the crude chlorogenic preparations (see p. 338) it was possible, from an examination of the ultraviolet absorption spectra of these crude preparations, to conclude that at least twice as much chlorogenic acid was present in the 70 kg. of apple pulp tissue as was isolated in a crystalline form, i.e. 6–7 g. in all or approximately 10 mg./100 g. of pulp tissue. The same pulp tissue contains 800 mg./100 g. of quinic acid (Hulme, 1952) and, in spite of the disparity in these quantities, it is tempting to conclude that the two acids are metabolically related in the fruit. It is possible that one role of quinic acid is, by combining with the relatively insoluble caffeic acid, to render the *o*-dihydroxyphenolic properties of this latter acid more readily available in the form of the soluble chlorogenic acid. Chlorogenic acid would then be analogous to the glycosides of the insoluble flavone and flavonol pigments, the quinic acid residue in the former playing the same 'solubilizing' role as the sugar residues in the latter. Glycosides of caffeic acid are not commonly known to occur; the

caffeic acid-phenol glycoside of olives of Cruess & Alsberg mentioned earlier appears to be the only one so far reported.

SUMMARY

1. The occurrence and possible function of chlorogenic acid in plants is discussed.
2. A method is described whereby the acid can be isolated free of catechins from the young fruits of Worcester Pearmain apples.
3. An estimation is made of the amount of chlorogenic acid present in these young apples.
4. Its possible relationship to quinic acid, also present in the fruits, is discussed.

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Separation and Estimation of Blood Keto Acids by Paper Chromatography

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Owing to the interest attaching to the level of pyruvic acid in the blood, and its variations under physiological and pathological conditions, a number of methods have been devised for its estimation. Based on the earlier work of Dakin & Dudley (1913), the 'hydrazone' method was introduced by Barrenscheen & Dreguss (1931) and Case (1932). Its modifications by Peters & Thompson (1934), Lu (1939a), Bueding & Wortis (1940) and others,

afforded a more specific method for the estimation of total keto acids than the 'bisulphite-binding' method of Clift & Cook (1932a). However, even the later precautions introduced by Friedemann & Haugen (1943), while improving still further the specificity of the method for the estimation of pyruvic acid, did not completely exclude interference from other keto acids present in blood or other biological materials.

The technique of paper chromatography (Consden, Gordon & Martin, 1944) seemed likely to be of particular value for the separation and identification of coloured compounds such as the 2:4-dinitrophenylhydrazones of keto acids, and Cavallini, Frontali & Toschi (1949*a*) have recently published a short account of the separation and estimation of these compounds by this technique. These workers later (Cavallini, Frontali & Toschi, 1949*b*) described the application of paper chromatography to the estimation of pyruvic acid and α -ketoglutaric acid in blood and urine. After extracting the coloured spots from the paper, colorimetric estimation in strongly alkaline solution yielded mean values of 0.28 mg. pyruvic acid and 0.21 mg. α -ketoglutaric acid/100 ml. blood in five samples from normal human subjects. Rossi & Di Leva (1951), using essentially the same method, found very much lower values for the pyruvic acid content of human blood, ranging from 0.06 to 0.13 mg./100 ml. for four normal subjects. α -Ketoglutaric acid was again found to be present in the blood extracts, and two further unidentified spots were also observed. LePage (1950) has separated the keto acids of normal and neoplastic rat tissues by chromatographic adsorption on columns of 'Hyflo Super-Cel', but he does not describe any application of this technique to human blood.

From a study of the blood 'pyruvate' levels, estimated by the Friedeman & Haugen (1943) technique, in a series of cases of polyneuritis (Joiner, McArdle & Thompson, 1950), we became interested in an exact identification of the raised levels of keto acids found in some of these patients in whom there was no reason to suspect a deficiency of vitamin B₁. In the case of a deficiency of this vitamin it is known that the raised level of blood keto acids is due largely to pyruvic acid, as this has been isolated and identified as its 2:4-dinitrophenylhydrazone (Thompson & Johnson, 1935; Johnson, 1936). In other types of polyneuritis, however, and particularly in those showing no biochemical response to massive parenteral thiamine therapy (i.e. no lowering of previously raised keto acid levels), it becomes important to identify the excess keto acid more precisely. We decided, therefore, to study the possibilities of paper-chromatographic techniques; in view of the low levels of pyruvic acid found in blood by the Italian workers, we began by re-investigating the procedure (methods of extraction, suitability of different solvents, etc.), outlined by them, comparing our chromatographic results with simultaneous estimations by the usual colorimetric technique.

