Proline catabolism in *Chlorella*

by

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LIST OF SYMBOLS AND ABBREVIATIONS

ϒAB	ϒ-aminobutyrate
oAB	ortho-aminobenzaldehyde
AHV	D,L-α-amino-δ-hydroxyvaleric acid
AmOH	ammonium hydroxide
$AmSO_4$	ammonium sulfate
BAW	n-butanol: acetic acid: water
BSA	bovine serum albumin
CAPS	cyclohexylaminopropane sulfonic acid
carb.	carbonate buffer
Ci	curie
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DEAE	diethylaminoethyl
2,4-DNPHine	2,4-dinitrophenylhydrazine
2,4-DNPHone	2,4-dinitrophenylhydrazone
DTT	dithiothreitol
EC	Enzyme Commission
FAD	flavin-adenine nucleotide
gln	glutamine
glu	glutamate
GSA	glutamate-ϒ-semialdehyde
hrs.	hours
hyp	4-hydroxyproline
IU	International unit of enzyme activity
αKG	α-keto glutarate

Km	Michaelis-Menten constant
min.	minutes
OAA	oxaloacetic acid
orn	ornithine
PCV	packed cell volume
phos.	phosphate buffer
POS	proline oxidase system
pro	proline
P5C	Δ^1-pyrroline-5-carboxylate
P2C	Δ^1-pyrroline-2-carboxylate
P5C/GSA	equilibrium product of P5C and GSA
2P5C	2-pyrrolidone-5-carboxylate
TCA	trichloroacetic acid
TCAP	tricarboxylic acid pathway (Krebs cycle)

INTRODUCTION

In recent years, evidence has been obtained which shows that there is a striking accumulation of proline in plants which are subjected to various environmental stresses. The major stress condition thus far examined, has been that of water stress. If the plant tissue does not dry out beyond its water recovery limit, and the tissue is then rehydrated, the proline concentration then decreases. It thus appears that free proline may serve unique purposes in plants, under the stimulation of adverse environmental conditions. Stresses seem to be intimately involved in the anabolic and catabolic pathways of proline. Investigations thus far, have been concerned chiefly with determining the amounts of proline accumulated, and the origins of the accumulated proline. Some work has been done on the enzymatic pathway which leads to the buildup of proline in plants. Scant information can be found in the literature on the metabolic details of proline catabolism in plants.

The overall metabolism of the α-ketoglutarate to proline pathway seems to be quite similar in all living systems thus far investigated. Work with microorganisms and with animal tissues, indicates that the final enzyme leading to proline biosynthesis is different from the first enzyme involved in the natural breakdown of proline. There is, of course, no guarantee that this situation holds for plant tissue.

The fundamental purpose of this investigation has been to examine the pathway of proline breakdown in plants. If proline is indeed an important metabolic compound during times of environmental stress in plants, it is imperative to elucidate both the anabolic and catabolic pathways of

proline. An attempt has been made to partially purify and to partially characterize the initial enzyme in the catabolic pathway.

LITERATURE REVIEW

Environmental stresses have profound physiological effects on plants. Many articles and reviews on these effects are available. Levitt (1972) has listed a number of these effects and has given a large bibliography of articles on the subject. Many stresses that have been investigated, have been shown to have a correlated buildup of free proline, often in huge amounts.

Accumulation of free proline in plant tissue subjected to environmental stresses

Thompson et al. (1960) found that turnips which were deficient in phosphorus and sulfur, accumulated free proline more than expected from proteolysis. Seitz and Hochster (1964) observed that free proline increased 70-fold in tobacco crown gall tumor tissue, as compared with the proline concentration in normal tissue. They also found a 22-fold increase of proline in diseased tomato plants. Strogonov (1964) has found increases of proline in a variety of plants as a result of salinity stress in both leaves and roots. Smith (1968) found that rubber dust in tobacco tissue cultures, was associated with up to 30 times the normal free proline level. Durzan (1968) demonstrated high proline levels in dormant white spruce buds. Roberts and Baba (1968) reported proline involvement in wound vessel member formation. Haas (1969) discovered that sun leaves of copper beech have higher proline content than do the shade leaves, suggesting a possible ultra-violet radiation stress. Le Saint (1969) reported that, in cabbage tissue subjected to freeze-hardening,

free proline increased from 2 to 4 % of total amino acid content in normal unhardened tissue, to 60 % on hardening. She found a better correlation between proline and plant hardiness than between sugars and hardiness.

Accumulation of free proline in wilted plant tissue

The most heavily investigated plant stress, resulting in proline accumulation, has been water stress. Water stress has many other effects also. Among the many reviews covering water stress, the reader is referred to those of Henckel (1964), Iljin (1957), Levitt (1951, 1972), and Vaadia et al. (1961). One effect of wilting in plants, is that of protein breakdown. With protein breakdown, considerable accompanying changes occur in the free amino acids which are present in plant tissue.

Kemble and Macpherson (1954) found that wilting in perennial rye grass, resulted in an accumulation of proline. Chen et al. (1964), using lemon and lime tissue, demonstrated that dehydration of roots and leaves, resulted in higher than expected amounts of free proline. Barnett and Naylor (1966) observed that, during water stress in Bermuda grass shoots, free proline characteristically accumulated 10 to 100 times the nonstress concentration. Sometimes the proline accumulation was up to 125 times the control value. Routley (1966), using wilted ladino clover leaves, found large increases of free proline. However, dead clover leaves had only about one-tenth as much free proline as was found in wilted leaves.

Thompson et al. (1966) observed proline accumulation in wilted turnip leaves. Turgid turnip leaves did not accumulate free proline. Stewart et al. (1966) extended the same findings to corn, sunflower, kidney bean, jackbean, tomato, and radish leaves. They found that the amount of

proline accumulation, exceeds the amount of proline released by protein breakdown, during the period of accumulation. Since 1967, there have been many reports of proline accumulation, under water stress, in a great variety of plants. Stewart (1972b) found that the increase of free proline occurring in wilted leaves, stopped immediately upon hydration of the leaves. Proline amounts then declined.

Mature pollen represent a highly dehydrated plant material. Britikov and Musatova (1964), in the investigation of around 200 species of plants, found free proline in all pollen, with many species showing enormous concentrations. In some pollen, they reported that more than 50 % of the free amino nitrogen was stored as proline.

Source of free proline not accounted for by proteolysis

Since the free proline accumulation during wilting, cannot be entirely from proteolysis, the question arose as to what other possible sources there could be. Routley (1966) found that leaves wilted in a nitrogen atmosphere, failed to produce any proline accumulation. He also discovered that, in an aerobic atmosphere, some proline accumulation came via the TCAP (tricarboxylic acid pathway). Further, he observed that leaves floated on a glucose solution, resulted in a greatly increased free proline accumulation. Thompson et al. (1966) and Stewart et al. (1966) further confirmed that wilted plant tissue, accumulated proline partly by synthesis of proline from other compounds. Probable compounds discussed were glutamic acid, arginine, and ornithine. The work of Oaks et al. (1970) suggested that proline accumulation in corn, could result from decreased protein synthesis, while de novo proline formation was occurring.

Stewart (1973) concluded that the higher proline content in wilted bean leaves, is due to a greater rate of proline synthesis. The source of nitrogen for this increased synthesis, conceivably, would be from other amino acids, both proteinogenic and nonproteinogenic. Since proline synthesis increased in starved leaves (leaves low in metabolizable carbohydrates), the source of carbon would likely be from noncarbohydrates, such as other amino acids. Stewart (unpublished data) found evidence that wilted bean leaves had considerable synthesis of proline from both glutamic acid and from arginine.

Proline utilization

Since Stewart (1972b) has shown that rehydration of wilted leaves, immediately results in a decrease of the accumulated free proline, one must ask about the fate of the disappearing proline. Wang (1968) found that ^{14}C-proline, in corn roots, produced glutamic acid and malic acid as the major products. Other amino acids stayed either as free amino acids or were incorporated into protein. Thus, proline was metabolized back into the TCAP. Barnard and Oaks (1970), Oaks et al. (1970), and Stewart (1972a) observed that the two major uses of the accumulated free proline, are protein synthesis, and oxidation to other amino acids, organic acids, and CO_2.

It should also be pointed out that proline has a unique and very important role when it is incorporated into polypeptide chains. It forms disruptions in the tertiary α-helical structure of proteins. These disruptions contribute greatly to protein stability.

Effects of carbohydrates on proline accumulation

There is a very complex relationship between metabolizable carbohydrates found in plant tissue, and the accumulation of proline. Stewart et al. (1966) found that proline accumulated in wilted leaves only when adequate carbohydrates were present. They also observed that addition of glycolytic and TCAP inhibitors, prevented proline accumulation. They concluded that a correlation exists between metabolizable carbohydrates in leaves and the amount of proline which can accumulate in the leaves when wilted.

Control of proline accumulation and utilization

Three major factors in the control of free proline accumulation and breakdown have been investigated. The first is that of the relative state of dehydration of the plant tissue. This has already been discussed. A second is that of the amount of free proline accumulated. The third is the effect of carbohydrates.

Stewart (1973) concluded that proline accumulation in bean leaves was not susceptible to feedback inhibition. Oaks et al. (1970) reported that proline inhibited the formation of free proline from glutamic acid in corn root tips. However, when glucose was added, proline did accumulate. They also discovered that the addition of large amounts of exogenous proline, did not affect the amount of proline converted to protein. Stewart (1972a) observed that adding large amounts of proline did increase the utilization of proline by oxidation. Thus, in the presence of metabolizable carbohydrates, accumulation of proline is unaffected by the free proline content. Protein synthesis from proline is unaffected

also, but proline oxidation is related to the amount of free proline which is available.

Considerable evidence has been obtained which shows that carbohydrates do exercise control over both the accumulation and the utilization of proline. As previously pointed out, Stewart et al. (1966) have shown that proline accumulates only when adequate carbohydrates are present. Thus, carbohydrates are involved in proline biosynthesis in plants during water stress.

Oaks et al. (1970) with corn tips and Stewart (1972a) with rehydrated wilted bean leaves, found that, with ample carbohydrates, plant tissue does not oxidize its accumulated proline. Thus, proline oxidation to other amino acids, organic acids, and CO_2, is inhibited. Stewart (1972a) has concluded that one role of carbohydrates is the prevention of proline oxidation. Thompson et al. (1966) and Stewart et al. (1966) showed that when wilted leaves were depleted of metabolizable carbohydrates, there was a net loss of free proline, indicating proline being metabolized. Stewart (1972a) demonstrated that after leaf carbohydrates are exhausted, then proline oxidation does occur.

Stewart (1972a) has advanced the idea that carbohydrates have a second role, in that the amount of carbohydrates in leaf tissue, alters the rate of proline incorporation into protein. Stewart (1972b) found that when carbohydrate content was high in rehydrated bean leaves, the free proline decrease was slow and proline was mainly converted to protein proline. When carbohydrate content was low, the rate of free proline decrease was rapid, with proline being mainly lost by oxidation. Thus, exogenous sugars applied to starved leaves, shifted proline utilization from oxida-

tion to protein synthesis. The presence of carbohydrates in leaves is necessary for proline to accumulate (Stewart, 1972b), because the absence of carbohydrates allows proline oxidation, which in turn lowers proline concentration.

Significance of proline accumulation in plant tissue during wilting and rehydration

Due to the unusually rapid accumulation of proline during water stress, and the rapid metabolism of proline into both protein and the TCAP, on hydration, there is a growing belief that proline serves several very important purposes in plants. Barnett and Naylor (1966) have speculated that accumulated proline may function as a storage compound for both carbon and nitrogen, during water stress. Barnard and Oaks (1970) believe proline has a unique role in rapidly growing tissue, possibly irregardless of the relative state of dehydration. In addition to being a carbon and a nitrogen source, free proline may also serve as a supplementary energy source (Barnard and Oaks, 1970). Levitt (1972) has suggested that proline may be less harmful to plants than are other amino acids or NH_3 which might accumulate. He also states that proline may be more readily translocatable than are other amino acids.

Proline, which appears to be readily metabolized to glutamic acid, could serve as an amino source for glutamic acid. As is well known, glutamic acid is the donor of amino groups to other amino acids, and plays a central role in the incorporation of nitrogen into a vast array of organic compounds.

Much of the literature which has dealt with the importance of free

proline in plant tissue, has been in the area of pollen physiology. Numerous workers have observed the unusually large content of proline in pollen. Britikov et al. (1965) in their work with pollen and with buds, have not only emphasized the possible importance of the above three uses of proline, but have advanced the concept of proline perhaps being a physicochemical mediator of metabolism in dormant and survival tissues. They speculated that free proline may protect and stabilize metabolism in the sites where the proline is stored. The work of Oaks (1965) with corn, seems to substantiate this regulatory role of proline.

Britikov et al. (1964) have pointed out that it is doubtful that pollen, due to its compactness, would contain nonessential material. Thus, the free proline in pollen appears to be a substance of high physiological activity, producing an exceptionally active biochemical substance. Tupý (1964) has shown that proline is the major amino acid in the pollen of most plant species thus far investigated, and that the amount of free proline is correlated with pollen fertility.

Thus, it is entirely possible that stored, free proline may serve at least four important functions: (1) nitrogen source, (2) carbon skeleton source, (3) energy source, (4) metabolic mediator of revived and of rapidly growing tissues. In short, there is ample evidence that higher plants accumulate proline during stress times, then use it when rejuvenated.

Proline metabolism in general

Figure 1 shows a diagram modified from Fowden (1965a, 1965b), and from Meister (1965), in which a proposed pathway linking αKG and proline, is given. Figure 2 shows branch linkages with several other important natural

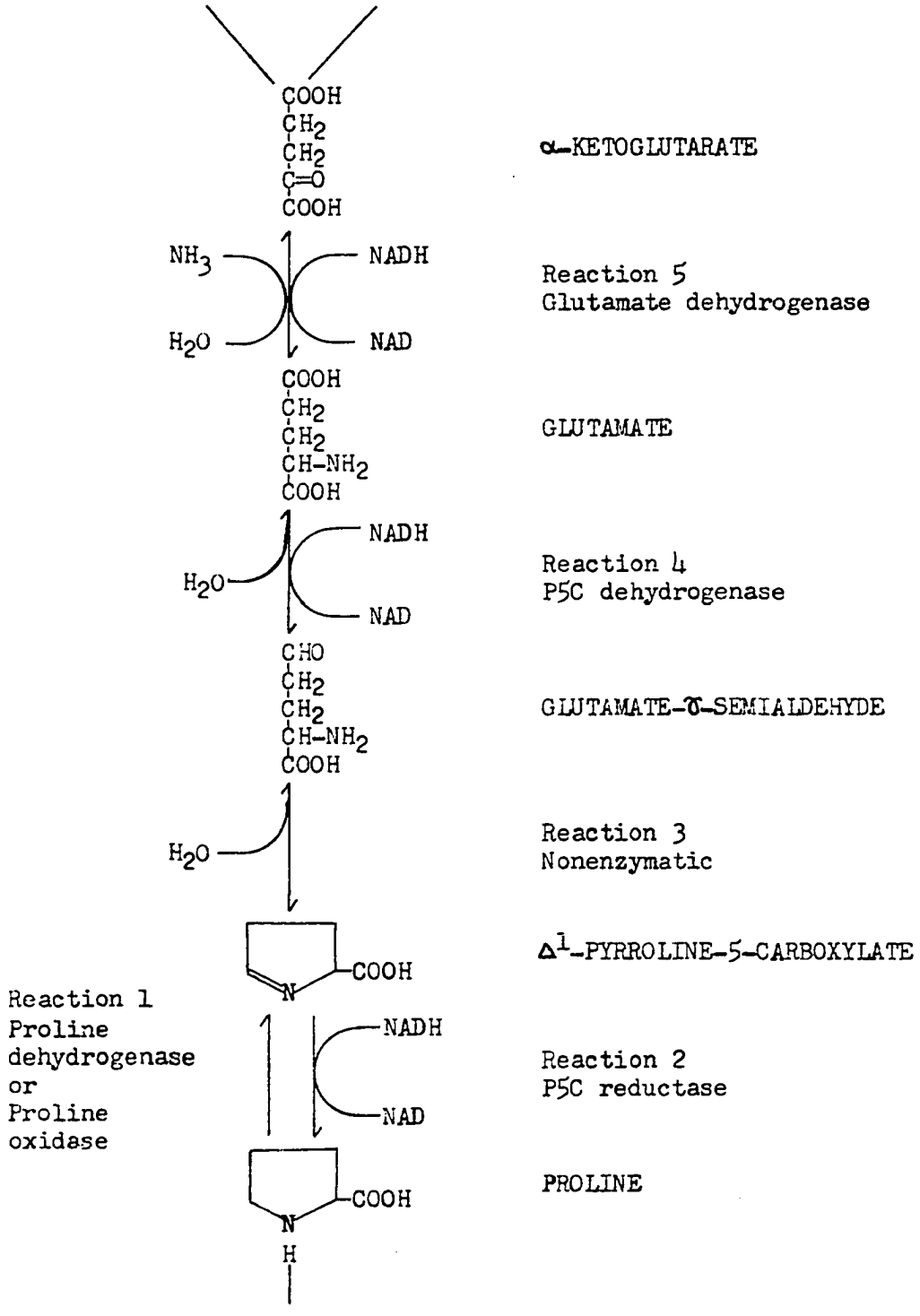


Figure 1. Possible pathways associated with the metabolism of proline. Adapted from Fowden (1965a, 1965b), and Meister (1965)

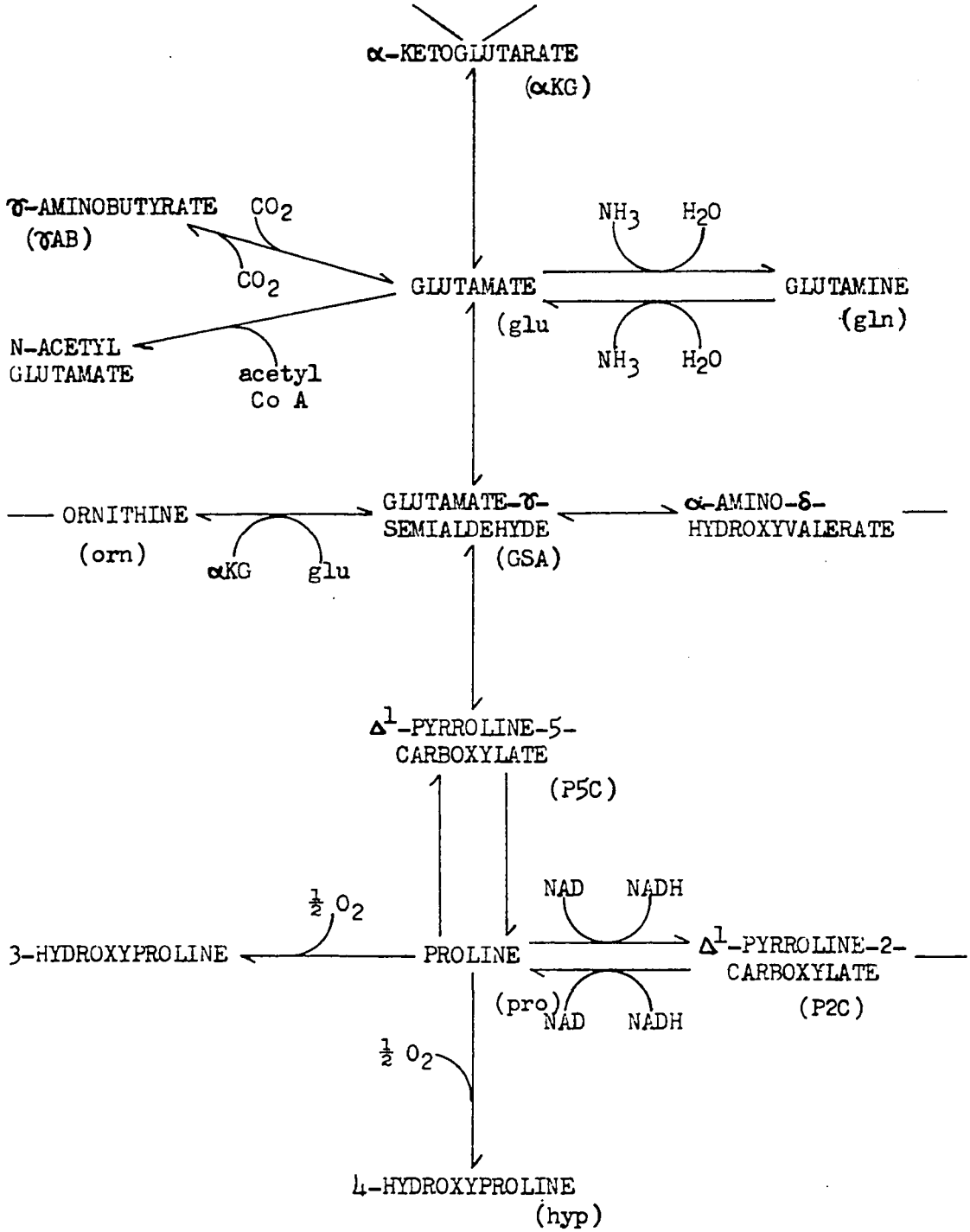


Figure 2. Amino acids and pathways which may be closely associated with the proposed direct metabolic pathways of biosynthesis and natural degradation of proline. Adapted from Steward and Durzan (1964), Fowden (1965a, 1965b), and Meister (1965)

amino acids, such as ornithine and GAB, since they are intimately related. All amino acids shown are of the L form. Except where otherwise noted, all amino acids in this text, will be assumed to be of the L form. Figure 1 will serve as a working pathway for the remainder of the text. There are other modifications, besides those indicated in Figures 1 and 2, that have been suggested for various microorganisms. However, the interconversions between proline and glutamate, as shown in Figure 1, may well hold for living systems in general. As presently viewed, the biosyntheses of proline, ornithine, and arginine, come only from glutamate.

As discussed previously, free proline can accumulate in large quantities. Thus, proline can be considered as an end product. In the pathway connecting αKG and proline, only one pair of metabolites, proline and P5C, are hypothesized to be controlled by two separate enzymes, depending on the direction of metabolism. Meister (1965) strongly suggested that the synthetic and degradative enzymes of proline, are probably different, since this allows better control of proline levels and uses. Meister's own research has shown that P5C reductase will not catalyze the oxidation of proline. He pointed out that other similarly accumulating end products, also appear to be controlled by two different one-way enzymes. Thus far, all evidence indicates separate enzymes (Meister, 1965). Although the purpose of this text is not to show that these two enzyme activities are different, it is convenient to discuss them as being two different enzymes. The unusual storage and utilization patterns of proline, by plants, would lead one to expect a highly controlled system. This control could well be on the enzyme level, with the P5C to proline and proline to P5C enzymes the most likely controllers. The work of Johnson and Strecker

(1962) also strongly suggested that different mechanisms operate when the same substrates and end products are involved in biosynthetic and degradative processes.

P5C and GSA

Before further discussing the pathways, a thorough examination of the two proposed metabolic intermediates between proline and glutamate, will be helpful. One of the major problems in the proline-glutamate interconversion pathways, has been that of providing evidence about the nature of the intermediates, that is satisfying to all researchers. Many articles which have made definite statements about the intermediates, have failed to provide the reader with convincing proof.

The two proposed intermediates between proline and glutamate, P5C and GSA, are shown in Figure 1. They may be the only intermediates between proline and glutamate. They may remain bound to enzymes, or they may exist as free amino acids in vivo. The proposed mechanism, stated very simply, is the following. Proline is dehydrogenated to P5C by removal of two hydrogens. P5C opens by breakage of the double bond to form GSA. GSA forms glutamate by oxidation of the aldehyde group to a carboxylic acid group. As later discussion will show, there is very good evidence that P5C is an intermediate between proline and glutamate, for animals and microorganisms. On the contrary, there is no good evidence for P2C being an intermediate in the main interconversion pathway.

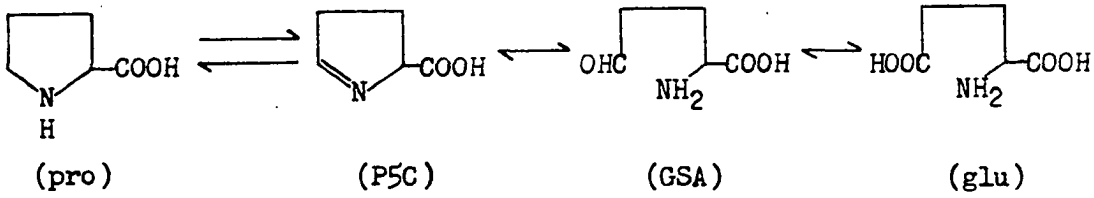
GSA is known to serve as a precursor for ornithine. Meister (1965) discussed possible mechanisms for GSA being channeled to both ornithine and proline (or glutamate). GSA's intramolecular cyclization product,

P5C, may also be involved in mediating this channeling. Vogel and Bonner (1954), and Davis (1968), using Neurospora, showed that ornithine and proline biosyntheses are independent, although both involve the common intermediate, GSA. The labeling experiments of Vogel and Kopac (1959) are consistent with GSA being in two separate locations, one for ornithine, and one for proline. Meister (1965) discussed other alternatives to channeling.

Two possible Δ^1-pyrroline carboxylic acids are shown in the pathways of Figure 2. Both isomers, P5C and P2C, are cyclic compounds, and differ structurally from proline, by having one double bond. The structure of P2C is shown in Figure 3. The open chain analogue of P5C, is GSA, shown in Figure 1. The structure of P2C's open chain analogue, α-keto-δ-amino-valeric acid (Meister, 1965), is shown in Figure 3. Meister (1954) demonstrated that E. coli mutant 55-25 would grow when supplied with exogenous P5C, but not with exogenous P2C. He used this as evidence that P5C and P2C are not in equilibrium with each other. No enzymatic interconversion of P5C and P2C has yet been found.

