provements in the determination of iron by the Nitroso-R Salt Method. Ind. Eng. Chem., Anal. Ed. 16: 272. 1944.

25. SIDERIS, C. P. and YOUNG, H. Y. Effects of iron on chlorophyllous pigments, ascorbic acid, acidity and carbohydrates of *Ananas comosus* (L.) Merr. supplied with nitrate or ammonium salts. Plant Physiol. 19: 52–75. 1944.

26. SIDERIS, C. P. and YOUNG, H. Y. Effects of potassium on chlorophyll, acidity, ascorbic acid and carbohydrates of *Ananas comosus* (L.) Merr. Plant Physiol. 20: 649–670. 1945.

27. SIDERIS, C. P. and YOUNG, H. Y. Effects of nitrogen on chlorophyll, acidity, ascorbic acid, and carbohydrate fractions of *Ananas comosus* (L.) Merr. Plant Physiol. 22: 97–116. 1947.

28. SIDERIS, C. P. and YOUNG, H. Y. Growth of *Ananas comosus* (L.) Merr., at different levels of mineral nutrition under greenhouse and field conditions. I. Plant and fruit weights and absorption of nitrate and potassium at different growth intervals. Plant Physiol. 25: 594–616. 1950.

29. SIDERIS, C. P. and YOUNG, H. Y. Growth of *Ananas comosus* (L.) Merr., at different levels of mineral

30. SIDERIS, C. P. Iron in pineapple growth and nitrogen utilization. Research Report 34: 1–27. 1955. (Private).

31. SMITH, E. L. Solutions of chlorophyll-protein compounds (phyllochlorins) extracted from spinach. Science 88: 170–171. 1938.

32. SMITH, E. L. Chlorophyll as the prosthetic group of a protein in the green leaf. Science 91: 199–200. 1940.

33. SMITH, E. L. The chlorophyll-protein compound of the green leaf. Jour. Gen. Physiol. 24: 565–582. 1941.

34. WILLSTÄTTER, R. and STOLL, A. Untersuchungen über die Assimilation die Kohlensäure. Pp. 1–424. Springer, Berlin, 1918.

35. WRISTON, J. C., JR., LACK, L. and SHEMIN, D. The mechanism of porphyrin formation. Further evidence on the relationship of the citric acid cycle and porphyrin formation. Jour. Biol. Chem. 215: 603–611. 1955.

nutrition under greenhouse and field conditions. II. Chemical composition of the tissues at different growth intervals. Plant Physiol. 26: 456–474. 1951.

KINETICS OF SULFATE ABSORPTION BY BARLEY ROOTS [1]

JAMES E. LEGGETT AND EMANUEL EPSTEIN

AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE,
BELTSVILLE, MARYLAND

This paper reports the results of short-term experiments on the absorption, by excised barley roots, of sulfate labeled with S^{35}. The findings are consistent with the view that the absorption of sulfate is mediated by carriers to which the ions are bound in their passage across a barrier impermeable to free sulfate ions. As regards kinetics and specificity, the results are interpreted in terms of the enzyme-kinetic hypothesis of the operation of carriers, as given by Epstein and Hagen (6).

MATERIALS AND METHODS

RADIOACTIVE SULFATE: To a 10-ml aliquot of a stock solution of K_2SO_4 was added carrier-free radiosulfate in dilute HCl, obtained from the Oak Ridge National Laboratory. The tagged solution was evaporated to dryness to free it from HCl, made to the original volume (10 ml) with water, and added to the stock solution of K_2SO_4. The specific activity was about 2 $\mu c/\mu eq$ SO_4.

