CHROMATOGRAPHY OF SOME 2,4-DINITROPHENYLHYDRAZONES IN ACID SYSTEMS

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The chromatographic separation of the 2,4-dinitrophenylhydrazones of carbonyl compounds was first reported in 1935[1,2] and was largely concerned with the derivatives of aliphatic ketones and aldehydes. Later, aromatic compounds were also studied[3,4]. This early work was done with adsorption columns, and paper was first employed by CAVALLINI *et al.*[5,6] who used either neutral (butanol or butanol–ethanol–water) or alkaline (butanol–3 % ammonia in water) solvent systems. Other systems subsequently proposed have been similar; thus, *tert.*-amyl alcohol was used with ethanol and water[7,8], or propanol[9] or *sec.*-butanol[10] was included; or sodium bicarbonate solution was substituted for the ammonia[11]. These systems were all designed to separate the derivatives of compounds found in blood or urine, such as pyruvic and α-ketoglutaric acids. RICE *et al.*[12] however, were mainly concerned with aliphatic aldehydes and ketones and their neutral systems had a rather different composition, with 5 % diethyl ether, or 30 % tetrahydrofuran, in light petroleum ether as the ascending solvent.

Systems which included a considerable amount of acetic acid (60 % v/v) have been used for the separation of the higher fatty acids by countercurrent distribution[13] and for the separation of bile acids[14]. Some useful properties of acetic acid systems in the paper chromatography of steroids have been discussed by BUSH[15]. It was decided to investigate the behaviour of some 2,4-dinitrophenylhydrazones in such systems, especially because of the hint of possible advantages given by WAGER's observation[16] that under slightly acid conditions, obtained by impregnating the paper with phosphate buffer of pH 6.2, the hydrazone of α-ketoglutaric acid ran faster than that of oxaloacetic acid while in alkaline systems the opposite occurred. In the only previous report[17] of the use of acid systems with 2,4-dinitrophenylhydrazones, 10 % acetic acid in heptane was the mobile phase but the system was unstable and broke down after about two hours when a second solvent front appeared.

The distribution of the 2,4-dinitrophenylhydrazones on a paper chromatogram has been determined either by inspection of the coloured compounds, by observation of their adsorption of U.V. light[10], or rarely by spraying the chromatogram with sodium[18,19] or potassium hydroxide[10,12]. RICE *et al.*[12] mentioned that different compounds may give different colours on spraying with alkali but only TOWERS *et al.*[19] found conditions, unfortunately unspecified, that distinguished by colour between the

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two isomers, *cis* and *trans*, of a single compound which often have different R_F values. STEWART[20] had made such a colour distinction with isomeric forms that he had separated with a column while ISHERWOOD AND JONES[21] also obtained different colours when the isomers, purified by paper chromatography and elution, reacted with 0.001 *N* aqueous sodium hydroxide solution. We were able to confirm such findings and found 1.0 *N* sodium hydroxide in 80 % aqueous ethanol more convenient because the alcoholic reagent did not weaken the softer grades of filter paper.

MATERIALS AND METHODS

The 2,4-dinitrophenylhydrazones of aldehydes, ketones, and α-keto-acids were prepared by adding approximately 0.07 mmoles of the parent compound to approximately 15 ml of a freshly prepared 0.2 % (w/v) solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. The 2,4-dinitrophenylhydrazones were purified where necessary by recrystallization from aqueous ethanol, approx. 80 % w/v, and their identity confirmed by determination of their melting-points. It was occasionally found useful to separate neutral from acid hydrazones by extracting a solution of the mixture in ether with approx. 3 ml of 10 % w/v sodium carbonate solution[22]. The acidic hydrazones were extracted from the aqueous wash by adding a slight excess of 2.5 *N* hydrochloric acid and re-extracting with ether.

The compounds were usually applied to the chromatogram dissolved in ethanol[23] but when necessary, as with the derivative of acetoacetic acid, they were applied dissolved in aqueous 1 *M* ammonium hydroxide[23].