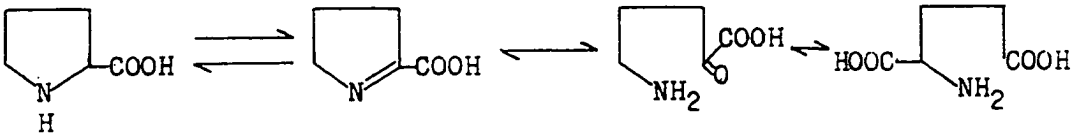
Figure 3 shows three possible sets of intermediates that have been considered, over the years. All three are discussed by Meister (1965), who lists evidence which indicates that pathway (A) is much more probable than pathways (B) and (C). Pathway (A) is also the simplest possible pathway and is hypothesized by the overwhelming majority of current investigators. P5C/GSA are the most chemically feasible intermediates. Pathway (A) served as the guiding theory in this text. P5C can be chemically oxidized to give the straight chain, GSA; and chemically reduced to give the saturated ring, proline. As will be seen later, there is overwhelming

(A)



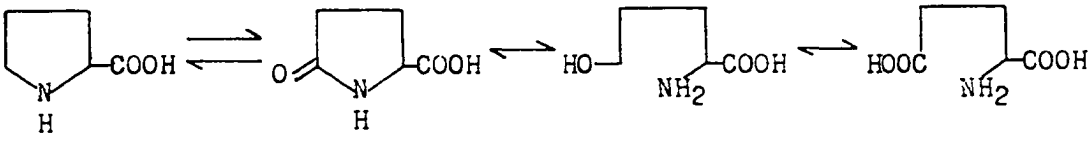
(pro) (P5C) (GSA) (glu)

(B)



(pro) (P2C) (α-keto-δ-aminovaleric acid) (glu)

(C)



(pro) (2-pyrrolidone-5-carboxylate) (α-amino-δ-hydroxyvaleric acid) (glu)

Figure 3. Possible intermediates in the proline-glutamate interconversion pathways. Adapted from Meister (1965).

evidence for pathway (A).

Pathway (C), involving 2-pyrrolidone-5-carboxylate, originally suggested by the general amino acid experiments of animals by Abderhalden (1912), has as evidence against it, the findings that exogenous 2P5C will not serve as substrate in animal extracts (Weil-Malherbe and Krebs, 1935; Neber, 1936) or in E. coli (Vogel and Davis, 1952). Pathway (B), involving α-keto-δ-aminovalerate, was first suggested by Krebs (1939), when he identified it from its 2,4-DNPHone, from animal extracts. Taggart and Krakaur (1949), as discussed later, presented strong evidence against pathway (B), as have many later investigators.

γAB is widespread in plant tissues (Steward and Pollard, 1956). As can be seen from Figure 2, γAB is closely associated with the metabolism of glutamate. γAB is probably not a possible intermediate in the direct proline-glutamate interconversion pathway, since the latter two compounds are 5-carbon amino acids, whereas γAB is a 4-carbon amino acid. Were γAB a direct-line intermediate, the pathways would require a decarboxylation step, and a carboxylation step, a highly unlikely situation.

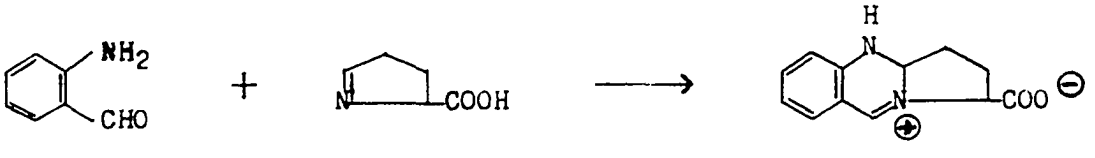
In solution, P5C/GSA are in equilibrium and interconvert nonenzymatically (Vogel and Davis, 1952). The symbol P5C/GSA will be used to indicate the equilibrium product. Strecker (1960a) determined many of the chemical and physical properties of P5C and of its complexes. He found that P5C/GSA is unstable in aqueous solution, but that P5C is stable for several years as a pure solid. Vogel and Davis (1952) suggested that P5C polymerizes readily, preventing isolation of pure, monomeric P5C. They reported this polymerization to be typical of aminoaldehyde derivatives

also. Strecker (1960a) also suggested this polymerization at room temperature, but reported that it did not polymerize when stored at 3 or 4 C. He found P5C to be more stable enzymatically, when stored at 3 or 4 C, than when stored above or below that temperature.

GSA is a δ-aminoaldehyde. δ-aminoaldehydes have been found to undergo spontaneous cyclization, which is reversible under certain conditions (Vogel and Davis, 1952). Adams and Goldstone (1960a) have pointed out, that in solution, equilibrium appears to usually favor the cyclized form. This is evidenced by frequent failure of aldehyde tests, and by the general success in reducing both synthetic and natural forms to proline. At physiological pH, P5C seems to dominate over GSA.

Schöpf and Oechler (1936) and Schöpf and Steuer (1947), described a color detection reaction for a variety of amine compounds. A trapping agent, oAB, is believed to complex with certain kinds of amine compounds, to form yellow molecules. Although Schöpf, an organic chemist, was primarily interested in the detection of specific alkaloids and alkaloid precursors, his oAB trapping method was adapted by Vogel and Davis (1952) for the detection of Δ^1-pyrroline carboxylates. Schöpf and Steuer (1947) showed that δ- and δ-aminoaldehydes undergo rapid spontaneous cyclization in neutral aqueous solutions.

The proposed reaction of Vogel and Davis (1952), as applied to P5C, is predicted to be:



(oAB) (P5C) (P5C-oAB)

The product structure is a 1,2-dihydroquinazolinium compound, and gives a clear, deep-yellow color in aqueous solution. They reported that 1,2-dihydroquinazolinium compounds are somewhat dissociable into their parent compounds. They found that P5C could not be isolated in pure form, so it had to be trapped as a complex. The high reactivity of P5C can be shown by its becoming biologically inactive when exposed to absolute alcohol.

P5C/GSA was first synthesized by Vogel and Davis (1952). They determined that it existed in the two configurations, straight chain, and the cyclized dehydration product. Using crystals purified with ion exchange resins, they determined P5C qualitatively be its reaction with oAB. Strecker (1957, 1960a) further purified P5C, quantitatively determined it by its reaction with oAB, and used the synthetic P5C as an aid in identifying P5C as a natural intermediate in the proline-glutamate interconversion pathways in animal tissues.

Many techniques have been employed in attempting to identify P5C as a natural intermediate in various cells and tissues. The most heavily used technique has been that of complexing P5C with oAB (Vogel and Davis, 1952; Fincham, 1953; Strecker, 1957; and many later investigators). Since P2C is another Δ^1-pyrroline compound that may possibly be an intermediate in the proline-glutamate interconversions, in some living systems, it is necessary to use both P5C and P2C as synthetic standards. P5C and P2C are the only likely candidates for oAB reaction in proline oxidation. Both P5C-oAB and P2C-oAB are deep yellow in acid solutions. Strecker (1957, 1960a, 1965) determined the absorption spectrum of P5C-oAB. Strecker (1960a) pointed out that an oAB positive reaction is insufficient for

for identifying the product, since both of the above complexes are yellow. The close structural similarity of P5C and P2C, make detection by any known technique, very difficult. Then too, there is the possibility that certain living systems may actually have P2C as a natural intermediate in the proline-glutamate interconversions.

The carbonyl complexing agent, 2,4-DNPHine, has been widely used in the past to complex the open chain aldehyde, GSA. However, results by different workers, have not been in full agreement. Taggart and Krakaur (1949), and Costilow and Laycock (1969), as discussed in more detail later, used the physical properties of the 2,4-DNPHones of P5C and of P2C, as evidence against P2C being a natural intermediate in the proline-glutamate interconversion pathways. Strecker (1960a) found that freshly synthesized P5C solutions did not react with 2,4-DNPHine. It was concluded that the unpredictable equilibrium of P5C/GSA, the proposed tendency of P5C to polymerize, and the ease of degradation of P5C, made identification by the 2,4-DNPHone, an unreliable technique.

Strecker (1960a) found that the P5C-ninhydrin complex was bright pink in acid solution, and the P2C-ninhydrin complex was purple. He concluded that occasional P5C-ninhydrin spots of yellow grey, were either polymerized P5C or a degradation product. Johnson and Strecker (1962) reported that biologically formed P5C behaved electrophoretically like synthetic P5C. Ling and Hedrick (1964) found that with BAW solvent paper chromatography, P5C migrates faster than P2C, with P5C traveling with the solvent front. As discussed previously, Meister (1954) was able to distinguish between P5C and P2C, by use of E. coli mutant 55-25, which could metabo-

lize P5C but not P2C. Treatment of P2C with H_2O_2, was reported by Meister (1954) to decompose the P2C completely to GAB and CO_2, and no glutamate. H_2O_2 decomposed P5C partially to glutamate, and no GAB. Strecker (1960a) and Ling and Hedrick (1964) also reported some success with this technique. However, Mazelis and Fowden (1971) found the H_2O_2 test to be inconclusive. Johnson and Strecker (1962) reported a small conversion of P5C to glutamate, but the P5C was largely unchanged.

Proline metabolism of vertebrate animals

Since living systems have generally been found to have many common intermediates, and many similarly functioning enzymes, it was deemed useful to review the work done on proline metabolism in all living systems. This was especially helpful, since most of the work on proline metabolism, has been done with nonplant materials. The earliest experiments were done with animal tissue. Much later, E. coli was examined; and then, other materials. Since amino acid catabolism in mammals, has been found to be most active in liver tissue, this has been the tissue of choice in many proline metabolism investigations.

Comprehensive reviews by Fowden (1965a, 1965b) and Meister (1965), give details on many of the workers who have explored both the proline to glutamate pathway, and the more heavily investigated glutamate to proline pathway. For the sake of brevity, only a few workers will be mentioned, primarily those who have made suggestions or found evidence of the intermediates involved, and those who have investigated the actual proline-involved enzymes. Greenberg (1961) and Rodman (1969) have surveyed the literature for animals and for certain microorganisms.

Abderhalden's (1912) and Dakin's (1913) experiments with dog liver, suggested that glutamate, proline, and ornithine, were closely related metabolically. The structural similarity of the 5-carbon amino acids, ornithine, proline, hydroxyproline, and glutamate, had provided early speculation and reason for experimentation on possible metabolic relationships. Bernheim and Bernheim (1932, 1934), using liver and kidney from seven different mammals, found that addition of proline to the tissue extracts, resulted in increased oxygen uptake, as measured with a Warburg apparatus. Among the earliest investigators who established that proline was degraded to glutamate, in vitro, were Weil-Malherbe and Krebs (1935), using rabbit and guinea pig kidney; and Neber (1936), using guinea pig liver and kidney.

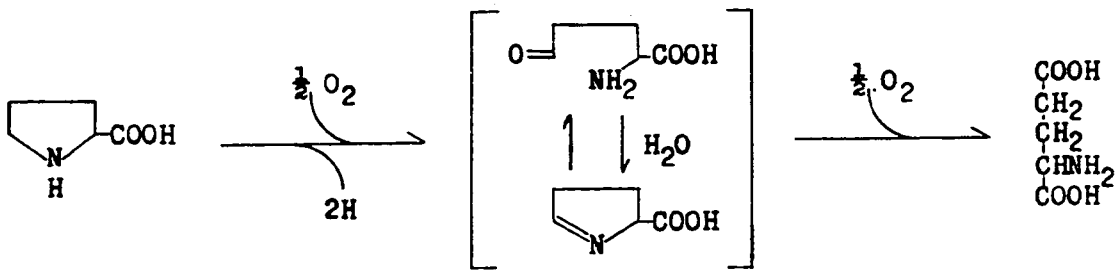
Krebs (1939) obtained a D-amino acid oxidase from sheep kidney. Blanchard et al. (1944), using rat liver and kidney, obtained an L-amino acid oxidase, which could oxidize 13 different amino acids. They found that one molecule of oxygen was used for each molecule of amino acid oxidized. Some of their preparations, but not all, would oxidize L-proline. Although not animal material, Stumpf and Green (1944) found a general L-amino oxidase in the bacterium Proteus vulgaris, which could oxidize 11 different L-amino acids. Proline oxidation was low. The work of the above three groups, seemed to implicate α-keto-δ-aminovalerate/P2C, shown in Figure 3, pathway (B), as intermediates in the interconversion of proline and glutamate. Krebs' (1939) proposed mechanism involved oxidation of proline to P2C, opening of the ring of P2C, deamination to α-ketoglutarate semialdehyde, oxidation to αKG, and finally, transamination to glu-

tamate.

Stetten and Schoenheimer (1944) showed that proline went to glutamate, in vivo, using labeled proline fed to rats. They demonstrated that both the nitrogen and the carbon skeleton of glutamate are derived from proline. They proposed P5C as an intermediate in the proline-glutamate interconversion pathways. Due to the powerful influence of the above mentioned Krebs mechanism, Stetten and Schoenheimer (1944), included P2C in their mechanism. They proposed that proline was dehydrogenated to P2C, which was in equilibrium with P5C. P5C was then directly oxidized to glutamate.

Taggart and Krakaur (1949), using rabbit kidney extracts, showed that proline went to glutamate, in vitro. They found the proline oxidase enzyme to be markedly different from the flavoprotein L-amino acid oxidase, which Blanchard et al. (1944) had isolated from rat tissues, differing in several properties, including specificity of substrates and the lack of an FAD group. It was also different from the D-amino acid oxidase reported previously. On the basis of the formation of a 2,4-DNPHone with a metabolic intermediate, and comparison with the 2,4-DNPHone of synthetic α-keto-δ-aminovalerate (in equilibrium with P2C), they concluded that α-keto-δ-aminovalerate/ P2C was not an oxidation product. This is strong evidence against pathway (B) of Figure 3, in which P2C is a proposed intermediate. P5C/GSA were predicted to be the sole intermediates.

On the basis of their findings, Taggart and Krakaur (1949) modified the proposed mechanism of Stetten and Schoenheimer (1944), by completely omitting P2C. The Taggart-Krakaur mechanism, shown immediately below, is still essentially the accepted pathway in animal metabolism.



(pro) (P5C/GSA) (glu)

They included an hydrolysis of P5C's ring between the nitrogen and the δ-carbon, to yield GSA. Note the proposed use of molecular oxygen to obtain glutamate from P5C/GSA.

Depocas and Bouthillier (1951) and Sallach et al. (1951) fed labeled glutamate to rats. Both groups of investigators recovered more label in proline than in any other amino acid, further evidence that glutamate was the precursor of proline in animals. Lang and Schmidt (1951), working with liver and kidney preparations of dog, rat, and rabbit, found evidence that proline was oxidized to a pyrroline carboxylic acid, which was in nonenzymatic equilibrium with its open chain analogue. The open chain compound was found to readily be oxidized to glutamate. Their work seemed to indicate the open chain compound to be GSA. Strecker and Mela (1955), and Roche and Ricaud (1955) reported the finding of a proline to P5C enzyme in rat liver mitochondria.

Smith and Greenberg (1957) showed that proline was metabolized to glutamate in rat liver, by an enzyme system requiring NAD. They also found that the reverse pathway of glutamate to proline, required a pyridine-linked enzyme system. Meister et al. (1957) also reported an NADH requirement for P5C to proline in extracts of rat tissue. They further

demonstrated that the extracts could convert P2C to L-proline. However, they found no evidence that P2C is in the actual glutamate-proline natural interconversion pathways. They reported that P5C reductase is a different enzyme than P2C reductase. They concluded that P2C has no significant role in proline-glutamate interconversions in vivo. Meister (1965) in discussing the P2C to proline enzyme, suggested that it may have as its main function, the formation of pipecolic acid, but not be a highly specific enzyme.

Lang and Lang (1958), using rat liver extracts for proline catabolism, suggested a molecular oxygen consuming system, which had relationships with the cytochrome system. They reported a proline oxidase system, POS, bound to insoluble particles. Strecker (1960b) found that beef liver extracts oxidized synthetic P5C to glutamate. His work seemed to indicate that P5C might be directly oxidized to glutamate, and found no evidence of the straight chain GSA existing in free form. However, GSA might be formed in association with the enzyme.

Johnson and Strecker (1962) demonstrated that rat liver extracts oxidized proline to P5C, stoichiometrically. Using mitochondrial band protein, they found that neither sonication nor detergent treatment, would solubilize the enzyme. This substantiated the finding of Lang and Lang (1958) that the POS is bound to an insoluble particle. P5C and GSA were both identified as intermediates. NAD and NADP were found to have no effect on the proline to P5C reaction. A molecular oxygen requirement was demonstrated for the reaction. The oxygen consumption was nearly equal to P5C production. Aerobic incubation resulted in P5C production,

anaerobic incubation resulted in nearly no P5C production. Cyanide completely inhibited the reaction and azide strongly inhibited. Thus, there was strong evidence of oxidase activity. They reported cytochrome system involvement for the proline oxidase enzyme. Since other L-amino acids were inactive with the enzyme, they concluded that proline oxidase was not a general amino acid oxidase. They found no evidence for its reversibility for use in the P5C to proline reaction. They suggested that the glutamate to proline and proline to glutamate pathways may be quite different, but with the common intermediates, P5C and GSA. Peisach and Strecker (1962), using calf liver, presented further evidence that the proline oxidase and P5C reductase enzymes, are different. Again, it was found that NAD was not involved with proline oxidase.

Peraino and Pitot (1962) showed that a tumor, murine hepatoma carcinoma, had an abnormally high content of free proline, generally 10 to 40 times the free proline concentration in liver of the same rats. They found that proline oxidase activity was much lower in the diseased tissue, and that P5C reductase activity was much higher. Thus, accumulation of free proline was due to alterations in both the catabolic and anabolic enzymes of proline. They concluded that the diseased cells seemed to be unable to control the enzymes immediately involved with proline.

Proline metabolism of invertebrate animals

A large amount of research has been done on proline metabolism in insects. As discussed later, proline seems to be of paramount importance in the process of insect flight. Some work has also been done on noninsect invertebrates, principally mollusks. Florkin (1954) found that the free

amino acid content in mollusk tissues, is often many times that of vertebrates. However, blood amino acid levels of lobster and crayfish, are similar to vertebrates. Greenberg (1962) reported finding P5C reductase in the slug Ariolimax Californicus brachyphallus. Hill and Chambers (1967) found that GSA and P5C are ornithine to proline pathway intermediates in the protozoan Tetrahymena. They found the enzyme P5C reductase would reduce synthetic P5C to proline.

Sacktor (1955) demonstrated that housefly flight muscle extracts, oxidized proline, and suggested that proline oxidation might provide energy for insect flight. He found that mitochondria isolated from the flight muscle, would oxidize proline. Addition of exogenous proline to the mitochondria, restored the oxygen uptake rates. Wyatt (1961) reported that insects, in general, have much higher concentrations of free amino acids in their hemolymph, than are found in mammalian blood. Sometimes, the concentrations are nearly one hundred times greater. Proline levels are especially high in hemolymph.

Corrigan and Kearns (1963) found that DDT caused a decrease of free proline in the hemolymph of cockroaches. The amount of decrease was related to the extent of DDT poisoning. Pyrethrum and dieldrin also produced decreases in free proline. When DDT poisoning symptoms were relieved, the amount of free proline increased to normal. They postulated that DDT poisoning shifts the demand for oxidizable carbon, to proline. Kirsten et al. (1963) reported on characteristic amino acid shifts in locust flight and jumping muscles. Glutamate and proline levels decreased, and alanine increased, in going from the rest stage to the completed ac-

tion stage. They concluded that normal carbohydrates were used first for flight muscle energy; then fats were used; then the two free amino acids, proline and glutamate. No other free amino acid was found to decrease.

Bursell (1963, 1966) demonstrated that proline was a substrate for flight metabolism in tsetse flies, that glutamate was an intermediate, and that alanine was the end product of flight processes. He found evidence that GSA was an intermediate. In resting flies, the proline level was high and the alanine level was low. During flight activity, large amounts of proline disappeared and large amounts of alanine accumulated. During 120 seconds of flight, in an individual, proline dropped from 1.3 µM to 0.4 µM in the hemolymph. Alanine increased from 0.3 µM to 1.2 µM.

Ray (1964) compared the free proline content in central nervous tissue of normal and of insecticide poisoned cockroaches. Various insecticides caused severe depletion of free proline in the central nerve cord, while free glutamine increased. He also reported that free proline was approximately 90 times greater in concentration in normal insect nerve cord, than in rat brain. Other amino acids had about the same concentrations in both insect nerve cord and in vertebrate nerve. His work strongly suggested that proline served as a metabolic reserve.

Brosemer and Veerabhadrappa (1965) found that grasshopper flight muscle contained P5C dehydrogenase activity, with an NAD requirement. Most of the activity was found to be particle bound. They reported a flavoprotein-linked proline oxidase, which catalyzed proline to P5C. All of this enzyme's activity was tightly bound to a particulate fraction. Addition of cytochrome c, increased activity. Sonication caused a total

loss of activity. The oxidase enzyme was inhibited both by anaerobiosis and by KCN. They called the enzyme, proline oxidase, in order to imply that the activity contained the entire ETS from proline to molecular oxygen. Their calculations showed that the locust wing muscle got 13 to 26 % of its in vivo energy, from proline, as determined by the amount of proline used and the total oxygen used.

Sacktor and Wormser-Shavit (1966) demonstrated great decreases in proline concentration and great increases of alanine concentration, in blowfly flight muscle, during activity. The relationship between proline used and alanine formed, was stoichiometric, suggesting that alanine was derived mostly from the large store of free proline. They found that P5C was an intermediate of proline oxidation by mitochondrial extracts. Sacktor and Childress (1967) reported that the proline to P5C enzyme, in blowfly flight muscle, was a flavoprotein-linked proline dehydrogenase, and that GSA was produced after P5C. Their published results did not indicate whether or not they checked the effects of oxygen or NAD in the dehydrogenase. They found participation by the TCAP, which suggested that proline's role might be to form precursors of OAA, thus allowing the complete oxidation of pyruvate via the TCAP. Exogenous proline, but not exogenous glutamate, can penetrate mitochondria. Thus, exogenous glutamate can not be substituted for exogenous proline in promoting insect flight.

Reddy and Campbell (1969), working with two different genera of silkmoths, demonstrated that proline was a major substrate in their flight muscle metabolism. They found the proline to be oxidized, within mitochondria, to TCAP intermediates. Again, in silkmoth, normal proline concentra-

tion was relatively high. Crabtree and Newsholme (1970) found high proline dehydrogenase activity in tsetse fly and cockchafer flight muscles. They measured ATP production, and reported that the 14 ATP produced from proline being oxidized to pyruvate, is more than is produced by TCAP oxidation of acetyl Co A. They concluded that the high ATP production observed, suggests that proline oxidation could provide most of the energy for a period of flight in these insects. They felt that their evidence indicated that the proline to P5C reaction, is the rate limiting enzyme activity of the entire proline to pyruvate pathway. This ties in with Meister's previously discussed statements about control of proline by means of the enzymes most closely associated with proline. Crabtree and Newsholme (1970) raised the important question, of why materials are converted to and stored as proline, rather than converted to and stored as glycogen or fat. The latter two substances, seemingly, could easily be converted to energy. The same question of why the high storage of proline, can also be applied to plant cells.

In summary, one of the most characteristic features of insect tissue associated with flight, is the very high concentration of free proline. This proline is believed to be an important energy source, in the rest-to-flight transition of insects. The remarkable functional features of flight muscles, are illustrated by the fact that insect muscles are capable of over one thousand contractions/second; and can use energy in amounts/gram, of up to 50 times those used in leg and heart muscles of Homo sapiens. Thus, proline appears to serve as a reserve metabolite, for use during periods of intense muscular activity, being converted to

glutamate, and thence to alanine, during the energy yielding reactions.

Proline metabolism in E. coli

Proline metabolism has been intensively studied in E. coli. Bernheim (1944) reported that E. coli can oxidize L-proline. He determined the molecular oxygen consumption, manometrically. Tatum (1945) found that proline and glutamate were interconvertible. Vogel and Davis (1952) were the first to show that GSA and P5C were intermediates, in E. coli, for the glutamate to proline pathway. They did so by devising a method of synthesizing P5C, so as to compare it with natural P5C produced enzymatically. Meister et al. (1957) further verified the role of P5C and GSA as intermediates. Strecker (1957), using highly purified P5C, determined the quantitative relationships between P5C, glutamate, and proline.

Frank and Rybicki (1961) used a Warburg apparatus to determine the molecular oxygen requirement for the proline to P5C reaction. 1 µatom of oxygen and 1 µmole of proline were needed to obtain 1 µmole of glutamate. They reported evidence that the POS was 90 to 95 % inducible, by growing the E. coli in proline enhanced medium. Kameda et al. (1963) presented evidence that 25 strains of E. coli, grew well with proline as the sole source of carbon. Alanine, aspartate, and glutamate combined could substitute for proline.

Frank and Ranhand (1964) found that NAD and $MgCl_2$ greatly stimulated the oxidation of proline, but that NAD or NADP, was required only for the P5C to glutamate reaction, and not for the proline to P5C reaction. They demonstrated that P5C reductase was in the supernatant fraction,

whereas POS was in the pellet. Thus, POS appears to be bound in E. coli; and hence, is distinctly different from P5C reductase. They also found proline oxidase activity in an E. coli mutant, which lacked P5C reductase activity. Their work indicated that the entire proline to glutamate oxidative enzyme system was particulate. Complete inhibition with cyanide and with anaerobiosis, indicated that an oxidase was involved. On the basis of the above investigations, Meister (1965) suggested that P5C reductase in E. coli, is totally different from proline oxidase, and that the latter enzyme is similar to proline oxidase in animal tissues.

