2. Chondroitin sulphate was found to play a part in the stabilization of tendon. It is suggested that a quarter of the stabilizing linkages are between chondroitin sulphate and collagen, consisting of 40 % salt-like links and 60 % hydrogen bonds.

3. Some other mucopolysaccharide or mucoprotein may also be concerned in this stabilization.

4. Salt-precipitated collagen is similar to fish skin in being stabilized mainly by salt-like cross-links.

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Chromatography of Pyruvic Acid and of Acetoacetic Acid as their 2:4-Dinitrophenylhydrazones

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The problem of the separation and the quantitative estimation of keto acids in the blood is of the greatest interest on account of the importance of these metabolic intermediates. Markees & Gey (1953) dealt with the quantitative paper chromatography of pyruvic acid as the 2:4-dinitrophenylhydrazone and showed that the derivatives of this α -keto acid form two sharply distinct spots with the R_F values of 0.36 (*A*) and 0.57 (*B*). This chromatographic separation has already been observed and described by other authors (Cavallini, Frontali & Toschi, 1949; LePage, 1950; Altmann, Crook & Datta, 1951; Seligson & Shapiro, 1952; Hockenhull, Hunter & Herbert, 1953; Stewart, 1953). The formation of these two spots *A* and *B* is most probably due to the formation of *cis* and *trans* isomerides.

The publication of El Hawary & Thompson (1953), in which the 2:4-dinitrophenylhydrazones of α -oxoglutaric acid, oxaloacetic acid, pyruvic acid, acetoacetic acid and acetone have been investigated chromatographically, prompted us to a renewed investigation of this problem. They found on analysis of their chromatograms a spot in the R_F region of 0.46–0.64 which they believed to be due to acetoacetic acid. Experimentally they proceeded in a very similar manner to ourselves, with the difference that we used ethyl acetate as a solvent for adding the substances to the chromatogram, while they, instead, employed sodium hydroxide plus phosphate buffer. The developing solvent was

identical in both cases (butanol:ammonia:water) except that they added 10 % (v/v) ethanol which we did not. The question now arises whether the spot in the R_F region of 0.46–0.64 can be considered as due to the 2:4-dinitrophenylhydrazone of acetoacetic acid, as has been suggested by El Hawary & Thompson (1953), or whether it has possibly been mistaken by them for the spot which we call *B*, and which appears to be due to a reaction product of 2:4-dinitrophenylhydrazine with pyruvic acid.

EXPERIMENTAL AND RESULTS

In order to clear up this point we spotted the chemically prepared 2:4-dinitrophenylhydrazone of acetoacetic acid directly, and we also carried out an analysis of blood to which acetoacetic acid had been added. In one case we also boiled the blood filtrate before the addition of 2:4-dinitrophenylhydrazine, and, in another case, we analysed it without boiling. The experimental details were exactly the same as described in our earlier work (Markees & Gey, 1953). Controls using buffered sodium hydroxide as the solvent did not show any change in the R_F values of the various 2:4-dinitrophenylhydrazones. The results of these experiments can be summarized as follows:

Chemically prepared acetoacetic acid 2:4-dinitrophenylhydrazone gives a spot with an R_F value of 0.48. We also find a spot with the R_F value of 0.94 which corresponds to the acetone hydrazone formed by decomposition.

Chemically prepared pyruvic acid 2:4-dinitrophenylhydrazone always forms the above-mentioned spots *A* and *B*. The R_F value of 0.57 of *B* is quite different from that of the dinitrophenylhydrazone of acetoacetic acid.

When substance *A*, isolated by extracting chromatograms, is spotted along with acetoacetic acid 2:4-dinitrophenylhydrazone, two spots are obtained which run into each other, thus forming a streak. A similar thing happens when the 2:4-dinitrophenylhydrazone of acetoacetic acid is combined with the extract of spot *B*, only in a correspondingly higher R_F region. When the chemically obtained pyruvic acid 2:4-dinitrophenylhydrazone (*A* + *B*) is added to acetoacetic acid 2:4-dinitrophenylhydrazone, a long, continuous, yellow streak stretching from *A* to *B* (Fig. 1) is formed.

