that an accurate representation of enzyme activity is afforded by a direct measurement of this fluid plus the small amount of solids contained by it. The "apparent" enzyme units actually state the over-all enzyme activity as found in the macerated tissues. This method is comparable to the widely used and accepted procedure of expressing ascorbic acid content of plants and plant parts on the basis of fresh weight. The argument in favor of this method of characterizing plants according to their ascorbic acid

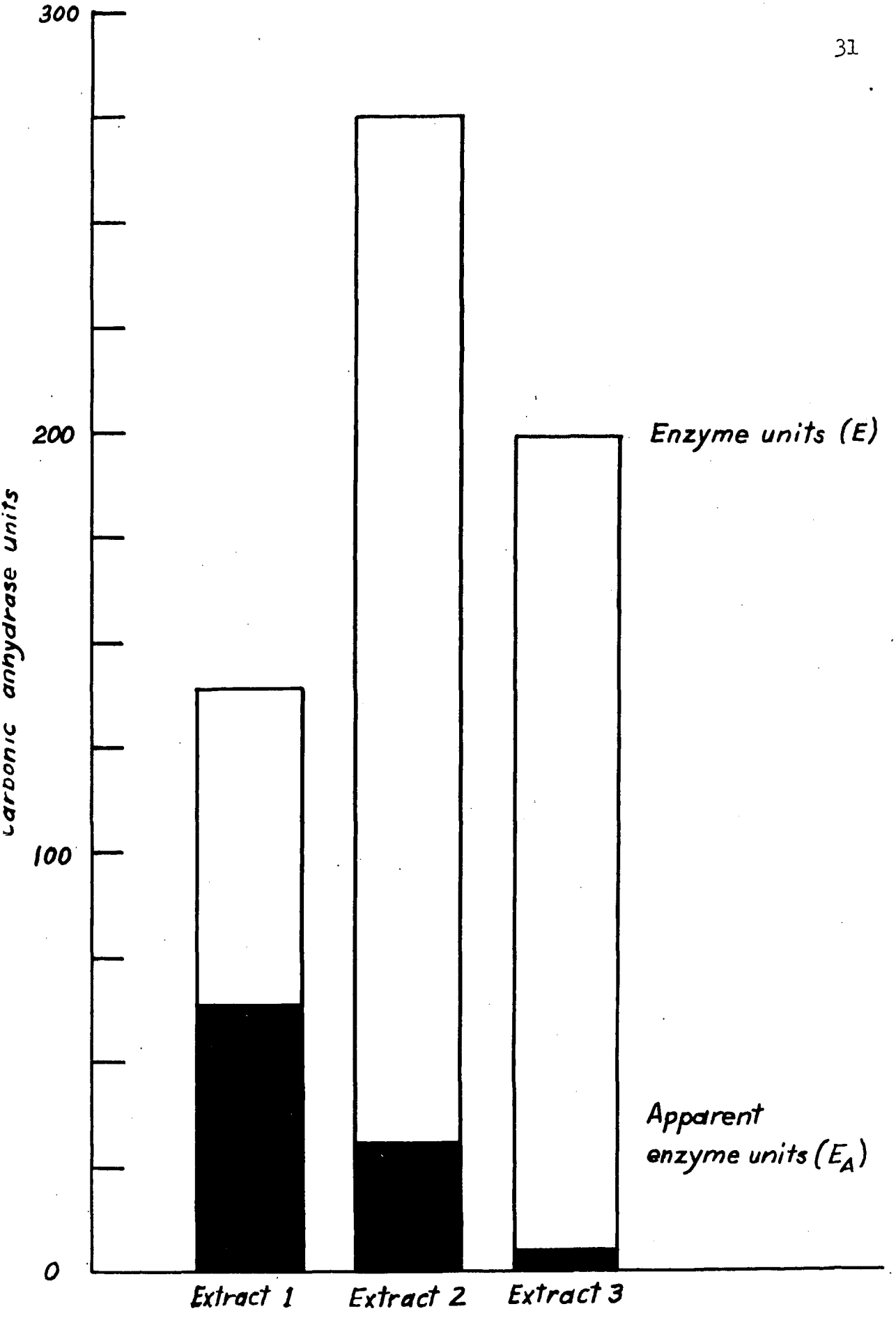
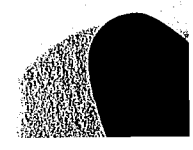


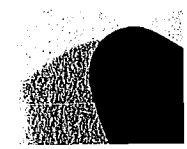
Figure III. Comparison between enzyme units calculated on the basis of total liquid (E_A) and dry weight (E)

content - and the writer finds himself in agreement with this line of thought - is essentially that plants are consumed with all the water they contain; hence, expressing ascorbic acid content on the basis of fresh weight does much more clearly indicate the available amount of ascorbic acid than using dry weight as the basis of the calculation. In contrast, such reasoning is much less to the point in the case of carbonic anhydrase and, for that matter, in the case of any other enzyme. If the enzyme were completely suspended or dissolved in the cell sap and cytoplasm and thus completely removed by simple extraction procedures, as is the case with ascorbic acid, fresh weight might constitute a reasonable basis for expression of enzyme activity. Much of the enzyme, however, is evidently adsorbed on colloid gels and especially on the living protoplasm which has the ability to hold substances in readiness for their use in physiological processes. Many earlier workers, like Hofmeister (22) and particularly the members of the Russian plant physiological school of the twenties and thirties, Palladin (45, 46), Oparin (40, 41, 42, 43, 44), Kurssanow (26) and their collaborators have done considerable theoretical and experimental work in support of this concept of enzyme release within the plant cell. Upon the theoretical assumption that only part of the actual enzyme is readily available by an average maceration and extraction procedure enzyme activity would be more logically expressed as a function of the total solids present in the extract.



It is possible, although not necessarily proven, that a preliminary acetone treatment had an enhancing effect on the efficiency of the subsequent water extraction. This could be in line with Chibnall's (7) and Chibnall's and Schryver's (8) observations that after treatment of leaves in bulk with ether it was possible to squeeze out most of the vacuole fluid with a hydraulic press. This procedure was based on the known fact that the vapors of chloroform or ether, or aqueous solutions of these and similar substances, would affect the permeability of plant cell membranes with the result that vacuolar fluid contents could exude. The immersion of leaves in ether would thus have made possible the removal of the vacuole fluid without rupture of the cell walls. Inasmuch as the complete extraction of carbonic anhydrase from the cells was considered a matter of major importance in the present work, some of Chibnall's procedures were adapted for this purpose.

Several preparations of frozen Chenopodium were made either by normal water extraction, in both a blender and the device described earlier in the paper, or by pretreatment with ether. Frozen leaves were immersed in quantities of ethyl ether for varying periods of time, usually not more than one-half hour. The leaves were then squeezed by means of a hydraulic press, blended or pounded in the special extraction device. As Vickery (61) has pointed out, the object of this operation, from the chemical point of view, was to remove water-soluble components before



attempting to bring the proteins into solution. The vacuole fluid expressed contains very little protein in solution but may contain 25 percent of the total leaf nitrogen. A substantial portion of the inorganic constituents could also be removed. It is interesting to note that Chibnall (7) attempted to separate two main types of protein components of the leaf, the chloroplastic protein and the cytoplasmic protein. This he accomplished by a procedure which, following the ether treatment, consisted of repeated grinding in a meat grinder and then in a plate-type mill. After straining through silk a green turbid suspension was obtained which was filtered through paper pulp whereby the green pigment and all of the turbidity were removed. It is in this step that the separation of protoplastic and cytoplasmic proteins was to be accomplished: The latter was said to have passed into the filtrate while the former remained in the paper pulp. Exact replicas of these experiments revealed interesting data. They are shown in Table 8.

The results show that what is assumed to be cytoplasmic fluid contains most of the enzyme activity. This result is in seeming discrepancy with the data presented in Table 7 and Figure III. Although the pre-treatment with ether is a distinguishing facter it is obvious that it had not inactivated the enzyme, at least in the cytoplasmic fluid. This pre-treatment could have had, as it was supposed to, an enhancing effect on the extraction of the cytoplasmic

TABLE 8

RELATIVE VALUES OF APPARENT ENZYME ACTIVITY IN VARIOUS TYPES OF EXTRACTS OF CHENOPODIUM ALBUM LEAVES

Experiment number	Percentage of apparent enzyme activity after ether treatment (Water extract = 100)		
	Expressed fluid	Water extraction	
		Filtrate	Re-extracted protoplastic material
1	125	125	30
2	115	125	25
3	110	100	20
4	118	125	35

components. In this respect, the repeated extraction of the enzyme from filter residues obtained without the ether pre-treatment could be explained satisfactorily. Furthermore, as Figure III brings out, the trend of apparent enzyme activity is identical in both instances. So far as the pre-treatment with acetone is concerned, experimental conditions varied in that considerable time elapsed between the consecutive extractions while in the case of the ether pre-treatment they followed each other immediately. This would leave open the possibility of enzyme regeneration within the chloroplastic portion (40), a point which is merely to be mentioned but which cannot be further discussed here.

In a similar experiment the results were calculated on the basis of total solids in the extracts. They are

presented in Table 9.

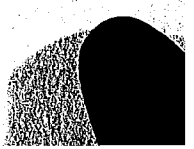
TABLE 9

RELATIVE VALUES OF ACTUAL ENZYME ACTIVITY IN VARIOUS TYPES OF EXTRACTS OF CHENOPODIUM ALBUM LEAVES

Experiment number	Percentage of actual enzyme activity after ether treatment (Water extract = 100)		
	Expressed fluid	Water extraction	
		Filtrate	Re-extracted protoplastic material
1	16	115	80
2	22	110	76
3	25	112	75

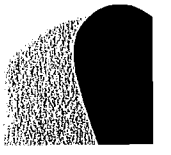
The data clearly indicate a different trend. While the expressed fluid yields a comparatively low activity because of the large amount of solids contained in it, the protoplastic material contains a considerable amount of the enzyme. It may be surmised that the value could have been even greater had the material been allowed to stand and autolyze for an extended period.

Precipitation experiments. Waygood and Clendenning (62) have shown in an interesting experiment that carbonic anhydrase activity was found in one plant species (Sambucus racemosa) to be associated with the chloroplast fraction. They reasoned that a tannin-like substance might have caused all of the cytoplasmic proteins, including carbonic anhydrase, to be precipitated with the chloroplasts. They



prevented this precipitation by raising the pH, which normally was between 6.0 and 6.2, to about 10.0. These authors did not mention that there might also be the possibility of a partial or even complete inhibition of enzyme activity because of the presence of tannins in plant cells. It was mentioned earlier in this paper that several Russian workers had dealt extensively with this subject. It was thought to be of theoretical as well as practical interest to find out whether their findings could be applied to the study of carbonic anhydrase. Oparin (40), in a review paper, questioned whether the conclusions drawn from his and his collaborators' experiments could be directly extended to other enzymes. This was mainly emphasized because most of their work was done with amylase which acts upon a substrate that is insoluble or at least of scant mobility. In the case of amylase adsorption the mere separation of the enzyme from the substrate could be the reason for its "inactivation". Nevertheless, there are results available which pertain to other enzymes and point in the same direction: Willstätter and Waldschmidt-Leitz (65) have shown adsorption of lipase by cholesterol; Hedin (20, 21) reported trypsin and rennin inactivation through adsorption by charcoal; and Miller and Bandemer (37) used the inactivation of invertase as the test reaction.

The main purpose of the set of experiments, which is reported in the following, was to establish whether different concentrations of tannic acid would inactivate carbonic



anhydrase in crude plant extracts. It was also intended to ascertain the details of such a reaction.

Chenopodium album leaves which had been frozen at sub-zero temperature since summer 1950 were used for the experiments. The leaves were brought to the laboratory and extracted with water in a blender before their temperature reached equilibrium with that of the room. A 5 percent stock solution of tannic acid was prepared and dilutions were made to give 1 and 0.1 percent solutions. To each 45 ml of plant extract 5 ml of tannic acid solution was added in the following manner:

a. Five ml of stock solution to each of three 45 ml portions to give a final tannic acid concentration of 0.5 percent.

b. Five ml of 1 percent solution to each of three 45 ml portions to give a final tannic acid concentration of 0.1 percent.

c. Five ml of 0.1 percent solution to each of three 45 ml portions to give a final tannic acid concentration of 0.01 percent.

One sample in each group was allowed to stand in a refrigerator for 30 minutes, another for 90 minutes, the third one for 6 hours. The preparations were centrifuged after the termination of each precipitation period mentioned and the supernatants decanted and tested (Table 10).

