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On the Isomerism of Hydroxyurea

VI. Paper-Partition Chromatography

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A paper-chromatographic technique for analytical separation and identification of the alleged isomers, melting at 72° C ("OU_{72}") and at 130—140° C ("OU_{140}") respectively, is described in detail. Samples of OU_{140}, prepared according to the usual ionic process (I) have been found to be contaminated with minor amounts of a closely related substance, provisionally named OU_X, the constitution of which is not yet known.

Heat-induced interconversion processes have been studied qualitatively in neutral, acid and alkaline aqueous solutions.

The irreversible isomerization $OU_{72} \rightarrow OU_{140}$, postulated by previous investigators, has been proved.

The discoverers of the lower-melting hydroxyurea (m. p. 72° C), Francesconi and Parrozzani, in their paper [1] stated that the substance, when heated in ethanolic solution, was easily converted to the previously known isomer, melting at 140° C. This was apparently inferred from the fact that an ethanolic solution, after having been boiled, produced a blue-violet colour with ferric chloride, similar in appearance to that produced by the isolated higher-melting hydroxyurea. The authors did not submit experimental documentation.

In 1907 Conduché [2], citing the Italian paper, added that in aqueous solution the lower-melting hydroxyurea was partly converted to the higher melting isomer and partly decomposed. He also reported that the inverse transformation of the higher-melting isomer into the lower-melting one had proved unsuccessful. Experimental evidence was lacking in this paper too.

The aim of the work, described in the present communication, was to supply direct proof — or disproof — that the process responsible for the vanishing of the lower-melting hydroxyurea (OU_{72}) in solution leads to the formation of the higher-melting hydroxyurea (OU_{140}). Since the paper-chromatographic technique under favourable conditions is an extremely sensitive analytical tool, it was also hoped to reveal a possible formation of other products and in general to obtain useful information on the nature of the conversion process. Such information was also desirable, because a planned kinetic investigation

by means of the polarographic method, developed in the preceding paper, would be profitable only if the process were relatively clear-cut. Finally there were still some doubts concerning the homogeneity of the isolated hydroxyurea, melting at 140° C (X-ray powder diagrams, *vide* Ref. 3; the difficulties in getting well-developed single crystals, Ref. 3 p. 486). Paper-chromatography should be well suited for settling this question.

In the search for a satisfactory spray detecting reagent 1 N ferric chloride was first tested. It is known to produce an intense blue-violet colour with OU_{140}, similar to that formed with hydroxamic acids, to which OU_{140} is probably closely related. The lower limit of sensitivity is approximately 5 μg in a spot 1 cm in diameter (Whatman No. 1 paper). However, OU_{72} gives but a faint red, quickly fading colouration in relatively high concentrations, and ferric chloride is therefore quite inadequate as a detecting reagent for this isomer.

A satisfactory universal spray reagent for both isomers is picryl chloride, originally recommended by Bremner [4] for the detection of hydroxylamine on paper chromatograms.

Upon spraying with a 1 % ethanolic solution of picryl chloride OU_{72} is revealed by a bright red colour, which gradually developes in the course of a minute or two, and which is further intensified by subsequent exposure of the dry chromatogram to ammonia vapour.

In contradistinction to OU_{72} the isomeric OU_{140} gives no colour reaction with picryl chloride alone. At high concentrations (>100 μg) the spot is discernible as a discoloured area on the yellow background. If, however, the dry chromatogram is subquently exposed to ammonia vapour, a bright orange, for higher concentrations a brown, colour is produced spontaneously. This colour is very similar to that produced by hydroxylamine salts but less dense for equal quantities of material in the spots.

The different behaviour of OU_{72} and OU_{140} toward picryl-chloride/ammonia is in itself of diagnostic value and permits safe differentiation. The lower limit of sensitivity is in either case approximately 1 μg in a spot, 1 cm in diameter. The most characteristic colours and best developed spots were obtained with 50 to 100 μg applied to the paper. The red colour produced by OU_{72} fades away in the course of some days, whereas the orange colour characteristic of OU_{140} is more stable. Presence of mineral acids in the paper inhibits the colour reaction. Care should therefore be taken to remove any trace of acid prior to spraying, when acidic solvents are being used for irrigation. With ethanol/hydrochloric acid it was sufficient to dry the chromatogram at room temperature overnight.

