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Berkeley, California

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ABSTRACT

The non-volatile, 80% ethanol-soluble products of fixation have been investigated in excised roots, using $C^{14}O_2$ and radiochromatography.

The main radioactive compounds separated were malic, citric (or iso-citric) aspartic and glutamic acids, asparagine and glutamine.

Less activity was present in serine, tyrosine, α-ketoglutaric acid and alanine, and in a number of unidentified compounds.

The uptake of $C^{14}O_2$ was inhibited by virtually anaerobic conditions.

From the above observations, it is considered likely that C^{14} is transformed through the reactions of the tricarboxylic acid cycle.

C^{14} in the soluble fraction was markedly increased by maintaining the root material in water rather than in a nutrient solution prior to exposure to $C^{14}O_2$. This increase was chiefly in malic acid.

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INTRODUCTION

Many non-green tissues, both plant and animal, are capable of assimilating carbon dioxide[1,2,3,4,5] and, in a number of heterotrophic micro-organisms, a certain concentration of the gas has been shown to be essential for growth.[6,7]

Ruben and Kamen[1] using short-lived radioactive carbon-11, were the first to demonstrate fixation of carbon dioxide by a preparation of barley roots, while the uptake of the bicarbonate ion was studied by Overstreet, Ruben and Broyer.[8] Owing to the short half-life of the tracer and to the absence at that time of a convenient and rapid micro-method of separation, neither of these groups of workers were able to determine in what compounds the radioactivity appeared and in what sequence.

Following the availability of carbon-14, Laties[9,10] studied fixation of $C^{14}O_2$ by barley roots during malonate inhibition and found the product to be carboxyl-labelled succinic acid. By means of the radiochromatographic technique, which has been used by Lynch and Calvin[11] in studies of carbon dioxide fixation in various micro-organisms, the present writer has determined the non-volatile, ethanol-soluble products of fixation in barley roots and has examined the effects of certain factors on fixation. A preliminary statement of the main results of this work has been published.[12]

GENERAL TECHNIQUE

Plant material

Barley seedlings (variety "Atlas") were grown on wire screens over unaerated

tap water in a greenhouse. At an early stage in the work, seedlings were also

grown in the laboratory at 24° C., the purpose being to control more closely the

conditions of growth and thereby to produce very uniform material. It was found,

however, that those roots grown in the greenhouse were superior for metabolic

studies, being more numerous, uniform and almost devoid of root hairs.

The roots were excised when one week old and placed in aerated Hoagland

solution[13] was modified by omitting ferric tartrate, as this tends to precipitate

on the roots and it was considered that an iron-deficient medium would not intro-

duce metabolic irregularities during the period of the experiments.

Exposure to $C^{14}O_2$

A quantity of excised roots was removed from the nutrient solution, lightly

blotted and an arbitrarily-chosen weight of 0.7 gm. taken. The roots in the

sample were cut at random into short lengths and placed in a modified Warburg

flask. In the preliminary experiments, the supporting medium in the flask was

fresh Hoagland solution minus iron, but in all the experiments referred to in this

paper, 5 ml. of phosphate buffer (pH 5.6) was used in order to maintain a stable

reaction during exposure to radioactive carbon dioxide. The side arm of the flask

contained 250 μl. of a solution of $NaHC^{14}O_3$ representing 5.75 micromoles of CO_2

and 51.25 μC. of C^{14}. The vessel was attached to a Warburg manometer and equili-

brated for 1 hour at 24° C., with shaking at approximately 130 oscillations per

min.. At the end of this period, the stopcock of the manometer was closed and the

bicarbonate solution discharged into the supporting buffer. Shaking was continued

for 1 hour. By means of the manometer, CO_2 pressure could be checked during the

period of exposure and any discrepancies in this respect between the various units

of an experiment detected.

Preparation of ethanol extract

Having removed the manometer from the shaker, the flask was opened in a well-ventilated fume cupboard and the buffer decanted off. The roots were rinsed rapidly with distilled water to remove residual salts, likely to interfere with the chromatographic separation, and boiling 80% ethanol poured on. Root material, with added ethanol, was then transferred with rinsings of 80% ethanol to a mortar and thoroughly ground. Quantitative transfer to a stoppered, graduated cylinder followed, the volume of the extract being recorded when cool.

