L-malate, citrate, α -ketoglutarate) found in solutions as a result of the interaction between acetoacetate and oxaloacetate were determined in aerobic and anaerobic experiments. The most notable effect of oxygen on the yields of various substances was a higher yield of citrate. Calculations based on the yields suggest that acetoacetate contributes towards the formation of the acids of the tricarboxylic cycle, a conclusion now firmly established by the tracer work of Buchanan *et al.* and of Weinhouse *et al.*

5. Erythro-1:2-dihydroxybutyric acid was not metabolized by sheep kidney cortex whilst the threo form was slowly oxidized. In accordance with Weil-Malherbe's findings, the slowness of the oxidation excludes this substance as an intermediate in the removal of ketone bodies.

6. Measurements of the rates of α -ketoglutarate formation from various substrates and substrate combinations showed that the tricarboxylic acids

yield α -ketoglutarate more rapidly than any other substrate. This supports the assumption that α -ketoglutarate arises via the tricarboxylic acids when formed in the presence of oxaloacetate and acetoacetate (or pyruvate). The rate of citrate formation via carboxylation of α -ketoglutarate (Ochoa, 1945) was relatively slow in sheep kidney cortex.

7. The sum of *cis*aconitate plus *isocitrate* was found to exceed 10.5% of the total tricarboxylic acids (maximum 49%) under suitable conditions. It is pointed out that this finding excludes the sequence (4) (p. 302) and supports the sequence (3) (p. 301), confirming conclusions earlier drawn from tracer work.

8. It was confirmed that barium (Wieland & Rosenthal, 1943) and bicarbonate (Breusch, 1943*a, b*) ions increase the yield of citrate from oxaloacetate and acetoacetate. Possible causes of this effect were investigated.

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The Isolation of Hydroxylysine Picrate from a Gelatin Hydrolysate

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Schryver, Buston & Mukherjee (1925) claimed the isolation, from isinglass hydrolysate, of an 'oxylysine' to which they attributed the formula $C_6H_{14}O_3N_2$; but they were unable, on repeated attempts, to prepare a crystalline picrate. Van Slyke & Hiller (1921) reported the presence of hitherto unknown material in the basic amino-acid fraction of hydrolyzed gelatin; this base was isolated by Van Slyke, Hiller, Dillon & MacFadyen (1938) who ascribed to it the formula of a hydroxylysine.

After removal of arginine and histidine, these workers freed the 'lysine fraction' from monoamino-acids, and then removed the greater portion of the lysine by the addition of sufficient picric acid to combine with three quarters of the amino nitrogen present. A picrate of composition $C_6H_{14}O_3N_2 \cdot C_6H_3O_7N_3$, which melted at 225° when heated slowly, was eventually isolated from the mother liquor. Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940) and Van Slyke, Hiller & MacFadyen (1941)

have since endeavoured to establish the structure of hydroxylysine, and to determine its presence in other proteins by adaptation of the periodate oxidation reaction of Nicolet & Shinn (1939).

Martin & Synge (1941), by the application of an acetylation-benzoylation procedure to the lysine fractions of gelatin and isinglass hydrolysates, isolated in each case a small amount of a crystalline picrate which exploded at 226–227°. Although their analytical figures for formaldehyde and ammonia evolved on periodate treatment were low, this work is the only independent confirmation, by isolation, of the existence of the base first described by Van Slyke *et al.* (1938).

Additional evidence has been forthcoming from the periodate reaction besides that supplied by Martin & Synge. Using Vorländer's (1929) technique for determining the formaldehyde produced by oxidation of the 'lysine fraction' of isinglass, Beveridge & Lucas (1944) obtained a value from which they deduced the probable presence of some 0.36 % hydroxylysine. It was not apparent whether the compound estimated was that described by Schryver *et al.* (1925), or by Van Slyke *et al.* (1938), and accordingly they attempted the isolation advocated by the American workers, but failed to obtain any hydroxylysine picrate. More recently, Rees (1946) has carried out estimations by periodate oxidation which have confirmed the presence of a basic hydroxy amino-acid in gelatin and collagen, but not in any other proteins examined. Desnuelle & Antonin (1947) have criticized Van Slyke's figures based on the production of ammonia in the periodate reaction when carried out in the presence of excess glycine, and have preferred a colorimetric estimation of the formaldehyde simultaneously liberated. They found no evidence for the presence of hydroxylysine in casein, which contains, according to Van Slyke and his co-workers, 0.33 %. Neither Rees (1946) nor Desnuelle & Antonin (1947) attempted the isolation of any derivatives.

