550. *The Constitution of Suint. Part II.* Organic Acids.*

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The following acids have been identified in suint: acetic, propionic, n-butyric, n-valeric, oxalic, succinic, and glutaric. Paper chromatography indicated the presence of adipic and pimelic acid.

Suint is that portion of the sheep's fleece that is soluble in cold water after the wax has been removed. Older work shows that it is a complex mixture of metallic ions, organic acids, peptides, weak bases, neutral substances, and inorganic cations. A total of 41 organic compounds has been said to be present in the aqueous extract of raw wool (for a review see Truter [1]). Unfortunately, in no case has identification been adequate experimentally. In the older work (before 1955) no experimental measurements have been adduced; in recent studies by Farnworth [2] and by Green and Preston [3] the evidence is entirely chromatographic. Such evidence is unconvincing because it is becoming increasingly clear that in complex mixtures of closely related substances chromatographic measurements alone do not permit unequivocal identification. For example, the behaviour of the peptides in suint very closely resembles that of amino-acids; [4] further, in the present study it was found that the lower normal and iso-acids could not be distinguished by partition chromatography. Hence, chromatographic evidence *alone* does not *prove* the presence of a compound.

In the present study, suint from Australian merino wool has been fractionated by means of ion-exchange resins, into four main fractions: inorganic cations 44%; organic acids and anions 36%; weak bases 4%; and ampholytes 3%. Complete recovery may not always be achieved with ion-exchange resins, but here the discrepancy is large enough to suggest the presence of the ammonium ion, and of water-insoluble fatty acids which were originally present as water-soluble potassium salts. Farnworth,[2] in a detailed analysis of the inorganic cations, has found K, Na, Ca, Mg, Al, and Fe, and we have shown that the ampholyte fraction contains at least eleven peptides each of which contains between eight and thirteen different amino-acids.[4]

The steam-volatile organic acids (2%) were separated from one another by partition chromatography on silica, and on a larger scale by countercurrent distribution between carbon tetrachloride and dilute aqueous alkali. The presence of acetic, propionic, n-butyric, and n-valeric acid has been confirmed by preparation of their *p*-bromophenacyl esters. Contrary to expectation, because most of the acids of wool wax are of the branched-chain type, there are no branched-chain acids in suint. The acids not volatile in steam and also insoluble in ether (18%) are dibasic, ranging from oxalic to pimelic but excluding malonic acid. Of these, succinic and glutaric acid were identified as their *p*-phenylazoanils, and oxalic acid was identified by Martin's method.[5] The presence of adipic and pimelic acid is less certain, but was indicated by paper chromatograms.

A small amount of material was precipitated as 2,4-dinitrophenylhydrazone; this was not investigated. It may be derived from the keto-acids suggested by Green and Preston.[3] No steroids could be detected by the Liebermann–Burchardt reaction.

The approximate amounts of the acids recovered from suint were: acetic, 1·1%; propionic 0·2%; n-butyric 0·3%; n-valeric 0·2%; oxalic 0·3%; succinic 10%; and glutaric acid 1%. Each of these was pure by chromatographic standards.

* Part I, *Biochim. Biophys. Acta*, 1955, **18**, 435.

[1] Truter, "Wool Wax," Cleaver-Hume Press, London, 1956, pp. 61—72.
[2] Farnworth, *Austral. J. Appl. Sci.*, 1956, **7**, 233.
[3] Green and Preston, *J. Textile Inst.*, 1956, **47**, T497.
[4] Deane and Truter, *Biochim. Biophys. Acta*, 1955, **18**, 435.
[5] Martin, *Chem. and Ind.*, 1955, 427.

Experimental

Preparation of Suint.—Australian merino wool of 64's quality was sorted to remove gross impurities such as vegetable matter, and treated with light petroleum (b. p. 60—80°) in a Soxhlet apparatus for 8 hr. After removal of the solvent from the wool, the latter was gently squeezed in cold water (4 × 300 ml. per 100 g.). The aqueous solutions were combined and centrifuged until clear, and the concentration of solute was determined by evaporation of aliquot parts.