Aliquots of 100 μl. of strongly-centrifuged extract were "plated" on aluminum discs for radioactivity assay, using a thin, end-window Geiger-Muller tube. The activity of the total volume of ethanol extract could thus be calculated.

Radiochromatography

The use of long-lived carbon-14 and the radioautography of filter paper chromatograms render the identification of the compounds in which fixed radiocarbon appears a simple matter. The general technique has been described elsewhere.[14, 15] In the present investigation, the ethanol extract, after determination of radio-activity, was filtered and concentrated to about 1 to 2 ml. in vacuo. Aliquots of the concentrate, varying in different experiments from 100 to 300 μl. according to activity, were placed on sheets (18" x 22 1/2") of Whatman No. 1 filter paper and run two-dimensionally in phenol-water and butanol-propionic acid-water. Radio-autographs were prepared on Eastman "No Screen" X-ray film. As the amount of C^{14} fixed in heterotrophic fixation is small by comparison with photosynthesis, long exposures are frequently required, sometimes of the order of a month or more, depending upon the activity of the aliquot of extract placed on the paper and upon the number of radioactive compounds separated.

THE PRODUCTS OF FIXATION

The typical non-volatile, 80% ethanol-soluble compounds found to be radio-active were malic, citric (or iso-citric) aspartic and glutamic acids, serine,

asparagine, glutamine and tyrosine (Figure 1). The identities of all the named radioactive spots have been confirmed by elution and co-chromatography with standard compounds. Unnamed spots have yet to be identified unequivocally. Although alanine produced a large, clear spot on the paper with ninhydrin, it contained little C^{14}. The activity of α-ketoglutaric acid was low and only a trace of radioactive threonine occurred. It is by no means certain, however, that these weakly-radioactive compounds are unimportant in the path of fixed carbon, since they may occur only in catalytic concentrations at any given instant. For example, the considerable activity located in glutamic acid and glutamine suggests that a rapid transfer of C^{14} through α-ketoglutaric acid takes place.

The pattern of products in barley roots is very similar to that obtained by Benson and Calvin[16] for dark, aerobic fixation of $C^{14}O_2$ by the leaves of the same plant, except that only a trace of activity appeared in asparagine and none in tyrosine in the experiments with leaves. In both roots and leaves, all the compounds are consistent with a sequence of C^{14} transformations through the tricarboxylic acid cycle, previously indicated by the work of Laties.[9,10] Aspartic, glutamic and malic acids were found to be radioactive in all the organisms studied by Lynch and Calvin,[11] but the percentage of the total soluble activity made up by these substances varied widely with different materials. Glutamine and asparagine were rarely encountered and then only in low activity. It would thus appear that although the general pattern of dark fixation is the same in a wide range of organisms and tissues, the relative rates of the intermediate reactions are very diverse.

FIXATION AND MINERAL NUTRITION

When, during the course of the research, phosphate buffer was substituted for modified Hoagland solution as the exposure medium, continued pre-equilibration in the nutrient solution appeared to be illogical, especially as the roots had been grown in tap water. In later experiments, therefore, the roots were placed in

aerated tap water for 24 hours after excision, all other conditions being un-
changed.

This treatment resulted in at least five times as much activity in the soluble
fraction (Table I). A similar effect followed equilibration in distilled water
(Table II).

TABLE I

The effect of pre-equilibration in Hoagland solution minus iron versus tap water on fixation of $C^{14}O_2$ by excised barley roots.

Roots: 0.7 gm. wet wt. Exposure medium: phosphate buffer, 5 ml. pH 5.6, 250 ul.
$NaHC^{14}O_3$ containing 5.75 μmol. CO_2 and 51.25 μC. C^{14}. Time of exposure: 1 hour.

