SUMMARY

By the Archibald approach to sedimentation equilibrium method, we have found a molecular weight of $4.2 \cdot 10^5$ g for either, (1) myosin (A) or, (2) the principal, (*ca.* 65% by weight), schlieren-visible component of 5-h extracted myosin B. This same value has also been estimated from the conventional sedimentation velocity-diffusion method, using sedimentation coefficients obtained at very low protein concentrations, and diffusion coefficients measured by observing boundary spreading in synthetic boundary cells. Although lower than hitherto reported molecular weights for myosin (A), this value is in line with minimum molecular weight estimates based on the present knowledge of myosin sub-units.

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Note added in proof

A condensed account of the foregoing results appeared on p. 14T, *Abstracts, 132nd Meeting, Am. Chem. Soc.*, New York, September, 1957. It is encouraging to note that since this paper has been in press, there have appeared preliminary reports of values for the molecular weight of myosin which agree closely with those reported here, *viz.*, the report of W. F. H. M. MOMMAERTS AND B. B. ALDRICH (*Science*, 126 (1957) 1294), who used the "Archibald method", and that of J. GERGELY (p. 46, *Abstracts, Biophys. Soc. Meeting*, Boston, February, 1958) who used light-scattering.

A STUDY OF THE α-KETO-ACIDS IN BLOOD

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INTRODUCTION

Several keto-acids are present in biological fluids and it is obvious that their individual quantitative estimation is necessary in order to understand their role in normal and pathological metabolic states.

A number of methods have been devised for their quantitative determination. The most recent among them is an enzymic procedure which is rapid and specific, but is limited, at the moment, to a few α-keto-acids[1,2].

The colorimetric determination of α-keto-acids, which is simple and fairly accurate, has been used for some fifteen years. The best known method, has been described by FRIEDEMANN AND HAUGEN[3] and consists in the colorimetric assay of the 2,4-dinitrophenylhydrazones of α-keto-acids in alcaline medium. These and other authors

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attempted, by various modifications in reaction time and extraction procedure, to make the method specific for some α-keto-acids (*e.g.* pyruvic acid).

These methods, however, have lost their interest since the chromatographic techniques proved to be not only simple and valuable in the separation and quantitative determination of each of the α-keto-acids present, but also in the detection of hitherto unidentified ones. This chromatographic separation has usually been performed with the 2,4-dinitrophenylhydrazones (DNP). These are stable under the usual laboratory conditions and their yellow colour permits their extraction and separation to be followed. The separation of the DNP of some keto-acids on columns of diatomaceous earth[1], aluminium oxide[5], cellulose powder[6] and also by means of paper electrophoresis[7,8,9] has been described.

The paper-chromatographic separation of keto-acid hydrazones, introduced by CAVALLINI *et al.*[10,11], proved to be the best method and was subsequently used by most authors[7,12–19].

It was in studying this original technique and a modification proposed later by the same authors[13] that we noticed the presence on our chromatograms of previously unidentified α-keto-acid hydrazones. Their identification and other problems involved in the chromatographic separation of the DNP of several α-keto-acids will be presented in this paper.

METHODS

Preparation and extraction of keto-acid hydrazones from blood

To 5–10 ml of freshly drawn venous blood (V_1), taken with a minimum of venous stasis and no clenching and unclenching of the hand, are immediately added at the bedside, 3 vol. of water, 1 vol. of 0.66 *N* sulfuric acid and 1 vol. of 10% sodium tungstate. After vigorous shaking, the mixture (V_2) is allowed to stand for 10 min at room temperature and subsequently centrifuged and filtered. To the total amount of filtrate (F), 1 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 *N* HCl is added and the solution is left for 30 min at room temperature. Several control experiments have shown that this reaction time is necessary and sufficient. The mixture is then extracted 4 to 5 times with 5 to 7-ml portions of ether until no more colour comes out. The ether fraction is evaporated under reduced pressure at a bath temperature of maximum 40°. To the dry residue, 1 ml *N* ammonia is added and the walls of the flask are carefully wetted during some 5 min. Then an equal amount of chloroform is added. The mixture, after shaking, is transferred to a small conical tube and centrifuged.

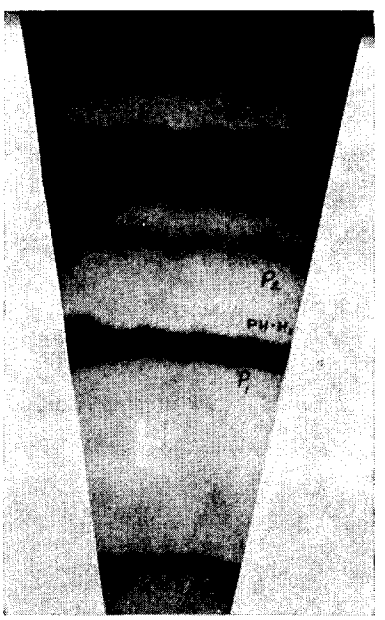

Fig. 1. A typical chromatogram of the α-keto-acid 2,4-dinitrophenylhydrazones of human blood.

