HISTAMINE AND ITS DERIVATIVES IN HUMAN URINE

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Extensive investigations by various workers indicate that histamine is excreted in human urine in a free and a conjugated form (ANREP *et al.*[1], ADAM[2]). Using the cationic exchanger Decalso ROBERTS AND ADAM[3] developed a method for the separate determination of free and conjugated histamine in the urine and found with this technique that on average 21.6 μg of free histamine and 125 μg of conjugated histamine were excreted daily in human urine. ROCKENSCHAUB[4] showed by the same method that the excretion of free histamine by pregnant women was of the same order as that by a male. Considering the large amounts of histaminase known to occur in the blood of pregnant women this finding is surprising. Moreover, in a recent publication[5] evidence was provided for the existence of minute amounts of histaminase in human urine, a finding which makes the presence of free histamine in urine difficult to understand. A re-investigation of the problem of histamine excretion in human urine therefore appeared desirable. For this purpose it was decided to combine the technique of ROBERTS AND ADAM[3] with a paper chromatographic one, in the hope that more information about the nature of the excreted histamine would be gained from a resolution on paper of the urinary material adsorbed on Decalso columns. It will be shown that in this procedure a material from urine is adsorbed on Decalso which, besides histidine, free histamine and acetylhistamine, has been found to contain two other iminazole compounds, not hitherto identified in urine. In paper chromatographic investigations these two substances have been identified as N-methylhistamine and N-dimethylhistamine. The significance of the occurrence in human urine of these histamine derivatives, methylated on the side chain amino groups will be discussed.

MATERIALS AND METHODS

Collection of urines

24 hours' specimens of male human urine were carefully collected in chemically clean flasks containing 5 ml of a 2 *N* hydrochloric acid.

Chromatography of urines for the detection of free histamine

The technique of ROBERTS AND ADAM[3] was essentially followed. 50 ml of urine, adjusted to pH 8, were percolated through Decalso, Grade F, columns (6 × 1 cm) in one hour. After that time the column was washed, five times with small amounts of 0.9% NaCl and then with 15 ml of absolute alcohol to remove most of the moisture. Elution was effected by adding to the column 3 ml of ammonium hydroxide (AR, sp. gr. 0.880) followed by 50 ml of AR chloroform which had previously been saturated with dry ammonia gas. The eluate obtained was evaporated *in vacuo* at 40°. The slight residue was treated with acidified alcohol to neutralize traces of alkali and the solution evaporated under reduced pressure at a low temperature. Aliquot amounts of the residue were assayed biologically for histamine.

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Investigation of urines by a combined technique of chromatography on Decalso and paper chromatography for the detection of free and conjugated histamine

Since the total amount of histamine-like activity extractable on Decalso from 50 ml of urine was too small for a paper chromatographic analysis larger quantities of urine (750 to 1000 ml) were put through larger Decalso columns (16 × 3.5 cm), a process which required 15 hours. To diminish the possibility of a partial decomposition of histamine during this long time at a high pH (8.0), and to facilitate also the adsorption of acetylhistamine, which may account for at least part of the urinary conjugated histamine, the pH of the urine was adjusted to 5.5. ROBERTS AND ADAM[3] showed that at that pH maximum adsorption on Decalso of both histamine and acetylhistamine could be achieved. After the completed percolation of the urine the columns were treated in the same way as described above. The dry residue from the Decalso eluate was taken up in 1 ml of distilled water, the solution was centrifuged, and 0.1 or 0.2 ml of the supernatant, in aliquots of 10 μl, were applied to the paper. These aliquots were applied on top of the initial small spot and after each application the spot was dried with the warm air blast from a hair dryer. The method used was an adaptation of that described by AMES AND MITCHELL[6] for the paper chromatography of iminazole compounds. A mixture of *n*-propyl alcohol and 0.2 *N* NH_4OH (3:1, v/v) was used as a solvent and the developed chromatograms were sprayed with a solution of diazotized sulphanilic acid. Whatman No. 4 paper, descending chromatography, room temperature, and a developing time of 4 hours were further essential features of the adapted technique.

