Isolation and Identification of α -Ketoaldehydes in Calf and Rabbit Livers[†]

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Carbonyl compounds in calf liver stored at 4°C for 5~6 hr after slaughter were investigated, with emphasis on α -ketoaldehydes. After conversion of carbonyls into their 2,4-dinitrophenylhydrazones (2,4-DNPs), they were fractionated by preparative thin-layer chromatography (TLC); at least, twenty-three fractions were separated, among which eighteen fractions contained 2,4-DNPs of α -dicarbonyls. From some of the fractions, crystalline 2,4-DNPs were isolated, and identified by TLC and infrared spectra: pyruvaldehyde, 3-deoxypentosulose, xylosulose, 3-deoxyglucosulose, 2,3-diketogulonic acid or/and dehydroascorbic acid, and formaldehyde were identified, and glucosulose was tentatively identified.

The carbonyl pattern, obtained from rabbit liver frozen in liquid nitrogen immediately after slaughter, was also similar as that from calf liver, and further, the amount of each α -ketoaldehydes remained at the almost same level during storage at 4°C for 24 hr. These results indicate that the identified α -ketoaldehydes are constituents or metabolic intermediates in calf and rabbit livers.

The formation mechanisms of the carbonyls in tissues are discussed.

For many years, pyruvaldehyde (methylglyoxal) has been known to be produced on incubating glycolyzing tissue homogenates with substrates, although its formation mechanism is not clear. Recently, Szent-Györgyi *et al.*¹⁾ has postulated that α -dicarbonyl compounds such as α -ketoaldehydes are involved in the regulation of cell division in animal tissues; Együd and Szent-Györgyi have shown that pyruvaldehyde inhibits growth of *Escherichia coli*,²⁾ protein synthesis,³⁾ and growth of ascites

tumor cells.⁴⁾ Some investigators^{5~7)} demonstrated the evidences of interaction between glyoxal derivatives and nucleic acids. Szent-Györgyi⁸⁾ pointed out that, calf livers contained several free and bound α -dicarbonyls, one of which revealed an inhibiting effect on protein synthesis in trace amounts. The most abundant bound ketoaldehyde, which was produced on treatment of calf and rat livers with

3) L. G. Együd and A. Szent-Györgyi, *Proc. Natl. Acad. Sci. U.S.*, **56**, 203 (1966).

4) *idem*, *Science*, **160**, 1140 (1968).

5) M. Staehelin, *Biochim. Biophys. Acta*, **31**, 448 (1959).

6) O. L. Klammer, *ibid.*, **155**, 271 (1968); B. R. Brooks and O. L. Klammer, *European J. Biochem.*, **5**, 178 (1968).

7) K. Tanaka, O. Takenaka, H. Horinishi and K. Shibata, *Biochim. Biophys. Acta*, **161**, 23 (1968).

8) A. Szent-Györgyi, *Proc. Natl. Acad. Sci. U.S.*, **57**, 1642 (1967).

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1) A. Szent-Györgyi, L. G. Együd and J. A. McLauphin, *Science*, **155**, 539 (1967).

2) L. G. Együd and A. Szent-Györgyi, *Proc. Natl. Acad. Sci. U.S.*, **55**, 388 (1966).

arsenite,⁹⁾ was identified as 3-deoxyglucosulose (3-deoxyglucosone).¹⁰⁾ This ketoaldehyde has, however, been considered to be an artifact arising from glucose and amino acids by arsenite- and phosphate-catalyzed nonenzymatic reaction.¹¹⁾ These findings are of great interest, though the formation mechanism and physiological significance of the α -dicarbonyls remain obscure.

In the present report, we describe the isolation and identification of carbonyls, especially of α -dicarbonyls, which are present in fresh calf and rabbit livers. Since α -dicarbonyls are known to be highly reactive and labile compounds, the isolation procedures have been carried out after converting them into their 2,4-dinitrophenylhydrazones (2,4-DNPs). As the results, four kinds of osuloses (osones) have been identified for the first time in animal tissues.

