CCLXV. REACTIONS OF PYRUVIC ACID WITH THIOLACETIC ACID AND CYSTEINE.

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Baumann [1885, 2] observed that the action of ethyl-, phenyl- and *p*-bromophenyl-mercaptans on pyruvic acid led to the exothermic formation of substances which could be recrystallized from benzene and possessed well-defined melting-points. Though stable in the solid state and in benzene solution, these compounds were rapidly broken down to their original constituents when dissolved in water, and were regarded by Baumann as having the general structure $RS.C(OH)(CH_3).COOH$. When treated with dry HCl the thiophenylpyruvic acid passes into the mercaptol $(RS)_2C(CH_3).COOH$. The mercaptals and mercaptols [Baumann, 1885, 1, 2] have been extensively studied both by Baumann and his school and by other investigators, but the unstable "hemimercaptals" and "hemimercaptols" were little investigated until the recent work of Schönberg & Schütz [1927], Levi [1932] and Schubert [1935; 1936] appeared. Their work is of interest both from the point of view of the chemistry of the formation of mercaptals [Fromm, 1889; Levi, 1932] on which the present paper and recent work [Giršavičius & Heyfetz, 1935; 1936, 1] throw some light, and also from a biological aspect. Lohmann's [1932] well-known discovery that the transformation of methylglyoxal into lactic acid by glyoxalase requires the presence of reduced glutathione as a necessary and specific co-enzyme, together with the observations of Kühnau [1931], of Lohmann [1932] and of Jowett & Quastel [1933], that glutathione reacts in aqueous solution with methylglyoxal to form a fairly unstable compound, have led to the hypothesis [Jowett & Quastel, 1933] that a hemimercaptal-like compound of methylglyoxal with glutathione forms a necessary intermediate stage in the enzymic reaction. Further evidence for this view has been advanced by Platt & Schroeder [1934] and by Giršavičius & Heyfetz [1936, 2]. Possibly the biological significance of reactions of this type may extend beyond their participation in glyoxalase action [see Kühnau, 1931; Bersin, 1935].

In the following experiments the compounds formed between pyruvic acid on the one hand and thiolacetic acid [Baumann, 1885, 2; Bongartz, 1886] or cysteine on the other have been investigated more closely, the process of their formation and its reversal being studied.

Reversible combination of pyruvic with thiolacetic acid.

Thiolacetic acid (2·12 g.) and pyruvic acid (2·03 g.) in substance were mixed; the mixture became hot, then set to a mass of white crystals mixed with a good deal of viscous liquid. After cooling, the product was stirred up with ether and filtered through a sintered glass funnel. Yield: 2·63 g. (64 %) of crystalline substance. The ether washings (20 ml.) gave on evaporation a small additional crop. Ice cooling during the reaction did not appreciably alter the yield.

[1] The work described in this paper was carried out in 1933, while both authors were working at the Cambridge Laboratory.

The following investigation of the crystalline compound shows that it is made up of the two reactants in equimolecular amounts and that it is exceedingly labile in aqueous solution.

Titration of —SH group. 61 mg. of the compound were dissolved in 5 ml. of ice-cold water and immediately titrated with 0·0996 *N* iodine to the first stable yellow tint. Iodine uptake was as rapid as speed of titration permitted. 3·50 ml. were required. Calculated for complete oxidation of the thiol group: 3·40 ml. [cf. Lucas & King, 1932].

Titration of —CO group. 59 mg. titrated according to Cook & Clift [1932]. Found: 6·58 ml. iodine. Calculated: 6·58 ml.

Isolation of components. (1) 5·647 g. of the addition product were dissolved in 30 ml. of water and 5 g. of $NaHSO_3$ added. The solution was repeatedly extracted with ether and the combined ethereal extracts dried over Na_2SO_4; evaporation of the ether left 2·95 g. of oily substance (102 % of original thiolacetic acid). Vacuum distillation (102–112°/10–16 mm.) gave 2·04 g. (70 %) of thiolacetic acid, identified iodimetrically and acidimetrically. (2) 0·4975 g. of the compound in 10 ml. of water was treated with 50 ml. of 2*N* HCl, containing 0·8 g. of 2 : 4-dinitrophenylhydrazine. The precipitate was twice purified by dissolving in *M* Na_2CO_3 and reprecipitating by acidification. Yield: 0·752 g., corresponding to 94·1 % of the theoretical amount of pyruvic acid dinitrophenylhydrazone. In another experiment 91·6 % was obtained.

