A Note on the Chromatographic Identification of Blood Keto Acids in Animals poisoned with Arsenite and Alloxan

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In agreement with the original observations of Cavallini, Frontali & Toschi (1949) on the separation of keto acid 2:4-dinitrophenylhydrazones by paper chromatography, El Hawary & Thompson (1953) demonstrated that when pure pyruvic acid 2:4-dinitrophenylhydrazone is applied to filter paper and chromatographed with a butanol–ethanol–ammonia–water mixture, a single spot, having R_F about 0·4, is obtained. With the samples of pyruvic acid and its 2:4-dinitrophenylhydrazone which we were then using we found no evidence at that time of the presence of a second spot due to pyruvic acid 2:4-dinitrophenylhydrazone, even when the pyruvic acid derivative was taken through the whole extraction process which we were using for the separation of 'hydrazones' from blood.

When the keto acid hydrazones extracted from human blood were separated chromatographically, however, we found, in addition to the α-oxoglutarate and pyruvate derivatives, a third spot having R_F slightly higher than that for the pyruvate hydrazone. We had further shown that the acetoacetic acid hydrazone, when applied directly to paper, has under our conditions, R_F similar to that for the third blood spot, although when it is carried through the extraction process it is partially decarboxylated to the acetone hydrazone, having R_F 0·90–0·95; this latter finding has been confirmed by Markees (1954). We therefore provisionally identified the third spot in the blood chromatograms as due to acetoacetic acid, although we stated that we had been unable to get good agreement between the absorption curve of the pure acetoacetic acid hydrazone and that of the solution eluted from the third blood spot. We suggested that this lack of agreement might have been due to the blood spot containing traces of the acetone hydrazone formed by decarboxylation of the acetoacetic acid derivative while running on the paper.

Several workers, however, have reported the presence of two spots in chromatograms of the hydrazone derived from pure pyruvic acid (LePage, 1950; Altmann, Crook & Datta, 1951; Seligson & Shapiro, 1952; Hockenhull, Hunter & Herbert, 1953; Stewart, 1953). In further work of our own we have confirmed this finding, using pure sodium pyruvate or freshly distilled pyruvic acid and extracting the hydrazone formed from this by the usual procedure used for the extraction of hydrazones from blood. However, when pyruvic acid 2:4-dinitrophenylhydrazone is applied directly to the paper, without preliminary extraction, we still regularly obtain one spot. When the hydrazone is taken through the extraction process, the relative intensity of the second spot varies widely under our conditions, corresponding in some cases to less than 5% of the total pyruvic acid, and in others to more than 50%.

It has further been stated that the third spot usually visible in chromatograms of blood filtrates has R_F and absorption curve similar to those for the more rapidly moving spot produced by the pyruvic acid hydrazone (Cavallini & Frontali, 1954). Kulonen, Carpén & Ruokolainen (1952), however, had earlier claimed that the R_F value for this faster pyruvic acid component corresponds to that for the acetoacetic acid hydrazone, although Markees (1954) has stated that under his conditions the R_F of the latter is intermediate between the values for the two pyruvate components.

Because of these conflicting findings we have studied chromatographically the blood of animals poisoned with sodium arsenite or alloxan, and containing therefore high levels of pyruvic acid (Peters, Sinclair & Thompson, 1946) and acetoacetic acid (Lackey, Bunde, Gill & Harris, 1944) respectively. By this means it was hoped to provide further evidence towards the identification of the blood constituent forming this third spot. These experiments form part of a more comprehensive study of the actions *in vivo* of a number of enzyme inhibitors on the level of various blood constituents, which will be reported later together with a fuller account of the physiological state of these poisoned animals.

EXPERIMENTAL

Chromatographic procedure. The preparation and extraction of keto acid 2:4-dinitrophenylhydrazones from blood, and the chromatographic procedure and subsequent estimation of the separated hydrazones, were carried out as described earlier (El Hawary & Thompson, 1953).

