

SULPHUR IN THE PROTEIN MOLECULE

by

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I. INTRODUCTION

Fleitmann (1846, 1848) working in Liebig's laboratory observed that only a part of the sulphur of egg albumin was removed by heating the protein with alkali and from this and similar observations on other proteins he concluded that sulphur was linked in proteins in more than one manner. At this time cystine and taurine were the only two sulphur containing compounds that had been isolated as products of animal metabolism. From the action of these two compounds with alkali he concluded that sulphur in proteins was present in two forms, oxidised and unoxidised, the unoxidised form being converted to hydrogen sulphide on treatment with alkali and the oxidised form remaining unchanged in the protein molecule.

The first real evidence that the terms oxidised and unoxidised sulphur could not wholly explain the linkages of sulphur in the protein molecule was shown by Krüger (1888). He showed that certain mercaptans and thioethers, unoxidised forms of sulphur, were not decomposed by treatment with alkali to give hydrogen sulphide, or, as he expressed it, these compounds were "nicht bleischwärend"; on the other/

other hand he showed that certain other forms of oxidised sulphur such as sulphonic and sulphinic acids were decomposed in the presence of alkali and gave as decomposition products alkali sulphites. As a conclusion from these experiments Krüger adopted the terms loosely bound sulphur (locker gebundener Schwefel) and firmly bound sulphur (fest gebundener Schwefel). Suter (1894 working in Baumann's laboratory recognised a similarity between the action of alkali on cystine and the proteins, but these observations did not become of real significance until after the independent discoveries of K.A.H.Mörner (1899) and G. Embden (1901)^x of cystine as a normal breakdown product on the hydrolysis of proteins. Mörner isolated cystine from the keratins, horn and/

x

R. Külz discovered cystine as a product of the pancreatic digestion of fibrin, but his untimely death prevented a complete identification and analysis of his product, but he was certain of the identity of his product being a student of Baumann and having worked with it in Baumann's laboratory. See E. Külz. Zeit.f. Biol. 27, 415, 1890.

and hair, and Embden isolated it from egg albumin, serum albumin and edestin. Mörner (1901-1902) then attempted to solve the question whether the sulphur linkage in this breakdown product, cystine, represented the whole or a part of the sulphur in proteins. He observed that approximately 75% of the sulphur of cystine was removed by heating with alkali under standard conditions and that approximately 75% of the sulphur of the proteins, horn, hair, serum albumin and serum globulin, was removed by alkali under these same conditions, and from this constant proportion of detachable sulphur he assumed that sulphur was combined in these proteins only in the form of cystine. On the other hand he found in the proteins, fibrinogen and crystalline ovalbumin, after the removal of the cystine there remained 66.7% and 83.8% of the sulphur respectively and that only 23.9% and 27.1% respectively of this sulphur was lead blackening, and from this he concluded that in certain proteins other sulphur compounds than cystine existed.

Osborne (1902) using the sulphur content of proteins in an attempt to determine the molecular weights did many accurate determinations of the loosely bound sulphur. Although he was not primarily concerned in the presence of non-cystine sulphur in proteins/

proteins his results led him to the conclusion that such sulphur is present in proteins. Particularly interesting was his determination of the loosely bound sulphur of casein in which he found that only 13% or roughly 1/8 of the sulphur was loosely bound sulphur. Casein was not examined in detail by K.A.H. Mörner who was not convinced of the purity of Hammersten's protein.

More conclusive evidence of the presence of non-cystine sulphur was shown by C.Th. Mörner (1914) by his studies of the oxidation of a number of proteins with nitric acid. By the oxidation of the following proteins he isolated methyl sulphonic acid as the barium salt $(\text{CH}_3\text{SO}_3)_2\text{Ba}$ (expressed as gm. per kilo of protein), serum albumin (3.1), egg albumin (5.8), casein (5.5), ovomucoid (12.4), haemoglobin (3.1), sheep's wool (1.8), gelatine (1.4). Mörner also repeated his technique of oxidation with nitric acid on cystine and the only organic product he was able to isolate was a small quantity of oxalic acid, thus showing that the mother substance of the methyl sulphonic acid could not be cystine, but some yet unknown form of sulphur in the protein molecule.

Mueller (1921) in the course of an investigation of the cultural requirements of certain pathogenic bacteria found that a substance which occurred in meat/

meat infusions and in certain of the proteins, notably casein, was essential for the growth of hemolytic streptococcus and certain strains of pneumococcus. Since this growth-stimulating substance was removed by boiling meat infusions and commercial preparations of peptone with charcoal (Norit) Mueller concluded that "It seemed most probable an amino acid or polypeptide was in question". Continuing this line of reasoning he added a neutral sulphuric acid hydrolysate of casein to the inactive charcoal treated meat infusion and found that the hydrolysate was as effective in growth promotion as an addition of commercial peptone. He then prepared other "active" hydrolysates from edestin and meat residue, but found that the hydrolysate from egg white was only weakly active and those from wheat gluten, gelatine, wool and silk were quite inactive. To test the possibilities of the constituents of nucleic acid he prepared hydrolysates of yeast and salmon sperm but found these to be inactive. Next he attempted a separation of the substance from the casein hydrolysate. The method used was the butyl alcohol method of Dakin (1920). He found the active substance among the monoamino fraction and then attempted further separation with mercuric sulphate in sulphuric acid solution and found that this reagent/

reagent precipitates the active material. At this point he also tested the other amino acids precipitated by this reagent both singly and in combination. The above mentioned amino acids are of course cystine, tryptophane, tyrosine and histidine, and all were found to be inactive. He next found that the mercuric sulphate reagent precipitates the active substance from the original hydrolysate.