Proline metabolism in other microorganisms

Vogel and Davis (1952) reported that synthetic P5C served as a substrate in both N. crassa and E. coli. Fincham (1953) detected both P5C and GSA in Neurospora extracts. Vogel and Bonner (1954), and Abelson and Vogel (1955) found evidence that GSA and P5C were intermediates in the glutamate to proline pathway of N. crassa. Yura and Vogel (1955, 1959) discovered P5C reductase in Neurospora. They devised an assay for the pyridine nucleotide-linked enzyme. NADPH proved to give more activity than NADH. Meister et al. (1957), using a N. crassa mutant, with the P5C to proline pathway blocked, demonstrated that P2C could serve as a precursor of proline, but found that P2C was not on the major pathway of proline biosynthesis.

Bonner (1946), using Penicillium fungal mutants, showed that ornithine was not a precursor of proline, but that both had the same precursors. Abelson and Vogel (1955) reported that the fungus, Torulopsis utilis, synthesizes proline by the same mechanism as do E. coli and N.

crassa; namely, that GSA and P5C are intermediates. Ling and Hedrick (1964) discovered two different L-proline oxidases, in the mitochondria of the yeast Hansenula subpelliculosa. One oxidized proline to P5C, and one oxidized proline to P2C. The straight chain equilibrium compounds, GSA and α-keto-δ-aminovalerate, were also detected. They reported, contrary to E. coli findings, that the NAD requiring enzyme which further oxidized P5C, was in the soluble, nonmitochondrial fraction. They declared the proline to P2C enzyme to be inducible; but the proline to P5C enzyme was found to be constitutive. Meister et al. (1957) reported finding P5C reductase in the bacterium Aerobacter aerogenes. Smith (1957) demonstrated that glutamate enzymatically produced GSA/P5C in pleuropneumonia-like organisms of human origin. Coleman (1958) fed labeled glutamate to the bacterium Rhodospirillum rubrum. Only a small amount of the label was recovered in proline, with several other amino acids being more labeled.

De Hauwer et al. (1964) used the Warburg apparatus to measure oxygen uptake for a proline oxidase in the bacterium Bacillus subtilis. P5C was produced and this was shown to produce glutamate by action of P5C dehydrogenase. Laishley and Bernlohr (1968) used the methods of De Hauwer et al. (1964) to demonstrate a POS in Bacillus licheniformis. They found P5C to be a key intermediate in the proline to glutamate pathway. P5C dehydrogenase was found. Dendinger and Brill (1970) reported that proline catabolism in the bacterium Salmonella typhimurium, gave P5C as the immediate product. An oxygen requirement was suggested by the use of an oxygen electrode. P5C dehydrogenase was found to require

NADP. Prival and Magasanik (1971), using the oxygen electrode and the methods of Dendinger and Brill (1970), demonstrated a proline oxidase in the bacterium Klebsiella aerogenes.

Costilow and Laycock (1969) identified GSA and P5C as intermediates in the proline-glutamate interconversion pathway, using two species of the bacterium Clostridium. They employed a variety of identification criteria, such as oAB complexing, paper chromatography using several solvent systems, and the melting points of 2,4-DNPHones. Synthetic P5C and synthetic P2C were used as standards, against the natural intermediates. Their evidence strongly ruled out P2C as an intermediate. In sharp contrast to the above discussed proline catabolizing enzyme in E. coli, other microorganisms, and in various animals, Costilow and Laycock (1969) found that the proline to P5C reaction, required NAD, and not molecular oxygen. Synthetic P5C served as an oxidant for oxidation of NADH, but P2C did not, when cell extracts were used.

Proline metabolism in plants

As discussed above, GSA and its cyclic form, P5C, are critical intermediates in the proline-glutamate interconversions of all living systems, in which the intermediates have been definitely established. As a consequence, these two intermediates must be considered as the major candidates for the corresponding intermediates in plants. Plant amino acid metabolism is far more complex than amino acid metabolism in animals. Plants must synthesize their own amino acids, in addition to providing degradative pathways. Plants also synthesize many nonprotein amino acids, which lead to many intricate interrelations of both proteinogenic

and nonproteinogenic amino acids. This complicated interlinking of pathways, has been one of the difficulties in establishing a definite pathway for proline degradation in plants. The roles of closely associated amino acids, such as the ubiquitous GABA, are still being investigated.

Meister et al. (1957) demonstrated that both P5C and P2C could be converted to L-proline, using bean and pea extracts. Their total work, with plant tissue, rat tissue, and several microorganisms, indicated that serious proline pathway examination, requires careful consideration of both P5C and P2C, as possible natural intermediates. McConnell (1959), using labeled glutamate, found that labeled proline was produced in wheat. Bone (1959) reported finding GSA as a product from ornithine, by mung bean mitochondria, but was unable to use the mitochondria to obtain GSA from glutamate.

Baker and Thompson (1962), using Chlorella vulgaris, found that the greatest amount of ^{14}C from labeled proline, went into GABA, or into a substance with a chromatography spot very similar to that of GABA. They pointed out that no known mechanism exists for this. It is possible that the chromatography spot could have been P5C. They demonstrated that proline was catabolized to glutamate. Mizusaki et al. (1964) showed that labeled proline went to glutamate, and that labeled glutamate went to proline, in tobacco leaves. Noguchi et al. (1966) partially purified P5C reductase from tobacco leaves. Stewart (1967) demonstrated that labeled glutamate readily went to proline in jack bean leaves, and that labeled proline went to glutamate. He also found that labeled GSA was incorporated into proline, which is evidence for GSA being an intermedi-

ate in jack bean leaves. Strong P5C reductase activity was observed in turnip leaves by Stewart (1967).

Wang (1968) found that corn leaves rapidly changed labeled proline to glutamate. No label was found in hydroxyproline, ornithine, or arginine. Morris et al. (1969) reported the existence of GSA/P5C as an intermediate in the glutamate to proline pathway, in Swiss chard leaf extracts. Durzan (1969) reported finding P5C/GSA in spruce buds. Barnard and Oaks (1970) observed TCAP involvement in proline catabolism of corn root tips, suggesting mitochondria as containing the proline oxidation enzyme(s).

Mazelis and Fowden (1971) obtained a proline dehydrogenase from peanut cotyledons, which had an NAD requirement for activation. NADP gave about 15 % as much activity. Durzan and Ramaiah (1971), using jack pine seedlings, reported that proline label was recovered as P5C, glutamate, and TAB. They found that TAB, GSA, and P5C, were separable by paper chromatography. Mazelis and Creveling (1972) reported an NAD dependent L-proline dehydrogenase in wheat germ. They found that NADP would not replace NAD as oxidant. The molecular weight of the enzyme was estimated to be over 100,000. Stewart (1972a) showed that proline was converted to glutamate in garden bean leaves.

Further information on the enzymes associated with proline metabolism

Several enzymes which can directly or indirectly oxidize amino acids, have been found in a variety of living systems (McKee, 1962). These range from polyphenol oxidase enzymes, to direct acting D- and L- amino acid oxidases. McKee (1962) discussed a number of mechanisms for oxidation. He

pointed out that, where comparable data are available, the mechanisms of breakdown in plants, resemble those in animals and microorganisms.

Webster and Bernheim (1936) reported a D-amino acid oxidase found in the bacterium Bacillus pyocyaneus. Since then, other nonspecific D-amino acid oxidases have been found in a variety of tissues and cells (McKee, 1962). D-amino acid oxidase (EC 1.4.3.3), a molecular oxygen requiring enzyme is listed by Barman (1969) as an FAD requiring enzyme, with broad specificity. It deaminates D-amino acids to their corresponding α-keto acids. Amino acids with good activities are D-proline, D-methionine, D-isoleucine, D-alanine, D-valine, D-phenylalanine, and D-lysine. L-proline is also acted upon. The enzyme's molecular weight has been reported to be from 90,000 to 125,000.

Stumpf and Green (1944) using bacteria, and Blanchard et al. (1944, 1945) using rat liver and kidney, found very general L-amino acid oxidases. L-amino acid oxidase (EC 1.4.3.2), is also a molecular oxygen requiring enzyme, and is catalogued by Barman (1969) as an FAD requiring enzyme, with broad specificity. It deaminates L-amino acids to their corresponding α-keto acids. Amino acids with good activities are L-alanine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tryptophane, L-tyrosine, and L-valine. Its molecular weight, in various living systems, has been found to range from 49,300 to 140,000. These very general forms of L-amino acid oxidases, have been found in animals and in microorganisms (Fowden, 1965b).

Thus, L-amino acid oxidases and D-amino acid oxidases appear to differ markedly, in enzymatic properties, from the highly specific L-pro-

line oxidases and L-proline dehydrogenases, discussed in previous sections. The L-proline oxidases/dehydrogenases, gave P5C/GSA as the direct product, and not α-keto acids. Neither of the L-proline oxidases/dehydrogenases, deaminated proline, as would be expected from the general D- and L-amino acid oxidases.

Although discussed in some detail in previous sections, a survey of the enzymes involved in the interconversions of proline and glutamate, should be helpful. The enzyme of reaction 1 of Figure 1, may be either proline oxidase or proline dehydrogenase, depending on the type of living system. Enzyme 1 can be called proline oxidase in mammalian liver (Taggart and Krakaur, 1949; Lang and Schmidt, 1951; Lang and Lang, 1958; Johnson and Strecker, 1962; Peisach and Strecker, 1962); in yeast (Ling and Hedrick, 1964); in E. coli (Bernheim, 1944; Strecker, 1957; Frank and Rybicki, 1961; Frank and Ranhand, 1964); in Salmonella (Dendinger and Brill, 1970); in Bacillus (De Hauwer et al., 1964; Laishley and Bernlohr, 1968); in insects (Brosemer and Veerabhadrappa, 1965); and in Klebsiella (Prival and Magasanik, 1971), since an aerobic requirement seems to be well established. In living systems where an NAD requirement has been shown instead of a molecular oxygen requirement, the enzyme can be referred to as proline dehydrogenase. This is the case for Clostridium (Costilow and Laycock, 1969), peanut (Mazelis and Fowden, 1971), and wheat (Mazelis and Creveling, 1972).

In every instance where evidence is available, proline oxidase/dehydrogenase appears to be particle bound. This is the case with rat liver mitochondria (Strecker and Mela, 1955; Lang and Lang, 1958; Johnson and

Strecker, 1962); beef liver mitochondria (Peisach and Strecker, 1962); E. coli (Frank and Ranhand, 1964); insects (Brosemer and Veerabhadrappa, (1965); and yeast mitochondria (Ling and Hedrick, 1964). This particulate nature is in accord with the investigations of Schneider and Hogeboom (1950), who presented evidence that mitochondria are centers of respiratory activity. Their work indicated that respiratory and respiratory-associated enzymes, such as proline oxidase/dehydrogenase may possibly be, are mitochondrial bound.

The enzyme of reaction 2 of Figure 1, generally can be called P5C reductase. The enzyme has EC number 1.5.1.2, and is considered essentially irreversible (Peisach and Strecker, 1962; Barman, 1969; Rodman, 1969). It seems to have a universal requirement for NADH in rat liver (Smith and Greenberg, 1957; Meister et al., 1957); bovine liver (Peisach and Strecker, 1962); N. crassa (Yura and Vogel, 1955, 1959; Meister et al., 1957); E. coli (Meister et al., 1957); Clostridium (Costilow and Laycock, 1969); silkmoth (Reddy and Campbell, 1969); slug (Greenberg, 1962); tobacco (Noguchi et al., 1966); jackbean (Stewart, 1967); and turnip (Stewart, 1967).

The evidence indicates that NADPH will function to various extents, in P5C reductase from certain tissues and cells. NADPH activation has been reported by Yura and Vogel (1955, 1959), Smith and Greenberg (1957), Meister et al. (1957), Peisach and Strecker (1962), Greenberg (1962), Noguchi et al. (1966), and Stewart (1967). In fact, Yura and Vogel (1955) with N. crassa, and Noguchi et al. (1966) with tobacco, reported NADPH to be more active than NADH, in P5C reductase. Hill and Chambers (1967)

found that NADH gave only 3 % of the activation that was obtained with NADPH in Tetrahymena P5C reductase. Costilow and Laycock (1969) found that NADPH would not substiute at all for NADH.

As mentioned before, there is, to date, no evidence that suggests that P5C reductase is the same enzyme as proline dehydrogenase/oxidase. On the contrary, evidence exists that these two enzymes are not only different (Adams and Goldstone, 1960b; Frank and Rybicki, 1961; Johnson and Strecker, 1962; Peisach and Strecker, 1962; Frank and Ranhand, 1964), but exist in different subcellular locations. As discussed previously, proline dehydrogenase/oxidase has been found generally to be particle or mitochondrial bound, whereas P5C reductase has been shown to appear as an unbound enzyme. Evidence for the latter exists for microorganisms (Meister et al., 1957; Frank and Ranhand, 1964), mammalian liver (Meister et al., 1957; Lang and Lang, 1958; Johnson and Strecker, 1962), insects (Reddy and Campbell, 1969), and Tetrahymena (Hill and Chambers, 1967). Meister (1965) reported that his own research has shown that P5C reductase will not catalyze the oxidation of proline.

Frank and Ranhand (1964) found proline oxidase activity in an E. coli mutant, which lacked P5C reductase activity. The proline oxidase activity was equal to that of nonmutants, strong evidence for two different enzymes. P5C reductase activity is very high relative to proline oxidase activity (Frank and Ranhand, 1964; C. R. Stewart, unpublished data). P5C reductase, in all living systems thus far investigated, has been shown to have a pyridine nucleotide requirement. Most proline dehydrogenases/oxidases appear to have no pyridine nucleotide involvement. Hill

and Chambers (1967) concluded that P5C reductase was the most active enzyme in the glutamate to proline pathway. Their inhibition studies, indicated that P5C reductase has no regulatory role. Proline especially does not inhibit P5C reductase. As discussed in previous sections, proline dehydrogenase/oxidase appears to be regulated by a variety of factors.

Reaction 3 of Figure 1, is, of course, nonenzymatic; and doubtlessly, involves a multitude of presently unknown factors which mediate the equilibrium between P5C and GSA. Reaction 4 involves an enzyme which, increasingly, is being called P5C dehydrogenase. It formerly was called aldehyde dehydrogenase. However, Strecker (1960b) has indicated that this enzyme is quite different from other known aldehyde dehydrogenases. He tested a variety of aldehydes to show that the enzyme involved in P5C oxidation, was not a broad-specificity type of aldehyde dehydrogenase. He also found evidence that the cyclic P5C is the oxidative substrate, and not the straight chain GSA. The enzyme is pyridine linked, with a seemingly universal NAD requirement. Strecker (1960b) found NADP only about one-fifth as effective as NAD. Brosemer and Veerabhadrappa (1965) found that NADP gave no activity at all.

Puzzling are suggestions that P5C dehydrogenase is particulate in the proline to glutamate pathway (Johnson and Strecker, 1962; Frank and Ranhand, 1964; Brosemer and Veerabhadrappa, 1965), but nonparticulate in the glutamate to proline pathway. Ling and Hedrick (1964) reported that it was not bound at all in yeast. The binding differences could indicate that the enzyme exists in two different subcellular compartments, or that

two different enzymes are operating between glutamate and P5C/GSA. Reaction 5 will not be discussed in this text.

PART I.

FACTORS AFFECTING PROLINE UTILIZATION IN *CHLORELLA*

INTRODUCTION

The overall problem of proline uptake, interaction with other materials, effects by environmental stresses, accumulation, utilization, and the pathways of proline, is a task which will require the work of many investigators for many years. It was decided to examine only a few of these many interrelated aspects at this time.

As was mentioned in the LITERATURE REVIEW, several workers (Thompson et al., 1966; Stewart et al., 1966; Routley, 1966; Oaks et al., 1970; Stewart, 1972a; Stewart, 1972b) have found a complicated interaction between proline and carbohydrates in plants. As a preliminary to examining the catabolism of proline in Chlorella, it was considered necessary to see how exogenous proline and exogenous glucose interact with each other, and how they affect the growth of Chlorella. It was decided not to thoroughly examine stress conditions at this time.

It was also desirable to examine the other conditions for obtaining suitable growth of the cells, for the later enzyme extraction work. Such factors as proper lighting, proper nutrients, temperature, etc., had to be determined. Along with these factors, came the miscellaneous details of finding proper shaking speeds, suspension volume to flask size ratios, and whether or not to use exogenous CO_2 for enhanced growth. A problem which consumed much time, was that of lowering the chances of bacterial and fungal contamination, since Chlorella is highly susceptible to contamination difficulties.

Another question that needed to be answered, was that of the general fate of exogenous proline applied to Chlorella. Although this had been shown with numerous higher plants (Wang, 1968; Barnard and Oaks, 1970;

Oaks et al., 1970; Stewart, 1972a), it needed to be demonstrated with Chlorella, that proline is metabolized into the TCAP.

METHODS AND MATERIALS

Choice of plant material

The experiments were done almost entirely with Chlorella pyrenoidosa Chick, which was obtained from the Culture Collection of Algae, Department of Botany, University of Indiana, Bloomington. Chlorella was chosen for several reasons. It has the organelles typical of higher plants, such as mitochondria, nucleus, nucleolus, starch particles, vacuole, etc. Many physiological and biochemical investigations by numerous workers, have shown that Chlorella has pathways and physiological responses that are almost identical to those of higher plants.

Chlorella grows rapidly, and culture flasks, containing enormous numbers of cells could be obtained within one week. Myers (1951) has shown that Chlorella cell mass can increase up to sevenfold in one day. Environmental conditions can be rather carefully and reproducibly controlled throughout the entire year. Cost of a growth environment is considerably less than for higher plants and little space is required. Although many investigators using Chlorella, have enhanced the growth by applying exogenous CO_2, this was found to be unnecessary for obtaining sufficient cellular material, when the cells were grown heterotrophically. Thus, large quantities of cellular material were easily obtained, using normal room CO_2 concentration. Chlorella has an unusually high nitrogen content, generally between 5.9 and 9.6 % (Myers, 1946). Hence, it can rapidly utilize many nitrogenous exogenous compounds such as proline.

A major difficulty of Chlorella, is its susceptibility to bacterial and fungal diseases. Application of antibiotics caused alteration of the Chlorella cultures. It was found necessary to use strict sterile techniques in handling. A quick means of determining contamination (discussed later) at any time, had to be devised, so that experiments could be discontinued when necessary. Chlorella vulgaris was found not to respond as well to biochemical investigations.

The nature of Chlorella pyrenoidosa

Since Chlorella's cell organelle system led to problems in enzyme extraction and purification, it is advisable to describe the cell and its organelles. Smith (1933) described Chlorella as a unicellular fresh water alga in the Oöcystaceae family of the Chlorophyta division in the plant kingdom. Eny (1950) reported that the cells usually divide into 2 or 4 autospores, each of which becomes an individual cell. Chlorella growth increases logarithmically, as with bacteria.

Figure 4 is a drawing of Chlorella, made from an electron micrograph by Reger and Krauss (1970). The cell is characterized by one huge, cup-shaped chloroplast. This probably accounts for Chlorella's tremendous photosynthetic capacity. It also led to a major problem, namely that of separating the enormous amount of chlorophyll from enzyme extracts. The mitochondria are ellipsoidal and are smaller than the mitochondria in higher plants. Due to the minuteness of the cells, a 1000-power light microscope was of little value in viewing organelles or in determining the results of cell fracturing. Myers (1946) reported that one ml of suspension can contain nearly 19 million cells. Ideally, one would like to lo-

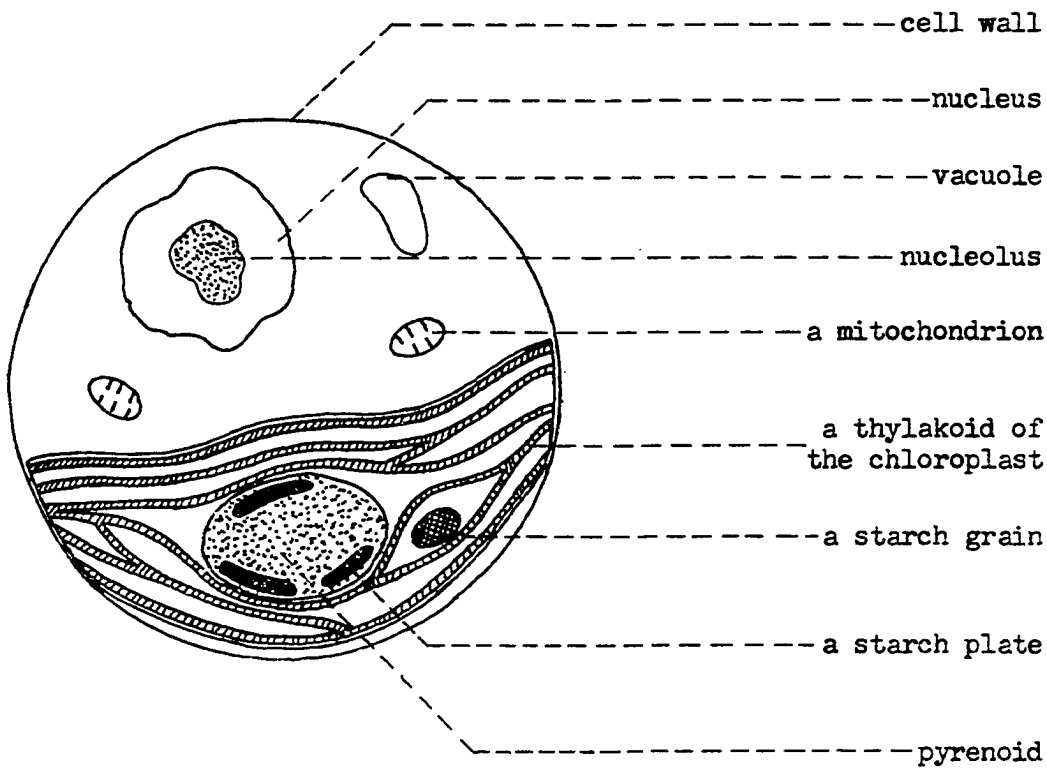


Figure 4. Subcellular organization of Chlorella pyrenoidosa. Magnification is approximately 24,000 times. Drawing adapted from an electron micrograph of Reger and Krauss (1970)

calize enzyme activity by separating intact mitochondria and other organelles, but this proved to be impractical in Chlorella.

Growth medium

The medium, shown in Table 1, was that of Ellsworth (1968). Initial

Table 1. Chlorella basal growth medium

Component	Final solution concentration
KNO_3	1.23 g/l
$MgSO_4 \cdot 7\ H_2O$	2.46 g/l
$Ca(NO_3)_2$	0.24 g/l
KH_2PO_4	1.13 g/l
K_2HPO_4	0.12 g/l
Iron EDTA	0.077 g/l
$MnCl_2 \cdot 4\ H_2O$	0.5 mg/l
$ZnCl_2 \cdot 7\ H_2O$	0.05 mg/l
$CuSO_4 \cdot 5\ H_2O$	0.02 mg/l
$NaMoO_4 \cdot 2\ H_2O$	0.01 mg/l
$CoCl_2 \cdot 6\ H_2O$	0.04 mg/l

pH was in the range of 5.6 to 5.8. EDTA maintained the iron concentrations at a proper level during growth (Myers, 1951). Glucose of various concentrations (usually 1 %) was added as discussed later. Solid medium contained the above components, along with 1 % glucose and 1.5 % agar.

Growing conditions

The master culture tubes, as obtained from Indiana University, were maintained, in darkness, at 15 C. The subcultures were maintained under the same conditions in 20 ml tubes, containing 6 ml of the basal growth medium, plus 1 % glucose, and 1.5 % agar.

Liquid pre-experiment cultures consisted of 100 ml of liquid growth medium with 1 % glucose, in oculated with a few Chlorella cells, by means of a wire loop, from the subculture tubes. These were grown in 250 ml Erlenmeyer flasks, in the light, until the cultures reached a stationary state of growth.

Experimental cultures consisted of either transferring the above light-grown cells in the ratio of one ml suspension to 20 ml of fresh medium, or centrifuging an entire light-grown flask of cells, and then resuspending the pellet in fresh medium. Glucose and proline of various concentrations, were added as required. 100 ml suspensions were always g. wn in 250 ml flasks, and usually reached their stationary phase of growth in approximately four days. 1500 ml suspensions, grown in two liter flasks, reached stationary phase of growth in approximately seven days. Small sampling, cell density, and contamination determinations, were done by pipeting one ml of cells from the flasks.

All pre-experimental and all experimental cultures were grown on New Brunswick rotary shakers, either the open Model VS, or the closed-box Model G-25. The cells grew best with vigorous shaking. The tem-

perature in all cases was maintained at 25 C. Light experiments were done with a bank of white fluorescent lamps producing 400 foot-candles at the upper surface of the cell suspensions. Complete darkness on the Model VS shaker, was obtained by fastening foil-covered boxes over the shaker.

Growth measurement

Growth was determined by a modification of the method of Myers (1946), who found this technique to be rapid and precise to within ±2 % in measuring Chlorella growth. Thrombocytocrit tubes, Van Allen type, were used. These were calibrated to give the packed cell volume (PCV) directly in µl of packed cells per ml of original cell suspension. In practice, one ml of suspension cells was pipeted into a thrombocytocrit tube. The cells were packed by centrifugation at 1400 x g for 20 minutes in an IEC Model UV centrifuge. PCV was found to be a convenient index of cellular growth. Cell growth of this unicellular alga, indicates the cell number, and does not necessarily imply that cell sizes have been increased.

PCV tubes also were used to obtain a quick indication of contamination. A sharp line between green, dense cells, and the white, fluffy material above, meant healthy Chlorella. A yellow band between the green and white layers, indicated bacterial contamination.

Determinations of proline and glucose

Exogenous proline in the cell medium, was determined spectrophotometrically by reaction with ninhydrin, according to Chinard's method (1952). Exogenous glucose in the cell medium, was determined spectrophotometrically by the dinitrosalicylic acid method of Bernfield (1955).

