PROPIONIC ACID METABOLISM IN SOME HIGHER PLANT TISSUES

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LIST OF ABBREVIATIONS

CoA	-	coenzyme A
ATP	-	adenosine triphosphate
DPN	-	diphosphopyridine nucleotide
TPN	-	triphosphopyridine nucleotide
GSH	-	glutathione
TCA cycle	-	tricarboxylic acid cycle
R.Q.	-	respiratory quotient
β-OH propionate	-	β-hydroxypropionic acid
CPM	-	counts per minute
Amino-N-	-	amino nitrogen

ABSTRACT

Zilkey, Bryan Frederick. M.Sc., The University of Manitoba, February, 1964. Metabolism of Propionic Acid In Some Higher Plant Tissues.

Major Professor: D. T. Canvin.

Enzymatic and intact plant tissue studies, (Giovanelli and Stumpf 1958) have provided evidence for a pathway of propionate metabolism in higher plant tissues which can be characterized as; propionic acid ⟶ propionyl-CoA ⟶ acrylyl-CoA ⟶ β-hydroxypropionate ⟶ malonic semialdehyde → malonyl-CoA ⟶ acetyl-CoA and CO_2. In intact tissues, however, the recovery of propionate-C^{14} in known products was never equal for all 3 carbons. These discrepancies suggested that a portion of the propionate may have been metabolized by an alternate pathway. The purpose of this thesis was to determine if any evidence could be obtained for alternate pathways of propionate metabolism or if the metabolism of this compound could be wholly accounted for by the above pathway.

Propionate-1-, -2- or -3-C^{14} was supplied to pea cotyledons, etiolated sunflower cotyledons and germinating castor bean endosperm for various periods of time. The tissues were extracted with 80% ethanol and the radioactive constituents separated by ion-exchange and paper chromatography.

Eighty-five to 100 percent of the added propionate-

C^{14} was absorbed and metabolized by the tissues in 2-4 hours.

In all 3 tissues, carbon 1 of propionate was released first as $C^{14}O_2$ and carbons 2 and 3 were released in a manner consistent with their metabolism as the methyl and carboxyl carbons, respectively, of acetate. β-hydroxypropionate-C^{14} was isolated from all 3 tissues and β-alanine-C^{14} was isolated from sunflower cotyledons. These facts were consistent with propionate metabolism via the modified β-oxidation scheme proposed by Giovanelli and Stumpf.

In the pea tissue, C^{14} from propionate-2- and -3-C^{14} accumulated mainly in organic and amino acids. Carbon 2 of propionate was extensively incorporated into the TCA cycle acids, citrate, malate and succinate. These findings indicate that carbon 2 of propionate was metabolized as acetate in this tissue.

In the sunflower tissue, propionate-2- and -3-C^{14} was incorporated mainly into a variety of unidentified acidic compounds and into the amino acids serine, alanine and tryptophan with small amounts of radioactivity in glutamate, aspartate and glutamine. In addition, about 15% of the C^{14} from carbons 2 and 3 were incorporated into sugars.

In the castor bean endosperm, propionate-1-C^{14} accumulated mainly by β-hydroxypropionate over a two hour time interval. Although some radioactive sugar was found from propionate-2-C^{14}, the majority of this carbon was present in

amino acids and organic acids. β-hydroxypropionate derived from propionate-2-C^{14} initially contained a considerable amount of C^{14}, but rapidly decreased in radioactivity with time. A large number of acidic unknowns were found in the organic acid fraction derived from propionate-1- and -2-C^{14} and very little C^{14} was found in the TCA cycle acids.

In all three tissues, propionate appeared to be metabolized completely or in part by the pathway that had previously been shown to exist in higher plants. However, the pattern of C^{14} distribution from propionate-2- and -3-C^{14} in the 80% ethanol soluble compounds from sunflower cotyledons and castor bean endosperm was not entirely consistent with their conversion to acetate and suggested that a portion of the propionate was metabolized by other reactions.

INTRODUCTION

There are only isolated instances in which odd chain fatty acids such as propionic acid have been found in higher plants, Paech and Tracey (1955). However, in animal tissues odd and branched chain amino acids are catabolized via propionate, (Fruton and Simmonds 1958), (Feller and Feist 1959, 1962, 1963) and presumably this could also occur in plants, Stumpf and Bradbeer (1959). In addition, β-oxidation of saturated odd chain fatty acids could also produce propionate, Callely et al (1958), Reisner et al (1961, 1962, 1963a).

In biological systems three distinct pathways have been elucidated for the metabolism of propionic acid. In animals, (Flavin and Ochoa 1957) and root nodule bacteria (De Hertogh and Evans 1963) propionate is oxidized via methylmalonyl-CoA to succinate. By this mechanism carbon 1 of propionate becomes the carboxyl group of succinate while carbons 2 and 3 become equivalent as the methyl carbons in the succinate molecule.

Fungal spores (wheat stem rust), Reisner et al (1963b) and certain bacteria, Vagelos et al (1959b) apparently metabolize propionate via lactic acid to CO_2 and acetate. Here, carbon 1 of propionate is converted to CO_2 while carbons 2 and 3 become the carboxyl and methyl groups, respectively, of acetate.

Giovanelli and Stumpf (1958) obtained evidence that propionate was catabolized in peanut mitochondria by the

following series of reactions; propionic acid → B-hydroxypropionic acid → malonyl-CoA → CO_2 and acetyl-CoA. Later, Hatch and Stumpf (1962a) obtained evidence that propionate was rapidly oxidized by this mechanism in a wide variety of intact plant tissues. In this oxidation carbon 1 of propionate was lost as CO_2 while carbons 2 and 3 were converted to the methyl and carboxyl carbons, respectively, of acetate. However, the recovery of propionate-C^{14} in known products was seldom equal for all 3 carbons. For example, in the pea cotyledon tissue 65% of the added propionate-1-C^{14} was accounted for in known products but only 34% and 38% of the added propionate-2- and -3-C^{14}, respectively, was reported in known compounds. This large discrepancy in propionate-C^{14} recovery may indicate that a portion of the propionate was metabolized by an alternate pathway.

Since propionate was formed during amino acid and fatty acid catabolism in other tissues, it might be assumed that propionate would be produced in germinating seeds such as the castor oil seed, the sunflower seed or the pea seed which are actively breaking down large storage reserves of fat or protein. Fortunately, the metabolism of acetate, a proposed product of propionate oxidation, is well known in the seeds decomposing reserve fat, Canvin and Beevers (1961), Bradbeer and Stumpf (1959). Hence, it would be expected that all of the carbons of propionate could be equally accounted for in

known products in a predictable manner, regardless of the route of propionate metabolism. This study was undertaken to determine if propionate metabolism could be wholly accounted for by the pathway which has been proposed for higher plants or if any evidence could be obtained for alternate pathways of metabolism.

LITERATURE REVIEW

Occurrence or Production of Propionic Acid

Information on the occurrence or production of propionic acid in biological organisms, especially in higher plants, is very sparse. In general, the evidence indicates that this short chain fatty acid may be an intermediate in the catabolism of amino acids and fatty acids.

Enzymatic and radioisotope studies in bacteria and higher animals have shown that the amino acids, alanine, threonine, serine, methionine, isoleucine and valine are metabolized to propionic acid via various pathways, Fruton and Simmonds (1958). In a series of papers, Feller and Feist (1959, 1962, 1963) have isolated propionic acid as an intermediate in the catabolism of uniformly labelled alanine-C^{14}, serine-C^{14}, valine-C^{14}, leucine-C^{14}, isoleucine-C^{14}, methionine-C^{14} and threonine-C^{14} in the mouse epididymal fat pad. The presence of unlabelled propionate reduced the conversion of C^{14} labelled amino acids to fatty acids and thus provided further evidence for the role of propionic acid in amino acid metabolism in this tissue. Since propionate was readily metabolized by plant tissues which were actively involved in protein catabolism, propionic acid may also arise from amino acids in higher plants, Hatch and Stumpf (1962a).

Branched or straight odd-chain fatty acids are not generally found in higher plant tissues, (Hilditch 1956), but

there are some isolated reports of their occurrence. The volatile material given off by apples contains esters of propionic, n-valeric and other short chain fatty acids and the volatile material from pineapples contained methyl and ethyl isovalerate and methyl valerate, Paech and Tracey (1955). The origin of these acids is obscure but in other tissues it has been shown that propionate can be produced from the β-oxidation of longer odd-chain fatty acids. Thus, propionic acid and acetic acid were detected in soluble extracts of a vibrio growing on nonanoic acid, (Callely et al 1958), whereas only acetate was found when octanoic acid was used as the sole source of carbon. Three moles of acetic acid and 1 mole of propionic acid were produced from 1 mole of nonanoic acid which indicates that the acid was oxidized by a β-oxidation sequence. Propionic acid was also produced by rat liver during the oxidation of C_5-C_{17} odd-chain saturated fatty acids, Huennekens et al (1951). In wheat stem rust uredospores Reisner et al (1961, 1962, 1963a) have also demonstrated that propionate was formed from the β-oxidation of an odd-chain fatty acid. The distribution of C^{14} in glutamate and γ-aminobutyric acid and the release of $C^{14}O_2$ from specifically labelled valerates definitely showed that carbons 1 and 2 of valerate were removed as acetate and that carbons 3, 4 and 5 were converted to propionate. The propionate was

subsequently metabolized by the pathway that is operative in this tissue. Thus, it is possible that in organisms possessing a β-oxidation mechanism for fatty acid metabolism that propionic acid could be produced from the oxidation of saturated longer odd-chain fatty acids, if these acids occur naturally or are produced during the breakdown of unsaturated fatty acids.

Propionic Acid Metabolism

Propionic acid may be metabolized by several pathways (Figure 1). At present, more than one pathway has not been found in any one organism and the diagram is a composite of propionate metabolism from several different tissues.

Propionate Metabolism in Bacteria

In the first of a series of papers on propionate metabolism, Vagelos *et al* (1949a) described the purification and properties of an inducible enzyme acrylyl-CoA aminase which aminated acrylyl-CoA to β-alanine. The enzyme system was apparently confined to *Clostridium propionicum* and had an absolute substrate specificity for acrylyl-CoA and ammonia.

Vagelos *et al* (1959b) also elucidated a second pathway of propionate metabolism in bacteria. An acrylyl-CoA hydrase which catalyzed the irreversible hydration of acrylyl thioesters to

lactyl thiolesters was found in a Pseudomonas species and pig heart muscle extracts. No lactyl-CoA dehydrogenase was detected, but the product formed using deacylase enzymes isolated from Cl. propionicum and Cl. kluyveri was L(+)-lactic acid because it served as a substrate for lactic dehydrogenase.

Cell free extracts of Cl. kluyveri, (Vagelos and Earl (1959c) catalyzed the oxidation of propionate by a series of reactions similar to those found in higher plants. However, propionate conversion to propionyl-CoA required acetyl phosphate, a TPN^+ specific β-hydroxypropionyl-CoA dehydrogenase was isolated which converts β-hydroxypropionyl-CoA to malonic semialdehyde-CoA directly and a TPN^+ specific malonic semialdehyde CoA dehydrogenase catalyzed the oxidation of malonic semialdehyde-CoA to malonyl-CoA.

Swick and Wood (1960) have elucidated another pathway for propionate oxidation which involves a novel biochemical reaction. CO_2 is transferred from a donor compound to an acceptor without the expenditure of energy. Thus, in Propionibacterium shermanii pyruvate was converted to propionate by the following series of reactions:

(1) Pyruvate + Methylmalonyl-CoA $\rightleftharpoons$ Oxalacetate + Propionyl-CoA

(2) Oxalacetate + 4H $\rightleftharpoons$ Succinate

(3) Succinate + Propionyl-CoA $\rightleftharpoons$ Succinyl-CoA + Propionate

(4) Succinyl-CoA $\rightleftharpoons$ Methylmalonyl-CoA

Sum: Pyruvate + 4H $\rightleftharpoons$ Propionate

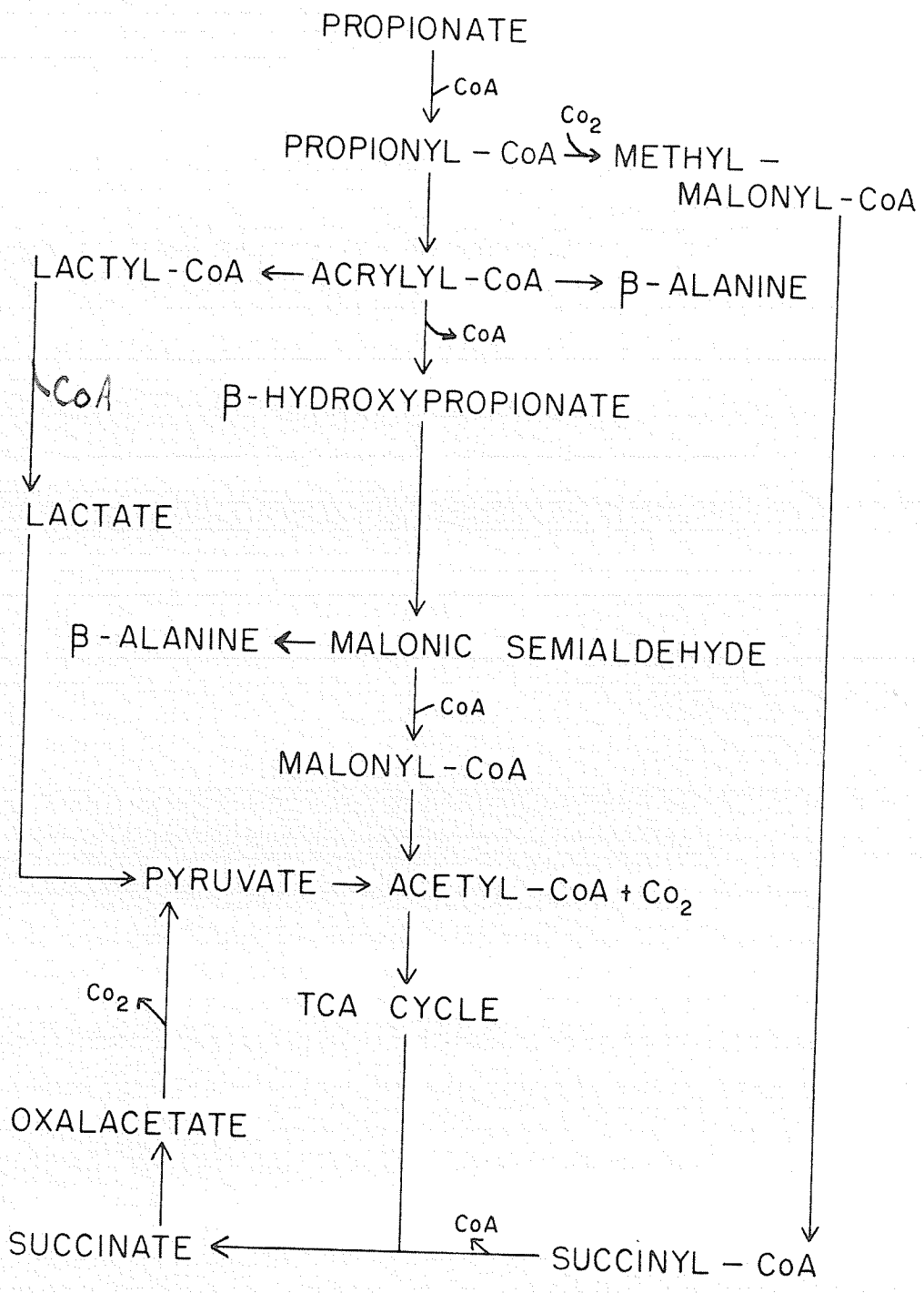

Fig. 1 The pathway of propionic acid metabolism in biological organisms.

Reaction (1) was catalyzed by a biotin-enzyme methylmalonyl-oxalacetic transcarboxylase, Wood and Stjernholm (1961).

Recently, De Hertogh and Evans (1963) have isolated propionyl-CoA carboxylase and methylmalonyl isomerase from cell-free extracts obtained from the nodules of several legumes and 5 pure cultures of Rhizobium sp. Respirometry studies with some of these systems showed that succinate was oxidized most rapidly and that propionate and other substrates were utilized at slower rates. The substrates were oxidized without apparent lag suggesting that the necessary oxidative enzymes were present within the cell. Apparently, propionate was metabolized in these bacteria via methylmalonyl-CoA and succinate, a pathway previously thought to exist only in animals.

Propionate Metabolism in Fungi

Reisner et al (1963b) have shown that propionate may be metabolized in fungi in a manner previously thought to exist only in bacteria, Vagelos et al (1959b). Wheat stem rust uredospores in a buffered solution containing propionate-1-, -2- or -3-C^{14} removed 55% of the added C^{14} from solution in 3 hours. Most of the C^{14} from propionate-1-C^{14} was released as $C^{14}O_2$ whereas only 19% and 8%, respectively, of added propionate-2- and -3-C^{14} was released as $C^{14}O_2$. However, approximately 20% and 31% of propionate-2- and -3-C^{14}, respectively,

were incorporated into spore constituents. Most of the incorporated C^{14} from carbons 2 and 3 of propionate was in amino acids, with smaller amounts of radioactivity in organic acids, carbohydrates, fats and spore residue.

Glutamic acid, γ-aminobutyric acid and alanine contained the major portion of the radioactivity in the amino acid fraction. Partial degradations showed that the glutamate-C^{14} derived from propionate-2-C^{14} contained 87% of the total radioactivity in the carboxyl carbons, whereas the glutamate-C^{14} derived from propionate-3-C^{14} contained 43% of the total radioactivity in carbons 3 and 4.

These findings were consistent with the idea that propionate metabolism in the rust uredospore involved the conversion of carbon 1 to CO_2 and utilization of carbons 2 and 3 as acetate with carbon 2 acting as the carboxyl carbon of acetate. The proposed mechanism (Figure 1) by which this could occur is the following:

Propionate → propionyl-CoA → acrylyl-CoA → lactyl-CoA → pyruvate → acetate and CO_2

Propionate Metabolism in Animals

In animal tissues the principle pathway by which propionate is degraded involves (a) conversion to propionyl-CoA (Figure 1), (b) an ATP dependent carboxylation to methyl-malonyl-

CoA and (c) a quantitative conversion of methylmalonyl-CoA to succinate which then enters the TCA cycle for further transformation, Flavin and Ochoa (1957a), Flavin et al (1957b), Beck et al (1957).

Reaction (b) was catalized by propionyl-CoA carboxylase, an enzyme that required biotin as a co-factor. Using a highly purified pig heart preparation, Kaziro et al (1960) have proposed that the formation of the complex enzyme-biotin-CO_2 is part of the enzyme catalyzed reaction.

