Separation of Saturated Straight Chain Fatty Acids III. Quantitative Paper Chromatography

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The lower fatty acids, separated by paper chromatography as their salts, may be determined quantitatively by the area method¹. The basis of another method of quantitative estimation of the ammonium salts of organic acids is that if an acid is added to a neutralized solution of an acid indicator the anion of which is distinctly coloured, then the depth of the colour due to the anion is inversely proportional to the amount of acid added between 20 and 80% neutralization². The value of both these methods is somewhat reduced because of the sensitivity of the indicators towards atmospheric (or dissolved) carbon dioxide. As hydroxamic derivatives, the acids may be estimated in more stable conditions. Stadtman³ describes a method of this kind for the acyl phosphates of $C_2 \dots C_6$, in which the chromatographed hydroxamic derivatives of the acids are extracted from the paper and estimated colorimetrically. Block⁴ used a corresponding procedure for the same acids, chromatographing them according to the method of Thompson⁵ and estimating them colorimetrically directly on the paper. Wagner⁶ describes a quantitative chromatographic method for the long chain acids ($C_{12} \dots C_{22}$) in which he used the chromatographic procedure of Kaufmann⁷ and estimated the amounts of the acids colorimetrically directly on the paper, first converting the acids by the method of Kaufmann into their copper salts and then the copper into the ferrocyanide complex, the amount of which was easily estimated colorimetrically. In the present study corresponding investigations were carried out. The amount of copper was determined colorimetrically by the dithizone method from the ash of the spot cut from the chromatogram. It was found that the amount of combined copper was not quite equivalent to the amount of fatty acid on the paper, but that very small amounts of acids combined with copper in a molar ratio. In the following, procedures now found suitable for the separation and

estimation of fatty acids from formic to cerotic acid are described.

The acids from formic to valeric acid may be separated from each other and estimated by converting them into their hydroxamic derivatives and chromatographing with the solvent *n*-butanol:benzene:water (1:1:1, upper layer) by the circular method, which is the most suitable in this case. The acids are located by spraying the paper with $FeCl_3$ solution. The spots are extracted in cuvette solutions containing $FeCl_3$ and the amounts of acids are estimated colorimetrically.

The acids from caproic to pelargonic acid are analysed in the same manner as in the former group, but chromatography is carried out with the solvent benzene:formic acid:water (1:1:1, upper layer).

The acids from capric to cerotic acid may be separated by the method previously described by the author⁸. Quantitative estimation of the acids is achieved by converting them into silver salts on the paper, removing the excess of silver and determining the amount of silver in each spot colorimetrically by the dithizone method.

Experimental: Separation and estimation of the acids from formic to valeric acid. A slight excess of diazomethane dissolved in ether was added at room temperature to the methanol solution of the fatty acids and the excess of diazomethane was evaporated. The methyl esters obtained were converted into hydroxamic derivatives by the method of Thompson⁵, adding to the solution of esters obtained alkaline hydroxylamine solution. The reaction was allowed to proceed for half an hour at room temperature, after which the solution was acidified with acetic acid. The sodium acetate precipitate was filtered off and the solution was concentrated in vacuum. Chromatography is most conveniently carried out by the circular method (cf. Perilä⁹) using as solvent the upper layer of the mixture *n*-butanol:benzene:water (1:1:1)⁹. Because the solubility of water in the solvent is poor, it is advisable to keep a sheet of filter paper wetted with the water layer of the above-mentioned mixture on the bottom of the chromatographic chamber. Any Whatman double-washed paper is suitable for these analyses. After development, the paper was dried at room temperature and sprayed with $FeCl_3$ solution (e. g. 50 grams of $FeCl_3 \cdot 6H_2O$ dissolved in one litre of $NHCl$ in ethanol³). For qualitative detection 0.05 micromoles of each acid are sufficient, but for quantitative estimation an acid concentration of 0.1–1 $\mu M/ml$ is needed. This concentration

then determines the amount of acids to be taken for the chromatography. The spots obtained were cut out, and the ferric complexes of the hydroxamic derivatives of the fatty acids were extracted with a FeCl_3 solution (5 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ + 25 ml conc. HCl + 575 ml water³). This solution was then analysed colorimetrically at a wavelength of 5200 Å. According to Goddu¹⁰ the accuracy of the measurement is $\pm 2\%$. The accuracy is somewhat reduced, however, because of the chromatographic procedure. In the conditions described the following R_F -values were obtained: formic 0.24, acetic 0.34, propionic 0.51, butyric 0.69, valeric 0.83, and caproic acid 0.90.

Separation and estimation of the acids from caproic to pelargonic acid. The analyses were carried out in the same conditions as those of the preceding group, except that the chromatographic solvent was the upper layer of the mixture benzene:formic acid:water (1:1:1)⁵. The following R_F -values were obtained: valeric 0.18, caproic 0.33, oenanthylic 0.49, caprylic 0.62, pelargonic 0.72, and capric acid 0.76.

Separation and estimation of the acids from capric to cerotic acid. The chromatography was carried out by the method previously described by the author⁸, using a paper saturated with hydrocarbon and as solvent a mixture of acetic acid and water (9:1) saturated with hydrocarbon. The hydrocarbon was evaporated and the paper treated for half an hour with an 1.5% aqueous solution of AgNO_3 . The excess of silver was removed by washing with deionized water, after which the paper was rapidly dried and sprayed with 0.05% ethanolic *p*-diethylaminobenzylidenrhodanine. After drying, the silver spots were conveniently visible in ultraviolet light. The spots were cut out and the paper was burned in the usual manner with sulfuric acid-perchloric acid-nitric acid. The amount of silver was determined colorimetrically by the dithizone method at a wavelength of 6250 Å¹¹. For qualitative detection with the silver reagent mentioned 0.05 μM of each acid is sufficient, but for quantitative estimation it is advisable to use 0.2–2 μM of each acid. According to several determinations the accuracy of the method is about $\pm 5\%$.

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Purification of Clearing Factor from Postheparin Plasma

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Since the original discovery by Hahn¹ of the lipemia clearing effect of heparin upon injection and the subsequent demonstration of an *in vitro*-active clearing principle in plasma withdrawn after heparin administration² much successful work has been done to elucidate the mechanism of this peculiar phenomenon. Evidence has accumulated to show that the clearing factor acts as a lipoprotein lipase³. In spite of these advances only a few attempts have so far been made for the purification of the active principle from postheparin plasma. Anfinsen *et al.*⁴ showed that the clearing factor falls in Fraction III-1,2,3 in the ethanol fractionation scheme of Cohn and they could thus achieve an about fourteen-fold purification of the activity. Later, Anfinsen and Quigley⁵ reported a purification up to 100- to 200-fold by a relatively laborious procedure based on a complex formation between the clearing factor and low density lipoproteins (enzyme-substrate complex). Earlier studies in this laboratory revealed that the clearing activity was precipitated quantitatively from plasma at a low ionic strength at pH 5.6 along with 1/20 to 1/25 of the plasma proteins⁶. This finding was confirmed by Hood *et al.*⁷ and the method has since been used by a