MATERIALS AND METHODS

Liver samples. Calf livers stored at 4°C for 5–6 hr after slaughter were used as a 4°C-stored calf liver sample. A rabbit of *ca.* 2.5 kg in body weight was killed, immediately the livers were removed, and a part of them was immersed in liquid nitrogen and the remaining parts were stored at 4°C for 5 hr and 24 hr; the nitrogen-immersed part was used as a freshly frozen liver sample.

Preparation of 2,4-DNPs of carbonyls from livers

a) From calf livers. One kilogram of 4°C-stored calf liver sample was homogenized with 1250 ml of methanol and 500 ml of water under cooling with ice. After 1 hr at room temperature (*ca.* 20°C), the mixture was centrifuged, the methanol concentration of the resulted supernatant being *ca.* 50%. The extract was diluted with two volumes of 10% trichloroacetic acid and centrifuged. The deproteinized supernatant

was treated with one-tenth volume of conc. HCl and one-tenth volume of 2,4-dinitrophenylhydrazine reagent⁹⁾ at room temperature for 30 min. The 2,4-DNPs formed were extracted with chloroform, and the extract was washed with 2N HCl and water subsequently to remove the excess reagent and then concentrated. To the concentrate was added hexane to precipitate the hydrazones. The precipitate was dissolved in ethyl acetate and fractionated by preparative thin-layer chromatography as described below. All α -dicarbonyl bis(2,4-DNPs) precipitated on adding hexane, but a part of formaldehyde 2,4-DNP remained in the supernatant, and then this fraction was similarly treated.

b) From rabbit livers. Freshly frozen or 4°C-stored rabbit liver sample was homogenized with methanol and water similarly as described above and immediately centrifuged. The resulted supernatant was treated similarly as in the case of calf liver sample to prepare 2,4-DNPs. All these procedures were carried out at 4°C.

Thin-layer chromatography (TLC). Glass plates (20 × 20 cm) coated with Silica Gel G according to Stahl (Merck) in 0.5 mm thickness were used for the preparative TLC. The first development was made with toluene-ethyl acetate, 2:1 v/v. Each separated band was cut off from the plates, extracted with ethyl acetate, concentrated under reduced pressure, and further fractionated by using similar plates. The second development was done with the following five solvent systems: toluene; toluene-ethyl acetate, 6:1, 2:1, and 1:1 v/v; benzene-ethylacetate-pyridine-methanol, 95:5:5:3 v/v. Double development was also applied, when it was necessary for the better separation of adjacent bands.

Isolation and identification of 2,4-DNPs. Each band, separated by the second development as described above, was cut off, extracted with ethyl acetate, and dried up. The residue was recrystallized from ethanol-ethyl acetate; in the case of formaldehyde 2,4-DNP, hexane-ethyl acetate was used as solvent. The isolated 2,4-DNPs were identified by their behaviors on thin layer chromatograms and by comparing their infrared spectra with those of authentic specimens. Infrared spectra were recorded in Nujol mull using a JASCO IR-S spectrophotometer.

Estimation of the amount of α -dicarbonyl. 2,4-DNP of the α -dicarbonyl separated on thin-layer chromatogram

9) L. G. Együd, J. A. McLaughlin and A. Szent-Györgyi, *Proc. Natl. Acad. Sci. U.S.*, **57**, 1422 (1967).

10) G. Fodor, J. P. Sachetto, A. Szent-Györgyi and L. G. Együd, *ibid.*, **57**, 1644 (1967).

11) H. Otsuka and L. G. Együd, *Biochim. Biophys. Acta*, **165**, 172 (1968).

was extracted with ethyl acetate, and the extract was concentrated, filled up to a certain volume, and the optical density was measured at 435 m μ . The approximate amount of the α -dicarbonyl was calculated from the standard curve of authentic 3-deoxy-D-glucosulose bis (2,4-DNP). In some cases, the amounts of carbonyls were calculated from the weight of 2,4-DNP isolated.