In order to identify the coloured spots the 2:4-dinitrophenylhydrazones of a number of pure keto acids which might reasonably be expected to occur in blood were first prepared and studied.

EXPERIMENTAL

Melting points are uncorrected. Micro-analyses were done by Weiler and Strauss, Oxford.

General method for the preparation of 2:4-dinitrophenylhydrazones. Excess of a saturated solution of 2:4-dinitrophenylhydrazine in 2*N*-HCl is added to an aqueous solution of the sodium salt of the keto acid, prepared by neutralizing 0.2 g. of the acid dissolved in 5 ml. water with the equivalent amount of NaHCO₃ dissolved in 5 ml. water. On standing at room temperature the 2:4-dinitrophenylhydrazones (referred to hereafter as 'hydrazones') crystallize out, and are purified by recrystallization. A further crop of crystals can be obtained by standing the separated mother liquor at 0-4° overnight.

Pyruvic acid 2:4-dinitrophenylhydrazone. Sodium pyruvate, prepared by the method of Robertson (1942), was used for the preparation of the hydrazone. This was recrystallized from glacial acetic acid: m.p. 218°. (Found: C, 40.2; H, 3.0; N, 20.5. Calc. for C₉H₈O₆N₄: C, 40.2; H, 3.0; N, 20.9%.)

Oxaloacetic acid 2:4-dinitrophenylhydrazone. The pure acid (m.p. 146°), prepared by standard methods, was used. The dry hydrazone was recrystallized from ethyl acetate in the cold by addition of light petroleum (b.p. 60-80°): m.p. 211°. (Found: N, 17.8. Calc. for C₁₀H₈O₈N₄: N, 17.9%.)

α -Ketoglutaric acid 2:4-dinitrophenylhydrazone. The pure acid (m.p. 110°) was prepared by the method of Schneider (1945); the hydrazone was recrystallized as for the oxaloacetic acid derivative: m.p. 219°. (Found: C, 40.8; H, 3.3; N, 17.0. Calc. for C₁₁H₁₀O₈N₄: C, 40.5; H, 3.1; N, 17.2%.)

Acetoacetic acid 2:4-dinitrophenylhydrazone. Acetoacetic acid was prepared as the sodium salt in aqueous solution by the method of Shaffer (1921). The hydrazone was prepared and recrystallized by the method of Clift & Cook (1932*b*), m.p. 124°. (Found: N, 20.3. Calc. for C₁₀H₁₀O₆N₄: N, 19.9%.)

Acetone 2:4-dinitrophenylhydrazone. This was prepared and recrystallized by standard methods: m.p. 126°.

Preparation and extraction of keto acid hydrazones from blood. Immediately after withdrawal from the body, 5-7 ml. blood are added to 20 ml. 5% (w/v) metaphosphoric acid in a weighed tube. It is important to collect the blood with the minimum of venous stasis, and no clenching and unclenching of the hand is allowed before collection. The tube is re-weighed, allowed to stand for 10 min. at room temperature, and then centrifuged.

To 20 ml. of the centrifugate 2 ml. 0.2% (w/v) 2:4-dinitrophenylhydrazine in 2*N*-HCl are added, and the mixture incubated for 20 min. at 38°.

The mixture is then extracted in a separating funnel with 5 ml. ethyl acetate. The layers are separated and the aqueous phase re-extracted with 5 ml. ethyl acetate. The two ethyl acetate extracts, which are in the form of a fine emulsion, are centrifuged for 20 min. at 4000 rev./min. The clear, upper ethyl acetate layer is then removed with a teat-pipette, and the combined aqueous layers re-extracted successively with three lots of 4 ml. ethyl acetate, centrifuging each time for 10 min. and adding the ethyl acetate layer to the first extract.

The combined ethyl acetate phase is next extracted successively with four lots of 2 ml. 10% (w/v) Na₂CO₃. The Na₂CO₃ solution is then extracted once with 2 ml. ethyl acetate to remove traces of free 2:4-dinitrophenylhydrazine. It is then neutralized by the dropwise addition of cold (0-4°) conc. HCl until there is no further evolution of CO₂.