Considering the losses involved in the isolation methods, these results, together with the above described titrations, demonstrate the easy dissociation in aqueous solution of the addition compound. No conclusion can however be drawn from these experiments as to whether the compound is actually incapable of existing in aqueous solution, or whether it attains an equilibrium, dissociating more or less according to the dilution and the extent to which one or other component is removed.

Reaction of pyruvic acid with cysteine.

Titration methods were incapable of telling us anything about the state of the pyruvic-thiolacetic compound in aqueous solution, or even whether any reaction takes place, when the components are mixed in solution. The lower reactivity of the products obtained by the interaction of pyruvic acid with cysteine permits a more profitable application of iodine titration. We attempted also to obtain a clearer picture by a parallel study of the changes in rotatory power undergone by natural *l*-cysteine in presence of pyruvic acid.

The readings were taken with Hg green ($\lambda = 5461$ Å.) in a 2 dm. tube. The times were measured with a stopwatch and alternate readings were taken approaching from the right and from the left.

Fig. 1 shows the course of change of rotation of 0·2 *M* cysteine in aqueous solution in presence of 0·2, 0·4 and 0·6 *M* pyruvic acid. The solutions were always kept long enough for the rotation to reach a final value (several days). These end-values were: for 0·2 *M* pyruvic acid −5·32°, for 0·4 *M* −7·2°, and for 0·6 *M* −7·44°. The experiments were carried out at 26–27°.

Fig. 2 shows a number of curves obtained with cysteine and pyruvic acid in alcoholic solution (in view of the possible dissociating effect of water), at 37° ±0·1° in a jacketed polarimeter tube. The cysteine concentration was again 0·2 *M* and those of pyruvic acid 0·2 and 0·4 *M*. The end-values, after several days in a thermostat at 37°, were: 0·2 *M* pyruvic acid −7·52°, 0·4 *M* −9·56°. An inspection of both sets of curves shows that they have their origin at some point corresponding to a weak negative rotation, which, as far as can be judged, is

the more marked, the higher the pyruvic acid concentration. Since cysteine itself is weakly dextrórotatory (arrow in Fig. 1 shows the rotation of 0·2 *M* cysteine alone), we are clearly dealing with two successive effects: a practically instantaneous shift of rotation (of about 1° with our concentrations) with reversal of sign, succeeded by the gradual development of a strong laevorotation.

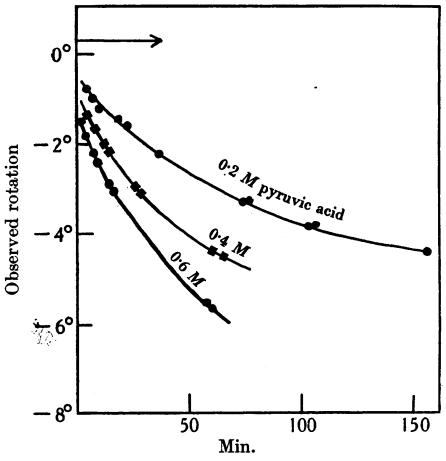

Fig. 1.

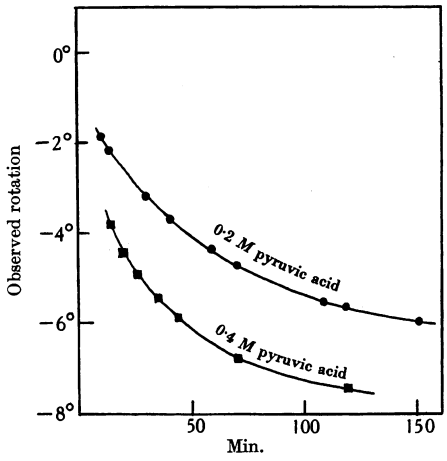

Fig. 2.