A suint solution (10·1 g./900 ml.) was passed through a column containing 550 c.c. of Zeo-Karb 225 in the H^+ form, at a rate of 18 ml./hr. The effluent contained anions, organic acids, and neutral substances (total 3·6 g.). The column was then washed with 2N-ammonia (1 l.), and the eluate, which contained weak bases and ampholytes, was concentrated to 500 ml. and passed through a column containing 250 c.c. of Deacidite FF in the OH^- form. The effluent from the second column contained only weak bases (0·4 g.). Further washing of the Deacidite FF column with N-hydrochloric acid (350 ml.) yielded the ampholytes (0·3 g.). Subsequently, treatment of the Zeo-Karb 225 column with N-hydrochloric acid (550 ml.) afforded a mixture of ammonium chloride (18·2 g.) and the cation chlorides (8·5 g.; equiv. to 4·4 g. of cations if the composition is assumed to be similar to that found by Farnworth).

Steam-volatile Acids.—The aqueous solution was adjusted to pH 2 with N-sulphuric acid. After the small amount of precipitate that was formed had been filtered off with a filter aid, the filtrate was treated with ether in a liquid–liquid extractor for 72 hr. The dried precipitate was also extracted with ether, and the combined ethereal extracts yielded a brown, sticky residue (3·0 g., 34·5%). The ether-soluble acids were extracted, as above, from a large amount of suint (32·9 g.) and distilled in steam. Titration of the distillate against ethanolic potassium hydroxide indicated that it contained 0·009 equivalent (*i.e.*, 0·27 mequiv./g.).

The volatile acids were examined on columns of silica which had been prepared according to Nijkamp's method.[6] Silica prepared by Sanger's method [7] was unsuitable.

Preparation of the Silica.—Commercial water-glass (*d* 1·35; 200 ml.) was mixed with water (400 ml.), acidified to Methyl Orange with 10N-hydrochloric acid, and stirred for 15 min. The resulting paste was ground with just sufficient 10N-hydrochloric acid to render it acidic to Thymol Blue, transferred to a Buchner funnel, and washed with water (about 4 l.) until free from chloride, without the filter-cake being allowed to become dry. The product was then set aside in 2% hydrochloric acid for 15 hr. and again washed free from chloride. Most of the water was removed by washing the filter-cake with 96% ethanol (about 1 l.), and the residue was dried at 80° for 18 hr. and then to constant weight at 150°.

Preparation of Columns.—Silica (0·6 g.) was mixed with a 0·8% solution of Bromocresol Green in dry methanol (0·12 ml.), and water (0·48 ml.) and 0·1N sodium hydroxide (0·3 ml.) were added. The apparently dry, blue-green product was mixed with carbon tetrachloride containing 6% of butan-1-ol, and the smooth slurry was transferred to a tube 270 × 7 mm. to give a column 6 cm. high. Preliminary experiments showed that a mixture of 0·01 milli-equivalent of each of the acids acetic, propionic, n-butyric, n-valeric, and hexanoic, could be separated on a 6 cm. column with an efficiency of 97%. Each acid, visible as a yellow band, was eluted and titrated, in an atmosphere of nitrogen, against 0·005N-sodium ethoxide with Cresol Red as indicator.

Potassium salts of the steam-volatile acids (190 mg.) were mixed with finely powdered Zeo-Karb 225 (500 mg.) in the H^+ form. Iso-octane (5 ml.) was added, and after the mixture had been shaken for 10 min. the mixture was centrifuged and the solvent decanted. The resin was washed with more iso-octane (3 × 5 ml.), and the combined washings were made up to 25 ml. Aliquot parts (1 ml.) of the solution were then chromatographed on silica (600 mg.) columns. Four clear bands were obtained, and the amounts of each, calculated on the dry suint, were: valeric (fastest band) 0·2%; butyric 0·3%; propionic 0·2%; and acetic acid 1·1%. Confirmatory evidence was obtained by collecting the acid from each band separately, mixing it with the appropriate known acid, and re-chromatographing the binary mixtures. In every case only a single band was observed, but the technique failed to separate the n-acids from the corresponding iso-acids. Hexanoic acid was absent.