The neutralized solution is then extracted successively with four lots of 2 ml. ethyl acetate, and the combined ethyl acetate extracts are evaporated to dryness *in vacuo* at temperatures not exceeding 40°.

Chromatographic procedure. The dry residue obtained above is dissolved in 0.1–0.3 ml. 0.1 N-NaOH. Phosphate buffer (0.1 M; pH 7.2) is then added until the red colour of the solution disappears (usually 0.5–1.0 ml. is required). In some experiments 0.2 ml. ethanol was added first, but this was found unnecessary. The total volume of NaOH and phosphate buffer added is noted.

0.2 ml. of the solution (or 0.1 ml. if the expected keto acid content is high) is then placed on a strip of Whatman no. 3MM filter paper with a micrometer syringe. The paper is hung in a sealed glass cabinet and the solvent mixture allowed to ascend for about 16 hr. A number of different solvents were tried, and the following mixture finally adopted: *n*-butanol 70 parts, ethanol 10 parts, 0.5 N-NH₄OH 20 parts.

In the case of the synthetic hydrazones 10 µg., dissolved in 0.02 ml. dilute phosphate buffer, were applied to the paper strips; as acetone 2:4-dinitrophenylhydrazone is insoluble in this medium ethyl acetate was used instead.

Elution and quantitative estimation of separated hydrazones. The coloured area on the paper occupied by the given hydrazone is cut out with scissors, and after cutting into small pieces is placed in a sintered-glass micro funnel.

1 ml. 10% Na₂CO₃ is added to the funnel, the contents stirred with a glass rod until the paper has disintegrated and then filtered under pressure into a small test tube. The residue on the funnel is re-extracted with a further 1 ml. Na₂CO₃.

To the combined filtrates 1 ml. 2 N-NaOH is added, the red colour measured after 15 min. in a 1 cm. cell using an Ilford Spectrum violet 601 filter and compared with a standard curve prepared from known amounts of the corresponding keto acid.

Colorimetric estimation of keto acids. Keto acids were also estimated in the blood samples by the method of Friedemann & Haugen (1943), using ethyl acetate as solvent.

RESULTS

Choice of chromatographic solvent

The final solvents chosen by the Italian workers were butanol-ethanol mixtures (Cavallini *et al.* 1949*a, b*; Rossi & Di Leva, 1951). Starting from their work, we have studied a series of mixtures of *n*-butanol, *isobutanol*, *n*-propanol, *isopropanol* and *n*-amyl alcohol with varying amounts of ethanol, water and 0.5 N-NH₄OH, and have compared the relative *R_F* values of our synthetic hydrazones. As mentioned in the Experimental Section, the final solvent which gave the best separation of these hydrazones was a mixture of *n*-butanol, ethanol and 0.5 N-NH₄OH. The relative positions taken up by the different pure hydrazones when run in this solvent mixture are shown in Fig. 1 (chromatograms *P₃* and *P₄*). In these chromatograms the hydrazones were not carried through the full extraction procedure used when assaying blood, but were applied directly to the paper. It will be seen

that under these conditions satisfactory resolution of these pure hydrazones is obtained; as is to be expected, the *R_F* values show slight variation from day to day, due probably to changes in environmental temperature and slight changes in the composition of the solvent mixture. The ranges of

Fig. 1. Chromatograms of pure keto acid 2:4-dinitrophenylhydrazones. *P₃*. α -Ketoglutaric, oxaloacetic, pyruvic and acetoacetic acid hydrazones and acetone hydrazone (10 µg. each). *P₃*. *A*, 30 µg. α -ketoglutaric acid hydrazone; *B*, α -ketoglutaric, pyruvic and acetoacetic acid hydrazones (30 µg. each); *C*, 30 µg. acetoacetic acid hydrazone; *D*, 30 µg. pyruvic acid hydrazone. *P₄*. *A*, hydrazones of α -ketoglutaric, pyruvic and acetoacetic acids (30 µg. each) applied direct to paper; *B*, hydrazones of same acids carried through complete extraction process used for blood analysis.

R_F values found for the different hydrazones are 0.08–0.15 for α -ketoglutaric acid, 0.19–0.29 for oxaloacetic acid, 0.35–0.47 for pyruvic acid, 0.46–0.64 for acetoacetic acid and 0.90–0.95 for acetone.