RESULTS

Carbonyls in 4°C-stored calf liver

2,4-DNPs prepared from 4°C-stored calf liver sample were separated by preparative TLC. As shown in Fig. 1, on the first development, nine bands were separated, which were named band A, B, C, D, E, F, G, H and I in the order of *R_f* value, respectively. On the second development of each band, at least twenty-three bands were observed in all. Most of them, *i.e.* eighteen bands, were those of bis-(2,4-DNPs) of α -dicarbonyls, which changed

their orange color into blue or violet on spraying with methanolic potassium hydroxide (Fig. 1). From some of the comparatively abundant bands were obtained crystalline 2,4-DNPs. Their infrared spectra are shown in Fig. 2.

Thus, the following carbonyls were isolated and identified as their 2,4-DNPs, respectively: pyruvaldehyde from band A, formaldehyde from band B, 3-deoxypentosulose from band E, xylosulose and 3-deoxyglucosulose from band G, and 2,3-diketogulonic acid or/and dehydroascorbic acid from band H; in addition, glucosulose was isolated from band I and tentatively identified by TLC. As shown in Table I, the approximate amount of each carbonyl was below 0.25 mg per 100 g of moist liver, except the amounts of formaldehyde and 2,3-diketogulonic acid.

The other bands, however, were less in quantity and were not obtained in crystalline form. Neither band corresponding to 2-

FIG. 1. Thin-layer Chromatograms of 2,4-Dinitrophenylhydrazones Prepared from the 4°C-Stored Calf Liver.

First development: toluene-ethyl acetate, 2:1 v/v. Second development:

A and B, toluene;

C and D, toluene-ethyl acetate, 6:1 v/v;

E, F and H, toluene-ethyl acetate, 2:1 v/v;

G, benzene-ethyl acetate-pyridine-methanol, 95:5:5:3 v/v;

I, toluene-ethyl acetate, 1:1 v/v.

Oblique-lined spots indicate bis-(2,4-DNPs) of α -dicarbonyls. Spots numbered with arrow were isolated and identified: 1, pyruvaldehyde; 2, formaldehyde; 3, 3-deoxypentosulose; 4, xylosulose; 5, 3-deoxyglucosulose; 6, 2,3-diketogulonic acid; 7, glucosulose.

FIG. 2. Infrared Spectra of 2,4-Dinitrophenylhydrazones of Carbonyls Isolated from the 4°C-Stored Calf Liver.

---, authentic; —, isolated.		
1, Pyruvaldehyde	2, Formaldehyde	3, 3-Deoxypentosulose
4, Xylosulose	5, 3-deoxyglucosulose	6, 2,3-Diketogulonic acid

furaldehyde 2,4-DNP nor 5-(hydroxymethyl)-2-furaldehyde 2,4-DNP was detected on the thin-layer chromatograms. Any 2,4-DNPs of aldehydes of longer carbon chain than that of acetaldehyde, of which *R_f* values were larger than that of formaldehyde or acetaldehyde, were also not detectable. Formaldehyde 2,4-DNP and acetaldehyde 2,4-DNP showed the similar behavior on thin-layer chromatograms, but only the former crystallized out from the corresponding fraction. Accordingly, the existence of acetaldehyde is doubtful. Attempts to determine D- or L-configuration of the isolated osulose bis(2,4-DNPs) were unsuccessful because of their low yields.

Inspection of α -dicarbonyl formation by using a model system

In order to check on the α -dicarbonyl form-

ation from ascorbic acid and glucose in livers during the isolation procedure and treatment with 2,4-dinitrophenylhydrazine reagent, the following experiment was carried out. L-Ascorbic acid (27 mg), D-glucose (100 mg) and L-valine (20 mg) were dissolved in Ringer's phosphate buffer solution (pH 7.0, 50 ml),¹²⁾ and kept for 4 hr or 24 hr at 4°C or 20°C. The resulted solution was treated with 2,4-dinitrophenylhydrazine reagent, and the 2,4-DNPs formed were analyzed by TLC similarly as described above. The results are shown in Table II. A spot of 2,3-diketogulonic acid bis(2,4-DNP) was detected after 24 hr at 4°C or after 4 hr at 20°C, and that of glucosulose bis(2,4-DNP)

12) V. Riddle and F. W. Lorenz, *J. Biol. Chem.*, **243**, 2718 (1968).

