*KETONE ALDEHYDES IN ANIMAL TISSUES**

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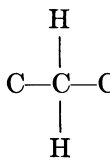
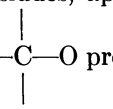
In earlier papers,[1-3] the preparation of a 2,4-dinitrophenylosazone from liver extracts was described. Precipitated from water, this has a mp 265°; $[\alpha]_D^{25}$ = +862° (in DMSO/c, 9.07 × 10^{-4} gm/ml). The IR spectrum (Fig. 3) indicates the presence of (bonded) hydroxyls at 3300 cm^{-1} and of two conjugated C=N double bonds at 1585 and 1620 cm^{-1}. Therefore, this is a bis-dinitrophenylhydrazone, as suggested earlier,[1] based on the Neuberg test.[5, 6]

If the molecule contains two 2,4-dinitrophenylhydrazone residues with eight nitrogens, then elementary analysis (Table 1) leads to the formula $C_{18}H_{20}O_{12}N_8$.

Recrystallization from ethyl acetate gave needles with the approximate composition $C_{22}H_{26}O_{14}N_8$, indicating the presence of ethyl acetate of crystallization. Drying (120°, 0.1 mm) gave an anhydrous product $C_{18}H_{18}O_{11}N_8$, which showed a very high optical rotation (Fig. 5). The NMR spectrum in both pyridine-d_5 (Fig. 1, curve 1, and Table 2) and dimethylsulfoxide-d_6 was rather simple. The aromatic signals and those belonging to the H—C=N—, imidoformyl proton were identified by comparing with the NMR spectrum of glucose-2,4-dinitrophenylosazone. The formyl proton was found at δ8.6 ppm. Taking this as unity, integration showed six aromatic protons. Five other protons were displaced to δ5.8 ppm corresponding to DO-H (Fig. 2, curve 4) on addition of D_2O. Thus, apart from the two NH protons of the 2,4-dinitrophenylhydrazone residue, a further three protons must be attached to oxygen in the liver osazone. The signal at the highest field (δ4.41 ppm) integrates as two protons, clearly belonging to a



system. Two more protons, strongly deshielded as a consequence of the nitroaryl residues, appeared at 7.13 and 7.61 ppm, respectively. We ascribed them to the



protons.

Acetylation of liver-2,4-DNP-osazone with acetic anhydride in pyridine (20° 24 hr) and evaporation of the solvent at 20° gave brown-colored crystals of mp 184°, after several recrystallizations. The elementary analysis and the molecular weight (664) corresponded to a triacetyl derivative $C_{24}H_{24}O_{14}N_8$ (mol wt 648), while the *O*-acetyl number (17.8%) was lower than calculated (19.9%). Modification of the acetylation technique (pouring onto ice instead of evaporating) gave light-yellow crystals with better analytical figures and fairly good acetyl value (19.02%). The NMR spectrum of the latter clearly integrates for nine H_3C—CO protons (at δ2.20, 2.01, and 1.98 ppm). Thus the presence of three acylable hydroxyls in the dinitrophenylosazone was shown.

TABLE 1

ELEMENTARY ANALYSES

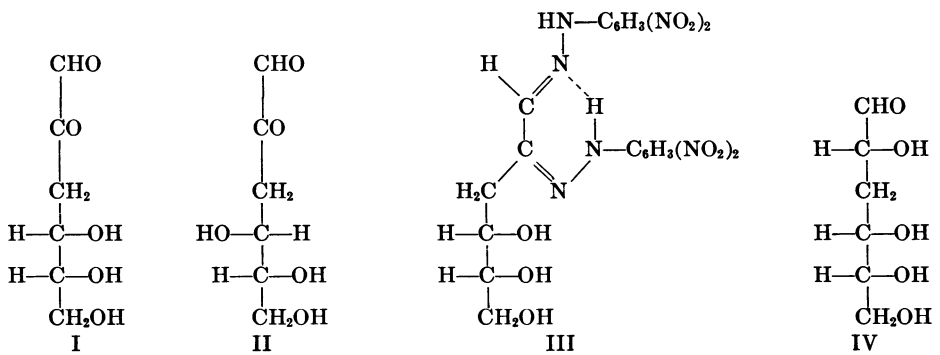
Sample	C	H	N	O	Mol wt	O-Acetyl
Liver-bis-2,4-dinitrophenylhydrazone *monohydrate*						
Found:	40.16	3.46	20.56	35.73	—	—
Calcd. for $C_{18}H_{20}O_{12}N_8$	40.2	3.7	20.78	35.80	—	—
Liver-bis-2,4-dinitrophenylhydrazone + ethyl acetate						
Found:	41.73	4.15	18.56	—	637	—
Calcd. for $C_{22}H_{26}O_{13}N_8$	43.00	4.30	18.4	—	610	—
Liver-bis-2,4-dinitrophenylhydrazone (anhydrous)						
Found:	41.32	3.42	21.43	33.74	—	—
	41.66	3.57	20.49	34.34	—	—
Calcd. for $C_{18}H_{18}O_{11}N_8$	41.40	3.50	21.50	33.60	—	—