In a later paper (1922) he reported that this substance was definitely an amino acid and contained sulphur and that on extreme purification by mercuric sulphate, it probably lost its growth-promoting character. To eliminate the possibility that this sulphur amino acid had been formed from the acid hydrolysis of casein he then prepared it from Aminoids ("a commercial enzyme digest of milk proteins to the amino acid stage"). The sulphur amino acid was isolated from the Aminoids without further hydrolysis by means of the mercuric sulphate reagent followed by silver sulphate for further purification. In this paper a description of the isolation is given (apparently du Vigneaud and Meyer (1931) overlooked this paper), but no analysis or physical properties beyond the description of the crystalline shape of the sulphur amino acid are given.

Mueller/

Mueller (1921) published a paper bearing the title "A new sulphur containing amino acid isolated from casein". He reported that he hydrolysed 30 lbs. of commercial Argentine casein with sulphuric acid, neutralised with sodium carbonate and after partial separation of the sodium sulphate he added the mercuric sulphate reagent, filtered off this precipitate, and reprecipitated with mercuric sulphate, the sulphur compound remaining in the filtrate. This filtrate was further purified by the addition of silver sulphate and barium hydroxide and then freed from silver and barium, the amino acid being obtained by fractional crystallisation. The yield was 10 gm. of a crude product. As a criterion of purity, amino nitrogen determinations were carried out after successive crystallisations until a constant value was obtained. His analysis of this product indicated the formula $C_{11}H_{23}SN_2O_4$. He then found when most of his material was exhausted that his substance contained a material forming a hydrochloride relatively insoluble in concentrated hydrochloric acid - most probably glutamic acid, see Plimmer

In a later paper (1923) Mueller described a greatly improved method of isolation of the sulphur/

sulphur amino acid whereby from the sulphuric acid hydrolysate of casein he was able to obtain about 1-2 grams of a fairly pure product from a pound of casein, i.e. from 0.2% to 0.4%. This represented about ten times the quantity obtained from the earlier investigation. This increase in yield was obtained by the observation that most of the sulphur amino acid remained in the acid filtrate from the precipitation with mercuric sulphate reagent. Therefore after the addition of the mercuric sulphate reagent in sulphuric acid solution he neutralised the hydrolysate carefully to congo red with sodium hydroxide and filtered off the now, very bulky, mercuric sulphate precipitate. Discarding the filtrate, he treated the washed precipitate with baryta until alkaline to litmus and further extracted with 2% baryta. To these combined extracts, after quantitative removal of mercury and barium and evaporation to 200 c.c. for each lb. of casein used, he added 30 gm. of mercuric chloride per lb. of casein used and obtained a precipitate. After an extraction of this precipitate with saturated barium sulphide solution and a quantitative removal of mercury, barium and electrolytes, the extracts were evaporated to crystallisation in vacuo, and redissolved in a small/

small quantity of water. On the addition of three or four volumes of 95% alcohol to this water solution crystals separated. Further purification was obtained by reprecipitation with mercuric chloride and by the preparation of the barium salts of the amino acids in the crude product, whereby glutamic acid was removed by taking advantage of the relative insolubility of its barium salt in alcohol. He was, however, unable to remove completely the phenylalanine from the crude product even by distillation of the ethyl esters, but by one experiment he was able to obtain a small quantity, 0.35 gm. from 6.8 gm. of ester, "which proved to be fairly pure", and from which he deduced the formula $C_5H_{11}SNO_2$ (Calc. C = 40.23%, H = 7.43%, S = 21.50%. Found C = 41.44%, H = 7.34%, S = 20.85%).

F.P. Coyne and the present writer were unable to obtain a crystallisable product after the distillation of the ester of methionine and it is most probable that this ester is for the most part decomposed by distillation.

From the beginning Mueller was extremely conservative as to the fact that this sulphur amino acid was "really a primary component of protein", and he reserved his final opinion until he had tested the following suppositions: 1. That the sulphur had been introduced into the molecule by the sulphuric acid used in the hydrolysis. (A) This seemed very unlikely as the amino acid did not have the properties of a sulphonic acid; (B) That this sulphur amino acid could be isolated from proteins (gelatin) by hydrochloric acid; (C) That it can be isolated by alkaline hydrolysis with sodium hydroxide from the proteins of egg white; (D) That this sulphur amino acid could be isolated from the products of enzymatic digestion.

2. That the sulphur had been introduced from the hydrogen sulphide used throughout the preparation. (A) This question was settled by the use of hydrogen selenide instead of hydrogen sulphide. In order to show further that this sulphur amino acid is a primary product of protein digestion he tested the ability of the human body to oxidise this compound, (1923) realising that the body has the ability to oxidise other than naturally occurring substances; a negative result, that is, the excretion of this sulphur/

sulphur in the neutral sulphur fraction of the urine would show that this compound was a substance foreign to the body. He found, however, that the body was easily able to split out the sulphur from this compound and oxidise it to the form of inorganic sulphate.