Methylmalonyl isomerase catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA. Gurnani et al (1960), observed that the activity of methylmalonyl isomerase was severely depressed in the liver of vitamin B_{12} deficient rats and that the activity could be restored completely by the addition, in vitro of 5, 6-dimethylbenzimidazole-cobaltamide coenzyme. If evidence could be found for the activity of this enzyme in higher plant tissues, it would provide evidence for the essentiality of the cobalt ion in plant metabolism.

Although the methylmalonate pathway appears to be the dominant one in animal tissues, other pathways have been demonstrated. Kendina and Coon (1957) have shown that (a) acrylyl-CoA is converted to β-hydroxypropionyl-CoA in the presence of crystalline crotonase, (b) β-hydroxypropional-CoA deacylase catalyzes the formation of free β-hydroxypropionate which is

(c) converted to malonic semialdehyde, a reaction which is catalyzed by a DPN specific β-hydroxypropionic dehydrogenase isolated from pig kidney extracts and (d) malonic semialdehyde can be transaminated to β-alanine.

Propionate condensation directly with malonyl-CoA to form odd-chain fatty acids has been shown to be the principle fate of propionate in lactating rat mammary glands, Cady et al (1963).

Propionate Metabolism in Higher Plants

Stumpf and Barber (1956) observed that 40% of the added C^{14} from propionate-1-C^{14} and 11% from valerate-1-C^{14} was converted to $C^{14}O_2$ in 2 hours by a peanut mitochondrial system supplemented with Mn^{++}, a TCA cycle acid, ATP and CoA. This rapid release of C^{14} by an apparent β-oxidation mechanism was examined in more detail in the same system by adding propionate-1-, -2- and -3-C^{14} and resulted in the elucidation of the only known pathway of propionate metabolism in higher plants, Giovanelli and Stumpf (1958). The evidence supported the reaction series: propionate → propionyl-CoA → acrylyl-CoA → B-hydroxypropionic acid → malonic semialdehyde → malonyl-CoA → CO_2 and acetyl-CoA, the latter compound was then incorporated into TCA cycle acids (Figure 1). The enzyme system was dependent on CoA and ATP in equimolar amounts and DPN, TPN, GSH, Mn^{++} and a TCA cycle acid stimulated the oxidation to varying degrees.

The reaction was inhibited by a lack of oxygen, but the site of oxygen requirement was not clear.

It was also shown that (a) propionate-1-C^{14} released all of its C^{14} as $C^{14}O_2$, (b) propionate-2- and -3-C^{14} yielded $C^{14}O_2$ only after a lag of 60 minutes and after the TCA cycle acids citrate, malate, succinate and fumarate became labelled, (c) propionate-1-C^{14} was oxidized to $C^{14}O_2$ more rapidly than propionate-3-C^{14} which in turn was oxidized to labelled CO_2 more rapidly than propionate-2-C^{14}, (d) succinate derived from propionate-2-C^{14} was labelled exclusively in the methylene carbons while succinate derived from propionate-3-C^{14} was labelled only in the carboxyl groups and (e) β-hydroxypropionic acid was an intermediate in the oxidation of propionate-1-, -2- and -3-C^{14}.

These facts were interpreted to indicate that the entire propionate molecule was converted to β-hydroxypropionate followed by the release of carbon 1 as $C^{14}O_2$ and the metabolism of carbons 2 and 3 as acetate in the TCA cycle. Carbon 3 of propionate would become the carboxyl carbon of acetate and carbon 2 the methyl carbon of acetate.

The failure of fluoride and malonate to inhibit the oxidation of propionate-1-C^{14} to $C^{14}O_2$ coupled with the failure to demonstrate a $C^{14}O_2$ dependent carboxylation of propionate indicated that propionate carboxylation to methylmalonate and thence to succinate did not occur.

To provide further evidence that the proposed scheme of propionate oxidation was operative in intact higher plant tissue propionate-1-C^{14} was supplied to seven-day-old wheat epicotyls, wheat roots, safflower cotyledons, safflower stems, safflower roots, pea leaves, pea roots, pea stems and pea cotyledons by Hatch and Stumpf (1962a). These tissues oxidized from 55-98% of added propionate-1-C^{14} in 3.5 hours, at 30°C, predominantly to $C^{14}O_2$ and β-hydroxypropionate-C^{14} with small amounts of labelled malate, citrate and β-alanine also being formed. Pea cotyledon, safflower root and wheat epicotyl systems were examined in more detail by incubating tissue sections with 0.5 μM propionate-1-, -3- and -3-C^{14} at 30°C. In all tissues the recovery of C^{14} as $C^{14}O_2$ was highest from propionate-1-C^{14}. Propionate-3-C^{14} released less $C^{14}O_2$ than did propionate-1-C^{14}, but more than propionate-2-C^{14}. The TCA cycle intermediates contained more radioactivity from propionate-2-C^{14} than from propionate-3-C^{14} and almost nothing from propionate-1-C^{14}. Isolated β-hydroxypropionate contained equal amounts of C^{14} regardless of the position of label in propionate. These findings were consistent with the mechanism that was previously proposed on the basis of enzymatic evidence.

However, the reported recovery of C^{14} from propionate-2- and -3-C^{14} was far below that from propionate-1-C^{14} in the pea cotyledon. In wheat epicotyl tissue carbons 1 and 3 of propionate

were not recovered as well as carbon 2. In contrast the reported recovery of C^{14} from carbon 2 was below that from carbons 1 and 3 in safflower roots. No explanations were offered for these large discrepancies and although they could be due to poor incorporation of propionate in these samples they could also indicate that a portion of the propionate was metabolized by an alternate pathway.

Hatch and Stumpf also reported that small amounts of C^{14} labelled β-alanine, malonate and methylmalonate were formed in most tissues. β-alanine became labelled at approximately the same rate from all three propionate carbons in the three tissues and they suggested that it was probably formed via the transamination of malonic semialdehyde. Although it has been shown that ammonia can add across the double bond in acrylyl-CoA, Vagelos et al (1959a), this reaction has only been shown to occur in bacteria. Although small amounts of methylmalonate-C^{14} was formed, this alternate pathway (Figure 1) appeared to be of little significance in these plant tissues because the expected equivalence of carbons 2 and 3 of propionate as CO_2 and TCA cycle intermediates was not observed. Presumably the small amount of labelled malonate could arise from the thiolysis of malonyl-CoA but the presence of adequate amounts of malonyl-CoA decarboxylase in most plant tissues, (Hatch and Stumpf 1962b), should favour the metabolism of malonate to acetate and CO_2.

It must be emphasized that oxidation of propionate via lactyl-CoA, a scheme described for Pseudomonas, (Vagelos et al 1959b) and for fungi, (Reisner et al 1963b), does not occur extensively in these tissues because carbon 3 of propionate was converted to CO_2 much more rapidly than carbon 2.

MATERIALS AND METHODS

Plant Materials

Castor bean seeds (Ricinus communis L.), variety Baker 296, sunflower seeds (Helianthus annuus L.), variety CM5 and pea seeds (Pisum sativum L.), variety Kelvedon Monarch, were soaked for 24 hours in water. The seeds were planted in moist vermiculite and allowed to germinate at 25°C. After 5 days the castor bean endosperm was separated from the testae and embryo and sliced into 0.5 - 1.0 mm. thick slices with a straight razor. Also, after 5 days the cotyledons from the sunflower seedlings were separated from the testa and excised. At the end of 7 days the pea cotyledons were excised from the seedling. All tissues were washed thoroughly in distilled water twice and blotted dry on paper toweling.

Radioactive Materials

Propionate-1-C^{14} (5.7 mc/mM) and propionate-2-C^{14} (4.25 mc/mM) were obtained from Atomic Energy of Canada Limited while propionate-3-C^{14} (2.0 mc/mM) was obtained from New England Nuclear Corporation. Before use propionate-1-C^{14} was diluted to a specific activity of 769,500 cpm/uM and 472,000 cpm/μM, propionate-2-C^{14} to 684,000 cpm/μM and 506,000 cpm/μM while propionate-3-C^{14} stock solutions had a specific activity of 367,000 cpm/μM and 478,000 cpm/μM.

Malonate-1-C^{14} (2.05 mc/mM) was obtained from New England Nuclear Corporation and diluted to a specific activity of 162,800 cpm/μM before use.

Incubation Conditions

In respiration studies, 250 mg (fresh weight) of castor bean endosperm slices were incubated with various concentrations of propionate in 15 ml Warburg flasks. The incubation medium consisted of 0.5 ml of 0.1 M phosphate buffer, pH 5.0, plus distilled water and propionate pH 5.0, to a total volume of 1.5 ml. Gas exchange was measured by standard manometric techniques Umbreit et al (1957). The final pH of the medium was measured before the tissue was discarded. The final results represent the mean of duplicate tissue lot measurements on gaseous exchange at each propionate concentration.

When C^{14} labelled substrates were used, 1 gm (fresh weight) of tissue was incubated at 25°C in 125 ml Warburg flasks for the requisite time period. The incubation medium consisted of 2.0 ml 0.1 M phosphate buffer, pH 5.0, radioactive substrate and distilled water to a final volume of 4.0 or 4.2 ml. $C^{14}O_2$ was trapped in 20% carbonate free NaOH in the centre well.

Collection of Evolved $C^{14}O_2$

At the end of the incubation period the NaOH containing

the $C^{14}O_2$ was transferred using a Weintrobe pipette to a 15 ml conical centrifuge tube containing 7 ml of a 10% $BaCl_2$ + 1% NH_4Cl solution. The centre well was washed twice with CO_2 free water. The flocculent $BaC^{14}O_3$ precipitate was sedimented by low speed centrifugation, resuspended in carbonate free water and plated on microporous porcelain planchets using a pasteur pipette.

Combustion of C^{14} Labelled Materials

Labelled compounds were converted to $BaC^{14}O_3$ for assay of radioactivity by either of two methods:

(1) combustion using the wet combustion reagents of Van Slyke and Folch in the Stutz and Burris apparatus, Canvin (1959).

(2) combustion using the persulfate method of Katz, Abraham and Baker (1954) as modified by Stiller, Canvin (1959).

Assay of Radioactivity

Almost all labelled compounds were assayed as $BaC^{14}O_3$ plated on microporous porcelain planchets. Count rates were corrected for background, self-absorption and coincidence when necessary.

The activity of some samples, e.g., lipids, was determined on nickel plated planchets after drying down under an infra red lamp. Glass planchets were used to assay the activity of each organic or amino acid fraction obtained by gradient elution

from formate and acetate columns.

The radioactivity of a sample was determined by means of a mylar window continuous gas flow automatic counter. Samples were usually counted to less than 2% error.

Extraction of Tissue

After the NaOH containing $C^{14}O_2$ was removed from the centre well, the tissue was killed immediately by the addition of 20 ml boiling 80% ethanol. The tissue was then successively extracted on a steam bath or hotplate with 20 ml 20% ethanol, 20 ml of distilled water and 20 ml 80% ethanol. The tissue remaining after this extraction procedure was designated the insoluble residue. Each extract was filtered through Whatman #1 filter paper into a 250 ml boiling flask. When it was desirable to retain propionic acid in this extraction procedure, the extraction reagents were made basic with NaOH.

The combined alcohol-water extracts were evaporated to dryness *in vacuo* on a water bath at 45°C. The dry residue was successively extracted 3 times with ethyl ether and water. The ether phase constituted the lipid fraction. The water soluble compounds were made up to 25 ml volumetrically.

Steam Distillation of Basic Ethanol-Water Extract

The steam distillable, volatile fatty acids were recovered from the basic ethanol-water extract by adding 5 ml extract to

a steam distillation apparatus, carrier propionate and enough 6 N sulfuric acid to produce an orange color with thymol blue, followed by rapidly distilling approximately 200 ml into a 250 ml boiling flask.

After neutralizing the solution to a phenol red end-point, the steam distillable fraction was taken to dryness on a flash evaporator at 45°C. *in vacuo*. This fraction was made up to 5 ml volumetrically with carbonate free water. After aliquots were removed for radioactivity determination, the remainder of the steam distillable fraction was taken to dryness in an air stream, taken up in a small volume of water and spotted on Whatman #1 chromatography paper. Paper chromatograms were developed in an ascending direction in

(1) ethanol:ammonia:water (16:1:13, v/v/v)

(2) n-butanol:acetic acid:water (12:3:5,v/v/v)

The paper chromatograms were sprayed with a solution of 0.4% bromocresol green in 95% ethanol. Radioactive areas were determined using autoradiography on Kodak (No Screen) X-ray film.

The steam distillable residue was neutralized with barium carbonate, centrifuged and the supernatant decanted into a boiling flask. The precipitate was washed twice with distilled water. The combined supernatant and washings were taken to dryness on a flash evaporator at 45° C *in vacuo*. Non-volatile

materials were extracted with warm water and made up to 25 ml volumetrically. Aliquots were removed for assay of total radioactivity.

Fractionation of Water Soluble Components

Ion-exchange resins were used to fractionate the water soluble compounds into three fractions. The extract was passed through a 6 x 1 cm column of Dowex 50-X8 (H^+) first and then through a 6 x 1 cm column of Dowex 1-X10 (formate). Basic compounds are retained on the Dowex 50 column, acidic compounds on the Dowex 1 column and neutral components pass through in the effluent. Basic compounds were eluted from the column with 50 ml 1N NH_4OH whereas acidic compounds were eluted with 30 ml 4N formic acid followed by 20 ml 8N formic acid.

Thus the three fractions obtained were:

(1) basic-composed mainly of amino acids and hence called the amino acid fraction.

(2) acidic-mainly composed of organic acids but also containing phosphorylated compounds. This fraction was termed organic acid fraction.

(3) neutral-almost exclusively composed of sugars, hence termed the sugar fraction.

These fractions were evaporated to dryness <u>in vacuo</u> on a water bath at 40°C, redissolved in 25 ml water and aliquots

removed for combustion to determine the total radioactivity present in each fraction.

Resolution of Sugars

After determination of total activity, the sugar fraction was taken to dryness in an air stream on a water bath at 45°C, taken up in a few drops of distilled water and streaked on Whatman #1 paper. The sugars were then separated by descending chromatography in ethyl acetate:acetic acid :water, 4:1:1 (v/v/v).

The locations of the various sugars on chromatograms were determined by dipping strips of the paper into the reagent of Zimmerman, Canvin (1959). Thus, equal quantities of 0.93 gm aniline in 50 ml 96% ethanol and 0.2 M oxalic acid in 96% ethanol were mixed. The strips were dipped in this solution and then heated above a hot plate until glucose had reacted to produce a brown spot and fructose had just begun to react. Then the strips were dipped in a second solution composed of equal quantities of 0.5% resorcinol in acetone and 5% HCl in 95% ethanol. The strips were reheated until all sugars reacted.

This procedure differentiates aldoses, ketoses, and non-reducing carbohydrates from each other. Aldoses yield a brown spot on the first heating. Ketoses and ketone containing non-reducing compounds react in the second heating to yield bright

red spots. Non-reducing aldoses yield brown spots in the second heating.

Autoradioagraphy using chromatogram strips on Kodak (No Screen) X-ray film was utilized to determine radioactive areas.

Resolution of Amino Acids

The resolution of amino acids from plant extracts or protein hydrolysates is usually very difficult. The large amounts of amino acids present cannot usually be resolved adequately using one and two-dimensional chromatography. If aliquots of the amino acid extract are used to circumvent the first problem, these aliquots usually do not contain enough radioactivity to facilitate the identification of labelled amino acids. For these reasons a procedure reported first by Hirs *et al* (1954) and modified by Canvin (1958) has been used in conjunction with one and two dimensional chromatography to resolve the amino acid fractions.

The amino acid extract was evaporated to dryness, taken up in 1 ml distilled water, pH 9.0, and added to a 6 x 1 cm column of Dowex 1-X10 (acetate) - generated from the chloride form with 1 M sodium acetate and 0.1 N acetic acid. The column was washed with ca.10 ml water, pH 9.0, and the fraction collected was called the column effluent. The remaining

amino acids were eluted with 0.1 N acetic acid and the effluent collected in 3 ml fractions.

Active fractions were determined by counting a 0.3 ml aliquot on a glass planchet.

Ninhydrin determinations were carried out on 1 ml aliquots of each fraction using a method outlined by Yemm and Cocking (1955) the reagents and procedure were:

(1) dilute 10 ml 0.01 M KCN to 500 ml with methyl cellosolve,

(2) 5% w/v ninhydrin in methyl cellosolve,

(3) 125 ml (1) mixed with 25 ml, (2) and allowed to stand overnight. This solution is stable at room temperature for at least one week.

(4) 0.2 M citrate buffer (pH 5.0).

The 1 ml sample was added to 0.5 ml (4) and 1.2 ml (3). This solution was heated for 15 minutes at 100°C and cooled for 5 minutes. Then after adding 3 ml of 60% ethanol and shaking, the optical density of each fraction was determined at 570 mu in a Bausch and Lomb spectronic 20 colorimeter.

The correspondence between the amount of ninhydrin positive material and radioactivity in each 3 ml fraction collected provided a basis for selecting a "peak" and pooling all fractions belonging to that "peak". The pooled fractions were dried down in an air stream on a water bath at 45°C.

The residue was dissolved in 5 ml distilled water and aliquots removed for persulfate combustions. The remainder of each "peak" was dried down in an air stream at 45°C.

The identity of the constituents of the three "peaks" that were obtained using this method was confirmed in three ways:

(1) descending co-chromatography with authentic samples in n-butanol:acetic acid:water, 12:3:5 (v/v/v).

(2) descending two-dimensional chromatography with ethanol:ammonia:water, 16:1:3 (v/v/v) in the first direction and phenol:water, 4:1 (v/v) in the second direction. The amino acid positions were determined by spraying the chromatogram with 0.2% ninhydrin in acetone.

(3) autoradiography using the chromatograms for (1) and (2).

The separation of glutamic and aspartic acids, using the above method, is shown in Figure 2. When radioactive amino acid fractions were added to the acetate column the recovery of amino acids was quantitative (Table 12).

The standard curves for the ninhydrin determination of glycine, aspartic acid and glutamic acid is shown in Figure 3. The curves shown are the average of duplicate measurements.