Subtracting two dinitrophenylhydrazone residues ($C_{12}H_8O_8$) and adding two oxygens to the empirical formula of $C_{18}H_{18}O_{11}N_8$ leaves $C_6H_{10}O_5$, which corresponds to the free α-ketoaldehyde. Since a hexosone would be $C_6H_{10}O_6$, the substance is a deoxyhexosone[7] (hexosulose[8]).

In order to find out which of the possible 20 positional and/or stereoisomeric deoxyhexosones is identical with the natural one, a degradation with sodium periodate in aqueous acetonitrile was carried out. This led to the consumption of 1.9 moles oxidizing agent and production of 0.9 mole formaldehyde (precipitated as methylene-bis-dimedone), indicating the presence of a methylol (CH_2OH) group. Thus, the eight possible 6-deoxyglucosones were ruled out. The other product of oxidation was a phenylosazone with *no optical activity* throughout the region of 400–700 mμ. The four possible 4-deoxyglucosulose-osazones were hence excluded, for they should give an *optically active* osazone.

The fact that the mother liquor of this new osazone is acidic and consumes nearly 1 mole of alkali clearly indicates the formation of 1 mole *formic acid* per mole osazone. This can only be reconciled with one of the four possible *3-deoxy*glucosones.

Fortunately, the preparation of the two diastereoisomeric *D*-3-deoxyhexosuloses (I and II) from glucosylamines[12] and difructosylglycine,[11] via an Amadori rearrangement, has been recently described,[9–12] together with their dinitrophenylosazones,[10, 11] having $[\alpha]_D = +800°$ and $+300°$, respectively. The L-forms were thus ruled out. Authentic samples of D-erythro-3-deoxyhexosulose-2,4-dinitrophenylosazone (III) were kindly provided by Drs. Anet and Kato. The authors are very grateful for their prompt cooperation.



Identification of Liver-2,4-dinitrophenylosazone as D-erythro-3-deoxyhexosulose.—Comparison of the bis-2,4-dinitrophenylhydrazone of the substance from liver with that of the two authentic samples of 3-deoxy-D-glucosulose was made by melting-point and mixed melting-point determinations; by NMR spectra; by UV, visible, and IR spectra; and by optical rotatory dispersion using the same instrument for each measurement.

Melting Points.—Liver-2,4-dinitrophenylosazone and the specimen obtained from Dr. Anet both melt at 269° (dec.) in an electrothermal melting point apparatus (speed no. 4) and gave *no depression* of melting points when mixed. Dr. Kato's specimen shows mp 266°; its decomposition in admixture with our 2,4-dinitrophenylosazone started at about 265°. Since melting was accompanied by decomposition, these observations alone cannot be regarded as decisive.

NMR Spectra.—Table 2 and curves 1–3 in Figure 1 show the spectra of the osazone from liver (curve 1) and Dr. Anet's specimen (curve 2) in 8 per cent and Dr. Kato's specimen in 4 per cent concentration (curve 3) taken in pyridine-d_5 with the Varian A-60 apparatus. Most signals are congruent. Interpretation is needed only for the large signal comprising five protons, which appears somewhat shifted: δ6.65 in curve 1, 7.52 ppm in curve 2, and 5.75 ppm in curve 3.

The effect of the addition of deuterium oxide is shown in Figure 2. The osazone from liver corresponds to curve 4, Dr. Anet's specimen to curve 5, and Dr. Kato's to curve 6. The mentioned signal disappears in curves 4 and 5 with simultaneous appearance of a broad signal at 5.85 ppm corresponding to H-OD. Dr. Kato's sample, obtained from *N*-butyl-aminoglucose,[10] has the same H-OD signal after deuteration at δ5.25 ppm.

The assignment of the somewhat shifted resonance to the N—H and O—H protons is hence correct. The identity of the NMR spectra of our 2,4-dinitrophenylosazone and the osazone of 3-deoxy-D-glucosulose (III) is likewise proved.