First of all the homogeneity of OU_{140} was investigated. The sample was prepared as previously reported [5] by the ionic process

$$HONH_3^+ + {}^-OCN \rightarrow CH_4O_2N_2 \qquad (I)$$

conducted at low temperature. The reaction mixture is known to contain $OU_{72} + OU_{140}$. The former was extracted from the freeze-dried mixture by means of anhydrous ether, then the latter was isolated by extraction with hot absolute ethanol, from which it crystallized upon cooling to room temperature. Fig. 1, No. 2 shows a typical paper-chromatogram of OU_{140}, deve-

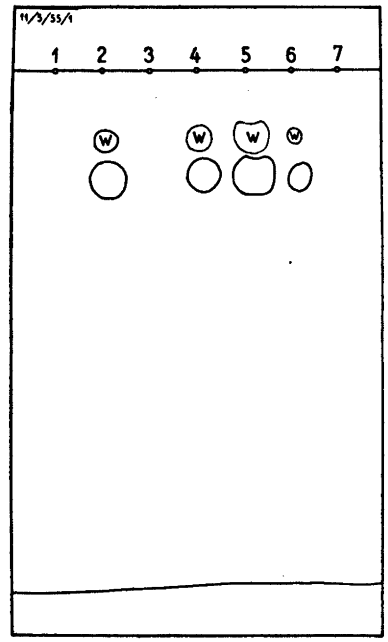


Fig. 1. Solvent isobutanol > water, 16 hours. Spray-reagent picryl chloride/ammonia. w weak, colour of all spots orange. 1. *hydroxylammonium chloride 50 µg;* 2. OU_{140} *100 µg;* 3. *nil,* 4. *freshly prepared reaction mixture from process I, see text, 10 µl;* 5. OU_{140} *100 µg, 6 months old sample;* 6. *as* 4., *5 µl solution;* 7 *as* 1.

loped with *iso*butanol, saturated with water. Two completely separated spots are formed, having the R_F-values *ca.* 0.20 and 0.14 respectively. The spots produced the same colour with picryl chloride/ammonia. The slow spot was always smaller and less densely coloured than the fast one. A duplicate chromatogram sprayed with ferric chloride revealed that only the fast spot responds to this reagent (blue-violet colour), occasionally the slow spot was just discernible as a very faint reddish colour on the dry chromatogram. This suggests that OU_{140} is responsible for the principle spot R_F 0.20 and in the following this spot will be denoted accordingly. The possible origin of the slow, subsidiary spot (for the sake of brevity denoted OU_X) will be discussed below.

In Fig. 1, No. 4 is reproduced a paper-chromatogram of a fresh reaction mixture from process (I). 5 millimoles of the reactants were separately dissolved in 25 ml of water, and the solutions mixed at room temperature. 10 µl of the solution, containing approximately 1 % hydroxyurea were applied to the paper. Two spots are developed, corresponding to OU_{140} and OU_X, whereas OU_{72} does not appear on this chromatogram. This is partly because it is formed by process (I) predominantly at low temperature, and partly because it is to a large extent converted to OU_{140} during the long period of development (16 h). More satisfactory chromatographic conditions for OU_{72} are described below.

It is obvious that the slow spot OU_X found in all our samples of OU_{140} cannot be due to a contamination with OU_{72}, since this substance responds in an entirely different manner to picryl chloride/ammonia. This conclusion was borne out by comparative paper-chromatography of isolated samples of OU_{140}

and OU_{72} on the same paper strip. These experiments showed that OU_{72} is distinctly different from either spot OU_{140} and OU_X also by flow rate (*cf.* Tables 1—3 and Fig. 2, No. 6). Furthermore, during ealier reported polarographic work[15], OU_{140}-samples have never been found to be contaminated with OU_{72}.

Since the hydroxylammonium ion and cyanate ion constitute the starting materials in the preparation of the hydroxyureas, it should be considered, whether OU_X be identical with hydroxylammonium cyanate; although this

Table 1. Paper-chromatography of hydroxyurea. Isobutanol > water, *25° C. 100 µg substance in initial spot 1 cm in diameter.*

Substance	R_F	Spray reagents		
		picryl chloride	picryl chloride /ammonia	ferric chloride
OU_X	0.14	no colour	orange	faint red
OU_{140}	0.22	no colour	orange	blue-violet
OU_{72}	0.27	weak red	red	faint red
Hydroxylamine		no colour	no colour	no colour

Table 2. Paper-chromatography of hydroxyurea. Ethanol-ammonia pH ca. 12—13; 25° C. 100 µg substance in initial spot 1 cm i diameter.