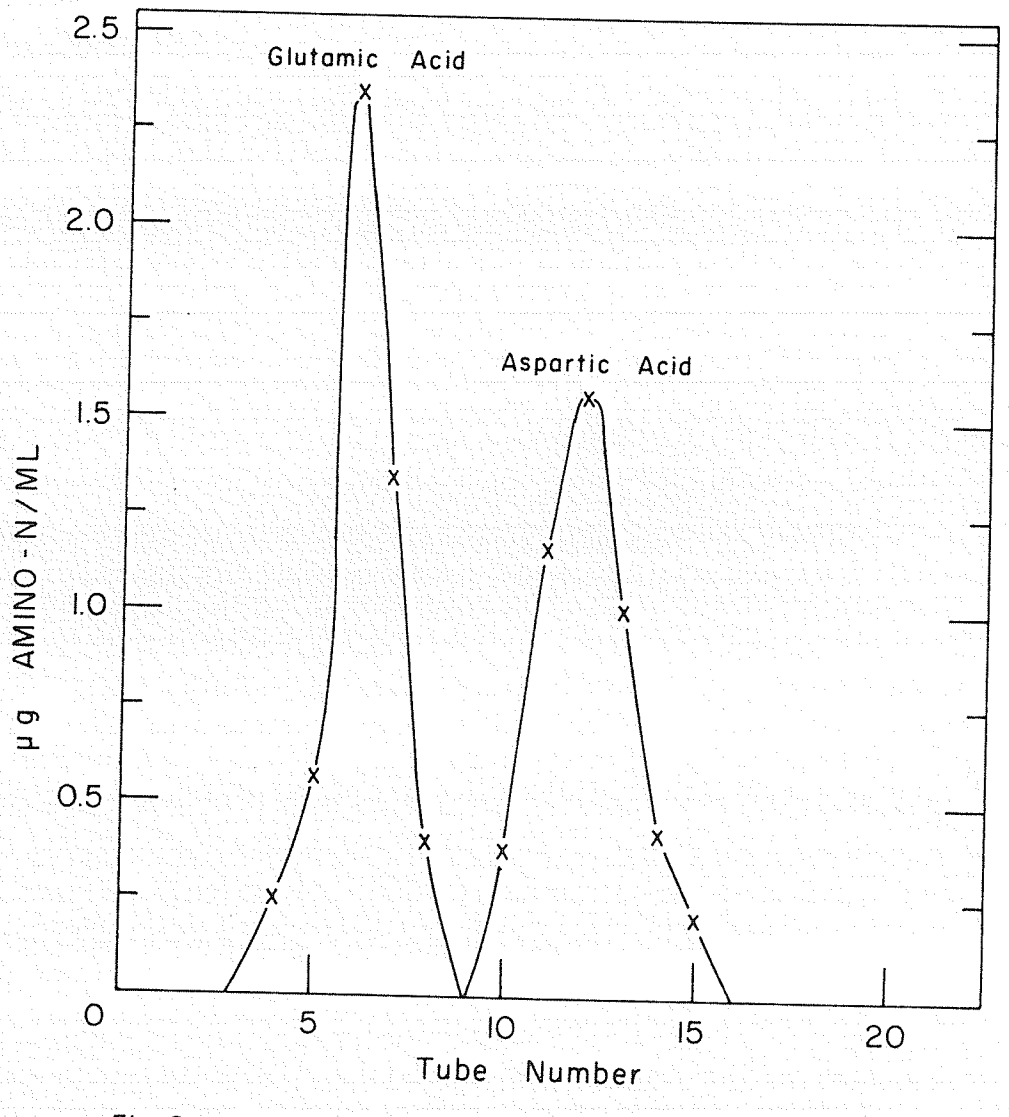

Fig. 2 Elution of glutamic and aspartic acids from Dowex 1-x 10 (acetate) with 0.1N. acetic acid.

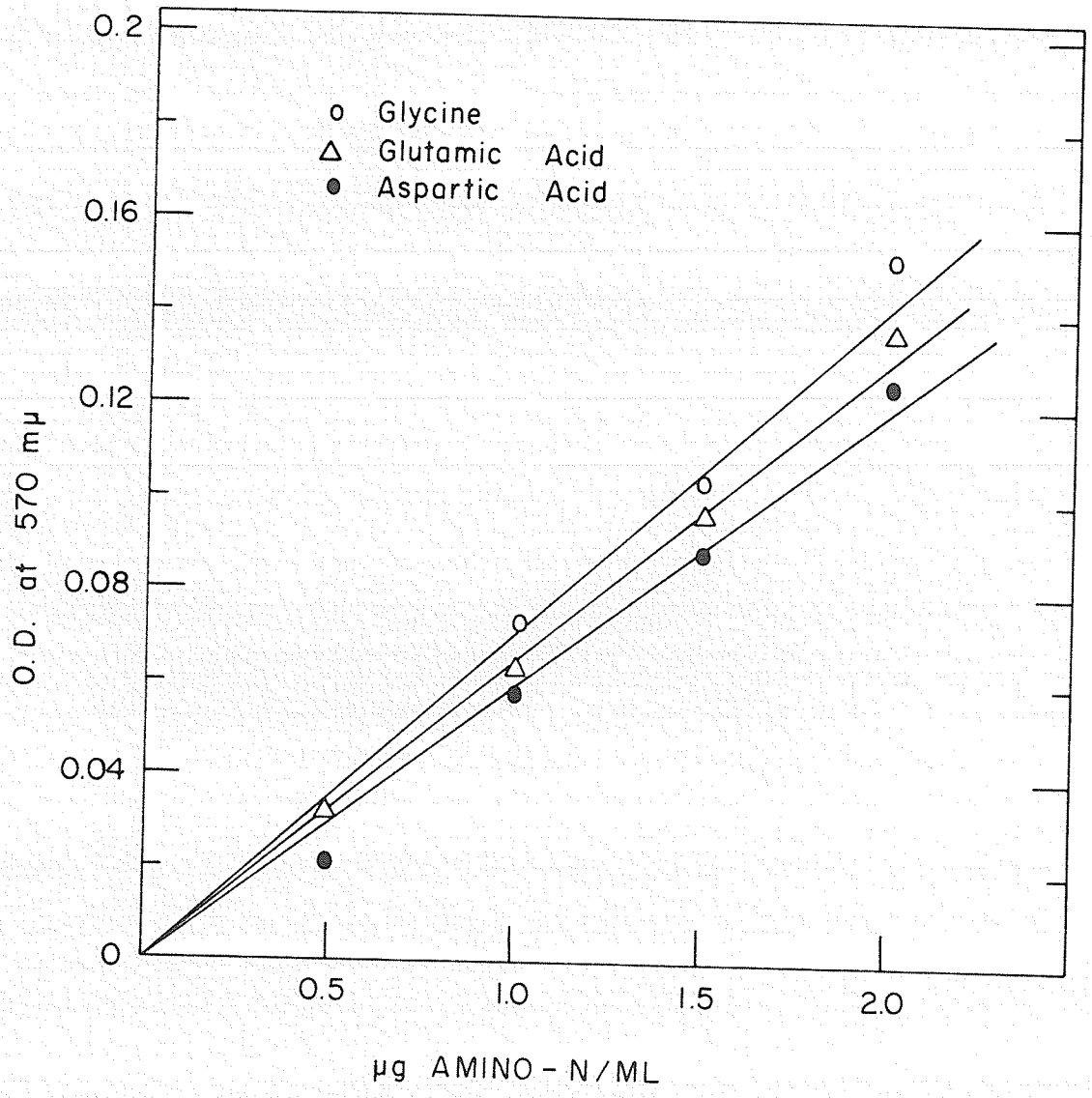

Fig. 3 Standard curves for glutamic acid, aspartic acid and glycine determined with ninhydrin.

Resolution of Organic Acids

Palmer (1955) has demonstrated that gradient elution from ion-exchange resins can be used to separate organic acids. However, by altering the apparatus to establish a linear gradient it has been shown that the resolution of organic acids can be improved, Canvin (1959). Thus concentrated acid can be mixed in such a manner that a linear increase in normality of eluant can be obtained (Figure 4). The gradient elution apparatus consists of two cylindrical containers, a mixing flask A which has twice the cross-sectional area of the reservoir B. A constant level siphon composed of capillary tubing connects the two cylindrical containers. Another siphon joins the mixing flask to the ion-exchange column. To begin with the mixing flask contains water and the reservoir contains the concentrated eluting acid. As water is siphoned from the mixing flask to the column, concentrated acid from the reservoir enters the mixing flask. It is immediately mixed with the water by means of a magnetic stirrer. Because the cross-sectional area of the mixing flask is twice that of the reservoir and since the level of liquid in both must remain constant, it follows that liquid leaves the mixing flask at twice the rate of that entering from the reservoir. Hence, the linear gradient of eluant established has a slope which depends solely on the

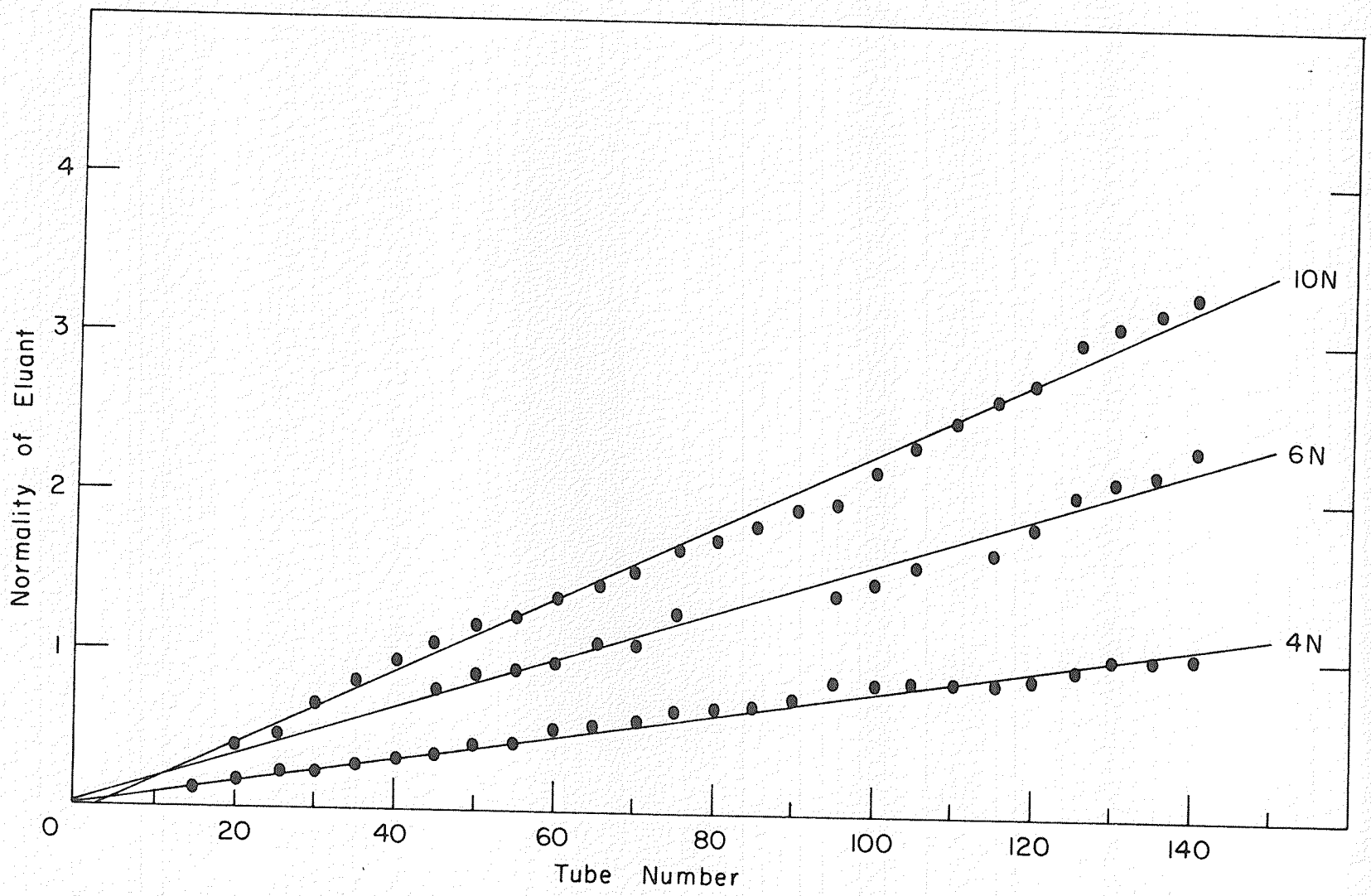

Fig. 4 The linear gradient of eluant. 4N, 6N and 10N refer to the initial concentration of formic acid in the reservoir.

initial concentration of acid in the reservoir, assuming that the initial volume of liquid in each container is constant. In this study, the mixing flask contained water initially and the reservoir contained 6N formic or acetic acid. The sample was usually transferred to a 11 x 1 cm Dowex 1-X10 (formate) ion exchange column, but 11 x 1 cm Dowex 1-X10 (acetate) columns were used occasionally. Initial column flow rates were ca. 1 ml/min. and the column effluent was collected in 3 ml fractions on a fraction collector.

The emergent pattern of authentic organic acids using this system is shown in Figure 5. A 6 N formic acid gradient was used.

The gradient was usually only continued to test-tube 150 because no radioactive compounds emerged from the column beyond this point in this study. The elution pattern for the separation of an organic acid sample from this study is shown in Figure 6.

Radioactive fractions were determined by counting a 0.3 ml aliquot of each test-tube on a glass planchet. The tubes containing labelled acids making up a distinct "peak" were pooled and the formic acid eluant blown off in an air stream at 45°C. The residue was redissolved in 5 ml carbonate free water and transferred volumetrically to suitable containers

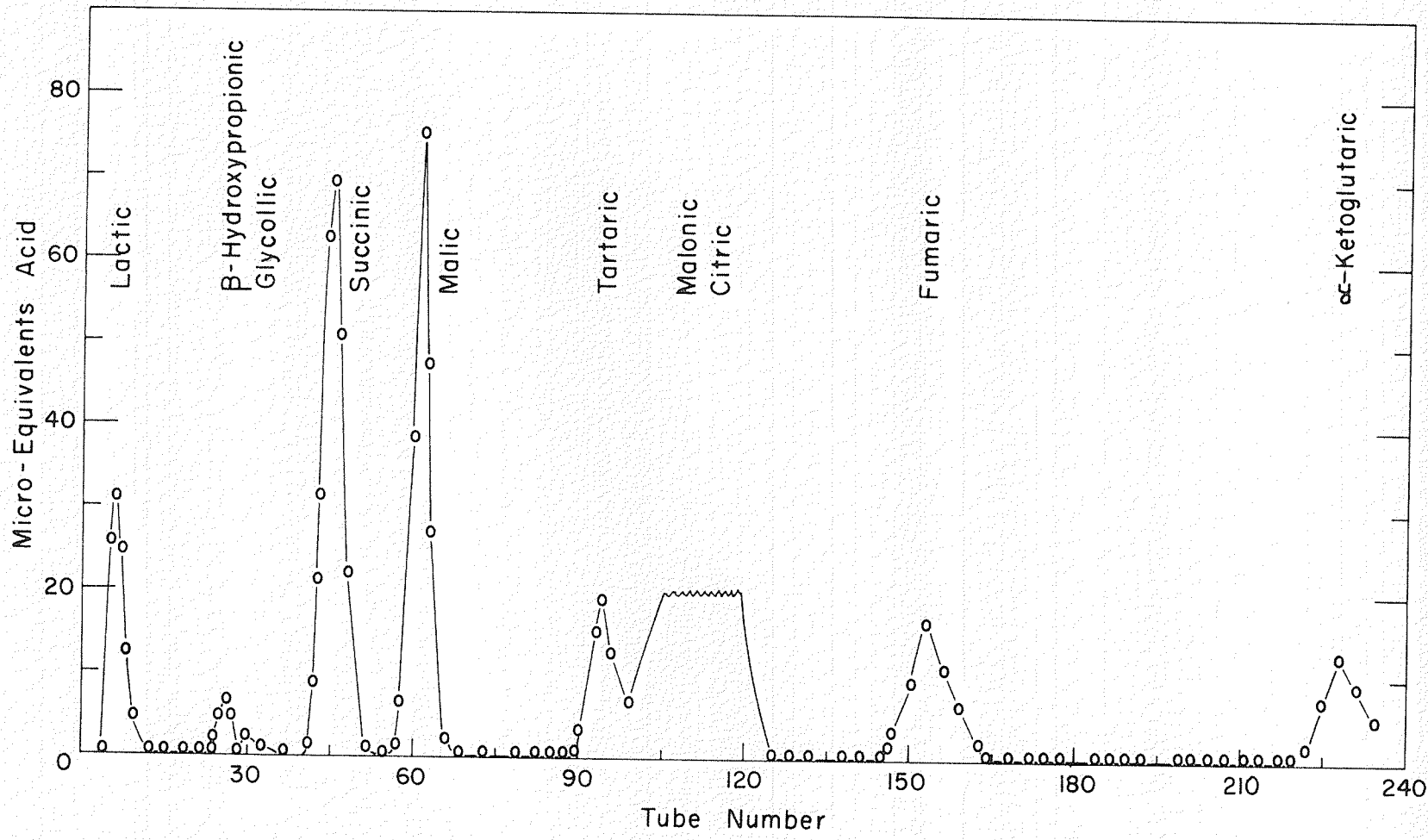

Fig. 5 Separation of known organic acids using ion-exchange chromatography. The peak for malonate and citrate is drawn based on the recovery of malonate, citrate and malonate - C^{14} from the column.