The results are clear-cut and indicate that only a concentration of 0.5 percent tannic acid effectively in-

TABLE 10

INACTIVATION OF CARBONIC ANHYDRASE BY CONTACT WITH DIFFERENT CONCENTRATIONS OF TANNIC ACID FOR DIFFERENT PERIODS

(Activity expressed as percentages of the activity of the crude extract)

	0.5% tannic acid			0.1% tannic acid			0.01% tannic acid		
	30 min.	90 min.	6 hrs.	30 min.	90 min.	6 hrs.	30 min.	90 min.	6 hrs.
Exp. 1	26	12	0	40	40	40	100	82	70
Exp. 2	24	15	0	54	37	28	98	83	74
Exp. 3	27	7	0	42	32	24	100	85	77
Exp. 4	21	11	0	44	39	36	100	80	71

activated the solution. The inactivation had progressed within 30 minutes to the point where only one-fourth of the enzyme activity was retained. It gradually declined, and after 90 minutes only one-sixth to one-tenth of the activity remained. After 6 hours no activity was recorded; it is likely, however, that complete inactivation is achieved within 2 hours. In the case of 0.1 percent tannic acid the remaining activity of the supernatant was between 25 percent and 40 percent even after 6 hours. About three-fourths of the activity was found in the supernatant after precipitation for 6 hours with 0.01 percent tannic acid.

All subsequent attempts to liberate the enzyme from the tannic acid precipitate have been unsuccessful. In this respect the similarity between the observations of Waygood and Clendenning and the present experiments ceases to exist. Oparin and co-workers (41, 44) have recommended the use of

peptone or albumin to aid in the elution of the enzyme from the precipitate. Scott (54) and co-workers (55, 56, 57) in a series of papers on the purification of the animal enzyme have made use of peptone as a stabilizer of the enzyme after it had been removed from its natural surroundings. In all these publications considerable success was reported. In its application to plant carbonic anhydrase, however, no protective action of peptone could be observed.

Since Neish (39) was the first one to isolate carbonic anhydrase from plant tissue and to determine activity of this enzyme in three different plants as a matter of routine merely to show the applicability of separation measures it was one of the first steps in the search for the enzyme to use some of his methods.

Lima bean leaves and sweet pea leaves were used in these experiments. After washing the leaves to remove any adhering foreign material - which was advisable especially in the case of the sweet peas since the greenhouse-grown plants always received dust treatments for mildew control - a given weight of them was blended with ten times the weight of distilled water. The resulting macerate was strained through cloth and centrifuged at 2000 rpm for 10 minutes. The purpose was the removal of the starch grains which sediment at a lower speed than the chloroplasts do. This was a separation designed to eliminate constituents which were considered unnecessary but to leave the chloroplasts in suspension. The supernatant liquid was decanted and a 2 M

solution of $CaCl_2$ was added to it, the volume of the precipitant being 5 percent of the total volume. After 30 minutes when the flocculated chloroplasts had settled down the supernatant liquid was decanted again. The chloroplasts were then precipitated by centrifuging at the speed of 2500 rpm, the supernatant decanted and the chloroplasts triturated with distilled water. Centrifuging and washing was repeated until the concentration of the flocculant was reduced to such a degree that the chloroplasts started to disperse. At this stage, according to Neish, only a trace of the flocculant remains. For the determination of enzymatic activity the chloroplasts were redispersed. The results obtained with a number of flocculating agents are shown in Table 11.

TABLE 11

CARBONIC ANHYDRASE ACTIVITY IN CHLOROPLAST FRACTIONS OF LIMA BEAN LEAF AND SWEET PEA LEAF EXTRACTS USING SEVERAL FLOCCULATING AGENTS

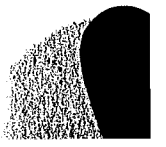
(Apparent enzyme activity of crude extracts: E (bean) = 82; E (pea) = 76)

Flocculant	Percent activity in final chloroplast suspension	
	Lima beans	Sweet peas
$CaCl_2$	31	27
$MgCl_2$	17	26
$Mg(NO_3)_2$	26	19
$MgSO_4$	30	21
$FeCl_2$	18	20
$AgNO_3$	0	0
$Pb(C_2H_3O_2)_2$	16	21

From the results it is quite obvious that Neish obtained only a fraction of the activity present in the plant material he used. The results are not necessarily indicative of a specific influence of the cations incorporated, except in the case of Ag^+ which no doubt is toxic. This is in agreement with the early findings about toxicity of metal ions toward animal carbonic anhydrase (35).

Further precipitation experiments were conducted using Menke's (36) methods. The preparation of the extracts proceeded in a manner similar to that outlined above but precipitation was carried out with 0.25 saturated ammonium sulfate. Very low activities, if any, were obtained.

Distribution in green organs. Generally speaking, there is very little evidence to support a prediction as to where in the plant and at what stages the activity of an enzyme might increase or decrease. Even in cases where the significance of an enzyme for physiological processes can be documented there is no sound basis for definite statements about the occurrence and intensity of the relative and much less the absolute enzyme activity. This is mainly due to the fact that the role of enzymes in physiological processes of biological entities can be understood in a general way (58) but has not been elucidated in the specific manner of classifying all the reactions in which the participation of a given enzyme is confirmed, nor is there any immediate prospect that this task will ever be accomplished.



In an attempt to provide clues for the action of an enzyme within a biological entity the determination of its presence or absence in certain parts of the organism can be helpful. The study of several plant species for distribution of carbonic anhydrase gave results which were rather uniform. Species high in carbonic anhydrase are more useful for such a study; for morphological reasons dicotyledonous species are more suitable than monocotyledons.

Freshly collected plants of Chenopodium album, 40-60 cm tall and just prior to flowering in June, 1950 and 1951, were separated into the following portions: 3-4 lowest leaves (blades only); leaves between the third (or fourth) lowest and the 7th to 9th (blades only); leaves above the 7th to 9th (blades only); axillary growth (blades only), not above 9th leaf; top above 9th leaf including petioles and apical meristem; stem with petioles of all leaves below the 9th and a point 24-30 cm above the stem base. These parts were separately extracted with water in the customary manner. The results are presented in Table 12.

It is apparent that the older leaves are carrying a greater amount of the enzyme than the younger ones. The activity decreases in the leaves toward the top but the youngest parts of the plant containing meristematic tissue have higher activity than the leaves just below. The activity found in the blades of the axillary leaves is in agreement with the correlation between activity and age of the primary leaves. Small but distinctive activity could be established in stems and petioles.

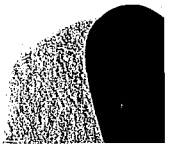


TABLE 12

CARBONIC ANHYDRASE ACTIVITY OF DIFFERENT PARTS OF FRESH *CHENOPODIUM ALBUM* PLANTS COLLECTED BEFORE FLOWERING

$$(E = \frac{R - R_o}{g} \cdot C \ \mu\text{moles of } CO_2)$$

Plant part	Dry weight Mg/ml extract	E
3rd or 4th lowest leaf blades	18.2	103**
Leaf blades between 3rd or 4th and 7th to 9th leaf	25.6	80**
Leaf blades above 7th to 9th leaf	27.3	72**
Top above 9th leaf	15.0	83**
Axillary growth, below 9th leaf (blades)	18.5	67*
Stem with petioles	10.0	20

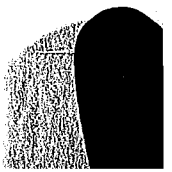
*Significant at the 5 percent level with respect to primary leaves of the same region

**Significant at the 1 percent level with respect to the adjoining parts examined

As might be expected, the literature on enzyme activity in plant parts of different age reveals a wide variety in the correlation of enzyme occurrence to kind and age of plant organs. Catalase, peroxidase and invertase activity appears to increase with increasing maturity, and to decrease with the onset of senescence (2). Oxidases (2) were reported to be maximal in the youngest leaves and declined as they developed. Knott (25) reported a relation between catalase activity of the apical bud of spinach and the type of growth of the plant. Ezell and Crist (13), investigating

the effect of certain nutrient conditions of the soil on the activity of oxidase and catalase in three species and the relation of this effect to growth, found a slight correlation between oxidase and growth or size of plants, with a tendency to be negative. They found a more pronounced correlation of significantly negative character between catalase activity and growth or size. They conclude that soil treatments, affecting growth and size of plants indirectly decreased oxidase and catalase activity when accelerating growth, and vice versa. This is in agreement with the repeated observation that carbonic anhydrase activity is, to a great extent, a function of the water content of the tissues.

Although the role of carbonic anhydrase in plants has not been established there was much theoretical support for the assumption that the enzyme, as a catalyst of a reaction which has definite relations to the process of photosynthesis, would be present primarily in photosynthesizing tissues. Bradfield (3) and Waygood and Clendenning (62) have shown that this is the case. The present findings indicate rather high enzyme activity in all leaves. The difference found in the leaves of different ages, though significant, thus appears a point of minor importance. The much lower enzyme activity in stems and petioles stresses the possibility of participation of the enzyme in photosynthesis.



Chlorophyll and carbonic anhydrase. The possible connection of the enzyme with photosynthesis has raised the question of its correlation with the chlorophyll content of tissues. Bradfield (3) has reported that roots are devoid of the enzyme and Waygood and Clendenning (62) have shown some correlation of carbonic anhydrase activity and chlorophyll content in green and albino leaf tissue of Tradescantia fluminensis and Hordeum vulgare. Exposing plants of Tropaeolum majus and Petroselinum hortense to darkness, they found that a depletion of carbonic anhydrase was accompanied, but not necessarily correlated with, a corresponding loss of chlorophyll.

Several species were examined to ascertain a possible correlation between chlorophyll and carbonic anhydrase concentration. The studies were carried out during the summer of 1951. The results are assembled in Table 13.

No relationship between chlorophyll concentration and carbonic anhydrase activity could be established. Both properties seem to be entirely independent of each other. Helianthus annuus and Vinca minor which did not show any carbonic anhydrase activity have medium chlorophyll content, while Acer pseudoplatanus, which has the highest chlorophyll content of the species tested, exhibits very moderate enzyme activity. On the other hand, the species showing the highest enzyme activity, Asclepias syriaca, Chenopodium album and Phytolacca americana, are in the low or intermediate chlorophyll range. In both cases where old and young leaves of

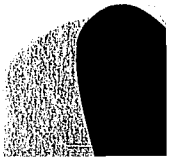


TABLE 13

CARBONIC ANHYDRASE ACTIVITY AND CHLOROPHYLL CONTENT* OF THE LEAVES OF VARIOUS PLANT SPECIES

Species	Milligrams of total chlorophyll per gram of fresh tissue	Enzyme units
Acer pseudoplatanus	4.36	24
Asclepias syriaca	1.67	152
Buxus sempervirens	1.52	
Chenopodium album	1.44	113
Convallaria majalis	3.00	19
Dahlia variabilis	2.24	32
Forsythia sp., young leaves	2.04	32
old leaves	2.21	42
Helianthus annuus	2.26	0
Helianthus decapetalus	2.32	44
Hypericum aureum	1.73	52
Koelreuteria paniculata	3.09	31
Laburnum vulgare	3.14	85
Maclura pomifera	1.93	35
Paeonia officinalis	2.65	41
Pelargonium hortorum	1.23	66
Phytolacca americana	2.44	110
Pseudotsuga taxifolia, young leaves	0.95	35
old leaves	1.77	10
Ulmus americana	2.00	55
Vinca minor	2.87	0

*Chlorophyll determinations made at the Department of Agricultural Chemistry through courtesy of Dr. E. J. Benne.

the same species were tested chlorophyll content of the older leaves, as was to be expected, was higher while only in one of these instances, Forsythia sp., was there a corresponding rise in enzyme activity. In the other, Pseudotsuga taxifolia, less enzyme activity was found in older leaves.

It must be mentioned, however, that this latter instance could be due to an inefficiency in extracting the enzyme from the tougher needles of the older growth. None of the extraction techniques used seemed to be sufficiently thorough to insure a complete removal of the enzyme. Likewise, the low enzyme readings in some other species could be due to the presence of interfering substances. This might be particularly true in the leaves of some of the woody perennials tested where tannins and tannin-like substances have been shown (41) to inactivate enzymes, partially or completely, during the process of extraction.

Distribution in chlorophyll-free organs. Although evidence was quite convincing that carbonic anhydrase is localized in photosynthetic tissues even though no correlation with chlorophyll could be established it seemed desirable to ascertain that chlorophyll-free tissues did not exhibit enzyme activity. Table 14 presents a survey made with roots of plants whose leaves had shown distinct enzyme activity. The negative results obtained confirm Bradfield's (3) findings.

In addition to roots the chlorophyll-free tissues of

TABLE 14

SEARCH FOR CARBONIC ANHYDRASE IN ROOTS OF PLANTS WITH CONSIDERABLE CARBONIC ANHYDRASE ACTIVITY IN THEIR LEAVES

Species	Apparent enzyme activity $E = \frac{R - R_o}{R_o} \times 100$	
	Leaves	Roots
Chenopodium album	105	0
Lathyrus odoratus	75	0
Polygonum persicaria	126	0
Raphanus sativus	114	0

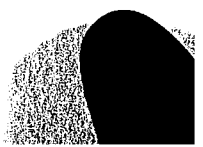
flowers were investigated. The results are assembled in Table 15.