Estimation of histaminase activity

The slightly modified microvolumetric technique (KAPELLER-ADLER[7]) was used. The histaminase activity is measured by an indirect determination of the H_2O_2 formed in the histaminase–histamine reaction, and is expressed in permanganate units (P.U.), one P.U. representing the amount of enzyme which under standard conditions produces H_2O_2, equivalent to 0.1 ml of 0.002 *N* $KMnO_4$. One P.U. corresponds to the destruction of 0.46 μg of histamine/60'/37°.

Estimation of histamine activity by bioassay in Decalso eluates and elutions from paper chromatograms

The solutions were assayed on the isolated guinea pig ileum against the respective control solutions.

Measurement of the effect of preparations of hog kidney histaminase on the histamine activity contained in material extracted on Decalso from urines, or from histamine solutions in 0.9% NaCl.

To 3 ml of a Decalso eluate, the histamine content of which has previously been assayed biologically, 1 ml of a solution of a purified pigs' kidney preparation in phosphate buffer pH 7.2, and 1 ml of Ringer solution are added and the mixture is incubated for 30 min at 37°. After that time 1 ml of Ringer solution is rapidly added, and the fluid is heated to 80°. The histamine content of the cool solution is assayed on the isolated guinea pig ileum.

RESULTS AND DISCUSSION

Chromatography of urines on Decalso

In a series of seven independent experiments urines, subjected to the original Decalso method of ROBERTS AND ADAM[3], showed in bioassays a mean histamine-like activity corresponding to a histamine content of 0.52 μg per 50 ml of urine (range 0.35 to 0.75 μg/50 ml). This histamine-like activity was inhibited by 0.1 ml of a very dilute solution of mepyramine maleate ($2 \cdot 10^{-8}$).

A series of five recovery experiments, in which histamine was added to the urine before extraction at the rate of 1 to 2 μg/50 ml, gave a mean recovery of 66.4% (range 50 to 75%). These results compare well with those reported by ROBERTS AND ADAM[3] and MITCHELL[8].

Table I shows that solutions of a partly purified histaminase preparation from hog kidney, when acting on the material extracted on Decalso from urines, failed to abolish completely the histamine activity in all seven urinary extracts investigated, 29% of the histamine activity remaining on average unaffected by the enzyme. ROBERTS AND ADAM[3] found in their experiments that about 3 to 4 times as much histaminase was required to break down the histamine-like compound from urine in a given time as for an equal amount of authentic histamine. Since in their experiments

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TABLE I

EFFECT OF A PURIFIED PREPARATION OF KIDNEY HISTAMINASE ON DECALSO EXTRACTS WITH HISTAMINE-LIKE ACTIVITY FROM HUMAN URINES (U_D) AND ON DECALSO EXTRACTS FROM SOLUTIONS OF AUTHENTIC HISTAMINE IN SALINE (H_D).

Decalso extracts from urine	*ng of histamine-like activity extracted on Decalso from 50 ml of urine*	*Residual histamine-like activity after incubation with histaminase* %	*Decalso extracts from pure histamine solutions (2 µg of histamine in 50 ml of 0.9% NaCl*	*µg of histamine extracted on Decalso from histamine solutions*	*Residual histamine activity after incubation with histaminase* %
U_{D1}	0.41	30.0	H_{D1}	0.70	0.5
U_{D2}	0.75	27.3	H_{D2}	0.85	0
U_{D3}	0.54	30.4	H_{D3}	1.20	0
U_{D4}	0.50	25.0	H_{D4}	0.92	0.3
U_{D5}	0.35	34.5	H_{D5}	1.00	0
U_{D6}	0.48	28.6	H_{D6}	0.75	0
U_{D7}	0.65	27.7	H_{D7}	1.15	0
	Mean: 0.52	Mean: 29.0%			

they found that authentic histamine which had been put through the Decalso procedure, was also decomposed less rapidly than histamine in pure solution, they suggested that some enzyme-inhibiting substance might be extracted from Decalso columns into the final extract. This finding could not be confirmed in the present paper, for it can be seen from Table I that in all seven control experiments, in which authentic histamine had been put through the Decalso procedure, the eluted histamine was, unlike the urinary histamine-like substance, completely destroyed by histaminase. This suggested, that the material from urine adsorbed on Decalso may possibly contain another compound whose pharmacological activity must be very similar to that of histamine, but which is not inactivated by histaminase.