The isolation of a hydroxylysine picrate, which agrees in melting point and other physical properties with the compound isolated by Van Slyke and which affords formaldehyde and ammonia on periodate oxidation is described in this paper. The separation was effected by means of fractional recrystallization of the product derived from the 'lysine fraction' of a purified gelatin catholyte.

MATERIALS AND METHODS

Gelatin (1 kg.) known commercially as 'Spa Brand', kindly supplied by Messrs B. Young and Co. Ltd., of Bermondsey and containing 2 % ash, 15 % moisture and 13.82 % (uncorr.) nitrogen was hydrolyzed by boiling for 24 hr. with 2.5 l. of 30 % (v/v) H_2SO_4 ; after dilution, the H_2SO_4 was removed first to pH 2.5 and finally to pH 5.5 with $Ba(OH)_2 \cdot 8H_2O$. The hydrolysate, together with washings of the precipitates,

was then concentrated *in vacuo* to c. 1 l., and subjected to fractional electrodialysis in a three-compartment cell to remove all but traces of monoamino-acids from the catholyte. From this purified basic fraction, arginine and histidine were completely removed with flavianic acid, and excess flavianic acid with baryta. Finally the excess Ba^{++} was entirely removed with H_2SO_4 and ammonia by concentration *in vacuo*.

EXPERIMENTAL

Main picrate fraction

Preparation. The 'lysine fraction' referred to above (700 ml.) was precipitated with 45 g. (excess) of picric acid, affording a picrate which, after recrystallization from 1 l. of water, weighed 24 g. and exploded at 267°, the usually accepted explosion point for lysine picrate. Recrystallization of this picrate from water produced no change in explosion point.

Conversion to dihydrochloride. A portion (10 g.) of this twice recrystallized main picrate fraction was converted into the dihydrochloride (Cox, King & Berg, 1929). Although free from arginine, histidine and ammonia, the product melted at 192–193°, i.e. about 10° below the accepted melting point of lysine dihydrochloride and was also very hygroscopic.

Analysis of the dihydrochloride by HIO_4 oxidation. The dihydrochloride (0.1 g.), prepared as above, was dissolved in water (5 ml.) and HIO_4 (0.1 g. in 10 ml. of water) added. The oxidation was carried out in the manner described by Van Slyke *et al.* (1940). Subsequent treatment with Vorländer's reagent, 'dimedone' (5:5-dimethylcyclohexane-1:3-dione) precipitated an insoluble condensation product which was identified by its m.p. (185°) as methylene bis-dimedone. The melting point of this compound was not depressed on admixture with authentic material (m.p. 189°). Assuming the formaldehyde to have been derived from a hydroxylysine, the yield of dimedone derivative, 1.61 mg., corresponds to the presence of some 1.3 g. of the hydroxylysine salt/100 g. of dihydrochloride. (This figure must be regarded as minimal and the true content may well be higher.)

The suggestive analytical evidence obtained from the main picrate fraction warranted a search for greater quantities of the presumptive hydroxylysine in the main picrate mother liquors. Fractionation of these did, indeed, lead to the isolation of a picrate having the properties of that of hydroxylysine.

Fractionation of the main picrate mother liquors

The mother liquor and the washings (c. 1 l.) from the main crop of picrate were evaporated to 50 ml. affording, after one recrystallization, a second crop P_1 (1.9 g.) of explosion point 254°. Recrystallization of P_1 from water yielded 1.76 g. of P_2 (explosion point 259°). From the filtrate and washings, containing 0.14 g. of mixed picrates, was obtained, after evaporation to a few ml. and cooling to room temperature, a picrate P_3 , paler yellow in colour than the picrate of lysine.