In view of the complexity of these reactions, of the initial shift of rotation which seems to imply a rapid first stage[1] and the variable end-point which seems to indicate the attainment of an equilibrium, it is not surprising that the reaction courses observed fail to fit the ordinary equations. With equimolecular concentrations of pyruvic acid and cysteine the bimolecular reaction constants increase with time; the unimolecular constant is reasonably uniform in the experiment in water, but rapidly falls in the alcoholic solution. In all the other experiments the bimolecular constant falls with time, the unimolecular constant of course even more so.

Some colour reactions.

An attempt to obtain some of the typical —SH reactions in presence of pyruvic acid provided additional evidence of a combination involving the sulphydryl group of the cysteine.

$FeCl_3$. Added to an alkaline solution of cysteine, $FeCl_3$ gives a purple colour, which fades on standing, but reappears on admitting oxygen [Harris, 1922; Michaelis & Barron, 1929]. To an alkaline (ammonia) mixture of 1 ml. *M*/4 cysteine + 2 ml. *M*/4 Na pyruvate was added 0·1 ml. *M*/100 $FeCl_3$. The purple colour faded more rapidly than in absence of pyruvic acid and reappeared less strongly on shaking; after repeating several times the cycle of reduction and re-oxidation the colour fails to reappear again. In absence of pyruvic acid the process can be repeated almost indefinitely. If the cysteine and pyruvic acid are allowed to stand a short while before adding $FeCl_3$ the colour reaction is faint and rapidly vanishes irreversibly.

[1] Unpublished observations by one of us with P. A. Heyfetz have demonstrated a similar phenomenon in the reaction of GSH with methylglyoxal (iodimetric titration).

Nitroprusside. In ammoniacal solution cysteine gives immediately the well-known purple colour; this slowly fades to a brownish yellow. Pyruvic acid, under the same conditions, gives a slowly developing blue colour. On adding nitroprusside to an ammoniacal solution of cysteine and pyruvic acid (in excess) a transient purple colour is observed, followed by the development of the blue pyruvic acid colour. Between the two a colourless interval is sometimes noticed, showing that the colour due to cysteine has actually vanished and is not merely covered by the deeper colour due to excess pyruvic acid.

Methylene blue reduction. The reduction of methylene blue by cysteine in alkaline solution is inhibited by pyruvic acid, though not very strongly (Thunberg tube).

Titration experiments.

Absolute alcoholic solutions of cysteine hydrochloride (0·19 *M*) and of pyruvic acid ($2\frac{1}{2} \times 0{\cdot}19 = 0{\cdot}475$ *M*), or mixtures of the two solutions were added by means of 0·5 or 1 ml. Ostwald pipettes to ice-cold 1–2 *N* HCl containing 1 ml. of 0·1014 *N* iodine and one drop of starch solution. The excess iodine was titrated with 0·1001 *N* $Na_2S_2O_3$ from a microburette allowing an accuracy of 0·001 ml.

(1) 3 ml. of 1·3 *N* HCl + 0·5 ml. cysteine solution + 0·2 ml. pyruvic acid solution were mixed ice-cold. The mixture was kept in ice for various periods before adding iodine and (as rapidly as possible) titrating. As in all further experiments, the iodine consumption is given in terms of 0·1 *N* iodine. Cysteine alone (no pyruvic acid): 0·958, 0·955 ml.; with pyruvic acid: after 2 min. 0·907 ml., after 10 min. 0·881 ml., after 15 min. 0·789 ml.

(2) 15 ml. of the cysteine solution + 6 ml. of the pyruvic acid solution (that is, an absolute alcoholic solution, 0·136 *M* in each of these substances) were kept for 4 days. 0·5 ml. samples were added to a mixture of 1 ml. 2 *N* HCl + 1 ml.