Chromatographic behaviour of synthetic 2:4-dinitrophenylhydrazones

As subsequent work (see below) showed that oxaloacetic acid is not present in normal human blood in amounts detectable by this technique, the hydrazone of this keto acid was not further studied.

Chromatograms were next prepared by taking the hydrazones through the complete extraction procedure as outlined above for blood. Chromatogram *P₄* in Fig. 1 shows the results obtained (*A*) when hydrazones were applied directly to the paper, and (*B*) when they were taken through the extraction process used for blood.

It will be seen that the *R_F* values for the hydrazones of α -ketoglutaric acid and pyruvic acid are unaffected by this treatment, whereas the picture given by acetoacetic acid is profoundly changed;

the spot having an R_F value slightly greater than that of the pyruvic acid hydrazone is largely replaced by a spot just below the solvent front, together with a faint, diffuse spot slightly in front of the original one. It will be noticed, however, that a faint spot also appears just below the solvent front in chromatogram P_4 (spot A) of Fig. 1, i.e. when the hydrazones are applied directly to the paper. It seemed likely, therefore, that acetoacetic acid hydrazone was undergoing some slight degree of decomposition during the chromatographic procedure, which was markedly accentuated when it was also subjected to the extraction process. Decarboxylation to acetone seemed a likely explanation of these findings. Such decarboxylation might take place to a small extent during the application

acetoacetic acid hydrazone, applied direct to the paper and also after being carried through the extraction procedure, was compared with that of the acetone hydrazone (Fig. 2). It will be seen that the extracted acetoacetic acid hydrazone has been almost completely converted to a substance giving the same R_F value as the acetone hydrazone.

Chromatographic behaviour of 2:4-dinitrophenylhydrazones extracted from blood

Fig. 3 shows two chromatograms, each comparing the picture obtained with blood with that given by the synthetic hydrazones. In paper P_{10} in this figure it will be seen that there are two spots corresponding to the hydrazones of α -ketoglutaric acid

Fig. 2. Chromatographic behaviour of acetoacetic acid 2:4-dinitrophenylhydrazone. P_7 . A, acetoacetic acid hydrazone ($30\text{ }\mu\text{g.}$) applied direct to paper; B, acetone hydrazone ($100\text{ }\mu\text{g.}$) applied direct to paper. P_8 . A, acetoacetic acid hydrazone ($30\text{ }\mu\text{g.}$) carried through complete extraction process used for blood analysis; B, acetone hydrazone applied direct to paper.

of the hydrazones to the paper, since a current of hot air from an electric hair drier is directed on to the paper while the hydrazone is being applied in order to prevent undue spreading of the hydrazone solution. When, however, the hydrazone is carried through the full extraction procedure it remains at room temperature in approx. 4% metaphosphoric acid for a short period, after which it is subjected to evaporation *in vacuo* at a temperature of up to 40° . It might be expected, therefore, that further decarboxylation would take place during these procedures. In order to test this possibility, chromatograms were prepared in which the behaviour of the

Fig. 3. 2:4-Dinitrophenylhydrazones extracted from human blood. P_{10} . A, hydrazones of α -ketoglutaric, pyruvic and acetoacetic acids ($30\text{ }\mu\text{g.}$ each) applied direct to paper; B, hydrazones extracted from blood. P_2 . A, hydrazones extracted from blood; B, 'tracers' (as in P_{10}) carried through complete extraction process used for blood analysis.

and pyruvic acid respectively. A third is visible in the blood picture slightly above the pyruvate spot; the former corresponds in position with that given by the acetoacetate tracer. In addition, it will be noticed that a certain amount of decarboxylation of this hydrazone has taken place, rather more marked in the case of the tracers than with blood, since a fourth spot is visible slightly behind the solvent front. The other chromatogram (P_2) in Fig. 3 again shows the α -ketoglutarate and pyruvate hydrazones clearly, but in this case the acetoacetate hydrazone, both in the tracer and in the blood, shows two somewhat indistinct spots just above the pyruvate, in addition to the acetone spot below the

solvent front. This variable behaviour of the acetoacetate hydrazone will be discussed later.

