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TRITIUM INCORPORATION AND RETENTION IN PHOTOSYNTHESIZING ALGAE

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SUMMARY

The ratios of incorporation and retention of tritium compared to protium into metabolites in *Chlorella pyrenoidosa* and in *Anacystis nidulans* growing in water labeled with tritium have been determined. The algae were continuously supplied during growth with CO_2 labeled with $^{14}CO_2$, and the ^{14}C content of metabolites were used to determine their concentrations. The tritium/protium ratios (R) of metabolites in *Chlorella* were determined following growth at 10°C, 20°C and 25°C.

As previously reported, variations in R in *Chlorella*, range from 0.5-0.7 for most metabolites, to values of R around 1 for metabolites of the tricarboxylic acid (TCA) pathway. The R value for fumarate has now been measured. The increased R values for TCA cycle intermediates and related amino acids can be accounted for in terms of specific isotope effects of several enzyme-mediated steps. Very different R values for certain metabolites were found in *Anacystis nidulans*--for example, R for citrate was 1.81 (the highest value observed in these studies) while aspartate was only 0.59, comparable to other metabolites in both organisms not related to the TCA cycle. This lower value for aspartate is explainable in terms of the incomplete TCA cycle in *Anacystis nidulans*.

No significant differences in R values for Chlorella pyrenoidosa grown at 20°C and 25°C were observed, but in cells grown at 10°C, there was a small but significant increase in R for TCA cycle metabolites.

If the increase in R from sugar phosphates to TCA cycle intermediates seen in these two types of algae may be taken as an indication of likely discriminatory retention of tritium in organisms higher in the food chain, it would appear that no serious concentration of tritium due to isotopic discrimination should occur in the biosphere. However, research workers using compounds labeled with hydrogen isotopes for studies of in vivo metabolism should take into account the likelihood of such discriminatory uptake and retention during specific metabolic steps.

INTRODUCTION

Given the release of large amounts of radioactive hydrogen (tritium, ^3H) during nuclear weapon testing, the low-level release of tritium from an increasing number of nuclear reactors, and the continual production of "natural" tritium by cosmic radiation in the atmosphere, it is necessary to know the fate of tritium in the biosphere. The way in which tritium is taken up from the environment by plants, especially algae, is particularly important, since these organisms are at the start of the food chain. Furthermore, long-term growth of algae in tritiated water is useful for the biochemical preparation of tritium-labeled compounds, like carbohydrates, fatty acids and amino acids[1]. For this purpose, a detailed knowledge of tritium isotope effects is needed.

When high levels of D_2O, together with $^{14}CO_2$ were administered to algae and other organisms, severe alteration in cell size as well as in cell components was observed[2-4]. These effects have not been found with HTO, if

the radioactivity does not exceed 20 mc/ml[5]. The first studies on discrimination between hydrogen isotopes taken up from the medium during photosynthesis have been described by Reitz and Bonhoeffer[6], who reported that the intracellular deuterium content of Scenedesmus was only 30 to 70% of that of the culture medium. Early tritium experiments with photosynthesizing algae were mostly short-term studies. Moses and Calvin showed that T from HTO was seen first in the same compounds as those in which ^{14}C was seen after feeding $^{14}CO_2$ [7]. The incorporation of tritium into carbohydrates and its intramolecular distribution in sugars were determined by Simon et al.[8] after a few minutes of photosynthesis. Their results indicated that the incorporation of tritium into the products of photosynthesis is mostly due to highly reversible reactions such as isomerization reactions. Isotopic discrimination during such incorporation led to different intramolecular tritium content in the different sugars and their derivates[8].

The first long-term experiments, done by Weinberger and Porter[9], showed that Chlorella pyrenoidosa discriminates against tritium during the incorporation of hydrogen from tritiated water; however, they did not look for the incorporation into the individual compounds. Kanazawa and Bassham[10] carried out long-term experiments using the double isotope technique (T and ^{14}C), which enabled them to measure exactly the concentration of the metabolic intermediates, and so to determine the non-exchangeable tritium incorporation into the different compounds. Their results showed that tritium incorporation compared with protium incorporation into the metabolites is highly variable, with the highest ratios of tritium to protium (R) found in compounds related to the tricarboxylic acid (TCA) cycle, and low values of R in lipids and some amino acids.