P_3 , after filtration, washing with ice water and drying *in vacuo*, melted at c. 234°, with decomposition and bubbling but without explosion. Recrystallization from the minimum amount of water gave picrate P_4 of similar melting point and so the solvent was changed to 50 % (v/v) aqueous

ethanol. This solution was boiled with charcoal, filtered and evaporated to dryness to give P_5 . The latter was taken up in boiling methanol containing a little water and allowed to crystallize at 5°. The picrate so formed melted with decomposition at 225°, and this melting point was unchanged on further recrystallization from aqueous ethanol.

Properties of the picrate isolated

The compound was finally isolated in a yield of some 80 mg. When heated at the rate of 1° every 3 sec., it showed the same m.p. (225°) as the hydroxylysine picrate isolated by Van Slyke *et al.* (1938). The change was fairly sharp, but, though decomposition took place at the same time, there was no explosion such as occurs with lysine picrate. The intermediate fractions, P_3 , P_4 , showed the typical decomposition of impure lysine picrate. The crystals of the new compound, which consisted of pale-yellow needles possessing a tendency to sheaf-like formation, appeared under the microscope very similar in shape to those of lysine picrate. Crystallization in the test tube took place rapidly from a supersaturated solution and a fluffy precipitate then formed. This dull, lemon-yellow precipitate was quite different in appearance from the characteristic, bright, golden-yellow colour associated with the picrate of lysine.

Oxidation by periodate. Samples of about 20 mg. each of the new picrate were treated with 0.5M- HIO_4 , and the resulting formaldehyde was precipitated as the dimedone derivative, washed and dried at 110° to constant weight. Duplicates agreed to within 2.4%, but the yield of methylene bis-dimedone varied with the conditions of condensation. It is now known, from the work of MacFadyen (1945), in which the dimedone-formaldehyde condensation was studied by means of the chromatotropic acid reaction that, for 'complete' (98%) precipitation, up to 29 hr. at 39°, may be necessary if the condensation is carried out at pH 3.5, though the time required rapidly diminishes as the pH rises. At the time that these experiments were being carried out, however, it was not realized that the conditions of precipitation were so important. In all experiments, precipitation was carried out in a medium which was acid to methyl red and at room temperature. In one set of experiments, where the mixture was cooled immediately after the addition of dimedone and then allowed to stand in the refrigerator for 18 hr., the yield corresponded to only 62% of theory for hydroxylysine picrate; when the mixture was allowed to stand at room temperature for about 3 hr. before refrigeration, the yield rose to 81.8%.

Lack of material prevented the carrying out of further analyses. There seems little doubt, however, when the results of the periodate oxidation are considered in conjunction with the other properties of the picrate isolated from these mother liquors, that the base in question was hydroxylysine. The claims of Van Slyke *et al.* (1938) are thus confirmed.

DISCUSSION

It appeared impossible to purify the picrate mixture P_3 , melting at c. 234°, by further recrystallization from water although, on changing the solvent, the

heterogeneity of the substance became apparent and eventually hydroxylysine picrate of lower melting point was separated from the mixture. Thus the evidence strongly indicates that a solid solution separates from the aqueous system.

In this connexion, it may be pointed out that Van Slyke *et al.* (1941) found that diamino-acids are precipitated from mixtures by phosphotungstic acid partly as mixed salts rather than as individual substances each with its own independent solubility. These authors have shown further that in a mixture of lysine and hydroxylysine so precipitated the amount of hydroxylysine in the filtrate varies inversely to the ratio of lysine/hydroxylysine in the precipitate. The observations contained in this paper on the picrates are in good agreement with the above, and are of some practical importance in indicating that the isolation of hydroxylysine, whether by fractional precipitation with picric acid or by fractional recrystallization, depends ultimately on the solubilities of the picrates in water and in one another.

In view of this evidence, it would seem unlikely that hydroxylysine of 100% purity could be obtained by ordinary purification procedures. Gale (1947) has recently tested a decarboxylase, claimed to be specific for L-lysine, on samples of hydroxylysine received from Van Slyke. These samples were slowly, but incompletely, attacked. Zittle & Eldred (1944), using the same organism on zein hydrolysates, have shown that hydroxylysine cannot be decarboxylated to any appreciable extent. Although the amounts of lysine and hydroxylysine in zein are somewhat uncertain, it is possible that the results of Gale (1947) are due, in part, to contamination of the hydroxylysine with lysine.