TABLE I. CARBONYL COMPOUNDS IDENTIFIED IN THE 4°C-STORED CALF LIVER

Compound identified ^{a)}	Approximate amount ^{b)} (mg/100 g of moist liver)
Formaldehyde	1.00
2,3-Diketogulonic acid	0.67 ^{c)}
Xylosulose	0.24
Pyrvaldehyde	0.21
3-Deoxypentosulose	0.19
3-Deoxyglucosulose	0.13
Glucosulose	0.25 ^{d)}

^{a)} The carbonyls were isolated and identified as their 2,4-DNPs.

^{b)} The amount of the carbonyl was calculated from the weight of 2,4-DNP isolated. In the cases of xylosulose, 3-deoxypentosulose and 3-deoxyglucosulose, the amount was estimated colorimetrically from the standard curve at 435 m μ of authentic 3-deoxy-D-glucosulose bis(2,4-DNP) in ethyl acetate.

^{c)} Dehydroascorbic acid also gives the same bis(2,4-DNP) as does 2,3-diketogulonic acid. Accordingly, this amount may be the sum of the both acids.

^{d)} Infrared spectrum of this fraction indicated a little contamination.

TABLE II. FORMATION OF DICARBONYLS FROM L-ASCORBIC ACID, D-GLUCOSE AND L-VALINE IN RINGER'S SOLUTION

Dicarbonyl	Reacting condition			
	At 4°C		At 20°C	
	4 hr	24 hr	4 hr	24 hr
2,3-Diketogulonic acid	—	+	+	††
3-Deoxypentosulose	—	—	—	—
3-Deoxyglucosulose	—	—	—	—
Xylosulose	—	—	—	—
Glucosulose	—	—	—	+

L-Ascorbic acid (27 mg), D-glucose (100 mg) and L-valine (20 mg) were dissolved in Ringer's phosphate buffer solution (50 ml),¹²⁾ and the mixture (pH 7.0) was stored at 4°C or 20°C for 4 hr or 24 hr. After treatment with 2,4-dinitrophenylhydrazine reagent, the 2,4-DNPs formed were extracted with chloroform and developed on silica gel plates using the same solvent system as described in Fig. 1.

—, not detected; +, slightly detected;

††, abundantly detected.

FIG. 3. Thin-layer Chromatograms of 2,4-Dinitrophenylhydrazones Prepared from the Freshly Frozen Rabbit Liver.

Conditions for TLC were the same as described in Fig. 1.

Oblique-lined spots indicate bis(2,4-DNPs) of α -dicarbonyls. The alphabets and the numbers with arrow correspond to those in Fig. 1.

appeared after 24 hr at 20°C. The latter was not detected after 24 hr at 4°C or after 4 hr at 20°C. After 4 hr at 4°C, any spot of hydrazone was not detected. Any of xylosulose, 3-deoxypentosulose or 3-deoxyglucosulose was not produced even after 24 hr at 20°C in this model system.

Carbonyl pattern in freshly frozen rabbit livers

Further inspection of dicarbonyl formation in livers after slaughter was made as follows. A freshly frozen rabbit liver sample, which was immersed in liquid nitrogen immediately after slaughter, was processed to prepare 2,4-DNPs under the condition at 4°C. The resulted 2,4-DNPs were developed on thin-layer plates using the same solvent systems as described in the case of calf liver sample. The chromatograms were as shown in Fig. 3. The carbonyl pattern is quite the same as that of 4°C-stored calf liver sample in Fig. 1.