Administration of labeled proline to cells

Two different experiments were done. In the first, Chlorella cells from stationary phase cultures, were centrifuged and resuspended in liquid growth medium, containing 0.1 % glucose. Six aliquots of 5 ml each of this new suspension, were placed in 50 ml centrifuge tubes, which were wrapped in foil to exclude light. A tight-fitting stopper with a wire embedded in its lower surface, was fitted to each tube. A CO_2 trap consisting of filter paper saturated with monoethanolamine, was hung on the wire of each tube. Throughout the 5-hour experiment, the tubes of cell suspensions were shaken on a mechanical shaker. Exogenous proline was added to give the initial suspensions a concentration of approximately 1 µmole/ ml. Approximately one-half µCi of uniformly labeled ^{14}C-proline was added to each reaction tube. At each sampling time, an entire reaction tube was used.

In the second experiment, several changes were made. A better trap for CO_2 was constructed. A 250 ml Erlenmeyer flask, with a glass side arm and an attached 4 ml vessel, containing one ml of ethanolamine, was used. The amount of ^{14}C-U-proline/ ml of cell suspension was the same as in the first experiment. 100 ml of cell suspension was placed in the 250 ml flask. 5 ml aliquots were removed at each sampling time, so as to correspond somewhat with the first experiment.

Separation of fractions in labeling experiments

Cell suspensions, containing labeled proline, were analyzed by separating a number of cellular and cell medium components. The methods of Thompson et al. (1959) and of Thompson and Morris (1959) were followed

rather closely. Figure 5 shows the general procedures used.

At the time of sampling, the ethanolamine, containing trapped CO_2, was placed in a counting vial. Liquid and solid components of the cell suspension were separated by centrifugation at 5000 x g for 5 minutes. The pellet was extracted by the methods of Thompson et al. (1959). The insoluble residue was hydrolyzed with a one-to-one mixture of 3 N HCl and glacial acetic acid, 16 hours, in an autoclave. Although the ^{14}C labeling of the major fractions were counted, only the free neutral and acidic amino acids were paper chromatographed. The paper chromatogram spots were cut out and eluted in counting vials.

Organic acids were separated on Biorad AG-1-X-10-formate anion exchange resin. They were eluted with a 0 to 4 N formic acid gradient. 5 ml samples were collected. The TCAP organic acid standards consisted of 10 µmoles each of malic acid, succinic acid, citric acid, and fumaric acid. Standard peaks were determined by titration with 0.005 N NaOH.

Determination of radioactivity

Samples were counted in scintillation vials, with 10 ml of scintillation solution added. The solution used was that of Stewart (1972a). The $^{14}CO_2$ fractions, containing ethanolamine, received an additional one ml of methyl cellosolve.

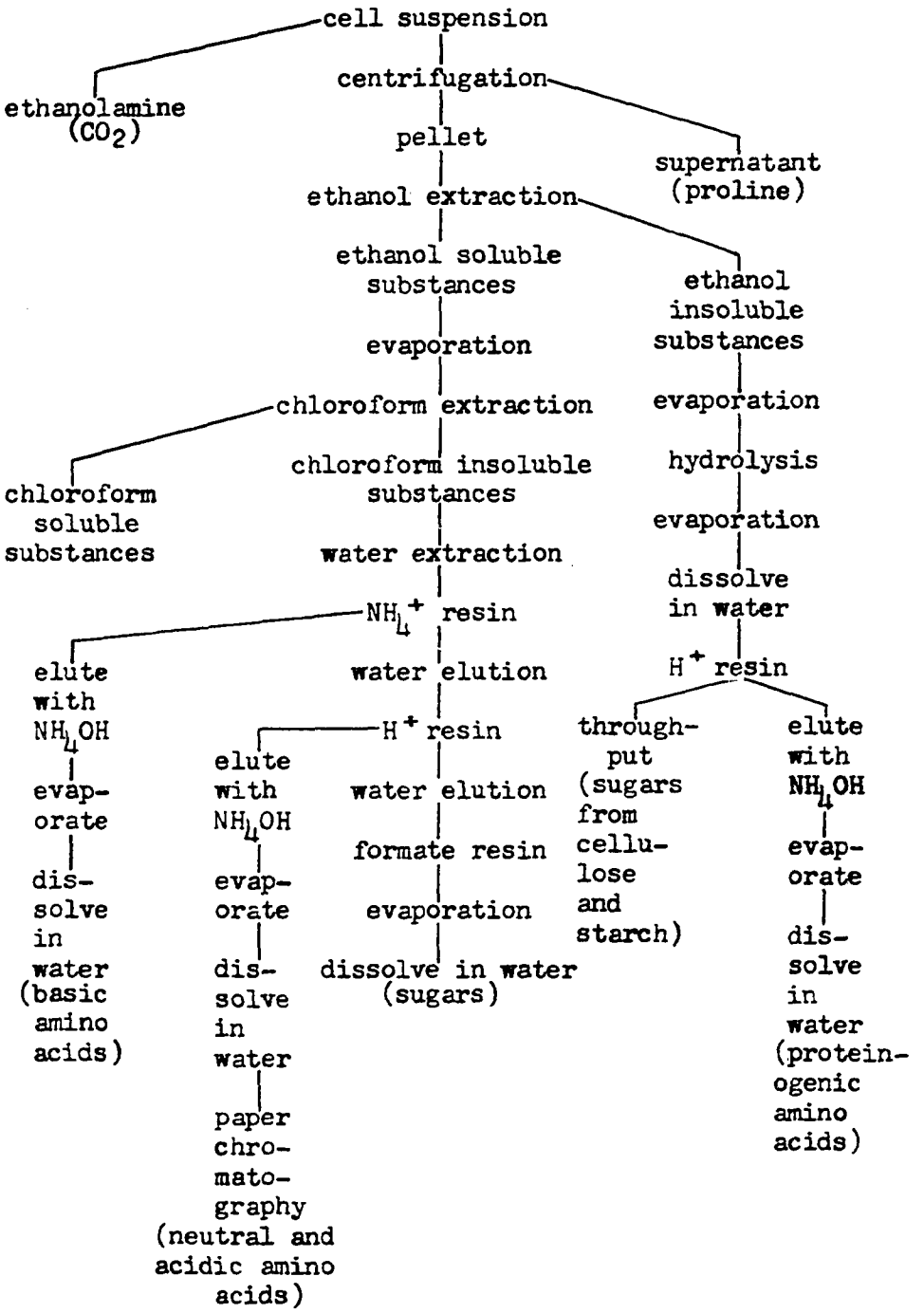


Figure 5. Flow diagram showing the general method of separation of fractions in the labeling experiments. Adapted from Thompson et al. (1959) and Thompson and Morris (1959).

RESULTS AND DISCUSSION

Uptake of proline and glucose and their effects on cell growth

In the early experiments, exogenous proline concentrations of approximately 10 μmoles/ml of cell suspension, were used. Figure 6 is typical of a number of these experiments. Chlorella cells were placed in the growth medium, containing 1 % glucose. Simultaneously, the proline was added. Cell growth, as measured by the PCV method, consistently began to level off when the proline was essentially gone. Figure 6 shows that the growth curve is like the expected ideal curve. The typical log phase has a doubling time of 12 hours. A stationary phase was reached after approximately 80 hours.

The glucose in the medium was depleted after 60 hours, somewhat before the cells entered the stationary phase. The most rapid depletion was during the period of most rapid cell growth. There was a lag in proline uptake until approximately 40 hours. At this time, the cells were growing most rapidly and the glucose was becoming depleted. As can be seen in Figure 6, proline uptake lags behind glucose depletion. All of the proline was utilized by 70 hours, which means before the cells reached their stationary phase. The above times were reproducible, with the above proline concentration.

The results shown in Figure 6 are from an experiment done in the dark. The uptake of proline and of glucose were found to be very nearly the same in the light. Table 2 shows the timed uptake of proline with respect to the presence and absence of glucose, and with respect to the presence and absence of light. Cell growth, as measured by the PCV

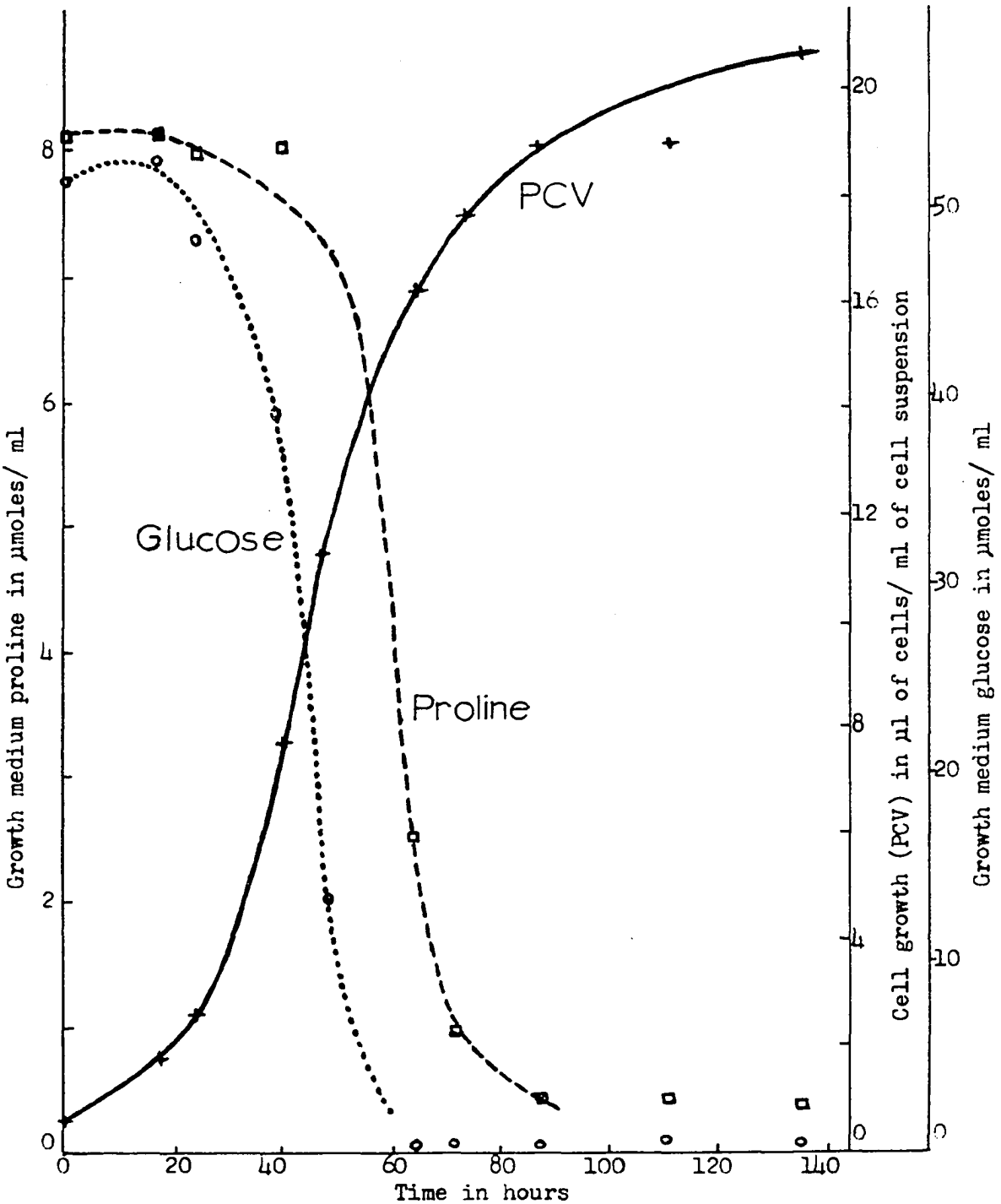


Figure 6. Relationships of proline uptake and glucose uptake with growth of Chlorella

Table 2. Relationships between cell growth, presence or absence of glucose, and presence or absence of light, with exogenous proline in the medium of Chlorella cells (PCV is in µl of cells/ml of cell suspension. External proline is in µmoles/ml. External glucose is in µmoles/ml.)

Conditions		Incubation time in hours 0	25	51	72	100
+ glucose, + light	PCV	0.6	1.8	2.1	8.1	20.6
	proline	11.4	9.7	11.5	0.3	0.3
	glucose	27.8	23.8	3.1	0.1	0.1
- glucose, + light	PCV	0.7	0.7	1.2	2.0	4.0
	proline	9.8	9.3	12.7	10.4	0.3
	glucose	0.1	0.1	0.0	...	...
+ glucose, - light	PCV	0.7	1.8	12.6	13.7	20.6
	proline	10.1	13.2	12.9	6.3	0.4
	glucose	26.1	24.0	0.1	0.0	...
- glucose, - light	PCV	0.5	0.6	0.8	0.8	0.9
	proline	10.4	12.7	11.7	0.3	0.3
	glucose	0.0	0.0	...	...	...

method, is also shown. Initial proline was approximately 10 µmoles/ml. By 100 hours, proline essentially was all taken up by the cells, regardless of light and exogenous glucose conditions. The results show that with glucose supplied, proline is taken up during the period of maximum cell growth, typically 50 to 70 hours. However, if no rapid growth occurs, the proline is eventually taken up, but generally requires longer

than 70 hours. Thus, even nongrowing Chlorella cells will ultimately take up proline.

Cell growth is markedly affected by the presence of glucose. Table 2 shows, that with glucose, cell growth went from 0.6 µl of cells/ml of suspension to 20.6 µl of cells/ml of suspension, in 100 hours, a 34-fold increase. Almost identical results occurred in the dark. Without glucose, growth was very slow, increasing about 6-fold in light and about 2-fold in the dark, in 100 hours. Thus, light affects growth but not proline uptake.

Determination of medium materials limiting cellular growth

Since the previous section has shown that glucose was needed for cellular growth, in dark-grown cells, the next question which needed to be asked, is that of what other components might be limiting in the medium at the time of glucose disappearance. The most likely possibility was that of nitrate.

Experiments were performed in which glucose and KNO_3, individually, and in combination, were added to stationary cells, to find possible effects on the algal growth. Figure 7 shows composites of the results of several experiments. The control flasks received only proline of approximately one µmole/ml of medium. All other flasks had about the same concentration of proline. As can be seen in Figure 7A, the flasks in which KNO_3, was added, had very little growth and the averaged growth curve was nearly identical to the control growth curve. By the end of 50 hours, both had PCV's of approximately 16.5 µl of cells/ml of suspension.

The addition of glucose to the starved cells, substantially increased

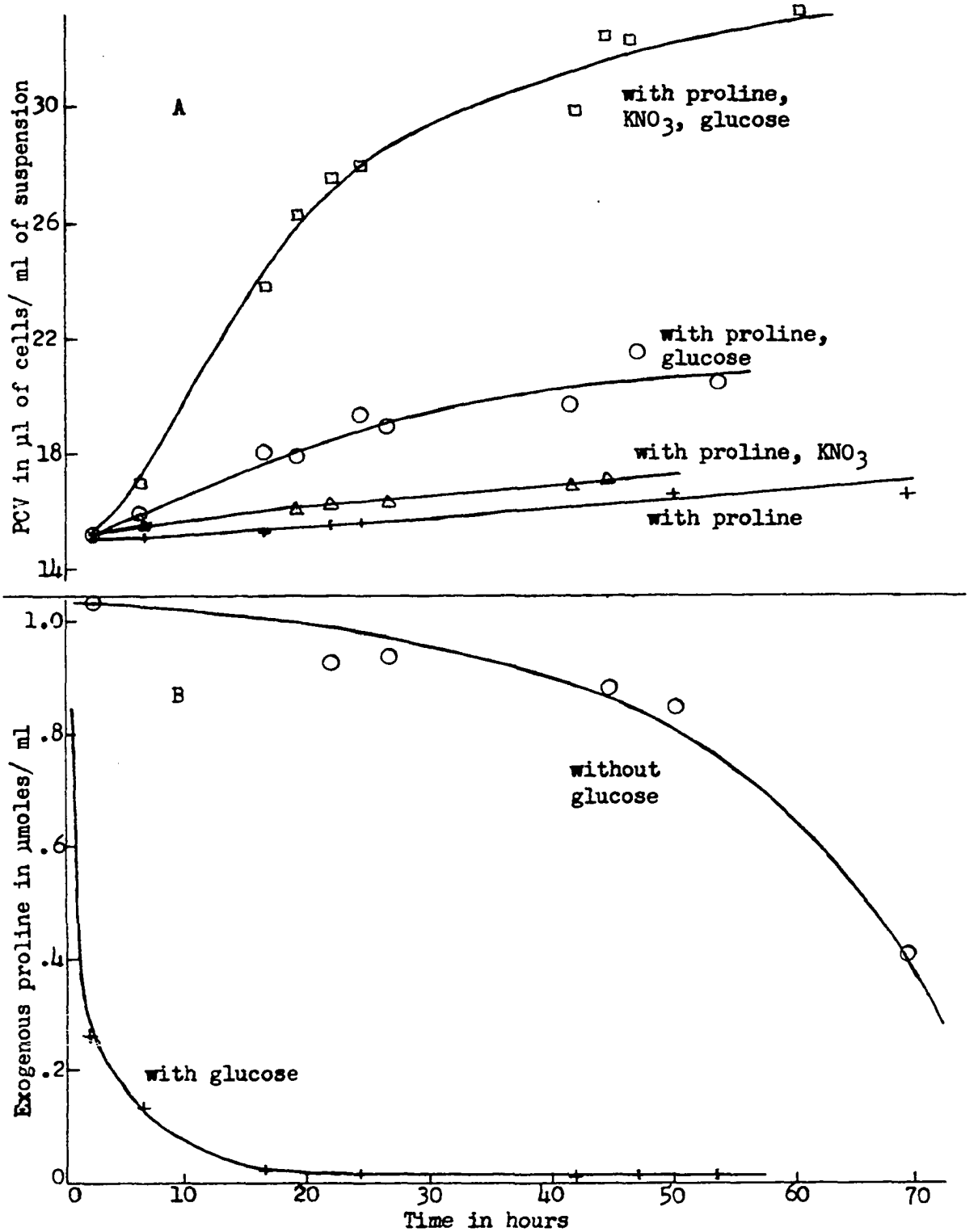


Figure 7. Effects of 1 % glucose and 12 mM KNO_3, on growth of Chlorella and on proline uptake

cell growth. The growth at the end of 50 hours, was nearly 1.25 times greater than the control. With the addition of both glucose and KNO_3, growth was still increasing at the end of 60 hours. By 70 hours, the projected growth, with glucose and KNO_3, would be more than double the growth of the control. These results show again that glucose is indeed limiting, and that nitrate is nearly limiting. With both additional glucose and additional nitrate, cellular growth is increased. This is taken as evidence that no other component in the growth medium, is limiting the growth of Chlorella in stationary cultures.

A substantially lowered initial proline concentration was used in these experiments, since the primary objective was to observe effects of glucose and nitrate on cell growth. Figure 7B shows that with the lowered proline concentration, a marked difference in the proline uptake pattern was noticed, depending on whether or not glucose was simultaneously added to the starved cells. With no addition of glucose, there was a lag of 40 hours before rapid uptake took place. However, when glucose and proline were added at the same time, the proline was rapidly taken up and was essentially gone from the medium by 15 hours.

Effects of glucose on the uptake of small amounts of proline

Since the last series of experiments indicated that the relationship between glucose and proline uptake, was more complex than at first believed, short-time experiments were conducted, using small concentrations of proline. Figure 8 shows the results of a 5-hour experiment on proline uptake, with different concentrations of glucose in the medium. Each glucose concentration was done in duplicate, with very similar results

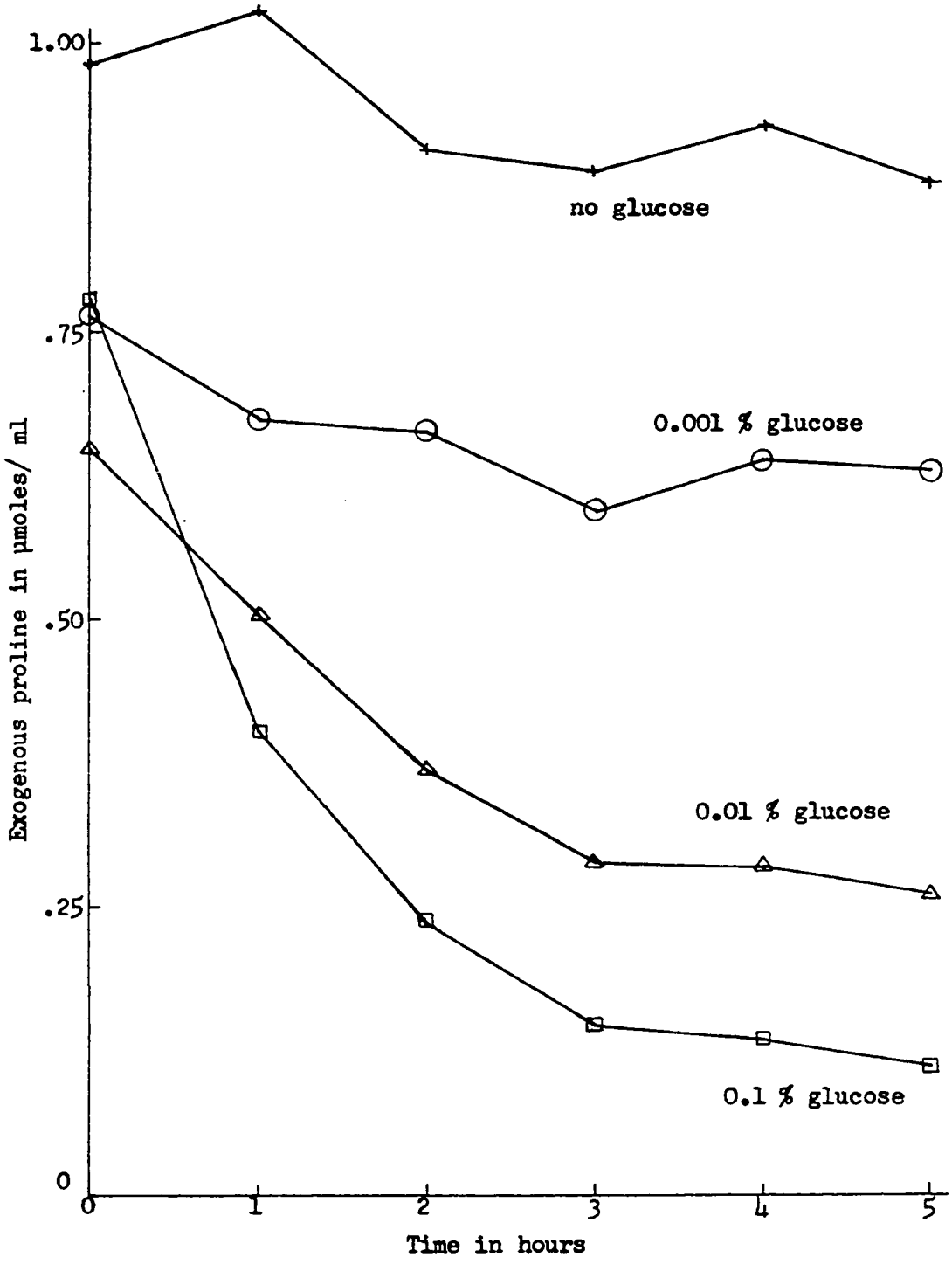


Figure 8. Proline uptake by Chlorella in media containing various concentrations of glucose

for each matching pair of flasks. Only the pair-averages are shown. The cells without glucose had nearly no proline uptake by the end of 5 hours. Cells with 0.1 % glucose, took up approximately 90 % of the exogenous proline by the end of 5 hours. Intermediate glucose concentrations resulted in intermediate rates of proline uptake. Clearly, the presence of sufficient glucose greatly accelerates proline uptake. Similar results were obtained with an experiment terminated at 22.5 hours. Presence of glucose resulted in almost complete uptake of proline by 3 hours, whereas lack of glucose resulted in no proline uptake in the 22.5 hour period.

Thus, the smaller concentration of proline, gave a finer view of the glucose-proline relationship. Previous work in which 10 µmoles of proline per ml of medium, were used, in growing cultures, had suggested inhibition of proline uptake by glucose. This was shown in Figure 6, with substantially no proline uptake until the glucose was gone. Figure 8 shows that, in stationary cultures, there is an uptake of about 0.6 µmole of proline per ml of suspension, and this required glucose. The results suggest that some proline should have been taken up during the early times shown in Figure 6. This small uptake amount was not noticeable in the previous high concentration proline experiments.

The results shown in Figure 8, led to the question of what kind of long-term proline uptake pattern might be obtained, using small amounts of proline, with one % glucose, carefully analyzed, over a longer period of time. Several experiments were done, using an initial proline concentration of approximately one µmole/ml of medium, again with dark-chamber growth. One particular experiment was done with quadruplicate

flasks, but similar experiments with less flasks, gave almost identical results. Figure 9 shows the results of the quadruplicate experiment. Glucose disappearance and growth curves were essentially the same as those shown in Figure 6 of the earlier high proline concentration experiments.

Proline typically showed a rapid initial uptake of approximately 0.3 to 0.5 μmoles/ml of suspension, within the first 10 to 20 hours. Then, there was a long lag of about 40 to 60 hours in which very little proline was taken up. At about the time that glucose had disappeared from the medium, there was a second surge of rapid proline uptake, which usually depleted the exogenous proline supply within another 10 to 20 hours. The results in Figure 9 indicate that proline did not completely disappear until after the external glucose was completely gone. Although several similar experiments gave depletion in 10 to 20 hours, Figure 9 indicates a longer time interval.

An hypothesis for the proline-glucose uptake relationship in Chlorella

An attractive explanation for the double-breaking proline uptake curve, shown in Figure 9, would be that glucose plays a dual role by being both a positive and a negative modulator. This can be best understood by means of a simple diagram. The diagram below depicts a nonquantitative proline uptake curve, obtained from Figure 9, and showing the hypothesized glucose mediation. In region A, starved cells have been introduced to fresh medium, containing both glucose and proline. Past experiments have shown that both were readily taken up. It was shown in Figure 8 that no proline uptake occurred in the first 5 hours, unless glucose was present.