Paper chromatography of material adsorbed from urines on Decalso

(a) *Identification of three iminazole derivatives: histidine, histamine and acetylhistamine**. Representative paper chromatographic results of many investigations obtained on Decalso columns are shown in Fig. 1. Five spots of iminazole derivatives were obtained from urinary Decalso extract (A). The spot with the smallest R_F (0.15) was verified as histidine in other paper chromatograms by comparison with spots of authentic histidine solutions. In seven different runs a mean R_F of 0.15 was obtained with the present technique for pure histidine preparations.

In Fig. 1 it can be further seen that the second spot (R_F 0.54) fully matches the spot of authentic histamine (D), and that the fourth one (R_F 0.79) matches that of acetylhistamine. In 14 independent paper chromatographic experiments mean R_F values of 0.53 and 0.79 were obtained for histamine and acetylhistamine respectively. The identification of the third spot (R_F 0.62) and that of the last one (R_F 0.85) will be discussed later. To confirm further the identity of the histamine spot in A, 90 µg of authentic histamine acid phosphate were added to the Decalso extract used in A, and the mixture was submitted to paper chromatography (B). The authentic histamine spot was detected in the same position in B as the presumed histamine in A and the pure authentic histamine in D. This seems to prove the presence of free histamine in Decalso extracts.

* A generous gift of acetylhistamine by Dr. H. TABOR is gratefully acknowledged.

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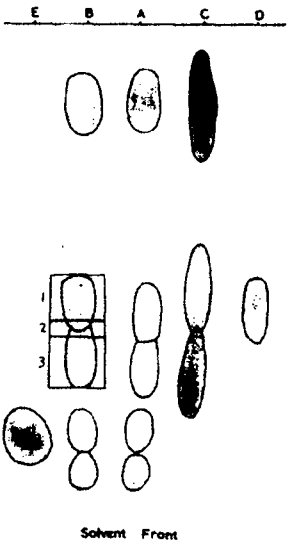

Fig. 1.

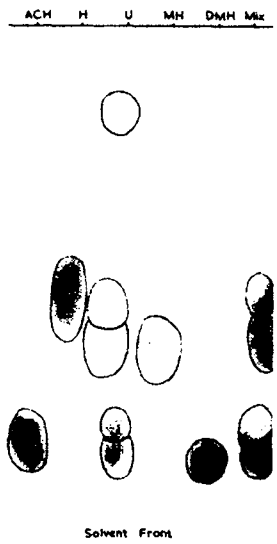

Fig. 2.

Fig. 1. A: Paper chromatogram of urinary material adsorbed on Decalso. B: To the same Decalso extract 90 μg of histamine acid phosphate were added before paper chromatography. The areas 1, 2, 3, were separately eluted in another paper chromatogram and investigated for pharmacological activity. C: Chromatogram of alcoholic washings (usually discarded) of Decalso columns on which urine had been adsorbed. D: Authentic histamine. E: Authentic acetylhistamine.

Fig. 2. Paper chromatogram of urinary material adsorbed on Decalso (U). Chromatograms of: acetylhistamine (ACH), histamine (H), N-methylhistamine (MH), N-dimethylhistamine (DMH) and of a mixture of above compounds (MIX).

A significant finding is shown in C. On the average, only 66.4% of added histamine could be recovered from urine. It was suspected, therefore, that a loss of pre-existing histamine was also to be expected during the procedure as described above. The Decalso column, before the final elution of the adsorbed material with chloroform-ammonia is washed with alcohol and this is discarded (ROBERTS AND ADAM[3]). These washings were collected, dried *in vacuo*, the residue taken up in alcohol and submitted to paper chromatography. The result is shown in C, in which there are three well defined spots, one in the histidine position, another one in the histamine position, and a third, unknown one, adjacent to the histamine spot, just as in A and B. No spot of acetylhistamine was detected in the chromatogram of the alcoholic washings, neither was there the second unknown spot with the highest R_F. The fact that in the alcoholic washings, so far discarded, these three iminazole spots were identified, may account for the lack of complete recovery of histamine and may also indicate that the amount of free histamine actually excreted in the urine may be higher than that so far detected with the Decalso method.