The objectives of the present study were: (1) To obtain additional information about the R values of TCA cycle intermediates and to determine the mechanisms leading to these ratios; (2) to determine the dependence (if any) of the R values on the temperature at which the algae were grown in water labeled with T; and (3) to compare R values for metabolites in Chlorella pyrenoidosa with those found in metabolites from the photosynthetic, autotrophic blue-green algae, Anacystis nidulans. The metabolic pathways, especially those involving TCA cycle intermediates, appear to be different in blue-green algae from the pathways in the eucaryotic, green algae, such as Chlorella (see DISCUSSION).

METHODS

As described earlier[10], Chlorella pyrenoidosa was grown for several generations in a closed system containing about 4% $^{14}CO_2$ of known specific radioactivity and tritiated water (1 curie in 50 ml). A pump recirculated the gas from a reservoir through the algae suspension. Four 6-watt fluorescent lamps illuminated the algae continuously for 4-6 days, a water jacket held the growing temperature at 10°, 20° or 25°C. The entire apparatus was placed in a large glove box with negative pressure to handle safely the HTO. At the end of the experiments the algae had doubled 5-6 times, so that more than 97% of the mass of cells were formed from $^{14}CO_2$ and HTO. To harvest the cells, they were filtered, killed in 80% methanol, and then extracted for 15 min in 100% methanol.

The blue-green algae, Anacystis nidulans, were illuminated for 8 days with two 6-watt fluorescent lamps. During this time they doubled 4-5 times, so that more than 94% of the cells were newly grown from the radioactive substrates. The growing temperature was 20°C.

The extracts of C. pyrenoidosa and A. nidulans were analyzed by paper chromatography in two dimensions (phenol-water-acetic acid and butanol-propionic acid-water) for 24 and 48 h. For analysis of pyruvate, a portion of the sample with added authentic keto-acid was reacted with 2,4-dinitro-phenyl-hydrazine, extracted, and the hydrazone chromatographed in the dark using n-butanol-ethanol-0.5 M NH_4^+, as described earlier[11]. Radioautography revealed the location of radioactive spots on the paper chromatogram. After identification, the compounds were cut out and combusted in a "Packard Automatic Combustion Apparatus" to give CO_2 and water. The tritium and ^{14}C content of these products were determined separately in a "Packard Scintillation Counter". This procedure made it possible for us to use relatively low specific radioactivity in the tritiated water.

Since the isotope effects of ^{14}C can be neglected under these conditions[12], the ^{14}C content of the spots was used as a measure of the amount of each compound. In a previous study using these techniques[10] it was found that the amounts of free amino acids measured in this way agreed well with the amounts measured by colorimetric determination.

Knowing the concentration and the non-exchangeable tritium content of the compounds allowed us to calculate the specific tritium radioactivity of the intermediates. This specific tritium radioactivity per hydrogen position of each compound was compared with the specific radioactivity of the hydrogen atom in HTO. This ratio gives the R value, or the relative incorporation of tritium compared with protium. For example, an R value of 0.5 indicates that the average tritium radioactivity of the non-exchangeable hydrogen positions of a compound is half that of the specific tritium radioactivity of the hydrogen atom in HTO. This in turn means that tritium is incorporated in those positions to only one-half the extent that protium is incorporated.

RESULTS

The ratio R (specific tritium radioactivity of non-exchangeable hydrogen positions, divided by the specific radioactivity of the hydrogen atom in tritiated water) is shown for different metabolites of Chlorella pyrenoidosa at different growing temperatures (Table I). Each R value in Table I is an average value of 4-6 different paper chromatograms. The maximal error (if not otherwise indicated) is ±0.05. The values of pyruvate show an error of about ±0.1, since their concentration in the extract is not high enough to give sufficient yields in the hydrazone formation.