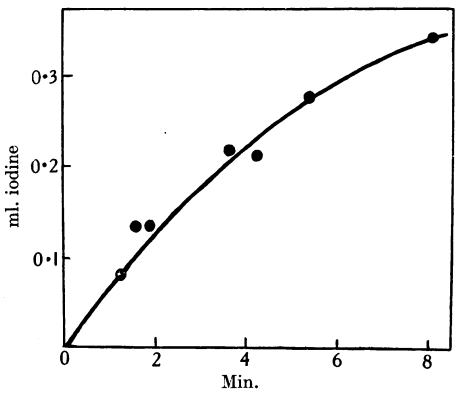

Fig. 3.

0·1 *N* iodine, cooled in a freezing mixture to −5° to −6°; on adding the alcoholic solution the temperature rose to about 0°. The titration vessel containing the mixture (a short wide tube with a tapered bottom) was transferred to a beaker containing ice in dilute brine, so that its temperature up to and during the titration was kept at −2° to 0°. At the moment of adding the cysteine-pyruvic acid mixture to the iodine a stopwatch was started, which was stopped at the

end of the titration. The titration was started after the iodine had been acting for various periods. Fig. 3 shows the course of iodine uptake up to 8 min. The contrast with the experiments with thiolacetic acid, where the whole of the iodine corresponding to the —SH introduced is immediately consumed, is striking.

It is not clear from these results whether the gradual uptake of iodine is entirely due to its action on the compound, or whether a certain amount of dissociation takes place spontaneously when the alcoholic mixture is added to water + HCl. The following experiment was designed to answer this question. 0·5 ml. of the alcoholic mixture of pyruvic acid and cysteine was added to 1 ml. HCl + 1 ml. iodine, as above, or else only to HCl, the iodine being added some time later. The total times up to the end of the titration, were kept as nearly as possible alike.

	Iodine uptake ml.
Iodine at once. Titration finished in 2 min. 12 sec.	0·082
Iodine at once. Titration finished in 8 min. 14 sec.	0·213
Iodine added after 6½ min. Total time 8 min. 15 sec.	0·097

In the following experiment the alcoholic solution was added to 4 ml. 2 *N* HCl + 3 ml. of water; iodine present or added later.

	Iodine uptake ml.
Iodine at once. Titration finished after 7 min.	0·476
Iodine after 6½ min. Total time 7 min. 4 sec.	0·173

Little, if any, sulphydryl is free in the aqueous HCl solution in absence of iodine. This accords well with the polarimetric observations, which showed that the reaction follows much the same course in water and in alcohol. (It must be noted, that on adding the alcohol mixture to the dilute HCl not only does a change in the nature of the medium take place, but also a considerable change in volume). Judging from the second series of figures higher dilution increases the effect of the iodine and perhaps has itself a certain dissociating effect.

Conclusions.

Giršavičius & Heyfetz [1935] have recently studied in some detail the reaction between glutathione and methylglyoxal in aqueous solution. Two main conclusions were reached: (*a*) a true equilibrium is established between the free components and the reaction product; (*b*) the reaction rates in both directions depend on the *p*H, being slow in strongly acid solution and extremely rapid as the solution approaches neutrality (compare Giršavičius & Heyfetz [1936, 1] where this observation is elaborated). The results described in the present paper fit in with the view that here too reactions of the same kind as that between glutathione and methylglyoxal may take place, and that such differences as are found, for instance between cysteine and thiolacetic acid, are expressions of different reaction rates and equilibrium constants. Baumann [1885, 1, 2], in investigating the products of spontaneous reaction between pyruvic acid and various mercaptans, already mentioned the differences in their properties, including their stability. Great differences in the reactivity (reaction rates) of the —SH group, in accordance with the structure of the molecule of which it forms a part, are also mentioned by Michaelis & Schubert [1934]. It must be admitted, however, that the great difference in behaviour shown by the compounds of pyruvic acid with thiolacetic acid on the one hand and with cysteine on the other suggests the possibility of a different type of bond. Schubert [1935] has shown that with

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