TABLE 15

SEARCH FOR CARBONIC ANHYDRASE IN CHLOROPHYLL-FREE TISSUES OF CERTAIN FLOWERS

Species	pH of		Apparent enzyme activity of petal extracts $E = \frac{R - R_o}{R_o} \times 100$
	Extract	Buffered test soln.	
Begonia Dregei	5.9	7.0	40
Calendula officinalis	6.5	7.3	8
Chrysanthemum cinerariaefolium	6.4	7.5	0
Dianthus Caryophyllus	6.4	6.9	18

The results of this test are somewhat unexpected but they have been confirmed by repeated determinations. Extension of this part of the work to cover other species has not been possible as yet; there is no question, however, that it should be done. Since in all these tests green parts were carefully eliminated this seems to be the first instance in which the occurrence of carbonic anhydrase has been found in other than chlorophyll-bearing tissues. It was surmised that the hydrogen ion concentration of the extracts might be responsible for the increased evolution of CO_2 in most of the extracts. For this reason pH measurements were taken in the original extracts as well as in the mixture of the reagents after the addition of the plant extract. The data of the table indicate that even the variation in the original extracts is probably too small to explain differences; moreover, the test solutions appeared well buffered and within the range where no differences in CO_2 evolution should be recorded. There is no explanation so far for these results from the hypothetical viewpoint. In the tissues mentioned carbonic anhydrase could certainly not be of use for the purpose of catalyzing any reaction of photosynthesis. No clue is apparent that the enzyme might be formed in the flowering parts since it is present in the leaves long before flowers form. This must lead to the assumption that some other physiological process might also require the presence of the enzyme.



Properties of the Enzyme

Stability in crude solutions

Considerable discrepancy is found in the literature (11, 19, 32, 39, 62) regarding stability of the enzyme in fresh preparations. A large number of experiments conducted for the purpose of determining the prevailing conditions resulted in a partial explanation of the discrepancies. Table 16 shows some of the results obtained, which represent averages of 25 individual experiments for each plant.

TABLE 16

THE STABILITY OF CARBONIC ANHYDRASE IN CRUDE WATER EXTRACTS OF FRESH PLANT MATERIALS AT ROOM TEMPERATURE AND AT 4° C

Species	Percent of enzyme activity after 6 hours							
	6		12		24		48	
	R.t.	4°C	R.t.	4°C	R.t.	4°C	R.t.	4°C
Antirrhinum majus	34	76	4	58	0	50	0	30
Chenopodium album	95	100	80	100	25	100	0	100
Lathyrus odoratus	60	100	55	100	19	100	0	100
Phaseolus vulgaris	56	91	17	78	0	34	0	13
Tulipa Gesneriana	63	100	18	99	0	79	0	72

The rate of destruction of the enzyme varies with the plant material but probably also with other factors. While

there is a definite ability of certain extracts, as those of Lathyrus odoratus and Chenopodium album, to retain their activity over long periods when refrigerated other plant extracts may at times retain activity and at other times lose it within much shorter periods. To illustrate this, the retention of carbonic anhydrase activity of 20 preparations of Tulipa Gesneriana, which were made identically and stored under identical conditions at 4° C, is presented (Table 17).

TABLE 17

DECLINE OF CARBONIC ANHYDRASE ACTIVITY OF WATER EXTRACTS OF TULIPA GESNERIANA IDENTICALLY PREPARED AND STORED AT 4° C

Apparent enzyme activity of fresh extract: E = 105

Extract no.	Percent activity after 6 hours	12 hours	24 hours	48 hours
1	100	100	93	86
2	100	102	73	71
3	100	99	81	65
4	100	100	78	70
5	100	99	74	70
6	100	96	81	80
7	100	101	85	78
8	100	103	69	64
9	100	97	91	85
10	100	99	73	65
11	100	91	80	80
12	100	100	77	70
13	100	100	79	73
14	100	100	85	74
15	100	100	68	54
16	100	102	90	83
17	100	100	78	72
18	100	100	73	69
19	100	93	81	76
20	100	97	75	75

It is concluded from these experiments that carbonic anhydrase stability in aqueous preparations is partly influenced by the presence of protective substances in the extract, partly, however, dependent on environmental conditions which cannot be fully explained at the present time. It seems possible that certain protective substances that counteract the deterioration of the enzyme are present in greater abundance in some extracts of the same species than in others, which in turn might be due to external factors of unknown nature.

Work by others (62) has shown that extracts of spinach could be stored without loss of activity for several months at -40° C. Similar observations resulted from experiments conducted to investigate the stability of extracts at very low temperatures. Several species were extracted with water by one of the standard methods and the extracts stored in screw-cap jars at temperatures ranging from -15° to -30° C. The results of tests conducted after varying periods are summarized in Table 18.

The values obtained indicate that retention of activity depends on the species. As the first column of data shows all extracts examined had a high initial activity. It is likely that the losses in these extracts will be smaller than in those with smaller initial enzyme activity. However, milkweed extract had decidedly less tendency to retain carbonic anhydrase activity than the two other preparations.

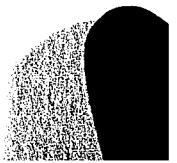


TABLE 18

RETENTION OF RELATIVE CARBONIC ANHYDRASE ACTIVITY IN AQUEOUS EXTRACTS STORED AT VERY LOW TEMPERATURES

Species	Apparent enzyme activity before storage	Percent of enzyme activity in extracts thawed after		
	E	3	6 months	12
Asclepias syriaca	116	76	70	*
Chenopodium album	72	100	100	97
Tulipa Gesneriana	76	96	95	94

*Not tested

Stability of frozen plant material

While Waygood and Clendenning (62) reported the application of procedures previously described for the storage of isolated chloroplasts for photochemical experiments no mention is made, in the meager literature pertaining to plant carbonic anhydrase, of the storage of fresh plant material in the deep-freeze. Extensive experimentation was carried out to show the effect of extended storage of freshly gathered plant material at very low temperatures. For experimentation with plant enzymes such a method is of considerable practical value because it would allow the practically unlimited use of uniform experimental material throughout the year if it were successful.

During the summers of 1949, 1950 and 1951 fresh plants of Chenopodium album, Tulipa Gesneriana and Asclepias syriaca were gathered and immediately compressed in No. 10 tin cans.

No attempt was made to pack the plant material as tightly as could have been possible by using devices to insure a great deal of pressure; mere hand pressure was exerted. The cans were not sealed but merely tightly closed by attaching wax paper lids with rubber bands. They were taken to a storage room having a temperature of -20° C immediately after each can had been filled. Consecutive sampling revealed the percentage of carbonic anhydrase in the extracted plant material. Care was taken in each instance that blending or grinding took place while the plants were still in a frozen state; thawing thus occurred during the process of extraction. Table 19 summarizes the results.

TABLE 19

CARBONIC ANHYDRASE ACTIVITY IN AQUEOUS EXTRACTS MADE FROM PLANTS FROZEN AND STORED AT -20° C

Species	Enzyme activity $E = \frac{R - R_o}{g} \cdot C$ μmoles			
	0	6	12	24
Asclepias syriaca	155	116	*	*
Chenopodium album	110	108	112	114
Tulipa Gesneriana	105	106	102	101

*Not tested

The outstanding retention of enzyme activity in the plant material stored according to the method described is evident from the table. This virtually insures the possibility of studying the enzyme throughout the year in sources

grown under natural conditions. This point is of importance since it had been questionable, not only on the basis of reasoning but also according to erratic results obtained from some greenhouse-grown plants, whether material from the greenhouse should be used at all for fundamental studies of the enzyme.

Interaction with chemicals *in vitro*

Before the publication of Waygood and Clendenning (62) appeared but after Day and Franklin (11) and Bradfield (3) had reported their work, experiments were conducted to show the effect of inhibitors, especially those which were known to inhibit animal carbonic anhydrase (19, 35, 53), on the plant enzyme. Sodium cyanide and sulfanilamide were used in different molarities. A special study was made with 2,4-dichlorophenoxyacetic acid (2,4-D) since the entire work with carbonic anhydrase was originally started to investigate the possibility of an interference with this enzyme by 2,4-D when applied to plants. The results of these studies are presented in Figures IV and V.

The addition of sodium cyanide in concentrations of 0.01 and 0.001 molarity in the final dilution inhibited the enzyme approximately 60 and 30 percent, respectively. These figures are in general agreement with the ones obtained by Bradfield (3) and Waygood and Clendenning (62) but do not agree with those of Day and Franklin (11).