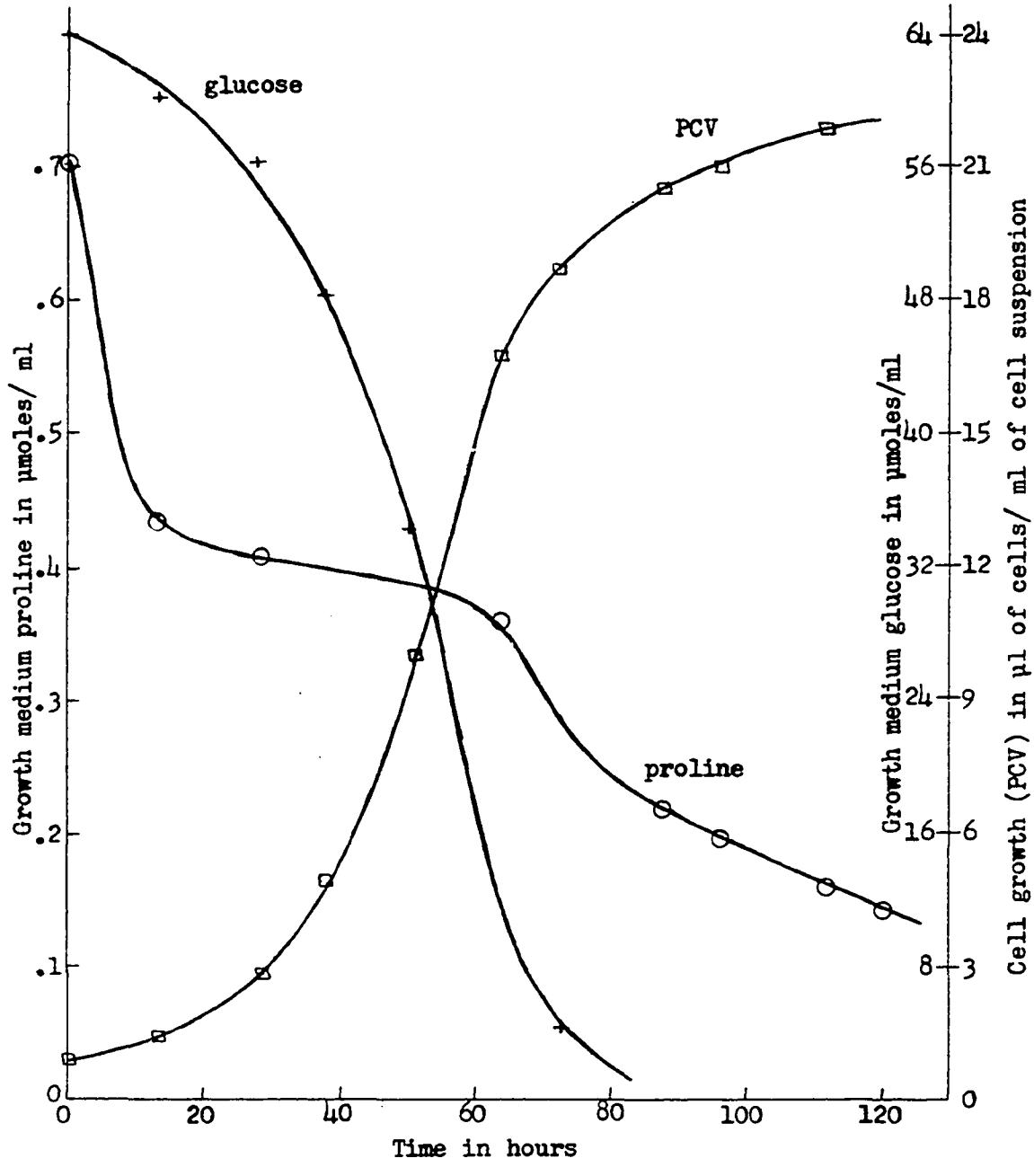
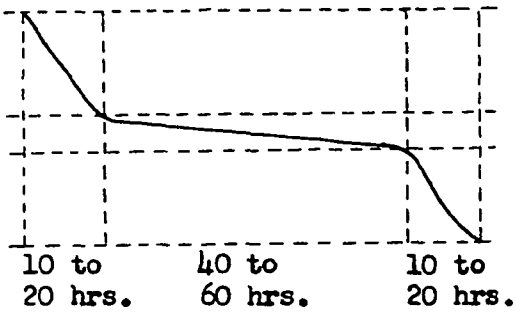


Figure 9. Relationships of proline uptake and glucose uptake with growth of Chlorella, using low initial proline concentration



A. Proline uptake

B. Proline uptake inhibited by glucose

C. Proline uptake

Thus, the glucose in the medium, in some way, would conceivably, promote the uptake of proline.

In region B, experiments have shown that considerable glucose had been taken up by the cells. The theory would be that this internal glucose inhibits the further uptake of proline. In region C, the internal glucose has been used up, thus releasing the inhibition of proline uptake. The results of Table 2 show, that given a very long time period, some proline uptake can occur irregardless of glucose involvement. Preliminary measurements of internal glucose are consistent with these ideas; however, it is difficult to accurately measure internal glucose to the exclusion of other internal carbohydrates, including hydrolyzed cell wall carbohydrates.

Thus, evidence has been presented for both promotive and inhibitory effects of glucose on proline uptake. Very careful examination of internal carbohydrate contents is needed. This possible double role of glucose is an area that needs more thorough study, but is beyond the scope of the present work, which has a central theme of proline catabolism.

Cellular use of proline that is taken up by Chlorella cells

The above experiments gave some information on growing satisfactory Chlorella cultures and some information on proline-glucose interaction. Since the experiments showed that the cells can take up large quantities of exogenous proline from their liquid growth medium, the next logical question is, what happens to this proline inside the cells? Is it simply stored? Is it oxidized? Or is it built into new protein?

To attempt to answer these questions, two labeling experiments were done, using uniformly labeled proline, added to the growth medium of the cells. The general procedures are given in METHODS AND MATERIALS. The experiment was first done with stationary (nongrowing) cells. The cells had been grown in the dark, with 1 % glucose, for 5 days. They were centrifuged, the pellet of cells was washed, recentrifuged, and finally resuspended in 0.1 % glucose. The labeled proline was added at this time. After separation of various fractions according to METHODS AND MATERIALS, the radioactive fractions were counted. Table 3 shows the raw count results and Figure 10 shows the information in percentage form; that is, each fraction's radioactivity as a percent of the total recovered radioactivity.

As can be seen from Figure 10, nearly 43 % of the recovered, labeled carbon atoms, are in the form of CO_2 by the end of 5 hours after the cells have been incubated with labeled proline. Nearly one-half of the proline taken up by starved Chlorella cells, was oxidized within 5 hours. CO_2, by one hour, accounted for over 12 % of the initial label. The hydrolysate curve of Figure 10, shows that nearly 29 % of the recovered

Table 3. Total radioactivity recovered from the various fractions of Chlorella cells, after incubation with ^{14}C-proline (Numbers indicate the actual counts per minute x 10^{-3}.)

	Incubation time in hours					
Fraction	0	½	1	2	3	5
CO_2	0.2	3.7	81.4	125.6	181.7	281.6
basic amino acids	0.6	2.3	28.1	41.0	28.7	11.8
neutral and acidic amino acids	52.6	260.1	376.7	217.8	164.5	112.0
organic acids	0.4	8.0	20.8	13.6	6.5	4.2
neutral sugars	0.3	0.7	2.1	1.2	0.9	0.7
hydrolysate	1.1	9.6	61.0	111.3	151.8	189.8
supernatant	883.3	658.0	58.1	60.4	56.6	63.6
total recovered	938.5	942.4	628.3	570.9	590.6	663.7

label is in protein. This means that a large amount of the exogenous proline carbon atoms that were taken up by starved Chlorella cells, were incorporated into protein within 5 hours. As can be seen, the neutral, acidic, and basic amino acids, reach peak concentrations in one or two hours, then decrease. Thus, the amino acids are acting as metabolic intermediates. The organic acids also behave as intermediates, reaching peak concentration between one and two hours. CO_2 and labeled protein do not peak in the five hour period. Hence, they behave as end products.

The large amount of basic amino acids, which reached a peak of over 7 % of the recovered radioactivity, in 2 hours, shows that proline is,

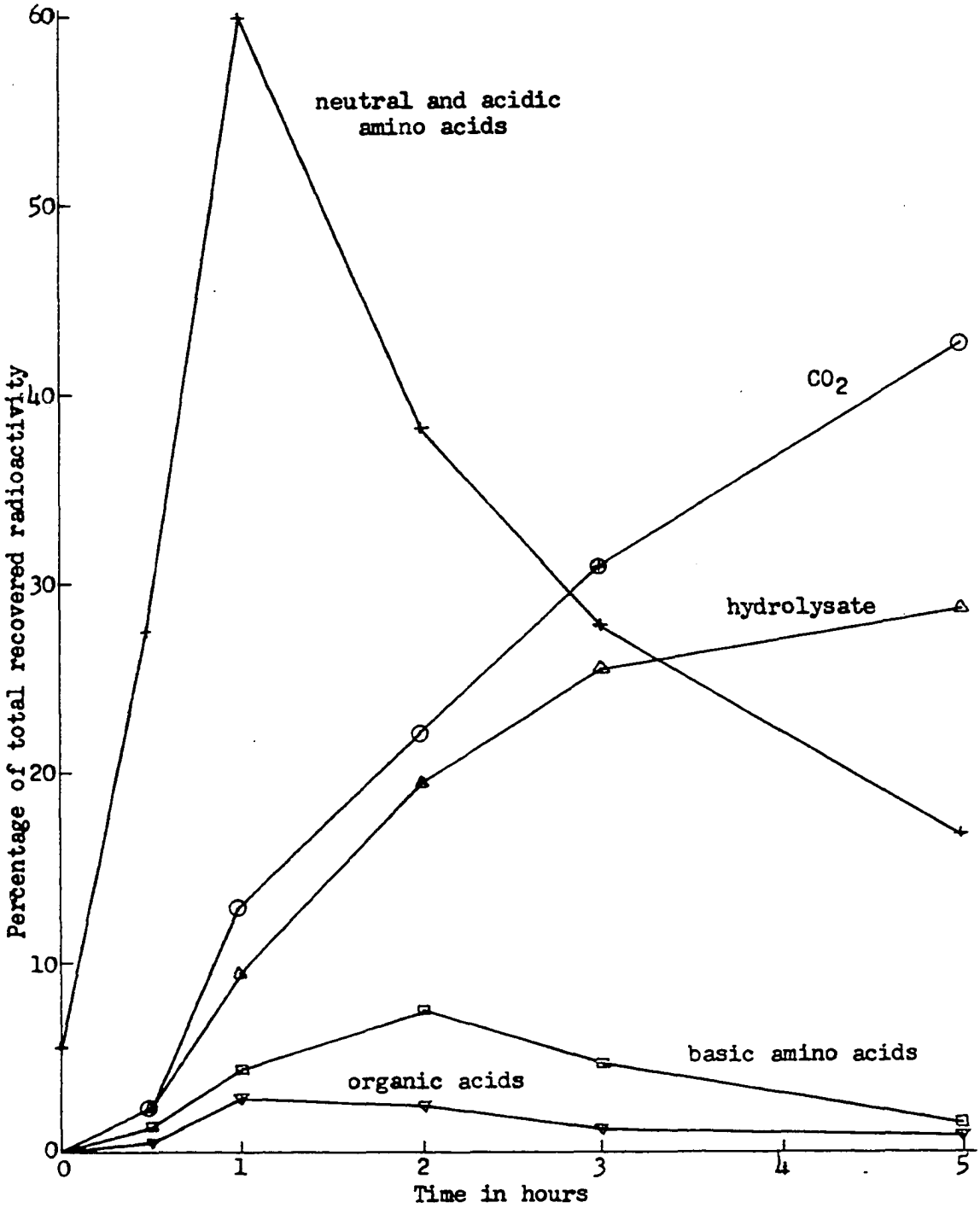
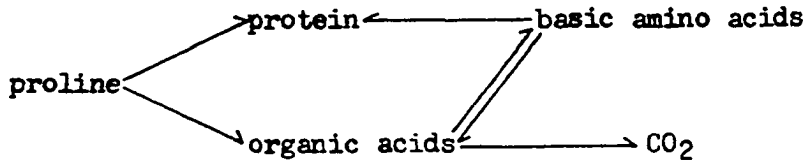


Figure 10. Percentages of ^{14}C found in various fractions of Chlorella, with respect to time, after incubation with uniformly labeled proline

at least partially, converted to other amino acids. The proposed relationships between the exogenous proline and its uses, are shown immediately below.



The labeling experiment also gave some indication of the pathway of proline oxidation. As one might suspect from the large amounts of CO_2 evolved, proline has been oxidized through the TCAP. Figure 10 shows that the organic acid fraction built up to a peak in approximately one hour, and then the total organic acid concentration decreased. Oxidation of proline by means of the TCAP was expected, since this had been shown previously to occur in corn (Wang, 1968; Barnard and Oaks, 1970; Oaks et al., 1970) and in beans (Stewart, 1972a).

To verify that the TCAP was indeed used, the organic acid fraction was analyzed by means of formate gradient elution. Labeled succinic acid and labeled citric acid, both key TCAP intermediates, were recovered in substantial amounts. Malic acid, although not prominent, was detected. From the acidic amino acids fraction, large amounts of labeled glutamic acid was also recovered.

Thus, in answer to previous questions, within 5 hours, most of the proline that was taken up, was either oxidized to CO_2, or was incorporated into protein. Only about 20 % remained stored inside the cells at the end of 5 hours, either as free proline or as other amino acids.

In an effort to further elucidate the fate of exogenous proline in

its protein and oxidative roles, a longer time labeling experiment was done. This second labeling experiment also differed from the first experiment in that actively growing cells were used. The first experiment had made use of stationary cells. In the second experiment, less fractions were separated. The general procedures are given in METHODS AND MATERIALS. Table 4 shows the label recovered from various components.

Table 4. Percentages of ^{14}C found in various fractions of Chlorella, after incubation of cells with uniformly labeled proline

time (hrs.)	CO_2 (%)	free amino acids (%)	hydrolysate (%)	organic acids (%)	neutral sugars (%)
0	0	91.7	4.5	2.4	1.4
8.3	10.8	50.1	34.7	4.0	0.5
23.5	56.6	15.0	27.3	0.8	0.3
32.3	45.6	22.4	31.5	0.5	0.2
47.8	43.3	6.9	49.4	0.2	0.2
56.3	45.5	13.0	41.0	0.4	0.2
72.5	43.3	13.3	42.9	0.5	0.2
80.0	46.6	12.3	40.4	0.6	0.2
96.0	64.2	11.4	23.6	0.5	0.3

Only the nonsupernatant, recovered radioactivity, was used in making the above table. The data have been adjusted to show the percentages on the basis of a 5 ml aliquot of sample.

By 8 hours, the label incorporated into protein, had reached about

35 %. This remained nearly constant through 80 hours. By 96 hours, the protein label had decreased markedly. This could be interpreted as meaning that the cells had exhausted their food supply, and cellular maintenance began to occur at the expense of the disintegration of other cells. Indeed, the increased percentage of label in CO_2, at 96 hours, is higher than at 80 hours. CO_2 production had an initially rapid rate until approximately 24 hours, leveled off, then experienced an increased rate beginning at around 72 hours. The initial rapid rate could be interpreted as meaning that free proline was being oxidized, while the later rapid rate could be interpreted as being oxidation of protein. The results are in agreement with those shown in Figure 6. By 96 hours, Chlorella had taken up all of its exogenous glucose and all of its exogenous proline, and growth had become stationary. This forced cell maintenance to occur at the expense of other cellular components.

As in the first labeling experiment, using stationary cells, the percentage of label in sugars was very low. Also, once again, the organic acid component showed the expected increase to a peak, then decrease, indicating that the organic acids produced from proline, were quickly oxidized through the TCAP. One might also note that, by 96 hours, more than 64 % of the nonsupernatant ^{14}C, had been recovered as CO_2. This indicates the existence of a very decisive oxidative pathway for proline catabolism in Chlorella.

PART II.

THE NATURE OF PROLINE CATABOLIC ENZYME ACTIVITY IN *CHLORELLA*

INTRODUCTION

PART II is concerned with the purification and characterization of the first enzyme of proline catabolism. The principal problems to which answers were sought, were (1) an assay for this first enzyme, (2) the nature of the oxidizing substance, (3) enzyme purification, (4) the immediate product, and (5) preliminary characterization of the enzyme.

As discussed in detail in the LITERATURE REVIEW, nearly all evidence of the past 25 years, has been in favor of P5C being the first intermediate of proline oxidation. Determining the product for Chlorella, was the primary goal of the investigation presented in this text. In the search for this key intermediate, P5C was given prime consideration. However, searches for other compounds, were also made.

The LITERATURE REVIEW has demonstrated, that in animals and many microorganisms, the first enzyme of proline catabolism is an oxidase. A molecular oxygen requirement has been found. However, three living systems, the bacterium Clostridium, and the plants peanut and wheat, were reported to have an NAD oxidant requirement, and not an oxygen requirement. The second most important goal of this investigation, has been to determine whether NAD, oxygen, or some other oxidant, is involved in the first catabolic reaction.

Purification of the enzyme was not carried out as far as desired. Nevertheless, a number of important facts about the characterization of the enzyme, have been established. Restrictions imposed by an impure enzyme extract, have prevented certain aspects of characterization, from being completed. Preliminary work with higher plants has been presented.

The last section of PART I, indicated the existence of a proline oxidation mechanism, which quickly metabolized proline to TCAP intermediates. A thorough examination is needed of all of the intermediates and enzymes between proline and the TCAP. It is hoped that PART II will at least shed some light on the most obscure part of the pathway, namely the reaction of proline to the first intermediate.

METHODS AND MATERIALS

Enzyme extraction and purification

Chlorella cells were grown as described in PART I. When the cells had reached approximately maximum growth, they were processed according to the scheme shown in Figure 11. Use of a manually operated Aminco French pressure cell gave better disintegration of cells than could be obtained by hand grinding with abrasives, freezing and thawing, sonification, or use of various mechanical grinding and disintegration equipment. Routinely, better enzyme activity was obtained when the extract was put through the French pressure cell a second time. A Branson sonifier, Model L S75, gave fair disintegration, but proved to be difficult in giving uniform results with different batches of Chlorella. Acetone powder extractions and $AmSO_4$ disruption, were unsuccessful, unless the cells were first passed through the French cell. Acetone powder results were generally poor in all situations.

The chelator, Na_2EDTA, and the protective agent, DTT, have been widely used in the extraction of enzymes, since they often result in greater enzyme stability. Various concentrations of both Na_2EDTA and DTT were examined for possible enhancement of proline dehydrogenase activity. It was observed that they consistently gave better enzyme activity. They were found to be effective in final concentration ranges of 0.3 to 1.0 mM. These concentrations were used routinely in extraction media. DTT was obtained from Sigma (St. Louis). Trial and error showed that the use of 0.05 M phos. (pH 7.2) as an extraction medium, gave optimum enzyme activity.

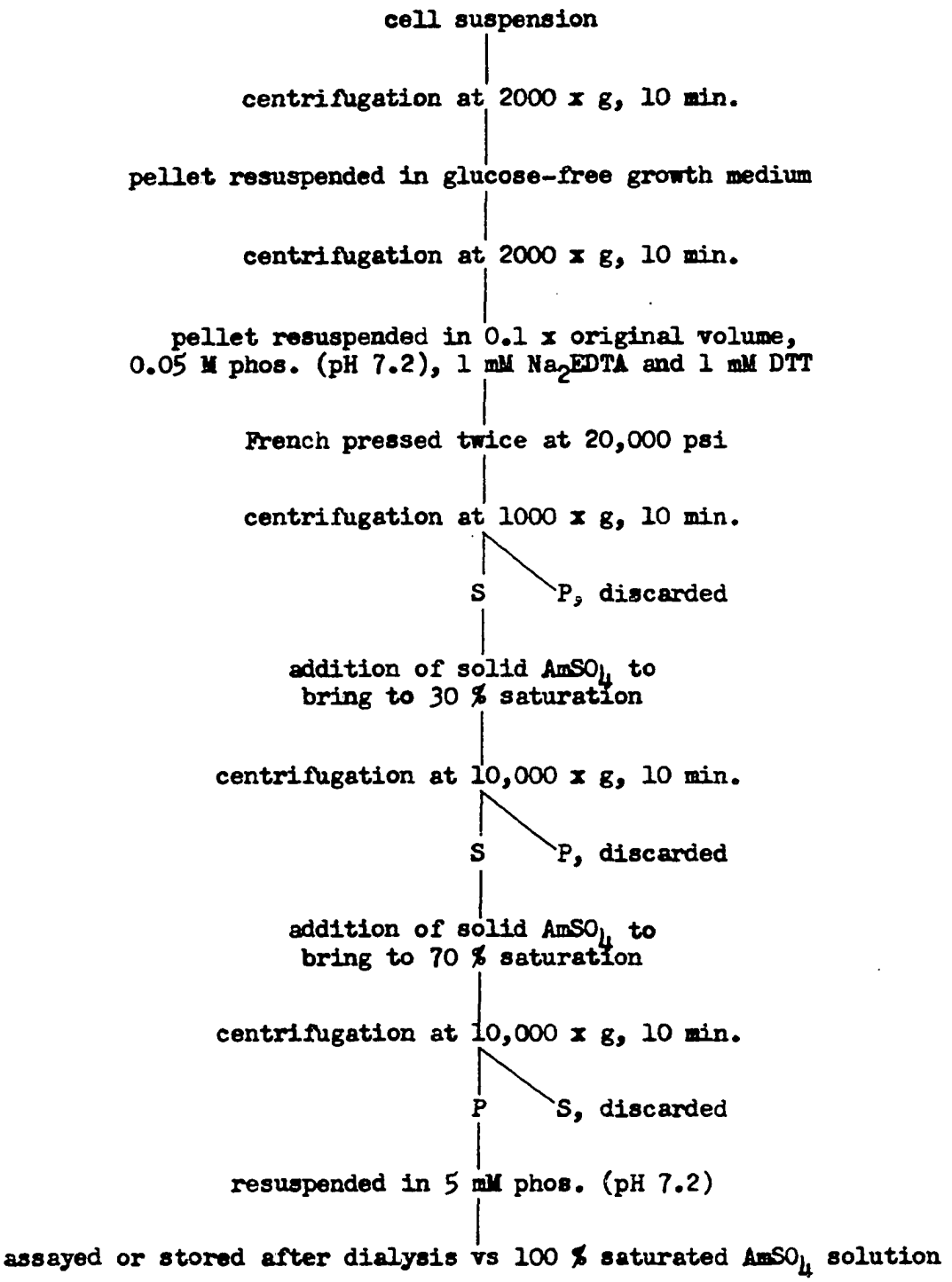


Figure 11. Method of harvesting Chlorella cells, and extraction of a crude enzyme fraction with proline dehydrogenase activity

The pH was adjusted to approximately 7.2, at various stages, by addition of 2 M AmOH. From the time of French pressing onward, the cell extracts were kept chilled on ice, or the operations were done in a coldroom with a temperature range of 3 to 10 C. A rather broad 30 to 70 % $AmSO_4$ cut was used because of variability in Chlorella batches. Use of higher centrifugation speeds proved unnecessary in isolating the desired enzyme activity. The crude enzyme preparation, was either used directly, or was concentrated and preserved by dialyzing against a 100 % saturated $AmSO_4$ solution in 0.05 M phos. (pH 7.2). This was preferred over storage by freezing, since $AmSO_4$ dialysis, also removed a considerable amount of water, without damaging the enzyme activity.

Due to the enormous amount of chlorophyll in the final supernatant fraction shown in Figure 11, numerous techniques were attempted to remove this spectroscopically interfering pigment. The most successful method, by far, was that of passing the enzyme extract through columns of DEAE-cellulose. This also resulted in further purification of the desired enzyme activity. Figure 12 shows the general scheme of DEAE-cellulose work. Approximately 5 ml of crude extract was loaded on a column for each run. Experimentation showed that proline dehydrogenase adheres to the cellulose under the influence of 0.05 M phos. (pH 7.2), and is completely removed with 0.2 M phos. (pH 7.2). Before use, the cellulose columns were equilibrated with 0.05 M phos. (pH 7.2). The actual threshold removal concentration was approximately 0.12 M. Presence of $AmSO_4$ caused some variation in the removal concentration. Where required, enzyme extracts were desalted by passing through a Sephadex G-25 column.

