Colour Tests for the Detection and Characterisation of Tertiary Amines

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According to Ohkuma,[1,2] a red, violet or blue colour is obtained when small amounts of tertiary amines are heated to 100° for a few minutes with a drop of a 2 % solution of citric acid in acetic anhydride. Similar colour tests are given with malonic acid, aconitic acid,[3] and with many other polycarboxylic acids,[4] such as tartaric acid, acetonedicarboxylic acid, ascorbic acid. We have studied reactions of this type to determine their sensitivity towards tertiary amines and have also investigated the nature of the colouring materials. Thin layer chromatography of the reaction mixtures shows that the colour formed in these reactions consists of many components. The chromatographic pattern depends both on the acid and the amine used. When malonic acid was used, the differences in the chromatograms were particularly significant.

The light absorption properties of the components of the reaction mixtures differ markedly in UV light (350 mμ) but less so in visible light. Hence, by working under standardized conditions we have been able to "finger-print" many tertiary amines simply by observing the developed chromatograms in UV light. A quicker and easier way to obtain this chromatographic pattern is to allow approximately 20 μl of reaction solution (5—15 μmoles tertiary amine per ml of acetic anhydride containing 2 % malonic acid warmed to 100° for 3—5 min) to be absorbed from a capillary onto a thin layer plate, coated with 0.25 mm silica gel. A round spot consisting of different coloured, concentric rings in a characteristic pattern is obtained. The identification of an unknown amine is simply a matter of comparing the spot with standard colour photographs. This method has been successfully applied to the tertiary amines listed in the table below; in each case a specific spot was obtained.

Aliphatic amines: N,N-dimethyldodecylamine, N-ethylpiperidine, tributylamine, 1-dimethylaminopropanol, triethanolamine.

Aromatic amines: tribenzylamine, N,N-diethylaniline.

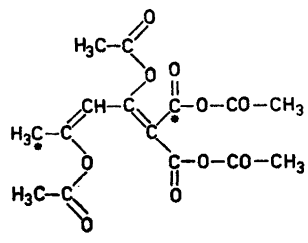
Heterocyclic amines: pyridine, 2-chloropyridine, quinoline, 4-benzylpyridine, 2-benzylpyridine, 2-benzylthiazole.

Alkoloids: LSD 25, quinine sulphate, brucine, strychnine, morphine, apomorphine, codeine, atropine.

The technique described above has been used satisfactorily to identify amines in 10—20 μg quantities taken from a gas chromatograph.

With regard to the mechanism of the reaction, there can be little doubt that it is a base catalyzed condensation. Here tertiary amines differ from primary and secondary amines in that they keep their basic qualities even in an acetylating medium like acetic anhydride. The intense colours, together with strong fluorescence, indicate that the products are aromatic. Bromination did not alter the colours or the infra-red spectra at 1500—1600 cm^{-1} (C—C skeletal vibration in aromatic compounds). Pyrolysis of the evaporated reaction mixtures at 500° produced phenols.

A deeply coloured condensation product was prepared from malonic acid, pyridine, and acetic anhydride (10:5:500) in the usual manner and then evaporated to dryness. Elementary analysis of the product indicated it to be a pyridine salt of a compound produced by the condensation of 2—3 moles of acetic anhydride and 1 mole of malonic acid with the loss of 5—6 moles of water. The final aliphatic material, prior to aromatization must have six adjacent carbon atoms, with a carbonyl group at one end of the chain and a reactive methyl or methylene group at the other. If we consider the malonic acid to react as the mixed anhydride with acetic acid, the compound that is cyclised should have the structure shown. Condensation



would occur between the two marked carbon atoms.

If this is in fact the case, the first aromatic compound formed would be a half-anhydride of a completely acetylated phloroglucinol carboxylic acid and acetic acid, the reaction being analogous to that whereby phenols are obtained from hexadiene-carboxylic acids by reaction in acetic anhydride.[5] The different colour components then have to be formed through further condensations with the phloroglucinol derivative, together with deacetylation. This could explain why the amines are bonded to the coloured reaction products.

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^{35}S-Cystamine as a Thiol Reagent for the Study of Oxidative Phosphorylation

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Rat liver mitochondria contain approximately 0.1 μmole of thiol groups per mg of protein.[1] A number of investigators have felt that thiol groups may be involved in oxidative phosphorylation.[1-3] This conclusion has mainly been reached through the study of the uncoupling effect of different thiol reagents, whereas variations in mitochondrial thiol levels in different metabolic states have not been demonstrated. A possible explanation for this may be that silver ions and other reagents used for the estimation of thiol groups are so reactive that they cause a rapid lysis of mitochondria even at concentrations amounting to only 10 % of mitochondrial thiol groups.

^{35}S-Cystamine by its ability to form mixed disulphides can be regarded as a specific and mild thiol-blocking agent.[4] Preliminary experiments showed that cystamine was bound to mitochondrial proteins much more slowly than were silver ions. Since cystamine, even at high concentrations (5 mM), does not inhibit the respiratory chain,[5] nor uncouples oxidative phosphorylation provided substrate is present,[6] this disulphide might prove to be a useful reagent in the study of mitochondrial thiol groups. It might be possible to maintain different states of electron transport and oxidative phosphorylation even during the reaction period which is required with cystamine (5—15 min at pH 7.0—7.5). Since Eldjarn and Bremer [7] showed that disulphides (*e.g.* cystamine) which penetrate into mitochondria slowly will become reduced to thiols, it was regarded essential to secure by thiol estimation that the disulphide concentration is not too much altered during experiments on the binding of cystamine to mitochondria.

In the present work it is shown that ^{35}S-cystamine is suitable as a reagent for the study of mitochondrial thiol groups, and that a relation seems to exist between the levels of reactive thiol groups and different states of oxidative phosphorylation.

Methods. ^{35}S-Cystamine was obtained from the Radiochemical Centre, Amersham, England. 0.1 M stock solutions of cystamine (specific activity approximately 50 000 cpm/μmole) were prepared by the addition of carrier cystamine purchased from Calbiochem, Los Angeles, California, USA (lot No. 30 177). Oligomycin and Antimycin A were products of Sigma Chemical Co., St. Louis, Mo., USA. All other reagents were commercial products of high purity. Rat liver mitochondria were prepared according to Myers and Slater.[8] A Zeiss RPQ 20 A Recording Spectrophotometer was used in the swelling experiments. The oxygen uptake was measured by Warburg techniques, and the reaction was stopped with 0.25 ml of 6 M $HClO_4$. Disappearance of P_i was determined by the method of Martin and