Changes in the amounts of α -dicarbonyls during 4°C-storage

The freshly frozen rabbit liver sample and

FIG. 4. Changes in the Amounts of the Dicarbonyls during 4°C-Storage of Rabbit Liver.

Zero hour means the sample frozen in liquid nitrogen immediately after slaughter. The amounts of dicarbonyls were estimated colorimetrically after isolating their 2,4-DNPs on thin-layer plates.

- 3-Deoxypentosulose
- Xylosulose
- ▲—▲ 3-Deoxyglucosulose
- △—△ Glucosulose
- ×—× Pyruvaldehyde
- 2,3-Diketogulonic acid or/and dehydroascorbic acid

the samples stored at 4°C for 5 hr and 24 hr after slaughter were treated similarly as described above, and the amounts of 2,3-diketogulonic acid, 3-deoxypentosulose, 3-deoxyglucosulose, xylosulose, glucosulose, and pyruvaldehyde were estimated in each sample. The results are shown in Fig. 4. 2,3-Diketogulonic acid or/and dehydroascorbic acid considerably increased after 24 hr at 4°C, but other dicarbonyls remained at the almost same level as that of zero hour (freshly frozen sample).

DISCUSSION

From the calf liver stored at 4°C for 5~6 hr after slaughter were detected 3-deoxypentos-

ulose, 3-deoxyglucosulose, xylosulose, and glucosulose in addition to 2,3-diketogulonic acid and pyruvaldehyde. These α -ketoaldehydes (osuloses) were not produced in the model system consisting of ascorbic acid, glucose, valine and phosphate under the condition at 4°C for 24 hr. They were detected also in the freshly frozen rabbit liver, and further, each amount of 3-deoxypentosulose, 3-deoxyglucosulose, xylosulose, glucosulose or pyruvaldehyde did not change during storage at 4°C for 24 hr. These experimental results lead us to the conclusion that the α -ketoaldehydes identified in the present study are the constituents or metabolic intermediates in calf and rabbit livers.

It is uncertain whether the α -ketoaldehydes are present in free form or not, because acid-labile linkages such as Schiff's bases or N-glycosidic type of linkages of osuloses would be possible to be cleaved through the treatment with 2,4-dinitrophenylhydrazine reagent even under the condition at 4°C. Anyway, it is very interesting that such highly reactive compounds as α -dicarbonyls have been detected in animal tissues.

The formation mechanism of the α -ketoaldehydes in tissues is the problem to be discussed. As widely known, 3-deoxyglucosulose is formed from glucose through Maillard reaction.¹³⁻¹⁵ Kato has disclosed that, 3-deoxypentosulose is similarly produced through the reaction between pentoses and amino compounds,¹³ and further, glucosulose is formed from glucose by Maillard reaction under oxidative condition.¹⁶ Xylosulose is

13) H. Kato, *Bull. Agr. Chem. Soc. Japan*, **24**, 1 (1960); *idem*, *Agr. Biol. Chem.*, **25**, 671 (1961); *idem*, *ibid.*, **26**, 187 (1962).

14) E. F. L. J. Anet, *J. Am. Chem. Soc.*, **82**, 1502 (1960); *idem*, *Australian J. Chem.*, **13**, 396 (1960).

15) T. M. Reynolds, "Advances in Food Research," Vol. 12, ed. by C. O. Chichester, E. M. Mrak and G. F. Stewart, Academic Press, Inc., New York, 1963, p. 1.

16) H. Kato, *Agr. Biol. Chem.*, **27**, 461 (1963).

known as a decomposition intermediate of dehydroascorbic acid.^{17,18)} Kurata and Sakurai¹⁹⁾ have shown that 3-deoxypentosulose is produced also from ascorbic acid under acidic condition. Carboxylate anions and polyvalent anions such as phosphate are known to be effective to accelerate Maillard reaction.^{20,21)} Riddle and Lorenz¹²⁾ investigated and discussed on nonenzymatic, polyvalent anion-catalyzed formation of pyruvaldehyde from trioses under physiological conditions. These accumulated knowledges suggest that nonenzymatic formation of α -ketoaldehydes may be possible to occur also in living tissues, where reducing sugars, ascorbic acid, various amino compounds and polyvalent anions, and others are coexisting.