A concentration of 0.1 M sulfanilamide in the plant

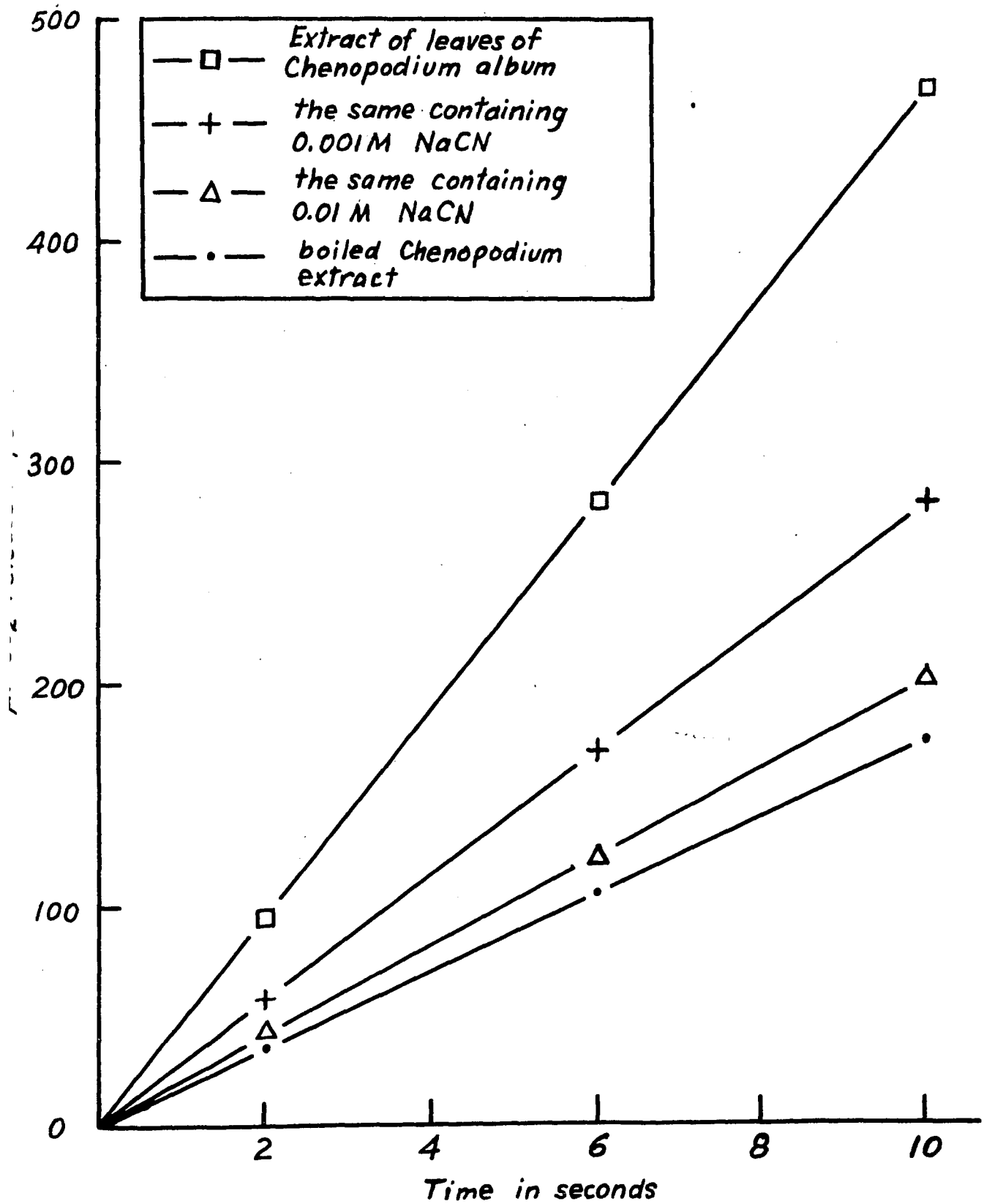


Figure IV. Inhibition of carbonic anhydrase by sodium cyanide

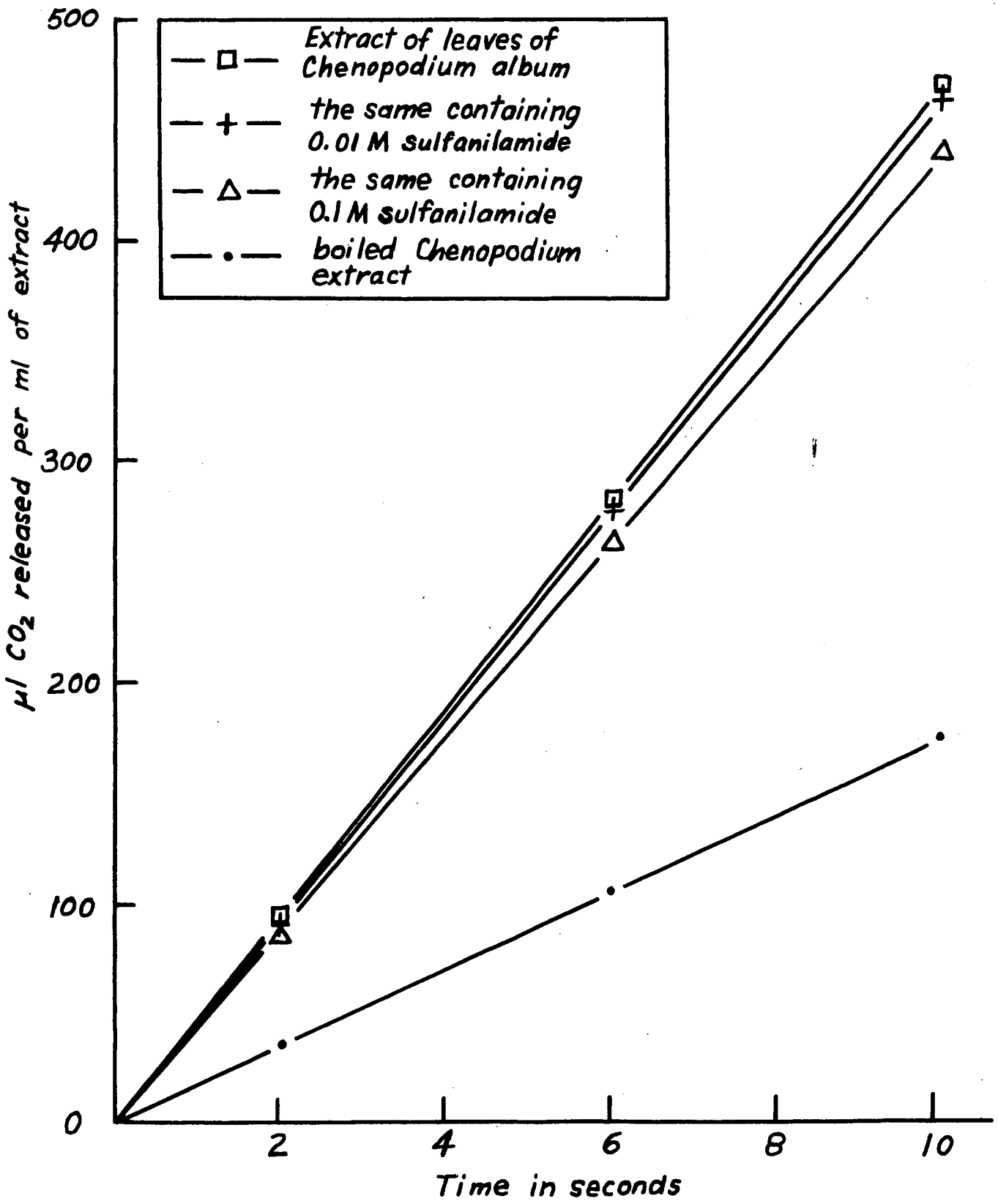


Figure V. Inhibition of carbonic anhydrase by sulfanilamide

extract tested inhibited only 15 percent while a molarity of 0.01 did not affect the enzyme activity at all. This agrees with Bradfield's (3) findings as well as with those of Day and Franklin (11). It does not agree, however, with Waygood and Clendenning (62) who reported an inhibition of 59 percent of Tradescantia leaf extracts by 5.2×10^{-4} M sulfanilamide.

It is likely that these discrepancies can be explained on the basis of the kind of plant material used, the time of contact of the inhibitor with the enzyme and the temperature at which the test is performed.

In the case of 2,4-dichlorophenoxyacetic acid no appreciable interaction could be found. In view of the fact that Waygood and Clendenning (62) reported 33 percent inhibition of carbonic anhydrase activity in extracts of Tetragonia expansa by 2,4-D, experiments were conducted with various plant extracts in order to ascertain whether earlier negative results were in error or whether the species was the determining factor in the discrepancies. No differences could be found, however, from the original tests in which leaves of Phaseolus vulgaris had been used. Table 20 shows that the enzyme activity of two species used in the present experiments was hardly more affected than that of beans. The greatest loss of activity was 10 percent but the inconsistency of the inhibitions resulting from different concentrations of 2,4-D used is evidence that any inhibitory effect recorded is of a minor nature.

TABLE 20

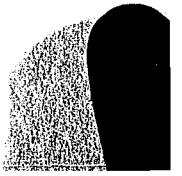
EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON CARBONIC ANHYDRASE

Source of enzyme	Original enzyme activity E_A	Molar conc. of 2,4-D in test solution	Relative enzyme activity Exp. 1	Exp. 2	Exp. 3
Chenopodium album - leaves	102	10^{-2}	95	99	100
		10^{-3}	90	91	94
		10^{-4}	90	96	94
		10^{-5}	91	93	94
		10^{-6}	90	96	98
		10^{-7}	96	93	98
Pisum sativum - leaves	88	10^{-2}	95	96	95
		10^{-3}	97	97	96
		10^{-4}	98	98	93

It is possible that the discrepancy between these results and the ones reported by Waygood and Clendenning (62) is based on the considerable difference of temperature at which the reaction took place (30° C as compared with 15° C). No attempt was made, however, to use the lower temperature selected by the Canadian workers.

Studies in the kinetics of the enzyme

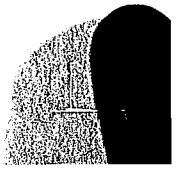
Since no work had been published to furnish information on the kinetics of the plant enzyme similar to the fairly extensive literature that exists on the kinetics of the animal enzyme, investigations were started by Byerrum and



Lucas in early summer of 1950. Part of the work done was published recently. The following is an account of some phases of this cooperative work.

Fresh leaves of Tulipa Gesneriana and frozen leaves of Chenopodium album (the freezing of the plant material done according to the description given earlier in this paper) were used in the experiments. Redistilled water was used for all preparations which were made in a Waring Blendor. Blending took place in a low-temperature room at 7° C. The normal time of blending was 5 minutes. Usually 100 g of fresh leaves were blended with 200 ml of water. The blended material was strained through 4 layers of cheesecloth and the residue rehomogenized in the blender with one weight of water based on the original weight of leaves. After straining this material the liquid was combined with the original. The total filtrate, usually 325-350 ml, was left at the same temperature for 4-5 hours and then centrifuged. In the case of the tulip leaves a complete separation of the chloroplastic fraction was possible and the supernatant appeared as a clear amber-colored solution. The lamb's-quarters preparation was slightly greenish after centrifugation. The supernatants were used in both cases for the studies. Dry weight was determined of each preparation used and the results were expressed in terms of dry weight.

A modified Warburg technique was used for the determination of the enzyme activity. The use of a Warburg apparatus for rapid enzyme determination is somewhat open



to question. As a matter of fact, testing would have been difficult if not impossible had the adjustment of the closed arm been necessary as in the conventional method. The modification used dispenses with this step. The accuracy of the method employed is amply shown by the fact that duplicates checked within 10 percent except when evolution or uptake of gas was less than 10 μl in which cases errors as high as 20 percent were recorded.

When CO_2 evolution was determined 0.5 ml of buffer solution of the desired pH was introduced into the main compartment of a Warburg vessel together with 0.1-0.5 ml of leaf extract. Water was added to make 1 ml. One-half ml of sodium bicarbonate solution in water was added to the sidearm. The vessel was attached to a manometer, placed in the constant temperature bath of the desired temperature and flushed with water-saturated N_2 for 10 minutes. The reaction was initiated by tipping the bicarbonate solution from the side-arm into the main compartment; this operation took about 4 seconds. Readings were made on the closed arm of the manometer at desired time intervals. During the reaction the flask was shaken 2 times per second at full amplitude on a GME circular Warburg apparatus.

For CO_2-uptake measurements 1 ml buffer and the desired volume of leaf extract were pipetted into the main compartment and water was added to make 1.5 ml. Flask and manometer were again placed in the constant temperature bath and allowed to stand for 8 minutes. CO_2-N_2 mixtures of the desired composition were introduced into the reaction

vessels by first evacuating the flask and then allowing the gas mixture to enter. Gassing was repeated 3 times and the reaction was initiated by shaking the flasks violently 6 times per second on an A. H. Thomas Warburg apparatus. Rapid shaking was necessary because diffusion appeared to become the limiting factor in the hydration of CO_2 when agitation was less violent; in this case no catalytic activity could be recorded. CO_2 uptake was measured on the closed arm of the manometer at desired time intervals. Cleaning of the flasks took place, according to recommendation by Roughton and Booth (52), in a sulphuric acid-nitric acid mixture.

Boiled preparations were run as controls. Boiling was accomplished in a water bath for 5 minutes; flocculated protein was redispersed by triturating with a glass rod to make the boiled preparation as homogeneous as possible and to facilitate pipetting.

Analyses were made in duplicate or triplicate, and several determinations were made with different leaf preparations.

Effect of leaf extract concentration on CO_2 evolution

Figure VI shows the effect of the concentration of the enzyme, expressed in terms of dry weight, on the rate of evolution of CO_2 from a buffered bicarbonate solution. The ordinate represents the first order rate constant for the reaction in the presence of the plant extract minus the first order rate constant in the presence of the boiled

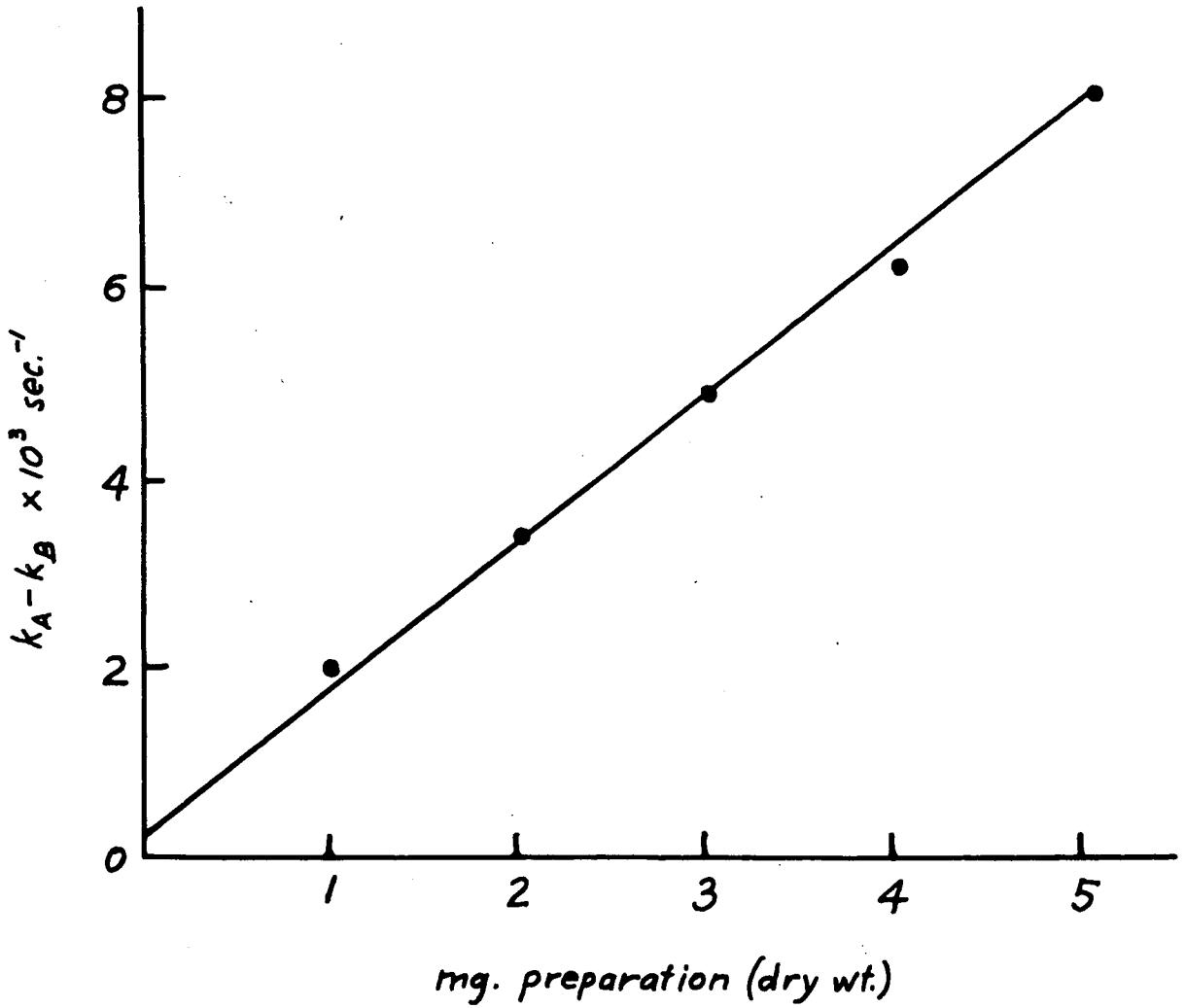


Figure VI. The influence of leaf extract concentration on the evolution of CO_2

preparation. This method of defining carbonic anhydrase activity was used by Brinkman, Margaria and Roughton (4) and subsequently by Kiese and Hastings (24). The first order rate constant can be used as a measure of activity only when hydration of CO_2 is negligible. The evolution of CO_2 within the first 15 seconds was used therefore to calculate the data. The abscissa expresses mg of dry weight of the preparation added.