Chromatograms were run in the descending manner using Whatman No. 3 MM filter paper, with the less-polar phase of one of the following systems as the mobile phase:

(a) Alkaline: *n*-butanol–0.5 *M* ammonia in water (1:1 v/v)[5] ($BuOH/NH_3$).

(b) Acid: (i) Toluene–acetic acid–water (4:3:1 v/v) (T/A75); (ii) light petroleum (b.p. 100–120°)–toluene–acetic acid–water (10:10:17:3 v/v) (LT11/A85); (iii) decalin–acetic acid–water (10:9:1 v/v) (D/A90).

The chromatography tanks were usually in a room maintained by a thermostatically controlled heater at a temperature of 25.6° ± 0.5°[24] but neither R_F values nor rate of solvent movement were appreciably altered when the chromatograms were developed either at room temperature or in a cold room (temperature about 4°). The solvent front moved 36 cm from the line of application of the compounds (which was about 6 cm from the mobile phase in the trough) in roughly 16, 2.3, 2.3, and 8 hours for the systems $BuOH/NH_3$, T/A75, LT11/A85, and D/A90 respectively. Before development with the mobile phase, the chromatograms were either placed in the chromatography tank for 3 hours or, with the acetic acid systems, were impregnated with a solution of the stationary phase in diethyl ether (15 vol. stationary phase: 85 vol. ether), after which only 15 minutes equilibration in the tank was necessary.

For the quantitative estimation of a compound separated by chromatography, one of three methods was used. In the first the chromatogram was inspected under U.V. light (Hanovia U.V. lamp Model II) and the dark zone marked with a pencil. This zone was cut from the chromatogram and chopped into pieces which were placed in a boiling tube (15 × 2½ cm) in 10 ml of 0.1 *M* phosphate buffer at pH 7.4[23]. The tube was stoppered with a foil covered cork and shaken occasionally during a

6 hour period after which the yellow solutions were filtered through sintered glass funnels (1.5 cm diam., Gallenkamp grade 3 porosity). The extinction coefficient of the solution was measured at 390 mμ on a Unicam SP600 Spectrophotometer. The phosphate buffer was used as the blank solution. Correction was made for the value obtained from a similar extract of a zone of blank chromatogram.

In the second method, the relevant zone on the chromatogram was again cut out but the compound on it was then eluted directly with a filtered solution of 1 ml of 5 % (w/v) metaphosphoric acid in water, 60 ml ethanol, and 40 ml ethyl acetate in an elutor similar to that described by BUSH[24]. The solution obtained was made up to a standard volume with ethanol and its extinction coefficient obtained as before.

Thirdly, the chromatogram was scanned directly by U.V. light at a wavelength approximately that of the λ_{max} of the compound in an apparatus described by BUSH[24,25]. The transmitted light was recorded as an automatic trace on a Sunvic (A.E.I.) recorder with automatic integration and recording of the area enclosed by the trace[25].

When chromatograms were treated with alkali, they were dipped over the surface of a solution of 1.0 *N* sodium hydroxide in 80 % (v/v) aqueous ethanol contained in a watch-glass. The colours that appeared were to some extent transitory and so they were recorded within five minutes of dipping; an hour later they had faded considerably.

RESULTS

The R_F values of a number of 2,4-dinitrophenylhydrazones are listed in Table I. Nine of the twenty-one gave two bands in the alkaline system as had previously been described (*e.g.* for pyruvate[10,11] and for acetone[2]). With the biologically important ketoacids this phenomenon is particularly inconvenient. Thus the predominant zone of pyruvate (*i.e.* that of lower R_F) is close to the acetoacetate zone while the second zone of pyruvate (with higher R_F) is in the region of the *o*- and *p*-hydroxyphenylpyruvate zones. In the least polar of the acid systems (D/A90) some separation of the tautomers of α-ketovaleric and α-ketoisocaproic acid dinitrophenylhydrazones occurred but with over-run chromatograms this should not cause serious confusion.