On the other hand, it would be also acceptable that, in living tissues, Maillard reaction might be partly or mostly under control by inhibitors such as thiol compounds. Lipid autooxidation in livers also seems to be under control, since aldehydes of long carbon chain such as butanal are not detectable as already mentioned.

Fructoseamino acids, *i.e.* Amadori rearrangement products from glucose and amino acids, have been detected in hog liver powder or extract.^{22,23)} Fructoseamino acids are known to be intermediates in osulose formation from glucose and amino acids.¹³⁻¹⁶⁾ However, Heyns and Paulsen²³⁾ showed that freshly frozen calf liver did not contain any fructoseamino acid, and suggested that enzymatic

reactions were involved in the formation of fructoseamino acids after slaughter. In other words, it may be considered that the formation of fructoseamino acids are under control in living tissues. In most cases of nonenzymatic reactions, either (both) 2-furaldehyde or (and) 5-(hydroxymethyl)-2-furaldehyde is (are) formed along with 3-deoxyosulose formation.^{13-15,19,24)} In the present experiments, however, none of them was detected in calf and rabbit livers. These facts may propose the possibility that α -ketoaldehydes in livers are produced *via* other paths than nonenzymatic reaction, *i.e.* paths involving enzymatic reaction.

Ikeda²⁵⁾ has described the formation of glucosulose from fructose by *Gluconobacter roseus*. In animal tissues, however, any enzyme system involving in α -ketoaldehyde formation has never been reported. On the other hand, some ketoaldehydes are known to be metabolized: pyruvaldehyde is converted into lactic acid by glyoxalase system,^{26,27)} and 3-deoxyglucosulose is oxidized into 3-deoxy-2-ketogluconic acid by a dehydrogenase in livers.²⁸⁾ Accordingly, there is a possibility that the levels of the α -ketoaldehydes in tissues are being controlled under enzyme systems. The biological significance of the ketoaldehydes will be an interesting subject for future investigation.

Formaldehyde was detected in 4°C-stored calf liver and also in freshly frozen rabbit liver. The formation mechanism and biological significance of this aldehyde also are unknown. Amano *et al.* have detected formaldehyde in fresh and stored tissues of some

17) G. C. Whiting and R. A. Coggins, *Nature*, **185**, 843 (1960).

18) T. Kurata and Y. Sakurai, *Agr. Biol. Chem.*, **31**, 177 (1967).

19) *idem*, *ibid.*, **31**, 170 (1967).

20) H. S. Isbell and H. L. Frush, *J. Org. Chem.*, **23**, 1309 (1958).

21) H. Kato, M. Yamamoto and M. Fujimaki, *Agr. Biol. Chem.*, **33**, 939 (1969).

22) H. Borsook, A. Abrams and P. H. Lowy, *J. Biol. Chem.*, **215**, 111 (1955).

23) K. Heyns und H. Paulsen, *Ann.*, **622**, 160 (1959).

24) H. Kato and M. Fujimaki, *J. Food Sci.*, **33**, 445 (1968).

25) Y. Ikeda, *Nippon Nogeikagaku Kaishi*, **26**, 90 (1952); *idem*, *ibid.*, **28**, 538 (1954).

26) C. Neuberg, *Biochem. Z.*, **49**, 502 (1913).

27) H. D. Dakin and H. W. Dudley, *J. Biol. Chem.*, **14**, 423 (1913).

28) E. Jellum, *Biochim. Biophys. Acta*, **165**, 357 (1968).

species of fish,²⁹⁾ and postulated its enzymatic formation from trimethylamine oxide.³⁰⁾

29) K. Amano, K. Yamada and M. Bito, *Nippon Suisan Gakkaishi*, **29**, 695, 860 (1963).

30) K. Amano and K. Yamada, *ibid.*, **30**, 430, 639 (1964).

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