Arsenite-poisoned animals. A solution of sodium arsenite, prepared by dissolving As_2O_3 in 0·1 N-NaOH, was neutralized

and injected subcutaneously into rats in 0·2 ml. doses equivalent to 12 mg. As_2O_3/kg. body wt. The animals were killed by decapitation 2 hr. later, and blood was drained directly into the appropriate protein precipitant. The amount of blood taken was then determined by reweighing the tubes.

Alloxan-poisoned animals. 200 mg. alloxan/kg. body wt. was injected intraperitoneally into rats. The animals were killed after hyperglycaemia had developed, i.e. 48–72 hr. later, and blood samples obtained as described above.

Acetoacetic acid was estimated in blood by a modification of the method of Thin & Robertson (1952). We have used 10% (w/v) trichloroacetic acid as a protein precipitant instead of the $Ba(OH)_2$ and $ZnSO_4$, $7H_2O$ used by these workers. We have also modified the colour reagent, using 1 ml. of a mixture of 0·5 ml. salicylic aldehyde with 8 ml. 4N-KOH. After incubation for 2 hr. at 37°, we have removed the 1 ml. from the centre well of the Conway units with a Pasteur pipette and have then washed out the well twice with 1 ml. of distilled water.

RESULTS

Chromatograms were prepared from the keto acid 2:4-dinitrophenylhydrazones extracted from the blood of seven normal rats, seven poisoned with arsenite and five with alloxan. The pyruvate spot (i.e. with R_F corresponding to that of the single spot obtained when the pure pyruvic acid 2:4-dinitrophenylhydrazone is applied to the paper) and the 'third spot' present in the blood filtrates (i.e. with R_F resembling both that of acetoacetate and that of the faster component derivable from pyruvic acid) were cut out from the papers and the hydrazone eluted and estimated colorimetrically. Further blood samples were taken from each animal for estimation of acetoacetate. The results are shown in Table 1.

In confirmation of Peters *et al.* (1946) it will be seen that sodium arsenite, administered in these doses, causes a fivefold increase in the blood pyruvate level; the level of acetoacetate however is only 1·8 times that in the normal animals. With alloxan, on the other hand, the level of acetoacetate is increased ninefold, under our conditions while that of pyruvate is only 1·9 times the normal level.

Furthermore, the rise in the pyruvate level in the arsenite-poisoned rats is accompanied by an increase in the third spot, whereas the marked rise in the acetoacetate level in the alloxan animals is associated with a fall in the mean level of the substance responsible for the third spot.

The level of α-oxoglutarate is also increased in both the arsenite- and alloxan-poisoned groups, but it is not proposed to describe in detail results obtained for this keto acid since it yields only one spot under our conditions (see also Cavallini & Frontali, 1954) and is widely separated from the pyruvate and acetoacetate spots.

DISCUSSION

Although it is now known that pure sodium pyruvate gives rise to two dinitrophenylhydrazone components having different R_F values, the presence of both pyruvate and acetoacetate in blood, together with the conflicting statements concerning the R_F values of the dinitrophenylhydrazones derived from these substances, made it desirable to determine with more certainty which of these two acids is responsible for the third component normally visible in chromatograms of blood extracts, or whether both acids contribute to variable degrees.

It is clear from the results described above that, in our earlier provisional identification of this third spot as due to acetoacetate (El Hawary & Thompson, 1953), we were misled by the close similarity under our conditions between the R_F values of the acetoacetate dinitrophenylhydrazone and of the faster pyruvate component. Despite the wide variations in the relative intensity of the second pyruvate spot which we have found in different experiments with pure sodium pyruvate, the present work supports the conclusion of Markees (1954) that this third component in chromatograms of blood extracts is due to pyruvate, since even when the

Table 1. *Blood keto acid levels in normal rats and in rats poisoned with sodium arsenite and alloxan*

Expressed as mg. acid/100 g. blood: 'third spot' expressed in terms of pyruvic acid.