TABLE I

R-Values in Chlorella pyrenoidosa

	Exp. I (10°C)	Exp. II (20°C)	Exp. III (25°C)
Threonine	0.54	0.49	0.55
Alanine	---	0.66	0.68
Serine	0.59	0.79	0.60
Dihydroxyacetone Phosphate	0.48	0.47	0.49
3-Phosphoglycerate	0.55-0.65	0.64	0.65
Sucrose	0.76	0.71	0.72
Uridine Diphosphoglucose	---	0.83	---
Citrate	0.99	0.94	0.92
Malate	1.05	0.98	0.95
Fumarate	1.16	1.08	1.04
Glutamate	1.00	0.87	0.88
Glutamine	1.03	0.90	0.91
Aspartate	0.99	0.89	0.93
Pyruvate	0.5-0.7	0.6-0.7	0.5-0.7

Table II shows the average ratio R for different metabolites of *Anacystis nidulans* for six different paper chromatograms of the same experiment. Several intermediates were lacking, as malate and fumarate could not be detected and the concentration of citrate was rather small. Thus, there were larger experimental errors ($\pm$0.10) during the determination of the tritium radioactivity in metabolites in *Anacystis nidulans*.

TABLE II

R-values in *Anacystis nidulans*

	average R
Threonine	0.50
Alanine	0.62
Serine	0.72
Dihydroxyacetone Phosphate	0.56
3-Phosphoglycerate	0.84
Sucrose	0.59
Uridine Diphosphoglucose	0.85
Citrate	1.81
Glutamate	0.77
Aspartate	0.59
Pyruvate	0.6

DISCUSSION

During the uptake of hydrogen as protons from the medium into the different compounds of the metabolic pathways, a much smaller amount of the higher hydrogen isotope is usually found in the products than in water.

This widely known effect is a result of isotope discrimination in the cleavage of the O-H bond[13]. These isotope effects may result in as much as a six-fold greater incorporation of H than T from HTO with isolated enzyme systems[14,15]. According to the results of our in vivo experiments with Chlorella pyrenoidosa (Table I), the ratio in reactions involving uptake of H from the medium may be about two, since in dihydroxyacetone phosphate and threonine there is about half as much tritium in non-exchangeable positions as in HTO..

During the subsequent steps of the metabolism there must be several reactions which discriminate for retention of the tritium radioactivity in the metabolic compounds. Such discriminations in uptake and retention lead to R values of 0.47 to 1.16 in Chlorella pyrenoidosa. We can attribute the discrimination and retention to certain steps in the metabolism.

Looking at experiment II and experiment III of Table I, we see that we can separate two groups of R values. Threonine, alanine, serine, dihydroxyacetone phosphate, 3-phosphoglycerate, sucrose and pyruvate show R values of about 0.7 or lower, while citrate, malate, glutamate, glutamine and aspartate show values of about 0.9 or higher. Citrate and malate are metabolites of the tricarboxylic acid cycle, while glutamate, glutamine and aspartate derive directly from the tricarboxylic acid cycle.

These results indicate that one or more steps in this cycle, or just preceding it, are responsible for the increased specific radioactivity of these compounds. Primary isotope effects may occur in all reactions involving the breakage of a C-T bondage. Secondary isotope effects can occur when tritium atoms are present in the molecule near the reaction center but not involved in the broken bond[13]. Such secondary effects are small. Primary

isotope effects may increase the tritium radioactivity either in the substrate or in the product of a discrimination step, depending on whether the discrimination is within the molecule (intramolecular) or between molecules (intermolecular). If a hydrogen is removed from a methyl group (intramolecular case), there is a competition between the different hydrogen isotopes, and if $\frac{k_H}{k_T} > 1$, there will be an increased radioactivity in the product.

When the hydrogen atom is removed from a unique position (intermolecular case), there is a competition between labeled and non-labeled molecules. When $\frac{k_H}{k_T} > 1$, the unlabeled molecule reacts faster than the labeled molecule, so that the radioactivity in the substrate increases. When this case occurs, the tritium content will increase in the pool before the discriminating step until the inflow of isotope equals the outflow. A steady state is then established. If we assume an isotope effect of 2, the tritium content in the substrate will be twice that of a substrate without an isotope effect[16].