SELENATE: Commercially available "CP" potassium selenate was found to be contaminated with selenite. The selenate was reduced to selenium with hydroxylamine hydrochloride, the selenium washed, dissolved in nitric acid, and after neutralization of the solution, oxidized to selenate with bromine, essentially according to the method of Dennis and Koller (1). The stock solution of selenic acid was titrated with standard KOH to pH 5.5. The concentration of the stock

solution was determined 1) from the amount of KOH added, 2) by evaporating an aliquot of the stock solution of K_2SeO_4 and weighing the salt, and 3) by reduction of selenate and weighing the selenium, according to Hillebrand, Lundell, Bright, and Hoffman (9). The results of all three methods agreed within 2 %. Selenite was less—probably much less—than 0.1 percent of the selenate, on an equivalent basis, according to the test of Dennis and Koller (1).

ASSAY FOR RADIOSULFATE: After an absorption experiment, the 1.00-gm (fresh weight) samples of excised roots were transferred to 30-ml Pyrex beakers. About 3 ml ethyl alcohol, 1 ml 0.1 N K_2SO_4 (nonradioactive), and 1 ml $Mg(NO_3)_2$ reagent (14) were added, the solutions evaporated to dryness, and the samples ashed overnight at 500° C. The ash was moistened, acidified with HCl, and the clear solution evaporated to dryness. The chloride salt was dissolved by adding 10 ml 0.35 M acetic acid at about 70° C. Benzidine sulfate was precipitated by addition of 2 ml benzidine reagent (8.0 gm benzidine di-hydrochloride dissolved in 500 ml water containing 5 ml 1 N HCl, filtered through #42 Whatman filter paper before using). After addition of 4 ml acetone, the beakers were tightly covered with aluminum foil and placed in the refrigerator at 8° C overnight. The suspension of benzidine sulfate was filtered, under suction, through circles of #1 Whatman filter paper, placed in the bottom of ¼-ounce seamless tin boxes (made by the Buckeye Stamping Company, Columbus, Ohio), 3.3 cm in diameter and 8 mm deep, the bottom

being perforated by 25 evenly spaced 1 mm holes. The beaker and the precipitate on the filter-funnel were rinsed with 2 ml cold acetone, whereupon the samples were ready for counting. A thin window (2.4 mg/cm^2) Geiger-Müller tube and conventional scaling circuit were used. Aliquots of the radiosulfate solutions used in the experiments were taken through the entire procedure outlined above to serve as standards in calculating radiosulfate absorbed by the root samples. The above method for precipitating benzidine sulfate is a modification of that given by Kahn and Leiboff (11).

EXCISED BARLEY ROOTS: Seeds of barley, var. Atlas 46, were germinated and seedlings grown as described earlier (5). After soaking in aerated water for 24 hours the seeds were planted over an aerated solution of Ca(H$_2$PO$_4$)$_2$, 2×10^{-4} M, and seedlings grown for 5 days, during which time the solution was renewed once. During the entire period the plants were kept in a dark chamber at 24° C. Just before the experiment proper, the roots were rinsed, excised, and suspended in 4 liters of water.

STANDARD EXPERIMENTAL PROCEDURE: The procedure of conducting the absorption experiments proper was essentially as described before (7). Excised roots were blotted, 1.00-gm portions weighed out and transferred to 100-ml test tubes containing 50 ml water. The absorption period was initiated by decanting the water and replacing it with 50 ml of the experimental solution, adjusted to pH 4.0 unless otherwise stated. After the absorption period, the solution was decanted and replaced by a solution of non-radioactive CaSO$_4$, 1 meq/l, or, in some experiments, by water, to free the roots of the diffusible fraction of sulfate in the "outer" space (5). During both absorption and desorption periods, the tubes were kept in a water bath at 30° C, and the solutions were constantly aerated.