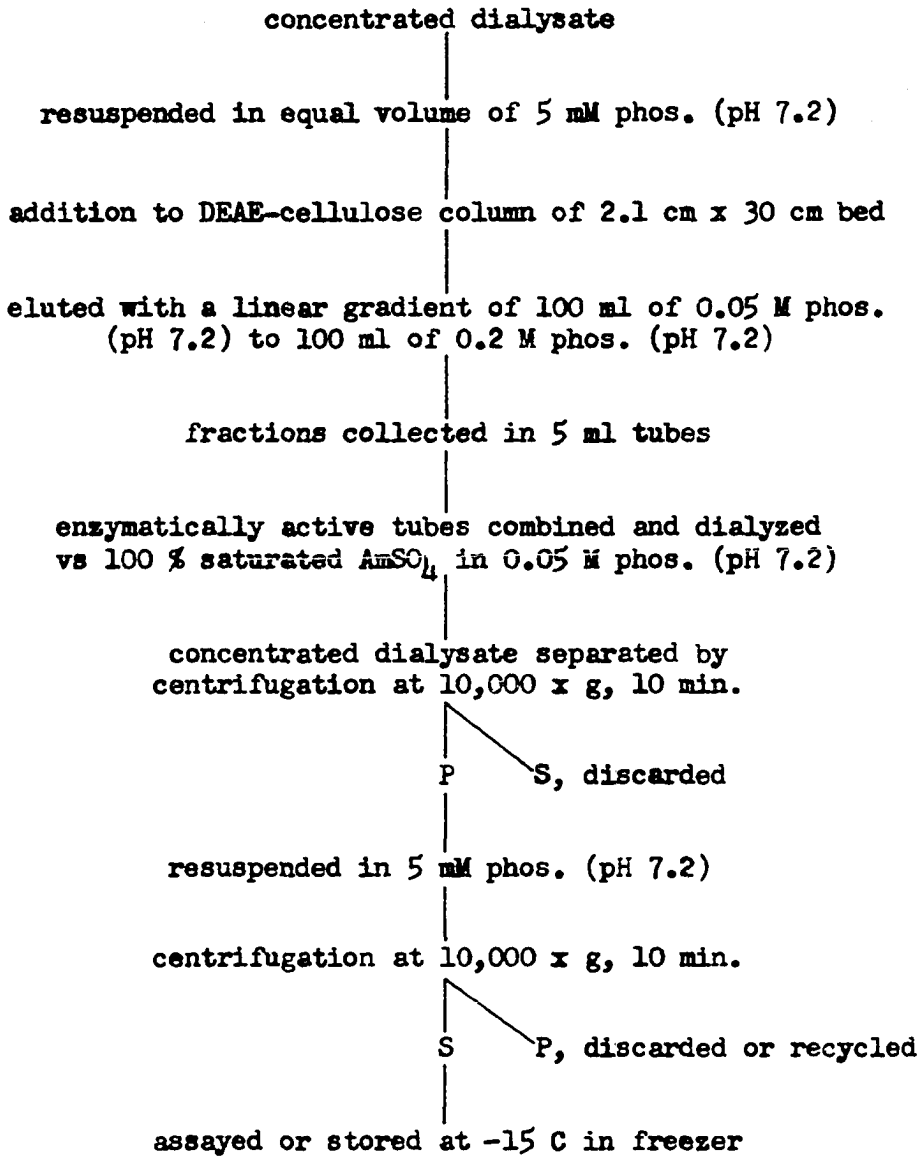


Figure 12. Method of removing chlorophyll and further purifying Chlorella proline dehydrogenase

DEAE-cellulose beds were of 2.1 cm ID, and ranged from 15 to 35 cm in height. Hydrostatic operating pressure was maintained at 40 cm. 5 ml fractions were collected with a fraction collector. Fractions which had proline dehydrogenase activity, were combined and saved. The activity of proline dehydrogenase in phos. (pH 7.2), at refrigerator temperature, declined considerably in a few days. It was necessary to preserve the enzyme with $AmSO_4$. Pigmented cellulose was removed from the bed tops periodically, and reprocessed.

Attempts to apply the above identical techniques to pea proline dehydrogenase, were unsuccessful. Output activity was very low compared with the activity of material loaded onto the column. The remaining activity was quickly lost when dialyzed against $AmSO_4$.

Assay for proline dehydrogenase by means of reduction of NAD

The proline dehydrogenase assay of Mazelis and Fowden (1971) was used, with slight modifications. A typical reaction mixture contained 2.5 ml of 0.2 M carb. (pH 10.2 to 10.5), 0.1 ml of NAD solution, 0.2 ml of enzyme extract, and 0.2 ml of 0.1 M proline solution. The reaction was started by the addition of the proline. Further modifications are described, as used, in RESULTS AND DISCUSSION. Assays were all done at room temperature, which fluctuated somewhat. Temperatures are usually given in RESULTS AND DISCUSSION, where critical.

The carbonate buffer was prepared from stock solutions of anhydrous Na_2CO_3 and $NaHCO_3$, and mixed to desired concentrations and pH's, according to the methods of Gomori (1955). NAD stock solutions of 10 mg/ml, 20 mg/ml, and 40 mg/ml, were of concentrations 14.3 mM, 28.6 mM, and

57.2 mM, respectively. 0.1 ml of NAD stock solution in 3.0 ml cuvet mixtures, gave initial reaction concentrations of 0.48 mM, 0.95 mM, and 1.91 mM, respectively. Proline stock solutions were always of 0.1 M. They were generally in 0.2 M carb., with the final pH carefully adjusted to 10.2. 0.2 ml of proline stock solution in 3.0 ml cuvet mixtures, gave an initial reaction concentration of 6.7 mM.

The reaction of proline to its first product, was followed spectroscopically, by measuring the reduction of NAD to NADH, at 340 nm, using either a Beckman DK-1A or a Beckman DB-G spectrophotometer. The principle involved was that of NADH having greater optical absorbance at 340 nm, than does NAD. Recorder slopes were converted to optical absorbance changes, and then to mμmoles of product per minute, by use of Beer's law, and from the acceptor extinction coefficient of NAD•NADH of 6.22 cm^2/mmole at 340 nm. Most results are expressed in mIU. One International Unit of enzyme activity, is defined by the relationship:

one IU ≡ one μmole of product formed per minute.

Hence, one mIU/ml would be equivalent to one mμmole of product formed per minute per ml of extract. Generally, the results reported, were the initial reaction rates, obtained in the first one or two minutes.

Anaerobic assays

Anaerobic experiments were done either with 19.5 cm OD Thunberg tubes, or with a square anaerobic spectrophotometer cuvet of one cm light path. Proline dehydrogenase assay mixtures and procedures were the same as those previously described. The general scheme was to place the substrate in the side chamber of the tube or cuvet, and the remain-

ing ingredients in the main chamber. The chambers were evacuated by aspiration. Nitrogen was forced in, with a total of three aspirations and three nitrogen flushings. A final nitrogen blanket was left in the gas-tight vessels. After the spectrophotometer was stabilized, the substrate was tipped into the main chamber, and mixed, to start the reaction.

The oxygen content in the reaction vessels, after thrice aspirating and nitrogen flushing, was tested by use of glucose oxidase. The enzyme, EC 1.1.3.4, is known to use molecular oxygen in aqueous solution. The commercial Glucostat prepared reagent of Worthington (Freehold, N. J.), was used according to its accompanying directions.

Assay for P5C by complexing with oAB

The immediate product of proline oxidation, P5C, was trapped by complexing with oAB, essentially according to the methods of Vogel and Davis (1952) and of Strecker (1957, 1965). This was the second successful assay found for Chlorella proline dehydrogenase. The P5C assay had the advantage over the NAD reduction assay, in that interference by other enzymes has a much lessened effect.

A typical reaction mixture consisted of 2.4 ml of 0.2 M carb. (pH 10.2 to 10.5), 0.1 ml of NAD solution, 0.3 ml of enzyme extract, and 0.2 ml of 0.1 M proline (pH 11.0). NAD concentration was generally 10, 20, or 40 mg/ml of water. The 3.0 ml mixture was incubated for 30 minutes at room temperature. When desired, the reaction was run until the maximum NADH absorbance change, at 340 nm, was recorded on a spectrophotometer chart. One ml of protein denaturing solution was then added. This solution consisted of 20 % TCA (w/v) and ½ % oAB (w/v) in 95 % ethanol.

After thorough shaking, the 4.0 ml mixture was incubated 30 minutes at room temperature, to allow any enzymatically produced P5C, to complex with oAB, and to allow protein to become denatured and precipitated. After centrifugation at 2000 x g for 10 minutes, the supernatant was carefully removed. The absorbance of the supernatant, at 443 nm, was spectroscopically determined. The complex was found to be yellow only at low pH, and was completely colorless at alkaline pH.

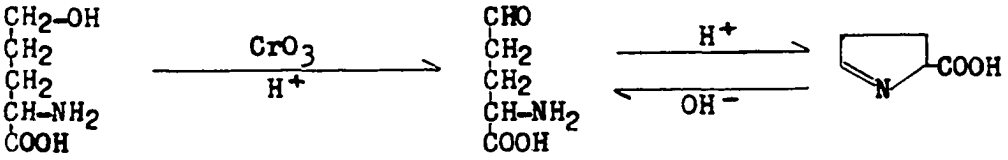
The oAB was obtained from Sigma (St. Louis). Care was taken to insure complete dissolving. Final solutions had to be clear for proper results. Absolute ethanol or $HClO_4$ solutions, of oAB, were occasionally used to stop the enzymatic reactions, and to deproteinize the mixtures. Strecker (1965) found the extinction coefficient of the P5C-oAB complex, to be 2.71 cm^2/mmole at 443 nm. He had found that the absorbance at 443 nm was linearly related to the amount of P5C complexed with oAB. The extinction coefficient, the absorbance change, and Beer's law, were used in all cases where P5C was quantitatively determined.

Synthesis of P5C

Standard P5C/GSA was synthesized according to the method of Stewart (1967). This method is similar to the synthesis of α-aminoadipic acid-δ-semialdehyde, as given by Jones and Broquist (1965). α-aminoadipic acid-δ-semialdehyde, whose structure is $CHO-(CH_2)_3-CHNH_2-COOH$, differs from GSA by having one more methyl group in the chain. Since Stewart's method (1967) of synthesis of P5C/GSA, is not known to have appeared in published form, it will be given here in entirety. 40 mg of DL-α-amino-δ-hydroxyvaleric acid (AHV), obtained from Cyclo Chemical Co. (Los Angeles),

and 20 mg of CrO_3, were dissolved in 5 ml of 4 N HCl. After heating in a water bath at 40 C, for 16 hours, a crude mixture, containing P5C, was obtained. As will be shown in RESULTS AND DISCUSSION, the presence of P5C was verified by paper chromatography, by the oAB complex's absorption spectrum, by ninhydrin complexing, and by use of the synthetic P5C as a substrate for P5C reductase.

Under the conditions of low pH and being in aqueous solution, it is believed that equilibrium favors P5C over GSA. The expected reaction is:



(AHV) (GSA) (P5C)

Further purification was found to be unnecessary for the purposes needed. The synthetic P5C was stored at refrigerator temperature, in the synthesis vessel, and was found to be unchanged after several months. The actual pH remained at less than 0.5. One batch of synthetate was still enzymatically active, when finally used up, nearly six months after synthesis. At time of use, the synthetic mixture was neutralized to pH 7, with NaOH solutions. The blue-green $Cr(OH)_3$ precipitate was then removed by centrifugation at 10,000 x g for 10 minutes. C. R. Stewart (unpublished findings) has estimated that the original synthetate contained approximately 2 mg of P5C/ml.

Synthesis of P2C

Standard P2C was synthesized by the methods of Burton (1955). The

reaction is by oxidation of D-proline with D-amino acid oxidase:

$$\text{D-proline} \xrightarrow[\text{D-amino acid oxidase}]{O_2} \text{P2C} + H_2O_2$$

H_2O_2 is removed by the action of catalase:

$$H_2O_2 \xrightarrow[\text{catalase}]{} H_2O + \tfrac{1}{2}O_2$$

D-proline was obtained from Calbiochem. D-amino acid oxidase and catalase were obtained from Sigma (St. Louis). Standard Warburg apparatus techniques were used.

As discussed in the LITERATURE REVIEW, P2C is in equilibrium with α-keto-δ-aminovaleric acid. As with synthetic P5C, further purification was found to be unnecessary for the purposes needed. As will be shown in RESULTS AND DISCUSSION, the synthetic P2C was identified by its paper chromatography migration properties, and by the complexing of the resulting spots with ninhydrin and oAB. The synthetic P2C mixture was stored in a refrigerator, without further preservation. It was found to be unchanged after several months. The synthetate was used directly without further purification or processing.

Calculations, using the concentrations and amounts involved, by C. R. Stewart (unpublished findings), gave an estimated 33 μmoles of P2C per ml of final synthetate. oAB complexing, determination of the absorption at 443 nm, and using P5C's extinction coefficient of 2.71 cm^2/mmole as a rough estimate, showed that 33 μmoles/ml was very close to reality.

Identification of P5C and P2C

As discussed in the LITERATURE REVIEW, identification of P5C and P2C, requires several criteria. The primary identification technique

used, was that of complexing the Δ^1-pyrroline carboxylates with oAB, as discussed previously. Both P5C-oAB and P2C-oAB have identical, clear, yellow colors. Preliminary work with 2,4-DNPHones, was not promising, so this complexing technique was discontinued. A third complexing process, reaction with ninhydrin, was used. The P5C-ninhydrin complex was found to be bright pink (Strecker, 1960a), and the P2C-ninhydrin complex was found to be purple.

Paper chromatography, employing a variety of solvent systems, was used in identification. The solvent system which proved most successful, was the descending chromatography, 4:1:5 (by volume) BAW system used by Mazelis and Fowden (1971). Whatman # 1 and Whatman # 3 chromatography papers were used. Both synthetic pyrroline carboxylates and enzymatically produced products, were chromatographed. Occasionally, oAB complexes were chromatographed. Amino acid standards used, were proline (2 µmoles/ml), TAB (2 µmoles/ml), and glutamate (2 µmoles/ml). When Rf estimations were desired, the chromatograms were run until the solvent front was nearly to the end of the paper. Where maximum separation was desired, solvents were usually run off the paper. After drying, the chromatographic spots were usually treated with 1 % oAB (w/v) in 95 % ethanol, or with 1 % ninhydrin (w/v) in n-butanol.

Identification of P5C as the product of proline dehydrogenase

The P5C assay described previously, was used in identifying the product of proline dehydrogenase. Generally, both the number of µmoles of NADH produced, and the number of µmoles of P5C produced, were determined. Paper chromatography was performed as described in the previous

section. The labeling experiments were performed in reaction vessels containing 0.066 ml of NAD (40 mg/ml), 0.010 ml of nonlabeled 0.1 M proline (pH 11.0), 0.4 ml of uniformly labeled ^{14}C-proline (NEN, Boston) of various activities, and 0.033 ml of enzyme extract. The labeled proline had been evaporated to dryness, and resuspended in 0.05 M carb. (pH 11.0). The low salt content was used to lower the amount of salt streaking, for purposes of paper chromatography.

The reaction mixtures were incubated one hour at room temperature, and were timed from the addition of enzyme. Vessel A received 0.05 ml of synthetic P5C, processed as described previously. Sufficient 95 % ethanol was added to each vessel to bring all final volumes to one ml, and to terminate all enzymatic reactions. After shaking for one minute, the vessels were left at room temperature for one-half hour. After chilling for a few minutes on ice, the mixtures were centrifuged at 2000 x g for 10 minutes. Supernatants were carefully removed, and one-fourth of each volume was spotted on Whatman # 3 chromatography paper. On top of each spot, 4 µmoles of unlabeled proline was added, for the purpose of making proline more visible after the later ninhydrin treatment. 0.25 ml of the processed P5C was placed on each spot, also for better later visualization.

Chromatography was terminated at 62 hours. When visualization of the amino acid-ninhydrin complexes, was complete, the chromatograms were cut into one cm, and larger, segments of uniform length, and eluted into counting vials. Counting procedures were the same as used in PART I, with the paper chromatograms counted directly in the toluene base solvent.

Assays for other enzymes involved in proline metabolism

P5C reductase was assayed according to Stewart (1967). 2.5 ml of 0.02 M carb. and phos. buffers of various pH, 0.2 ml of NADH, and 0.1 ml of enzyme extract of various dilutions, were added to the same cuvet. The stock concentration of NADH was 1.4 mM (1 mg/ml). The initial reaction concentration was 0.1 mM. The reaction was followed spectrophotometrically, by the oxidation of NADH, through the decrease in absorbance at 340 nm. After stabilization, 0.3 ml of the P5C substrate was added. P5C was prepared as described earlier.

The final number of µmoles of NADH oxidized per minute, was obtained by subtracting the number oxidized without P5C, from the number oxidized with P5C, and by use of the extinction coefficient of 6.22 cm^2/mmole. Since the enzymatic determination of P5C reductase is coupled to NADH oxidation, and the theoretical yield is one µmole of P5C reduced for each µmole of NADH oxidized, the final results are expressed in mIU/ml of extract. When activity was very high, dilution of the extract was necessary.

P5C dehydrogenase was determined as was proline dehydrogenase, except that synthetic P5C was used as substrate, instead of proline. The usual assay consisted of 2.5 ml of buffer, 0.1 ml of NAD (40 mg/ml), 0.2 ml of enzyme extract, and 0.2 ml of P5C. Various buffers were used as described in RESULTS AND DISCUSSION. The reaction was followed spectrophotometrically at 340 nm. NADH oxidase activity was assayed spectrophotometrically at 340 nm. The usual reaction mixture, consisted of 2.6 ml of 0.2 M carb. (pH 10.2), 0.2 ml of enzyme extract, and 0.2 ml of NADH.

Determination of the properties of proline dehydrogenase

The pH work was done primarily with an Instrumentation Laboratory Model 265 pH meter. A micro combination electrode was used directly in cuvet mixtures. pH determinations were made at the end of reaction periods. The pH meter was standardized at pH 10.0. Inhibition studies generally were done with an assay mixture containing 2.4 ml of 0.2 M carb. (pH 10.5), 0.1 ml of NAD of 10 mg/ml concentration, 0.2 ml of enzyme extract, and 0.1 ml of inhibitor of various concentrations. These were added to both the sample and reference cuvets. After spectrophotometer stabilization, 0.2 ml of water was added to the reference cuvet and 0.2 ml of 0.1 M proline (pH 10.5) to the sample cuvet.

Substrate assays were the usual NAD reduction assay with various substrates of 0.1 M concentration used in place of proline. Cofactor assays were done in the same manner, with NADP used instead of NAD. The NADP was obtained from Calbiochem. Stock concentration was 10 mg/ml, which gave an initial reaction mixture concentration of 0.44 mM. Metal cofactors of 0.1 M stock concentrations were added in the same manner as inhibitors.

Km determinations were done by means of the NAD reduction assay for proline dehydrogenase. 0.2 M carb. (pH 10.5) was the buffer used in all determinations. NAD stock was 10 mg/ml (14.3 mM) for Km of NAD, and 20 mg/ml (28.6 mM) for Km of proline determinations. 100 mM proline (pH 11.0) was the stock solution for proline Km use. 0.2 ml of enzyme extract was used in every sample, with the final assay volume being 3.0 ml. Fisher λ pipets were used to dispense the components, for maximum accu-

racy. The temperature for all assays was 33 C.

Molecular weight estimations were done with glass columns of various sizes, in a coldroom. Both Bio-Gel P-100 and Sephadex G-100 were used. Constant hydrostatic pressures were maintained by use of a Mariotte flask. The eluting buffer in all cases, was 0.2 M phos. (pH 7.2). Downward elution was used. A one % aqueous solution (w/v) of Blue Dextran T-2000 (Pharmacia) was used to determine void volumes. Generally, two to five ml of enzyme extract was applied to the column. A fraction collector was used to collect eluent samples. Procedures used were those of Mazelis and Fowden (1971), and of the brochures of the companies selling Bio-Gel (Bio-Rad) and Sephadex (Pharmacia).

Inducibility experiments involved cells grown as in PART I, with proline added at the time of final resuspension of the centrifuged cells. Duplicate flasks were used for each proline concentration. The cells were then grown for four days in darkness. At the time of harvest, the duplicate flasks were combined and thoroughly mixed. The regular harvest and extraction procedure, through two French pressings, and low centrifuging, were completed, as shown in Figure 11. Final supernatants were assayed by the NAD reduction method.

RESULTS AND DISCUSSION

Assay for proline dehydrogenase by means of reduction of NAD

Initial attempts to assay for the first catabolic enzyme of proline, involved the use of a Yellow Springs Instrument Comp. O_2 electrode, Model 53. As discussed in the LITERATURE REVIEW, previous investigators had successfully used both the Warburg apparatus, and the oxygen electrode, in demonstrating the oxygen requirement for the proline to P5C enzyme, in animals and in various microoganisms. Use of the oxygen electrode, with Chlorella extracts, gave no success. This was surprising, since the oxygen electrode is considerably more sensitive than the Warburg apparatus, which had been used successfully by many workers with other kinds of living systems. Early attempts to use the oAB complexing assay, discussed later, were negative, due to a variety of problems which had to be overcome.

The first successful assay for Chlorella proline dehydrogenase, was the NAD reduction assay of Mazelis and Fowden (1971). They had found that the use of a high pH system, gave maximum detectable plant proline dehydrogenase activity. This high pH assay is believed to be successful, due to the lessened effects of interfering enzyme activities at alkaline pH's. Evidence for this will be given later. Repeated attempts to assay proline dehydrogenase at neutral pH's, were unsuccessful. This was in sharp contrast to the excellent results obtained with crude enzyme extracts from animals and microoganisms, as discussed in the LITERATURE REVIEW.

Other buffers, such as CAPS, borate, phosphate, and tris, did not

give as consistent results, as were obtained with carbonate buffer. Since CAPS was the buffer system of Mazelis and Fowden (1971), it was carefully evaluated against other buffer systems, for Chlorella proline dehydrogenase. Table 5 shows the results of a comparison of carbonate buffer with

Table 5. Comparison of carbonate and CAPS buffers, in the assay of Chlorella proline dehydrogenase
(The assay mixtures also contained 0.3 ml of enzyme extract and 6.7 mM proline, to give a final volume of 3.0 ml.)

buffer	amount of buffer (ml)	initial NAD concentration (mM)	enzyme activity (mIU/ml)	number of runs
0.2M carb. (pH 10.2)	2.45	0.24	30.1	2
0.2M carb. (pH 10.2)	2.40	0.48	41.6	4
0.2M carb. (pH 10.2)	2.35	0.72	28.9	2
0.2M carb. (pH 10.2)	2.30	0.95	29.9	2
0.2M CAPS (pH 10.3)	2.45	0.24	11.6	2
0.2M CAPS (pH 10.3)	2.35	0.72	23.6	2
0.2M CAPS (pH 10.3)	2.30	0.95	11.8	3

CAPS buffer.

As can be seen, repeated trials established the superiority of carbonate buffer for assaying Chlorella dehydrogenase. It was also on the basis of the results of Table 5, and of similar experiments, that the decision was made to use 0.48 mM initial reaction concentrations of NAD. Lower NAD concentrations, gave lower activities. This was the NAD concentration used in most later experiments. No activity was obtained unless NAD

was in the reaction mixture. Activities with carbonate buffers of various pH, will be discussed later. Through trial and error, it was found best to extract with phos. (pH 7.2) buffer, but to assay with carb. (pH 10.2 to 10.5) buffer. Extraction or storage in high pH buffers, resulted in immediate loss of activity.

Figure 13 shows the linearity of Chlorella proline dehydrogenase activity, as compared with the amount of enzyme extract. The extract used was quite crude. The excellent linear relationship is evidence that NAD dependent Chlorella proline dehydrogenase, represents the activity of a single enzyme.

Attempts to find an oxygen requirement for Chlorella proline dehydrogenase

As discussed in the LITERATURE REVIEW, an oxygen requirement has been found for the first enzyme of proline oxidation in animal tissues and in many microorganisms. Many investigators reported the enzyme to be an oxidase, with no NAD requirement. As reported in the previous section, attempts to measure oxygen utilization in Chlorella enzyme extracts, by means of an oxygen electrode, were completely negative.

Because of the seemingly low activity of Chlorella proline dehydrogenase, as compared with the corresponding proline oxidation reported in animal tissues, it was considered necessary to further investigate the effects of oxygen. A considerable number of attempts were made, using evacuated, nitrogen-filled Thunberg tubes and an anaerobic spectrophotometric cuvet, to determine if molecular oxygen was involved in the activity of Chlorella proline dehydrogenase. The glucose oxidase test reaction, showed that most of the molecular oxygen was removed by the aspi-

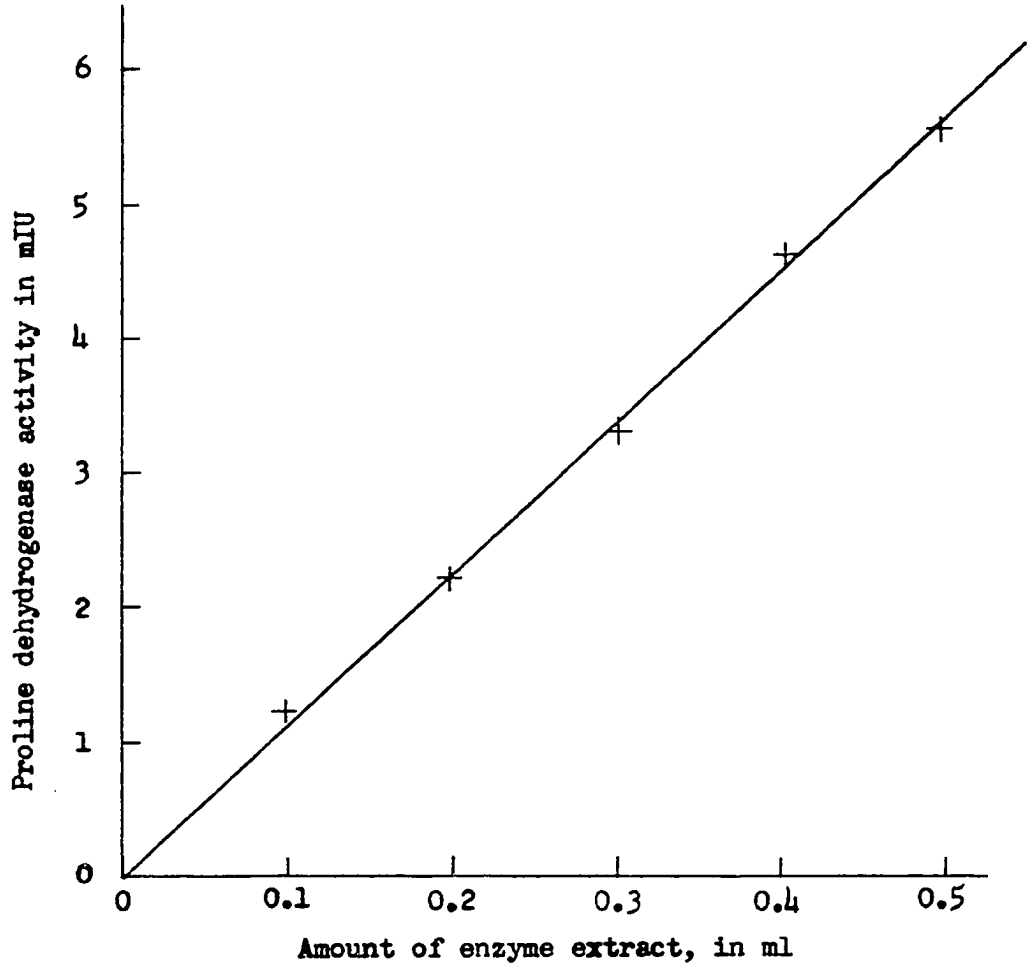


Figure 13. Chlorella proline dehydrogenase activity compared with amounts of crude enzyme extract
(Assays were by the NAD reduction method.)

ration-flushing process.