Countercurrent Separation of the Volatile Acids.—The steam-volatile acids (5·2 milliequivs.) were subjected to thirty transfers in a countercurrent apparatus, with carbon tetrachloride

[6] Nijkamp, *Analyt. Chim. Acta*, 1951, **5**, 325; 1954, **10**, 448.
[7] Sanger, *Biochem. J.*, 1945, **39**, 507.

(50 ml.) and aqueous sodium hydroxide (0·1N; 50 ml.) as the solvents. Titration of the lower phases showed that the acids had separated into four distinct groups containing a total of 0·43 milliequiv. of acid. Correspondingly, the upper phases were combined into four fractions and the free acids which were liberated by treatment with Zeo-Karb 225 were titrated against 0·1N-alkali.

Fraction 1. n-Valeric acid, 0·81 milliequiv. Its *p*-bromophenacyl ester had m. p. and mixed m. p. 75° (Found: C, 52·3; H, 5·2. Calc. for $C_{13}H_{15}O_3Br$: C, 52·2; H, 5·1%). The isovaleric ester has m. p. 66° and when mixed with the derivative from suint the m. p. was 48—51°.

Fraction 2. n-Butyric acid, 0·33 milliequiv. Its *p*-bromophenacyl ester had m. p. and mixed m. p. 63° [mixed m. p. with the isobutyric ester (m. p. 77°) 54—58°] (Found: C, 50·4; H, 4·4; Br, 28·0. Calc. for $C_{12}H_{13}O_3Br$: C, 50·5; H, 4·6; Br, 28·0%).

Fraction 3. Propionic acid, 0·4 milliequiv. Its *p*-bromophenacyl ester had m. p. 63° (lit., m. p. 63°) (Found: C, 48·5; H, 4·4; Br, 29·2. Calc. for $C_{11}H_{11}O_3Br$: C, 48·7; H, 4·1; Br, 29·5%).

Fraction 4. Acetic acid, 2·5 milliequiv. Its *p*-bromophenacyl ester had m. p. 84° (lit., m. p. 85°) (Found: C, 47·4; H, 3·8. Calc. for $C_{10}H_9O_3Br$: C, 46·7; H, 3·5%).

Succinic Acid.—The acids not volatile in steam (33% of the suint) were treated with ether, and the yellow residue (18%) was decolorised with charcoal. Sublimation of the product under reduced pressure afforded needles, m. p. (sealed tube) 181° (12%) [Found: Equiv., 56·5. *M* (in camphor; extrapolation to zero concentration), 114]. After re-sublimation it had m. p. and mixed m. p. 185° (Found: C, 40·9; H, 5·3. Calc. for $C_4H_6O_4$: C, 40·7; H, 5·1%; *M*, 118; equiv., 59). Its *p*-bromophenacyl ester had m. p. and mixed m. p. 214·5—215° (Found: Br, 30·5. Calc. for $C_{20}H_{16}O_6Br_2$: Br, 31·2%). Paper chromatograms of succinic acid and the acid from suint had identical R_f values and a mixture gave only one spot, in the following solvents: (i) ethanol–aqueous ammonia (*d* 0·88)–water, 8 : 1 : 1 (Brown [8]); (ii) propan-2-ol–t-butanol–benzyl alcohol–water–90% formic acid, 1 : 1 : 3 : 1 : 2 (Stark *et al.*[9]); (iii) phenol–water, 3 : 1, containing 1% of 90% formic acid (Stark *et al.*[9]); (iv) tetrahydrofuran–3N-ammonia, 4 : 1 (Kalbe [10]).