RESULTS

Sulfate absorption was influenced by the pH of the test solutions. Highest absorption rates were observed from solutions adjusted to pH 4. The test solutions were not buffered, but solutions adjusted to pH 4.0 with HCl did not change by more than 0.2 unit during a 3-hour absorption period. All subsequent experiments were conducted at pH 4. After a period of absorption of S*O$_4$,[2] the total S*O$_4$ within the root can be cleanly separated into two fractions, 1) diffusible and 2) actively absorbed S*O$_4$. This is demonstrated in figure 1. Before zero time, the roots were exposed to a solution of K$_2$S*O$_4$, 0.5 meq/l, for an absorption period of 60 min. Root samples were then blotted, for determination of total labeled sulfate, and others exposed to water or 1 meq/l solutions of non-radioactive CaSO$_4$ for desorption periods ranging up to 120 min. A rapid loss of S*O$_4$ from the roots is followed by a period in which there is no further loss of S*O$_4$, either to water or solutions of CaSO$_4$. The loss is due to diffusion of S*O$_4$ from the "outer" space of the tissue, i.e., a space to which inorganic ions have free and reversible access by diffusion

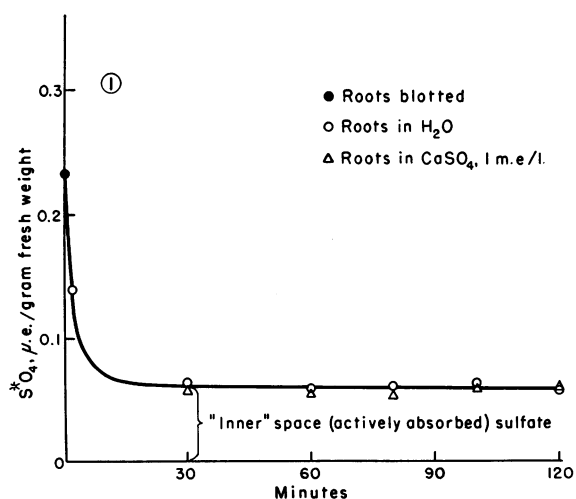[2] Asterisks indicate radioactive labeling.

FIG. 1. Loss of labeled sulfate by excised barley roots. Before zero time, the roots had been exposed to K$_2$S*O$_4$, 0.5 meq/l, for 60 min.

(5). The magnitude of the "outer" space, in these roots, is 0.23 ml/gm fresh weight (5). The fraction remaining after the rapid, initial loss has been absorbed by the metabolic, active transport mechanism, or in other words, has been transferred to the "inner" space. The present paper is concerned with this fraction only. For routine measurements of "inner" space, or actively absorbed sulfate, the roots, after the absorption period in S*O$_4$ solutions, were exposed to solutions of CaSO$_4$, 1 meq/l, for a period of 60 min. All subsequent measurements refer to roots freed of the diffusible S*O$_4$ fraction in this manner.[3] For determinations of the rate, v, of S*O$_4$ absorption, 3-hr absorption periods were used. Absorption was a linear function of time, and the 3-hr value gave an adequate measurement of the rate of absorption.[4]

In figure 2, the reciprocal of the absorption rate, 1/v, has been plotted against the reciprocal of the substrate ion (S*O$_4$) concentration, 1/(S) (cf Epstein and Hagen, 6). The S*O$_4$ concentration, (S), was varied from 0.005 meq/l to 0.05 meq/l. Straight lines were obtained, evidence that the rate, v, of S*O$_4$ absorption varied as a function of S*O$_4$ concentration, (S), according to Michaelis-Menten kinetics (13). Selenate competitively interfered with S*O$_4$ absorp-

[3] In the earlier experiments on absorption of bromide (2), complete removal of the diffusible bromide was not essential because the rate of active absorption of bromide is so high that diffusible bromide remaining in the "outer" space of the tissue, after 3 water rinses, represents a negligible proportion of the total measured uptake.