Substance	R_F	Spray reagents		
		picryl chloride	picryl chloride /ammonia	ferric chloride
OU_X	0.27	no colour	orange	faint red
OU_{140}	0.53	no colour	orange	blue-violet
OU_{72}	0.60	weak red	red	faint red
Hydroxylamine		no colour	no colour	no colour

Table 3. Paper chromatography of hydroxyurea. Ethanol-hydrochloric acid, pH ca. 0; 25° C. 100 µg substance in initial spot 1 cm in diameter.

Substance	R_F	Spray reagents		
		picryl chloride	picryl chloride /ammonia	ferric chloride
OU_{72}	0.43	weak red or yellow	red	faint red
Hydroxylamine	0.48	no colour	orange	no colour
OU_{140}	0.67	no colour	orange	blue-violet

substance has never been isolated in solid state, it might be present in samples of OU_{140} either as an impurity, due to incomplete reaction (I) or possibly existing in equilibrium with hydroxyurea in solution, analogous to the well-known equilibrium ammoniumcyanate ⇌ urea. This explanation was, however, disproved by the observation that hydroxylamine salts cannot be detected at all on paper chromatograms run with neutral and alkaline solvents in the concentrations applied in the experiments, *vide* Fig. 1, No. 1 and 7 and Fig. 2, No. 4, *cf.* Ref. 4. On chromatograms run with acidic solvents (Fig. 4, No. 3) the hydroxylammonium ion is easily recognizable, whereas the spot OU_X has vanished. This may also be taken as an indication of non-identity.

It therefore appears that the spot OU_X must be due to a new molecular species, possibly a third isomer. This conclusion cannot, however, be drawn from the paper-chromatograms without due precautions. When a supposedly pure substance produces one single spot, this is generally taken as an evidence of homogeneity, particularly when this behaviour can be replicated with different solvents or solvent mixtures. The converse is often tacitly assumed, that the production of multiple spots is an indication of inhomogeneity. During the last few years, however, it has become evident that a single pure substance may well under certain conditions give rise to more than one spot on the paper. When weak acids and bases are chromatographed with neutral solvents they will be partly ionized and the flow rate of the ionized and of the unionized form will very often differ [6-8,11]. Though this phenomenon usually gives rise to diffuse trails rather than to resolved spots, there are some reports to be found in the literature, in which the existence of multiple spots are attributed to ionization [11]. The fact that OU_{140} has been shown to be a weak acid [9] ($pK_a \approx 10.6$) merits a consideration of an explanation of the slow-moving spot OU_X along these lines. The question was settled by running some chromatograms with basic solvents. In Fig. 2 is shown a paper strip irrigated with ammonia/ethanol in which OU_{140} is present almost entirely as its anion. The observation that OU_X, recognizable by its very weak response to ferric chloride, has *not* increased in size at the expense of OU_{140} clearly indicates that the phenomenon cannot be interpreted as being due to ionization.

Another cause of "ghost spots" in paper-chromatography is particularly associated with water-rich, partially miscible solvent systems, such as for instance *iso*butanol>water. Due to either careless equilibration or to accidental drop in temperature during the irrigation the flowing solvent may contain traces of suspended water, which virtually constitutes a second phase, and the result may be the occurrence of multiple spots [10]. During the present work *iso*butanol and water were saturated one with the other by vigorous shaking, the phases were carefully separated by standing and these procedures as well as the development of the chromatograms were carried out in an air-thermostat at 25.0° ± 0.5. Water-logging of the paper should therefore be improbable. Finally the slow spot has also been found in chromatograms run with completely miscible solvents (*e. g.* Fig. 2), in which this phenomenon cannot arise.