Glutaric Acid.—The ether-insoluble acids which were not volatile in steam (230 mg.) were converted into their anhydrides by heating them with acetic anhydride (2 ml.). After the excess of acetic anhydride had been distilled off, the residue was heated in chloroform (2 ml.) with *p*-aminoazobenzene (300 mg.) in chloroform (2 ml.). When the mixture cooled, the anilic acids were precipitated; these were filtered off and heated under reflux with acetyl chloride (2 ml.) for 1 hr. to bring about cyclisation of the succinyl- and glutaryl-derivatives. Removal of the solvent gave a dark red solid (271 mg.). Part of this material (102 mg.) was placed on an alumina column (Spence type H; 100 × 1·4 cm.) and developed with benzene–ether (3 : 1). Three yellow bands were collected separately and a red band, possibly the anilic acids of homologous dibasic acids, remained on top of the column. Fraction 1: glutaro-*p*-phenylazoanil (5·3 mg.), m. p. and mixed m. p. 225° (Found: C, 69·1; H, 5·2. Calc. for $C_{17}H_{15}O_2N_3$: C, 69·6; H, 5·2%). Fraction 2: succino-*p*-phenylazoanil (81 mg.), m. p. and mixed m. p. 219° (Found: C, 68·7; H, 4·7. Calc. for $C_{16}H_{13}O_2N_3$: C, 68·8; H, 4·7%). Fraction 3: *p*-acetamidoazobenzene, m. p. 144°.

Other Dibasic Acids.—Hyflo-Supercel (40 g.) was washed with ether and with water, dried at 105°, mixed with 0·05N-sulphuric acid (28 ml.) and packed into a column (60 × 1·7 cm.) as a benzene slurry. The non-volatile acids of suint (466 mg.) were applied to the column and washed successively with benzene (200 ml.), ether (800 ml.) and butan-1-ol (600 ml.). The effluent was automatically collected as 10 ml. fractions, and each was titrated against 0·05N-sodium ethoxide. A group of six distinct, but incompletely separated fractions, followed by a seventh well-defined fraction, was obtained, but the recovery was only 86%. The first peak (approx. 15% of suint) contains higher aliphatic acids, and the next five peaks contain hydroxy-acids. It was expected that the dibasic acids would be eluted with butanol in the seventh fraction, so this material was chromatographed on paper after regeneration of the free acids by treatment of the sodium salts with Zeo-Karb 225. One-dimensional chromatograms in two solvents, (*a*) tetrahydrofuran–3N-ammonia, 4 : 1 (Kalbe [10]), and (*b*) propan-2-ol–t-butanol–benzyl alcohol–water, 1 : 1 : 3 : 1, containing 1% of 90% formic acids (Stark *et al.*[9]), indicated

[8] Brown, *Nature*, 1951, **167**, 441.
[9] Stark, Goodban, and Omen, *Analyt. Chem.*, 1951, **23**, 413.
[10] Kalbe, *Z. physiol. Chem.*, 1954, **297**, 19.

the presence of oxalic, succinic, glutaric, adipic, and pimelic acid. Malonic acid was not found.

Oxalic Acid.—The presence of oxalic acid was confirmed by Martin's specific test. The paper was sprayed with 10% aqueous potassium ferrocyanide, dried in the oven, and then sprayed with 0·5% ferric ammonium sulphate followed by ammoniacal ethanol. Oxalic acid was shown to be present by the development of a bright blue colour.

After, but not before, reduction of the non-volatile acids with magnesium and dilute sulphuric acid, followed by treatment with warm 2,7-dihydroxynaphthalene in concentrated sulphuric acid, a purple solution was obtained, indicating the presence of glycollic acid. This could be derived from oxalic or glyoxylic acid by reduction. Because only a trace of 2,4-dinitrophenylhydrazone could be obtained, it must be inferred that glyoxylic acid is not an important constituent, and that the colour reaction is due to the presence of oxalic acid.

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