S. Odake (1925) reported the presence of a sulphur-containing amino acid in the alcoholic extraction of yeast. From 6000 kg. of yeast he isolated 0.6 gm. of a substance, the formula of which he deduced to be $C_5H_{11}O_2SN$. This compound coloured at 250° (uncorr.) and melted with decomposition at 283° (uncorr.). Odake's α -naphthyl-carbamido derivative melted at 187° (uncorr.) and Mueller's α -naphthyl-carbamido at 186° (uncorr.). From both of these derivatives the same empirical formula was deduced, that is, $C_{16}H_{18}SN_2O_3$. The copper salts prepared by these two workers also agreed in analysis, crystalline structure, and solubility. The above evidence was considered sufficient proof of the identity of Mueller's and his products. He extracted the fresh yeast with 80% alcohol, and precipitated this alcoholic extract with tannin solution; this tannin precipitate/

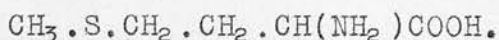
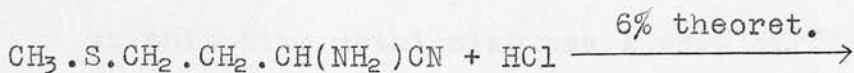
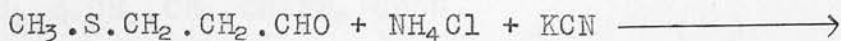
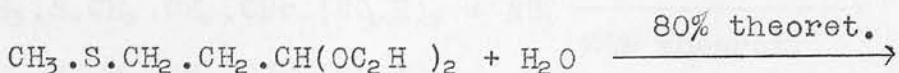
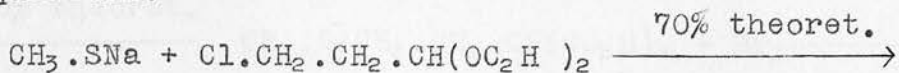
precipitate was treated in the usual manner with baryta. From the filtrate, after removal of barium, there separated in the cold crystals of adenyl-thio-methylpentose and calcium sulphate. To the mother liquor of these crystals alcohol was added to a concentration of 80% and there separated a precipitate which was filtered off and washed with alcohol. This precipitate was again dissolved in water, decolorised by charcoal, and reprecipitated by 70% alcohol. By repeated recrystallisations from 60% and 70% alcohol, white plates separated. From 6000 kg. of yeast 15 gm. of a crude product containing a large amount of leucine together with the sulphur-containing acid, were obtained. For the purification of this crude product it was boiled with 20% sulphuric acid, sulphuric acid removed, and boiled up again with 15% hydrochloric acid and this also removed. The copper salts were prepared and the practically insoluble leucine copper salt removed. The copper was removed and the resulting solution precipitated with mercuric chloride; after two successive precipitations with this reagent an analytically pure product weighing 0.6 gm. was obtained. Microphotographs of these crystals showed them to be hexagonal plates. This substance gave a rotation of $[\alpha]_D^{16} = -11.77^\circ$.

Barger/

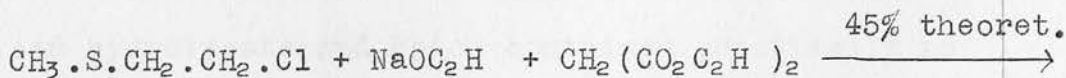
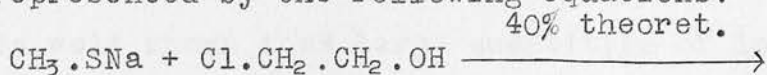
Barger (1928) while engaged in the investigation of a possible precursor (thiolhistidine) of the interesting metabolite ergothioneine, became interested in Mueller's sulphur amino acid. A sample of this substance was isolated from casein by Mueller's method and found to be stable to boiling hydrochloric acid but it was found that boiling hydriodic acid liberated methyl iodide and a trace of hydrogen sulphide from the compound. The quantitative method of Kirpal and Böhn (1914, 1915) for the determination of CH_3S - groups was applied to this compound and one such group was found to be present. This of course limited the possible formulae to derivatives of butyric and isobutyric acid, and as all of the known naturally occurring amino acids contain the amino group in the α -position, only four formulae remained. These were: (I) α -, (II) β - and (III) γ -methylthiolbutyric acid, and (IV) methylthiolisobutyric acid. Of these possible formulae the γ -methylthiol- α -aminobutyric seemed the most probable because of its analogy with the compound cheirolin, $\text{CH}_3\text{.SO}_2\text{.CH}_2\text{.CH}_2\text{.CH}_2\text{.N:C:S}$, isolated by Schneider (1910) from wallflower seeds (Cheiranthus cheiri) which would be, if this analogy were correct, the/

the sulphone of a mustard oil derived from Mueller's acid. This most probable formula (III) was synthesised and found to be identical with Mueller's acid in all of its properties except, of course, optical activity. After consultation with Dr J.H. Mueller, Professor Barger and Dr Coyne decided on the very appropriate name Methionine for this interesting substance. All attempts to synthesise methionine by Erlenmeyer's methods were unsuccessful. It was found impossible to condense methylthiolacetaldehyde, $\text{CH}_3 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHO}$, with hippuric acid and with diketopiperazine and condensation with hydantoin was only accomplished with the very poor yield of 20%, and on the condensation product, methylthiolethylidene hydantoin, no reduction could be effected. Condensation of phenoxyacetaldehyde, $\text{C}_6\text{H}_5\text{O} \cdot \text{CH}_2 \cdot \text{CHO}$, with hydantoin was also unsuccessful. The Zelinsky and Stadnikoff (1908) modification of Strecker's synthesis was next attempted and was successful, but the yield was only 6% (based on the β -methylthiolpropaldehyde used) and involved the use of the rather inaccessible intermediate, acrolein. This synthesis is represented by the following equations/

equations:

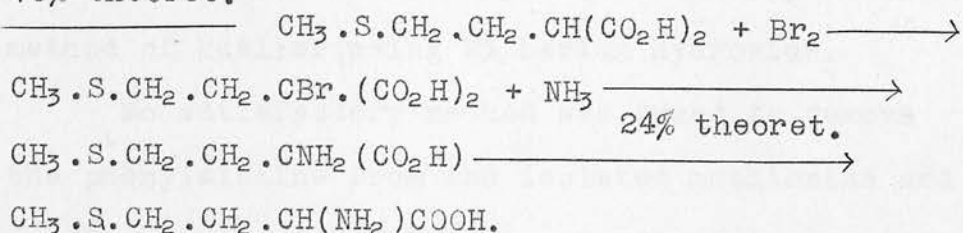


In 1930 Windus and Marvel (1930) using the well known malonic ester synthesis, published a method of preparation of methionine which they considered more satisfactory than that of Barger and Coyne, but their synthesis did not result in a greatly improved yield (7% based on the α -methylthiol- β -chloroethane used) due to the oxidation of the methylthiol group in the bromination of β -methylthiolethylmalonic acid. Their synthesis is represented by the following equations:



73%/

73% theoret.



At this time methionine was a very difficult substance to obtain in any quantity either by isolation from proteins or by synthesis. The primary purpose of this research was first to make this compound readily available, and secondly to make a study of its optical properties and its function in animal metabolism.

An isolation of methionine from casein was carried out by the general method of Mueller with the following modifications: (1) The greater part of the sulphuric acid used in the hydrolysis of the casein was removed by barium hydroxide before precipitation by the mercuric sulphate reagent, as it is well known that large quantities of inorganic salt greatly hinders the efficiency of this reagent; (2) The acid precipitate from the partially neutralised hydrolysate and which contained practically no sulphur was discarded, and after complete removal of sulphuric acid by means of barium hydroxide a further precipitation with mercuric sulphate was carried/

carried out and this precipitate worked up after the method of Mueller, using 2% barium hydroxide.

No satisfactory method was found to remove the phenylalanine from the isolated methionine and any advantage gained by the removal of inorganic salts was vitiated by the loss of methionine incurred in the handling of the large barium sulphate precipitates. Although these precipitates were repeatedly washed, the loss in this manner was considerable, and the filtration of these precipitates considerably lengthened an already slow and laborious procedure. The yields of relatively pure substance were no improvement on those of Mueller, but were in fact less than his best reported yields. Mueller reported from 0.2% to 0.4% yield of a 75% to 90% pure substance from casein, and our best yield was 0.18% of a substance giving a sulphur content of 17.88% or approximately 83% pure.

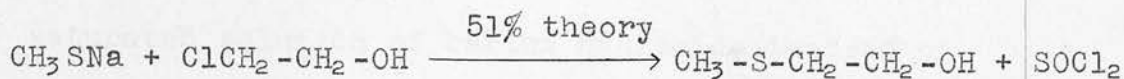
Finding that the isolation technique then known would not satisfy our purposes we then began to look for a synthetic method that would avoid the miserably low yields and the main difficulties of the preceding two syntheses; these were a yield of 6% (1929) and the use of the rather inaccessible and expensive/

expensive intermediate acrolein and a yield of 7% (1930) and the oxidation of the methylthiol group by bromination.

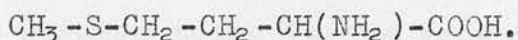
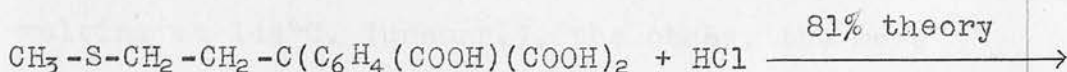
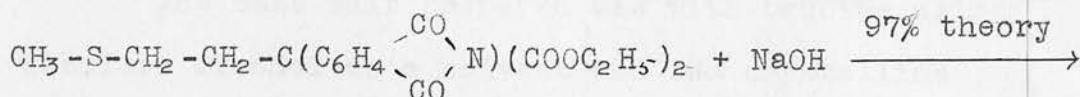
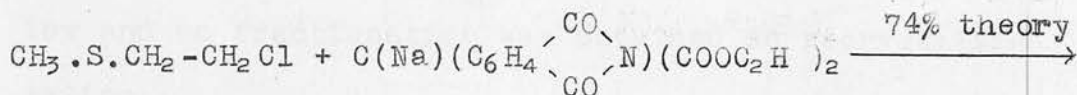
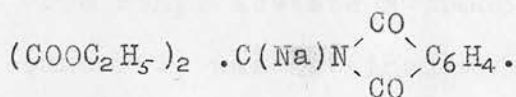
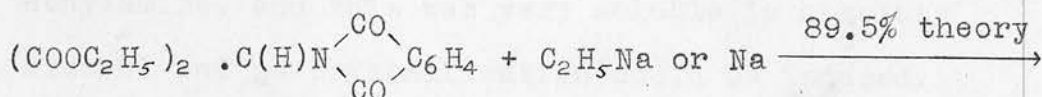
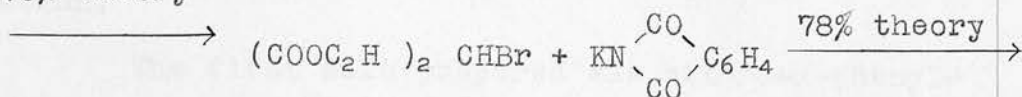
The sodiochloromalonate ester method as used by Pyman (1911) for the synthesis of histidine was tried, but neither ethyl sodiochloromalonate in alcohol solution nor crystalline methyl sodiochloromalonate would condense with α -methylthiol- β -chloroethane under any conditions.