With these principles in mind, let us consider the stereospecific pathways of the hydrogens of the different compounds in the tricarboxylic acid cycle. Pyruvate has a low R value, and since oxidative decarboxylation of pyruvate to give acetyl CoA involves no breakage of C-H bonds of pyruvate, we may assume a low R value for acetyl CoA. The first step in which isotope discrimination seems likely to occur is the citrate-synthase enzyme reaction (condensing enzyme). During this step one hydrogen of the methyl group is removed, while the two others are retained at the C-5 position in citrate and stay in the molecules of isocitrate, 2-ketoglutarate, succinate, glutamate and glutamine without being exchanged with the protons of the medium[17]. An isotope effect of this reaction may therefore give a common, partial explanation for labeling of all TCA substrates.

Eggerer et al.[18] investigated the citrate-synthase reaction in vitro with tritiated acetate and showed that 82% of the acetate radioactivity was retained in citrate instead of 66.7% as expected (due to an intramolecular isotope effect). Such an effect may contribute to the high R values found in the present studies, but, as the results of the in vitro experiment show, it is not large enough to account for an R value of 0.94 for citrate. Since pyruvate has an average radioactivity of 0.6 and since in the in vitro experiments 82% of the methyl group radioactivity was retained in citrate, we would expect a value of 3 x 0.6 x 0.82 = 1.48 for the two C-5 hydrogen of citrate--that is, 0.74 per hydrogen.

Thus we have to look for further reactions which can explain the observed high tritium incorporation values. From in vitro experiments it is known that oxidoreductases show isotope effects if the transformation of the ternary complex is the rate-determining step in the reaction sequence[19-21]. In the tricarboxylic acid-cycle there are three dehydrogenasereactions in which a hydrogen in a C-H bond is transferred to a coenzyme (isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase). In in vitro experiments with isolated enzymes from different sources for all three reactions, primary isotope effects have been reported[22-24]. Since discrimination during a dehydrogenase reaction results in intermolecular isotope effects, we expect an increased tritium radioactivity in the pools before the reaction.

We did not find a chromatographic spot for isocitrate, probably because the aconitase-citrate equilibrium is far in the direction of citrate (about 90%). Looking at the hydrogens of citrate we find an increase from about 0.6 in 3-PGA and pyruvate to about 0.95 in citrate,

though this is not the immediate substrate of the dehydrogenation (Table I). An explanation for this fact may be found in looking at the fate of the hydrogens in the aconitase and isocitrate dehydrogenase reactions[17]. An assumed discrimination in the isocitrate dehydrogenase reaction should increase the tritium content in the C-2 position of isocitrate (Ⓗ, Fig. 1). As there is a rapid equilibrium, the isocitrate should equilibrate (via cis-aconitate, or directly) with citrate, and in this reaction the tritium-enriched hydrogen position does not exchange with the protons of the medium[25,17]. Thus we find an increased tritium radioactivity in citrate.

It is not possible to calculate precisely the magnitude of the kinetic isotope effect from our *in vivo* results. Palm *et al.*[22], who investigated the isotope effects of isocitrate dehydrogenase from yeast, found a value of about 4 (for low substrate concentrations). An effect of this range would be large enough to increase the tritium radioactivity of citrate to the values we found. The tritium content of the products of isocitrate dehydrogenase should not be enhanced by this isotopic discrimination. Of course, part of the enhanced R value in citrate could be due to the citrate synthase step, as already discussed. Such enhancement could be carried through to the products.

The high tritium specific radioactivity of malate (0.95-0.98) could result from a discrimination in the malate dehydrogenase reaction, where the hydrogen on C-2 is transferred to NAD. Thus, the malate pool would have an increased number of molecules with a tritium on C-2. Since there is a rapid equilibrium between malate and fumarate and the critical hydrogen does not exchange with the water (Fig. 2), we also find an increased tritium radioactivity in fumarate (1.04-1.08). The fumarase reaction

itself seems to show no primary isotope effect, since the malate and fumarate tritium contents are not so different. For the isolated enzyme, no primary isotope effect could have been shown[26], while small secondary isotope effects have been reported[27]..

Several explanations are possible to account for the increased radioactivity in glutamate and glutamine (both are made in Chlorella pyrenoidosa by reductive amination of 2-ketoglutarate). Glutamate contains 5 hydrogens, two of which come into the TCA cycle from the methyl group of pyruvate. Two are taken up from the medium during hydrations, and the fifth comes from the reduced coenzyme of the glutamate dehydrogenase reaction. A reverse reaction could increase the radioactivity at the C-2 bound hydrogen position of glutamate. However, Palm et al.[30] detected only small secondary isotope effects for the isolated enzyme from beef liver.