The rate of evolution of CO_2 is shown to be directly proportional to the concentration of solid matter in the added plant extract.

It was found that during the conversion of bicarbonate to CO_2 and H_2O, H^+ also reacts and a phosphate buffer of about 0.1 molarity is required to maintain the pH during the reaction. Roughton and Booth have pointed out that inorganic ions in many buffers catalyze CO_2 hydration in the absence of carbonic anhydrase (52). It was necessary therefore to select a buffer which would be of sufficiently low concentration but would still maintain a constant pH during the time required for the experiment. A 0.05 M phosphate buffer as suggested by Roughton and Booth (52) met these requirements; it gave a final concentration in the flask of 0.017 M. It was noted that the pH rose during the time of measurement not more than 0.15 unit. The pH change after the reaction had proceeded to equilibrium, however, amounted to approximately 0.5 unit.

Effect of leaf extract concentration on CO_2 uptake

The hydration of CO_2 was also measured as a function of the concentration of the enzyme expressed as dry weight in the plant extract. The pertinent data appear in Figure VII. The ordinate represents the first order rate constant for the hydration of CO_2 in the presence of the enzyme preparation minus the first order rate constant in the presence of the boiled extract. The abscissa represents the concentration of dry matter contained in the leaf extract.

There appears to be a linear relationship between CO_2 uptake and concentration of the preparation. The pressure of CO_2 used as substrate was 300 mm Hg. Two other pressure values, namely 748 mm Hg and 37 mm Hg, did not provide suitable conditions for the demonstration of a catalytic effect on the CO_2 uptake in this experiment. Kiese and Hastings (24) reported similar conditions in kinetic studies with animal carbonic anhydrase where the range of CO_2 pressure at which enzyme activity could be shown in uptake experiments was equally narrow.

Effect of concentration of the substrate on the evolution of CO_2

Figure VIII demonstrates the effect of concentration of sodium bicarbonate which was used as substrate on the evolution of CO_2 in the presence of a leaf extract. The ordinate expresses μmoles of CO_2 liberated during the first 15 seconds of shaking minus μmoles of CO_2 liberated in the

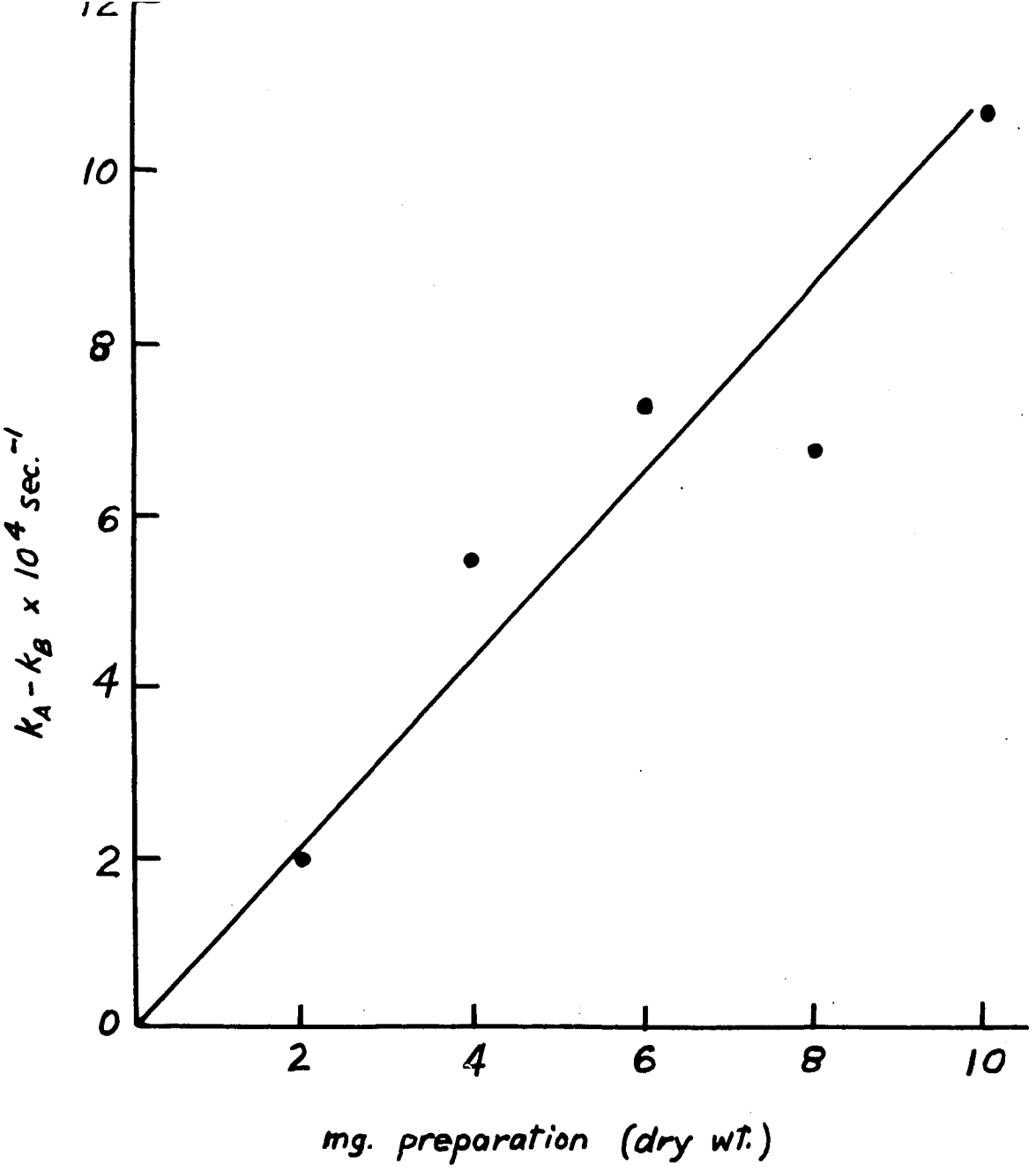


Figure VII. The influence of leaf extract concentration on

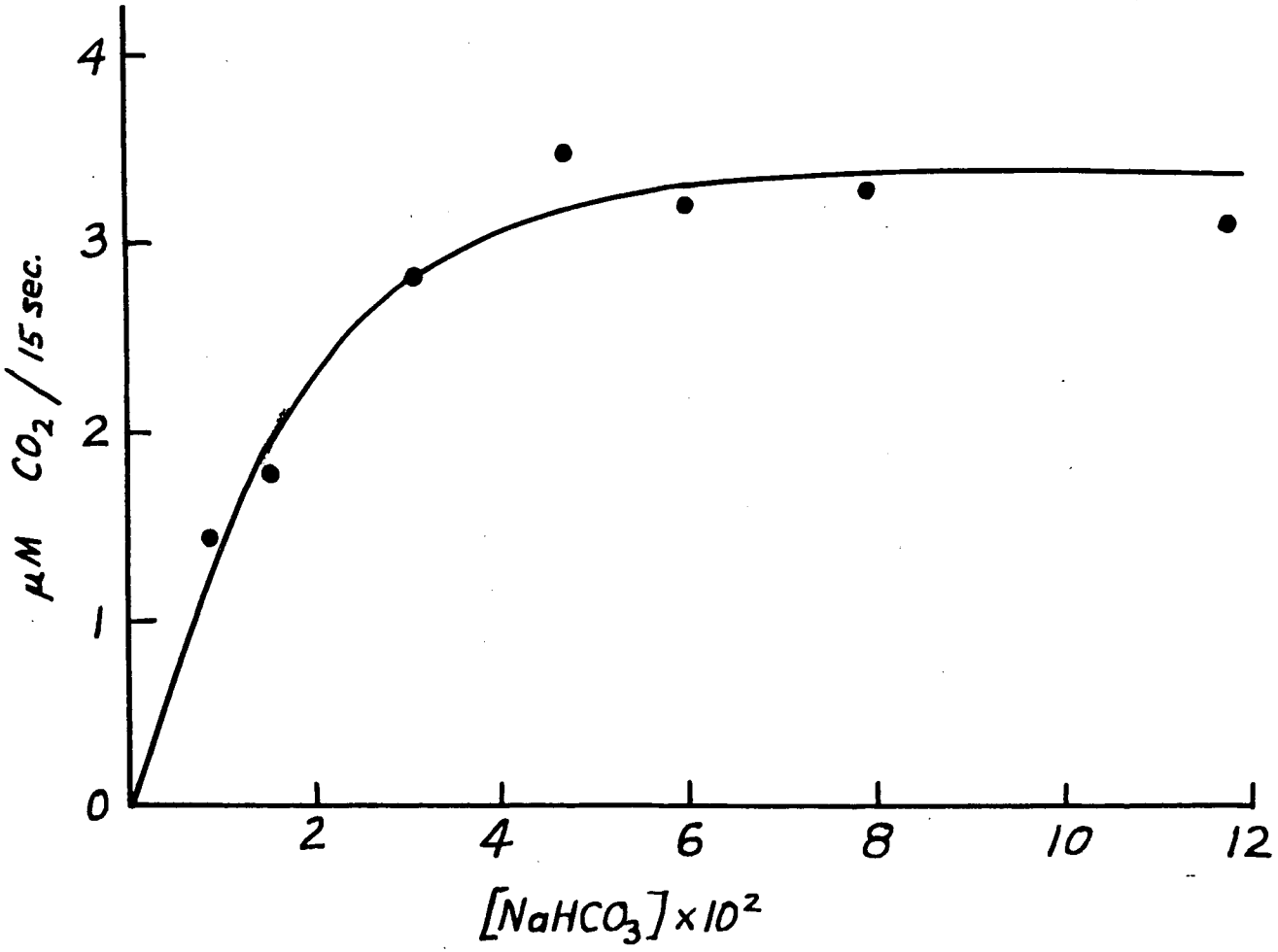


Figure VIII. The influence of substrate concentration on the evolution of CO_2

presence of a boiled preparation. The first order rate constant cannot be used as a measure of activity when initial substrate concentrations are varied. The abscissa indicates the molarity of the substrate.

When 6 mg of leaf preparation was used as enzyme source the rate of evolution of CO_2 increased with the substrate concentration to about 4×10^{-2} M bicarbonate. Any further increase in substrate concentration gave no further increase in the rate of reaction. It could be assumed that the levelling off of the curve be due to limitation of the rate of evolution of CO_2 by diffusion. This is improbable, however, since evolutions of CO_2 which were two to three times greater than those recorded in this experiment have been observed.