Finally, to gain further insight into the nature of the iminazole compound which in all paper chromatograms was very close to the histamine spot, a chromatogram of a urinary Decalso extract and of an authentic histamine solution was prepared on one sheet in duplicate. After development, half of the paper, to be used as a test strip, was cut off, and sprayed with the diazo reagent. After drying, the strip with the coloured spots was placed alongside the other half of the chromatogram and the regions cor-

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responding to the coloured spots were marked with pencil, and cut out in three sections as shown in B. These were eluted separately with 3 ml of a mixture of a Ringer solution and phosphate buffer solution, pH 7.2 (3:1). In aliquot parts of each eluate the biological activity was measured before and after incubation with a highly active histaminase solution. The two eluates from areas 1 and 3 (Fig. 1, B) showed a fairly strong biological activity before incubation with histaminase. This biological activity was inhibited by mepyramine maleate. It is noteworthy, however, that between these areas, which correspond to the two diazo spots, there was a narrow band (section 2) which proved to be almost pharmacologically inactive. Whereas the activity of the region of the standard histamine chromatogram, and of the corresponding area (1) of the urinary extract was completely abolished by histaminase, the biological activity of the area of the unknown iminazole derivative (3) was hardly affected by histaminase.

It was concluded from the results of manifold experiments that Decalso adsorbs from human urine a material, which besides histidine, free histamine and acetylhistamine was found to contain two other, so far unidentified iminazole compounds. One of these two compounds, whose spot in all the chromatograms prepared was adjacent to that of histamine displayed, after elution from the developed paper chromatogram, pharmacological and chemical characteristics, very much like those of histamine, from which it differed only in that it was not attacked by histaminase.

(b) *Paper chromatographic identification of two unknown iminazole compounds occurring in the material adsorbed from urines on Decalso.* In his extensive study of the pharmacological behaviour of histamine derivatives VARTIAINEN[9] found that of the two derivatives N-methylated on the side chain, the monomethyl compound was twice as active as histamine, and N-dimethylhistamine possessed 75% of the activity of histamine on the isolated guinea pig ileum. SCHILD[10] reported that mepyramine maleate inhibited the biological action of N-monomethylhistamine and histamine alike, and WERLE AND PALM[11] showed that another antihistamine compound, Antistin, interfered with the pharmacological activity of both N-methylhistamine and N-dimethylhistamine to the same extent as with that of histamine. Like histamine, both N-methylhistamine and N-dimethylhistamine couple with aromatic diazo compounds with the formation of red coloured azo dyes[12]. This close similarity in the pharmacological and chemical behaviour of histamine and its N-methyl derivatives along with the observations made in the present work suggested that the two unknown iminazole compounds detected in all paper chromatograms of Decalso extracts from human urine (R_F 0.62 and 0.85 respectively) might be identical with the two N-methylhistamines. Moreover, it was impossible to disregard the analogy with adrenaline and noradrenaline with respect both to the chemical structure in the NH_2-group of the side chain and the biological relationships. A simultaneous paper chromatographic investigation of Decalso extracts from human urine and of N-methylhistamine and N-dimethylhistamine as reference compounds therefore appeared desirable. A generous gift of these compounds by Dr. B. GARFORTH (Boots Pure Drug Co., Nottingham) gratefully acknowledged here, made it possible to investigate further the substances obtained from urine. The dihydrochlorides of N-methylhistamine and N-dimethylhistamine prepared from the picrates and submitted to the paper chromatography showed in 15 independent experiments an average R_F of 0.62 for N-methylhistamine and an R_F of 0.85 for N-dimethylhistamine. These R_F figures appeared to be identical with those obtained for the two unidentified iminazole derivatives (Fig. 1, A and B, third and last spot).

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Several experiments were then carried out in which the material adsorbed on Decalso from human urine was simultaneously chromatographed with histamine, N-methylhistamine, N-dimethylhistamine and acetylhistamine as reference compounds as well as with a mixture of all four of these substances. It can be seen from Fig. 2, which is representative of those trial experiments, that the two hitherto unidentified substances from urine take up the same position as N-methylhistamine (MH) and N-dimethylhistamine (DMH). Moreover, a mixture of the synthetic reference compounds (MIX) gave a picture which was almost identical with that of the material from urine (U). As mentioned above, the iminazole compound with the smallest R_F corresponds to histidine.