In those instances in which one compound occurred as two isomers of different R_F values it was shown that each isomer was to some extent interchangeable with its partner by cutting one zone from the chromatogram, eluting the compound with ethanol–ethyl acetate (1:1, v/v) and applying the solution so obtained to a second chromatogram. On developing this in the same way as the first, the same two zones seen on the original chromatogram again appeared. Following the arguments of BRYANT[26], GORDAN *et al.*[4], ISHERWOOD AND CRUICKSHANK[9], ISHERWOOD AND JONES[21], and STEWART[20], these two zones are regarded as the *cis* and *trans* isomers of one 2,4-dinitrophenylhydrazone. The *cis* isomer moved the faster in both alkaline (where the effect may be marked) and acid (where it is slight) systems. Also, the *cis* isomer showed little colour change on dipping over the alcoholic solution of sodium hydroxide whereas the *trans* isomer of α-ketoacids turned a darker colour, often of a donkey-brown hue, while with aldehydes and ketones a redder colour was seen, which was rusty with acetone or acetaldehyde or a pillar-box red with propionaldehyde. When the isomers of a compound have virtually the same R_F value an olive colour replaced the natural lemon after dipping. The bis derivative of glyoxal gave a blue colour.

TABLE I

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONES

Parent compound	$R_F \times 100$			
	BuOH/NH₃ *trans, cis*	*T/A75* *trans, cis*	*LT11/A85* *trans, cis*	*D/A90* *trans, cis*
Pyruvic acid	50, 68	75	44	3
α-Ketobutyric acid	55, 70	81	58	9
α-Ketovaleric acid	59, 70	86	69	13, 17
α-Ketoisovaleric acid	73		70	18
α-Ketoisocaproic acid	67		74	15, 20
α-Keto-β-methylvaleric acid	67		74	21
Glyoxylic acid or glyoxal,				
mono-hydrazone	44, 61	49, 64	14, 28	
bis-hydrazone			0	
Acetaldehyde	95, 93		74, 86	
Acetone	93		75, 86	22
Propionaldehyde		94	86	
Acetoacetic acid	50			
Laevulinic acid	63		58	
Oxaloacetic acid	10	21	6	0
α-Ketoglutaric acid	5	34	9	0
Methyl ethyl ketone	92		74, 85	
Phenylacetaldehyde	93	95	88	
Phenylpyruvic acid	73, 84	88	71	11
o-Hydroxyphenylpyruvic acid } *p*-Hydroxyphenylpyruvic acid }	67, 77	46	14	0
Methyl glyoxal (pyruvic aldehyde)	88	92	70	

The key to these different colours is the use of a weak alkaline solution, as used by ISHERWOOD AND JONES[21], and this is no doubt why they have not always been noticed after treatment of a chromatogram with alkali. Sodium hydroxide dissociates less in 80 % (v/v) aqueous ethanol than in water so that a 1 *N* solution was found to be suitable; the ethanolic solution allowed rapid drying of the chromatogram after dipping. A 2 *N* solution of sodium hydroxide in 70 % (v/v) aqueous ethanol did not give these different colours nor did a 1 *N* solution of potassium hydroxide in 80 % (v/v) aqueous ethanol; instead all the dinitrophenylhydrazones gave the rusty-red colour.

In Table II are listed the ΔR_M values[27] for some pairs of compounds. The values include those for the addition of a methylene group, the change from a methyl to a carboxyl group, and the change from the *cis* to the *trans* isomer of one compound.