The ethyl sodiophthalimidomalonate method of Sørensen (1903-1906, 1905) was then tried and condensation with α -methylthiol- β -chloroethane was successful in the yield of 74% (based on the α -methylthiol- β -chloroethane used). The subsequent steps of this synthesis were quite straightforward and resulted in good yields. The final yield of methionine, as shown below, was 58% of the theory (based on the α -methylthiol- β -chloroethane used). By means of this synthesis racemic methionine was made readily available (1931, 1934). The complete synthesis is represented by the following equations:





70% theory



Full details of this synthesis are given in the experimental part.

For the study of the optical properties of dl-methionine the general method of Fischer (1905) was used. This method was modified only by adding the alkaloid in two stages and the hydrolysis of the alkaloidal salts was carried out by means of a saturated/

saturated solution of barium hydroxide instead of strong sodium hydroxide to avoid possible reracemisation.

The first salt prepared was with d- α -phenylethylamine, and this was very soluble in absolute alcohol and no crystallisation could be induced. From ethyl acetate a product melting at 87° C. (uncorr.) was obtained which gave the approximately correct sulphur percentage but the rotation was very low and no fractionation was obtained on recrystallisation.

The next salt prepared was with brucine using absolute alcohol as a solvent and two crystalline fractions were obtained: one, the less soluble, melting at 144°C. (uncorr.), the other, the more soluble, melting at 194-195° C. (uncorr.). A very strange thing is the fact that the rotation of these two salts was approximately the same and in the same direction, laevo. This fact made it impossible to determine which isomer we were working with until after the brucine was split off. The less soluble salt, rotation $[\alpha]_D^{20} = -22.42^\circ$, proved to be the dextro isomer, and the more soluble salt, rotation $[\alpha]_D^{20} = -19.86^\circ$, proved to be the laevo isomer.

The/

The rotation of the d-methionine was $[\alpha]_D^{20} = +8.3^\circ$, the rotation of the l-methionine was $[\alpha]_D^{20} = -7.94^\circ$. A sample of the natural l-methionine isolated by the method of Mueller had a rotation of $[\alpha]_D^{20} = -7.23^\circ$; the formyl derivative of this sample had a m.p. 98-99°C. (uncorr.). The m.p. of synthetic l-formylmethionine was 98-99°C. (uncorr.). Mixed m.p. of the two l-formyl-methionines was 98-99°C. (uncorr.). Our results show that it is not probable that much racemisation occurs in the isolation of l-methionine from proteins. Full details of this resolution are given in the experimental part.

Very shortly after this resolution was completed Windus and Marvel (1931) published a resolution of dl-methionine also using the method of Fischer (1905) and reported the following figures: $[\alpha]_D^{25} = +8.7^\circ$ and 8.1° , as the highest figures of their attempts; others of their samples were appreciably lower probably showing that slight racemisation takes place under the experimental conditions used. An interesting observation made by these workers is that the direction of d-methionine changes to laevo in both 5% sodium carbonate solution and normal hydrochloric acid. Other rotations published for natural l-methionine are those of: Mueller $[\alpha]_D = -7.2$ (1923); Odake (1925) $[\alpha]_D^{16} = -11.77^\circ$; du Vigneaud and Meyer (1932) $[\alpha]_D^{25} = -7.3$; Pirie (1932) $[\alpha]_D^{21} = -7.9^\circ$.

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II. EXPERIMENTAL.

(a) Experimental description of the resolution of dl-methionine.

dl-Formylmethionine:

To 10 g. of dl-methionine in a 100 c.c. bolt-head flask fitted with a reflux condenser and calcium chloride trap, there were added 15 g. (12.4 c.c.) of absolute formic acid. This mixture was refluxed for 3 hours on a boiling water bath and the excess formic acid afterwards distilled off in vacuo. This mixture was treated twice more in the above manner with 15 g. portions of absolute formic acid and on final removal of the excess formic acid and water the residue was taken up in hot ethyl acetate and the solution filtered. The filtrate on standing overnight in the ice-chest crystallised in the form of prismatic white needles. After working up the mother liquor of these crystals a total yield of 9.5 g. (80.5% theory) of a product melting at 99° (uncorr.) was obtained. (All sulphur estimations in these experiments were done by the method of H.T. Clarke and/

and H. Zahnd (1930).