As will be discussed later, the P5C product can be determined by an assay. An experiment was conducted in which both NADH and P5C, produced during the reaction, were determined under aerobic and under anaerobic conditions. Table 6 shows the results of this experiment. The results

Table 6. Comparison of NADH and P5C production under both aerobic and anaerobic conditions

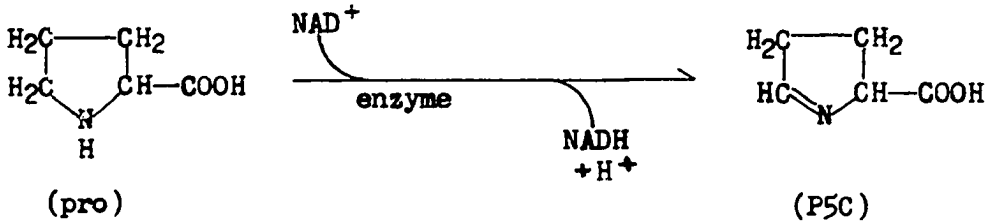
run condition	NADH production (μmoles/min)	P5C production (μmoles/min)	P5C production / NADH production
aerobic	0.0164	0.0127	0.78
anaerobic	0.0132	0.0125	0.95

indicate that Chlorella proline dehydrogenase does not require oxygen. The fact that the aerobic ratio of P5C/NADH is 0.78, while the anaerobic ratio is 0.95, suggests that oxygen may be interfering with the assay, possibly by allowing oxidation of NADH back to NAD. This experiment will be discussed later from another viewpoint.

Inhibition studies were semisuccessful, and need to be redone with more highly purified enzyme extract. As discussed in the LITERATURE REVIEW, several investigators obtained 100 % inhibition of proline oxidation, by using KCN. Use of KCN over a broad range, 0.36 to 3.33 mM initial reaction concentration, failed to show that KCN had any effect on Chlorella proline dehydrogenase. These findings are presented as further evidence that Chlorella proline dehydrogenase is not an oxidase,

nor does it involve the use of molecular oxygen. Azide does not affect proline dehydrogenase activity, either. Due to the negative responses obtained with KCN, azide, and molecular oxygen, no attempt was made to test the cytochrome involvement, discussed in the LITERATURE REVIEW, which was found to be involved in animal tissue proline oxidation.

The aerobic/anaerobic experiments and the inhibition experiments, suggest that Chlorella proline dehydrogenase is an NAD requiring enzyme, and that oxygen is not required. In fact, indications are, that oxygen lowers the amount of observed enzyme activity. Preliminary investigations with pea proline dehydrogenase, also indicate that the reaction proceeds more steadily under anaerobic conditions. Considering the results of this section, and the previous section, in which an NAD requirement has been demonstrated, it can now be proposed that the reaction of Chlorella proline dehydrogenase may be:



Proline dehydrogenase purification

Chlorella extracts were prepared and processed as given in METHODS AND MATERIALS. Table 7 shows the results of purification of proline dehydrogenase, at various stages of the purification process. Considerable enzyme was lost in the purification process. An 11-fold purification was achieved. Early attempts to dialyze the cell-free extracts, resulted in complete and rapid losses in proline dehydrogenase activity. Pure wa-

Table 7. Purification of Chlorella proline dehydrogenase (Extraction was as described in Figures 11 and 12. Assays were by the NAD reduction method. Activities were corrected to a temperature of 30 C.)

fraction	total[a] volume (ml)	protein[b] (mg)	total[c] enzyme activity (mIU)	specific[d] activity (mIU/mg)	purif-[e] cation factor	recov-[e] ery (%)
1. French press supernatant	134.0	892.5	1569	1.8	1.0	100.0
2. After solid $AmSO_4$ treatment and resuspension	19.1	68.7	1038	15.1	8.6	66.2
3. After first $AmSO_4$ dialysis	11.1	42.5	756	17.8	10.1	48.2
4. After DEAE-cellulose chromatography, second $AmSO_4$ dialysis, and removal of $AmSO_4$	9.4	23.4	478	20.3	11.5	30.4

[a] Total initial volumes were corrected for amounts previously used for activity and protein determinations.

[b] Determined by the method of Lowry et al. (1951), using crystalline BSA as the protein standard. The Folin phenol reagent was obtained from Fisher (Pittsburgh).

[c] Corrected as in the total volume column.

[d] Total enzyme activity divided by the mg of protein.

[e] Based on Fraction 1.

ter, phosphate buffers, and buffers reinforced with various metal ions, proved unsuccessful. No comprehensive attempt was made to ascertain which dialyzable factor(s) is required to restore full activity. As of this writing, it must be said that Chlorella proline dehydrogenase is undialyzable, except against saturated $AmSO_4$ solutions.

Stability of proline dehydrogenase in storage

Two methods of long-term storage of Chlorella proline dehydrogenase, were found to give satisfactory results. The first was storage in saturated $AmSO_4$, and the second by freezing in 5 to 50 mM phos. (pH 7.2). All possible storage situations were not exhaustively examined. The merits of Na_2EDTA and DTT in improving storage stability, were erratic, and use was discontinued.

Table 8 shows the stability of one batch of proline dehydrogenase

Table 8. Stability of Chlorella proline dehydrogenase when stored at 4 C in 50 mM phosphate buffer (pH 7.2)
(Assays were by the NAD reduction method. Assay temperatures were 27 C.)

time (hrs.)	proline dehydrogenase activity (mIU/ml)
0	33.5
28	29.5
98	31.2
456	23.6
936	14.2

stored at refrigerator temperatures. Not all batches retained activity this well. As can be seen, activity had been lowered to less than one-half by the end of 39 days (936 hrs.).

Table 9 shows the activity of the enzyme which had been frozen at

Table 9. Stability of Chlorella proline dehydrogenase when stored at -15 C in 5 mM phosphate buffer (pH 7.2) (Assays were by the NAD reduction method. Temperatures have been corrected to 30 C.)

time (days)	proline dehydrogenase activity (mIU/ml)
0	42.3
8	39.8
16	41.0
33	41.4
54	38.9
78	49.4

-15 C. These results and those of similar experiments, show that proline dehydrogenase activity does not diminish when frozen and thawed repeatedly.

Identification of P5C and P2C

Synthetic P5C was complexed with oAB as described in METHODS AND MATERIALS. The predicted, clear, yellow color was produced. The absorption spectrum between 200 and 760 nm, was made, and compared with the spectrum made by Strecker (1960a, 1965). Excellent agreement was found. A flexnode was found at 443 nm. This has been heavily used for identifi-

cation by many investigators. Maxima were found at 227, 293, and 364 nm. Strecker had not reported a maximum at 364 nm. Minima were found at 209, 273, and 320 nm. The great stability of the P5C-oAB complex in aqueous solution, was shown by the fact that rerunning the refrigerated solution six months later, gave an almost identical absorption spectrum. The color was still deep yellow. An additional maximum at 255 nm, and an additional minimum at 249 nm, had developed.

Proline and P5C solutions were found to have no maxima or minima between 260 and 760 nm. Carefully filtered, aqueous oAB appeared to have maxima at 240, 260, 282, and 364 nm. No flexmode was apparent for oAB. Thus, the discrepancies with Strecker's results, can be accounted for, by uncomplexed oAB in the solution of the P5C-oAB complex. The maxima at 364 and 255 nm, and the minimum at 249 nm, were probably due to uncomplexed oAB. Synthetic P2C was found to also react with oAB, as expected.

Paper chromatography of the synthetic P5C solution, showed several materials were present. P5C was identified by its yellow reaction product with oAB, and by its bright pink reaction product with ninhydrin. P2C was identified by its yellow complex with oAB, and its purple complex with ninhydrin. The oAB complexes had a brighter, and more orange, yellow color, than the yellow of the proline-ninhydrin complex. Results from BAW system chromatography, indicate that free P5C migrates appreciably ahead of free P2C and proline. Free P2C migrates behind proline. These results were reproducible. The oAB complexes migrated faster than the free pyrroline carboxylates, with P2C-oAB slightly ahead of P5C-oAB.

These results are consistent with those of Costilow and Laycock (1969).

Colors of the paper chromatography complexes were investigated, using visible fluorescent light, so-called "long" ultraviolet with a peak at 365 nm, and so-called "short" ultraviolet at 254 nm. A greenish tint of the P2C-oAB complex, as seen under ultraviolet, was reproducible, and was detected in every chromatographic run. P5C-oAB never displayed the greenish tint. Under exposure of five minutes or more, of ultraviolet, the greenish tint disappeared, resulting in the P2C-oAB complex having the same apparent color as P5C-oAB. The greenish tint was not altered in darkness over a period of several weeks. Due to the unknown concentrations of the complexes, care must be used in employing color under ultraviolet, as a criterion of distinction. However, this is an area in need of investigation by physical chemistry techniques.

Identification of P5C as the product of proline dehydrogenase

As mentioned previously, one of the fundamental problems of this investigation, has been that of identifying the first intermediate of proline catabolism. As discussed in the LITERATURE REVIEW, in all animals and microorganisms in which a product of proline oxidation has been identified, that product has been P5C/GSA. However, there existed the possibility that for plants, the product might be P2C, a non-Δ^1-pyrroline carboxylate compound, or there might be direct conversion to glutamate without isolatable intermediates.

To attempt to determine whether or not P5C is the product of Chlorella proline dehydrogenase, three series of experiments were performed. In the first series, labeled proline was used to enzymatically produce labeled

P5C. In the labeling experiments described in METHODS AND MATERIALS the enzymatically produced P5C was readily located by its bright pink ninhydrin complexing color on the paper chromatogram. Table 10 shows

Table 10. Distribution of radioactivity on chromatograms of reaction mixtures of active and boiled enzyme incubated with ^{14}C-proline
(Reaction conditions and chromatography are described in the text. The chromatograms were cut into segments and counted by liquid scintillation, using a toluene based solvent.)

distance from origin (cm)	active enzyme (CPM x 10^{-3})		boiled enzyme (CPM x 10^{-3})
0 to 5	2.4		1.8
5 to 10	1.2		0.8
10 to 15	2.5		1.0
15 to 20	2.2		0.8
20 to 25	2.0		0.8
25 to 30	1.5		0.6
30 to 37	2.6		2.1
37	1.1		7.3
38	14.9		60.8
39	82.0		91.0
40	112.0	center of visual	67.7
41	82.6	proline-ninhydrin	38.8
42	46.2		12.2
43	15.7		1.9
44	2.8		0.6
45	1.1		0.3
46	2.5	center of visual	0.3
47	3.3	P5C-ninhydrin	0.3
48	2.5		0.2
49	1.2		0.3

the counts per minute of two runs of the labeling experiments.

As discussed in the LITERATURE REVIEW, P5C is labile in solution, at room temperature. However, enough P5C was added to last during 62 hours

of chromatography. Table 10 shows a labeling peak at the center of the visual P5C-ninhydrin complex, in the active enzyme chromatogram. No such peak occurs for the boiled enzyme preparation. This indicates that a nonproline, labeled product, has migrated at a rate faster than proline. Comparison with previous chromatograms of synthetic P2C and synthetic P5C, showed that no accumulation of label occurs where P2C would be expected. No purple complex was found for P2C. Thus, there is no evidence that P2C is being produced by proline dehydrogenase.

The fact that visualized P5C coincides with the product labeling peak, is presented as evidence that P5C is the natural product of proline dehydrogenase in Chlorella. The product spot on the chromatogram, has the same position relative to proline, as was obtained by Mazelis and Fowden (1971), using peanut proline dehydrogenase, incubated with labeled proline. They had concluded that the product was not P5C, because it did not react with H_2O_2 to yield glutamate. However, as mentioned previously, Johnson and Strecker (1962) had reported that only a small amount of P5C is ordinarily converted to glutamate, when P5C is treated with H_2O_2; and that most of the P5C is not converted. Thus, it is probable that the product which Mazelis and Fowden (1971) obtained, was P5C.

A comparison of the labeling results for the active enzyme and the boiled enzyme, shown in Table 10, indicates a large amount of label scattered between the chromatogram origin and the proline peak, for the active enzyme. This scatter did not occur for the boiled enzyme. This can be interpreted as being due to the polymerization of the product, P5C, during the chromatography. As discussed in the LITERATURE REVIEW,

P5C is known to polymerize readily at room temperature.

In a second series of experiments, paper chromatography showed that the enzymatically produced product, complexed with oAB, had nearly the same migration rate as synthetic P5C-oAB. In one experiment, synthetic P5C-oAB had an Rf of 0.73, while synthetic P2C-oAB had an Rf of 0.76. The natural product, complexed with oAB, had an Rf of 0.72. Although this is not as precise as desired, it is consistent with P5C being the natural product. The natural product complex did not have the greenish tint, as viewed under 254 nm ultra violet radiation, which appears to be a characteristic of the P2C-oAB complex.

In the third series of experiments, stoichiometric comparisons of NADH and P5C production, were made. Table 11 shows the stoichiometric

Table 11. Stoichiometric relationships between NADH produced and P5C produced, by Chlorella proline dehydrogenase (Assays were by the NAD reduction method and by the oAB complexing method. Different enzyme preparations are indicated by different letters.)

enzyme preparation	conditions	NADH produced (μmoles/min)	P5C produced (μmoles/min)	P5C produced / NADH produced
A	aerobic	0.0162	0.0176	1.08
B-1	aerobic	0.0164	0.0127	0.78
B-2	anaerobic	0.0132	0.0125	0.95
C	aerobic	0.0079	0.0067	0.85

relationships between NADH and P5C. Some of the information was presented

previously in Table 6, but is included here to emphasize its importance in identifying P5C as the product of Chlorella proline dehydrogenase.

Since the ratios of P5C produced to NADH produced, were constant for each enzyme preparation, there is evidence that the ratio of P5C to NADH is a fixed number. Various enzyme preparations are believed to have varying amounts of interfering enzymes. Other experiments gave comparable results. The results shown in Table 11, especially the anaerobic run, suggest that a one to one relationship exists between NADH produced and P5C produced. This is evidence for not only P5C being the product of proline oxidation, but for one mole of NAD being reduced for each mole of proline oxidized. In runs where more NAD was observed than P5C, several explanations can be presented. The next two enzymes in the oxidative pathway of proline, are believed to be NAD requiring. Thus, in a crude extract, some NAD may be reduced by enzymes other than proline dehydrogenase. The possibility also exists that not all of the P5C is complexed, since some P5C may be converted to other compounds. Three lines of evidence have been presented in this section, to support the belief that P5C is the product of proline dehydrogenase.

P5C is produced only when NAD is oxidized

In the first section of RESULTS AND DISCUSSION, it was shown that NAD was required to oxidize proline. The previous section has demonstrated P5C to be the first oxidation product. In the LITERATURE REVIEW, investigations of animals and certain microorganisms were reported which showed that proline produced P5C, without an accompanying reduction of NAD. If the same were true in Chlorella, then P5C should accu-

mulate without adding NAD. The opposite was found to be true, namely that P5C accumulated only when NAD was added to enzyme extracts. As shown previously, there is nearly a one to one relationship between the moles of P5C produced and the moles of NADH produced.

Assays for other enzymes involved in proline metabolism

As discussed in the LITERATURE REVIEW, other enzymes involved in proline metabolism are also known to have pyridine nucleotide requirements. This could be a source of interference in the assay of proline dehydrogenase, where crude extracts are used. Three enzyme activities were investigated. P5C reductase catalyzes P5C to proline. P5C dehydrogenase is believed to catalyze P5C to glutamate. A possible NADH oxidase activity, was also examined.

Table 12 shows Chlorella P5C reductase activity and the final pH's

Table 12. Chlorella P5C reductase activity with respect to pH (The reaction was followed by the decreased absorbance of NADH, at 340 nm. The temperature was 31.5 C.)

number of runs	average P5C reductase activity (mIU/ml)	final pH
4	228	10.23
1	325	9.90
2	1718	9.40
1	2630	8.89
1	5700	8.41
1	7600	7.47

of the reaction mixture. Comparable proline dehydrogenase activities were approximately 100 mIU/ml at pH 10.23 and 2.8 mIU/ml at pH 7.62. The opposing enzyme, P5C reductase, is extremely active at neutral pH's, which were the pH's used to assay proline oxidation in animals and microorganisms. The results in Table 12 explain why the reaction rate of proline dehydrogenase declined with time. The NADH produced by proline dehydrogenase, was being used by P5C reductase, to change P5C back to proline. This was the reason why it was necessary to measure the reaction rate of proline dehydrogenase during the first minute of time, before P5C reductase activity became appreciable. The high activities, shown in Table 12, made use of synthetic P5C as substrate. This might be an indication of P5C being an intermediate in the glutamate-proline pathways in Chlorella. The use of synthetic P2C with the same enzyme extracts, gave no activity whatsoever. This is evidence against P2C being in the glutamate-proline pathways.

Comparable P5C dehydrogenase activity is shown in Table 13. P5C dehydrogenase activity was found to be low in comparison to proline dehydrogenase activity, generally about 30 times lower at pH 10. It can be concluded that P5C dehydrogenase offers very little interference to the results of the proline dehydrogenase assays, both the NAD assay and the P5C assay. This is evidence that most of the NAD which is reduced, is used for proline dehydrogenase, and not for enzymes between P5C and the TCAP.

NADH oxidase activity, which was found to be high at neutral pH's, was quite low at high pH's. The results of the above three assays, give

Table 13. *Chlorella* P5C dehydrogenase activity with respect to pH (The reaction was followed by the increased absorbance of NADH, at 340 nm. The temperature was 31.5 C.)

number of runs	average P5C dehydrogenase activity (mIU/ml)	final pH
2	6.0	10.53
1	3.6	10.29
2	3.2	10.09
1	2.3	9.77
2	2.2	8.81
1	1.2	8.10
1	0	7.17

an indication of why alkaline pH's are more suitable for assaying *Chlorella* proline dehydrogenase, than are neutral pH's. A pH dependence examination for proline dehydrogenase activity, was not reliable for the crude extracts, due to the interferences of the above mentioned enzyme activities. In general, however, a rather broad pH optimum between pH 10 and 10.5, was found to give maximum proline dehydrogenase activity.

Substrate and cofactor specificity of proline dehydrogenase

Various substrates were assayed in an attempt to find the specificity of *Chlorella* proline dehydrogenase. The results are shown in Table 14. Table 14 shows that D-proline and various L-amino acids have negligible activities. Hence, *Chlorella* proline dehydrogenase does not appear to be either of the general amino acid oxidases, discussed in the LITERATURE

Table 14. Substrate specificity of Chlorella proline dehydrogenase (All substrates, except for the final sample, had an initial reaction concentration of 6.7 mM. The final sample contained 3.4 mM L-proline and 3.4 mM L-glutamate. Activities were determined by means of the NAD reduction assay. Temperature was 32.5 C. Relative activity was determined on the basis of the activity of L-proline.)

substrate	final pH	enzyme activity (mIU/ml)	relative activity (%)
L-proline	9.73	48.2	100
D-proline	9.78	4.8	10
4-hydroxy-L-proline	9.73	4.8	10
L-glutamate	9.20	26.7	55
L-cysteine	9.67	7.2	15
L-serine	9.63	3.1	6
L-alanine	9.66	3.1	6
L-threonine	9.66	0	0
L-proline and L-glutamate	9.59	96.8	201

REVIEW, and typically cataloged as EC 1.4.3.2 and EC 1.4.3.3. The combined activity of L-proline and L-glutamate, is greater than the sum of the individual substrate activities. Since the enzyme extract was crude, no interpretation can be made of this unusually high activity. A later assay, using a different enzyme preparation, was done with 6.7 mM L-proline and 6.7 mM D-proline, together, with conditions otherwise as above. The average activity of two runs, was 59.5 mIU/ml. Two control runs, using 6.7 mM L-proline alone, gave an average activity of 50.8 mIU/ml. Thus, D-proline does not inhibit use of L-proline.

As discussed in detail previously, a requirement of the cofactor NAD, has been established for Chlorella proline dehydrogenase. An experiment was done to see if NADP would substitute for, or inhibit, NAD activity in the reaction. Table 15 shows the results. As can be seen,

Table 15. Effects of NAD and NADP on Chlorella proline dehydrogenase activity
(Activities were determined by means of the NAD reduction assay.)

NAD (mM)	NADP (mM)	enzyme activity (mIU/ml)
0.48	0	50.1
0.48	0.44	54.4
0	0.44	0
0	0	0

NADP will not substitute even partially for NAD, in the proline dehydrogenase reaction. This is in agreement with the findings of Mazelis and Creveling (1972) that NADP would not substitute for NAD in wheat dehydrogenase. It is in contrast to peanut proline dehydrogenase, in which NADP was found to partially substitute (Mazelis and Fowden, 1971). Simultaneous addition of both NAD and NADP, did not affect the enzyme activity. Thus, it can be concluded that NADP does not inhibit the use of NAD.
No metal ion requirement was found. Co^{++}, Fe^{++}, Fe^{+++}, Mg^{++}, Mn^{++}, Zn^{++} were tested.

Km of NAD and Km of proline for proline dehydrogenase

The general procedures for determination of Km's, are given in METHODS AND MATERIALS. The Km of NAD was determined for an NAD concentration range of 0 to 1.43 mM, with an initial proline concentration of 6.7 mM. The proline concentration range used for finding the Km of proline, was 0 to 10.0 mM, with an initial NAD concentration of 0.95 mM. This concentration of NAD, was more than 10 times the Km of NAD. Thus, NAD was not limiting in the determination of Km of proline. Typically, samples were run in triplicate and the final derived values were averaged.

The data were graphed by four versions of the Michaelis-Menten equation. The best graphs, considering the nature of the data involved, were obtained by using the Hanes (1932) form of the Michaelis-Menten equation. The Hanes equation is:

$$\frac{S}{v} = \frac{Km}{V_{max}} + \frac{S}{V_{max}}$$

with S representing substrate concentration, v the enzyme activity, Km the Michaelis-Menten constant, and V_{max} the maximum activity. Figure 14 shows the Hanes graph for the Km of NAD. The Eadie-Hofstee graph (also known as the Woolf graph), and the Lineweaver-Burk graph, are not shown. They proved to be less satisfactory than the Hanes graph, for the data involved. Figure 15 shows the traditional Michaelis-Menten graph. As can be seen, the data fit reasonably well to a hyperbola-like graph.

On the Hanes graph, shown in Figure 14, the x-intercept represents the negative of the Km. This gave a value for the Km of NAD, for Chlorella proline dehydrogenase, of 0.08 mM. The Hanes line slope represents the reciprocal of V_{max}. Thus, V_{max} was found to be 131 mIU/ml.

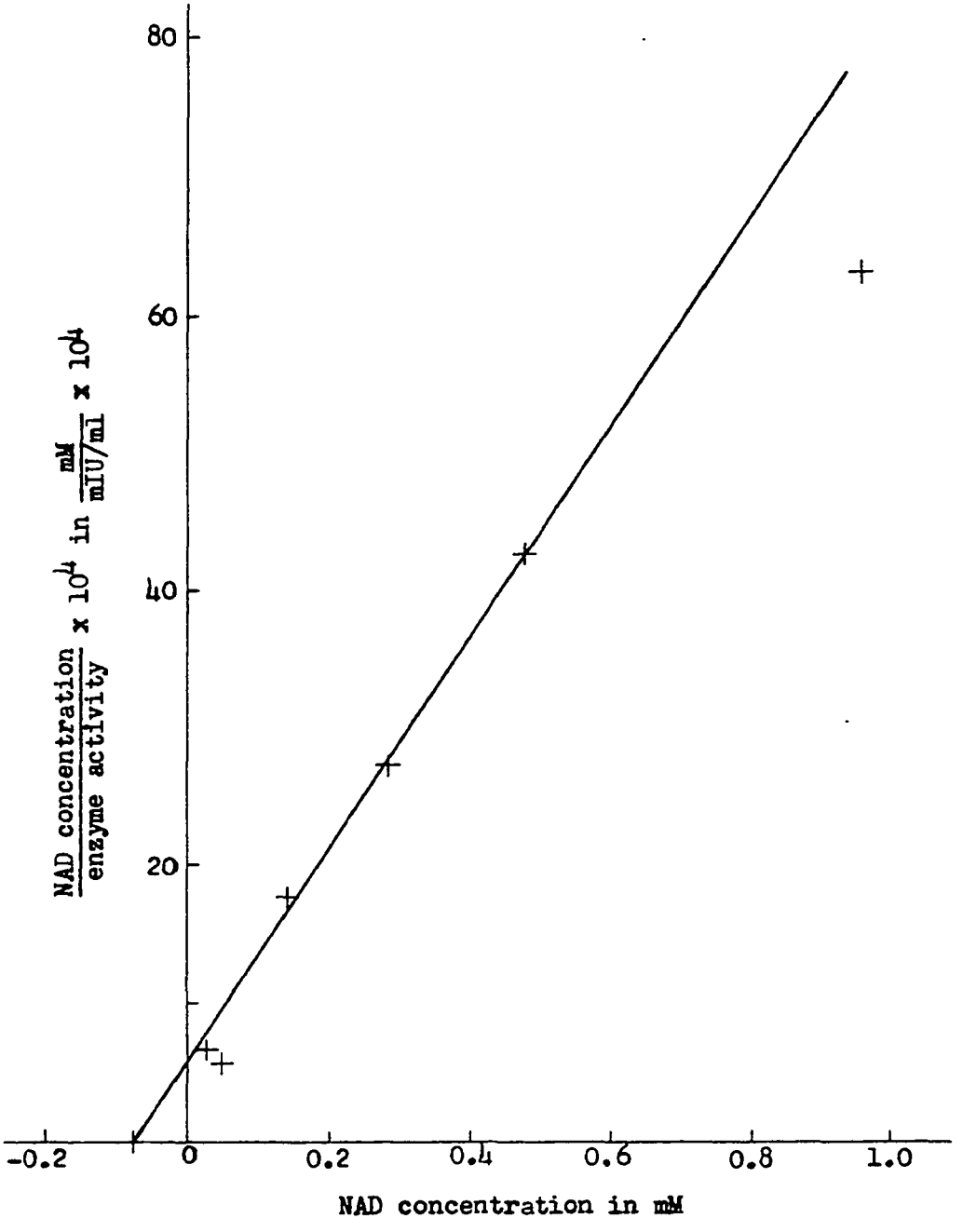


Figure 14. Determination of the Km of NAD for Chlorella proline dehydrogenase
(Proline of saturating concentration 6.7 mM was used. The activities were determined by means of the NAD reduction assay.)