The NMR spectra taken in dimethylsulfoxide-d_6 are less clear as to the aromatic regions (6.8–9.6 ppm) than in pyridine-d_5.

IR spectra of the three osazones show a very close resemblance (Fig. 3), with the best resolution in the livers 2,4-dinitrophenylosazone in KBr. The carbonyl region (curve 1) shows a double peak at 1620 and 1590 cm^{-1}. The samples of Anet and Kato (curves 2 and 3) also show the peak at 1620 cm^{-1} with a strong shoulder at 1590 cm^{-1}, leaving no doubt about the presence of an identical group in all three specimens. The same multiplicity of the carbonyl region was indicated by Kato.[10]

UV and Visible Spectra.—These spectra of the osazone from liver and of 3-deoxyglucosulose 2,4-dinitrophenylosazones at 10^{-5} *M* concentration in DMSO

TABLE 2

NMR SPECTRA IN PYRIDINE-D_5 OF DINITROPHENYLOSAZONES

Signal δ (ppm)				
	3-Deoxyglucosulose			
Liver	(Anet)	(Kato)	No. of protons	Assignment
8.60	8.64	8.64	1	H—C=N—
6.65	7.52	5.75	5	NH and OH
8.17 and 8.29	8.20 and 8.32	8.22 and 8.33	4	Aryl, C_5 and C_6
9.13	9.15	9.18	2	Aryl, C_3, deshielded
7.61	7.65	7.65	2	H—CO—
7.23	7.25	7.28	2	H_2>CO—
4.41	4.45	4.46	2	C—CH_2—C=

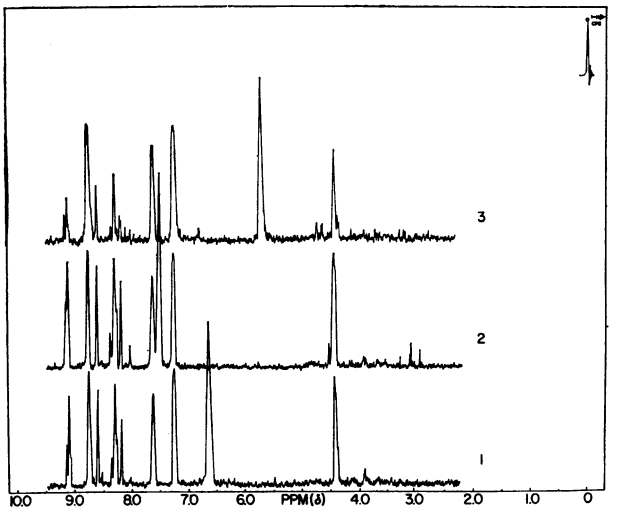


FIG. 1.—NMR spectra of the 2,4-dinitrophenylhydrazone from liver (*curve 1*), and of 3-deoxy-D-glucosulose synthesized by Dr. Anet (*curve 2*) and by Dr. Kato (*curve 3*) in pyridine-d_5.

are given in Figure 4. The curves are completely superimposable, including the fine structure from 450–475 mμ.

Optical rotatory dispersion curves of the three samples were measured in DMSO (C = 0.08%) (Fig. 5). Three different sensitivities have been used for each of the samples, using angles 0.2, 0.3, and 0.5, each of which were adjusted to full-scale deflection. The three curves have exactly the same shape with closely corresponding numerical values: +754° for the substance from liver, +732° and +730°

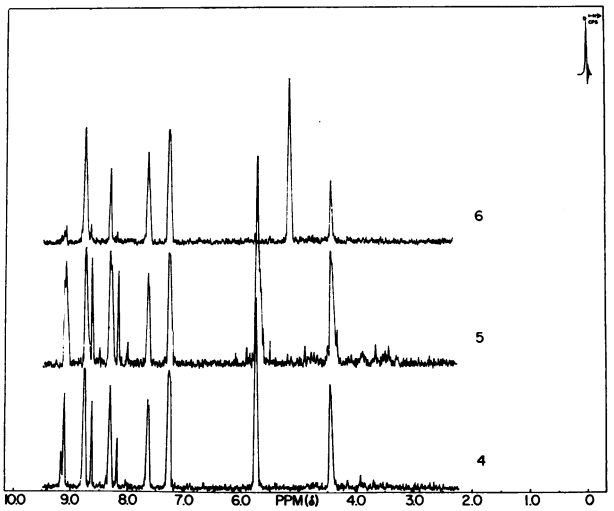


FIG. 2.—NMR spectra of the 2,4-dinitrophenylhydrazone from liver (*curve 4*), and of 3-deoxy-D-glucosulose synthesized by Dr. Anet (*curve 5*) and by Dr. Kato (*curve 6*) in D_2O.