The 2,4-dinitrophenylhydrazones of some compounds were unstable in acid media. Thus, the derivative of acetoacetate readily underwent decarboxylation to acetone 2,4-dinitrophenylhydrazone; indeed this occurred even during its application to a chromatogram if it was dissolved in a neutral organic solvent such as ethanol or ethyl acetate[7]. The derivative of oxaloacetic acid similarly decomposed to pyruvic 2,4-dinitrophenylhydrazone in acid systems, but only partially, to about 30 %. Apart from such large changes, a small loss of compound was observed during chromatography in the acid systems. This loss was measured with the pyruvate derivative and the recovery of the compound under various conditions is shown in Table III. The amount of hydrazone applied to the chromatogram varied from

TABLE II

ΔR_M VALUES FOR SUBSTITUENTS AND TRANSFORMATIONS OF KETOACID 2,4-DINITROPHENYLHYDRAZONES

Parent compound	ΔR_M				
		BuOH/NH₃	*T/A75*	*LT11/A85*	*D/A90*
(a) ΔR_{Mg}[15] *per methylene group*					
α-Ketobutyric—pyruvic	*trans*	−0.05	−0.23	−0.24	−0.40
	cis	−0.07			
α-Ketovaleric—α-ketobutyric	*trans*	−0.08	−0.17	−0.16	−0.29
	cis	−0.08			
α-Ketoisocaproic—α-ketoisovaleric		—	—	−0.11	−0.14
α-Ketoglutaric—oxaloacetic		0.32	−0.29	−0.21	—
Laevulinic—acetoacetic		−0.23			
(b) ΔR_{Mr}[15] (*methyl — carboxyl*)					
α-Ketobutyric—oxaloacetic	*trans*	−1.03	−1.19	−1.31	—
	cis	−1.28			
α-Ketovaleric—α-ketoglutaric	*trans*	−1.43	−1.17	−1.31	—
	cis	−1.68			
Acetone—pyruvic	*trans*	−1.09	−0.59	−0.69	−0.93
	cis	−0.72			
(c) ΔR_{Mr} (*trans isomer—cis*)					
Pyruvic		0.37	—	—	0.10
α-Ketobutyric		0.26	—	—	—
α-Ketovaleric		0.27	—	—	0.05
α-Ketoisocaproic		—	—	—	0.06
Phenylpyruvic		0.27	—	—	—
o- and *p*-Hydroxyphenylpyruvic		0.20	—	—	—
Acetone		—	—	0.31	—
Acetaldehyde		—	—	0.30	—
Methyl ethyl ketone		—	—	0.29	—

TABLE III

RECOVERY OF PYRUVIC 2,4-DINITROPHENYLHYDRAZONE AFTER CHROMATOGRAPHY

	Recovery %		
	BuOH/NH₃	*T/A75*	*LT11/A85*
Equilibrated only	100	96	92
Equilibrated and developed	96	85	85

10 to 60 γ. A similar degree of loss was found with the α-ketoglutarate derivative. The loss could be reduced by chromatography at 4°; thus, after equilibration and development in the LT11/A85 system at this temperature, the recovery of the pyruvate derivative was 95%. Under all circumstances, for the range 5 to 70 γ of compound, Beer's law held good for both the T/A75 and the LT11/A85 systems within the limits ± 3%.

This is illustrated in Figs. 1–4 which show results of the determination of both the pyruvate (Figs. 1–3) and the α-ketoglutarate (Fig. 4) derivative after chromatog-

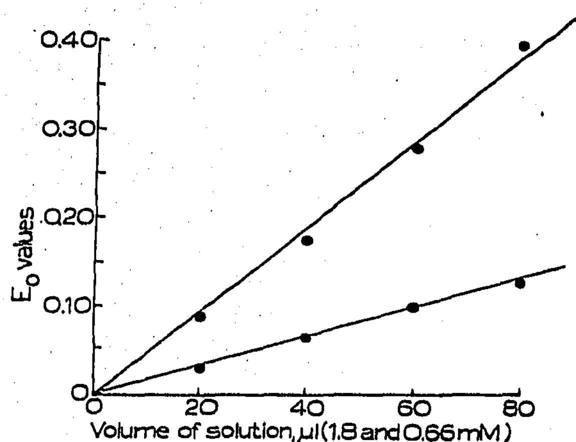

Fig. 1. Determination of pyruvic 2,4-dinitrophenylhydrazone in two solutions (ranges *ca.* 8–32 and 3–12 γ) by chromatography in LT11/A85 and elution.