[4] When amounts absorbed were plotted against time, the straight line obtained extrapolated to a positive value at zero time, for the higher concentrations used. This introduces an error in our rate measurements never exceeding 10% of the total measured absorption. The nature of the sulfate binding which this value represents has not been further investigated.
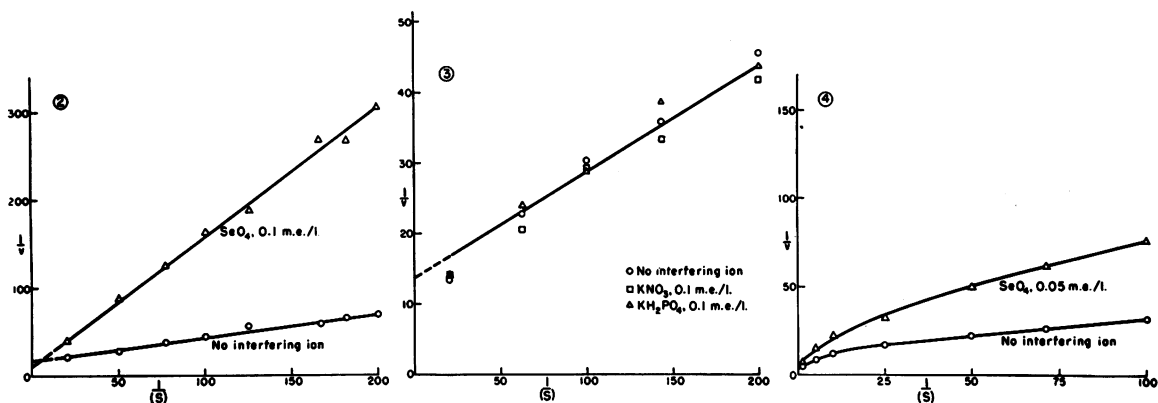
FIGS. 2 to 4. Double reciprocal plots of the rate of absorption of S*O₄ and the effects of other anions. The rate, v, of S*O₄ absorption is in μeq/gm fresh wt × 3 hrs. The concentration, (S), of S*O₄ in the solution is in meq/l.

FIG. 2. The effect of selenate; the concentration of S*O₄ varied over the range 0.005 meq/l to 0.05 meq/l.

FIG. 3. The effects of nitrate and phosphate; the range of S*O₄ concentrations was as in figure 2.

FIG. 4. The effect of selenate; the concentration of S*O₄ varied over the range 0.01 meq/l to 1 meq/l.

tion, indicating identical binding sites on the carriers for these two ions. Neither nitrate nor phosphate, at a concentration of 0.1 meq/l, had a significant effect on the rate of S*O₄ absorption (fig 3). Figure 4 shows that at S*O₄ concentrations in excess of 0.05 meq/l, approximately, rates of absorption are higher than would be expected on the basis of the extrapolation indicated in figures 2 and 3.

Viets (18) has shown that calcium ions increase the rate of absorption of both monovalent cations and anions by excised barley roots. Tanada (16) made similar observations in experiments with excised mung bean roots. The rate of absorption of S*O₄ was markedly increased by additions of CaCl₂ to the solution (fig 5). At a S*O₄ concentration of 0.5 meq/l, maximal activation (to 179 % of the controls) was obtained at a CaCl₂ concentration of 3 meq/l. At 0.05

meq/l S*O₄, a CaCl₂ concentration of 1 meq/l gave maximal activation, to 203 % of the control value. At higher concentrations of CaCl₂, the rate of S*O₄ absorption declined somewhat. However, even at the highest concentration of CaCl₂ used, 10 meq/l, uptake of S*O₄ from the 0.05 meq/l solution of S*O₄ was still 172 % of the control. Under these conditions, the ratio Ca/S*O₄ in the solution was 200/1, on an equivalent basis.

DISCUSSION

The rate of absorption of sulfate was found to depend on the sulfate concentration in the manner expected for reactions following classical enzyme kinetics (13), for the concentration range 0.005 meq/l to 0.05 meq/l. The implication is that sulfate absorption proceeds in three steps (cf Epstein and Hagen, 6): 1) sulfate ions reversibly combine with reactive sites of carriers; 2) the carrier-sulfate complex traverses a membrane not permeable to free sulfate ions; 3) sulfate ions are released internally in a rate-limiting, essentially irreversible step.