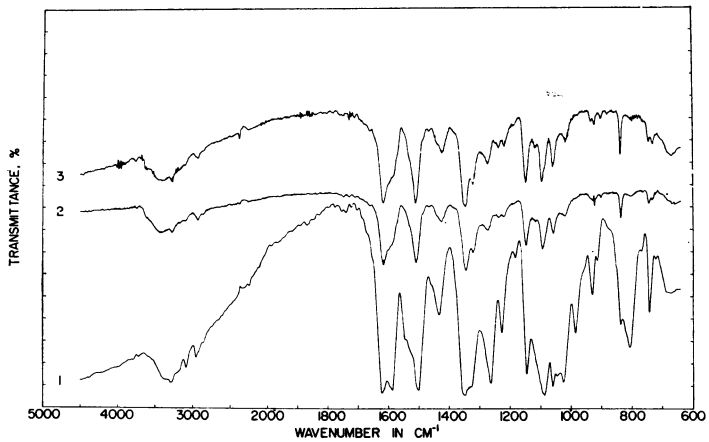


FIG. 3.—IR spectra of the 2,4-dinitrophenylhydrazone from liver (*curve 1*), and of 3-deoxy-D-glucosulose synthesized by Dr. Anet (*curve 2*) and by Dr. Kato (*curve 3*) in KBr micropellets.

for the two authentic samples of 3-deoxy-D-glucosulose-2,4-dinitrophenylosazone at the sodium D line. The latter two figures are somewhat lower than the first, as measured with the Perkin-Elmer electropolarimeter.

Acetyl-dinitrophenylosazone of the liver substance gave $[\alpha]_D$ +614° on the JASCO apparatus in DMSO. Anet[9, 11] reports +655° (in pyridine-acetic acid 1:1) for his acetyl-derivative.

The colored, solid product of degradation, which is very probably identical with the pyrazole derivative obtained by Kato[10] on periodic *acid* oxidation of the compound III, is optically inactive throughout the same spectral region, using DMSO as a solvent.

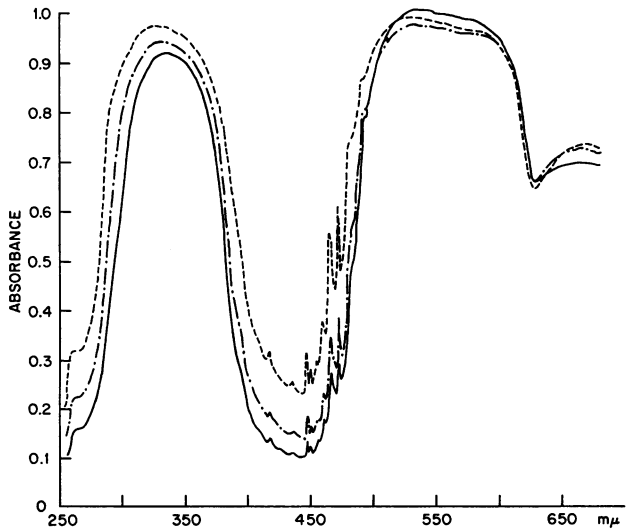


FIG. 4.—Spectra in the UV and visible region of the 2,4-dinitrophenylhydrazone from liver(——), and of 3-deoxy-D-glucosulose synthesized by Dr. Anet (·····) and by Dr. Kato (- - -).

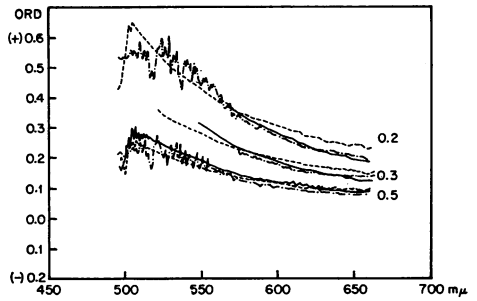


FIG. 5.—Optical rotatory dispersion curves of the 2,4-dinitrophenyl osazones from liver (——), and of 3-deoxy-D-glucosulose synthesized by Dr. Anet (-·-·-·-) and by Dr. Kato (---).

Identity of Osazone from Liver with 3-Deoxy-D-glucosulose.—Based on the data given above, the liver-2,4-dinitrophenylosazone is identical with D-*erythro*-3-deoxyhexosulose-2,4-dinitrophenylosazone. The question may arise whether this bis-2,4-dinitrophenylhydrazone was formed by a condensation from an α-ketoaldehyde or by an oxidation with condensation from 3-deoxy-D-glucose (IV).