Ghost spots are sometimes interpreted as being due to impurities in the paper (alkali salts [12], complex-forming heavy metals). Kennedy and Barker [13] have described a method of eliminating them in paper-chromatography of organic acids. The paper was washed with a 1 % aqueous solution of oxalic

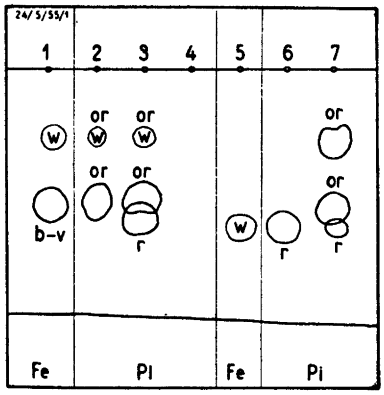


Fig. 2. Solvent ethanol-ammonia, 3½ h; spray-reagents 1 and 5 ferric chloride, 2—4 and 6—7 picryl chloride/ammonia; w weak, or orange, r red, b—v blue-violet. 1. OU_{140} 200 µg; 2. OU_{140} 100 µg; 3. OU_{140} 100 µg + OU_{72} 100 µg (mixture of isolated solids); 4. hydroxylammonium chloride 100 µg; 5. OU_{72} 200 µg; 6. OU_{72} 100 µg; 7. OU_{72}, 10 days old solution 1 %, 10 µl.

acid and subsequently rinsed with copious amounts of water and dried prior to the spotting-out. This treatment had, however, no effect on the double spot produced by OU_{140}, *vide* Fig. 3.

Finally "ghost spots" may arise when the solvent contains impurities, which can react with parts of the moving solute. Since one of the hydroxyureas (OU_{72}) readily forms carbamide-oximes with aldehydes [2], the *iso*butanol was carefully tested for a possible presence of aldehyde. The conventional test with 2,4-dinitrophenylhydrazine was negative.

With acidic flowing solvent (Table 3; Fig. 4) OU_{140} was found to give one spot only, detectable with picryl chloride/ammonia and by its blue-violet colour, when sprayed with ferric chloride. This spot seems to correspond to the principle spot in neutral and basic solvents. Although the absence of the slow-moving spot in acidic solvent may admittedly be correlated with the non-

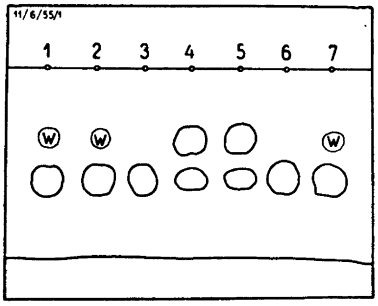


Fig. 3. Paper previously treated with oxalic acid. Solvent ethanol-ammonia, 2 hours; spray-reagent picryl chloride/ammonia; 100 µg substance in each spot. 1. and 2. OU_{140}; 3. OU^{II}_{140} (prepared according to process II); 4. and 5. Aged 1 % aqueous solution of OU_{72} (10 days) 10 µl; 6 as 3; 7 as 1.

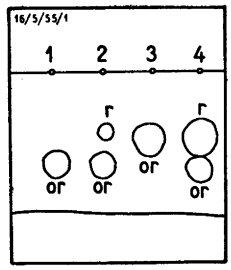


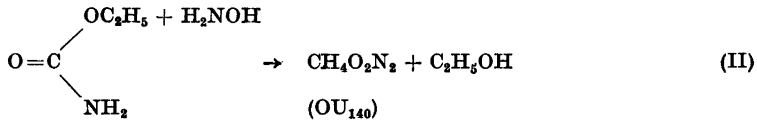
Fig. 4. Solvent ethanol-hydrochloric acid, 3 hours; spray-reagent picryl chloride/ammonia; r red, or orange. 1. OU_{140} 100 µg; 2. OU_{72} 100 µg; 3. hydroxylammonium chloride 100 µg; 4. OU_{72} 400 µg.

existence of the hydroxyurea anion at low pH values, it may as well be a result of insuffient resolution, *i. e.* the second spot is masked by the main spot.

It is seen that the order of the red OU_{72}-spot and the orange OU_{140}-spot on the chromatograms is reversed when the solvent is shifted from acidic to basic (*cf.* Tables 2 and 3 or Figs. 2 and 4). This is in accordance with the established acid-base-properties of the isomers [9], OU_{72} being a weak base, OU_{140} a weak acid. The ionic form is in either case the slower moving, in agreement with current rules [6].

