CCXXXIII. ISOLATION OF ACETIC ACID FROM MAMMALIAN LIVER.

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IN view of the generally implied importance of acetic acid as a degradation or fission product in intermediary metabolism, it is remarkable that no significant quantities of this substance have been found in mammalian tissues. The work which is described in this paper arose from an investigation of the alcohol-soluble constituents of ox liver, in the course of which 2:4-dinitrophenylhydrazine was added to the suitably concentrated extract in the hope of isolating carbonyl compounds of importance in intermediary metabolism. In addition to finding as a hydrazine derivative one compound which evidently possesses carbonyl groups, acetic acid was noticed in the form of its 2:4-dinitrophenylhydrazone. The results are also of interest as much for what was not discovered as for what was: acetaldehyde, pyruvic acid and kindred compounds appeared to be totally absent, although the experiments were done in the presence of sodium bisulphite (the "Abfang" method of Neuberg). The use of bisulphite has been successful in the investigation of muscle metabolites [Case & Cook, 1931] and there is no reason to suppose that intermediates containing the CO grouping would fail to be detected in mammalian liver using the same technique.

EXPERIMENTAL.

Isolation of hydrazone. About 4 kg. (10 lb.) of ox liver obtained from the slaughter-house less than an hour after killing were minced into a solution of 100 g. sodium sulphite (dissolved in 250 ml. water and saturated with sulphur dioxide). The mass was ground with sand (1 kg.), allowed to stand 15 min., and the protein removed by precipitation with 2 litres of 97 % alcohol. The thick paste was squeezed as free from liquid as possible by means of a hand screw-press and the extraction repeated with a further litre of alcohol. The united filtrates were kept at 0° overnight, centrifuged from protein, filtered through kieselguhr and evaporated down to small bulk (about 500 ml.) *in vacuo* at 30–40°. A certain amount of protein separated and was removed by filtration. A slight excess of conc. HCl was then added and most of the SO₂ blown off in a current of air. The solution was adjusted with HCl to make the solution approximately 2N, and 30 g. of 2:4-dinitrophenylhydrazine dissolved in 20 ml. conc. HCl were added. After a further passage of air through the solution it was allowed to stand for 24 hours at 37°.

The precipitate, which included some unchanged reagent, was centrifuged off, boiled several times with 10 % alcohol and filtered. The residue was reserved for the osazone preparation. The cooled united filtrates were extracted 4–5 times with 50 ml. lots of ethyl acetate. After drying as far as possible over anhydrous sodium sulphate, the extract was evaporated *in vacuo* to small bulk and set aside to crystallize. In 2–3 days orange-yellow crusts separated. After draining from the dark red mother-liquor, the semicrystalline mass was dissolved in boiling

10% alcohol, a little blood charcoal added, filtered and allowed to cool; yellow plates, which were sometimes tinged a pale reddish brown, separated out. Recrystallization was effected from 10% alcohol containing a trace of HCl; m.p. 195–196° (uncorr.). Analysis: found (Weiler) C, 37.39, H, 3.91; N, 22.07%. $C_8H_{10}O_6N_4$ requires C, 37.19; H, 3.90; N, 21.71%. Dissolved in pyridine-ethyl acetate the substance showed no optical activity. Attempts to determine the molecular weight in nitrobenzene or glacial acetic acid gave anomalous results, which suggested that the substance was hydrated or contained water of crystallisation. Drying at 100° *in vacuo* over phosphorus pentoxide confirmed this. Loss in weight found 7.08% (Weiler), 7.10%; $C_8H_8O_5N_4 \cdot H_2O$ requires 7.01%. Analysis of anhydrous substance: found (Weiler) C, 40.24, 40.20; H, 2.89, 3.09; N, 23.31%. $C_8H_8O_5N_4$ requires C, 39.98; H, 3.36; N, 23.34%. Mol. wt. (cryoscopic) in dry nitrobenzene; found 252; calc. 240.

The compound is evidently the 2:4-dinitrophenylhydrazide of acetic acid [Curtius & Dedichen, 1894; Purgotti, 1894]. The melting-points given in the literature vary from 193 to 197°. A specimen prepared by refluxing reagent and glacial acetic acid for one hour, pouring into water and recrystallizing, melted at 195°; mixed with the substance from liver, 195–196°. In crystalline forms and solubilities the compounds from both sources are identical. The compound gives a red colour with alcoholic potash. It is soluble in a solution of sodium carbonate and in this resembles the acid hydrazones. Unlike them, however, it cannot be titrated with alkali [Clift & Cook, 1932, 2]. The hydrazide is fairly easily hydrolysed and care must be taken that it is not heated in the presence of excess acid.