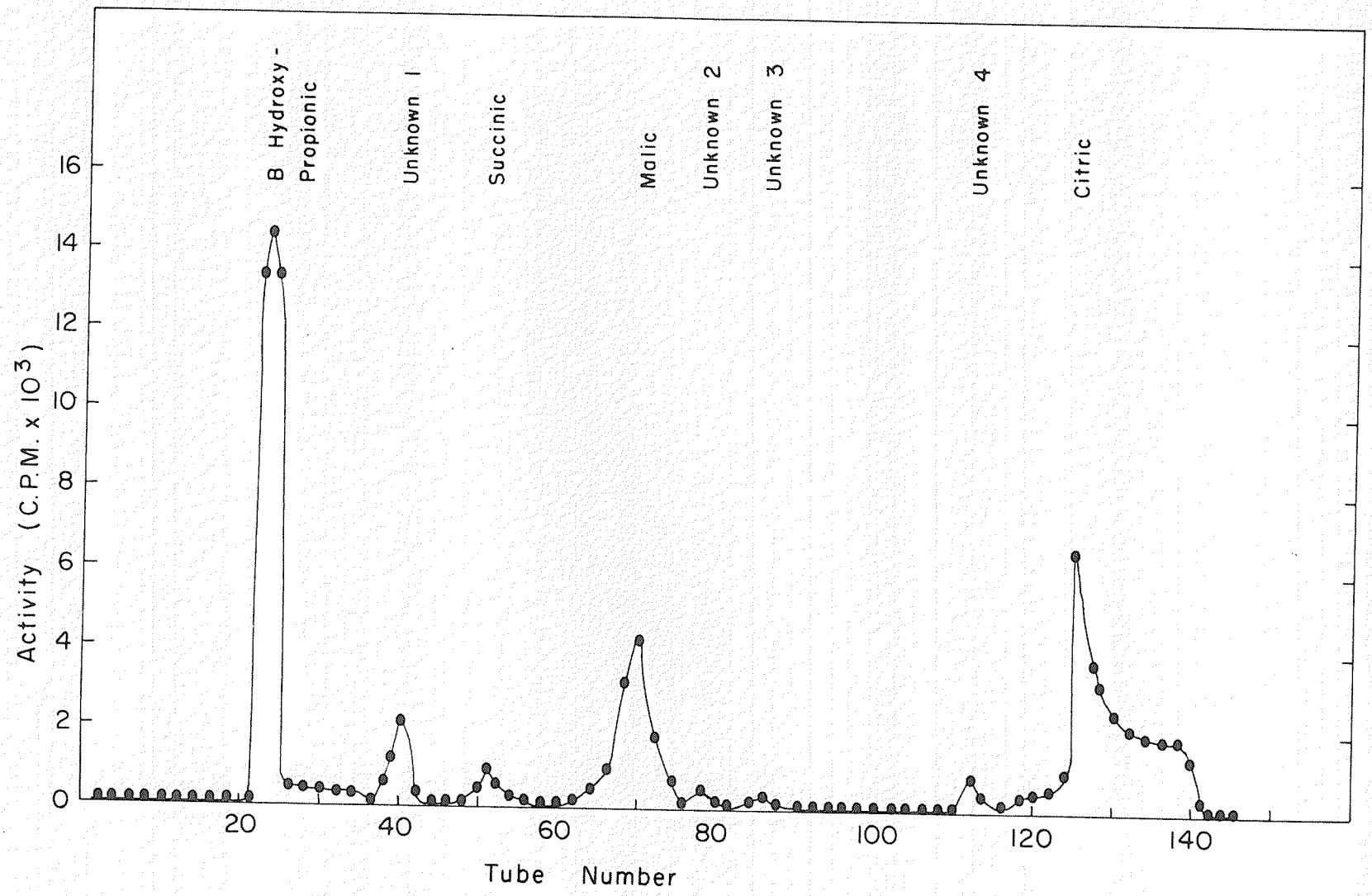

Fig. 6 Separation of the organic acid fraction from pea cotyledon tissue incubated with propionate $-2-C^{14}$.

for storage and handling.

Aliquots of each organic acid fraction were removed for persulfate combustions, usually for titrations, but not always, and the remainder was dried down in an air stream at 45°C to be used for chromatography.

The resolution of organic acids can be reproduced to within a single test-tube if sufficient care is taken initially to obtain identical starting conditions. The recovery of most of the organic acids from these formate columns is quantitative, but a few are poorly recovered. (Table 1).

TABLE 1. Recovery of known organic acids from ion-exchange chromatography.

Organic Acid	Meq Added	Meq Recovered	% Recovery
Lactic	114	107	94
Succinic	269	297	110
Malic	207	236	114
Fumaric	130	146	112
α- Ketoglutaric	198	116	59
Tartaric	159	110	69

Poor recovery is most probably due to volatility losses, especially if the organic acid is exposed to prolonged periods under the constant air stream after it has been dried down,

Palmer (1955). Quantitative organic acid recovery from formate columns was also reported by Canvin (1959) and Palmer (1955). Although the radioactivity of labelled organic acid fractions could not be recovered quantitatively (Table 2), it is believed that this was due to volatility losses. Support for this comes from the fact that the total recovered activity of certain column "peaks" was often up to 50% lower than the calculated activity from glass planchet results.

Identification of the Radioactive "Peaks"

The radioactive "peaks" were identified and characterized by:

(1) position of elution from ion-exchange column

(2) co-chromatography on paper with known samples

(3) the fluorescence of the derivatives formed with resorcinol and H_2SO_4

(4) digestion followed by inorganic phorphorus analysis

(5) Polidase-S hydrolysis of suspected phosphate

The organic acids were co-chromatographed with known samples on Whatman #1 chromatography paper. Ascending and descending chromatograms were developed in the following solvents:

TABLE 2. Activity of various fractions separated from the total organic acid fraction using ion-exchange chromatography. The first sample was the organic acid fraction extracted from 1 gm pea cotyledons incubated with 1 µM propionate-2-C^{14} (506,000 cpm) for 2 hours. Gradient elution chromatography on Dowex 1-X10 (formate) with 6N formic acid in the reservoir was used to separate this fraction. The second sample was the organic acid fraction separated from 1 gm castor bean endosperm slices incubated with 1 µM propionate-3-C^{14} (367,960 CPM) for 3 hours. This sample was separated by Dowex-1-X10 (acetate) using 6 N acetic acid in the reservoir.

Propionate-2-C^{14}		Propionate-3-C^{14}	
Fraction	Activity (cpm)	Fraction	Activity (cpm)
β-OH propionate	66,700	β-OH propionate	58,900
Unknown 1	6,970	Unknown 1	2,245
Succinic	7,350	Unknown 2	1,028
Malic	23,450	Unknown 3	1,691
Unknown 2	2,730	Unknown 4	6,520
Unknown 3	1,492	Unknown 5	3,720
Unknown 4	2,005	Unknown 6	4,165
Citrate	29,700	Unknown 7	1,006
		Unknown 8	858
		Unknown 9	844
		Unknown 10	1,023
Column Effluent	3,110	Column Effluent	2,134
Residual acids	3,370	Residual acids	345
Total	146,877	Total	84,479
Total added	178,250	Total added	119,320
% Recovery	82.3	% Recovery	70.7

(1) n-butanol:acetic acid:water, 12:3:5 (v/v/v)

(2) ether:formic acid:water, 5:2:1 (v/v/v)

(3) isoamyl alcohol saturated with 4N formic acid

(4) propanol:formic acid:water, 8:1:1 (v/v/v)

(5) ethanol:ammonia:water, 16:1:3 (v/v/v)

After development, ascending chromatograms were steamed for 3-5 minutes to remove solvent vapor. Descending chromatograms were exposed for sufficient periods of time to moving, warm air in an oven. Acid areas were determined by spraying the paper with 0.4% bromocresol green in 95% ethanol.

Autoradiography with Kodak (No Screen) X-ray film was used to localize radioactivity areas on chromatograms.

Dicarboxylic organic acids, when heated at 130°C for 5 minutes with resorcinol and H_2SO_4, react to form specific fluorescent derivatives, Feigl (1946). One to two µg of acid can readily be determined by this test, Canvin (1959).

A sample of the unknown was added to a test-tube and evaporated to dryness by an air stream at 45°C. 0.04 ml concentrated H_2SO_4 was added followed by approximately 0.1 mg resorcinol. The tube was heated in an oil bath at 130°C for 20 minutes, cooled in a water bath and 5 ml 0.2N NaOH added. The specific fluorescences were readily determined under the ultra violet lamp.

Since the acidic fraction obtained by the fractionation of water soluble components could contain phosphorylated compounds most unknown "peaks" were assayed for inorganic phosphorus by the Fiske and Subbarow procedure, Leloir and Cardini (1957). In this procedure the extra-labile compounds are estimated as inorganic phosphate due to the relatively high acid concentration and because molybdate accelerates the hydrolysis of some organic phosphates. Inorganic phosphate can be detected in the range 4-40± 0.2 µg., Le Page (1957).

In this procedure the inorganic phosphorus must be released for estimation by digestion, Le Page (1957). The sample was dried down in a test-tube in an air stream at 45°C, 0.4 ml 10 N H_2SO_4 was added and it was heated at 130-160°C for 30 minutes. After cooling partially, 1-2 drops of 30% H_2O_2 was added and the sample was put back in the hot oil bath for 15 minutes. Then it was partially cooled, 1 ml water added and heated at 100°C in a steam bath for 10 minutes.

The reagents and procedure for inorganic phosphate estimation are as follows:

(1) reducing agent - 0.25 gm of 1-amino-2-naphthol-4-sulfonic acid in 15% $NaHSO_3$ made up to 92.5 ml. Add 2.5 ml 20% Na_2SO_3 and heat until all reagents are in solution.

(2) 10 N H_2SO_4

(3) 2.5% ammonium molybdate

(4) standard solution - 1.3613 gm KH_2PO_4 in 1l. water. When diluted 1:10, 1 ml = 1 uM PO_4.

The digested sample was made up to 3.4 ml with water, 0.4 ml 10 N H_2SO_4 was added followed by 0.8 ml 2.5% ammonium molybdate and 0.4 ml of reducing agent, in that order. The solution was allowed to stand for 10 minutes, diluted to 10 ml with water and after 10 minutes at room temperature the optical density was read at 660 mu using a Bausch and Lomb spectronic 20 colorimeter.

Polidase-S hydrolysis of phosphate-esters, Benson (1957), coupled with ion exchange chromatography provides an effective means of detecting sugar phosphates if they are present in an organic acid sample.

Suspected phosphate esters eluted from chromatograms were incubated with 2 mg Polidase-S obtained from Schwarz Bioresearch Inc. The solution was layered with toluene and incubated in an oven at 35°C for 2 days. The toluene layer was removed and the sample was dried down at 45°C in an air stream. An aliquot of the redissolved sample was removed to determine the total activity of the sample on metal planchets. The remainder was added to a 6 x 1 cm Dowex 1-X10 (formate) column, washed with 10 ml water and eluted with 25 ml formic acid. The activity of the neutral and acidic fractions was

determined on metal planchets. If the compound was a sugar phosphate, then the radioactivity which was previously adsorbed on an acidic column should appear in the neutral fraction after Polidase-S treatment.

Preparation and Characterization of β-Hydroxypropionate

β-hydroxypropionic acid was prepared from propiolactone by two methods. In the first case, the propiolactone was hydrolyzed with an equimolar quantity of 0.5 N KOH, Giovanelli and Stumpf (1958). The solution was acidified with H_2SO_4 and the free acid extracted with ether overnight. In the second method, the propiolactone was hydrolyzed with excess $Ba(OH)_2$ and CO_2 was bubbled through the solution to precipitate the excess barium as $BaCO_3$. The solution was centrifuged and the precipitate discarded. The supernatant was acidified with H_2SO_4 to remove the barium as $BaSO_4$. The precipitate was removed by centrifugation. The supernatant then contained β-hydroxypropionic acid. The prepared product was characterized by ascending paper chromatography in five solvents (Table 3).

In addition, β-hydroxypropionate yields malonic acid when oxidized with acid dichromate, Giovanelli and Stumpf (1958). Thus, the β-hydroxypropionate preparations were oxi-

dized with acid dichromate and the product of oxidation was extracted in a liquid-liquid continuous extraction apparatus. Malonic acid was shown to be present by descending chromatography in ethanol:ammonia:H_2O, 16:1:3 (Rf 20-25).

TABLE 3. Characterization of β-hydroxypropionic acid with ascending paper chromatography.

Solvent	Rf of Acid
n-Butanol:acetic acid:H_2O, 12:3:5	71
Ether:formic acid:H_2O, 5:2:1	79
Isoamyl alcohol saturated with 4 N HF	57
Ethanol:ammonia:H_2O, 16:1:3	45
Ethanol:ammonia, 100:1	49

RESULTS AND DISCUSSION

Propionate Metabolism in Pea Cotyledons

One gram of pea cotyledon tissue was incubated with 2.0 ml of 0.1 M phosphate buffer, pH 5.0, 1 μM of propionate-1-C^{14} (472,000 cpm,) or propionate-3-C^{14} (478,000 cpm) for 4 hours or 1 μM propionate-2-C^{14} (506,000 cpm) for 2 hours in a total volume of 4.2 ml. The time course release of $C^{14}O_2$ from each propionate carbon was examined.

TABLE 4. Total radioactivity in various fractions of pea cotyledon tissue incubated with propionate-1-, -2- and -3-C^{14}. The tissue in the flask containing propionate-2-C^{14} was killed after 2 hours, whereas the incubation period was 4 hours for propionate -1- and -3-C^{14}.

Fraction	Propionate-1-C^{14}		Propionate-2-C^{14}		Propionate-3-C^{14}	
	Activity (cpm)	% of Added	Activity (cpm)	% of Added	Activity (cpm)	% of Added
CO_2	280,350	60.1	3,374	0.7	68,301	14.3
Organic acid	34,500	7.3	178,250	35.2	104,000	21.8
Amino acid	150,650	31.9	269,400	53.2	280,750	58.7
Sugar	2,245	0.5	2,020	0.4	1,850	0.4
Lipid	2,730	0.6	900	0.2	6,620	1.4
Insoluble	5,636	1.2	5,384	1.1	20,084	4.4
Total	476,111	101.6	459,328	90.8	481,605	101.0

Propionate was readily utilized by this tissue (Table 4) since all the added radioactivity from carbons 1 and 3 could

be accounted for after 4 hours in labelled CO_2, organic acids, amino acids, sugars, lipids, and insoluble material. Since unmetabolized propionic acid would be volatile under the extraction conditions utilized, this probably explains why 9% of the added propionate-2-C^{14} was unaccounted for after 2 hours.

The major fate of propionate-1-C^{14} in this tissue was incorporation into labelled CO_2 and amino acids. Organic acids accumulated a small, but significant amount of radioactivity in 4 hours. In contrast to the results obtained for carbon 1 of propionate, carbons 2 and 3 accumulated almost exclusively in organic and amino acids after 2 and 4 hours, respectively. A significant amount of propionate-3-C^{14} also accumulated in CO_2 and insoluble material in 4 hours. Lipids and sugars contained a negligible amount of radioactivity from each of the 3 propionate carbons.

Figure 7 shows the time-course release of the various propionate carbons as $C^{14}O_2$ in pea cotyledon tissue. Carbon 1 of propionate was released slowly for the first 30 minutes but subsequently was rapidly incorporated into $C^{14}O_2$. There was a very long lag in the release of carbons 2 and 3 of propionate as $C^{14}O_2$. Since less than 1% of the added C^{14} from propionate-2-C^{14} was released as $C^{14}O_2$ after 2 hours, carbon 2 of propionate appears to be retained within tissue components.

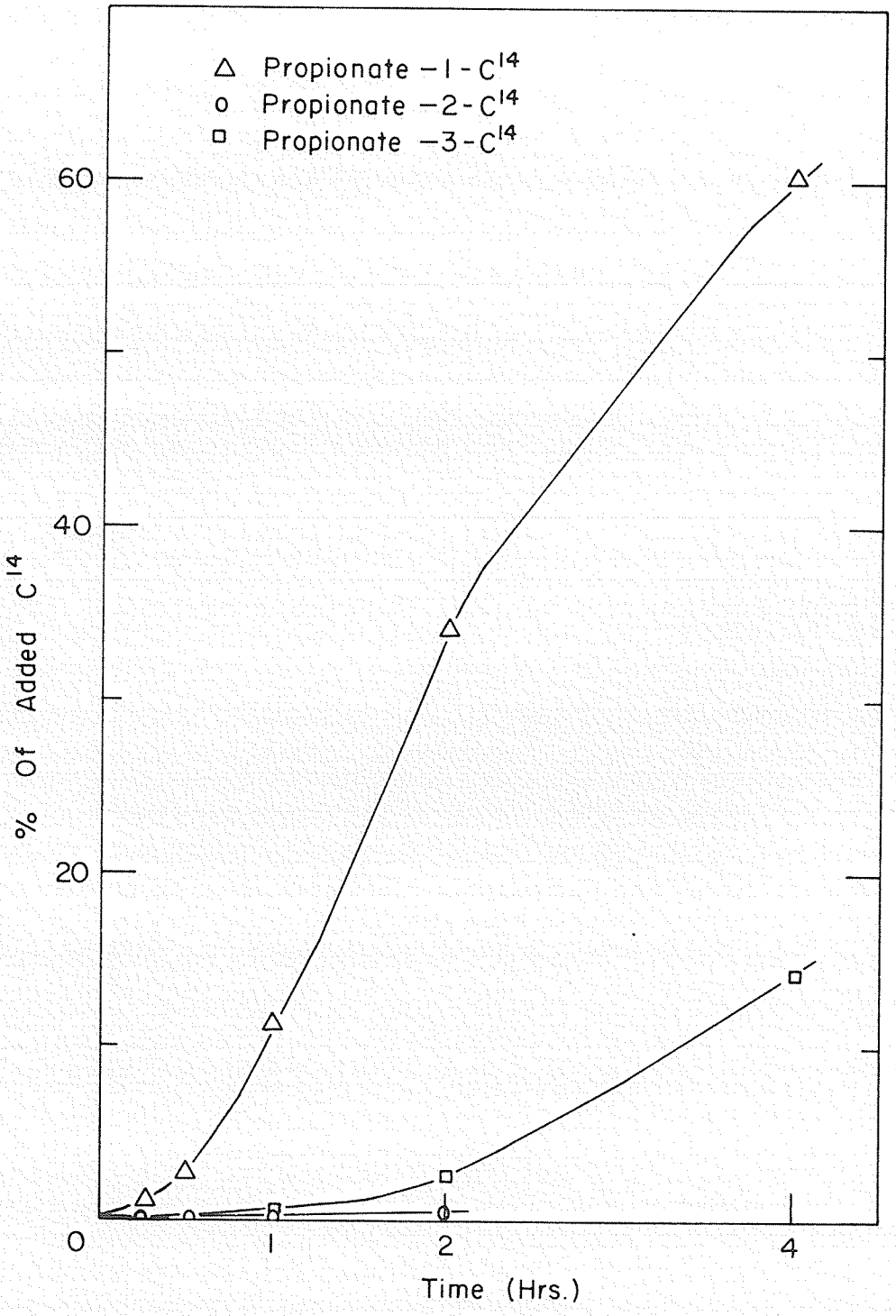

Fig. 7 Time-course release of various propionate carbons as $C^{14}O_2$ in pea cotyledon tissue.

2.5% of the added propionate-3-C^{14} was incorporated into $C^{14}O_2$ after 2 hours while after 4 hours, 14% of carbon 3 of propionate was released as $C^{14}O_2$. Thus, carbon 3 of propionate was released more rapidly as labelled CO_2 than carbon 2.

The organic acid fraction from propionate-2-C^{14} was separated by ion exchange chromatography (Table 5). Only 82% of the added radioactivity was recovered and it appears probable that this low recovery is due to the loss of a volatile component from the citrate peak (Figure 6). The activity of this peak was ca. 50,000 cpm on glass planchets while only 29,700 cpm was recovered subsequently and this recovered radioactivity was exclusively in citric acid.

Most of the radioactivity in the organic acid fraction derived from propionate-2-C^{14} was isolated in β-hydroxypropionate and TCA cycle intermediates (Table 5). Thus, 37%, 17%, 13% and 4% of the total radioactivity in the organic acid fraction was isolated in β-hydroxypropionate citrate, malate and succinate, respectively. Unknown acidic compounds accumulated a very small amount of the added C^{14}. Unknown 1 co-chromatogramed with glycollic acid in 3 solvents, but did not emerge from the ion exchange column in the proper position (Figure 6). Hence, it may not be glycollic acid. Unknowns 2, 3 and 4 did not co-chromatograph with any known standards and no further tests were performed to identify them.