A step further towards an interpretation of the slow-moving spot, OU_X, was made, when it was incidentally observed, that aqueous solutions of OU_{72}, when stored at room temperature and analyzed from time to time by paper-chromatography, at first produced the characteristic red spot (picrylchloride/ammonia), this then gradually vanished and two new spots appeared, which with respect to colour reaction and R_F-values were identical with the respective spots found for OU_{140}, Fig. 2, No. 7. It is seen that the slower moving spot OU_X is much more predominant in the aged solution of OU_{72} than in the isolated sample of OU_{140} (No. 2). It therefore appears a reasonable hypothesis that the spot OU_X is due to a new substance, possibly an isomer, which is present as a minor impurity in OU_{140} and is formed during the synthesis or during the isolation by heat-induced conversion of OU_{72}, *cf.* Fig. 6, Nos. 1—4.

In order to obtain further evidence for this general picture attention was drawn to a different route (II) for preparing the higher melting hydroxyurea OU_{140}. This has recently been reported by Runti and Deghenghi [14]. An alkaline solution of ethyl urethan and hydroxylamine was left standing for some days, then carefully neutralized and evaporated to dryness *in vacuo*. The hydroxyurea was extracted by means of absolute ethanol. In the hands of



(II)

the present author this product crystallized notably better than the product obtained by process (I) and showed a somewhat sharper melting point, suggesting a higher degree of purity. On paper-chromatograms developed with acidic (Fig. 5, Nos. 2—5) and basic solvent (Fig. 7, Nos. 2—5) one spot only was revealed. This was identical with the fast moving spot OU_{140} by R_F value and by colour reactions. Several fractions isolated from the reaction product of process (II) including the evaporated mother-liquors were analyzed without further purification, and in no one was any trace of OU_X detectable. These results corroborate the postulated explanation of the spot OU_X and further seem to indicate that in contrast to the ionic process (I) the molecular process (II) leads only to the formation of OU_{140}. Any simultaneous formation of OU_{72} would almost certainly have been revealed in the form of its conversion product OU_X. (The reaction mixture was exposed to 50° C for some hours during the evaporation.)

Additional information on the interrelationship of OU_{140}, OU_{72} and OU_X was obtained by the following experiments. A 1 % aqueous solution of freshly

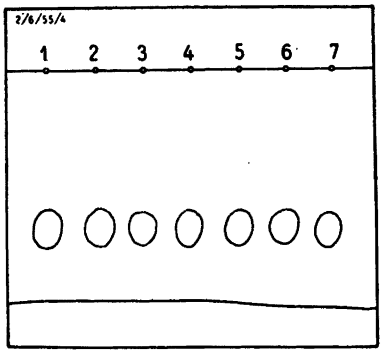


Fig. 5. Solvent ethanol-hydrochloric acid, 4 hours. Spray-reagent picryl chloride/ammonia; 100 µg in each spot. Colour orange. 1. OU_{140}; 2–5 OU^{II}_{140}, four successive fractions isolated from process II; 6. OU_{140}, 100 µg substance dissolved in 5 µl water, heated at 100° C for 10 minutes. 7. OU_{140}, 100 µg substance dissolved in 5 µl 1 N hydrochloric acid (20°), applied to paper.

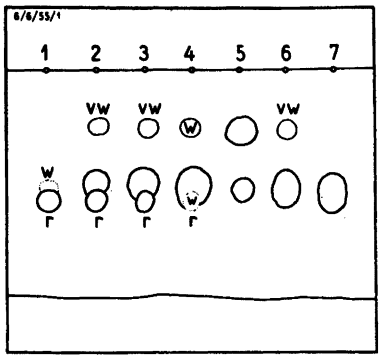


Fig. 6. Solvent ethanol-ammonia, 3 hours. Spray-reagent picryl chloride/ammonia; w weak, vw very weak; r red, other spots orange; 100 µg substance in each spot. 1. OU_{72} 1 % aqueous solution 10 µl; 2–4. the same solution heated at 100° C for 2, 5 and 10 minutes respectively; 5. OU_{72} 1 % aqueous solution aged at room temperature for 20 days; 6. OU_{140}; 7. OU^{II}_{140}.