Effect of a purified pigs' kidney histaminase on authentic preparations of N-methylhistamine, N-dimethylhistamine and acetylhistamine

It has been shown above that only part of the histamine-like activity of the urinary material was destroyed by a highly active histaminase, and that the eluate of the spot with R_F 0.62, identified now as N-methylhistamine, was entirely resistant to histaminase. The authentic preparations of N-methylhistamine and N-dimethylhistamine were therefore subjected to the action of this enzyme, using the slightly modified microvolumetric technique for the estimation of histaminase[7]. The effect of pig's kidney histaminase on acetylhistamine was also investigated.

In many experiments it was found that neither of the two side chain N-methylhistamines nor acetylhistamine was attacked by pigs' kidney histaminase. These results do not confirm the findings of WERLE AND PALM[11] who obtained some enzymic effect on N-methylhistamine and N-dimethylhistamine with a crude histaminase preparation from pea seedlings. The claim of MILLICAN *et al.*[13], however, that acetylhistamine is not attacked by histaminase is fully confirmed in the present work. ALLES *et al.*[14] have found that α-methylhistamine is not affected by histaminase, and TABOR AND BAUER[15] have shown a similar resistance of the lower homologue of histamine, aminomethyliminazole. All these observations seem to suggest that an intact aminoethyl side chain in iminazole derivatives is essential for histaminase activity.

In competition experiments, representative results of which are shown in Table II N-methylhistamine, N-dimethylhistamine, and acetylhistamine were all found to inhibit strongly the oxidation of histamine by histaminase. This, with the stability of N-monomethylhistamine and N-dimethylhistamine towards histaminase may well account for the fact that the entire urinary histamine-like material, adsorbed on Decalso was not inactivated by histaminase. These facts also explain why it is possible to encounter both histamine and histaminase in one and the same biological medium.

TABLE II

INHIBITORY EFFECT OF N-METHYLHISTAMINE, N-DIMETHYLHISTAMINE AND ACETYLHISTAMINE ON THE ENZYMIC DEGRADATION OF HISTAMINE (2.7×10^{-4} *M*) BY PIGS' KIDNEY HISTAMINASE.

$2.7 \cdot 10^{-4}$ *M*	*Inhibition* %
N-Methylhistamine	36.5
N-Dimethylhistamine	48.3
Acetylhistamine	49.4

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In a very interesting series of experiments SCHAYER *et al.*[16] using isotope techniques and ^{14}C histamine studied the *in vivo* degradation of histamine. They demonstrated that in all mammalian species tested, including man, two histamine-metabolizing enzymes exist: one is diamine oxidase (histaminase) which leads to the formation of iminazole-4-acetic acid (ImAA) and to 1-ribosyliminazole-4-(5)acetic acid (ImAA-riboside). The other enzyme, which SCHAYER *et al.*[16] called the histamine-metabolizing enzyme II is considered to be composite and to bring about methylation as well as oxidation. It is said to methylate histamine, on the ring-N remote from the side chain, to 1-methyl-4-(β-aminoethyl)iminazole (1,4-methylhistamine) and to oxidize it to 1-methyliminazole-4-acetic acid (1,4-methyl ImAA). All these histamine metabolites were detected by SCHAYER *et al.*[16] by isotope dilution assays in the urine of various species after feeding or injecting ^{14}C histamine. As to the nature of the oxidizing enzyme of this system SCHAYER AND KARJALA[16] are inclined to suggest monoamine oxidase on account of some inhibition experiments, although monoamine oxidase preparations from mouse liver had no *in vitro* effect on 1,4-methylhistamine. These authors think it improbable that the oxidizing enzyme is histaminase, because aminoguanidine, considered to be the most powerful inhibitor of histaminase did not in their *in vivo* experiments prevent the formation of 1,4-methyl ImAA.

Effect of pigs' kidney histaminase on methylhistamines with ring-N substitution

Having investigated the effect of histaminase on the N-methylhistamines with side chain substitution it appeared to be essential to include in this work a study of the action of histaminase on methylhistamines with ring-N substitution.

Sincere thanks are due to Dr. SCHAYER for his very generous gift of the picrates of 1,4-methylhistamine and 1,5-methylhistamine. From these picrates the corresponding hydrochlorides were prepared and these, when subjected to the action of a highly active histaminase, were found to be oxidized almost to the same extent as equimolar histamine solutions. In mixtures of histamine with either of these two compounds no additive effect was obtained, which indicates that only one enzyme was responsible for the destruction of the mixtures of the substrates (Table III).