Chromatographic procedure

0.3 ml of the supernatant ammonia solution (*S*) is put on a wedge-shaped strip (Fig. 1) of Whatman 3M+M filter paper, which had previously been dipped in 0.1 *M* Veronal–NaHCl buffer at pH 8.6,and air-dried.

The narrow end of the paper is put in the following solvent mixture; *n*-butanol–ethanol–water (40:10:50) (by vol.), which was adopted after several attempts with other solvents. The solvent mixture is allowed to ascend for 14–16 h, and the paper is subsequently dried in a current of dry air at room temperature.

Elution and quantitative estimation

The yellow-coloured areas are exactly delineated under a Wood lamp, cut into pieces and put in centrifuge tubes. To each of them, a convenient amount (3–6 ml)

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of 1 *N* NaOH is added; the paper is disintegrated by stirring with a glass rod and centrifuged. After decanting the clear supernatant solution, the residue is re-extracted with a further amount of 1 *N* NaOH (1–2 ml). 2 h after the first addition of NaOH, the extinction of the combined portions is read on a Bausch & Lomb Spectronic colorimeter at 420 mμ wavelength, against a blank prepared in the same manner from a chromatographed blank paper.

Standard extinction curves for different hydrazones were obtained from known amounts of pure α-ketoacids which had been carried through the full extraction and chromatographic procedure. The X_1 and X_2 DNP have been determined, using phenylpyruvic acid as a standard.

The amount of keto-acids present in blood is expressed in μg% according to the equation:

$$\text{Keto-acid in } \mu g\,\% = R.\ \frac{V_2 \times 100}{S \times V_1 \times F}$$

where: V_1 = volume of blood sample
V_2 = total volume of deproteinization mixture
F = volume of filtrate after deproteinization
S = volume put on the paper strip
R = amount of keto-acid as read on the standard curve.

RESULTS

General pattern of blood chromatograms

Under the conditions described above we obtained well delineated and sharply separated spots of blood keto-acid hydrazones as seen in Fig. 1. From bottom to top we noted the presence of the DNP of α-ketoglutaric acid, the first isomer of pyruvic acid, (P_1), the second isomer of pyruvic acid (P_2), and then, near the solvent front, two intense and distinctly separated spots of previously unidentified hydrazones (X_1 and X_2).

Close to the upper border of the first pyruvic acid isomer, there is a rather weak yellow band (PH-H) which has been identified as 1-hydroxy-6-nitro-1,2,3-benzotriazole, an artefact that arises from 2,4-dinitrophenylhydrazine in alkaline medium[21]. This artefact is, with most techniques, overlapped by the first isomer of pyruvic acid, but is neatly separated on our buffered and wedge-shaped chromatograms. When not separated, however, this product does not interfere with the colorimetric assay, as it does not give a red colour with sodium hydroxide.

Identification of unknown hydrazones

The first indication that X_1 and X_2 did not correspond to the isomers of the DNP of phenylpyruvic acid, as has been claimed[22], was their behaviour during the colorimetric estimation. The intensity of the colour of X_1 and X_2 in 1 *N* NaOH and their absorption spectra in this medium are fairly stable, as it is the case with other DNP derivatives of keto-acids, while the extinction of the two isomers of the DNP of phenylpyruvic acid rapidly decreases (Figs. 2 and 3).

Subsequently the X_1 and X_2 spots of many chromatograms were separately cut out and eluted with ethanol. The solution of X_1 presented a maximum of absorption at 355 mμ and contained some 100 μg of DNP of keto-acid per ml. The solution of X_2 had a maximum at 368 mμ and contained some 250 μg ml. The concentrations were based on the value $E^{1\%}_{1\,cm} = 9{,}000$ which was determined for the DNP of α-ketovaleric and α-keto-β-methylvaleric acid.

1 ml of each of these solutions was evaporated *in vacuo*, and the residue dissolved in 1 ml of water. A few mg of platinum oxide (Adams catalyst) were added and the mixture shaken overnight under hydrogen pressure in a Parr apparatus[17,20].

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This hydrogenation achieves the transformation of the DNP of the α-keto-acids into their corresponding amino acids, which may be identified chromatographically.

One-dimensional paper chromatograms were run in *n*-butanol–acetic acid and two-dimensional in butanol–acetic acid and phenol–water. After revealing the amino acid with ninhydrin, we found that X_1 gave one spot corresponding to one of the leucines and that X_2 gave two spots corresponding to valine and to one of the leucines.