isolated OU_{72} was divided into four parts. They were heated in a closed capillary in a steam bath for 0, 2, 5 and 10 minutes respectively. 10 µl of each batch was applied to the paper and chromatographed with a basic solvent mixture, Fig. 6, Nos 1—4. A similar experiment was made with extended heating periods, Fig. 9, Nos. 1—4. It is seen that heating has qualitatively the same effect as standing at room temperature. The characteristic red spot assigned to OU_{72} gradually disappears and OU_{140} and some OU_X are formed. The identification of the individual spots in Fig. 6 was accomplished by comparative runs of an aged solution of OU_{72} (room temperature, No. 5), of a pure sample of OU_{140} (No. 7), and of a sample of OU_{140}, containing some OU_X (No. 6). It appears that heating of the solution predominantly gives OU_{140}, whereas ageing at room temperature preferably gives OU_X (*cf.* Fig. 2, No. 7). In old

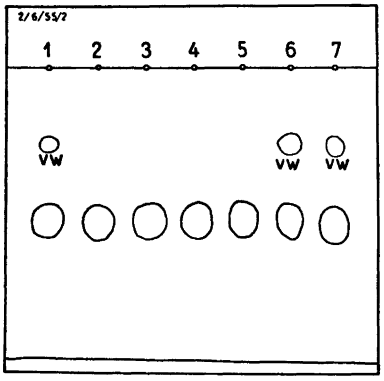


Fig. 7. Solvent ethanol-ammonia, 4 hours. Spray-reagent picryl chloride/ammonia; vw very weak. 1–7 as in Fig. 5.

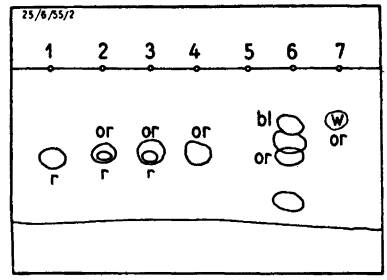
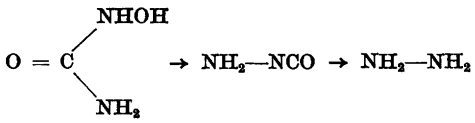


Fig. 8. Solvent ethanol-ammonia, 2 hours. Spray-reagent picryl chloride/ammonia. 1.—4. and 6. OU_{72} 1 % solution in 2 N ammonia, heated at 100° C for 0, 4, 6, 20 minutes and 20 hours respectively; 10 µl applied to paper in each case. 7. OU_{72} 1 % aqueous solution aged at room temperature for 40 days, 10 µl; r red, or orange, bl blue.

aqueous solutions of OU_{72} (40 days) only a weak OU_X spot is detectable, whereas OU_{72} and OU_{140} are completely destroyed (Fig. 8, No. 7).

At extreme pH values aqueous solutions of the hydroxyureas are very sensitive to heat. Thus in an acid solution of OU_{72} heated for one hour neither the starting material nor any conversion product was detectable (Fig. 9, Nos. 5—7). In an alkaline solution heated at 100° C for 20 minutes (Fig. 8, Nos. 1—4) all OU_{72} was converted to OU_{140} or decomposed. No OU_X was found. Further heating for 20 hours of the same solution gave rise to a rather complicated chromatogram (Fig. 8, No. 6) showing four substances, one of which was OU_{140}. The blue spot may be due to hydrazine, which is known [4] to produce a blue colour with picryl chloride/ammonia. Hydrazine may be formed by a Lossen-rearrangement of hydroxyurea followed by hydrolysis in the alkaline medium



This interesting possibility will be subjected to a closer examination.

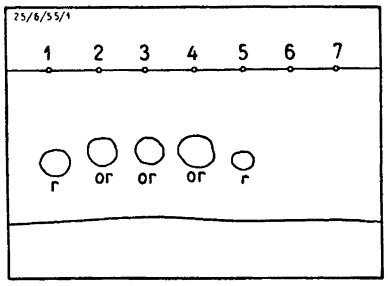


Fig. 9. Solvent ethanol-ammonia, 2 hours. Spray-reagent picryl chloride/ammonia. 1—4. OU_{72} 1 % aqueous solution heated at 100° C for 0, 30, 60 minutes and 20 hours respectively; 5. OU_{72} 1 % solution in 1 N hydrochloric acid heated at 100° C for 0 and 60 minutes and 20 hours respectively; 10 µl applied to paper.

