three are heat resistant. There is no relationship between migration and heat sensitivity.

These findings indicate that mycobacterial catalases are present in multiple molecular forms. All of these forms appear to have a lower molecular weight than the previously studied mammalian and bacterial catalases. Studies are currently underway to determine if these zymograms can be used for classification of different mycobacteria.

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Separation of isomeric lysine derivatives by ion-exchange chromatography

The biochemical literature contains numerous references to the chemical modification of gelatin[1,2]. The present interest in the reaction between gelatin and N-acetylsulphanilyl chloride was twofold. Firstly, that it should provide information regarding the reactivity of the reagent with this particular protein. In addition, that the acid hydrolysate of the modified gelatin should contain in fair yield Nε-(*p*-aminosulphanilyl)-lysine, a sulphonamide and potentially at least, a useful anti-inflammatory agent.

During the course of the work considerable difficulties were experienced in locating the Nε-(*p*-aminosulphanilyl)-lysine in the hydrolysate when employing the normal column chromatographic procedures of MOORE AND STEIN[3]. To resolve this problem, pure lysine derivatives were prepared for use as model substances. Reaction of L-lysine monohydrochloride with an excess of N-acetylsulphanilyl chloride gave

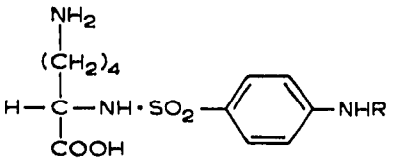
a mixture of the α-, the ε- and the α,ε-disubstituted derivatives of lysine. This paper outlines a procedure which effects the separation of the α and ε lysine derivatives from the free amino acids present in gelatin hydrolysates. At the same time this method makes it possible to calculate accurately the degree of reaction of the lysine in gelatin with N-acetylsulphanilyl chloride.

Experimental and results

Preparation of lysine derivatives. 1 g L-lysine hydrochloride in 8 ml water was heated to 60°, the pH of the solution was adjusted to 9.0 with 1.0 *N* sodium hydroxide and a suspension of 1.4 g N-acetylsulphanilyl chloride in 20 ml dioxane was added in small portions. The pH was kept at 9.0 by means of a "Radiometer" automatic titration apparatus. At the end of the reaction (1.5 h) the solution was acidified and evaporated to dryness at 60° on a rotary evaporator. The white residue was warmed with methanol, all insoluble material filtered off and the filtrate again evaporated to dryness.

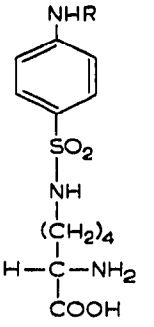
In a typical run a mixture of the lysine derivatives (9 g) obtained as above was dissolved in a minimum of 1-butanol–ethyl methyl ketone–water (2:2:1). This was adsorbed on to a column (4.5 × 80 cm) packed with cellulose (250 g) and eluted with the above solvent mixture. 10-ml fractions were collected. Little or no material was present in tubes 0–80, tubes 81–126 contained mainly α,ε-disubstituted lysine, tubes 127–162 contained a mixture of α- and ε- monosubstituted material, tubes 163–174 contained nearly pure α-derivative (crystallisation in these tubes) and unchanged lysine appeared from tube 175 onwards.