TABLE 5. The incorporation of propionate-2-C^{14} into the organic acid constituents isolated from pea cotyledon. 1 μM propionate-2-C^{14} (506,000 cpm) was incubated with 1 gm of tissue for 2 hours.

Fraction	Activity (C.P.M.)	% of Total Organic Acid Activity	% of Added Activity
β-hydroxy-propionate	66,700	37.4	13.2
Unknown 1	6,970	3.9	1.4
Succinate	7,350	4.1	1.5
Malate	23,450	13.1	4.6
Unknown 2	2,730	1.6	0.5
Unknown 3	1,492	0.8	0.3
Unknown 4	2,005	1.1	0.4
Citrate	29,700	16.6	5.9
Column Efflu-ent	3,110	1.8	0.6
Column resi-due	3,370	1.9	0.7
Total	146,877	82.3	29.1

If propionate were metabolized in pea cotyledon tissue via the modified β-oxidation pathway proposed by Giovanelli and Stumpf (1958), the relative rates at which each of the propionate carbons appear as CO_2 are of critical importance. Carbon 1 of propionate should appear exclusively as CO_2 while

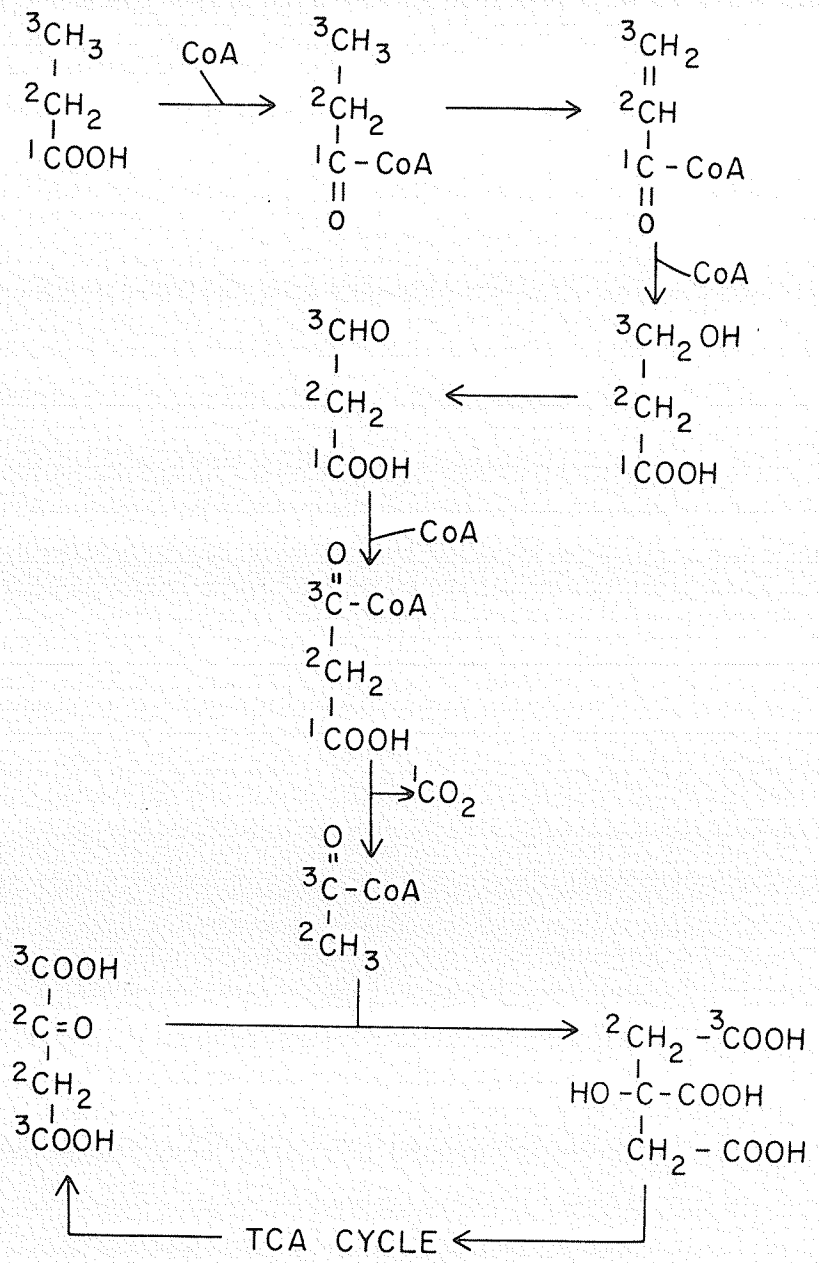

Fig. 8 Propionic acid metabolism; Pathway of propionate to TCA Cycle intermediates as outlined by Giovanelli and Stumpf (1958).

carbons 2 and 3 would be converted to the methyl and carboxyl carbons, respectively, of acetate (Figure 8). When the acetate enters the TCA cycle the carboxyl carbon of acetate would appear much more rapidly as CO_2 than the methyl carbon. As the number of cycles increase, the difference in rate would diminish. The over-all rate of oxidation of acetate formed from propionate would depend upon the activity of the TCA cycle and the endogenous pool size of acetate and TCA cycle intermediates.

The results discussed above are in accord with the operation of a modified β-oxidation pathway for propionate metabolism. The rapid release of carbon 1 of propionate as CO_2 with time coupled with the long lag in the appearance of carbons 2 and 3 in CO_2, especially the former, was quite consistent with propionate oxidation in such a manner that carbon 1 was lost as CO_2 and carbons 2 and 3 behaved as the methyl and carboxyl carbons, respectively, of acetate. The accumulation of much larger quantities of radioactivity in organic and amino acids from carbons 2 and 3 of propionate than from carbon 1 provides additional support for the above proposal. The identification of β-hydroxypropionate-2-C^{14} indicated that propionate was metabolized via this intermediate. In addition, the isolation of labelled citrate, malate and succinate from the organic acid fraction derived from propionate-2-C^{14} suggests

that carbon 2 of propionate entered the TCA cycle at citrate and was metabolized via the TCA cycle. Since the sugar fractions derived from propionate-2- and -3-C^{14} contained a negligible amount of activity, glyoxylate cycle activity was probably of little, if any, importance in this tissue.

Since β-hydroxypropionate contained the major portion of the activity in the organic acid fraction derived from propionate-2-C^{14} while the majority of the added radioactivity from carbons 2 and 3 of propionate was found in amino acids it seems highly probable that once the acetate moiety of propionate entered the TCA cycle at citric acid it was rapidly oxidized to keto-acids which could be incorporated into amino acids via transamination. The very long lag in the release of $C^{14}O_2$ from propionate-2- and -3-C^{14} relative to that from carbon 1 of propionate tends to support the idea that TCA cycle intermediates are being rapidly utilized for synthetic purposes. Hautanen (1946) has demonstrated that transaminase activity in this tissue was very high. Hatch and Stumpf (1962a) reported β-alanine formation from propionate metabolism in pea cotyledons. Thus, it is possible that part of the activity in amino acids derived from propionate-1-, -2- and -3-C^{14} could be due to β-alanine synthesis probably via transamination involving malonic semialdehyde (Figure 1). Presumably, the

amino acids could be used in protein synthesis. The accumulation of 4.4% of the added radioactivity from propionate-3-C^{14} in the insoluble fraction after 4 hours adds support to this view.

It should be emphasized that in these experiments complete recovery of the C^{14} from all the propionate carbons was achieved. Although the amino acids were not identified and there was a small amount of C^{14} in unknown acidic compounds, the pattern of $C^{14}O_2$ release and the incorporation of C^{14} into known TCA cycle intermediates was entirely consistent with the modified β-oxidation pathway of propionate metabolism proposed by Giovanelli and Stumpf (1958).

Propionate Metabolism in Etiolated Sunflower Cotyledon

One micromole of propionate-1-C^{14} (769,500 cpm or 472,000 cpm), propionate-2-C^{14} (684,000 cpm or 506,000 cpm) or propionate-3-C^{14} (367,960 cpm or 478,000 cpm) was incubated with 0.5, 1.0 or 1.5 gm of 5-day-old etiolated sunflower cotyledon tissue for various periods of time. Two millilitres of 0.1 M phosphate buffer, pH 5.0, and water were added to a final volume of 4.2 ml. Basic extraction reagents were usually used at the end of the incubation period to facilitate the determination of unmetabolized propionate-C^{14}. Two

$C^{14}O_2$ time-course experiments were carried out. Several experiments specifically designed to obtain information regarding the reduced recovery of propionate-1-C^{14} from this tissue were also carried out.

TABLE 6. The incorporation of specifically labelled propionate carbons into etiolated sunflower cotyledon constituents. 1 µM of each substrate was metabolized for 48 hours by 1.5 gm tissue.

	% of Added Activity (C.P.M.)		
Fraction	Propionate -1-C^{14}	Propionate -2-C^{14}	Propionate -3-C^{14}
CO_2	77.0	37.6	71.5
Organic acid	2.7	8.4	3.2
Amino acid	2.3	17.6	0.5
Sugar	0.6	1.7	0.9
Lipid	0.1	0.3	0.4
Insoluble residue	1.7	9.2	8.6
Total	84.4	74.8	85.1

Propionic acid was rapidly incorporated into and metabolized by etiolated sunflower cotyledon tissue (Figure 9, Tables 6 and 7). Seventy-five percent to 85% of the added radioactivity could be accounted for after 48 hours (Table 6) in labelled CO_2, organic acid, amino acid, sugar, lipid and insoluble residue components. Seventy-seven percent of the added propionate-1-C^{14} was released as $C^{14}O_2$ while 71.5% and

TABLE 7. The distribution of C^{14} in the various fractions obtained from 1 gram etiolated sunflower cotyledons incubated with propionate-1-, -2- or -3-C^{14} for 4 hours.

Fraction	Propionate-1-C^{14}		Propionate-2-C^{14}		Propionate-3-C^{14}	
	% of Added C^{14}	% of Incorp. C^{14}	% of Added C^{14}	% of Incorp. C^{14}	% of Added C^{14}	% of Incorp. C^{14}
CO_2	17.8	50.1	11.2	12.5	40.5	42.9
Organic acid	7.2	20.1	15.9	17.7	8.7	9.2
Amino acid	5.3	14.9	30.5	34.1	19.9	21.0
Lipid	0.4	1.3	1.7	11.8	0.8	0.8
Sugar	0.5	1.5	15.1	16.8	15.5	16.4
Insoluble residue	2.4	6.8	10.4	11.6	4.9	5.2
Steam distillable	0.4	1.2	0.9	1.0	0.5	0.6
Steam dist. residue	1.5	4.1	4.0	4.4	3.7	3.9
TOTAL	35.5	100.0	89.7	100.0	94.5	100.0

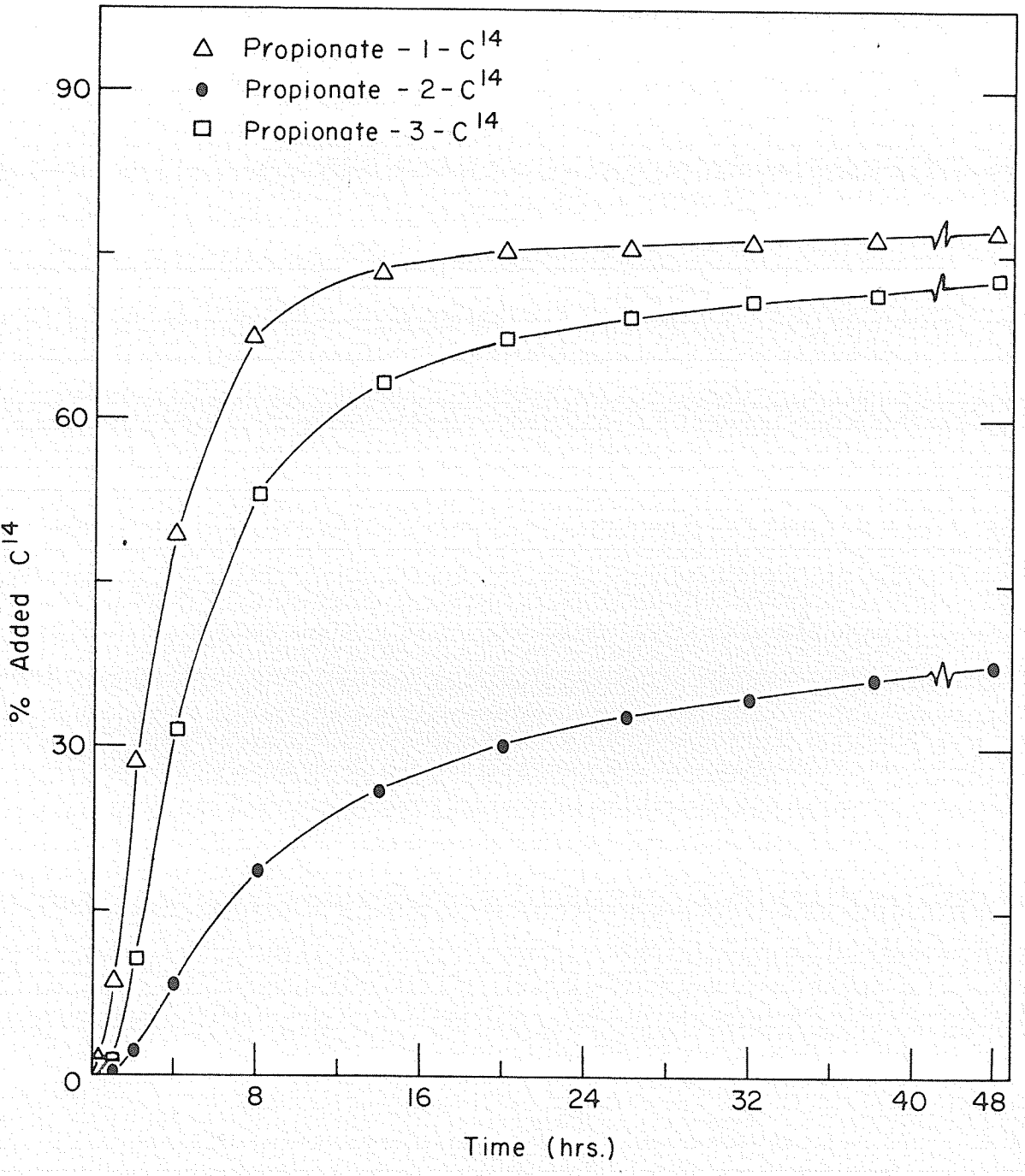

Fig. 9 Progress of $C^{14}O_2$ release from specifically labelled propionate-C^{14} incubated with etiolated sunflower cotyledons.

8.6% of the added propionate-3-C^{14} was incorporated into $C^{14}O_2$ and insoluble components respectively. Only 38% of the added propionate-2-C^{14} was released as $C^{14}O_2$ after 48 hours whereas 17.6%, 9.2% and 8.6% of the added radioactivity accumulated in amino acids, insoluble components and organic acids, respectively. In this experiment carbon 1 of propionate was immediately released as $C^{14}O_2$ while carbons 2 and 3 were evolved as $C^{14}O_2$ after a lag (Figure 9). Carbon 3 of propionate was released as $C^{14}O_2$ before carbon 2 and in addition, was incorporated into $C^{14}O_2$ much more rapidly with time than carbon 2. This pattern is consistent with the view that propionate is oxidized in plants to CO_2 and acetate, carbon 1 appearing as CO_2 first and carbons 2 and 3 appearing in the methyl and carboxyl carbons, respectively, of acetate.

In a short term experiment 90% and 95% of the added propionate-2- and -3-C^{14} were recovered but only 36% of the added propionate-1-C^{14} was recovered (Table 7). Because the added propionate-1-C^{14} was so poorly accounted for after 4 hours, the results are more reasonably viewed in relation to the incorporated radioactivity rather than the added radioactivity. Hence, 50%, 20%, 15% and 7% of the incorporated propionate-1-C^{14} accumulated in CO_2, organic acids, amino acids and insoluble residue after 4 hours. In contrast, 13%, 18%, 34% and 12% of the incorporated propionate-2-C^{14} was

metabolized to CO_2, organic acids, amino acids and insoluble components, respectively. These same fractions accumulated 43%, 9%, 21% and 5% respectively, of incorporated propionate-3-C^{14} after 4 hours. In addition, while only 1.5% of the incorporated propionate-1-C^{14} accumulated in sugars, approximately 17% of the total incorporated C^{14} from propionate-2- and -3-C^{14} was isolated in this fraction. The lipid fraction accumulated an insignificant amount of the incorporated propionate-C^{14}.

The unaccounted for radioactivity in the long-term incubation experiment (Table 6) was probably due to losses from CO_2 or volatile organic compounds. Very little loss could occur due to the volatility of unmetabolized propionate-C^{14} because in a short term experiment (Table 7) the steam distillable fraction, composed entirely of propionate-C^{14}, accounted for less than 1% of the added radioactivity. Since basic tissue extraction conditions were used, this radioactivity probably represents non-metabolized propionate-C^{14} from both the tissue and buffered incubation solution.

The low recovery of propionate-1-C^{14} (Table 7) is largely unexplainable. This occurred only when the base in the centre-well of the incubation flask was removed at the time the tissue was killed. If the base in the centre-well was removed at various time intervals and fresh carbonate free

base added, the recovery of propionate-1-C^{14} after 48 hours was extremely high (Table 6). In addition, using this latter method 50% of the added propionate-1-C^{14} could be recovered in $C^{14}O_2$ after 4 hours (Figure 9). Thus, the problem of propionate-1-C^{14} recovery appears to be due to the loss of $C^{14}O_2$ or some volatile organic compound.

The sugar fraction was separated into its constituents by paper chromatography. Sucrose was the only labelled sugar that was detected by autoradiography of the chromatograms. The glyoxylate cycle has been shown to operate in this tissue, Bradbeer and Stumpf (1959) and sugar can be synthesized from acetate via this cycle coupled with a reversal of glycolysis. If propionate was metabolized to acetate and CO_2 by the Giovanelli and Stumpf pathway, it can be predicted that twice as much label from propionate-2-C^{14} (acetate-2-C^{14}) should be incorporated into sugars than from propionate-3-C^{14} (acetate-1-C^{14}). In these experiments carbons 2 and 3 of propionate were equally incorporated into the sugar fraction. Bradbeer and Stumpf (1959) also reported that carbons 1 and 2 of acetate were almost equally incorporated into sugars, but no explanation was provided for this inconsistency with respect to the operation of a glyoxylate cycle. If propionate were oxidized via methylmalonyl-CoA, carbons 2 and 3 could be equally incorporated into sugar, but they would also have to

be equally incorporated in amino acids, organic acids and CO_2. The results clearly do not support such a pathway as being significant in sunflower cotyledon metabolism of propionate.