Analysis: 4.158 mg. of sample gave 5.482 mg. BaSO_4 .

Found 18.11% S; calculated for $\text{C}_6\text{H}_{11}\text{O}_3\text{NS}$ 18.10% S.

The brucine salts of dl-formylmethionine:

l-brucine-d-formylmethionate.

5 Gm. of dl-formylmethionine were dissolved in 1000 c.c. of hot absolute alcohol and to this solution there was added anhydrous brucine (7 g.) m.p. 177°C . (uncorr.) (approximately 0.6 amount to form neutral salt). This alcohol solution was placed in the ice-chest and after two days filtered and 8 g. of a white crystalline substance melting at $143\text{--}144^\circ\text{C}$. (uncorr.) were obtained. This substance was recrystallised from hot alcohol and now had a m.p. 144°C . (uncorr.).

Rotation: 0.1505 g. of sample made up to 25 c.c.

with water at 20°C . 2 dm. tube.

$$[\alpha]_{\text{D}} = -0.27^\circ$$

$$[\alpha]_{\text{D}}^{20^\circ} = -22.42^\circ$$

Analysis:

8.452 mg. of sample gave 3.408 mg. BaSO_4 .

Found 5.54% S; calculated for $\text{C}_{29}\text{H}_{37}\text{O}_7\text{N}_3\text{S}$ 5.61% S.

β -Formylmethionine:

7 g. of this salt were dissolved in 25 c.c. of water and to this solution, in an ice-salt bath, there were slowly added with stirring about 100 c.c. of a saturated solution of $\text{Ba}(\text{OH})_2$. After the precipitation seemed complete the brucine was filtered off and the filtrate extracted with chloroform until a sample of the aqueous solution no longer gave the brucine reaction with nitric acid (an intense red colour). After a final extraction with ether the aqueous solution was quantitatively freed of barium ions by the addition of normal sulphuric acid, and after removal by filtration of BaSO_4 , the water was removed in vacuo and the residue taken up in the minimum quantity of hot ethyl acetate and filtered, a very small insoluble fraction remaining on the filter. The ethyl acetate solution on cooling in an ice-bath and the addition of a few drops of ether began to crystallise and 0.85 g. of a product (white prismatic needles) melting at $98-99^\circ\text{C}$. (uncorr.) was obtained.

Rotation: 0.1530 sample made up to 25 c.c. with water at 20°C . 2 dm. tube.

$$[\alpha]_D = +0.12^\circ$$

$$[\alpha]_D^{20^\circ} = +9.8^\circ$$

Analysis: /

Analysis: 4.530 mg. of sample gave 5.986 mg. of BaSO₄

Found 18.12% S; calculated for C₆H₁₁O₃NS 18.10% S.

d-Methionine.

0.5 G. of d-formylmethionine was dissolved in 10 c.c. of 5 N. HCl and heated on a boiling water-bath for one and a half hours. HCl was then removed in vacuo and the residue taken up in the minimum quantity of water, 2 c.c. pyridine added and 3 volumes of hot alcohol. On cooling the alcohol solution in the ice-bath d-methionine separated. After recrystallisation from alcohol-water 0.210 g. of substance having the following rotation and analysis was obtained.

Rotation: 0.1505 g. of sample made up to 25 c.c. with water at 20°C. 2 dm. tube.

$$[\alpha]_D = +0.10^\circ$$

$$[\alpha]_D^{20} = +8.3^\circ$$

Analysis: 6.645 mg. of sample gave 10.380 mg. BaSO₄

Found 21.46% S; calculated for C₅H₁₁O₂NS 21.50% S.

l-brucine/

l-Brucine-l-formylmethionate:

To the filtrate from the l-brucine-d-formylmethionate a further 7 g. of anhydrous brucine were added, and after standing in the ice-chest for two days a small quantity of a white crystalline substance was deposited having no definite melting point, and was discarded. The filtrate from this substance was distilled in vacuo to a volume of about 300 c.c. and after standing in the ice-chest for 2 days 4.5 g. of a white crystalline substance melting at 193-195° C. (uncorr.) were obtained. By working up the mother liquor another 0.5 g. was obtained. These two fractions were combined and after recrystallisation from the minimum quantity of hot absolute alcohol had a m.p. 194-195° C. (uncorr.).

Rotation: 0.1510 g. of sample made up to 25 c.c. with water at 20°C. 2 dm. tube.

$$[\alpha]_D = -0.24^\circ$$

$$[\alpha]_D^{20^\circ} = -19.86^\circ$$

Analysis: 8.540 g. of sample gave 3.450 mg. BaSO₄

Found 5.55% S; calculated for C₂₉H₃₇O₇N₃S 5.61% S

l-Formylmethionine:

4.5 g. of the above salt were dissolved in 20 c.c. water and treated with 75 c.c. saturated solution $\text{Ba}(\text{OH})_2$ in the same manner as described for the d- salt. The same procedure was subsequently used as with the d- salt and the only difference found was a greater difficulty in getting the isomer to crystallise from ethyl acetate and ether had to added to the point of turbidity to induce crystallisation to begin. 0.480 G. of a substance (white prismatic needles) m.p. $98-99^\circ\text{C}$. (uncorr.) was obtained.