Repeated crystallisation (ethanol–water) of the crystals from tubes 163–174 finally gave a pure Nα-(*p*-acetylsulphanilyl)-lysine, m.p. 278° (I). (Found: C, 46.82; H, 6.77. Calculated for $C_{14}H_{21}N_3O_5S \cdot H_2O$: C, 46.53; H, 6.42 %.) Hydrolysis with 6.0 *N* hydrochloric acid yielded the crystalline Nα-(*p*-aminosulphanilyl)-lysine dihydrochloride, m.p. 195° (II). Both these compounds had been obtained previously by KUCHEROV AND IVANOV[4]. Re-crystallisation of the mixture of isomers from tubes 127–162 proved only partly successful in separating the α from the ε derivative. The latter was then prepared in high yield from L-lysine hydrochloride and N-acetylsulphanilyl chloride (in dioxane) following the method of ROESKE and his co-workers[5]. This method involves prior protection of the α-amino group by means of copper complex formation. Pure Nε-(*p*-acetylsulphanilyl)-lysine (III) had a m.p. of 257° (ref. 6 quotes 251–252°). (Found for sample dried at 110°: C, 48.94; H, 6.76. Calculated for $C_{14}H_{21}N_3O_5S$: C, 48.98; H, 6.17 %.) Hydrolysis with acid as above gave Nε-(*p*-aminosulphanilyl)-lysine dihydrochloride (IV), m.p. 205°. Neutralisation of IV with dilute sodium hydroxide, followed by acidification with acetic acid gave Nε-(*p*-aminosulphanilyl)-lysine (V), m.p. 224° (ref. 6 quotes 205°). The identity of this latter compound was proved beyond doubt by comparing its NMR spectrum (in deuterium oxide) with that of lysine hydrochloride[7] in the same solvent. The two are very similar apart from the appearance downfield of the A_2B_2 system of benzenoid protons. The CH (triplet) and adjacent CH_2 groups can all be assigned. The Nα,Nε-bis-(*p*-acetylsulphanilyl)-lysine (VI), m.p. 185° (from tubes 81–126) was obtained pure by small scale countercurrent separation using water saturated 2-butanol as mobile phase. (Found for sample dried at 100°: C, 48.60; H, 5.79. Calculated for $C_{22}H_{28}N_4O_8S_2$: C, 48.90; H, 5.22 %.)



I, R = $CO \cdot CH_3$

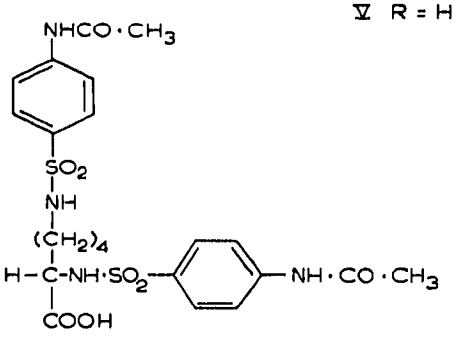
II, R = H, Dihydrochloride



III R = $CO \cdot CH_3$

IV R = H, Dihydrochloride

V R = H



VI

Reaction of N-acetylsulphanilyl chloride with gelatin. The method was similar to that employed for lysine. A large excess of N-acetylsulphanilyl chloride as a 35 % solution in dimethylformamide was added to the gelatin (limed hide, Bloom strength at 6 2/3 % w/w, 166 g; viscosity at 6 2/3 % w/w and 40°, 3.9 cS; ash content 1.2 %; moisture content 8.2 %, pH 4.8) at pH 9.0 and 60°. Reaction was complete in about 1 h and the product was washed and dried by standard procedures[8]. The substituted gelatin was hydrolysed in a sealed tube under vacuum with 6.0 *N* hydrochloric acid for 16 h at 110°.

Thin-layer chromatography of lysine derivatives. The three pure acetylsulphanilyl derivatives of lysine (I, III and VI) obtained as above were well separated from one another on thin-layer plates coated with Kieselgel GF_{254} (Merck) and with 1-butanol–ethyl methyl ketone–water (2:2:1) as solvent. The R_F values are shown in Table I.

Ion-exchange chromatography. All amino acid analyses were carried out on a "Beckman" Model 116 Analyser. The short column (6 cm) was packed with "Beck-

TABLE I

R_F VALUES OF ACETYLSULPHANILYL DERIVATIVES OF LYSINE

Derivative	R_F
Nα- (I)	0.29
Nε- (III)	0.42
Nα, Nε- (VI)	0.51

man" resin Type PA 35 and the long column (55 cm) with "Beckman" resin Type M 72. Analyses were done at 55°.