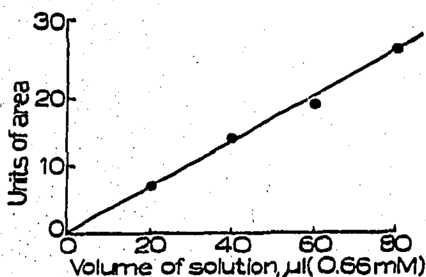

Fig. 2. Determination of pyruvic 2,4-dinitrophenylhydrazone (range *ca.* 3–12 γ) by chromatography and direct scanning.

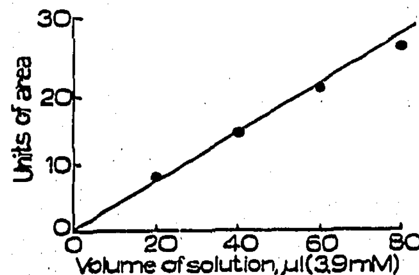

Fig. 3. Determination of pyruvic 2,4-dinitrophenylhydrazone (range *ca.* 18–72 γ) by chromatography and direct scanning.

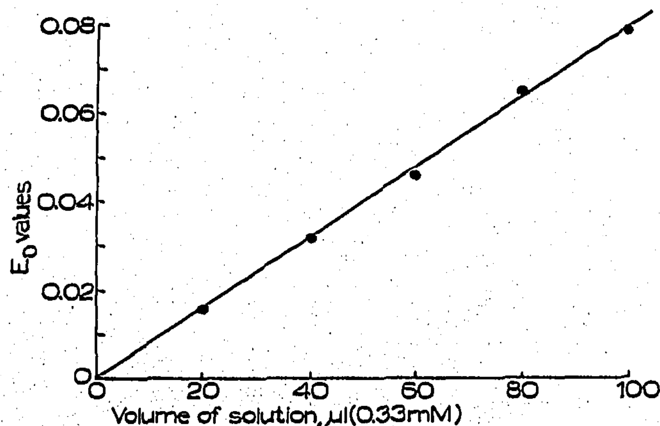

Fig. 4. Determination of α-ketoglutaric 2,4-dinitrophenylhydrazone (range *ca.* 2–10 γ) by chromatography in T/A75 and elution.

raphy. The determinations were made by elution and spectrophotometry in Figs. 1 and 4 and by mechanical scanning with automatic recordings in Figs. 2 and 3. For 21 pairs of determinations of a standard solution of pyruvic 2,4-dinitrophenylhydrazone by elution the average deviation of the readings from the mean for the pair was 2.4 % ± S.E.M. 0.5 %.

DISCUSSION

The behaviour of the 2,4-dinitrophenylhydrazones in acid systems was investigated with a view to the determination of pyruvic acid. Two obvious advantages of the acid systems over the usual neutral or alkaline systems were found: first, the solvent system ran an adequate length in two hours rather than sixteen; secondly, the *cis* and *trans* isomers of the pyruvate derivative had such similar R_F values that they formed a single band. On treating the chromatogram with the alcoholic solution of sodium hydroxide, however, it could be seen that this band had a lemon tint at its forward edge and a browner tinge behind, thus showing a slight difference in the R_F values of the two isomers.

The disadvantage of the greater loss of the derivatives during chromatography (which was 15 % as compared to 5 % in the alkaline system) was not serious since the loss was always proportional to the amount on the chromatogram (Figs. 1–4). The other disadvantage of the acid systems was a much greater loss of certain compounds by decarboxylation, for example, the two β-ketoacids, acetoacetate and oxaloacetate.