The organic acid fractions shown in Table 7 were separated into the acidic compounds shown in Tables 8, 9 and 10 by gradient elution ion exchange chromatography. An accident caused the loss of most of the activity from the ion exchange column fractions containing β-hydroxypropionate and unknown 1 derived from propionate-2-C^{14}. This probably explains the extremely low recovery obtained from that column (Table 9). Part of the reduced recovery of the organic acid fractions may also be due to losses of volatile compounds during prolonged periods of drying in an air stream at 45°C.

β-hydroxypropionate was identified as an intermediate in the metabolism of propionate-1- and -2-C^{14}, but not from propionate-3-C^{14}. Radioactive succinate was the only TCA cycle acid isolated from the metabolism of all 3 propionate carbons in etiolated sunflower cotyledons. Malate-C^{14} and citrate-C^{14} accounted for a large proportion of the radioactivity of the organic acid fraction derived from propionate-3-C^{14} and a small amount of citrate-C^{14} was detected in the fraction that contained unknown 6 from the metabolism of propionate-2-C^{14}. The differences between the amount of propionate-2- and -3-C^{14} incorporated into TCA cycle acids

and β-hydroxypropionate indicates that these two carbons may be metabolized via different pathways.

The majority of the radioactivity recovered from the organic acid fractions was present in unknown compounds (Tables 8, 9 and 10). Unknown 1 in Tables 8 and 9 and unknown 2 in Table 10 co-chromatographed with glycollic acid (Table 11), but did not emerge from the column in the correct position (Figure 5). Unknowns 1, 2a and 6a derived from propionate-1-C^{14}, unknowns 1 and 2 derived from propionate-1-C^{14} and unknowns 1 and 2 derived from propionate-3-C^{14} were present in both quantitative and radioactive amounts on paper chromatograms. When these compounds were polidased, no activity was obtained in the neutral fraction from the formate ion exchange column. It is assumed, therefore, that they are not phosphorylated sugar compounds.

None of the isolated unknowns (Tables 8, 9 and 10) are dicarboxylic acid compounds because they did not yield fluorescent derivatives in the test of Feigl (1946).

Unknown 2 derived from propionate-1- and -2-C^{14} emerged from the ion exchange column in a position which corresponded to malate (Figure 5), but this "peak" did not contain radioactive malate (Table 11). This unknown accumulated a large portion of the total radioactivity in the organic acid fractions, most of which was in unknown 2a (Tables 8 and 9).

TABLE 8. Radioactivity and characterization of acidic compounds isolated from etiolated sunflower cotyledon incubated with propionate-1-C^{14}. (1) refers to butanol:acetic acid:water solvent, (2) to ethanol:ammonia:water solvent and (3) to isoamyl alcohol saturated with 4N formic acid. Asterisks refer to the activity of the unknown relative to the total activity in the fraction. Thus, #=high, ##=medium and ###=low.

Compound	Activity (cpm)	Specific Act. (cpm./μ eq.)		Rf(X-100)in (1)	(2)	(3)	Relative Activity
β-OH propionate	13,970	6,260					
Unknown 1	2,120	1,040		62	55	30	
Succinate	987	807					
Unknown 2	8,400	1,265	a)	36	2	9	#
			b)	-	10	0	###
Unknown 3	3,580	1,750		87	26	84	
Unknown 4	1,110	880		77	-	67	
Unknown 5	715	934		92	-	88	
Unknown 6	5,980	3,340	a)	35	2	0	#
			b)	69	18	57	###
Column effluent	2,559						
Column residue	708						
Total	40,129						
Total added	50,645						
% recovery	79						

TABLE 9. Radioactivity and characterization of acidic compounds isolated from etiolated sunflower cotyledon incubated with propionate-2-C^{14}. (1), (2) and (3) refer to the same solvents as in Table 8. Asterisks refer to activity of the unknown relative to the total activity in the fraction. Thus #=high, ##=medium, ###=low, St=streak.

Compound	Activity (cpm)	Specific Act. (cpm./μ eq.)		Rf(X-100)in (1)	(2)	(3)	Relative Activity
β-OH propionate	6,710	5,250	a)				#
Unknown 1			b)	56	48	25	###
Succinate	3,120	2,445					
Unknown 2	11,300	11,070	a)	44	6	12	#
			b)	88	18	70	###
Unknown 3	2,020	1,730		89	21	80	
Unknown 4	742	968		10	0	0	
Unknown 5	2,265	2,220	a)	71	7	52	###
			b)		0	0	#
Unknown 6	2,700	621	a)	31	0	0	#
Citrate			b)	25	4	0	###
Unknown 7	1,400	1,095	a)	33	0	0	#
			b)	13	3	St	###
			c)	0		St	###
Column effluent	361						
Column residue	1,325						
Total	31,943						
Total added	103,000						
% Recovery	31						

TABLE 10. Radioactivity and characterization of acidic compounds isolated from etiolated sunflower cotyledons incubated with propionate-3-C^{14}. (1), (2) and (3) refer to the same solvents as in Table 8. Asterisks refer to the activity of the unknown relative to the total activity in the fraction. Thus #=high, ##=medium, and ###=low.

Compound	Activity (cpm)	Specific Act. (cpm./μ Eq.)		Rf(X-100)in (1)	(2)	(3)	Relative Activity
Unknown 1	3,370	89		-	55	76	
Unknown 2	5,420	1,930		60	48	30	
Succinate	1,578	685					
Malate	4,045	480	a)				#
			b)		8	0	###
Unknown 3	3,635	356	a)	-	-	70	##
Citrate			b)				#
Column effluent	344						
Column residue	1,665						
Total	20,057						
Total added	30,768						
% Recovery	65						

This compound was not detected as an intermediate in the metabolism of propionate-3-C^{14}.

TABLE 11. Characterization of known organic acids via descending paper chromatography. (1), (2) and (3) refer to the same solvents as in Table 8.

Organic Acid	Rf(X100) in (1)	(2)	(3)
Malic	54	13	22
Tartaric	40	10	10
Malonic	62	23	48
Citric	45	2	18
Glycollic	62	55	42
Glyoxylic	60	50	-

Unknown 6a in Table 8 emerged from the column in the malonate and citrate region (Figure 5), but it did not co-chromatograph with these compounds (Table 11).

Unknowns 3, 4 and 5 in Tables 8 and 9 emerged from the anion exchange column in the fractions between malic and citric acid (Figure 5). They did not co-chromatograph with malate, citrate, malonate or tartarate (Table 11). Unknowns 3, 4 and 5 derived from propionate-1-C^{14} had very high Rf values in acidic solvents. Unknown 3 derived from propionate-2-C^{14} appears to be the same compound as unknown 3 obtained from propionate-1-C^{14}. However, unknowns 4 and 5 obtained from propionate-2-C^{14} are

composed of different acidic compounds compared to those isolated from propionate-1-C^{14} metabolism.

It is obvious that carbons 1, 2 and 3 of propionate accumulated in at least one similar unidentified compound (unknown 1 for carbons 1 and 2 and unknown 2 for carbon 3) and carbons 1 and 2 of propionate accumulated in at least 2 more unknowns (unknowns 2a and 6a).

The amino acid fractions derived from propionate-1-, -2- and -3-C^{14} were resolved into 4 fractions (Table 12). Fraction 2 represents the water washings of the Dowex X10 (acetate) column. Fraction 1, glutamic acid and aspartic acid were eluted from the column with 0.1 N acetic acid and the pattern with which they were eluted is shown in Figure 10.

TABLE 12. Radioactivity of various fractions obtained from ion exchange chromatography of the amino acid fraction isolated from etiolated sunflower cotyledons incubated with propionate-1-, -2- or -3-C^{14}

Fraction	Activity (C.P.M.)		
	-1-C^{14}	-2-C^{14}	-3-C^{14}
Fraction 1	5,760	103,200	13,200
Glutamic	2,240	11,550	6,900
Aspartic	615	2,935	1,900
Fraction 2	28,600	70,489	46,618
Column residue	785	3,480	-
Total	38,000	191,654	68,618
Total added	39,200	200,000	70,130
Percent recovery	97.0	95.7	97.2

The recovery of amino acids was quantitative (Table 12)

By paper chromatography it was shown that fraction 1 and 2 contained labelled serine, β-alanine, alanine, tryptophan and glutamine. Tryptophan-C^{14} was not produced from propionate-1-C^{14}. Serine contained most of the radioactivity in the amino acid fraction. β-alanine also contained a considerable amount of radioactivity from all 3 propionate carbons. Carbons 2 and 3 of propionate were incorporated into alanine to a greater extent than was carbon 1.

The results that were obtained for propionate metabolism in sunflower cotyledons are not readily amenable to a clear interpretation.

The pattern of $C^{14}O_2$ release from the variously labelled propionates was entirely in agreement with the metabolism of propionate to CO_2 and acetate via the Giovanelli and Stumpf (1958) pathway. The isolation of C^{14} labelled β-hydroxypropionate from propionate-1- and -2-C^{14} metabolism and the isolation of labelled β-alanine are also in agreement with the operation of this pathway. C^{14} labelled β-alanine was formed from propionate-1-, -2- or -3-C^{14}, probably via the transamination of malonic semialdehyde, Vagelos *et al* (1959c). The high specific activity of the isolated β-hydroxypropionate also indicated that it was one of the first detectable organic acid intermediates in propionate metabolism.

However, the incorporation of carbons 2 and 3 of

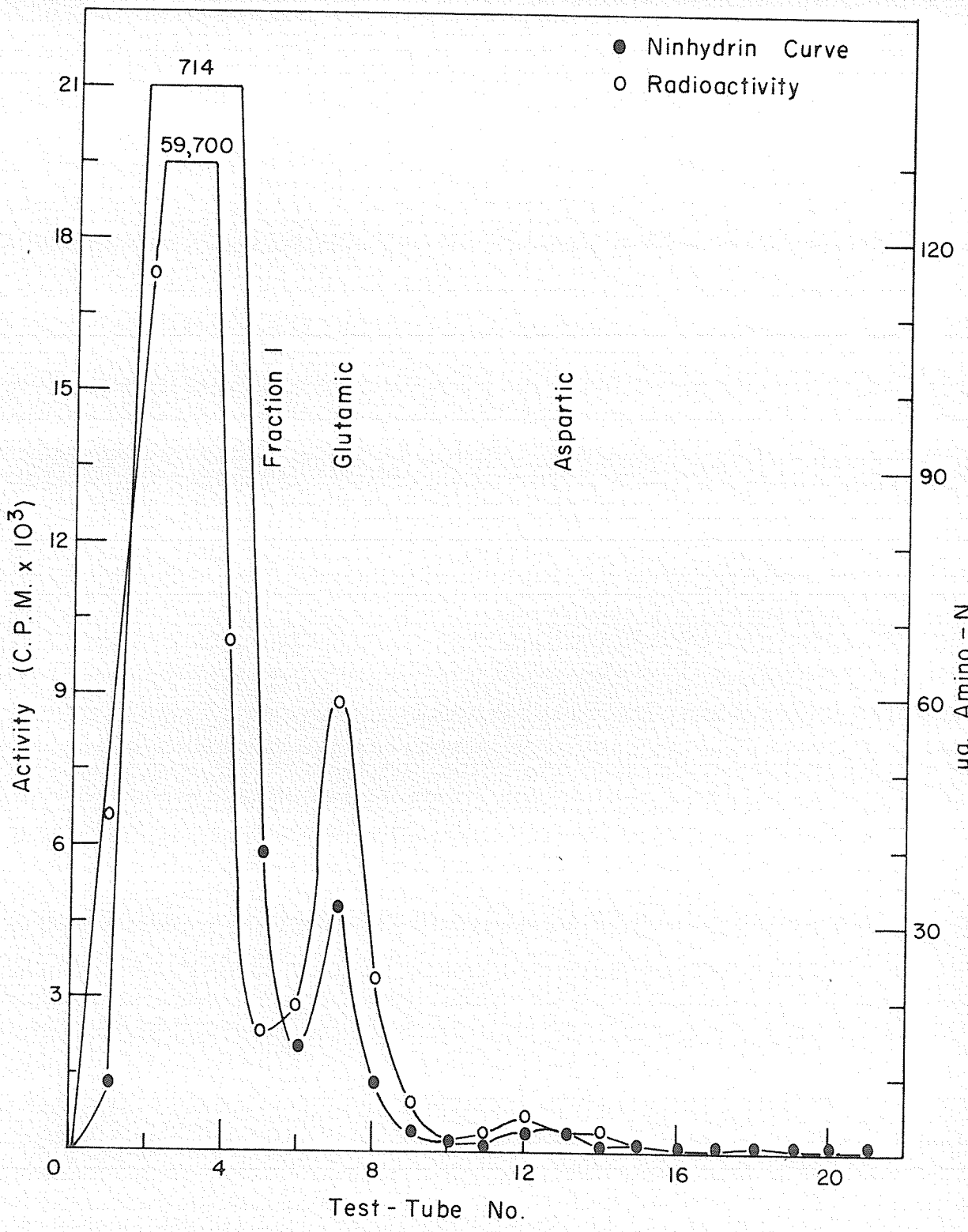

Fig. 10 Separation of amino acid fraction derived from propionate - 2 - C^{14} metabolism in etiolated sunflower cotyledons.

propionate, i.e. the acetate portion, into a variety of unknown organic acid components (Tables 8, 9 and 10) and amino acids was not at all consistent with the known metabolism of acetate in this tissue. Acetate was primarily converted to sugar by this tissue and malate and citrate were the most radioactive organic acids detected, Bradbeer and Stumpf (1959). The amino acids only accumulated small amounts of acetate-C^{14} and glutamate and aspartate were the most radioactive amino acids.

In contrast to the results obtained with acetate-C^{14}, the major portion of the C^{14} in the ethanol soluble fraction derived from propionate-2- or -3-C^{14} was found in the amino acid and organic acid fractions instead of the sugars. The amino acids that contained the most radioactivity were serine, alanine and tryptophan with very little C^{14} in glutamate and aspartate. The C^{14} of the organic acid fraction was largely found in a variety of unknown acidic compounds (Tables 8, 9 and 10) and, except for propionate-3-C^{14}, very little radioactivity accumulated in TCA cycle acids. In addition, carbons 2 and 3 of propionate were equally incorporated into sugars, a result not in agreement with acetate metabolism via the glyoxylate cycle. These facts suggest that propionate was, at least in part, metabolized by reactions not envisaged by the pathway of Giovanelli and Stumpf (1958).

Propionate Metabolism In Germinating Castor Bean Endosperm

Propionate at concentrations of 1.25×10^{-4} M or greater inhibited the respiration of germinating castor bean endosperm slices. Thirty-five to 50 percent inhibition of respiration was obtained at concentrations of 1.25×10^{-3} M or greater. Ten to 30 percent inhibition of respiration was obrained with no change in R.Q. at propionate concentrations in the range $1.25 - 6.25 \times 10^{-4}$ M. This degree of inhibition could always be obtained when the tissues were rapidly respiring. Because of this behaviour C^{14} labelled propionate was supplied at concentrations of 3×10^{-5} M and it was assumed that this concentration did not affect tissue respiration.

The Kinetics of Incorporation

One gram of 5-day-old germinating castor bean endosperm slices was incubated for various lengths of time with propionate-1-, -2- or -3-C^{14}. The incubation mixture consisted of 2 ml 0.1 M phosphate buffer, pH 5.0, 1 µM of propionate-1-C^{14} (472,000 cpm), propionate-2-C^{14} (506,000 cpm) or propionate-3-C^{14} (478,000 cpm) and water to a total volume of 4.2 ml.

The various incubation times reported constituted one

experiment. The carbon dioxide time course results constituted another experiment. However, the pattern with which CO_2 was released from the shorter time interval experiment was exactly the same as that from the time course experiment to be discussed. The remarkable agreement in the proportional amount of propionate incorporated into CO_2 in the two separate experiments is indicative of the consistency of propionate utilization by the germinating castor bean endosperm.

After 16 hours, 53% of propionate-1-C^{14}, 29% of propionate-3-C^{14} and 9% of propionate-2-C^{14} were released as $C^{14}O_2$ (Figure 11). Carbon 1 of propionate was released first into CO_2, and was followed by carbons 3 and 2, respectively. This pattern of $C^{14}O_2$ release was consistent with the view that carbon 1 was initially released as $C^{14}O_2$ and the remaining carbons were metabolized as acetate.

Propionate was rapidly incorporated into stable water soluble components by castor bean endosperm tissue (Figure 12). This incorporation was more rapid for propionate 2 than for propionate 1 during the initial 15 minutes and the last 90 minutes of incubation. However, there was an apparent lag in the incorporation of propionate 2 into water soluble components during the 15-30 minute time interval. It is possible that this apparent lag may have been due to a non-typical

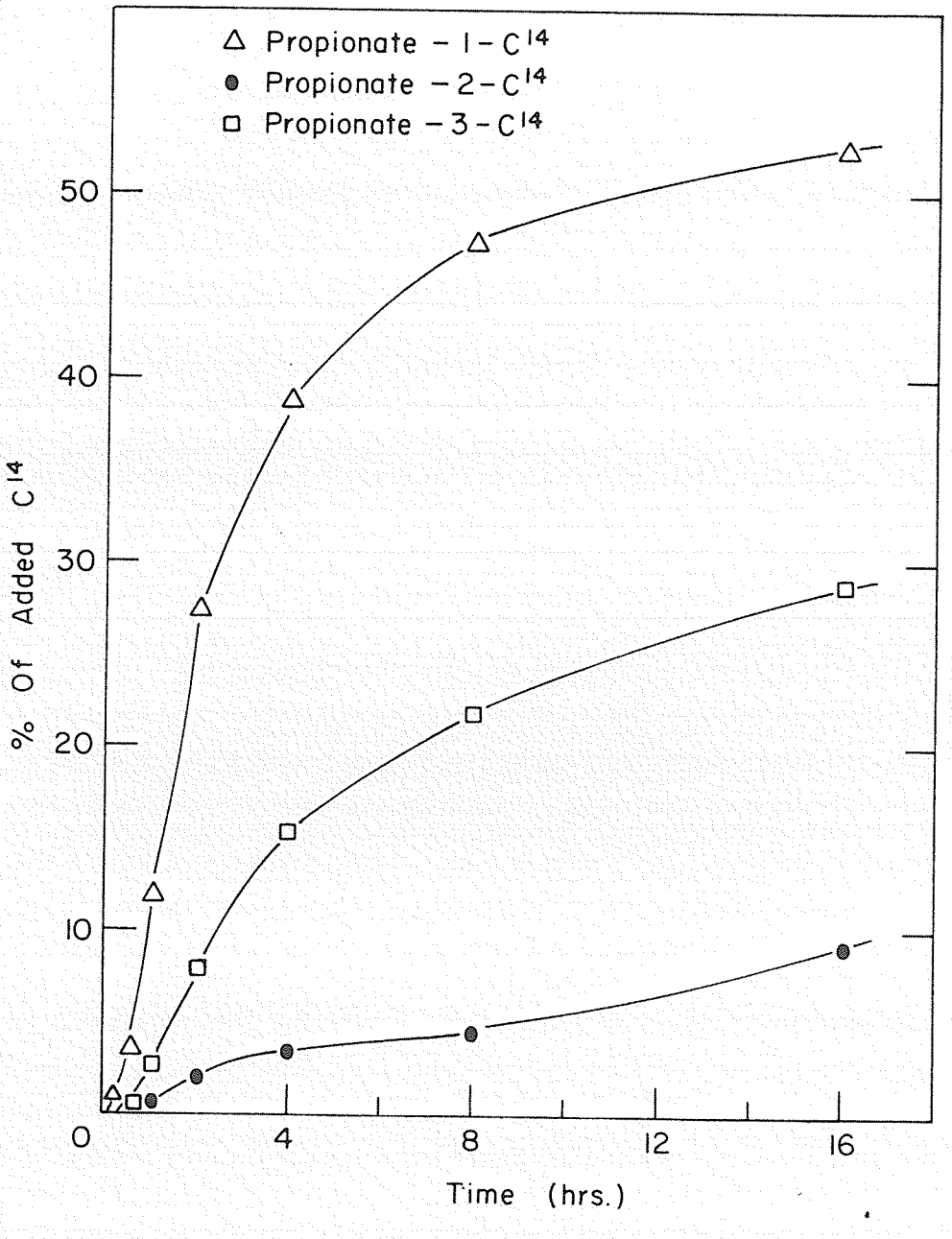

Fig. II The release of $C^{14}O_2$ from specifically labelled propionate – C^{14} incubated with germinating castor bean endosperm. One gram tissue was incubated with 1 μM propionate in 2 ml. 0.1 M (pH 5.0) phosphate buffer.