The monosubstituted derivatives of lysine (about 3 mg per 5 ml of pH 2.2 buffer) were applied (0.2 ml) to the long column but instead of eluting with the standard pH 3.25 buffer, followed by the pH 4.25 buffer, the pH 5.26 buffer was used from the start of the run. Table II shows the elution times (average of several determinations) of the four derivatives.

TABLE II

ELUTION TIMES FOR SOME AMINO ACIDS AND ACETYLSULPHANILYL DERIVATIVES OF LYSINE

Amino acid	*Elution time (min)*
Phenylalanine (reference)	54
Nα-(*p*-aminosulphanilyl)-lysine (II)	70
Nα-(*p*-acetylsulphanilyl)-lysine (I)	81
Nε-(*p*-aminosulphanilyl)-lysine (IV)	100
Nε-(*p*-acetylsulphanilyl)-lysine (III)	153
Lysine (reference)	173
Histidine (reference)	199

The derivative of greatest interest was IV since it is one of the components resulting from acid hydrolysis of the modified gelatin. When the gelatin hydrolysate was run under "normal" conditions on the long column IV remained undetected and no additional peak (compared with parent gelatin) was evident even after an elution time of 260 min. Attempts to find a peak due to this derivative on the short column also met with no success. In this case it was obviously being eluted ahead of lysine together with the large peak constituting the combined acid and neutral amino acids.

When the parent gelatin hydrolysates were chromatographed using the modified procedure (*i.e.* buffer pH 5.26 on long column, but maintaining the standard flow rate of 100.7 ml/h) lysine appeared after 173 min. No peak was apparent at the 100 min mark. In the hydrolysates of the modified gelatin however, a prominent peak appeared after 100 min. Co-chromatography of the modified gelatin hydrolysates and synthetic IV showed a single peak of increased intensity at 100 min and there is little doubt that the two products are identical. Also, by calculating the amount of lysine present in a known quantity of unmodified gelatin and comparing it with: (i) the amount of unchanged lysine remaining in a sample of modified gelatin and (ii) the quantity of IV formed, it was possible to establish accurately the extent to which lysine had reacted with N-acetylsulphanilyl chloride. If a 200% excess of reagent was used (based on the free amino content of gelatin) then 62% of the lysine reacted and this could be estimated quantitatively by assessing the peak area of IV appearing at the 100 min mark.

Discussion

Several methods have been developed to determine the degree of substitution at the available amino groups of gelatin. Thus LEACH and his co-workers[8] applied three methods to determine the degree of substitution in gelatins modified with benzenesulphonyl chloride. One of these, the ninhydrin colorimetric procedure, while

subject to certain minor sources of error, proved to be particularly useful as a result of its simplicity. This method is based on the assumption that if all the free amino and imino groups react with the substituting agent to the same extent, then the fractional decrease in colour resulting from the "loss" of these groups will be unaffected by the relative proportions of these groups in the parent protein. The degree of substitution can then be calculated from this decrease in colour. This procedure, which determines the substitution achieved at the α-amino plus ε-amino groups was subsequently modified to correct for the hydrolysis of the peptide chain which takes place during colour development[9]. In none of the above methods is the reactivity of any particular constituent (amino acid) of the protein examined. Much earlier however, GURIN AND CLARKE[10] had isolated in 50 % yield the copper salt of Nε-monobenzenesulphonyl-*d*-lysine from the hydrolysates of gelatin treated with benzenesulphonyl chloride. The present work is more closely allied to that of GURIN AND CLARKE with the difference that the lysine derivative could be determined without actual isolation.

Variation of the pH of the buffers in order to obtain the required separations is not an unknown technique. An example of this is cited in a recent paper by RONCA and his co-workers[11]. By lowering the pH of the first buffer from 3.25 to 3.19 it becomes possible to separate ε-carboxymethyl lysine from methionine and other ninhydrin-positive peaks. By using the 5.26 buffer on the long rather than the short column an equally successful separation of lysine derivatives from free amino acids has now been achieved. A surprising and as yet unexplained feature of these results is the fact that both acetylated bases I and III are eluted *after* the corresponding free bases II and IV.

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