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ABSTRACT

1. Acetyl thioctic acid has been prepared chemically and its chromatographic and acetylating behavior is described.
2. A C^{14} -containing substance has been found in Scenedesmus, photosynthesizing in the presence of $\alpha-C^{14}$ -pyruvate, which has properties suggesting that it is acetyl thioctic acid.
3. A C^{14} -containing substance has been found in Aerobacter aerogenes, metabolizing $\alpha-C^{14}$ -pyruvate, which shows the properties of a labile conjugate of thioctic acid with some relatively polar groups.
4. Acetyl thioctic acid is formed in vitro when light acts on a solution of thioctic acid and pyruvate.

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INTRODUCTION

The participation of thioctic acid in the oxidative carboxylation of pyruvic acid is well established. In addition, it is suggested that it may also participate in either the fission or formation of other α -keto alcohol or α -keto acid carbon-to-carbon linkage.¹ This knowledge, together with some observations on the behavior of certain intermediates in photosynthetic organisms, seems to closely implicate thioctic acid with primary photochemical quantum conversion.² For these reasons it seemed desirable to obtain additional information about the forms and compounds into which thioctic acid may be converted, both in photosynthetic systems and in other organisms in which the fission of the α -keto carbon-to-carbon linkage as well as the formation of the α -keto alcohol carbon-to-carbon linkage takes place. It is the purpose of this paper to report a number of observations on the occurrence of thioctic acid--particularly acetyl thioctic acid and some of its derivatives--in a photosynthetic organism (Scenedesmus) and in an acetoin-forming organism (Aerobacter aerogenes), together with a description of the direct chemical acetylation of the dithiol form of thioctic acid.

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Thioctic Acid Derivative in *Aerobacter Aerogenes*

Aerobacter aerogenes, strain VC, was grown in a medium containing 1% glucose, 0.3% proteose peptone, 0.8% secondary potassium phosphate, and 10% tap water. After 20 hours of growth at 30° C, the cells were harvested by centrifugation, washed twice with 0.1 M phosphate buffer, and a 10% (wet weight) suspension was disintegrated in a phosphate buffer at pH 6 for 20 minutes in the Raytheon apparatus. This disintegrated solution was centrifuged at 20,000 rpm and the supernatant was used for the following experiment.

Products of pyruvate-2-C¹⁴ metabolism

The enzymatic extract (0.2 to 0.4 cc) was diluted to 1 cc, and 0.30 mg of sodium pyruvate with a specific activity of 17.5 $\mu\text{c}/\text{mg}$ were added. After 10 minutes, an aliquot of the mixture was chromatographed on Whatman No. 4 paper in the solvent systems phenol-water and butanol-propionic acid-water.

Aside from a small amount of residual pyruvate, the major products consisted of a group of three spots in an area on the radiogram characteristic of phosphate esters. These were eluted and tested for thioctic acid biological activity on *S. faecalis*.³ The middle spot of the three (R_f 0.22 x 0.45) was the only one to show such activity. None of these areas, either before or after hydrolysis, showed biological activity for thiamin on the *Neurospora crassa* mutant G185, which requires thiamin or cocarboxylation for growth.⁴

A transformation of the conjugate

The above enzyme-pyruvate product was stored three weeks at -15° C and rechromatographed. The middle area, which has biological activity for thioctic acid, had diminished markedly, and a radioactive area in the position of thioctic acid (or acetyl thioctic acid) appeared. The new radioactive compound possessed a radioactivity approximately equal to the diminution of that in the conjugate which disappeared. An identical reaction, with a greater degree of transformation, was obtained when the enzyme-pyruvate mixture was boiled for a few seconds and then rechromatographed. No quantitative information is available as to the amount of biological activity that migrates in association with the migration of radioactivity.

Formation from C¹⁴-pyruvate of a thioctic conjugate in presence of excess thiamin pyrophosphate (TPP)

Another enzymatic experiment was performed with the addition of 1 mg TPP and 0.5 mg thioctic acid, phosphate buffer pH 6.5. No thiamin was detected by bioassay in the conjugate or in any of the other labeled products before or after acid hydrolysis. A new radioactive spot did appear in these radiograms at R_f 0.9 x 0.15. No thiamin was found associated with this radioactivity.

Acetoin cannot be detected on the paper chromatogram, but if one adds acetoin carrier to an aliquot of the enzymatic solution and isolates the crystalline dinitrophenylosazone (after washing with dilute sodium hydroxide to remove pyruvic hydrazone), the activity of the three-times-recrystallized derivative shows that 87 percent of the pyruvate was converted into acetoin.

Acetyl Thioctic Acid from Feeding Experiments with *Scenedesmus*

After a 40-minute light photosynthetic fixation of pyruvate-2-C¹⁴, three spots appeared in the lipid region of the radiogram. The middle one (R_f 0.8 x 0.8; the fats move with the front in both solvents) had thioctic acid activity with *Streptococcus faecalis*; the others were inactive. The bio-active spot was eluted with ethanol-chloroform mixture and an aliquot hydrolyzed for 30 minutes in 1 N sodium hydroxide in the absence of air. Then the mixture was acidified using 2 N sulfuric acid. After addition of 30 mg of acetic acid as carrier it was distilled. The acetate was isolated as the benzylthio-uronium salt, and after two crystallizations the activity remained constant. The fraction isolated as labeled acetate was 20 percent of the original activity, which was contaminated with phospholipids and similar compounds. Attempted hydrolysis of the bio-active spot in sulfuric acid indicated that the compound was more stable to acid conditions.