Glutamate and α-ketoglutarate are linked by a second reaction, the glutamate-oxaloacetate transaminase reaction. This pathway is used to make aspartate from oxaloacetate. The transaminase reaction may discriminate against tritium, as discrimination against deuterium was proved for the reaction between glutamate and oxaloacetate to make aspartate and α-ketoglutarate, catalyzed by the isolated transaminase[29].

The reason for the high R values of aspartate appears to relate back to the T-levels in fumarate and malate (see Fig. 2). The two hydrogens in fumarate are stereochemically similar. Malate may have an increased tritium content due to discrimination during the malate dehydrogenase reaction. If malate equilibrates with fumarate, the higher specific radioactivity would be transmitted into C-2 hydrogen of fumarate, where it is symmetrical with the C-3 hydrogen. So there would be an increased tritium content in both

carbon-bound hydrogens of fumarate (H and $\boxed{H}$). Only one of these hydrogens (H) is transferred to NAD during the malate dehydrogenase reaction, while the second one ($\boxed{H}$) remains bonded to carbon in oxaloacetate and from there is transmitted to aspartate. Thus there could be an increased tritium radioactivity in aspartate.

Temperature dependence of tritium retention

Theoretical considerations predict a linear decrease of log isotope effect vs. $\frac{1}{T^{o}K}$ [30,31]. Lowering the temperature should increase the isotope effect, and this has been found for a number of isolated enzymes[22]. Therefore it was interesting to see how the R values might vary *in vivo* with growing temperature. Table I shows that there is no significant difference between 20° and 25°C, but that lowering the growing temperature to 10°C changed the R values. While all substrates not related to the tricarboxylic acid cycle showed no change within the experimental error, the R values of citrate, malate, glutamate, glutamine and aspartate increased about 10%, showing that the isotope discrimination is significantly larger at low temperatures. The fact that the R values of the intermediates not related to the TCA cycle do not change with lower growing temperatures suggests that the tritium incorporation is not temperature dependent, whereas the tritium retention is.

Retention of tritium in *Anacystis nidulans*

The results of studies with the procaryotic, blue-green algae, *Anacystis nidulans* (Table II), were expected to be dissimilar from those with *Chlorella pyrenoidosa*, since these two organisms, though both photosynthetic, have important differences in metabolism and subcellular structure. When *Anacystis nidulans* was fed with ^{14}C-acetate, incorporation of

^{14}C was found into only four amino acids (glutamate, proline, arginine, leucine)[33]. No incorporation into aspartate was observed. Incorporation studies with ^{14}C-pyruvate gave a similar incorporation pattern[34], which suggested that the 2-ketoglutarate oxidation is blocked. Enzymatic analyses confirmed the absence of 2-ketoglutarate dehydrogenase[35] and extremely low levels of malate dehydrogenase and succinate dehydrogenase, while isocitric dehydrogenase was found to be present at high level[34].

Furthermore, glutamate dehydrogenase could not be detected in A. nidulans[33]. This is somewhat disturbing, since this enzyme is responsible for the nitrogen assimilation in most photosynthetic organisms. The transaminase enzyme connecting 2-ketoglutarate and glutamate was detected[33]. The absence of 2-ketoglutarate dehydrogenase prevents cyclic flow of carbon in the tricarboxylic acid cycle, explaining the entry of ^{14}C-acetate into only a limited number of amino acids. The synthesis of oxaloacetate and aspartate (and the amino acids connected with aspartate) could occur via carboxylation of phosphoenolpyruvate (Fig. 3).

The most striking result of the present study (Table II) is the high R value of citrate (average 1.81). This must result from a rather high discrimination against tritium during the isocitrate dehydrogenase reaction. An isotope effect between 7 and 9 for the hydrogen transfer might fit our results.

The fact that we find a much lower isotope effect for Chlorella is not entirely unexpected. Studies with enzymes isolated from different sources but catalyzing a given reaction have shown different isotope effects. For example, a low secondary isotope effect was found for the reaction mediated by isocitrate dehydrogenase from beef heart[32], whereas

the same reaction mediated by the enzyme from yeast shows large primary isotope effects[22]. Such differences can be attributed to differences in reaction mechanism and kinetic constants of the enzymes involved.