Absorption of sulfate in the present experiments was followed by means of the radioactivity of S³⁵, after exposure of the roots to solutions of K₂S³⁵O₄. It is possible, therefore, that a fraction of what has here been called, for convenience, "inner" space or "absorbed" sulfate was not sulfate at all, but represented S³⁵ incorporated into organic compounds. However, the subsequent fate of sulfate, after its absorption, is beyond the scope of this paper, which deals only with the rates at which sulfate is actively absorbed from the solution. These rate measurements lend themselves to interpretations of the mechanism of absorption, regardless of the eventual destinations of the absorbed sulfur atoms. Kylin (12) has demonstrated incorporation of S³⁵ into organic fractions of wheat seedlings. The process was slow compared with the rate at which S*O₄ was absorbed.
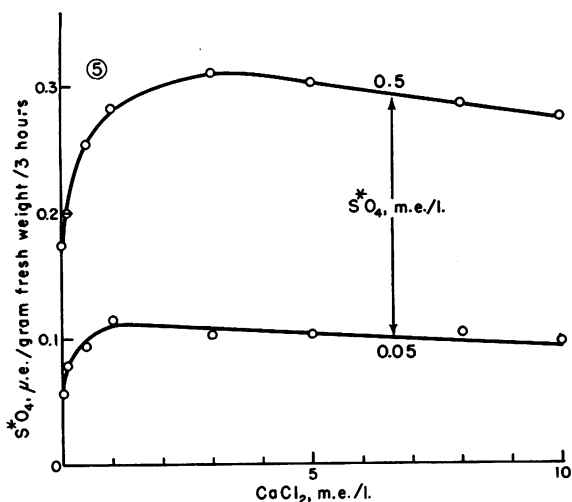
In the earlier experiments on the kinetics and

FIG. 5. The effect of calcium on the rate of S*O₄ absorption.

selectivity of ion absorption, the substrate ions whose rate of absorption was determined were bromide (2, 3), rubidium (6), and strontium (7), elements not normally present in significant amounts in the roots. Such elements were chosen to make sure that the measurements of absorption, made by means of radioactive tracing, represented values of net absorption. The radioactivity appearing in the roots could not be due to mere exchange of labeled ions for stable ones of the same chemical species initially present in the tissue, without any net change in the concentration of that ion in the tissue. Not so in the present instance. The measurements of absorption of labeled sulfate reported here may represent net absorption, or exchange with a pool of sulfur so large that the resulting isotopic dilution would render subsequent loss of radiosulfur to solutions of non-radioactive sulfate undetectable by our methods. The possibility of such exchange, emphasized by one of the reviewers of this paper, is being investigated. In either case, the absorption is an active, metabolic process. It is abolished by anaerobic conditions.[5]

Interference by other ions with the absorption of S^*O_4 was studied by exposing the roots to solutions of $K_2S^*O_4$ containing, in addition, the potassium salt of the ion whose effect on S^*O_4 absorption was to be investigated. Selenate competed with S^*O_4 (fig 2), but phosphate and nitrate were without effect (fig 3). The Michaelis constant for S^*O_4, i.e., the concentration giving half the maximal rate obtainable, was 0.019 meq/l. The calculated value for selenate, from the same experiment (fig 2) was 0.015, indicating that the affinity of these two ions for their common binding site is of the same order of magnitude.

At S^*O_4 concentrations above 0.05 meq/l approximately, the experimental points in the double-reciprocal plot (fig 4) fall below the values expected on the basis of the extrapolation indicated in figure 2, i.e., rates of absorption are higher than expected. The same phenomenon has been observed in absorption of rubidium in the presence of sodium (6) and in absorption of strontium (7). The implication is that at higher concentrations of the ions additional sites are involved which differ in their affinities for ions from the sites which mediate absorption at the lower concentrations.