We have prepared chromatograms from forty blood samples from healthy subjects, and have in each case found (a) the spot having the same R_F value as the hydrazone of the α -ketoglutarate, (b) the spot with the pyruvate R_F value, and (c) a spot or spots which we have provisionally identified as due to acetoacetate and acetone.

*Identification of the separated blood
2:4-dinitrophenylhydrazones*

Since slight variations in the R_F values of substances separated on paper chromatograms are customarily found, we felt it desirable to obtain further evidence of the identity of these blood spots with the three keto acids mentioned above. An attempt was made, therefore, to compare the absorption curves (in the visible region of the spectrum) of the eluted blood spots with those of the pure hydrazones. The measurements were carried out in a Unicam D.G. spectrophotometer.

Fig. 4. Absorption curves of 2:4-dinitrophenylhydrazones extracted from 'pyruvic' and ' α -ketoglutaric' spots, compared with curves obtained with pure pyruvic and α -ketoglutaric acid hydrazones. Upper curves, pyruvic acid; lower curves, α -ketoglutaric acid. O-O-O, pure hydrazone; ●-●-●, extracted material.

The curves obtained with pyruvic and α -ketoglutaric acid hydrazones are shown in Fig. 4, from which it will be seen that the agreement between the extinctions obtained with the eluted and with the pure hydrazones is reasonably good. The curves for the two hydrazones agree well with those obtained by Friedemann & Haugen (1943). These results may be taken to support our conclusion, based on the R_F values, that these spots in the blood chromatograms are due to α -ketoglutaric and pyruvic acid hydrazones respectively.

On the other hand, we were unable to obtain good agreement between the curve of pure acetoacetic acid hydrazone and that of the solution eluted from the blood spot presumed, from evidence

described above, to be due to this hydrazone. This lack of agreement may be due to the blood spot containing traces of the acetone hydrazone formed by decarboxylation of the acetoacetic acid derivative while running on the paper.

*Quantitative estimation of chromatographically
separated blood keto acids*

The coloured spots identified as corresponding to the pyruvic and α -ketoglutaric acid derivatives were next extracted with 10% sodium carbonate. After addition of sodium hydroxide to the resulting solutions they were estimated colorimetrically by comparison with the appropriate standard curve prepared from the pure hydrazone of the acid.

As is to be expected there are differences in the colour intensities per unit weight of the hydrazones of the three keto acids in alkaline solution; thus, the colour intensities of the hydrazones formed from equal weights of pyruvic acid, acetoacetic acid and α -ketoglutaric acid are in the ratio of 1.00:0.74:0.48.

Estimation of the 'total keto acids' in the respective blood samples was carried out both by the method of Friedemann & Haugen (1943) and also by applying to filter paper a measured amount of the extract prepared for chromatography, eluting it immediately without chromatographic separation, and estimating colorimetrically against a standard curve prepared from pure pyruvic acid hydrazone. The results obtained on blood samples from fifteen normal subjects in the post-absorptive state are shown in Table 1. Owing to the varying degrees of decarboxylation of the acetoacetic acid hydrazone in different experiments no attempt was made to extract and estimate the amount of this acid present in the samples.

From Table 1 it will be seen that the level of the total keto acids as estimated in the final concentrate applied to the papers for chromatography (column B of Table 1) is rather less than the level as estimated by the method of Friedemann & Haugen (1943), amounting to 63–105% (mean 84%) of the latter. This discrepancy is probably due to slight losses in the lengthy extraction and elution process necessary for obtaining the final concentrate for chromatography.

The level of pyruvic acid in the different samples varied from 0.26 to 0.69 mg./100 ml., with a mean of 0.44 mg./100 ml. Pyruvic acid accounts, therefore, for from 42 to 75% (mean 60%) of the total keto acids present in the final concentrate. The level of α -ketoglutaric acid varied from 0.13 to 0.24 mg./100 ml. with a mean of 0.16 mg./100 ml.

Assuming that acetoacetic acid is the only other acidic hydrazone-forming substance present in normal blood (and no other acidic hydrazones are visible by this technique) the approximate level of this acid in blood may be obtained by difference.

Table 1. *Blood keto acid levels in normal subjects in the post-absorptive state*

(Results expressed as mg. keto acid/100 ml. blood; total keto acids expressed as pyruvic acid.)