Effect of pH on CO_2 evolution

The effect of hydrogen ion concentration on the evolution of CO_2 in the presence of leaf extract is shown in Figure IX. The ordinate indicates the difference in first order rate constants as explained in the discussion of Figure VI. The abscissa represents the initial pH of the buffer. This pH did not rise more than 0.15 unit during the period of the experiment. The pH change in the individual experiments was approximately the same. The evolution of CO_2 under the conditions described is pH dependent. The two curves represent the values recorded with two different buffers - the standard phosphate buffer as described earlier

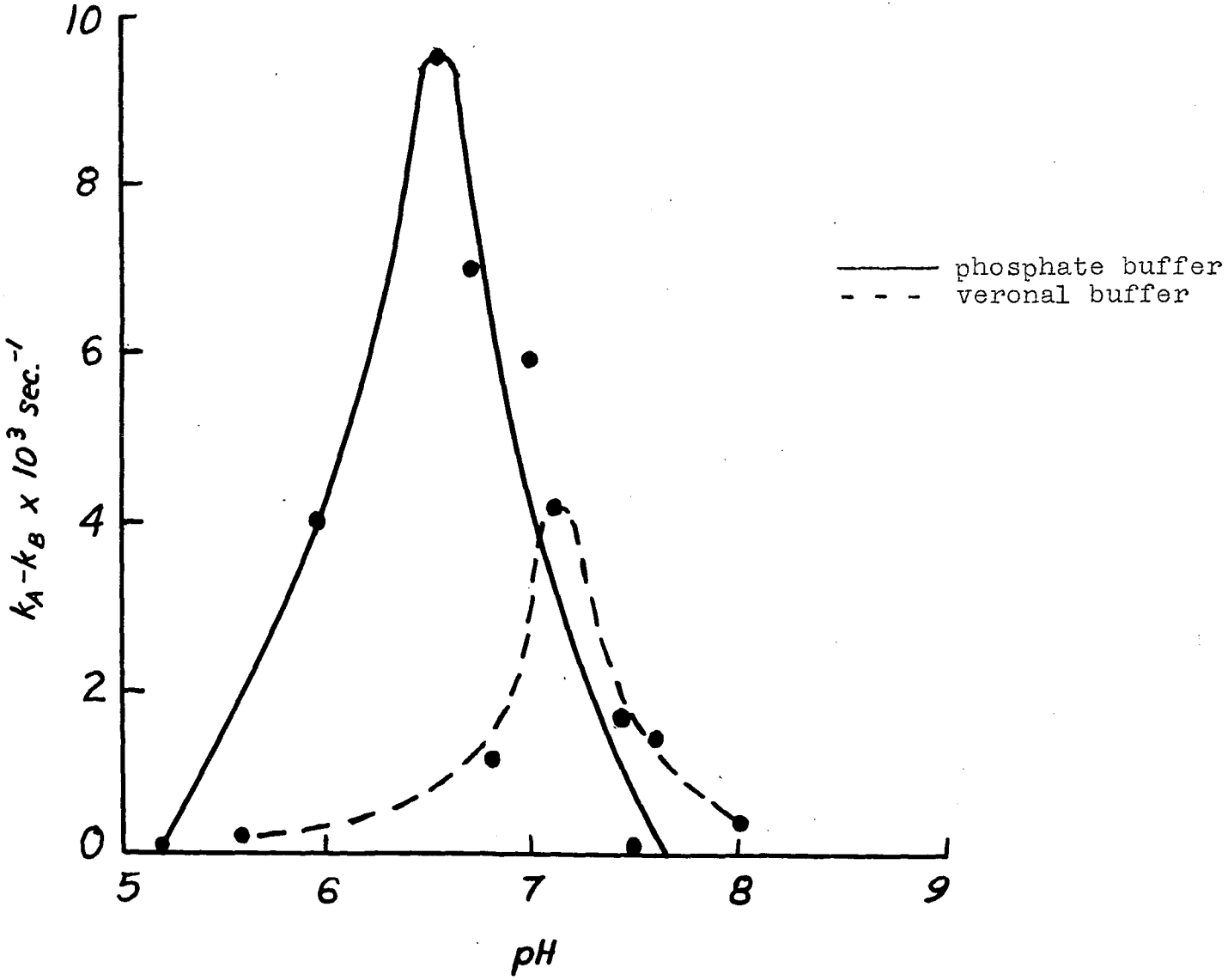


Figure IX. The influence of pH on the evolution of CO_2

in this paper and a veronal buffer. It can be seen that the peaks of these curves lie at different pH values. However, the general similarity of the curves is very evident. While under the standard conditions of the test the highest enzyme activity is recorded at approximately pH 6.55, it is at pH 7.1 in veronal buffer. Waygood and Clendenning (62) seemed to have some misgivings about testing the evolution of CO_2 at different pH levels; they assume that the change of the pH of the reaction medium involved a change in the method. They therefore incubated plant extracts with buffer solutions at various pH levels for several hours prior to testing and found no effect from exposures to buffers within the range of pH 5.0-7.0. Exposures to more acidic or more alkaline conditions, however, resulted in lower activities when the test was performed under standard conditions. It cannot be seen why testing the enzyme activity according to the description given above should not convey a true picture of the reactibility of the enzyme. It is assumed that incubation at varying pH levels with subsequent tests under standard conditions does not give the information desired and obtained in the studies presented here.

The comparison of the two curves indicates a much lower enzyme activity in the case of veronal buffer. The difference not only depends on the buffer used but primarily on the fact that the extract used in the veronal buffer experiment had been held in the refrigerator for about one week while the phosphate buffer tests were performed with

8-hour old extract.

Effect of temperature on CO_2 evolution

The evolution of CO_2 from a bicarbonate substrate in the presence of a leaf extract as influenced by temperature is shown in Figure X. The ordinate is the difference of first order rate constants as previously described. The abscissa represents the temperature at which the experiment was conducted. To obtain the conditions outlined the Warburg vessels were placed in a water bath of the desired temperature and allowed to remain for 10 minutes which insured that a temperature equilibrium between bath and test solution was attained. The release of CO_2 was then measured as described in the other experimental procedures. An increase in the activity of the system was observed up to a temperature of 40° C. Further temperature increases resulted in a sharp decline of the enzyme activity until at 50° C no catalytic effect was noticeable. It can be seen that the reaction rate is directly proportional to the temperature between 0° and 40° C.

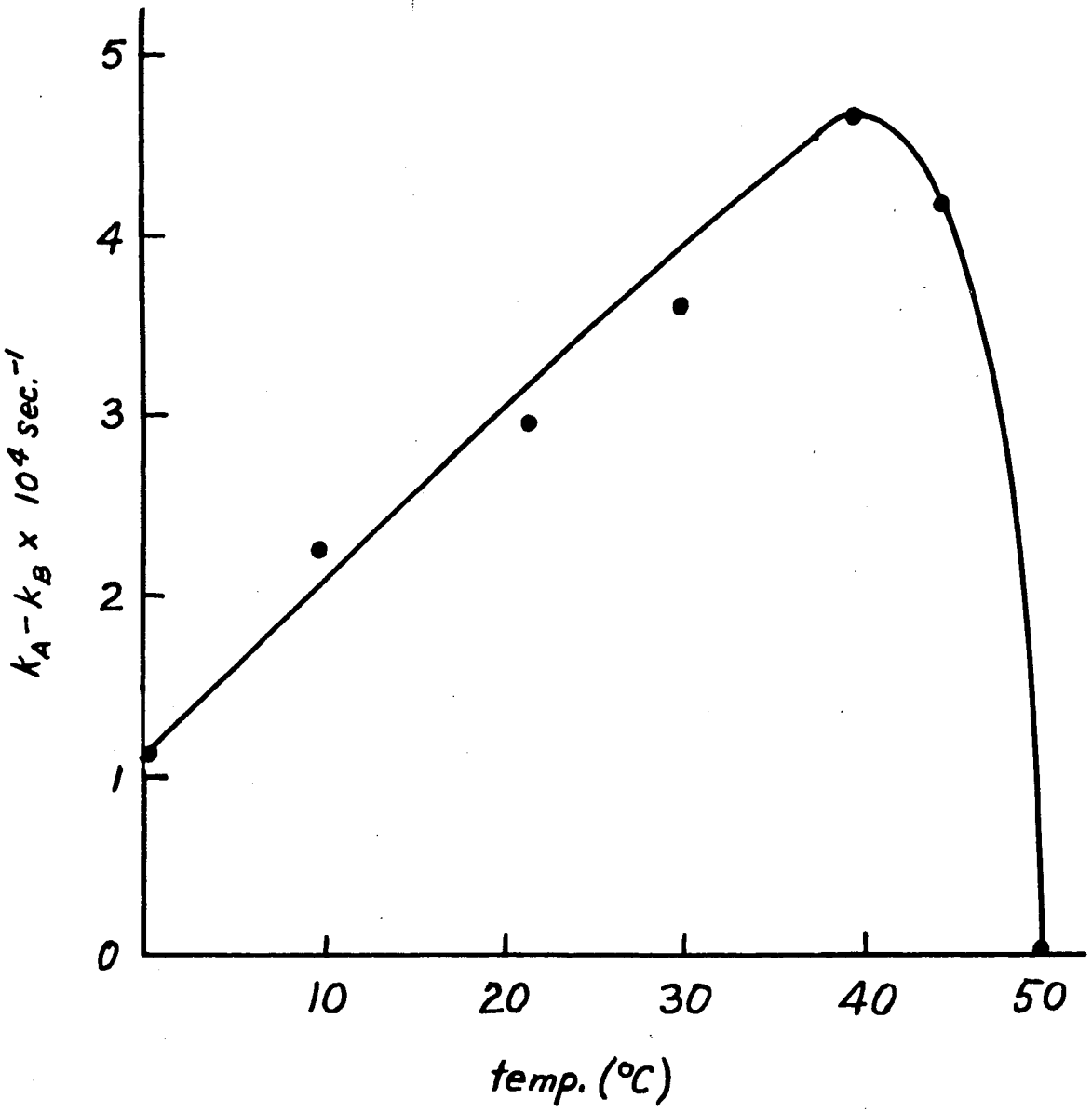


Figure X. The influence of temperature on the evolution of CO_2

Purification of the Enzyme

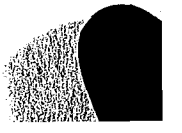
A number of attempts were made to prepare the enzyme in a purified form. Earlier experiments had established that salt precipitation had a destructive effect on the enzyme, and tannin precipitation led, in most instances, to an irreversible inactivation. It was necessary, therefore, to find procedures which would free the enzyme from as much of the extraneous matter as possible with the smallest possible loss of activity.

Materials

In many of these experiments frozen Chenopodium album leaves were used. As has been pointed out, this material could be made available in uniform quality throughout the year by keeping the freshly gathered plants in tin cans at a temperature of about -20° C.

When used, this material was removed from the cold storage shortly befcre preparations were made. Many of the preparations were made at temperatures between 3° and 5° C; when room temperature was chosen, the material under treatment was held cold by means of ice, ice-cold water or, in some instances, dry ice. This latter procedure was utilized whenever small quantities were prepared by means of the homogenizer described earlier in this paper.

In more recent experiments spinach leaves which had been frozen soon after being collected in the field were used. It was found that spinach, as could be expected from



the results of earlier work by others (62) and because of the close relationship to lamb's-quarters, was a suitable source of the enzyme.

The use of proteolytic enzymes

Oparin and collaborators (41, 44) have emphasized that plant enzymes are frequently adsorbed on colloidal surfaces, many of which are, as has been mentioned earlier, of proteinaceous nature. It appeared logical that proteolytic enzymes might be utilized to advantage in releasing enzymes from such an association provided that the application was so dosed that the enzymes themselves were not attacked by the proteolases. Preliminary experiments indicated that such a possibility existed. The following proteolytic enzymes were used: Protease, a commercial proteolytic enzyme; U.S.P. pancreatin; trypsin 1-300; and a commercial preparation of papain. The enzymes were added to the crude plant extract, which was strained and filtered as described before, in a tentative concentration of 1 percent. Digestion at room temperature was allowed to proceed for 1 hour. During this time the suspensions were stirred at slow speed by an electric stirrer. The material was centrifuged and the supernatant liquid tested. Table 21 presents the results obtained.

Neither protease nor pancreatin treatment left any enzyme activity while the treatment with trypsin diminished it by two-thirds. In contrast, treatment with papain

TABLE 21

CARBONIC ANHYDRASE ACTIVITY OF *CHENOPODIUM ALBUM* LEAF EXTRACT AFTER TREATMENT WITH PROTEOLYTIC ENZYMES

Treatment	Relative enzyme activity		
	Exp. 1	Exp. 2	Exp. 3
Untreated	100	100	100
Protease	0	0	0
Pancreatin	0	0	0
Trypsin	33	40	36
Papain	137	152	146

enhanced the enzyme activity by more than 40 percent. Similar results were obtained when acetone precipitates from crude leaf extracts were treated with enzymes. Precipitation with acetone at the levels of 50 and 85 percent of the final volume yielded a material which was inactive or of reduced activity compared with the original extract. When 1 percent papain was added to the original extract the acetone precipitates showed varying degrees of activity (Table 22).

The comparative values obtained are somewhat different if they are based on dry matter. Table 23 indicates this altered relationship.