The present demonstration of competition between sulfate and selenate for common sites which have little or no affinity for other ions (phosphate, nitrate, chloride) is the last in a series of investigations which have consistently revealed a high degree of specificity in the mutual effects of inorganic ions in the absorption process. The previous instances were 1) competition among potassium, rubidium, and cesium for sites having little affinity for sodium and lithium (6); 2) competition among bromide, chloride, and iodide, and lack of competition by nitrate for the halide bind-

---

[5] Recent unpublished experiments by one of us (E. E.) indicate that the absorption of S^*O_4, measured by radioactive tracing, represents a net uptake rather than an exchange with native, non-radioactive sulfur.

ing sites (2, 3); 3) competition among calcium, strontium and barium for binding sites which have but little affinity for magnesium ions (7).

In all these cases, the specificities observed were concordant with previous evidence on ion selectivity and mutual interferences, as discussed by Epstein (4). The same is true in the present instance. Cases of sulfate-selenate antagonism have been described for wheat (10), corn (17), yeast (8), *Chlorella vulgaris* (15), and *Aspergillus niger* (19). The specificity of the carrier sites of the roots for sulfate and selenate is very high. Nitrate and phosphate failed to interfere with S^*O_4 absorption, although their concentration was 0.1 meq/l—20 times the lowest concentration of S^*O_4 tested (fig 3).

SUMMARY

The absorption, by excised barley roots, of sulfate labeled with S^{35} has been studied in experiments lasting 3 hours. Diffusible labeled sulfate present in the tissue at the end of the absorption period was removed by exposing the roots to solutions of non-radioactive sulfate for 1 hour. This procedure leaves for final measurement the actively absorbed labeled sulfate only.

Rates of absorption were highest at pH 4 and the experiments reported were done at this pH. The rates of sulfate absorption varied in response to the external sulfate concentration in a manner consistent with the view that the absorption of sulfate involves the attachment of the ions to specific sites of carriers. Selenate competed with sulfate on approximately even terms. Phosphate, nitrate, and chloride had no measurable affinity for the sulfate-selenate binding sites. Calcium increased the rate of sulfate absorption.

LITERATURE CITED

1. DENNIS, L. M. and KOLLER, J. P. Selenic acid and copper selenate. Jour. Amer. Chem. Soc. 41: 949–970. 1919.

2. EPSTEIN, E. Mechanism of ion absorption by roots. Nature 171: 83–84. 1953.

3. EPSTEIN, E. Ion absorption by plant roots. Proc. Ann. Oak Ridge Summer Symp. 4: 418–434. 1953.

4. EPSTEIN, E. Absorption of ions by plant roots. In: Electrolytes in Biological Systems, A. M. Shanes, ed. Pp. 101–111. Amer. Physiol. Soc., Washington, D. C. 1955.

5. EPSTEIN, E. Passive permeation and active transport of ions in plant roots. Plant Physiol. 30: 529–535. 1955.

6. EPSTEIN, E. and HAGEN, C. E. A kinetic study of the absorption of alkali cations by barley roots. Plant Physiol. 27: 457–474. 1952.

7. EPSTEIN, E. and LEGGETT, J. E. The absorption of alkaline earth cations by barley roots: Kinetics and mechanism. Amer. Jour. Bot. 41: 785–791. 1954.

8. FELS, I. G. and CHELDELIN, V. H. Selenate inhibition studies. III. The role of sulfate in selenate toxicity in yeast. Arch. Biochem. 22: 402–405. 1949.

9. HILLEBRAND, W. F., LUNDELL, G. E. F., BRIGHT, H. A. and HOFFMAN, J. I. Applied inorganic analysis,

2nd ed. P. 336. John Wiley and Sons, Inc., New York 1953.

10. HURD-KARRER, A. M. Relation of sulphate to selenium absorption by plants. Amer. Jour. Bot. 25: 666–675. 1938.

11. KAHN, B. S. and LEIBOFF, S. L. Colorimetric determination of inorganic sulfate in small amounts of urine. Jour. Biol. Chem. 80: 623–629. 1928.