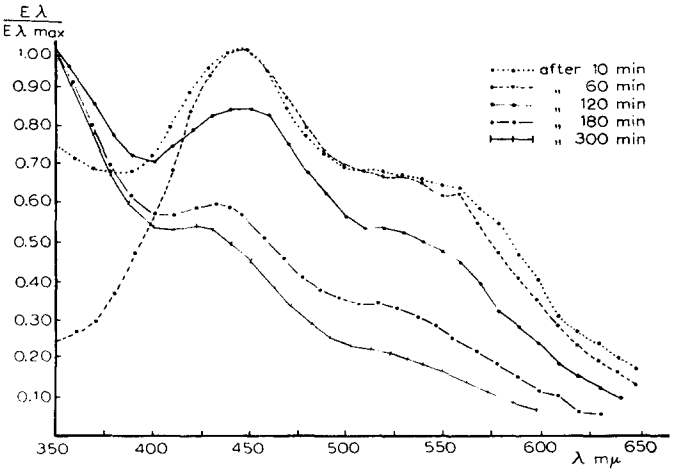

Fig. 2. Absorption curve of one of the isomers of the phenylpyruvic acid DNP (PP_1) in NaOH 1 *N* as a function of time.

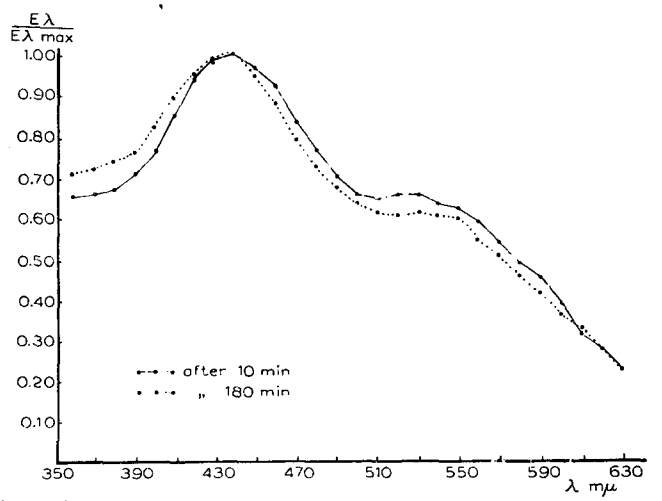

Fig. 3. Absorption curve of component X_1 DNP in NaOH 1 *N* as a function of time.

In order to refine the identification, chromatograms were performed in the system pyridine–isoamylalcohol[21]. Mixtures of the amino acids valine, norvaline, leucine, isoleucine and norleucine were run on the same paper in order to check the R_F-values. The X_1 yielded one spot corresponding to leucine, while the X_2 yielded valine, isoleucine and leucine. From these results, it may be concluded that α-keto-isovaleric, α-keto-β-methylvaleric and α-keto-isocaproic acid are present in the blood.

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Furthermore, we found that the DNP of the first two of these α-keto-acids gave one spot on our chromatogram, whose location did correspond to that of X_2, while the last-mentioned keto-acid gave two spots, corresponding to X_1 and X_2.

Some chromatograms run in the butanol–acetic acid system were, after treatment with ninhydrin, dipped into a 1% sodium bicarbonate solution. No blue colour was observed, and the spots could be eluted with water. With this technique, phenylalanine gives a blue colour that is not washed out with water[23].

As presumed, hydrogenolysis of P_1 and P_2 yielded alanine, and α-ketoglutaric acid yielded glutamic acid.

Concentration of α-keto-acids in blood

The average values for blood of healthy persons, with our technique, are:

— α-Ketoglutaric acid: 35–165 μg%.
— Pyruvic acid ($P_1 + P_2$): 380–900 μg%.
— $X_1 + X_2$: 930–1900 μg%.

The amount of $X_1 + X_2$ is expressed as phenylpyruvic acid which, at the beginning and during the major part of our study, was thought to be the keto-acid

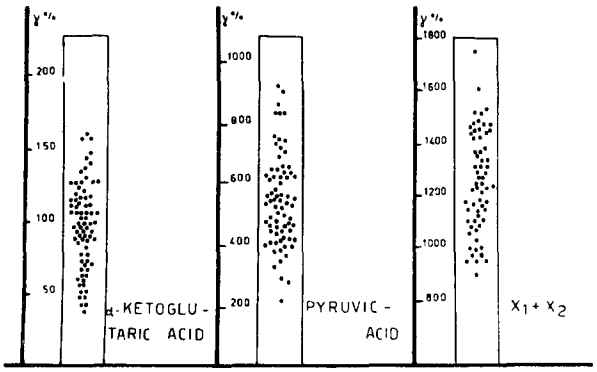

Fig. 4. The range of the α-keto-acids in human blood.