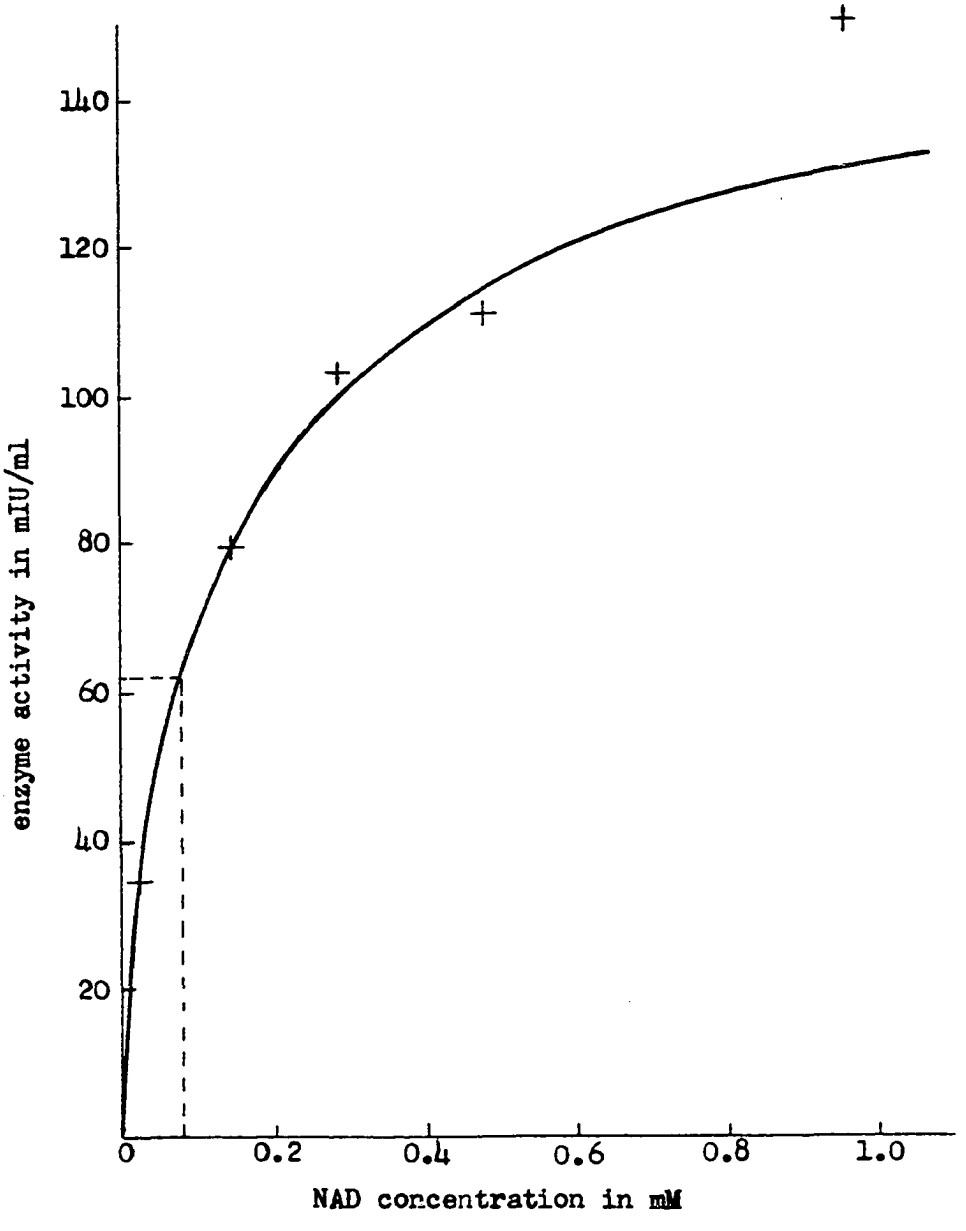


Figure 15. Michaelis-Menten graph of the Km of NAD for Chlorella proline dehydrogenase
(Assays were by the NAD reduction method.)

No attempt was made to estimate the Km from the Michaelis-Menten graph. The Hanes value of Km was used on the Michaelis-Menten graph, as shown in Figure 15, to obtain the V_{max} value. On the Michaelis-Menten graph, V_{max}, graphically, is represented by double the ordinate on the point on the graph, which has the Km value as its abscissa.

Figure 16 represents the Hanes graph for the Km of proline. The Km of proline is shown to be 0.73 mM, with a V_{max} of 106 mIU/ml. Figure 17 shows the graph of the Michaelis-Menten equation. As with the Km of NAD graph, the Michaelis-Menten graph for Km of proline, is hyperbola-like. Table 16 shows a summary of Km and V_{max} information, obtained from the

Table 16. Summary of Km and V_{max} values of NAD and proline, for Chlorella proline dehydrogenase, as determined by various graphic forms of the Michaelis-Menten equation

graph form	Km of NAD (mM)	V_{max} of NAD (mIU/ml)	Km of proline (mM)	V_{max} of proline (mIU/ml)
Hanes	0.08	131	0.73	106
Eadie-Hofstee	0.09	132	0.74	111
Lineweaver-Burk	0.10	135	...	...
Michaelis-Menten	...	126	...	106

different graphic forms of the Michaelis-Menten equation. As expected, the corresponding values are in agreement with each other.

It can be concluded that Chlorella proline dehydrogenase, for the required cofactor NAD, obeys typical Michaelis-Menten kinetics. The Km of NAD value of 0.08 mM is of the same order of magnitude as two other

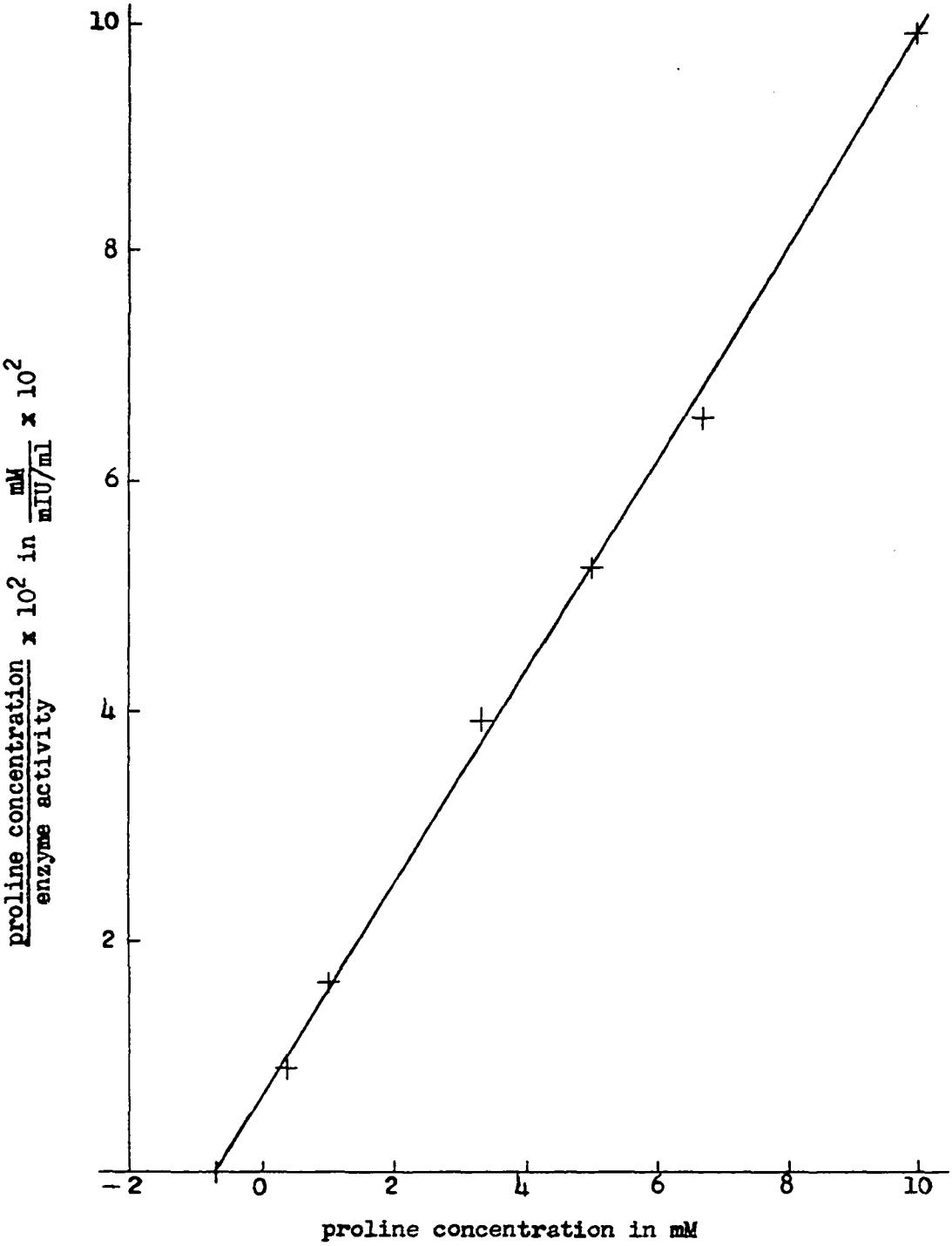


Figure 16. Determination of the Km of proline for Chlorella proline dehydrogenase
(NAD concentration was 0.95 mM. Assays were by the NAD reduction method.)

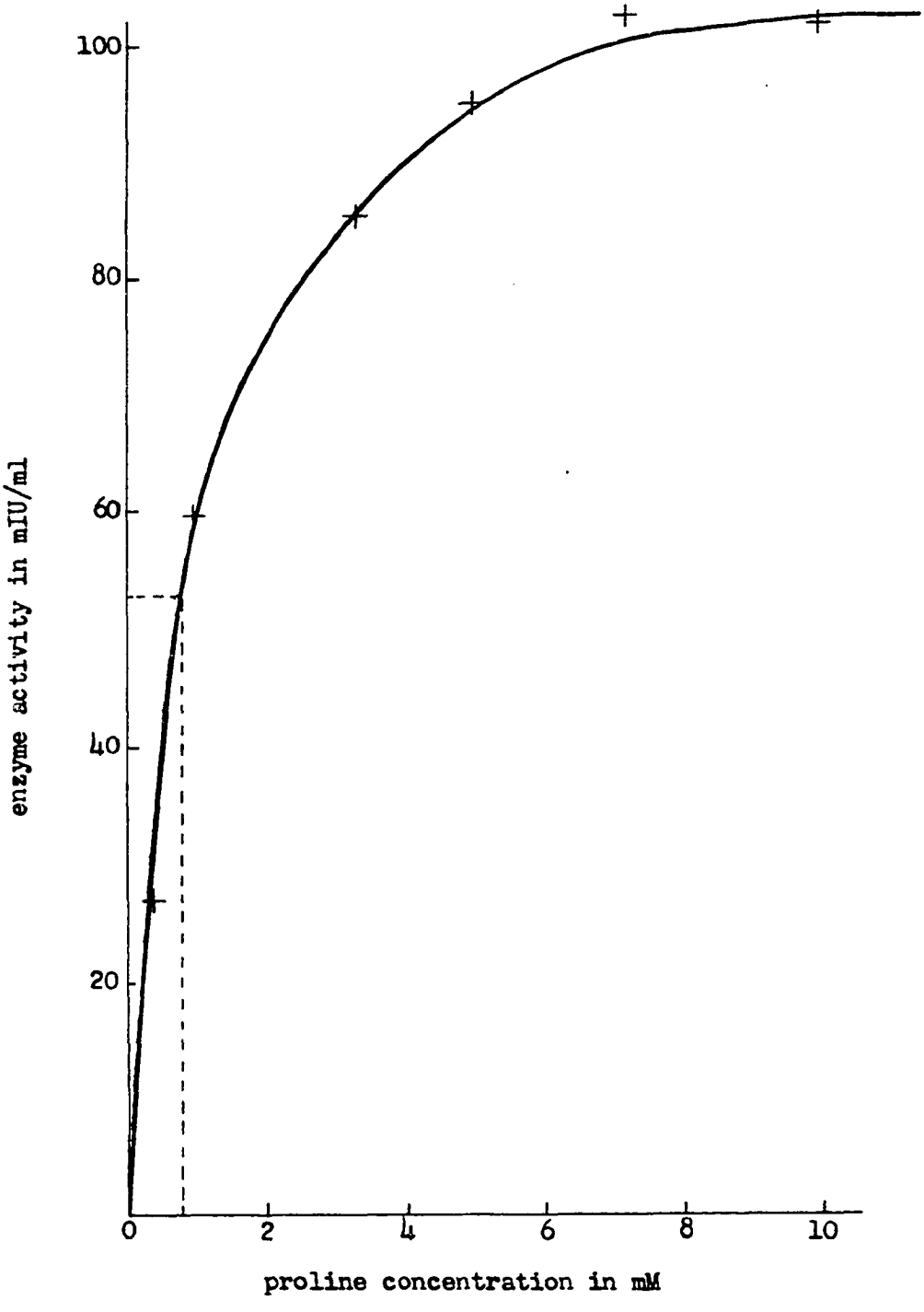


Figure 17. Michaelis-Menten graph of the Km of proline for Chlorella proline dehydrogenase
(Assays were by the NAD reduction method.)

Km values, reported for plant proline dehydrogenases. Mazelis and Fowden (1971) found that peanut proline dehydrogenase had a Km of NAD of 0.25 mM. Mazelis and Creveling (1972) reported that the Km of NAD for wheat proline dehydrogenase was 1.0 mM.

As with the Km of NAD, it can be concluded that the Km of proline for Chlorella proline dehydrogenase, obeys typical Michaelis-Menten kinetics. The Km value of 0.73 mM compares favorably with other plant proline dehydrogenases' Km's. Mazelis and Fowden (1971) found that peanut proline dehydrogenase had a Km of 0.30 mM. Mazelis and Creveling (1972) reported the Km of proline for wheat proline dehydrogenase, to be 1.0 mM.

Miscellaneous properties of Chlorella proline dehydrogenase

Since Mazelis and Fowden (1971) had reported that the molecular weight of peanut proline dehydrogenase, was less than 100,000, an attempt was made to apply their methods to Chlorella proline dehydrogenase. Repeated attempts, using both Bio-Gel P-100 and Sephadex G-100, indicated that proline dehydrogenase activity came off with the void volume. This is evidence that the enzyme either has a molecular weight of over 100,000; or that the enzyme is bound to a complex with a combined molecular weight of over 100,000. Later, Mazelis and Creveling (1972) demonstrated that the molecular weight of wheat proline dehydrogenase, was in excess of 100,000. Thus, it may be that plant proline dehydrogenases have molecular weights larger than 100,000.

Frank and Rybicki (1961) reported that 90 to 95 % of proline oxidase activity, in E. coli, was inducible by the presence of exogenous proline in the growth medium. With that consideration in mind, attempts

were made to induce proline dehydrogenase in Chlorella. The final supernatants of the cells, processed as described in METHODS AND MATERIALS, were assayed, with the results shown in Table 17. The results indicate

Table 17. Effects of various exogenous proline concentrations in inducing proline dehydrogenase activity in Chlorella (Activities were determined by the NAD reduction assay.)

proline concentration (µmoles/ml)	PCV (µl packed cells ml suspension)	final volume supernatant (ml)	enzyme activity (mIU/ml)	enzyme activity (mIU/ml PCV)
0	32.0	186	28.9	0.96
1	32.6	173	31.3	0.96
10	31.8	189	30.0	0.94
100	32.0	199	28.1	0.88

that the presence of proline, at all tested concentrations, exhibited no inducibility of Chlorella, within four days after being supplied to the cells.

Proline dehydrogenase in higher plants

As discussed in the LITERATURE REVIEW, proline dehydrogenase has been reported in peanut (Mazelis and Fowden, 1971), and in wheat (Mazelis and Creveling, 1972). A. B. Rena (unpublished data, University of Illinois, 1972) has demonstrated proline dehydrogenase activity in pumpkin. In all three of these higher plants, a requirement for NAD has been found. Since the main thrust of this investigator's research, was done with

Chlorella, only preliminary experiments were done with higher plants. Often, materials prepared by other workers, were assayed for proline dehydrogenase activity.

In this laboratory, preliminary work has shown that NAD requiring proline dehydrogenase activity, is present in castor bean, cauliflower, corn, garden bean, mung bean, soybeans, and turnip. In all cases, alkaline pH assays gave maximum detectable activity. Pea extracts gave a yellow oAB complex, so it is probable that P5C is the product of proline oxidation in higher plants. In garden bean tissues, acetone powder extraction, resulted in complete loss of proline dehydrogenase activity. However, excellent P5C reductase activity was retained. This is an indication that these two activities are produced by two different enzymes. Work with higher plants is actually beyond the scope of this report, but all findings about higher plants, have been consistent with the results obtained from Chlorella.

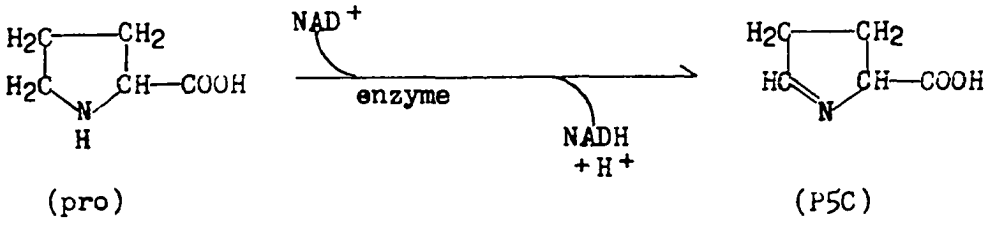
CONCLUSIONS AND SUMMARY

The Chlorella growth and proline uptake experiments, showed four salient features. First, exogenous proline was demonstrated to be readily taken up and used by the cells. Second, it was found that glucose must be present in the growth medium, for rapid proline uptake. Third, there was a complex pattern of inhibitive and promotive effects, by glucose, upon proline uptake. Proline typically showed a rapid initial uptake during the first 10 to 20 hours. Experiments in which no glucose was added to the medium, resulted in no proline uptake during the first 20 hours. Thus, glucose appeared to be promotive during this early period. The early surge, was then followed by a long lag in proline uptake, of 40 to 60 hours. It was during this latter period that an accumulation of internal carbohydrates was believed to have been inhibitory to proline uptake. After the glucose had disappeared from the medium, there was a second period of rapid uptake of proline that usually resulted in all proline being removed from the medium in a matter of 10 to 20 hours. Fourth, the proline taken up by the cells, was shown to be rapidly oxidized to CO_2 and tricarboxylic acid pathway intermediates.

The important first enzyme of Chlorella proline catabolism, has been purified 11.5 fold, by use of $AmSO_4$, and by DEAE-cellulose chromatography. A specific activity as high as 20.3 mIU/mg, has been achieved. The presence of Na_2EDTA and DTT in the extraction medium, was found to enhance enzyme activity. The enzyme will keep indefinitely when stored in saturated $AmSO_4$, or when frozen. The enzyme activity, graphed against time, was found to be linear. This indicated that a single enzyme is in-

volved. The enzyme has been found to be undialyzable, except against saturated $AmSO_4$.

Two different assays for the first enzyme have been modified for use. The first is by reduction of NAD to NADH, which can be followed spectrophotometrically at 340 nm. The enzyme has been found to require NAD, but not oxygen. Hence, the name proline dehydrogenase seems more suitable for the enzyme described, than does proline oxidase. Several approaches were used in an attempt to demonstrate a molecular oxygen requirement, but all results were negative. In fact, the reaction was found to proceed more smoothly, and with better stoichiometry, under anaerobic conditions. KCN and NaN_3, appeared to have no effect on the enzyme. In view of the NAD requirement, and the nonessentiality of oxygen, the first reaction of proline catabolism, has been proposed to be:



(pro) (P5C)

Interfering enzyme activities, necessitated the use of an alkaline pH assay, which was found to be optimum between pH 10.0 to 10.5. NADP will not substitute at all for NAD.

The second assay for proline dehydrogenase, utilized the complexing of the product with o-aminobenzaldehyde (oAB). This reaction can be followed by the intense yellow color of the newly formed complex, and is monitored spectrophotometrically at 443 nm. The product of the reaction has been identified as Δ^1-pyrroline-5-carboxylate (P5C). The following

evidence has been presented which leads to this conclusion: (1) The specific reaction of the product with oAB. (2) The product's bright pink reaction with ninhydrin. (3) The enzymatically produced product, chromatographed with synthetic P5C, but not with synthetic P2C. (4) The oAB complex of the enzymatically produced product, chromatographed with the oAB complex of synthetic P5C, but not with the complex of P2C. (5) One mole of P5C was produced for each mole of NAD reduced. (6) The ultraviolet observations of the oAB complexes, indicated P5C as the enzymatic product.

A number of properties of proline dehydrogenase have been investigated. Substrate determinations, indicate that proline dehydrogenase is highly specific for L-proline. It was found that D-proline, L-hydroxyproline, L-cysteine, L-serine, L-alanine, and L-threonine, serve poorly, or not at all as substrates. The substrate specificity and the lack of molecular oxygen requirement, indicate that this enzyme is not one of the two general amino acid oxidases, described for animals and microorganisms. The Km of NAD has been found to be 0.08 mM, and the Km of proline is 0.73 mM. Both NAD and proline, obey typical Michaelis-Menten kinetics. Gel chromatography has shown the molecular weight to be over 100, 000 daltons. The enzyme was found not to be inducible.

Proline dehydrogenase activity, with an NAD requirement, has been found in castor bean, cauliflower, corn, garden bean, mung bean, peas, soybean, and turnip. Thus it appears to be widespread in angiosperms, as well as in the alga examined.

FURTHER STUDIES

Since PART I, on growth and proline uptake, constituted the minor part of this thesis, only a cursory examination was made. Important questions were raised, whose investigation could well become another thesis. Of paramount importance, is the necessity of determining the internal glucose concentration during (a) the initial uptake period of proline, (b) the lag period of proline uptake, and (c) the later period of proline uptake. The effects of light and dark grown cells, on proline uptake, were not thoroughly investigated. Other sugars could be investigated to find their effects on proline uptake.

The most important problem, is that of further purification of proline dehydrogenase. The many techniques of chromatography, heat treatment, pH changes, etc., remain to be explored. After further purification, more information can be obtained on dialysis, cation requirements, and storage. Better stoichiometric work relating P5C, proline, and NADH, remains to be done. More precise work on paper chromatography and paper electrophoresis of the product, is needed. Obtaining more substantial amounts of enzyme, will allow the production of enough P5C product, to begin the task of purification of this product. A thorough comparative analysis of P2C and P5C, by the methods of physical chemistry, should be done.

A hitherto unreported difference between P5C and P2C, has been the discovery of an apparent ultraviolet difference. This needs to be thoroughly investigated with a fluorescent spectrophotometer. The pH and inhibitor work were not successful, primarily due to the interfering en-

zyme activities, which were not separated from proline dehydrogenase. Further purification will be needed before the pH optimum and inhibitor effects, can be thoroughly examined. Precise determination of the molecular weight of the enzyme, remains to be done. Only preliminary work was done on the localization of proline dehydrogenase. There are indications that the enzyme is mitochondrial associated, but this needs to be investigated more thoroughly. Since proline dehydrogenase activity has been demonstrated in higher plants, they might be more convenient for mitochondrial work, since mitochondrial isolation techniques are presently available for several higher plants.

A problem of importance, is the demonstration that the two opposing enzymes, proline dehydrogenase and P5C reductase, are indeed two different enzymes. It is currently suspected that they are different, but unequivocal proof is yet to be presented. As shown in this text and by others mentioned in the LITERATURE REVIEW, proline dehydrogenase/oxidase activity is very low, relative to the reverse activity of P5C reductase. One wonders why this should be, and whether or not the situation can be reversed. Investigation is needed to determine if certain conditions can be found, which would greatly increase proline dehydrogenase/oxidase activity within living cells.

A fundamental question which can be answered only by sequencing the enzymes and determining the active sites, is whether or not the first proline catabolic enzyme in plants and certain microorganisms, is fundamentally different from that in animals and other microorganisms. The differences could be in active site binding of NAD and oxygen, or by a

few amino acids, or by many amino acids in the enzyme sequence. The gigantic task of determining all of the intermediates and enzymes in the overall proline-glutamate pathways, in plants, remains to be fulfilled.

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