Subject	Total keto acids		Pyruvic acid	α -Ketoglutaric acid
	A. Method of Friedemann & Haugen (1943)	B. As estimated in final concentrate applied to paper for chromatography		
1	0.79	0.57	0.34	—
2	1.23	1.07	0.63	0.24
3	1.02	0.82	0.62	—
4	0.69	0.62	0.26	—
5	0.77	0.74	0.37	0.13
6	0.79	0.58	0.33	—
7	0.65	0.46	0.24	0.13
8	0.59	0.37	0.26	—
9	0.95	0.89	0.52	0.16
10	1.14	0.89	0.52	—
11	1.05	0.89	0.55	—
12	0.73	0.54	0.39	0.16
13	0.99	1.05	0.69	—
14	0.88	0.74	0.44	—
15	0.73	0.66	0.44	—
Mean	0.87	0.73 (=84% of A)	0.44	0.16
S.E.	± 0.19	± 0.21	± 0.14	

Thus, in Table 1 the mean level of total keto acids, expressed as pyruvic acid, is 0.73 mg./100 ml., as determined colorimetrically in the concentrate prepared for chromatography and estimated against a standard curve prepared from pure pyruvic acid hydrazone. Of this amount, pyruvic acid accounts for 0.44 mg./100 ml. There is also present 0.16 mg. α -ketoglutaric acid/100 ml., estimated against a standard curve prepared from the pure hydrazone of this acid; when estimated against pure pyruvic acid hydrazone the α -ketoglutaric acid level is equivalent in colour intensity to 0.08 mg. pyruvic acid/100 ml. Therefore, the mean acetoacetic acid concentration = $0.73 - (0.44 + 0.08) = 0.21$ mg. pyruvic acid/100 ml.

As pointed out above, however, the hydrazone formed from 1 mg. pyruvic acid yields the same colour intensity as does the hydrazone formed from 1/0.74 mg. acetoacetic acid, so that the concentration of the latter acid in blood = $0.21 \times 1/0.74 = 0.28$ mg. acetoacetic acid/100 ml. blood.

Table 2. *Mean concentrations of keto acids in post-absorptive human blood*

	As mg. of each keto acid/100 ml. blood	As mg. pyruvic acid/100 ml. blood
Pyruvic acid	0.44	0.44
α -Ketoglutaric acid	0.16	0.08
Acetoacetic acid	0.28	0.21
Total keto acids		0.73

The mean concentrations of these three keto acids in post-absorptive human blood are therefore as

shown in Table 2. Pyruvic acid is therefore the most plentiful of the three keto acids which we have demonstrated in human blood under post-absorptive conditions.

Effect of exercise on blood pyruvate level

Johnson & Edwards (1937) and Lu (1939*b*) have demonstrated that in exercise there is a considerable elevation of the blood pyruvate level, estimated by Peters & Thompson's (1934) modification of the method of Case (1932). By isolating the pyruvic acid 2:4-dinitrophenylhydrazone from 400 ml. of blood drawn from four subjects after they had run to exhaustion, Johnson & Edwards (1937) concluded that most of the keto acid accumulation in the blood was in fact due to pyruvic acid or to some unstable precursor.

It seemed of interest, therefore, to estimate the chromatographically separated keto acids from blood samples taken from four healthy subjects before and after exercise (running up and down stairs till exhausted). The results obtained (Table 3 and Fig. 5) confirmed Johnson & Edwards's earlier conclusion that the excess keto acid present after exercise is very largely pyruvic acid; in our experiments pyruvic acid accounted for 83–99% of the total keto acids accumulating. The excess pyruvic acid is clearly shown in the chromatogram (Fig. 5); it will also be seen from this figure that there is some increase in acetoacetic acid, whereas the α -ketoglutaric acid is not noticeably changed in amount.

Table 3. *Increase in pyruvic acid and total keto acids in blood after exercise*

Subject	Increase in total keto acids		Increase in pyruvic acid	Pyruvic acid as % of total keto acids
	Method of Friedemann & Haugen (1943)	As estimated in final concentrate applied to the paper for chromatography		
1	0.55	0.40	0.36	90
2	1.81	1.84	1.53	83
3	1.25	1.14	1.13	99
4	1.63	1.47	1.45	99

DISCUSSION

The results described above confirm the earlier findings of the Italian workers that it is possible to separate and identify both pyruvic and α -ketoglutaric acids in extracts of human blood by means of paper chromatography. Our identification of these acids rests primarily on the agreement between the R_f values of pure 2:4-dinitrophenylhydrazones of these keto acids and the values obtained on the same chromatogram for the two most slowly running spots in the blood extracts. Rough measurements of the absorption curves given by the extracted hydrazones in alkaline solution support this identification.