Rotation: 0.1520 g. of sample made up to 25 c.c. with water at 20°C . 2 dm. tube. (0.120 g. of the above sample recovered)

$$[\alpha]_D = -0.11^\circ$$

$$[\alpha]_D^{20^\circ} = -9.04^\circ$$

Analysis:

4.130 mg. of sample gave 5.451 mg. BaSO_4

Found 18.13% S ; calculated for $\text{C}_8\text{H}_{11}\text{O}_3\text{NS}$ 18.10% S.

l-methionine/

l-Methionine:

0.440 g. of l-formylmethionine was dissolved in 10 c.c. of 5 N. HCl and heated on a boiling water-bath for $1\frac{1}{2}$ hours; HCl was removed in vacuo and the residue taken up in a minimum of hot water, 2 c.c. of pyridine added and 3 volumes of alcohol. On cooling the alcohol solution in an ice-bath l-methionine separated. After recrystallisation from a water-alcohol mixture 0.175 g. of a substance having the following rotation and analysis was obtained.

Rotation: 0.1510 g. of sample made up to 10 c.c. with water at 20°C. 1 dm. tube.

$$[\alpha]_D = -0.12^\circ$$

$$[\alpha]_D^{20^\circ} = -7.94^\circ$$

Analysis: 7.152 mg. of sample gave 11.163 mg. BaSO₄.
Found 21.44% S; Calculated for C₅H₁₁O₂NS 21.50% S.

Natural Methionine:

A sample of natural methionine isolated by the method of Mueller and with a sulphur analysis of 20.80% which indicated 96.7% purity (this was obtained, in a poor yield, from a sample of 88% purity/

purity by taking advantage of the relative insolubility of phenylalanine hydrochloride in concentrated HCl).

Rotation: 0.1520 g. of sample made up to 10 c.c. with water at 20°C. 1 dm. tube.

$$[\alpha]_D = -0.11^\circ$$

$$[\alpha]_D^{20^\circ} = -7.23^\circ$$

Analysis: 8.153 mg. of sample gave 12.346 mg. BaSO₄. Found 20.80% S; calculated for C₅H₁₁O₂NS 21.50% S.

0.3 g. of the above sample was converted to the formyl derivative in the same manner as was the racemic methionine. M.p. 98-99°C. (uncorr.). Mixed melting point with the resolved synthetic l-formylmethionine 98-99°C. (uncorr.).

(b) Experimental description of the synthesis of dl-methionine.

α -Methylthiol- β -chloroethane.

Methyl isothiourea was prepared according to the method of Arndt (1921) in a yield of 95% of the theory. 440 G. of this substance were hydrolysed by 670 c.c. of 5 N. NaOH and the methyl mercaptan obtained was directly absorbed in a solution of 55.0 g. of Na in 1185 c.c. of commercial alcohol. This solution/

solution was heated to boiling and 207 g. of ethylene chlorohydrin were added in such a manner that the solution continued to reflux gently. After the addition of the ethylene chlorohydrin the solution was boiled for 10 hours. It was found inadvisable to distil the alcohol from the solution since the large amount of NaCl present caused considerable danger of breakage from bumping. The solution was centrifuged and the salt shaken up with successive quantities of absolute alcohol until it retained no appreciable odour of the thiol compound. The alcoholic solution of the thiol compound was then distilled in vacuo, the yield of α -methylthiol- β -hydroxyethane being 126 g. of a product boiling at 59-70°/16 mm. (51% of the theory based on the ethylene chlorohydrin employed).

The α -methylthiol- β -hydroxyethane was converted to the chloro compound after the method of Bennett (1929) for similar compounds. 126 G. of thionyl chloride were dissolved in 700 g. of dry chloroform and the solution was added dropwise to the dimethylaniline solution of the thiol compound. The solution was kept at room temperature for 2 hours and then heated for 20 minutes on the steam-bath. It was then washed three times with concentrated HCl and/

and twice with water, dried over calcium chloride, filtered and freed from chloroform by distillation. The residue which consisted of the crude chloro-sulphide, was distilled in vacuo. The yield was 115 g. of a product boiling at 50°/25 mm. (70% of the theory).

Ethyl phthalimidomalonate.

This compound was prepared according to Sørensen (1905). This preparation is also given in "Organic Syntheses" (1927). The average yield of several experiments was 78% of the theory.

Ethyl sodiophthalimidomalonate.

This compound was prepared by the method of Sørensen (1905) as follows: 4.6 g. of clean sodium were made to react with 150 c.c. of absolute alcohol (freshly distilled from over sodium). After the sodium had completely reacted with the alcohol and the temperature of the solution was about 60°, 63 g. of ethyl phthalimidomalonate were added and the mixture was shaken. A clear orange-yellow solution was produced and on cooling the flask with ice-water, the substance crystallised quite rapidly into an almost solid mass. The sodium compound was quickly/

quickly filtered at the pump and was twice washed with absolute alcohol and twice with absolute ether and was then dried in a vacuum desiccator over sulphuric acid. The yield of this substance was 67 g. and by neutralisation of the mother liquors with hydrochloric acid 5.5 g. of the ester were recovered. A sample of the sodium compound was dried to constant weight (alcohol of crystallisation). This corresponds roughly to $1\frac{1}{2}$ of alcohol (17.43%). As a result of the loss of the alcohol of crystallisation the colour of the substance changed on drying from a rather deep orange-yellow to a very light yellow. This is quite convenient as the colour change can be successfully applied as a gauge when drying large quantities of the compound. It is essential to obtain an almost alcohol-free product if one wishes to obtain the highest yield possible in the subsequent step. In order to drive off the alcohol the substance was placed in a bolt-head flask fitted with a good rubber stopper and a glass tube outlet and heated in an oil-bath at 145-155°/12 mm. with occasional shaking, for 8 hours or until the colour of the substance changes from the orange-yellow colour to a light yellow one. Yield was 89.5% of the theory.