	Pyruvate	'Third spot'	Aceto-acetate
Normal	0·52	0·38	0·40
	0·42	0·38	0·74
	0·51	0·35	0·62
	0·33	0·37	0·38
	0·46	0·19	0·72
	0·56	0·27	0·53
	0·65	0·08	0·30
Mean ± S.E.M.	0·49 ± 0·04	0·29 ± 0·04	0·53 ± 0·07
Arsenite-poisoned	1·70	0·86	0·55
	1·15	1·06	0·67
	1·78	0·64	0·90
	3·22	0·31	0·95
	4·23	1·26	1·30
	2·73	1·27	1·07
	2·05	0·42	1·06
Mean ± S.E.M.	2·46 ± 0·37	0·83 ± 0·15	0·93 ± 0·10
Alloxan-poisoned	1·00	0·35	5·10
	0·63	0·39	4·75
	0·97	0·06	5·95
	0·85	0·04	4·05
	1·32	0·05	4·22
Mean ± S.E.M.	0·95 ± 0·11	0·18 ± 0·08	4·81 ± 0·34
Ratio of means			
Arsenite-poisoned / Normal	5·0	2·9	1·8
Alloxan-poisoned / Normal	1·9	0·6	9·1

acetoacetate level is markedly raised it does not contribute to any increase in the third spot. The absence of any detectable acetoacetate spot is probably due to decarboxylation, both during the extraction process and during the run on the paper, and it was noticed that an acetone 2:4-dinitrophenylhydrazone spot was regularly seen on the chromatograms from the alloxan-treated animals.

In view of these findings, together with the resemblance between the absorption curve of the substance giving the third spot in blood filtrates with that of the second pyruvate spot (Cavallini & Frontali, 1954), which finding we have also confirmed, we conclude that this third blood component does not result from acetoacetic acid but corresponds to the more rapidly moving pyruvate component. It would seem therefore that a safe estimate of the pyruvate content of blood can be obtained by summing the two more rapidly moving spots, even when high levels of acetoacetate are present.

SUMMARY

1. A chromatographic study has been made of the keto acids present in the blood of normal rats and of rats poisoned with sodium arsenite and alloxan respectively.

2. It has been shown that a rise in the blood pyruvate level is accompanied by a rise in the 'third spot' visible in chromatograms of dinitrophenylhydrazones from blood, and that no increase in this occurs when the acetoacetate level is high.

3. It is concluded that this third spot is due to the more rapidly running component of the pyruvic acid 2:4-dinitrophenylhydrazone, and that high acetoacetate levels are not likely to interfere in the chromatographic estimation of the blood pyruvate level.

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Analogues of Diaminopimelic Acid as Inhibitors of Bacterial Growth

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Many analogues of amino acids have been synthesized and tested as potential bactericides. β-2-Thienylalanine inhibits growth of *Escherichia coli*, *Streptococcus faecalis* and *Lactobacillus arabinosus*, but it has no chemotherapeutic value, since it is also toxic to animals (du Vigneaud, McKennis, Simmonds, Dittmer & Brown, 1945; Dittmer, Ellis, McKennis & du Vigneaud, 1946; Ferger & du Vigneaud, 1948). This, and much other work, has made it evident that chemical analogues of the amino acids which are normal constituents of animal proteins are unlikely to display sufficient selective toxicity to make them valuable chemotherapeutic drugs.

The demonstration (Work, 1951; Work & Dewey, 1952, 1953) that $\alpha\alpha'$-diaminopimelic acid was confined to the bacterial kingdom, suggested that analogues of this compound might inhibit bacterial metabolism without being toxic to animal cells. The present communication records the synthesis of a series of such analogues and their derivatives, together with tests on these compounds as inhibitors of bacterial growth. Their behaviour as competitive inhibitors for the specific diaminopimelic acid decarboxylase of bacterial cells is recorded in the following paper (Dewey, Hoare & Work, 1954).

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