Fig. 5. Effect of exercise on blood keto acids. P_5 , 2:4-Dinitrophenylhydrazones extracted from 0.7 ml. blood *before* exercise. P_6 , Hydrazones extracted from 0.5 ml. blood *after* exercise.

Rossi & Di Leva (1951) observed two further spots in the blood extracts, which they did not identify. We have regularly found one or more spots

moving more rapidly than the pyruvic acid hydrazone in post-absorptive human blood. We think that the most slowly moving of these spots is acetoacetic acid hydrazone, but owing to decarboxylation of this compound another rapidly moving spot of acetone hydrazone also sometimes appears just behind the solvent front. The intensity of this acetone spot differs noticeably in different chromatograms. We feel that varying degrees of decarboxylation of the acetoacetic acid hydrazone also take place while the hydrazone is actually travelling up the paper; thus, whereas the spots due to α -ketoglutaric and pyruvic acids are in most cases small and show little evidence of spreading, the acetoacetate spot is in nearly all cases markedly elongated, with an indefinite and gradually fading upper edge.

As mentioned earlier, we have not obtained any evidence of the presence of oxaloacetic acid in blood in amounts detectable by this technique. In this connexion it is of interest to note that Frohman, Orten & Smith (1951) reported that the level of oxaloacetic acid in rat blood is less than 0.1 mg./100 ml., while LePage (1950) concluded that if oxaloacetic acid is present in rat blood it is below the level of detection by his method. Our failure to detect oxaloacetic acid in human blood is, in our opinion, not due to its decarboxylation to pyruvic acid during the extraction. Thus, when a solution of pure oxaloacetic acid hydrazone is placed on the paper, a single spot with a low R_f value is found; when it is carried through the full extraction procedure some decarboxylation evidently occurs, since a spot with an R_f value the same as that of the pyruvate hydrazone is also present; this, however, is always less intense than the unchanged oxaloacetate spot; if it is present in blood, therefore, one would expect to find an oxaloacetate spot, which under our conditions is not present.

The mean level of pyruvic acid in blood which we have estimated colorimetrically after chromatographic separation from the other keto acids, is considerably greater than that reported by the Italian workers, and amounts to about 60% of the

total keto acids. As our figures for the acetoacetic acid content of post-absorptive blood are obtained by difference, they will include the variable amount of acetone formed by decarboxylation during the chromatographic run. Our mean value of 0.28 mg. acetoacetic acid/100 ml. blood agrees well with the range of values (0.08–0.40 mg./100 ml.) found in five healthy subjects by Stark & Somogyi (1943), although it is somewhat higher than the five normal values (0.06–0.26 mg./100 ml.) reported by Rosenthal (1949). Our mean estimated level for α -ketoglutaric acid in blood also agrees reasonably well with the value of 0.21 mg./100 ml. found by Cavallini *et al.* (1949b).

Although the estimation of acetoacetic acid by this procedure is inaccurate when applied to blood owing to its instability and low concentration, it is possible, we feel, to obtain a more exact estimate of the pyruvate level by means of preliminary chromatographic separation than by the Friedemann & Haugen technique. In studying the changes in the level of blood keto acids in disease it not only provides us with a more specific method for the estimation of pyruvic acid, but also affords a means of detecting visually and of estimating

roughly keto acids other than pyruvic acid, particularly if these are present in raised amounts as a result of the disease.

SUMMARY

1. A method is described for the separation and identification of blood keto acids by paper chromatography, and for the extraction and quantitative estimation of the separated pyruvic and α -ketoglutaric acids.

2. In post-absorptive blood from healthy human subjects pyruvic acid, α -ketoglutaric acid and acetoacetic acid are the only keto acids detectable by this method. Under these conditions pyruvic acid accounts for about 60 % of the total keto acids.

3. The rise in the level of blood keto acids due to exercise has been shown to be almost entirely due to pyruvic acid, although the acetoacetate level is also increased.

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