It is evident that, although a smaller amount of enzyme was originally precipitated when the final volume contained 50 percent acetone than when it contained 85 percent, the proportion of enzyme in the precipitate is far greater in

TABLE 22

CARBONIC ANHYDRASE ACTIVITY OF ACETONE PRECIPITATES OF CHENOPODIUM ALBUM LEAF EXTRACTS WITH AND WITHOUT PAPAIN DIGESTION

Treatment	Relative enzyme activity		
	Exp. 1	Exp. 2	Exp. 3
Original water extract	100	100	100
Precipitation with acetone			
50 percent of final volume			
undigested	0	0	0
papain digested	35	62	47
85 percent of final volume			
undigested	69	75	72
papain digested	114	102	126

TABLE 23

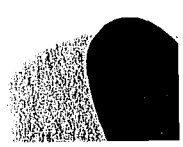
CARBONIC ANHYDRASE ACTIVITY OF ACETONE PRECIPITATES OF CHENOPODIUM ALBUM LEAF EXTRACTS DIGESTED WITH PAPAIN

Treatment	Relative enzyme activity		
	Exp. 1	Exp. 2	Exp. 3
Original water extract	100	100	100
Precipitation with acetone			
50 percent of final volume	145	139	156
85 percent of final volume	88	78	88

the former case. If dry matter is used as a basis of calculation, the yield of enzyme from the precipitation with the greater amount of acetone is less than in the case of computation on the basis of volume of liquid. This con-

dition seems to indicate that while precipitation at the level of 85 percent of acetone separates a larger amount of proteinaceous material, the papain treatment, under the conditions of the experiment, was more effective when a smaller quantity of the proteinaceous material was digested. There is little doubt that suitable conditions can be established under which a maximum of enzyme can be released from a maximum of proteinaceous material but thus far the optimum seems to lie at the level indicated by the table.

It appeared of importance to investigate the possibility of increasing the total yield of carbonic anhydrase by means of papain treatment in a series of subsequent precipitations. It has been pointed out earlier in this paper (cf. Table 7) that under certain conditions repeated extraction will result in the liberation of greater amounts of enzyme which is particularly expressed by a greatly increased enzyme activity based on dry weight of the preparation. In any purification procedure it is of importance to ascertain the conditions under which repeated removal of residual amounts of enzyme can be accomplished without deterioration of the enzyme activity. Acetone-precipitated material has, in general, exhibited decreasing activity but precautions in the process of extraction, particularly the maintenance of the material at a low temperature, may hold losses at a tolerable level. An extraction and precipitation procedure was devised which served to compare total activities in subsequent precipitations and yields of carbonic anhydrase in a partly purified state obtained with and without



pretreatment of the crude extract with papain. Figure XI indicates the course of fractionation.

Twenty g of frozen leaves of Chenopodium album was macerated in a frozen state in the stainless-steel tube of the device shown in Figure I. Distilled water was added when necessary to facilitate this process until a total of 20 ml was used. The macerate was homogenized in the Cori-type stainless-steel homogenizer, also depicted in Figure I. The material was strained through nylon, centrifuged, and filtered. The filtrate was made to 40 ml. The carbonic anhydrase activity was: $E_A = 93$. An aliquot of 10 ml was precipitated by adding acetone until precipitation occurred; in this process 4 ml acetone was used. After centrifuging at 2000 rpm for 15 minutes the precipitate was dissolved in 10 ml water and tested. The carbonic anhydrase activity was determined ($E_A1 = 52$). The same process of precipitation was repeated twice. In either case an amount of 6 ml acetone was required to bring about precipitation. The carbonic anhydrase activities were: $E_A2 = 43$ and $E_A3 = 20$.

An identical run was performed which differed from the one just described merely by the addition of 1 percent papain to the crude extract and digestion in a water bath of 30° C for 10 minutes. The consecutive carbonic anhydrase activities thus obtained were: $E_A1(P) = 73$, $E_A2(P) = 47$ and $E_A3(P) = 20$. The results in terms of percentage yields of enzyme in the case of both procedures are summarized in Table 24.

20 g frozen *Chenopodium album* leaves
pound in macerating device
add distilled water in small portions until total is 20 ml
homogenize in Cori-type homogenizer
strain, centrifuge, filter

Residue
(discard)

Filtrate
make to 40 ml
Carbonic anhydrase activity:
$E_A = 93$
10 ml aliquot, precipitate with small amount of acetone until precipitation occurs (4 ml)
centrifuge

Supernate
add acetone until precipitation occurs (6 ml)
centrifuge

Precipitate
dissolve in 10 ml water
Carbonic anhydrase activity:
$E_A1 = 52$
$E_A1(P) = 73$

Supernate
add acetone until precipitation occurs (6 ml)
centrifuge

Precipitate
dissolve in 10 ml water
Carbonic anhydrase activity:
$E_A2 = 43$
$E_A2(P) = 47$

Supernate
vaporize acetone under reduced pressure
add water to residual liquid to make 10 ml
Carbonic anhydrase activity:
$E = 0$

Precipitate
dissolve in 10 ml water
Carbonic anhydrase activity:
$E_A3 = 23$
$E_A3(P) = 20$

Figure XI. Scheme for the fractionation of *Chenopodium album* leaves

TABLE 24

CARBONIC ANHYDRASE YIELDS FROM CONSECUTIVE ACETONE PRECIPITATIONS WITH AND WITHOUT PAPAIN DIGESTION

	Yields in percentages of original activity	
	Without papain	With papain (1 percent)
First precipitation	55.9	78.5
Second precipitation	46.2	50.5
Third precipitation	24.7	21.5
Total recovery	126.8	150.5
Original extract	100.0	100.0

The remarkable increase in recoverable enzyme activity by successive papain treatment cannot be explained satisfactorily as yet. If the explanations so far proposed were correct it was to be assumed that an increase of total nitrogen in the preparation would indicate that papain had digested part of the proteinaceous material and thus allowed the liberation of more carbonic anhydrase from the plant tissues. Determination of Kjeldahl nitrogen in two preparations of Chenopodium album leaves showed that this assumption was not correct. For this determination aqueous extracts were prepared in the usual manner by blending. The extracts were divided in the following way: Sample 1 did not receive further treatment; sample 2 was digested with 1 percent papain for 1 hour at room temperature; sample 3 was prepared by using the same amount of papain which, however, had been

inactivated by boiling for 10 minutes. In the second experiment a fourth sample was added which was a 1 percent aqueous papain solution. The results are given in Table 25.

TABLE 25

KJELDAHL NITROGEN DETERMINATIONS* OF AQUEOUS EXTRACTS OF CHENOPODIUM ALBUM WITH AND WITHOUT PAPAIN PRETREATMENT

Sample no.	Description of sample	Kjeldahl N Mg/ml
Experiment I		
1	Aqueous extract	0.41
2	Aqueous extract digested with 1 percent papain	1.12
3	Aqueous extract with 1 percent heat-inactivated papain	1.29
Experiment II		
1	Aqueous extract	0.23
2	Aqueous extract digested with 1 percent papain	0.96
3	Aqueous extract with 1 percent heat-inactivated papain	0.99
4	1 percent aqueous papain solution	0.82

*Made at the Department of Agricultural Chemistry through courtesy of Dr. E. J. Benne.

It is obvious that the increase in nitrogen is solely due to the addition of papain. The greater amount of nitrogen in the sample containing heat-inactivated papain is explained by the fact that the amount of dry matter was greater; the nitrogen content expressed on the basis of dry weight in the three samples of this experiment were 0.42, 0.77, and

0.79 percent, respectively.

The logical explanation that remains is that the papain treatment freed bound and thus inactivated carbonic anhydrase from its association with proteins without making the extraction method of nitrogenous constituents used more efficient. Under this assumption the increased yield of carbonic anhydrase in repeated precipitations would indicate that more enzyme was available in active form than in the extract without papain pretreatment. The fact that the greatest difference in enzyme yield was noticeable after the first precipitation supports this idea.

Theories and findings of earlier workers concerning adsorption of plant enzymes on colloidal surfaces and, consequently, inactivation are evidently confirmed in the case of plant carbonic anhydrase.

Precipitation with organic solvents

Extensive experiments were carried out using organic solvents as precipitants. Experiments with acetone have been described before. They have shown that the precipitation of crude protein fractions containing carbonic anhydrase is possible but that separation of the enzyme invariably met with the difficulty that continued procedures involved gradual losses of activity until the preparation that might have been fairly purified was completely inactivated. Similarly, negative results were obtained with ethanol and methanol. Mixtures of these solvents were also

used but the results were equally unsuccessful. Precipitation experiments with glycerol and dioxane gave negative results but it was found that both were solvents for carbonic anhydrase. Furthermore, it was found that carbonic anhydrase retained its activity in these solvents for a considerable period at temperatures between 4° and 10° C. In both instances an enhancement of enzyme activity was recorded similar to that reported previously with respect to chloroform. As in the case of chloroform no interpretation of these results has been found, and further work must be conducted to clarify this condition.

Extraction under nitrogen

In order to rule out the possibility that oxidative or other catalytic destruction of the enzyme might interfere with purification procedures an attempt was made to let the entire extraction process take place in a nitrogen atmosphere. Extraction of frozen spinach leaves was made by means of a Waring Blendor in a coldroom. N_2 was introduced from a tank by bubbling through the water before blending until all air was removed from the vessel. This was repeated after the macerate had been poured into a beaker and during filtration. The preparation failed to yield higher activity than other crude extracts made without nitrogen protection.

Purification by means of dialysis

Several papain-digested preparations were dialyzed against running tap water and against distilled water, both at room temperature and in the cold. The results indicated that only brief duration of dialysis under sufficiently low temperature can be used in the process of purification. Even at the temperature of running tap water, which was approximately 17° C at the time of the experiments, destruction of the enzyme takes place when dialyses are carried on for more than 4 hours. Table 26 illustrates a typical case.

TABLE 26

CARBONIC ANHYDRASE ACTIVITY IN DIALYZED EXTRACTS OF SPINACH LEAVES

	Dry matter mg/ml	Enzyme activity
No treatment	16.6	105
Dialyzed against running tap water for 4 hours	7.0	208
Further dialyzed against distilled water for 2 hours	6.5	160

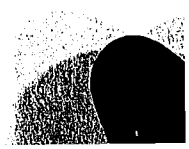
This experiment, which is representative of a large number conducted with similar results, shows that extended dialysis further reduces dry weight by removal of diffusable substances. The accompanying destruction of the enzyme, however, precludes any gain in enzyme activity.

Separation by chromatography

It has been known through experiments conducted as early as 1948 that carbonic anhydrase contained in plant extracts could be adsorbed on paper strips. This work was done to give preliminary information about the stability of the enzyme. When paper strips were brought into contact with aqueous extracts of leaves and the liquid was allowed to either descend or ascend enzymatic activity could be recovered from portions of the strips. The activity thus found, however, disappeared within a very short time, and it became clear that a procedure which would pursue preparative purposes would have to be modified considerably. In the subsequent years occasional attempts of chromatography were made. Several adsorbents - charcoal, Fuller's earth, alumina, magnesium silicate, tricalcium phosphate and bauxite - were used according to recommendations contained in handbooks on chromatography and in monographs. Satisfactory results could be obtained in no instance. The slowness of passage through the columns resulted in enzyme destruction because of high temperature and exposure to air. The only method which proceeded with sufficient rapidity, namely paper chromatography, could not be considered for preparative application because of the minute quantities of enzyme solution recovered.

The difficulty that had arisen in this respect was successfully overcome by the use of a column of Seitz filter pads of the brand Steriflo. It was found that these

disks were more suitable in every respect than the paper disks used and described by Zechmeister (67) in a similar setup. The method used must be termed provisional since equipment which would have made it more efficient was lacking. It is also most likely that other Seitz filter pads might give better results. In the present experiment 60 Steriflo disks #D-8, of 35 mm diameter, were packed into a glass tube of identical inner diameter and of approximately 330 mm length. This tube was inserted in a cylinder slightly wider and longer than the tube. The lower end of the tube was closed by means of glass wool. No great effort is required to pack the disks because of their smooth texture and considerable thickness (3 mm). Packing can be accomplished by means of a cylindrical pestle slightly smaller than the tube. The surface texture of the disks which contain paper and asbestos insures close contact between them. The device was placed in a coldroom, the plant extract poured onto the column and an inverted beaker was used as cover. An aqueous extract of spinach leaves which was digested with papain in the manner previously described was used for this adsorption experiment. During a period of 12 hours 114 ml of the extract was incorporated into the column by gravity flow. The column was extruded with a pestle although considerable friction between the disks and the glass somewhat hampered this operation. The removal of single disks or groups of disks is comparatively easy, however, with a suitable device, like a long-stem corkscrew. The disks were then immersed in varying amounts



of ice-cold water for short periods, thoroughly expressed and the liquid filtered by suction through Schleicher & Schuell analytical filter paper (Shark-Skin). All procedures described were performed at temperatures between 4° and 7° C. Results are presented in Table 27.