Pre-equilibration medium	Expt. no.	Radioactivity of 80% ethanol extract (counts per min.)	% of activity supplied
Hoagland minus iron	15/1	158,700	1.0
	16/1	129,250	0.8
	16/2	142,220	0.9
	Means:	143,390	0.9
Tap water	15/2	868,770	5.6
	16/3	783,000	5.0
	16/4	626,240	4.0
	Means:	759,337	4.9

TABLE II

The effect of pre-equilibration in distilled water versus

tap water on fixation of $C^{14}O_2$ by excised barley roots.

Roots: 0.7 gm. wet wt. Exposure medium: phosphate buffer, 5 ml. pH 5.6, 250 ul. $NaHC^{14}O_3$ containing 5.75 μmol. CO_2 and 51.25 μC. C^{14}. Time of exposure: 1 hour.

Pre-equilibration medium	Expt. no.	Radioactivity of 80% ethanol extract (counts per min.)	% of activity supplied
Distilled water	17/3	814,000	5.2
	17/4	982,600	6.3
	Means:	898,300	5.8
Tap water	17/1	878,080	5.6
	17/2	942,656	6.0
	Means:	910,368	5.8

The radioautographs for tap and distilled water are indistinguishable and an example is reproduced in Figure 2. The characteristic feature is the very obvious predominance of malic acid. This suggests that, as a direct or indirect result of mineral starvation, transformation of C^{14} is impeded. Restriction of amination as a direct consequence of nitrogen deficiency might be a cause of such blockage. Clearly, especial significance attaches to the amount and nature of the ethanol-insoluble activity in deciding whether the increased soluble activity following water equilibration represents an overall increase in fixation or, as appears more

likely, an accumulation of activity in soluble products. The insoluble residues
remaining from these experiments will be investigated. Moreover, it is hoped later
to study the entire problem of carbon dioxide fixation in relation to mineral nutri-
tion in greater detail, since this may have important bearings on the inter-
relationships of mineral nutrition and the metabolism or organic acids, carbohydrates
and proteins.

FIXATION AND OXYGEN DEFICIENCY

Further information on how tracer carbon becomes dispersed through the range
of compounds revealed by the radioautographs was obtained by studying the effects
of virtually anaerobic conditions on fixation.

The same quantities of root material, phosphate buffer and $NaHC^{14}O_3$ as before
were used, the excised roots being pre-equilibrated in tap water. Cylinder nitrogen
(containing approximately 0.5% oxygen) was passed through the reaction vessel for
30 min., following which the stopcocks were closed and the flask immersed in the
constant temperature bath (24° C.) with shaking at 130 oscillations per min.. The
C^{14}-labelled bicarbonate, introduced into the vessel's side-arm prior to flushing
with nitrogen, was then discharged into the buffer and shaking continued for 1 hour.
Control experiments were run using air instead of nitrogen.

The results for soluble activity are shown in Table III. No data are yet
available for insoluble activity, nor have chromatograms been prepared.

The aerobic nature of the mechanism whereby C^{14} is incorporated appears to be
clearly established by these experiments and this may be interpreted as additional
evidence for the operation of the Krebs cycle in barley roots.

TABLE III

Fixation of $C^{14}O_2$ by excised barley roots in air and in cylinder nitrogen.

Roots: 0.7 gm. wet wt. Exposure medium: phosphate buffer 5 ml. pH 5.6, 250 ul. NaHC^{14}O$_3$ containing 5.75 μmol. CO$_2$ and 51.25 μC. C . Time of exposure: 1 hour.

Atmosphere	Expt. no.	Radioactivity of 80% ethanol extract (counts per min.)	% of activity supplied
Air	20	512,120	3.4
	22	504,240	3.2
	Means:	508,180	3.3
Cylinder	21	4,440	0.03
nitrogen	23	4,480	0.03
	Means:	4,460	0.03

ACKNOWLEDGEMENTS

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CAPTIONS TO FIGURES

Fig. 1   Radioautograph showing the typical 80% ethanol-soluble products of $C^{14}O_2$ fixation.