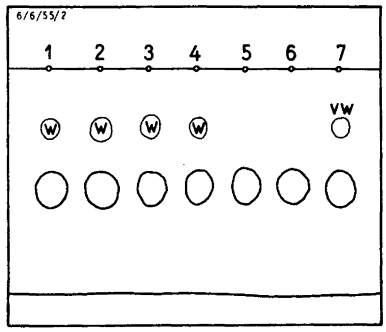


Fig. 10. Solvent ethanol-ammonia, 3 hours. Spray-reagent picryl chloride/ammonia. 1—4. OU_{140} 1 % aqueous solution heated at 100° C for 0, 2, 13 and 30 minutes, respectively; 5—7. OU^{II}_{140} 1 % aqueous solution heated at 100° C for 0, 10 and 30 minutes, respectively. 10 µl applied to paper.

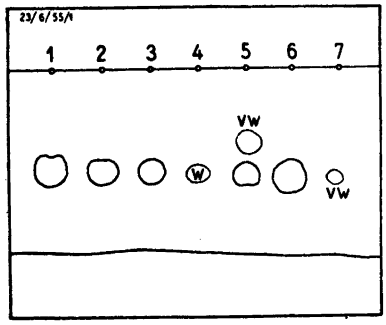


Fig. 11. Solvent ethanol-ammonia, 2½ hour. Spray-reagent picryl chloride/ammonia. 1—4. OU^{II}_{140} 1 % aqueous solution heated at 100° C for 0 and 30 minutes, 3 and 20 hours, respectively; 5—7. OU^{II}_{140} 1 % solution in 1 N hydrochloric acid, heated at 100° C for 30 minutes, 0 minutes and 3 hours, respectively; 10 µl applied.

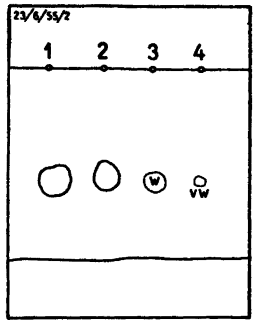
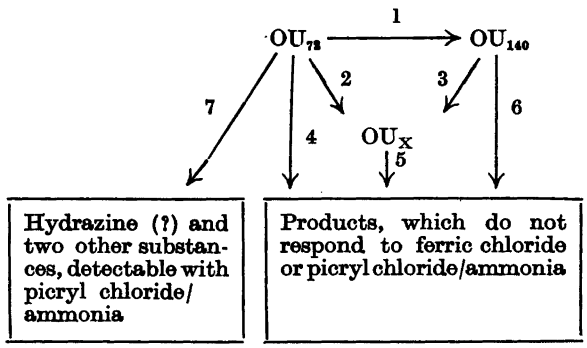


Fig. 12. Solvent ethanol-ammonia, 2½ hour. Spray-reagent picryl chloride/ammonia. 1—4. OU^{II}_{140}, 1 % solution in 2 N ammonia, heated at 100° C for 0 and 30 minutes, 3 and 20 hours respectively; 10 µl solution applied to paper.

The postulated[2] irreversibility of the conversion $OU_{72} \rightarrow OU_{140}$ was tested by a similar series of experiments in which OU_{140} was heated in neutral, acid and alkaline solution. A 1 % aqueous solution of pure OU_{140} was heated at 100°. After 20 hours heating the OU_{140}-spot was still discernible; neither OU_X nor any other conversion product was found (Fig. 11, Nos. 1—4). This experiment was repeated with a OU_{140}-sample containing OU_X (Fig. 10). The relative densities of the two spots are seen to remain unaffected by the heating of the solution and no novel spot is formed. A similar experiment with an acid solution of pure OU_{140} indicated that some OU_X was formed after 30 min. After a total of 3 h heating the OU_X spot had vanished and only traces of OU_{140} were still detectable (Fig. 11, Nos. 5—7). When an alkaline solution of pure OU_{140} was heated under similar conditions (Fig. 12, Nos. 1—4) the OU_{140} spot disappeared almost equally fast as in acid solution, but neither OU_X nor any other compound were found on the chromatogram.