However, the acid systems have advantages with other compounds than the pyruvate derivative. Thus, in T/A75 the α-ketoglutarate derivative was quickly separated from that of oxaloacetate while in $BuOH/NH_3$ this could be achieved only by a lengthy over-run. Again, in LT11/A85 the derivatives of pyruvate, phenylpyruvate, and *o*- or *p*-hydroxyphenylpyruvate gave single bands which were clearly separated. In contrast, each of these gave two bands (*cis* and *trans* isomers) in $BuOH/NH_3$ and certain *cis* isomers were mixed with the *trans* isomers of other derivatives. Separation of α-ketovalerate from α-ketobutyrate could be achieved by over-running in the less polar D/A90 system. The values of ΔR_M[27] in Table II illustrate the superior resolving power of the acid systems, in particular of D/A90, for the α-ketoacids that were investigated.

In alliance with an alkaline system the acid systems provide a useful tool in the analysis of an unknown compound. Thus, the ΔR_M value (oxaloacetate — α-ketoglutarate) may change from + 0.29 in T/A75 to — 0.32 in $BuOH/NH_3$. Similarly, the ΔR_M (phenylpyruvate — *p*-hydroxyphenylpyruvate) changes from —0.13 and —0.20 (for *trans* and *cis* isomers respectively) in $BuOH/NH_3$ to —1.16 in LT11/A85.

Some of the differences in R_F values between the various systems are easily explained on structural grounds. In the acid systems the compounds run mainly in the unionized state and, as in other series of homologues, the higher member, α-ketoglutarate, has a higher R_F than the lower, oxaloacetate. However, in $BuOH/NH_3$ the derivative of the stronger acid (oxaloacetic) has the higher R_F, contrary to the usual expectations. As expected, however, the ΔR_M values of the hydroxyl groups of *o*- and *p*-hydroxyphenylpyruvate are much smaller in $BuOH/NH_3$ than in the acid systems[15].

Among the α-ketoacids that have been examined ΔR_M *per* methylene group appears to decrease with increasing molecular size. For any pair of compounds, also,

it is greater in D/A90 than in LT11/A85 than in T/A75. Thus, in D/A90 ΔR_M (α-ketobutyrate—pyruvate) is —0.40 but ΔR_M (α-ketoisocaproate—α-ketoisovalerate) is only —0.14; in LT11/A85 the corresponding values have fallen to —0.24 and —0.11.

In other instances ΔR_{Mr}[15] may remain the same, in accordance with theory[28]. Thus, for the substitution of a carboxyl for a methyl group (*e.g.* α-ketobutyrate to oxaloacetate and α-ketovalerate to α-ketoglutarate) the ΔR_M value is —1.18 in T/A75 and —1.31 in LT11/A85 in either case. In the alkaline system the ionization of the added carboxyl group is an important factor: in this system the ΔR_{Mr} (methyl—carboxyl) is quite variable (Table II).

ΔR_{Mr} for the change from the *cis* to the *trans* isomer is also fairly constant in any one system, especially when the parent compounds are closely related (*e.g.* α-ketobutyrate and α-ketovalerate). In the *cis* form hydrogen bonding between the imino group and a carboxyl of the parent radical will occur more readily than in the *trans*[21, 29], and so the *cis* form may be expected to have the higher R_F value. In an acid system such hydrogen bonding will occur less readily because of suppression of the ionization of the carboxyl group. That the two isomers often have closely similar R_F values in the acid systems implies that the great differences in the R_F values of the isomers seen in neutral or alkaline systems depends mainly upon differences of intra-molecular hydrogen bonding.

SUMMARY

The chromatographic properties of the 2,4-dinitrophenylhydrazones of some ketones, aldehydes, and ketoacids in three acid systems are described. The behaviour of various compounds in the three systems is compared and contrasted with that in an alkaline system. Running time is less in the acid systems and the two isomers of some compounds (*e.g.* the derivative of pyruvic acid) have almost the same R_F value. *Cis* and *trans* isomers can be distinguished on the chromatogram by dipping it through the surface of a suitable alkaline solution.

The acid systems have properties which are convenient for the quantitative determination of α-ketoacids. Quantitative estimation has been carried out successfully either by elution of the zones and absorptiometry in solution or by direct scanning of the paper strips.

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