Acetyl transfer

Ethyl thioacetate carrier (200 µg) was added to the eluted bio- and radioactive compound. Excess hydroxylamine at pH 6.5 reacted to form the acetyl hydroxamic acid, which chromatographs unidimensionally⁵ in butanol-propionic acid solvent with an R_f of 0.66. The ferric chloride color reaction with the hydroxamate corresponded exactly to the position of the radioactivity. Of the initial eluted radioactivity, 10 to 15 percent was finally obtained as acetyl hydroxamic acid.

A similar transfer to sulfanilamide⁶ was attempted, but the R_f values for acetyl sulfanilamide and the starting acetyl thioester were too similar (R_f in phenol, 0.79; R_f in butanol-propionic acid, 0.70). It is therefore probable that the compound (R_f 0.8 x 0.8) is acetyl thioctic acid. The biological activity characteristic of thioctic acid, the acid stability and alkali lability, formation of authentic acetyl hydroxamic acid, and the chromatographic coincidence with synthetic acetyl thioctic acid (see section on chemical acetylation of 6,8-dithioloctanoic acid) all support this conclusion.

Chemical Acetylation of 6,8-Dithioloctanoic Acid

Synthesis

Labeled acetic anhydride was prepared by an exchange reaction with carboxyl-labeled sodium acetate. Sodium acetate (5 mg) with a specific activity of 15 $\mu\text{c}/\text{mg}$ was added to 10 λ of acetic anhydride and allowed to stand 24 hours at room temperature. The activity of the liquid anhydride was 154,000 cpm/ λ , determined in the acetanilide formed. Then, 1 mg of dithiol was dissolved in anhydrous pyridine and 1/2 λ of acetic anhydride was added. The product was isolated by paper chromatography in butanol-propionic acid-water, and the yield was 84 percent. The diacetyl derivative was prepared, in the same way, by adding 1 mg dithiol to 5 λ acetic anhydride (ten times excess), with a yield of 90 percent.

Paper chromatography

The mixture obtained from the anhydrous pyridine-acetic anhydride monoacetylation of the dithiol for 10 hours was chromatographed on paper, using butanol-propionic acid-water as solvent, and one radioactive spot was detected with an R_f of 0.88. In 70 percent methanol-water, the R_f of the acetyl thioctic acid was 0.78; in butanol-ethanol-water (80:20:20) the R_f was 0.89. It was not possible to separate the monoacetyl derivatives from the dithiol or the diacetyl thioctic acid by simple chromatography. They were easily distinguished, however, by the nitroprusside color, which is purple for the monoacetyl, orange for the dithiol, and negative for the diacetyl compound.

The separation of the dithiol from the monoacetyl derivatives was achieved by addition of arsenite just before the application of the compound to the paper chromatogram; this gives a material with a negative

nitroprusside test. With radioactive As^{73, 74}, the arsenite-thioctic complex has the following R_f values: in butanol-propionic acid-water, 0.94 - 0.96; in butanol-ethanol, 0.4; in butanol-ammonia, 0.25 - 0.28. By comparison, the trimethylenedithiol arsenite shows the following R_f values: in butanol-propionic acid-water, 0.97; in butanol-ammonia, 0.46. Radioactive arsenic itself moves in butanol-propionic acid-water with an R_f of 0.48; in butanol-ethanol, 0.56; and butanol-ammonia, 0. The migration of the monoacetyl thioctic acid is not changed after either addition of arsenite at pH 7.5, or chromatography in butanol-ethanol-water containing 0.1 M amount of arsenite.

Stability

Seventy-eight percent of the compound was hydrolyzed at 100° C in 1 N sodium hydroxide for 30 minutes. In 2 N sulfuric acid, 17 percent was hydrolyzed after 2 hours at 100° C. In both cases, the liberated acetic acid was isolated after addition of carrier and acidification, by means of a vacuum distillation and crystallization of the benzylthiouronium salt.⁷

Reaction with hydroxylamine

This reaction was first examined by using ethyl thiolacetate prepared by addition of 28 g of ethyl mercaptan to an ice-cold mixture of 32.5 g of acetylchloride in 80 cc of anhydrous pyridine. After 14 hours, ice was added to the reaction mixture, then water, then 3 N sulfuric acid in excess. The ethyl thiolacetate was extracted with ether and distilled. The yield was 67 percent of a compound boiling between 114° - 116° C. Upon the addition of hydroxylamine, this compound gives acetyl hydroxamic acid, which can be detected by its purple color after addition of ferric chloride. Paper chromatography shows that in phenol-water the acetyl hydroxyamic acid has an R_f of 0.71; in butanol-ammonia, 0.54; in butanol-propionic acid-water, 0.66.

With ethyl thiolacetate used as carrier, the monoacetyl and diacetyl thioctic acid gave rise to labeled acetyl hydroxamic acid, and the radioactivity was exactly coincident on a paper chromatogram run in butanol-propionic acid-water with the ferric chloride color test.

Photolysis of thioctic acid in the presence of labeled sodium pyruvate

Thioctic acid (200 γ) was dissolved in 100 γ of 95 percent alcohol, 100 γ of sodium pyruvate (specific activity 17.5 $\mu\text{c}/\text{mg}$) were added, and the mixture was evacuated on the high-vacuum line. The ultraviolet light was turned on

for one hour at room temperature, and an aliquot of the mixture was run on a paper chromatogram with butanol-propionic acid-water as solvent. One radioactive spot could be detected, with an R_f of 0.88. This spot, after elution, gave acetyl transfer with hydroxylamine, which could be seen by the appearance of labeled acetyl hydroxyamic acid on the paper chromatogram. This compound must be, therefore, a thiol ester, and could not be detected if the thioctic acid and sodium pyruvate were kept in the dark. The radioactive spot was completely absent from the pyruvate chromatogram and did not appear during photolysis of the pyruvate alone. The yield varies between 2 and 5 percent.

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