The conditions of the reaction with 2,4-dinitrophenylhydrazine are extremely mild (20°, 20 min), which precludes the second alternative. Furthermore, paper chromatography [*n*-butanol, acetic acid, water (4:1:1)] of purified liver extracts shows the same spots, r gluc 2.0–2.7, 1.37, and 1.06, as does 3-deoxy-D-glucosulose, prepared according to Kato.[12]

The similarity of the IR spectra of free ketone aldehyde from liver (a solid foam) and those of Kato's[12] 3-deoxy-D-glucosulose (a colorless white powder) are significant. A broad band centered at 3400 cm^{-1} shows a number of H-bonded hydroxyl groups, and another between 1010 and 1105 cm^{-1} is ascribed to the —C—O absorption. There is a clear indication of an aliphatic aldehydic carbonyl around 1745 cm^{-1}, and of a ketonic carbonyl about 1640 cm^{-1}; however, their intensity is low. This seems to support the generally accepted view[13, 14] that osones exist in a number of cyclohemiketal and hydrogen-bonded forms.

Conclusion.—Retine, the growth inhibitor found in tissues,[1] was supposed to be a ketone aldehyde.[4] The osazone prepared from liver is that of a ketone aldehyde. Its relation to retine awaits clarification. The suggestion[15] that retine is a diketene (carbon suboxide) is wrong. Such a compound would not be stable in the aqueous solutions.

The substance discussed in this paper represents the first example of the occurrence of a deoxysugar α-ketoaldehyde (osone or osulose) in animal tissue. 3-Deoxyglucosone was only reported to occur in soya beans[10] as a possible intermediate in plant-melanoidine formation.[12] The positive 2-thiobarbituric acid test[16] for 3-deoxyhexosones[10] found in deteriorating food lends support to the possibility[10] of their formation on cooking or storage of foods. 3-Deoxy-D-glucosulose (I) also reacts with amino acids causing Strecker-degradation[17] to an aldehyde.

In the tissue, the substance giving the osazone is linked to another, unidentified compound, the study of which is in progress.

Summary.—The bis-2,4-dinitrophenylhydrazone isolated from liver is the osazone of D-erythro-3-deoxyhexosulose. The free ketone aldehyde is identical with 3-deoxy-D-glucosone (deoxy-D-glucosulose). Chemical degradation, identification of formaldehyde, formic acid, and of an optically inactive osazone among the

oxidation products, together with NMR, ORD, IR, visible, and UV spectrometry and chemical analyses led to this conclusion.

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[1] Szent-Györgyi, A., L. G. Együd, and J. A. McLaughlin, *Science*, **155**, 539 (1967).
[2] Együd, L. G., J. A. McLaughlin, and A. Szent-Györgyi, these PROCEEDINGS, **57**, 1422 (1967).
[3] Szent-Györgyi, A., *Science*, **149**, 34 (1965).
[4] Együd, L. G., these PROCEEDINGS, **54**, 200 (1965).
[5] Neuberg, C., and E. Strauss, *Arch. Biochem.*, **7**, 211 (1945).
[6] Keeney, M., *Anal. Chem.*, **29**, 1489 (1957).
[7] Fischer, E., *Ber. deut. chem. Ges.*, **22**, 87 (1889).
[8] "Rules of Carbohydrate Nomenclature," *J. Org. Chem.*, **28**, 281 (1963).
[9] Anet, E. F. L. J., *J. Am. Chem. Soc.*, **82**, 1502 (1960).
[10] Kato, H., *Agr. Biol. Chem. (Tokyo)*, **24**, 1 (1960).
[11] Anet, E. F. L. J., *Australian J. Chem.*, **13**, 397 (1962).
[12] Kato, H., *Agr. Biol. Chem. (Tokyo)*, **26**, 187 (1962).
[13] Bayne, S., and J. A. Fewster, *Advan. Carbohydrate Chem.*, **11**, 43 (1956).
[14] Petuely, F., *Monatsh. Chem.*, **83**, 765 (1950).
[15] Marmasse, Cl., *Nature*, **209**, 1346 (1966).
[16] Patton, S., and G. W. Kurtz, *J. Dairy Sci.*, **34**, 669 (1951).
[17] Schoenberg, A., R. Moubacher, and A. Mostafa, *J. Chem. Soc.*, **1948**, 176.