Several criticisms may be levelled against the procedure. (a) The acetic acid may arise from the ethyl acetate. This possibility is unlikely for several reasons: (i) If ethyl acetate be used to extract the reagent from its solution in 2*N* HCl and then the extract be dried and allowed to evaporate spontaneously in the usual way, the crystals obtained are those of the unchanged reagent, uncontaminated with hydrazide. Many repetitions of this experiment have given negative results. (ii) The Neuberg-Case method for the estimation of pyruvic acid in muscle employs ethyl acetate for extraction: yet no appreciable quantities of acetic acid are formed as an artefact. (iii) The hydrazide is soluble in sodium carbonate, yielding a deep red coloured solution. This reaction may be employed to isolate the compound without the intervention of any organic solvent whatever. Incidentally this fact and the deep red colour obtained with alcoholic potash throw considerable doubt on the validity of the Neuberg-Case method for the specific estimation of pyruvic acid. The red colour may as well be due to acid hydrazides as to acid hydrazones and is therefore no proof of the presence of carbonyl compounds.

Alternatively (b) the acetate radical might have been derived from the alcohol used or from impurities in the sulphite. Control experiments were performed using purified sodium bisulphite (from pure sodium carbonate saturated with SO_2). The ethyl alcohol was refluxed for some hours with barium hydroxide and redistilled. For extraction, butyl alcohol redistilled from KOH was used. Under these conditions the same hydrazide was formed and it is difficult therefore to resist the conclusion that acetic acid occurs in some quantity in the liver. The amount depends on the speed with which it can be treated with bisulphite. Under favourable conditions 4 kg. of liver yield about 16 g. of hydrazide, or 4 g. of acetic acid, which is therefore present to the extent of nearly 0.1% of the wet weight.

Isolation of the osazone. The solid residue from the hydrazide preparation, which still contains unchanged reagent, is boiled several times with 2*N* HCl,

washed well, dissolved in pyridine, filtered and reprecipitated by acidification with HCl. The process is repeated and the brick-red osazone finally recrystallized from 97% alcohol (from which it separates in microcrystalline form): M.P. 175° (decomp.) Analysis: found (Weiler) C, 42.32; H, 3.58; N, 20.12%. $C_{20}H_{20}O_{12}N_8$ requires C, 42.5; H, 3.54; N, 19.85%. Mol. wt. in nitrobenzene: found 482; calc. 564.

It may be remarked that the determination of the molecular weight of this compound is not facilitated by the fact that its solubility in the ordinary organic solvents is very slight. We therefore should like to make a reservation as to any definite statement on the exact molecular weight. The compound appears to be the osazone of a substance of empirical formula $C_8H_{12}O_6$. It gives a blue colour with alcoholic potash. It is worth remarking that the osazone has the same empirical formula as that of the hydrazone of acetoacetic acid [Clift & Cook, 1932, 2].

A peculiar fact was noticed during the preparation of the concentrated liver extract. The bisulphite-binding power was estimated by the method of Clift and Cook [1932, 1] and it was found that the titration invariably doubled on concentration. Whether this is due to a reversal of enolisation or to a change involving two molecules has not yet been decided. The bisulphite-binding power is largely destroyed on heating the solution with an excess of alkali. The table gives the iodine equivalent of bisulphite-binding substances in various batches of liver extract.

Table I.

No.	Wet weight of liver g.	ml. of <i>N</i> /10 iodine		Calculated figures for 100 g. liver as ml. of <i>N</i> /10 iodine for bisulphite-binding compounds before concentration
		Before concentration	After concentration	
1	2300	735	1200	32
2	4000	1840	3960	46
3	2300	783	1585	34
4	280	133	270	47.5

DISCUSSION.

The most obvious source of the acetic acid is from higher fatty acids either by splitting of the chain or by successive β -oxidations. In the absence of definite observations, however, we are not prepared to hold this view to the exclusion of other possibilities. The absence of the simpler carbonyl compounds is remarkable for these have frequently been postulated in schemes for the intermediary metabolism of fats and carbohydrates. The bisulphite-binding power is almost wholly, if not entirely, accounted for by the compound which has been isolated as an osazone and which appears to contain 8 carbon atoms. This is not improbably a condensation product: attempts are being made to determine its constitution and the mode of origin. Glucosazone has not been found under the experimental conditions employed.

SUMMARY.

1. Acetic acid, as its 2:4-dinitrophenylhydrazide, has been isolated from fresh ox liver.
2. The osazone of a compound apparently containing 8 carbon atoms has been shown to represent the major, if not the entire, part of the bisulphite-binding compounds of liver extract.

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