TABLE 27

CARBONIC ANHYDRASE ACTIVITY RECOVERED FROM A SEITZ FILTER DISK COLUMN

(60 disks, Steriflo #D-8, numbered from top to bottom)

E of crude extract = 115

Range of disks	E
1 - 6	141
7 - 10	670
11 - 13	990
14 - 16	1664
17 - 23	1020
24 - 27	114
28 - 33	0
34 - 40	0
41 - 50	0
51 - 60	0

The accumulation of enzyme in one portion of the column indicates the successful separation. Further development of the chromatogram which was not attempted here should further concentrate the enzyme and increase the peak activity

recorded. Differences in the color of the column and the eluates indicated that other constituents undergo separation also. All green and greenish pigments were adsorbed above disk 10 while an intense yellow pigmentation sets in at disk 11 decreasing down to disk 24, below which a cream-colored zone appeared. Below disk 42, disks and eluates appeared colorless. The liquid which had passed the column did not reach disks 57-60 in considerable quantity. Below disk 51 amounts of less than 0.1 ml were expressed from individual disks while disk 1 contained the maximum of 1.9 ml.

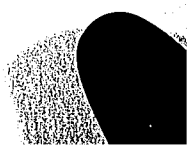
Carbonic anhydrase concentrates thus obtained can be further purified by precipitation, ultracentrifugation or electrophoresis. The eluates obtained have shown considerable stability under refrigeration.

DISCUSSION AND CONCLUSIONS

Presentation is made of the status of investigation of the newly discovered enzyme, plant carbonic anhydrase, as an initial contribution which is to be followed by additional studies that the author and his collaborators have partly started, partly planned. It is perhaps not out of place to point out that the first confirmation of the existence of plant carbonic anhydrase as a common constituent of green plants had been obtained early in 1947, at a time when Bradfield's publication (3) had not yet appeared. For more than six months the author continued work on this enzyme before he obtained the British publication which likewise confirmed the presence of carbonic anhydrase in many plants. Thus, the unequivocal evidence of the general occurrence of the enzyme in plants was obtained simultaneously at Oxford and at East Lansing.

The data submitted in this paper are based on a selection from rather voluminous records. They have been partly published in three separate papers. Another part is expected to be utilized in subsequent papers which are to continue the series of publications on this enzyme.

Details of the findings presented here have been discussed in connection with the enumeration of experimental data. It remains to evaluate the evidence accumulated and

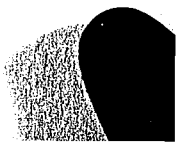


to draw conclusions regarding the possible role that the enzyme may play in the green plant.

Matters of methodology have been covered extensively in this investigation. The controversial results, however, obtained by so many workers over a considerable period, have stressed the intrinsic value of such an approach. Without it, such instances as the occasional occurrence in plant materials of heat-stable catalysts of the dehydration of carbonic acid might cast considerable doubt on the assumption that an enzyme of carbonic anhydrase character exists in plants. The employment of rigid methods has eliminated any possibility of confusion. Where catalysts of the type mentioned exist they must be considered separately from the catalytic activity exhibited by plant carbonic anhydrase.

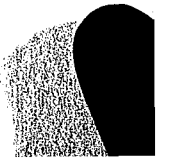
Since a number of such instances is known further work in this direction appears profitable and will be conducted in a subsequent series of experiments.

It has been pointed out by Waygood and Clendenning (62) that the failure of many investigators to find the enzyme can be attributed to the techniques used. The author shares this opinion as the only possible explanation. Yet, the interesting fact remains that none of the workers who were successful in demonstrating the existence of the enzyme have used more elaborate techniques than those who failed to do so. Practically all workers who started the investigation used an apparatus similar to the one employed by



Meldrum and Roughton (35) in their early work on the animal enzyme. In particular, it should be pointed out that the author utilized this method during the first two years of his investigation. Only in later phases was the Warburg apparatus employed in the manner which has been described. Similarly, Waygood and Clendenning (62) used a method for which they adapted certain elements of the Warburg instrument.

It is noteworthy that, while Meldrum and Roughton in their early work determined the activity of the animal enzyme at a temperature of 15° C, later investigators used lower temperatures, Waygood and Clendenning (62) 10° C, others, as has been reported by van Goor (19), and Roughton and Clark (53) 0° C. The reason for the use of lower temperatures has been claimed to be the greater difference between the catalyzed and uncatalyzed rates of hydration which allowed greater accuracy and demonstration of lower enzyme activities. The author has retained a water-bath temperature of 30° C for the reason that this temperature more closely approximates temperatures under which plants are normally grown. Of course, the objection is obvious that a temperature of 20° to 25° C would better satisfy such a requirement. However, it has been found, under the experimental conditions in the author's laboratory, that the most consistent results were obtained at 30° C. As has been shown in the presentation of the kinetic studies enzyme activity at 30° C lies



in the upper third of the ascending part of the curve (Figure X). It was felt that this expresses a favorable condition for the determination, and in view of the fact that difficulties in detecting the presence of the enzyme were never encountered, the temperature was retained.

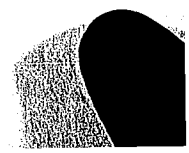
For kinetic studies the use of the Warburg apparatus seems appropriate. It must be emphasized, however, that the simpler equipment was used throughout the course of this investigation, in spite of the fact that Warburg machines were available; this not only indicates the greater rapidity attained with this equipment but also the general fact that simple equipment if properly used can be as valuable as elaborate instruments.

A deviation from the procedures employed by all other investigators who so far have studied plant carbonic anhydrase is in the use of sodium carbonate as reagent instead of sodium bicarbonate, which was first recommended by Meldrum and Roughton and subsequently used in every instance known to the author. Comparisons between sodium bicarbonate and sodium carbonate runs indicated a greater consistency of the results obtained with the latter; it was therefore retained except in the case of the kinetic studies.

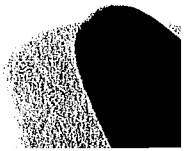
A survey of the quantitative occurrence of carbonic anhydrase in different species shows no lead for correlation with common properties of high and low carbonic anhydrase plants. Results have been obtained from 46 species representing 27 families all of which are listed in this paper.

The leaves of five species have failed to yield any measurable carbonic anhydrase activity; ther are Allium cepa (Liliaceae), Cyclamen persicum (Primulaceae), Cytisus genista (Leguminosae), Urtica urens (Urticaceae) and Vinca minor (Apocynaceae). The fact that these species belong to five different families and the observation that other species of two of these families were examined and found to contain carbonic anhydrase indicate that the enzyme is as ubiquitous as many other enzymes present in plants. Further support of this assumption is given by papers of other workers (3, 5, 39, 62) who found the enzyme in still other species. An obvious explanation for the occasional absence of enzyme activity from leaf extracts is the interference of inactivators. It has been pointed out earlier that there is a very definite possibility of inactivation although this may be a temporary and reversible state. The presence of precipitants as tannins (40, 41) is one of the most likely reasons for the possible disappearance of the enzyme in extracts. Enzyme activity could be restored in such cases by methods as those pointed out by Oparin and co-workers (41, 42, 43, 44) and by Waygood and Clendenning (62). The present investigation was less concerned with the proof that carbonic anhydrase activity be present in all leaf extracts than with presenting evidence that a significant majority of the plants examined show this activity. This has been accomplished.

In addition to green plants, the protozoa Euglena



gracilis, var. bacillaris and Euglena deses have been shown to contain small and irregular amounts of the enzyme. These low activities have their counterparts in the weak activities recorded and reported by Waygood and Clendenning (62) for several aquatic plants. These authors do not draw any conclusions regarding the conspicuous difference in carbonic anhydrase activity observed in aquatic species and most land plants. The papain treatment in the present study does not indicate that adsorption phenomena account for the low activity of Euglena. Waygood and Clendenning (62) have cited sources giving maximum photosynthetic capacities of plants. The maximum rates of photosynthetic oxygen production was reported in various Chlorella species (66). Hence, it may be postulated that the greatest need for catalytic activity would also occur in these species. The fact, however, that carbonic anhydrase activity in algae and other lower organisms is low or hardly detectable might point to the possibility that photosynthesis may not be an identical sequence of chemical reactions in different plants. As far as the author is aware no such hypothesis has been brought forward to date. There are anologies in the animal kingdom, however, where the very fundamental process of digestion assumes entirely different forms in different species. It has been pointed out by Burr (6) that during photosynthesis at high light intensities assimilation of carbon dioxide greatly exceeds the uncatalyzed rate. If the hypothesis first expressed by

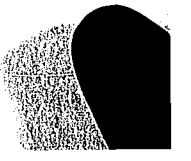


Willstätter and Stoll (64), namely that CO_2 entering the cell combines, after hydration, with chlorophyll as magnesium carbonate, is still partly accepted, the presence of the enzyme would be essential in those organisms that photosynthesize at high light intensities. Algae and other lower forms of plant growth are adjusted to comparatively low light intensities, and it seems quite possible that the enzymatic mechanism provided for such an environment is entirely different from that of higher plants. The ability of these organisms to show unusually high rates of oxygen production when exposed to high light intensities does not necessarily contradict such an assumption.

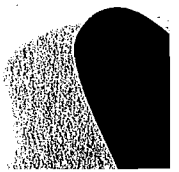
The preliminary findings concerning presence of a catalyst with carbonic anhydrase activity in flowers should perhaps caution one from drawing any premature conclusions as to the function of the enzyme in plants. It would not be uncommon, however, to find an enzyme performing several physiological functions or being part of an enzymatic adaptation system (58). The presence of carbonic anhydrase in certain plant parts may be of signal importance but its interpretation will have to await more specific studies involving the application of the enzyme. For this reason it was thought more important to investigate the chemical properties of the enzyme than the physiological aspects of its function. In this respect the following remarks by Davenport (10) are as applicable to the study of the plant enzyme as they are to the animal enzyme with respect to

which they were written: "..... excesses of speculation and the bitterness of much polemical literature could be avoided if several rules were kept in mind. These rules are 1, an enzyme only speeds the rate at which a reaction attains equilibrium and has no effect on the equilibrium; 2, whether an enzyme actually catalyzes a reaction depends on the presence and concentration of its substrate as well as on the presence of the enzyme; 3, the concentration of an enzyme in a particular cell or part of a cell is not evidence for the relative importance of the reaction the enzyme catalyzes; and 4, the amount that a catalyzed reaction is reduced by inhibition or destruction of an enzyme is not necessarily proportional to the degree of inhibition or destruction of the enzyme."

The investigation of certain properties of the enzyme as it occurs in plants and the development of methods to study them was the first endeavor in the phase of work on which this paper reports. The next step was the study of means to remove the enzyme from its natural surrounding in the purest possible form. The author is aware of the difficulties involved and the possibility that the isolation of the enzyme which is the main goal of further work may produce a biologically inactive material. Recent progress in this work, however, has given encouragement that an active and highly purified product can be obtained. The extreme solubility of the enzyme in water is an aid in extraction which had not been recognized in earlier work. What had



been thought to be a difficulty of extraction actually proved to be the problem of freeing the enzyme from colloidal adsorption. The papers of Oparin (40) and Ford and Guthrie (17) provided the most valuable clues for new approaches. As soon as the superior action of papain digestion and the extreme mobility of the enzyme after its liberation had become obvious, the way for application of subsequent rapid procedures of purification appeared to be open. Several procedures of separation have been too slow but improved methods of chromatographic separation using aqueous solutions of the enzyme promise to aid the accomplishment sought.



SUMMARY

1. Methods for the investigation of a new enzyme in plants, carbonic anhydrase, have been developed and described in detail.
2. The enzyme has been shown to be present in the leaves of most green plants studied, but it has been present in only minute amounts in green parts other than leaves. It was also found in the flowers of some plants. Roots of all plants tested were devoid of it.
3. In studying its distribution in the plant it was found that old leaves contain a higher concentration of the enzyme than young leaves but meristematic regions showed more enzyme activity than adjoining differentiated tissues.
4. The chlorophyll content of several species tested did not have any direct correlation to their carbonic anhydrase content.
5. Cyanide inhibited the enzyme but neither sulfanilamide nor 2,4-dichlorophenoxyacetic acid noticeably affected its activity.
6. Kinetic studies showed that the enzyme catalyzed the hydration of carbon dioxide and the dehydration of carbonic acid in direct proportion to the weight of plant material extracted.

7. Hydrogen ion concentration as well as temperature affected the rate of dehydration of carbonic acid. Optima were found between pH 6.5 and 7.1, depending on the buffers used, and at 40° C.
8. The rate of CO_2 evolution increased as the substrate concentration increased but levelled off beyond a sodium bicarbonate concentration of 4×10^{-2} M.
9. Purification studies resulted in the preparation of a chromatographic fraction having approximately 20 times the strength of the crude enzyme extract.

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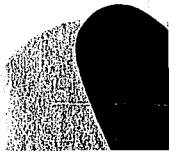

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