Normal blood without any addition gives besides the spots *A* and *B* a spot for α -oxoglutaric acid with the R_F value 0.07, which can be disregarded. When acetoacetic acid is added to the blood, it is almost completely transformed by the analytical procedure into acetone, even if the blood filtrate is not boiled before the addition of the 2:4-dinitrophenylhydrazine, and the spot of acetone 2:4-dinitrophenylhydrazone appears in its usual position. The same results are obtained whether the blood is boiled or not. If the blood contains large amounts of acetoacetic acid (large addition or diabetic acidosis), a fainter spot located between *A* and *B* appears when the blood filtrate is used unboiled.

Fig. 1. Chromatograms of pure keto acid 2:4-dinitrophenylhydrazones. *a*, acetoacetic acid 2:4-dinitrophenylhydrazone ($R_F = 0.48$); *b*, pyruvic acid 2:4-dinitrophenylhydrazone (spot *A*, $R_F = 0.36$; spot *B*, $R_F = 0.57$); *c*, substance *A* + acetoacetic acid 2:4-dinitrophenylhydrazone; *d*, substance *B* + acetoacetic acid 2:4-dinitrophenylhydrazone; *e*, substances *A* + *B* + acetoacetic acid 2:4-dinitrophenylhydrazone.

DISCUSSION

From our experimental results we conclude that in the ordinary chromatography of the 2:4-dinitrophenylhydrazones of the keto acids in blood, that of acetoacetic acid only appears if the latter is in great excess. The 2:4-dinitrophenylhydrazone of acetoacetic acid has an R_F value of 0.48 and is sharply separated from the spot *B* of pyruvic acid which has an R_F value of 0.57. El Hawary & Thompson (1953), like ourselves, observed that the acetoacetic acid 2:4-dinitrophenylhydrazone spot almost completely disappears, owing to the analytical procedure, and that in its place acetone dinitrophenylhydrazone

appears. Moreover, they were unable to obtain absorption curves identical with those of the chemically pure acetoacetic acid hydrazone with an extract of the spot which they ascribed to the acetoacetic acid hydrazone. We believe it probable that these authors have mistaken the spot *B* due to the pyruvic acid 2:4-dinitrophenylhydrazone for the acetoacetic acid 2:4-dinitrophenylhydrazone.

In a recent publication of Neish (1953), concerning the separation of the dinitrophenylhydrazones of the α -keto acids by ionophoresis and their polarographic estimation, there is also evidence that two spots may be found with the dinitrophenylhydrazone of pyruvic acid. The author says that this chromatographic separation is only found when old solutions are chromatographed and that with the freshly prepared dinitrophenylhydrazone of pyruvic acid only one spot appears. He explains this phenomenon as the formation of two isomers. With absolutely freshly prepared 2:4-dinitrophenylhydrazone of purest pyruvic acid, we have also observed a strong preponderance of fraction *A*. In the blood the relative intensity of the two spots is liable to marked variations, but complete absence of *B* has never been observed.

SUMMARY

1. Paper chromatography of the 2:4-dinitrophenylhydrazone of pyruvic acid shows two sharply separated spots with R_F values 0.36 (*A*) and 0.57 (*B*).

2. Acetoacetic acid dinitrophenylhydrazone has an R_F value of 0.48 and is only formed in the ordinary analytical procedure when acetoacetic acid is present in high concentration in the blood. In most cases, owing to decomposition, acetone 2:4-dinitrophenylhydrazone with the R_F value 0.94 is formed exclusively.

3. Attention is directed to the possibility of error in mistaking the spot *B* due to pyruvic acid for the spot due to acetoacetic acid.

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