The above results may be summarized in the following diagram



1 and 2 proceed simultaneously in neutral aqueous solution, 1 seems to predominate at 100° C; 2 at room temperature. In alkaline solution at 100° 1 proceeds rapidly, 2 and 3 do not seem to occur.

3 has been noticed only in acid solution heated to 100°.

4 takes place rapidly in acid solution at 100°, probably *via* 2—5, 1—3—5 or 1—6.

5 and 6 proceed slowly at 100° in neutral, rapidly in acid and alkaline solution.

7 occurs on prolonged heating of an alkaline aqueous solution.

There are no indications that any of the above processes can proceed in the opposite direction and especially no signs of tautomerism. It should, however, be remembered that paper-chromatography is a slow method of analysis. Rapid processes may therefore have escaped notice.

Ethanol-ammonia was preferred as the irrigation solvent-system, because it gives good resolution of the three spots with only 2—4 hours development. Addition of ammonia was indispensable, irrigation with ethanol-water of the same composition showed very poor separation of spots. In the alkaline solvent some OU_{72} will be converted during the development period, (*cf.* Ref. 15), this should be remembered when making quantitative estimates from the chromatograms. The neutral solvent system *iso*butanol-water showed no advantage in this respect, because a considerably longer development period was required (14—16 hours) in order to get good separations with this slower moving liquid, and more OU_{72} appeared to be converted on these conditions than during a short-time development with ethanol-ammonia. The application of ethanol-hydrochloric acid, in which OU_{72} is known to be perfectly stable [15] is limited to analyses of OU_{72}—OU_{140} mixtures, because OU_X is not separated from OU_{140} with this solvent mixture.

Attemps to isolate the substance responsible for the spot OU_X by preparative paper-partition chromatography are in progress.

MATERIALS, APPARATUS AND GENERAL TECHNIQUE

The isomeric hydroxyureas denoted OU_{72} and OU_{140}, respectively, without a roman numeral, were prepared according to the ionic process (I). Details have been published earlier [5]. The samples of the higher-melting isomer denoted OU^{II}_{140} were prepared strictly according to the directions given in the paper by Runti and Deghenghi [14] (process II). The melting interval of our sample was 135—140° (decomp.); (lit.[14] 139—141° C).

The samples were stored at 0—5° C under dry conditions. The lower melting isomer OU_{72} was used for the experiments within 2 weeks from the preparation of the sample. Unless otherwise stated freshly prepared solutions were employed.

*Solvents. Iso*butanol-water. Commercial, pure *iso*butanol was thoroughly shaken with an equal amount of water at 25° C and the phases were carefully separated. The chromatograms were equilibrated in an atmosphere, provided by the water-rich lower phase and developed with the upper phase, *iso*butanol > water.

Ethanol-ammonia was a mixture of 20 ml of *ca.* 10 N ammonia solution and 80 ml of 96 % ethanol.

Ethanol-hydrochloric acid was a mixture of 30 ml of 4 N hydrochloric acid and 70 ml of 96 % ethanol.

Spray Reagents. The ferric chloride solution was a 1 N aqueous solution of an analytical grade product.

Picryl chloride was prepared from picric acid and phosphorus pentachloride according to Jackson and Gazzolo [16]. It was employed in the form of a 1 % (w/v) solution in ethanol.

Picryl chloride/ammonia. The developed chromatogram was sprayed with the picryl chloride solution, mentioned above, allowed to dry in the air and subsequently exposed to the vapours of 10 N ammonia solution.

Paper Chromatographic Procedure. Whatman paper No. 1 was used throughout, without any previous treatment, unless otherwise stated. The sheets were normally 22 by 28 cm, except for chromatograms, developed with *iso*butanol-water, which required a length of 55 cm in the travelling direction. The chromatograms were run by the descending technique and the substances were applied to the paper, normally in the form of a 1 % solution, as circular spots along a marked starting line as usual. In most cases 10 μl solution containing 100 μg substance were used.

The development of the chromatograms was carried out in conventional all-glas chambers maintained at 25.0° ± 0.5 in an air-thermostat. With the *iso*butanol-water system as a developer the paper was equilibrated with the lower-phase atmosphere for 18 hours. With the ethanolic mixtures 2—3 hours sufficed, and the same mixture was used for equilibration and development.

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