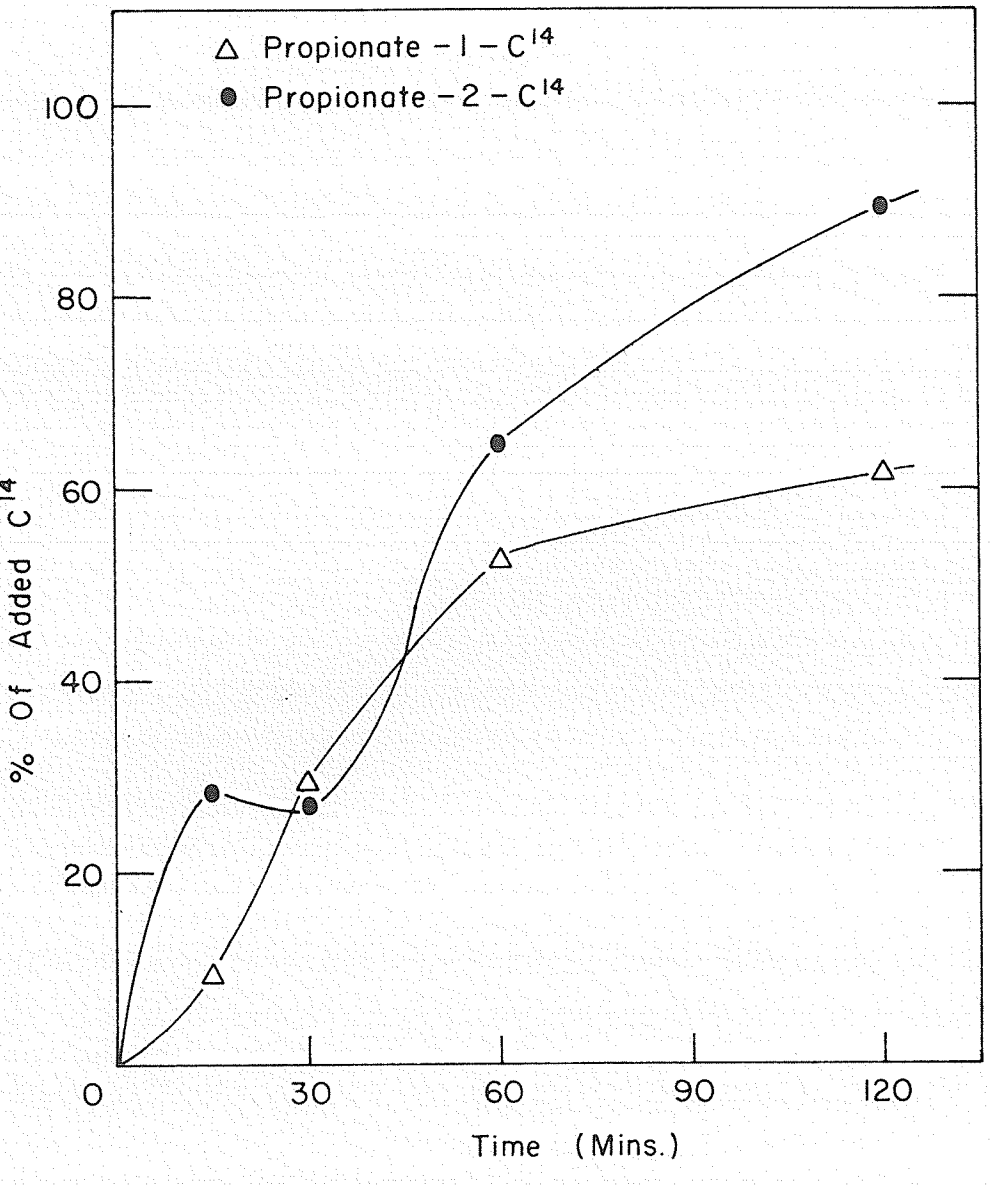

Fig. 12 Incorporation of propionate – 1 – and – 2 – C^{14} into water soluble constituents of germinating castor bean endosperm.

sample of plant material. At the end of 2 hours, both propionate-1- and -2-C^{14} had been completely metabolized (Figures 13 and 14) and 62% and 90% of carbons 1 and 2, respectively, of propionate was in water soluble components.

Expressing the radioactivity present in the various fractions of endosperm material as a percentage of the total added counts (Figures 13 and 14), the amino acid and organic acid fractions derived from propionate-1-C^{14} initially contained the greatest percentage of the added radioactivity (Figure 13). The amount of C^{14} in the organic acid fraction increased rapidly up to 1 hour and levelled off to reach 66% of the added radioactivity after 2 hours. The amount of C^{14} in the amino acids increased slowly to 8% of the added C^{14} after 2 hours. CO_2 initially contained very little radioactivity, but rapidly accumulated C^{14} after a short lag period so that after 2 hours it contained 21% of the added C^{14}. Sugars accumulated label very slowly during the 2 hour incubation period. The radioactivity in the lipid fraction increased to a maximum of 4.9% of that added after 1 hour and then decreased to less than 1% of the added radioactivity at the 2 hour time interval. Insoluble residue components accumulated a negligible amount of radioactivity from propionate-1-C^{14} metabolism after 2 hours. Thus, propionate-1-C^{14} was incorporated first into organic acids or amino acids and then was metabolized to CO_2.

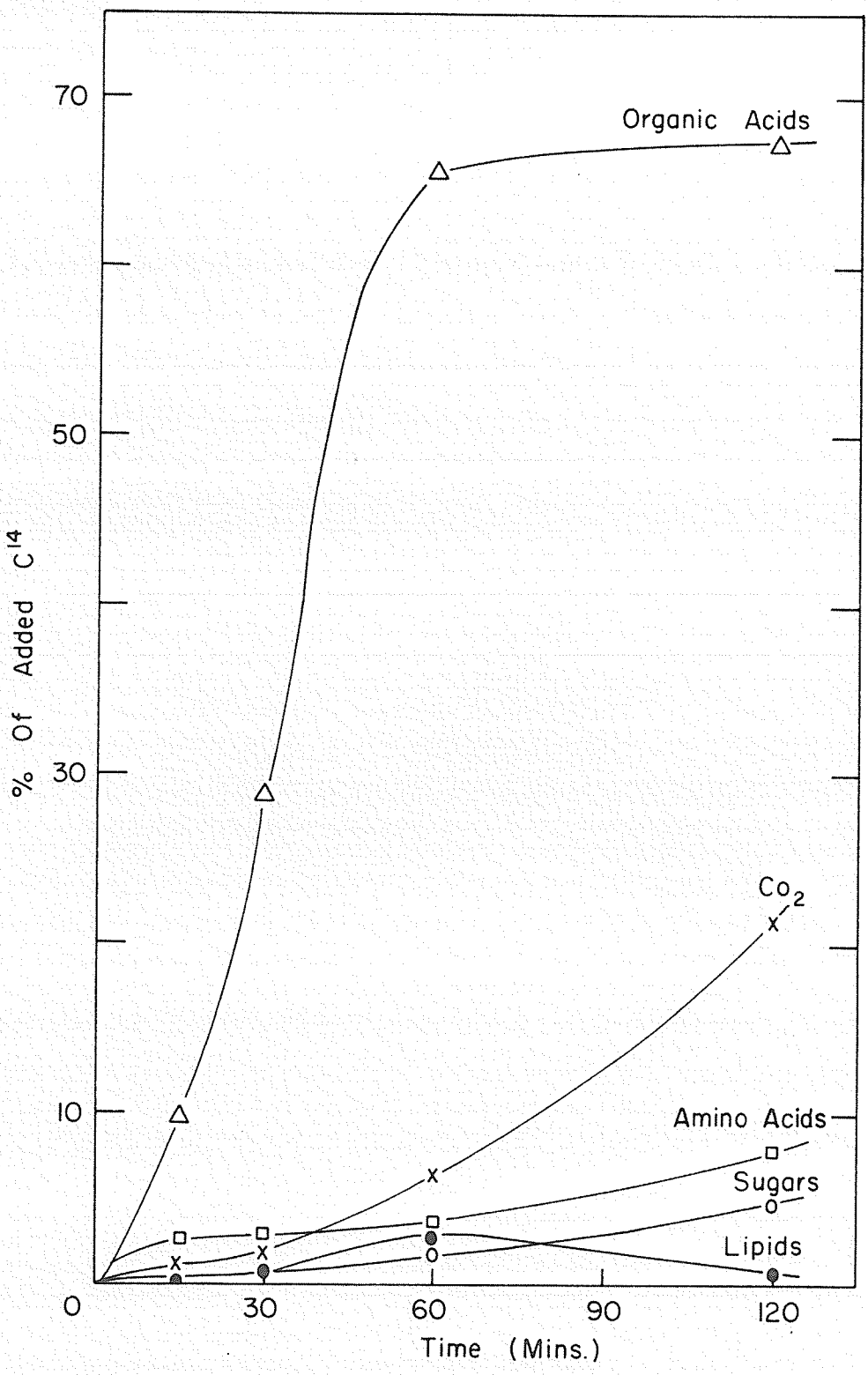

Fig. 13 Progress of C^{14} incorporation from propionate – 1 – C^{14} into the components of germinating castor bean endosperm.

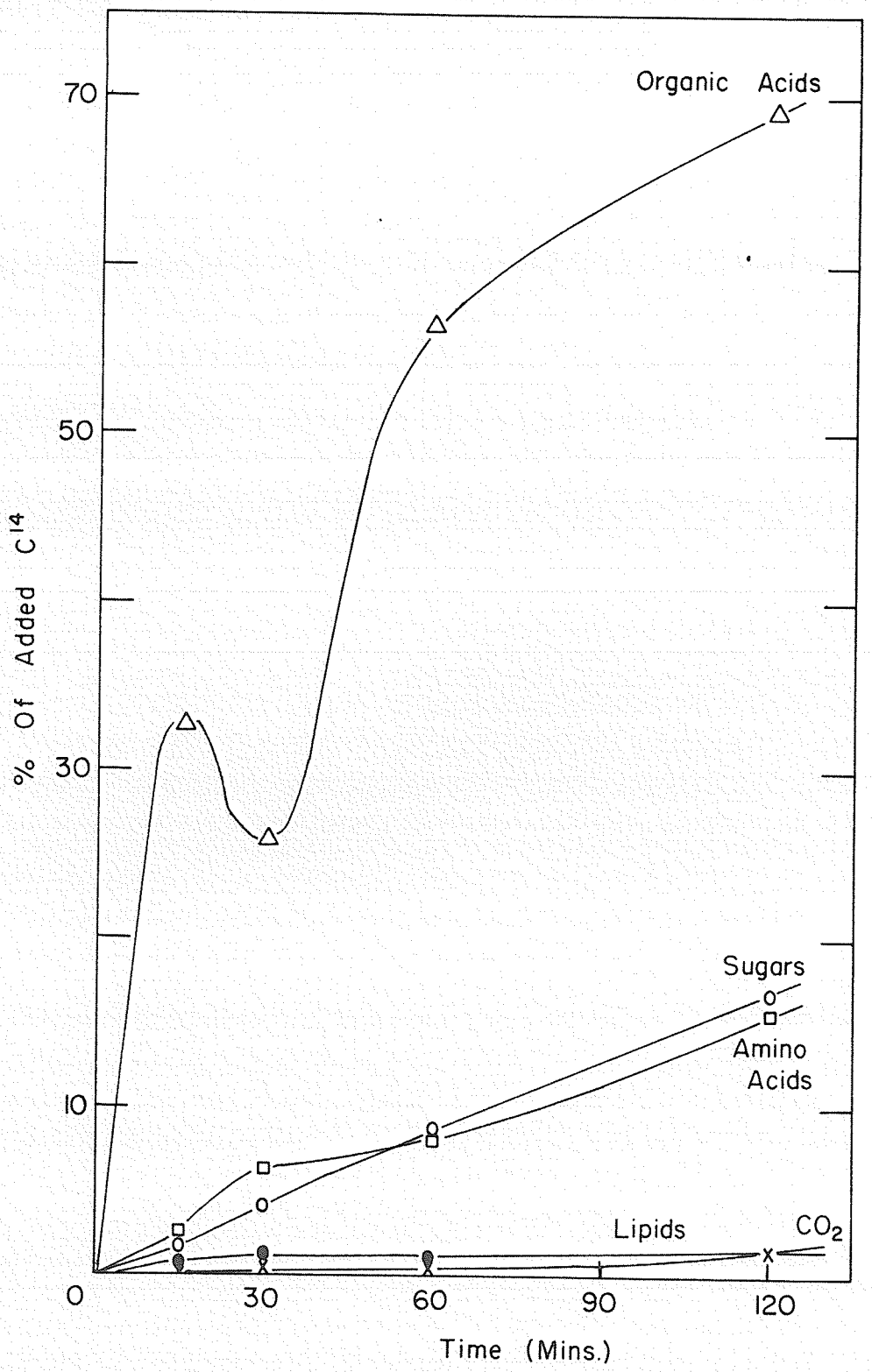

Fig. 14 Progress of C^{14} incorporation from propionate-2-C^{14} into the components of germinating castor bean endosperm.

Propionate-2-C^{14} was incorporated initially into organic acids and then was metabolized to amino acids and sugar (Figure 14). The incorporation into organic acids was halted briefly, but continued on to 69% of the added radioactivity in 2 hours. Amino acids accumulated carbon 2 of propionate fairly rapidly at first, but this incorporation soon became linear and reached 14% of the added C^{14} after 2 hours. The incorporation of propionate-2-C^{14} into sugars was linear and after 2 hours this fraction contained 16% of the added radioactivity. Very little of the added radioactivity from carbon 2 of propionate was incorporated into CO_2, insoluble residue or lipids after 2 hours.

The initial relationship between the organic acids and amino acids derived from propionate-1-C^{14} is more clearly shown in Figure 15. Here the radioactivity in each fraction is plotted as a percentage of the total radioactivity incorporated into the tissue. While the amino acids contain only 18% of the incorporated activity in 15 minutes, the organic acids contain 68% of the incorporated activity. More radioactivity was incorporated into the organic acid fraction up until 30 minutes after which the incorporated radioactivity slowly decreased to 66% after 2 hours. The amount of incorporated C^{14} in sugars increased from 3% initially to 5% after 2 hours. Six percent of the incorporated activity accumulated

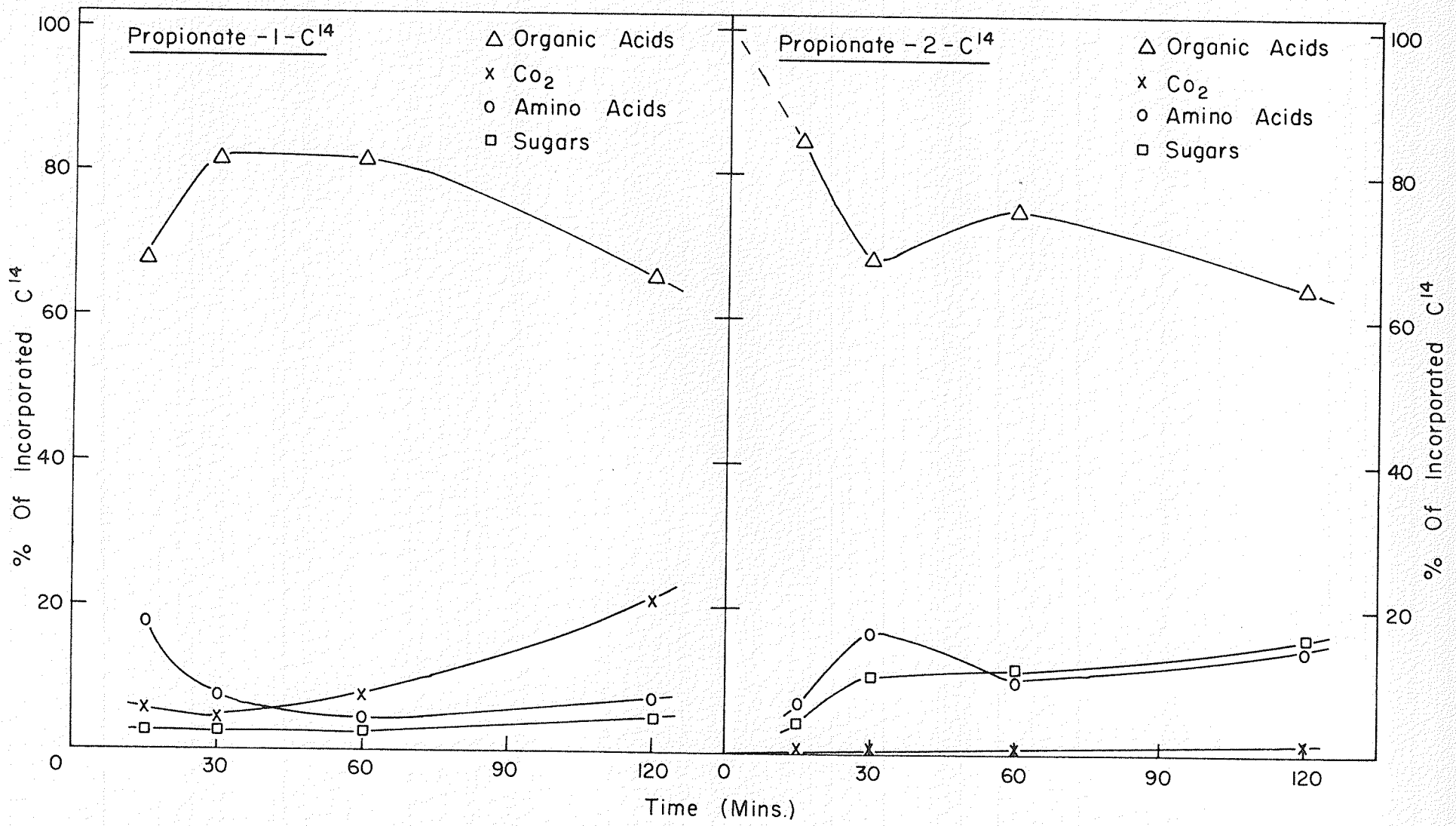

Fig. 15 The time course distribution of C^{14} among the various fractions obtained from germinating castor bean endosperm slices incubated with propionate $-1-$ or $-2-C^{14}$.

initially in CO_2 and after 2 hours, this fraction contained 21% of the incorporated radioactivity. Clearly, as the percentage of incorporated C^{14} into organic acids decreased there was a corresponding increase in the percentage of propionate-1-C^{14} incorporated into $C^{14}O_2$.