Fig. 2   Radioautograph showing the 80% ethanol-soluble products of $C^{14}O_2$ fixation, following pre-equilibration of the roots in tap water.
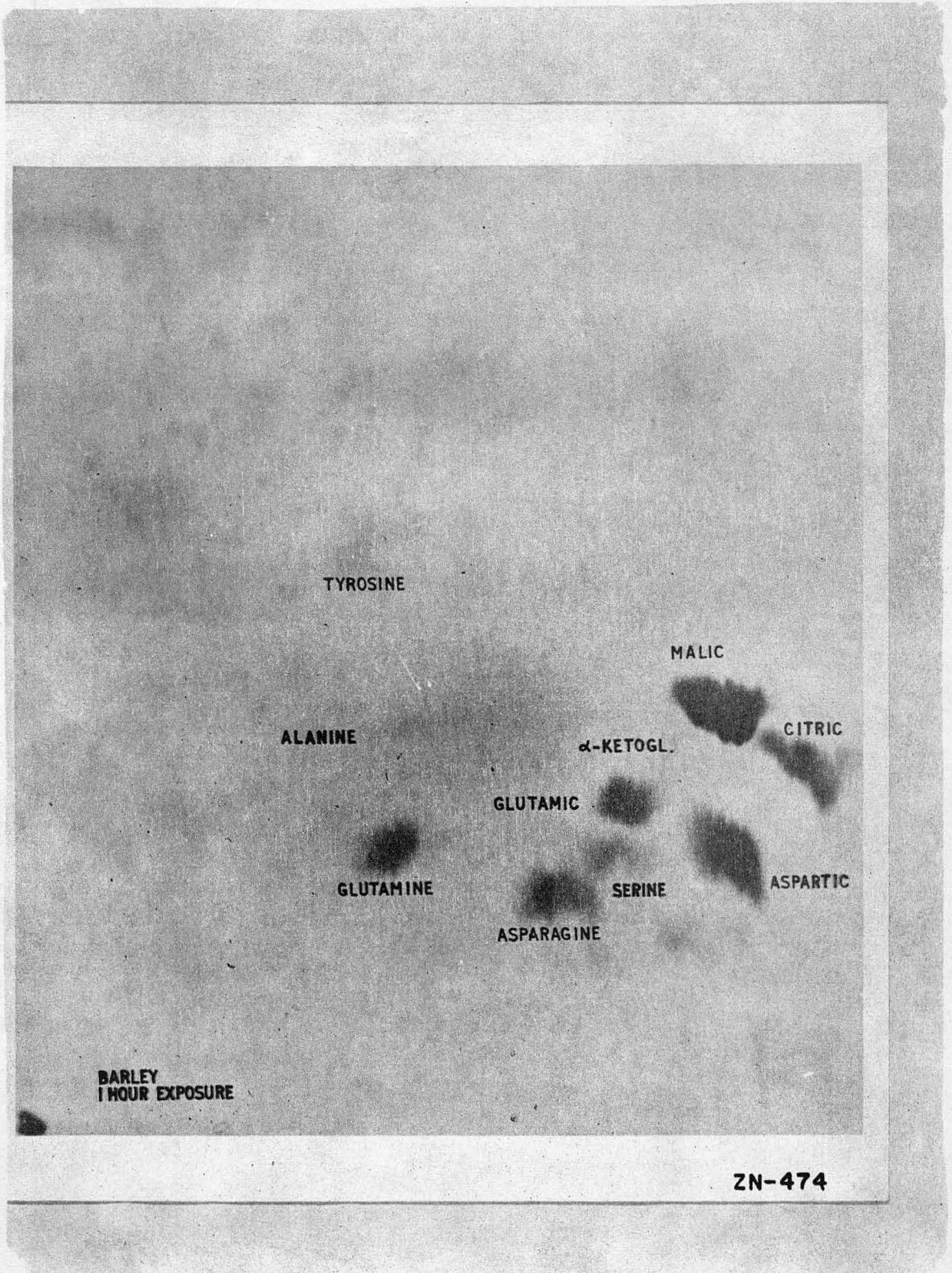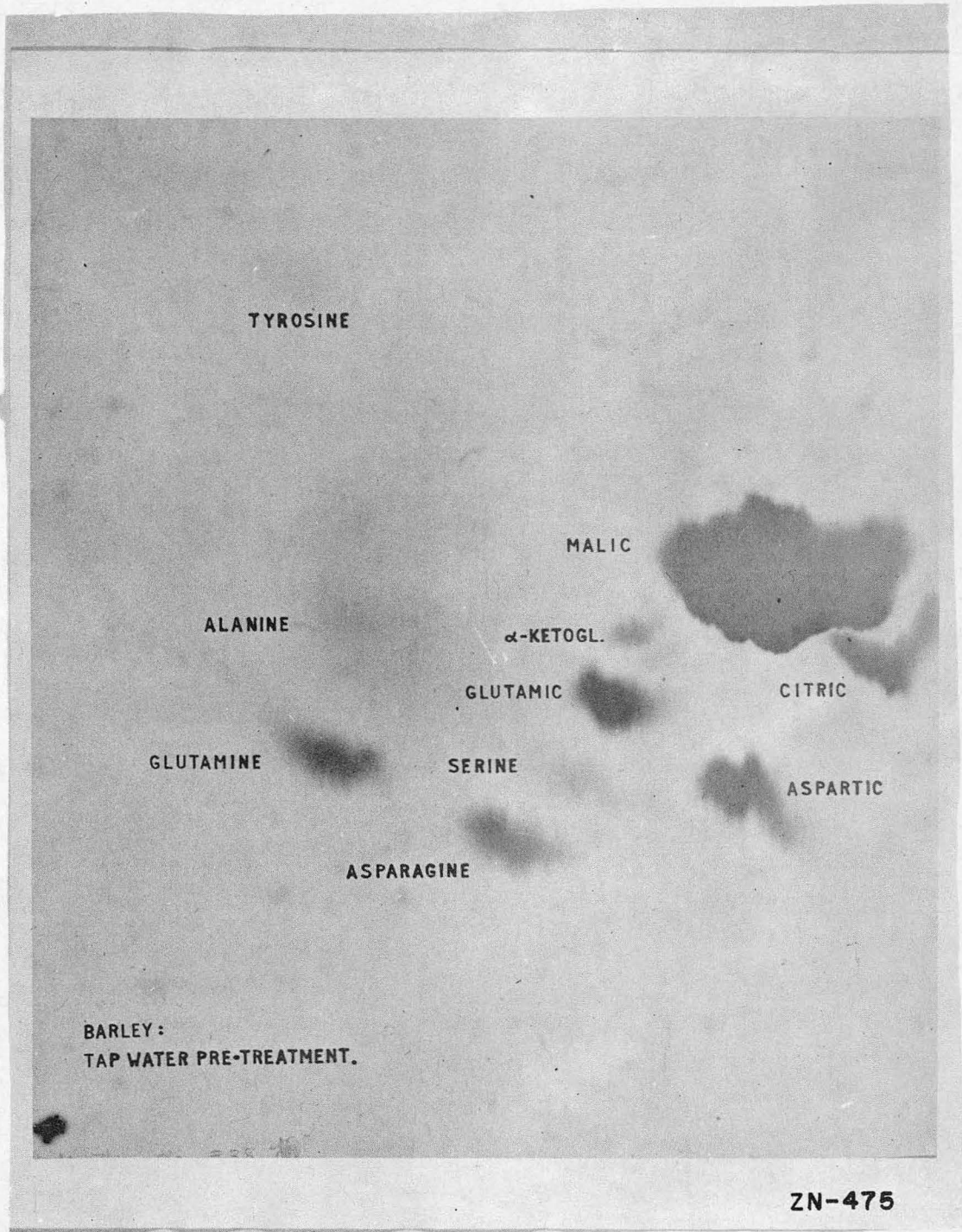
TYROSINE

MALIC

ALANINE

CITRIC

α-KETOGL.

GLUTAMIC

GLUTAMINE

SERINE

ASPARTIC

ASPARAGINE

BARLEY
I HOUR EXPOSURE

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Fig. 1

TYROSINE

MALIC

ALANINE

α-KETOGL.

GLUTAMIC

CITRIC

GLUTAMINE

SERINE

ASPARTIC

ASPARAGINE

BARLEY:
TAP WATER PRE-TREATMENT.

ZN-475

Fig. 2