12. KYLIN, A. The uptake and metabolism of sulphate by deseeded wheat plants. Physiol. Plantarum 6: 775–795. 1953.

13. LINEWEAVER, H. and BURK, D. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56: 658–666. 1934.

14. RICHARDS, L. A., ed. Diagnosis and improvement of alkali soils. P. 129. U. S. Dept. Agr., Handbook No. 60. 1954.

15. SHRIFT, A. Sulfur-selenium antagonism. I. Antimetabolite action of selenate on the growth of Chlorella vulgaris. Amer. Jour. Bot. 41: 223–230. 1954.

16. TANADA, T. Effects of ultraviolet radiation and calcium and their interaction on salt absorption by excised mung bean roots. Plant Physiol. 30: 221–225. 1955.

17. TRELEASE, S. F. and BEATH, O. A. Selenium. Pp. 1–292. Published by the authors, New York 1949.

18. VIETS, F. G. Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol. 19: 466–480. 1944.

19. WEISSMAN, G. S. and TRELEASE, F. S. Influence of sulfur on the toxicity of selenium to Aspergillus. Amer. Jour. Bot. 42: 489–495. 1955.

PLASTID PIGMENT CHANGES IN THE EARLY SEEDLING LEAVES OF ZEA MAYS L.[1]

ROBERT E. KAY [2] AND BERNARD PHINNEY

DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, LOS ANGELES, CALIFORNIA

Most publications dealing with the analyses and interrelationships of the plastid pigments of maize and Avena have been confined to groups of pigments rather than specific plastid pigments (3, 4, 5, 13). Since the changes in a group of pigments do not necessarily reflect the behavior of the individual pigments, knowledge about the specific pigment changes is desirable.

Moster et al (7, 8) have determined the kinds, as well as the amounts, of the major carotenoid pigments in young maize plants grown in the light and then exposed to different light intensities. However, information is lacking on the development of the specific carotenoid pigments in dark-grown corn seedlings and in etiolated corn seedlings exposed to light. This paper will present information on these subjects.

METHODS

All seedlings were planted in quartz sand and grown in a constant temperature chamber equipped with fluorescent lamps. The culture methods, as well as a description of the light source and constant temperature chamber, have been described (9).

Leaf samples for analyses were obtained in the following manner: the second seedling leaf was removed from 15 different plants and the 15 leaves divided into 3 samples of 5 leaves each. Using a steel die, a section 7 by 52 mm was punched from the distal end of each leaf. These 5 leaf punches were pooled to form one sample. The distal end of the second leaf was selected because it was found to be fully expanded at the time the analyses were started. Thus the pigment content is actually based upon a constant area. How-

ever, in order to make the units of concentration more easily comparable to values found in the literature, all pigment concentrations have been expressed as μgm/gm of initial dry weight of leaf. This is a valid method of expressing the pigment content, since the initial dry weights of the leaf sections in the various experiments were not significantly different and there was less than a 2 % difference between the dry weights of leaf sections taken over a 7-day period.

Determinations of initial dry weights were made from material collected 6 days after planting. Three collections of 5 leaf punches were made in the same manner as collections for pigment analyses. Each sample of 5 leaf punches was dried at 75° C for 24 hours, weighed and the weights averaged to give the initial dry weight.

The carotenoid pigments were extracted and chromatographically separated on magnesia columns using methods similar to those employed by Strain (11). The leaf samples were immersed in hot water (90 to 100° C) for 3 minutes and ground in a mortar containing sand, 15 ml of methanol and 0.1 gm $CaCO_3$. The resulting brei was filtered through a medium sintered glass filter and the filtrate was transferred to a separatory funnel. The chlorophylls were saponified by treating the filtrate with 15 ml of methanol containing KOH (35 gm/100 ml). Thirty ml of a 1 : 1 (v/v) mixture of petroleum ether and ethyl ether was then added to the methanol solution and the mixture was shaken gently. The carotenoids were transferred to the petroleum ether–ethyl ether solution by the addition of 25 ml of saturated salt water accompanied by gentle shaking, followed by the addition of 10 to 20 ml of water with further gentle shaking. The methanol-water solution was drained into a second separatory funnel, further diluted with saturated salt water, and again extracted with 15 ml of a 1 : 1 mix-