TABLE III

EFFECT OF PIGS' KIDNEY HISTAMINASE ON HISTAMINE AND ON THE TWO HISTAMINES METHYLATED ON THE RING-N AND ON MIXTURES OF HISTAMINE WITH THESE SUBSTANCES

Substrate (approx. $2.7 \cdot 10^{-4}$ M)	*Histaminase activity P.U.*
Histamine	8.6
1,4-Methylhistamine	6.1
1,5-Methylhistamine	5.2
Histamine + 1,4-methylhistamine	7.4
Histamine + 1,5-methylhistamine	8.0

ALLES *et al.*[14] found that 5-methylhistamine with the methyl group on the carbon atom close to the side chain was also oxidized by histaminase. It seems to follow that substitution in the iminazole nucleus does not interfere with the histaminase activity. The fact that 1,4-methylhistamine is oxidized by histaminase makes it possible that

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the metabolizing enzyme II (SCHAYER *et al.*[16]) is solely a methylating enzyme, the reaction product of which is oxidized by histaminase. As to observations of SCHAYER *et al.*[16] that in their *in vivo* experiments aminoguanidine did not prevent the formation of 1,4-methyl ImAA, a recent paper by MITCHELL[8] should be recalled here; he showed that in the human body histaminase is not very readily inhibited by aminoguanidine. SCHAYER[17,18] has recently reported on the metabolism of ^{14}C histamine in man. He found that after ingestion of microgram quantities of ^{14}C histamine to humans 81 to 84% of the total administered radioactivity was excreted in the urine in the form of free histamine, and various histamine metabolites, the principle excreted compound being 1,4-methyl ImAA. From his work SCHAYER claims that in man methylation to 1,4-methylhistamine is the fundamental reaction in histamine metabolism, oxidation to 1,4-methyl ImAA being subsequent to this; this is supported by the failure in the present work to find, in any of the paper chromatograms, material from urine corresponding to ImAA which TABOR *et al.*[19] considered to be a major product of the enzymic oxidation of histamine. It is true that in man some histamine may be converted to ImAA by histaminase but this may well be excreted not as such but in the form of ImAA-riboside which like 1,4-methyl ImAA itself, is not detectable by the diazo reaction. The two new metabolites of histamine, N-methylhistamine and N-dimethylhistamine, excreted in human urine, may be among the hitherto unidentified substances which may account for SCHAYER'S failure to recover the total radioactivity in the urine excreted after ingestion of ^{14}C labelled histamine.

In all the paper chromatograms of this work acetylhistamine was encountered and this seems to support the finding of MILLICAN *et al.*[13] that enzymic acetylation of histamine is also an important part of histamine metabolism.

The paper chromatographic detection of N-methylhistamine and N-dimethylhistamine in material adsorbed from human urine on Decalso leads to speculation on the origin and physiological significance of these two compounds, which are, pharmacologically highly active.

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SUMMARY

1. By means of a combined technique of ion exchange chromatography and paper chromatography two new iminazole compounds have been identified in the material adsorbed from human urine on Decalso. This material has been found to contain besides histidine, free histamine and acetylhistamine, two other compounds, N-methylhistamine and N-dimethylhistamine, which are pharmacologically highly active.

2. The effect of histaminase on these two compounds as well as on 1,4-methylhistamine and 1,5-methylhistamine and acetylhistamine has been investigated and the significance of the results obtained is discussed.

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THE AMPEROMETRIC TITRATION OF SULFHYDRYL GROUPS WITH SILVER NITRATE

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In the amperometric titration of SH groups of proteins and related compounds with $AgNO_3$, it is usually assumed that the number of SH groups in the titration mixture is equal to the number of Ag ions added up to the point where the two branches of the titration curve intersect. In the author's experience, however, this is not correct for some compounds when these are titrated in two media suitable for proteins.

EXPERIMENTAL

The electrode, containing a protruding Pt wire (length 10 mm, diameter 0.5 mm), was rotated at 1400 r.p.m. It was cleaned daily in hot nitric acid and coated with silver for 5–10 min at 10–20 μA, using the same buffer and the same potential as in the titrations (ammonia buffer −250 mV, tris buffer* −100 mV *versus* saturated calomel electrode).

* Tris(hydroxymethyl)aminomethane.

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