SUMMARY

1. Hydroxylysine, the amino-acid prepared from gelatin by Van Slyke and co-workers has been independently isolated by fractional recrystallization of its picrate, from a sample of crude lysine picrate. Some of the properties of hydroxylysine picrate have been described.

2. The difficulties encountered in the separation of hydroxylysine picrate from that of lysine by fractional crystallization are stressed, and evidence is produced indicating that even the best samples of hydroxylysine so far prepared are not entirely free of lysine.

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The Reaction of Nucleic Acids with Mustard Gas

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The delayed healing of lesions caused by mustard gas (*H*) provoked speculation that the cell nuclei are especially concerned, and confirmation of this view has accumulated from various sources. Auerbach & Robson (1942, 1946) showed that the exposure of *Drosophila melanogaster* to *H* in sublethal doses caused reduced fertility and induced visible mutations in succeeding generations. Robson (1943) observed that pollen grains of *Tradescantia* after treatment with *H*, showed widespread disorganization of chromosomes, and Fell & Allsopp (1943) noted similar behaviour in the cells of *H*-treated mouse skin. Koller (1943) records that during the 48 hr. following a short (30 sec.) exposure of *Tradescantia* pollen grains to concentrated *H* vapour, chromosome breakage, structural changes including new rearrangements, and adherences of adjacent chromosomes were induced at various loci in the chromosomes. He observed that the separation of the chromosomes at anaphase and telophase of mitosis was prevented by these attachments. These results focused attention on the action of *H* on nucleoproteins, and Berenblum (1940 *a, b*) and Berenblum & Schoental (1947) found that aqueous extracts of skin or solutions of thymus nucleoprotein underwent irreversible precipitation when treated with *H*, the precipitate having a higher sulphur content than the original nucleoprotein.

The action of *H* on proteins has been studied by many investigators, e.g. Berenblum & Wormall (1939); for reviews see Rydon (1943 *a, b*). The purpose of the present investigation was therefore to investigate the reaction of *H* with nucleic acids and their components.

EXPERIMENTAL

Choice of nucleic acids for investigation. The ribose nucleic acid of yeast (YNA) and the deoxypentose nucleic acid of calf thymus gland (TNA) were employed in this investigation because of their availability, and because more is known of their structures than of those of nucleic acids from other sources.

Preparation of materials. The samples of yeast ribonucleic acid were obtained commercially and were deproteinized by a combination of the method described by Sevag, Lackmann & Smolens (1938) and the purification process employed by Fletcher, Gulland, Jordan & Dibben (1944). Yeast ribonucleic acid (20 g.), stirred vigorously into water (250 ml.), was brought into solution at pH 7 by the addition of *N*-NaOH (about 48 ml.). This solution was shaken vigorously with the following volumes of a mixture (4 : 1) of chloroform and amyl alcohol: 200 ml. four times, 100 ml. ten times. After each shaking, the mixture was centrifuged and the aqueous layer containing the nucleic acid was separated from the chloroform layer which contained the protein as a stable gel. When gel formation no longer occurred after shaking, the solution of protein-free nucleic acid was slowly added to a stirred mixture of ethanol (1180 ml.) and concentrated HCl (6.6 ml.). The precipitated acid (12 g.) was centrifuged and washed three times with 66% aqueous ethanol when it was found to be free from chloride ions; it was then washed successively, once with 80 and 100% ethanol, finally with ether, and dried in a vacuum over P_2O_5 -KOH at room temperature. Analyses of various samples are recorded in Table 2.

Deaminated yeast ribonucleic acid (DYNA) was prepared from YNA as described by Fletcher, Gulland, Jordan & Dibben (1944). The samples of the sodium salt of deoxypentose nucleic acid of low molecular weight were obtained commercially and deproteinized by the procedure described above. The sample of high molecular weight had