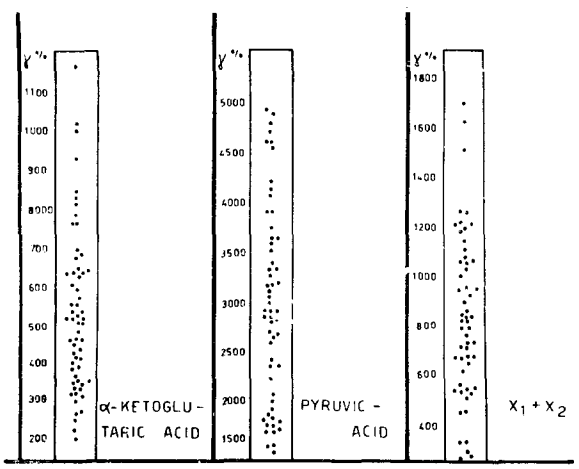

Fig. 5. The range of the α-keto-acids in rabbit blood.

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present[22]. After their identification we have found that the DNP of α-keto-isovaleric, -β-methylvaleric and -isocaproic acid in *N* sodium hydroxide solution have a much higher specific absorption (by a factor of 1.5 to 2) than phenylpyruvic acid. This means that all results for X_1 and X_2 should be reduced to nearly one half the mentioned value.

It would be interesting to determine the amount of each of these three α-keto-acids, especially since a significant change of X_1 and X_2 could be observed in some pathological conditions[28]. Unfortunately, sufficient separation could not be achieved by a simple modification of our chromatographic procedure.

The range of the values for human- and rabbit-blood keto-acids is given in Figs. 4 and 5.

DISCUSSION

We did not find evidence for the presence of oxalacetic acid or glyoxylic acid, confirming the results of other authors[4,7,14,19]. Acetoacetic acid is usually not present. (The second isomer of pyruvic acid P_2 should not be confounded with acetoacetic acid[14,15].) In some cases of severe diabetic state, however, we found the DNP of its decarboxylation product, *viz.* acetone, which runs close to the solvent front. At the same place, there is always a faint spot of some 2,4-dinitrophenylhydrazine; this spot does not give the typical red colour of hydrazones with sodium hydroxide and must not be taken for the acetone-hydrazone.

The presence of the unknown spots X_1 and X_2 had already been observed before[7,22,25–27]. They were always clearly visible on our chromatograms of human blood. This is due, in part to our chromatographic technique, but mainly to the extraction procedure. We used the modified CAVALLINI method[13], which does not involve a re-extraction of the DNP from ethylacetate into sodium carbonate solution as described in the original method[11] and which has been used or adapted by most authors. In this process, an important amount of these DNP is not removed from the organic solvent. This is certainly the reason why BISERTE AND DASSONVILLE[7], who employed the same technique as we used, had their attention drawn to these spots and correctly identified X_1. It is not clear, however, why these spots are not mentioned by CAVALLINI himself, but it may be due to the fact that he worked on blood and organs of rats. We have observed that these spots X_1 and X_2 were much weaker with the blood of rabbits.

Because the location of X_1 and X_2 on the chromatograms did correspond to that of the two isomers of the DNP of phenylpyruvic acid, TURNOCK identified them as this keto-acid[22]. KULONEN[27] submitted them to Al-amalgam reduction but only obtained indefinite spots of aminoacids. BISERTE AND DASSONVILLE[7] excluded the presence of phenylpyruvic acid, because in the solvent system isoamylalcohol–ethanol–water, the R_F-values did not correspond. After catalytic hydrogenation in the presence of platinum, they obtained leucine from the X_1 hydrazone. X_2 gave a negative result but they thought that it could be an isomer of X_1 and yet contain other keto-acids.

Using the same method, we have been able to prove that X_1 was one isomer of the DNP of α-keto-isocaproic acid, and that X_2 contained the other isomer, and also the DNP of α-keto-isovaleric and α-keto-β-methylvaleric acid. It could be shown, by a

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specific reaction, that no phenylalanine was obtained, thus excluding the presence of phenylpyruvic acid.

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We wish to express our thanks to Prof. A. MEISTER, Tufts University School of Medicine, for providing us with a sample of α-keto-β-methylvaleric acid, and to Profs. J. VANDENBROUCKE AND J. V. JOOSSENS, University of Louvain, for their help and interest during the course of this study.

SUMMARY

The α-keto-acids of blood have been separated by paper chromatography of their 2,4-dinitrophenylhydrazones. Their amount has been determined by measuring the extinction of their DNP in *N* sodium hydroxide solution. Three new α-keto-acids: α-ketoisovaleric, α-keto-β-methylvaleric and α-keto-isocaproic acid have been identified in human blood.

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