The high tritium incorporation in citrate in Anacystis suggests that it would be worthwhile to try to isolate the isocitrate dehydrogenase from the blue-green algae and to measure its isotope effect in vitro with tritiated citrate as substrate.

Those compounds not related to the tricarboxylic acid cycle have about the same R values in Anacystis as in Chlorella (except 3-phosphoglycerate). This indicates that the discrimination against tritium during the uptake from HTO is about the same as in Chlorella. Considerable variations in R values for 3-phosphoglycerate have been noted in earlier studies with Chlorella[10]. These may be due to the fact that 3-phosphoglycerate is involved in both photosynthetic and respiratory metabolism, and may have rather different expected R ratios, depending on whether it is formed by carboxylation of ribulose-1,5-diphosphate or by oxidation of glyceraldehyde-3-phosphate. Small variations in illumination of cells during killing could produce relatively larger effects on the R value of 3-phosphoglycerate.

The average R value of 0.59 for aspartate is a clear result of the incomplete TCA cycle (Fig. 3). In Anacystis, oxaloacetate is not formed via fumarate and malate, and therefore does not carry a tritium-enriched hydrogen (derived from the malate dehydrogenase reaction), which seemed to be the reason for the higher R values in Chlorella. Aspartate is made via oxaloacetate, which results from a carboxylation reaction of either pyruvate or PEP[33].

Glutamate, the second amino acid closely related to the tricarboxylic

acid cycle, shows low tritium incorporation too (R = 0.77). In Chlorella the glutamate dehydrogenase reaction seemed to be responsible for the retention of tritium in the C-2 hydrogen position of glutamate. From our results it seems possible that in Anacystis nidulans, glutamate dehydrogenase is lacking, since the glutamate radioactivity is low. Since glutamate dehydrogenase could not be detected in Anacystis nidulans[33], perhaps 2-keto-glutarate and glutamate are connected only via the transaminase reaction, while the nitrogen assimilation in this blue-green algae occurs via reductive amination of pyruvate leading to alanine.

CONCLUSIONS

1. The results of these studies suggest that there is no danger of high tritium levels occurring in specific compounds in algae as a consequence of accumulation and retention by reactions of known biochemical pathways. Even in the case of intermediates of the tricarboxylic acid cycle and related amino acids, where the highest retention has been observed, the levels of tritium found are not much higher than in the water from which they are formed. The highest value found (1.8 for citrate in blue-green algae) represents about a threefold increase over the average ratio of tritium in non-exchangeable positions of primary products of photosynthesis to tritium in water. Citrate represents only a miniscule part of the organic carbon in algae which could be eaten by organisms higher in the food chain. It does appear from the earlier studies of Kanazawa et al.[10] and the present studies that the tritium level of primary products of photosynthesis is about 0.6 that of the water, and that the level rises to about 1.0 in amino acids derived from the tricarboxylic acid cycle (in Chlorella).

Even if one made the unrealistically pessimistic assumption that these compounds alone were assimilated and metabolized with the same discriminatory retention in organisms higher in the food chain, the tritium enrichment through five trophic levels could be only $(1/0.6)^5 = 13$. Considering that most of the organic material transmitted from one organism to another will be made up of other compounds (fats, carbohydrates, and proteins containing amino acids with low R ratios), even that tritium increase is unlikely to occur.

2. It is clear that if tritium-labeled compounds were manufactured by growth of algae in tritium-labeled water, the resulting compounds would not be equally labeled in all positions, nor would the labeling be the same for different compounds. This inequality of labeling would have to be taken into consideration when these compounds are used in research.

The evidence for discriminatory retention of T *in vivo* in several oxidative steps involving dehydrogenases and transaminases makes clear the necessity for taking such effects into account in studies of metabolism, transport, compartmentation, etc. when substrates labeled with hydrogen isotopes are employed.

3. The high tritium labeling of citrate in *Anacystis nidulans* suggests that isocitrate dehydrogenase isolated from the organism might exhibit an unusually high isotopic retention of tritium and would be of interest for further study of isotope effects in enzymatically-catalyzed reactions.