The organic acids derived from propionate-2-C^{14} contained 85% of the incorporated C^{14} after 15 minutes and this curve readily extrapolated to 100% at zero time (Figure 15). Thus, the organic acids were the initial recipients of propionate-2-C^{14}. The percent incorporated C^{14} in this fraction decreased, except for a 30 minute period, then to a final value of 64% after 2 hours. After 15 minutes the percent of incorporated propionate-2-C^{14} was very low in amino acids and sugars. These fractions rapidly incorporated C^{14} during the 15-30 minute time interval indicating that the organic acids were probably the precursors of both sugars and amino acids derived from propionate-2-C^{14}. An increased percentage of incorporated C^{14} continued to accumulate in sugars with time. The proportion of C^{14} in amino acids decreased slightly during the 30-60 minute time interval and then slowly increased to 14% after 2 hours. Less than 2% of the incorporated propionate-2-C^{14} accumulated in $C^{14}O_2$ after 2 hours.

It must be noted that a considerable quantity of C^{14} from propionate-1- and -2-C^{14} was still in the organic acid fraction

after 2 hours (Figure 15). Since both carbons 1 and 2 of propionate were initially incorporated into organic acids it was of considerable importance to determine the specific compounds that were labelled, the organic acid fractions derived from propionate-1- and -2-C^{14} were then separated into their constituents by gradient elution from an anion exchange resin. The radioactivity and number of compounds present in the various acidic compounds is shown in Tables 13 and 14. The headings referred to as "No. Cmpds." indicate how many radioactive components were contained within the various "peak" fractions obtained by elution from the anion exchange column. For example, after 60 minutes unknown 3 in Table 14 was composed of 9 distinct radioactive compounds.

The recovery of radioactivity from the columns varied from 41-83%. This degree of recovery was not strictly quantitative. However, with time the unknown acids constitute a greater portion of the total radioactivity incorporated into these fractions while at the same time the percentage recovery decreases. These two facts suggest that losses may be primarily due to oxidation or volatilization of these compounds during the removal of the formate eluant.

β-hydroxypropionate accumulated the major portion of the C^{14} derived from carbon 1 of propionate (Table 13).

TABLE 13. The total radioactivity and characterization of various acidic compounds isolated from germinating castor bean endosperm incubated with propionate-1-C^{14}.

Compound	Time of Incubation (Mins.) 15 Activity (C.P.M.)	15 No Cmpds.	60 Activity (C.P.M.)	60 No Cmpds	120 Activity (C.P.M.)	120 No Cmpds		Rf (1)*	Rf (2)**
β-hydroxy-propionate	11,750	2	124,300	3	171,700	2			
Unknown 1	809	1	5,220	1	15,900	2			
Unknown 2	14,810	2	28,050	2	15,420	2	(a)	64	32
							(b)	85	38
Unknown 3	3,344	2	37,790	2	14,170	2	(a)	64	25
							(b)	71	51
Unknown 4	1,832	1	1,890	2	-	-			
Unknown 5	2,364	2	7,700	1	4,365	1			
Unknown 6	-	-	1,810	1	-	-			
Column effluent	30		979		3,480				
Column residue	-		1,363		1,189				
Total	34,999		202,092		226,224				
Total added	42,000		285,000		292,000				
% Recovery	83.3		70.3		77.5				

* N-butanol: HA_c: H_2O, 12/3/5
** Ethanol: NH_3H_2O, 16/1/13

TABLE 14. The total radioactivity in various acidic compounds isolated from germinating castor bean endosperm incubated with propionate-2-C^{14}

Compound	Time of Incubation (Mins.) 15 Activity (C.P.M.)	15 No Cmpds	30 Activity (C.P.M.)	30 No Cmpds	60 Activity (C.P.M.)	60 No Cmpds	120 Activity (C.P.M.)	120 No Cmpds
β-hydroxy-propionate	42,500	2	64,400	1	18,560	3	19,780	3
Unknown 1	-	-	-	-	2,190	3	2,780	3
Unknown 2	4,280	2	1,433	2	31,930	3	45,600	3
Unknown 3	10,170	4	5,690	3	40,050	9	50,100	9
Unknown 4	-	-	2,265	1	-	-	-	-
Unknown 5	-	-	1,878	1	3,935	3	-	-
Unknown 6	7,650	2	3,620	1	7,795	1	7,900	1
Unknown 7	-	-	-	-	5,055	2	-	-
Column effluent	500		416		1,638		1,859	
Column residue	350		195		1,160		1,547	
Total	115,450		79,897		113,113		129,566	
Total added	152,000		120,000		262,000		320,000	
% Recovery	76.0		66.5		43.2		40.5	

Unknown 2 contained more radioactivity than any other acidic compound initially, but accumulated very little radioactivity with time. Malate-C^{14} constituted a very small portion of the radioactivity incorporated into unknown 3 at the 2 hour time interval. This was the only TCA cycle acid isolated from propionate-1-C^{14} metabolism in the germinating castor bean endosperm.

Unknown 1 accumulated C^{14} rapidly with time. Unknown 4, while being present initially, did not accumulate radioactivity and disappeared with time. Unknown 6 was isolated only at the 60 minute time interval and contained a small amount of activity. Unknown 5, present initially in the acidic fraction, accumulated radioactivity up until 60 minutes and then decreased in activity.

The major acidic compound identified from propionate-2-C^{14} oxidation was β-hydroxypropionate (Table 14). This intermediate in propionate metabolism contained much more radioactivity initially than any other compound, but decreased rapidly in activity with time. Unknowns 1, 4, 5 and 7 appeared in the organic acid fraction only after a lag in time, indicating that they are not likely immediate acidic intermediates produced during propionate oxidation. Unknown 6 contained as much radioactivity after 2 hours as it had accumulated during the first 15 minutes of incubation. Unknowns 2 and 3 contained a small amount of propionate-2-C^{14} initially and

continued to accumulate radioactivity after 2 hours. Succinate-C^{14} appeared in unknown 2 after 1 hour, but comprised only a small portion of the radioactivity in this compound. Labelled malate constituted a very small portion of the radioactivity in unknown 3.

Unknown 1 (Tables 13 and 14) emerged from the column in a position which corresponded to glycollic acid (Figure 5) but did not co-chromatograph with glycollic or glyceric acid. While unknowns 2 and 3 were eluted from the column in fractions corresponding to succinate and malate, respectively, the major portion of the radioactivity in these compounds was present in unknown acids. Unknown 4 was not malate or tartarate. Unknowns 5 and 6 emerged from the column in positions which suggested that they might contain isocitrate, citrate or malonate. However, they failed to co-chromatograph with these known acids. Unknown 7 did not co-chromatograph with fumaric acid.

The large number of unknown organic acids isolated from germinating castor bean endosperm incubated with propionate-1- and -2-C^{14} has been a characteristic feature of propionate metabolism not only in this tissue, but also in the etiolated sunflower cotyledon. Most of them do not accumulate much radioactivity individually, but unknowns 1, 2 and 3 (Tables 13 and 14), do contain a considerable portion of the C^{14} in the

organic acid fraction. All attempts to identify these unknown compounds have failed completely.

The relationships between some of the organic acid compounds can be more clearly visualized when their activity is plotted as a percentage of the total activity in the organic acid fraction (Figure 16).

β-hydroxypropionate derived from propionate-1-C^{14} initially contained 28% of the C^{14} incorporated into organic acids and over the 2 hour incubation period accumulated 58%, almost in a linear fashion. Unknown 2 initially contained 35% of incorporated C^{14} in organic acids, but declined to steady state conditions with time. It seems probable that unknown 2 may be a precursor of β-hydroxypropionate derived from propionate-1-C^{14} in this tissue. It is also possible that propionate-1-C^{14} could be initially incorporated into both of these organic acid compounds via two separate pathways.

β-hydroxypropionate derived from propionate-2-C^{14} initially contained 61% of the label in organic acids. Unknowns 2 and 3 contained 3% and 5%, respectively, after 15 minutes. The percentage incorporated C^{14} decreased initially and then accumulated in these unknowns until steady state conditions were reached at 12-16% after 1 hour. β-hydroxypropionate reached a steady state at 6% of the incorporated

C^{14} in organic acids after 2 hours. Thus, propionate-2-C^{14}, in contrast to propionate-1-C^{14}, was largely incorporated initially into β-hydroxypropionate which was metabolized rapidly in this tissue. Whether propionate-2-C^{14} was incorporated into unknowns 2 and 3 by a pathway separate from that incorporated C^{14} into β-hydroxypropionate or was incorporated into these unknowns from β-hydroxypropionate is not clear. The time at which unknowns 1, 4, 5 and 7 appear as C^{14} labelled compounds from propionate-2-C^{14} metabolism (Table 14) corresponds to the rapid disappearance of β-hydroxypropionate-2-C^{14} (Figure 16) and suggests that these unknowns are produced from the metabolism of β-hydroxypropionate-2-C^{14}.

It should be emphasized that in these experiments complete recovery of the C^{14} from propionate-1- and -2-C^{14} was initially achieved. The pattern with which each propionate carbon was released as $C^{14}O_2$ and the incorporation of carbon 2 of propionate into sugars was consistent with the operation of the modified β-oxidation pathway for propionate metabolism proposed by Giovanelli and Stumpf (1958). Carbon 1 was primarily converted to $C^{14}O_2$ and not incorporated into sugars. If carbon 2 was converted to acetate it could be metabolized to sugar via the well established glyoxylate cycle coupled with a reversal of glycolysis, Beevers (1957), Canvin and Beevers (1961).

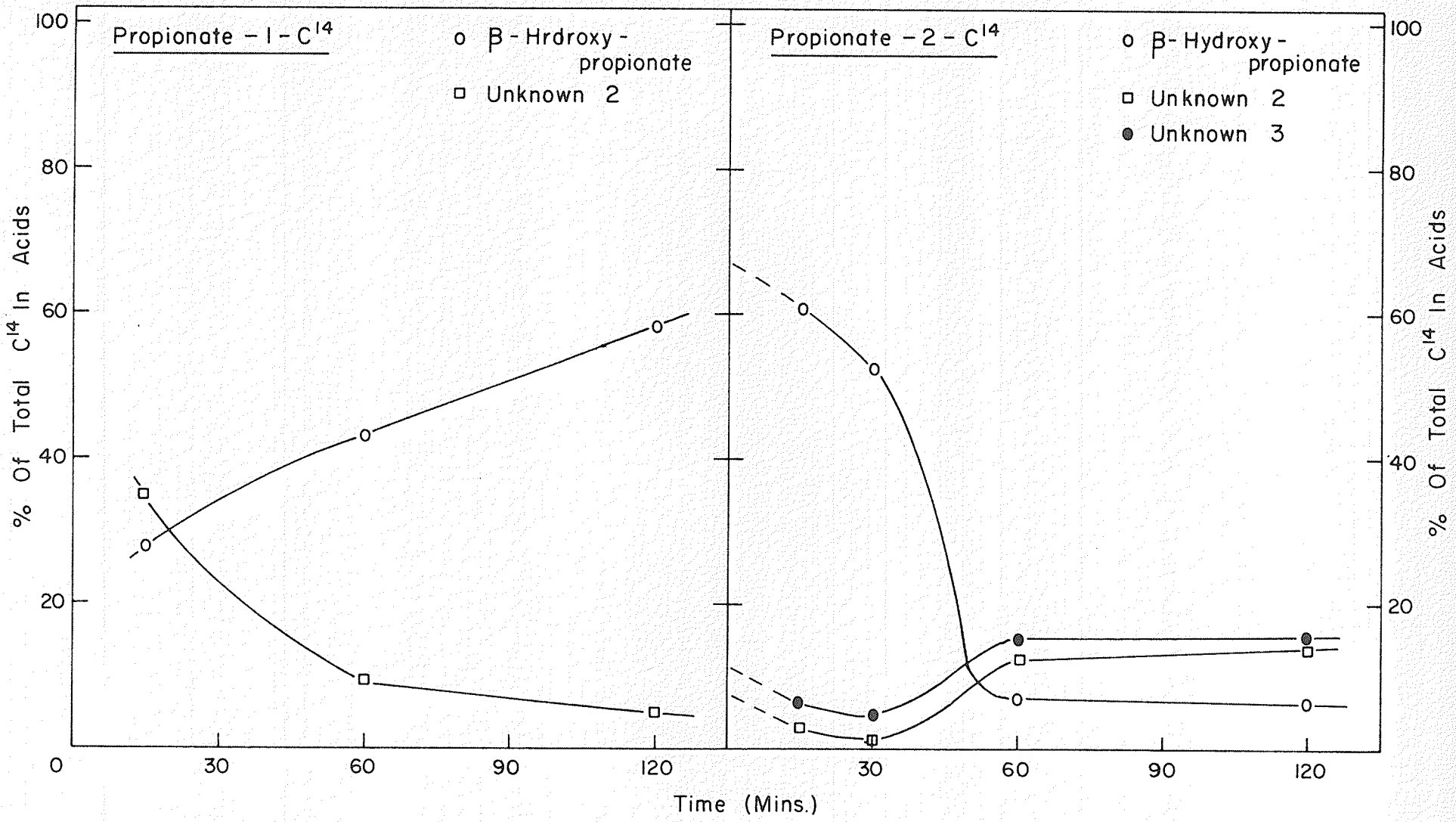

Fig. 16 Changes in the distribution of C^{14} among certain constituents of the organic acid fraction derived from propionate – 1 – and – 2 – C^{14}.

However, the unequal pattern with which propionate-1- and -2-C^{14} were incorporated into B-hydroxypropionate, the presence of a large number of radioactive acidic unknowns and the absence of significant amounts of C^{14} in TCA cycle acids suggests that a sizeable portion of the propionate was not metabolized by the above pathway. The alternate reactions by which propionate was metabolized could not be characterized and their elucidation will require the identification of some of the unknown radioactive acidic compounds.

SUMMARY AND CONCLUSIONS

The experiments described in the preceding sections were designed to determine if propionate metabolism could be wholly accounted for by the Giovanelli and Stumpf pathway in higher plant tissues which might produce propionate or if any evidence could be obtained for alternate pathways of metabolism. To evaluate these possibilities propionate-1-, -2-, or -3-C^{14} was supplied to 7-day-old pea cotyledons, 5-day-old etiolated sunflower cotyledons and 5-day-old germinating castor bean endosperm slices. These tissues are catabolizing large fat or protein reserves, and based on the mechanism of fat or amino acid breakdown in other tissues, it appeared likely that propionate could be produced in these tissues.

When propionate was supplied to these three tissues, 85 - 100% of the added radioactivity was metabolized to other products in 2-3 hours. Thus, these tissues did, in fact, possess an efficient mechanism for the utilization of propionic acid.

In all 3 tissues, the kinetics of $C^{14}O_2$ release established that carbon 1 of propionate was released first as $C^{14}O_2$ and carbons 2 and 3 were released in a manner consistent with their metabolism as the methyl and carboxyl carbons of acetate. C^{14} labelled β-hydroxypropionate was isolated from all 3 tissues and radioactive β-alanine was isolated from sunflower tissue. The isolation of these intermediates and the pattern of $C^{14}O_2$

release argue in favour of propionate metabolism to β-hydroxy-propionate and malonic semialdehyde and thence to CO_2 and acetate as envisaged by Giovanelli and Stumpf.

In the pea tissue, C^{14} from propionate-2- and -3-C^{14} accumulated mainly in organic and amino acids. The TCA cycle acids, citrate, malate and succinate contained a sizeable portion of the C^{14} in the organic acid fraction isolated from tissue supplied with propionate-2-C^{14}. This is entirely consistent with the metabolism of carbon 2 of propionate as an acetate molecule.

In sunflower tissue, the major portion of the C^{14} in the ethanol soluble fraction derived from propionate-2- or -3-C^{14} was found in the amino acid and organic acid fraction. Serine, alanine and tryptophan accumulated most of the radioactivity in the amino acid fraction while a small amount of C^{14} was found in glutamate, aspartate and glutamine. Unknown acidic compounds incorporated most of the radioactivity in the organic acid fraction and, except for propionate-3-C^{14}, very little label accumulated in TCA cycle acids. In addition, carbons 2 and 3 of propionate were equally incorporated into sugars, a result which clearly disagrees with acetate metabolism via the well established glyoxylate cycle in this tissue. Thus, at least a portion of carbons 2 and 3 of propionate was metabolized by reactions not consistent with their conversion to

acetate.

In the castor bean endosperm tissue, propionate-1-C^{14} accumulated in β-hydroxypropionate over a two hour time interval. However, considerable C^{14} from propionate-2-C^{14} was initially incorporated into β-hydroxypropionate, but this compound decreased rapidly in radioactivity subsequently. A large number of acidic unknowns were also found in the organic acid fractions derived from propionate-1- or -2-C^{14} while TCA cycle acids contained very little C^{14}. Although some radioactive sugar was found from propionate-2-C^{14}, the majority of this carbon was present in amino and organic acids. This pattern is not consistent with the conversion of propionate--2-C^{14} to acetate and suggests that a sizeable portion of the added propionate was metabolized by reactions other than those found by Giovanelli and Stumpf.

In general, it would appear that in pea tissue propionate is metabolized entirely by the pathway proposed by Giovanelli and Stumpf, whereas in sunflower and castor bean tissue only a portion of the propionate is dissimilated by this pathway. The alternate reactions that propionate undergoes in the sunflower and castor bean were not indicative of the operation of the pathways of propionate oxidation that have been found in bacteria and animals and their elucidation must await the isolation and identification of some of the unknown compounds.

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