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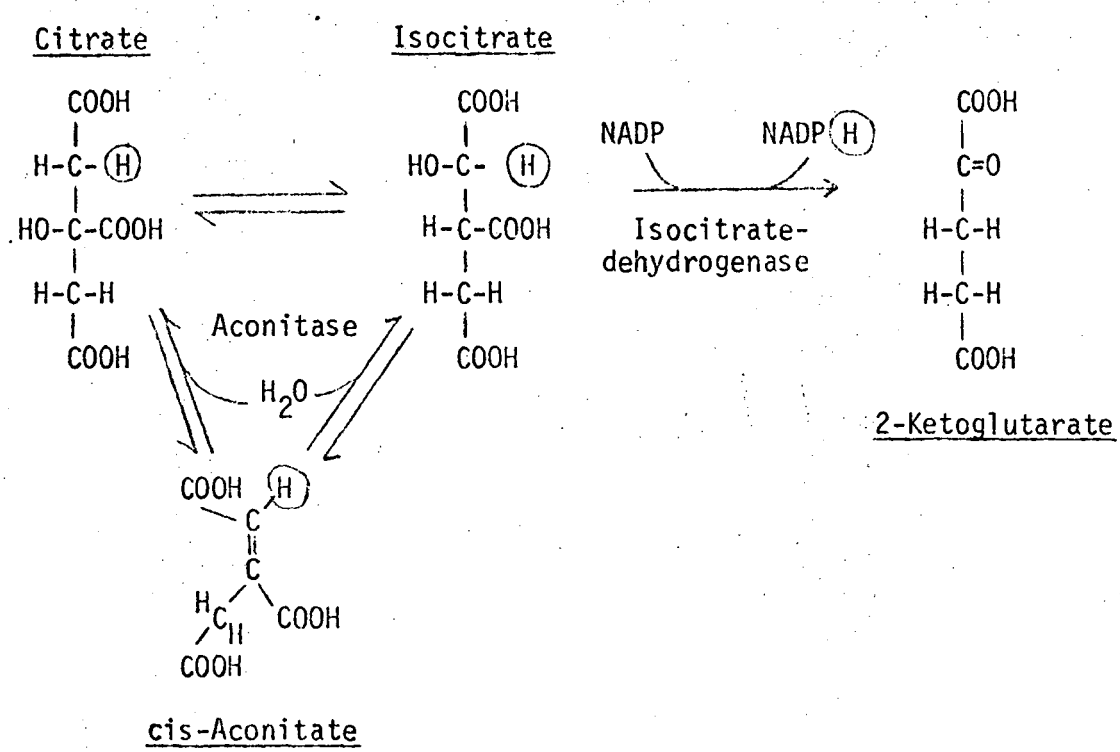

Fig. 1. Fate of hydrogen from citrate to 2-ketoglutarate

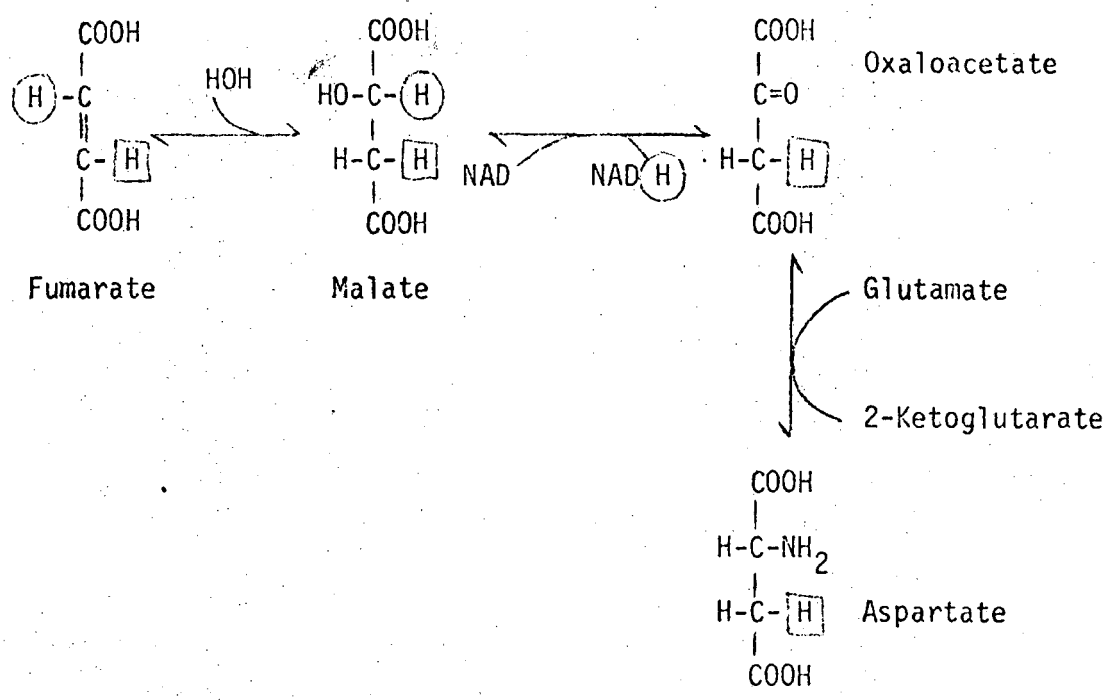

Fig. 2. Fate of hydrogen from fumarate to aspartate

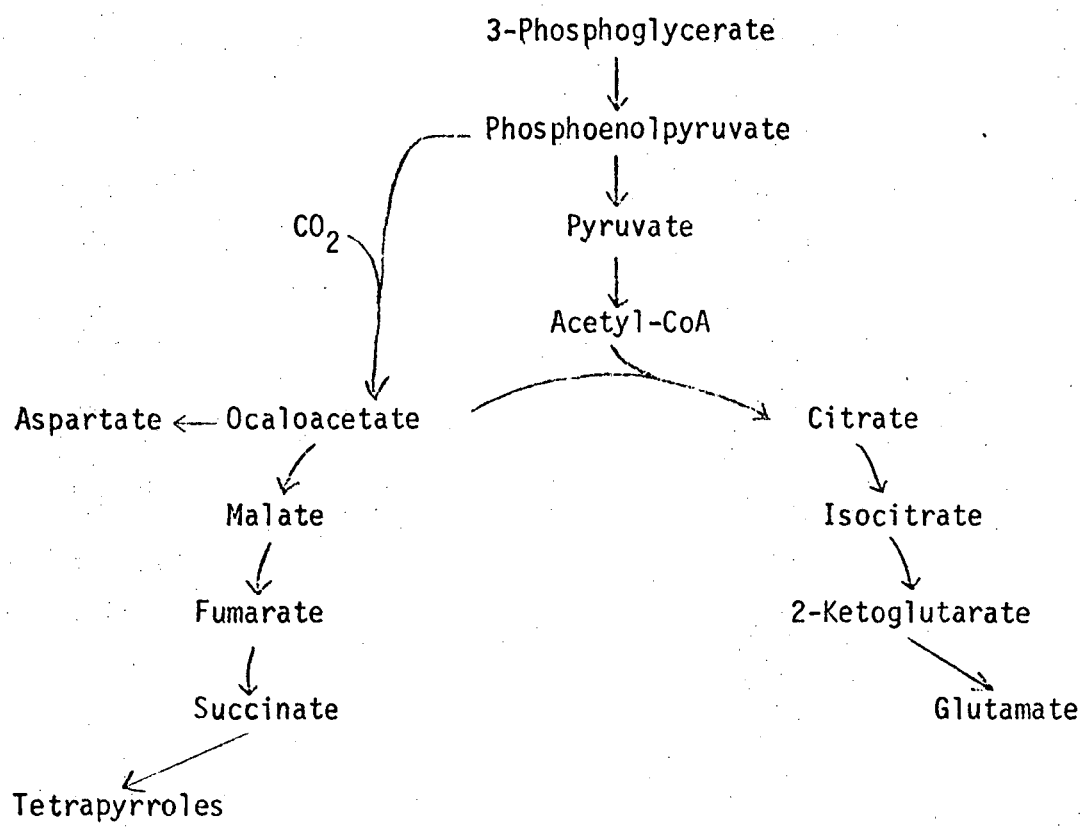

Fig. 3. Incomplete TCA-cycle in <u>Anacystis nidulans</u>

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