Analysis/

Analysis: 5.491 mg. of sample gave 1.194 mg. Na_2SO_4 .

Found 7.036% Na; calculated for $\text{C}_{15}\text{H}_{14}\text{O}_6\text{NNa}$ 7.04% Na.

In order to avoid the inconvenience of having to rid the substance of its alcohol of crystallisation, the method of Dunn and Smart (1930) was tried, but was found not to be as satisfactory as the above as particles of unreacted sodium were often found in the finished substance.

Ethyl β -methylthiol- α -phthalimidoethylmalonate.

85 G. of the ethyl sodiophthalimidomalonate were placed in a 250 c.c. bolt-head flask fitted with a three-holed stopper for condenser, thermometer and stoppered glass tube for sampling. 43 G. of α -methylthiol- β -chloroethane (60% excess) were also placed in the flask and the flask, fitted as described above, was heated in an oil-bath at 150° (temp. of the bath) until the reaction mixture no longer gave an alkaline reaction to tumeric paper. In this case the time was one hour and forty-five minutes for completion of the reaction. The flask was then fitted with a still head and the excess of the chloro compound distilled off in vacuo (after redistillation/

redistillation 12 g. of a pure product were obtained). The residue, which consisted of a yellow oil, was mixed with warm water and poured into an evaporating dish where it almost immediately crystallised. The crystalline substance was again extracted with water to remove any remaining sodium chloride and recrystallised from the smallest quantity of alcohol possible. The crystals were white prisms having a m.p. 67° (uncorr.). The yield was 79 g. (74% of the theory, based on the chloro compound used).

Analysis: 5.300 mg. of sample gave 11.070 mg. CO_2
and 2.64 mg. H_2O .

Found 56.96% C, 5.52% H; calculated for $\text{C}_{18}\text{H}_{21}\text{O}_6\text{NS}$
57.14% C, 5.58% H.

10.010 mg. of sample gave 6.210 mg. of BaSO_4 .

Found 8.53% S; calculated for $\text{C}_{18}\text{H}_{21}\text{O}_6\text{NS}$ 8.44% S.

β -Methylthiol- α -phthalaminoethylmalonic acid.

25 G. of the ester were dissolved in 30 c.c. of alcohol in a 200 c.c. bolt-head flask, placed on a boiling water-bath and 70 c.c. of 5 N. NaOH were added. The cloudy liquid was heated until a sample gave a clear solution when diluted with water. The sodium salt of the acid is very insoluble in/

in an excess of NaOH but very soluble in water. For the preparation of methionine it is unnecessary to isolate the free acid, though this was actually done. After the hydrolysis was completed the solution was cooled to 0°C. in an ice-salt mixture and carefully neutralised with 0.2 N HCl to Congo red paper. To this neutral solution, still at 0°C., 75 c.c. of 5 N HCl were added carefully during which the acid began to separate in the form of white crystals. In order to complete the salting-out process 60 c.c. of concentrated HCl were added slowly. The white crystalline mass was then filtered at the pump and ridded of NaCl by trituration with ice-cold water. The yield was 21.5 g. and 0.5 g. more was obtained by working up the mother liquors (Yield 97% of the theory). M.p. 142-143° (uncorr.).

Analysis: 4.810 mg. of sample gave 8.740 mg. CO₂
and 2.07 mg. H₂O.

Found 49.55% C, 4.78% H; calculated for C₁₄H₁₅O₇NS
49.27% C, 4.40% H.

11.947 mg. of sample gave 8.305 mg. BaSO₄.

Found 9.56% S; calculated for C₁₄H₁₅O₇NS 9.38% S.

Methionine/

Methionine.

21.5 G. of the above acid were placed in a litre flask and suspended in 350 c.c. of water. The flask was then placed on a boiling water-bath and 40 c.c. of concentrated HCl were added. Almost immediately CO₂ was given off and the substance began to go into solution. The heating was continued for about one and a half hours and 200 c.c. more of concentrated HCl were added which did not produce a further precipitate. It was then heated for 45 minutes to complete the reaction. The water and acid were then removed by distillation first at ordinary pressure and then in vacuo to remove the last traces of acid. In the cool solution, before the above distillation, about 75% of the resulting phthalic acid crystallises and is filtered off and it is advisable to rid this solution of as much phthalic acid as possible as pyridine phthalate separates with the methionine and is very troublesome to separate.

The residue from the vacuum distillation was taken up in warm water, 18 c.c. of pyridine were added, and three volumes of hot alcohol. On cooling the methionine separated as white crystals.

The/

The methionine was then suspended in 200 c.c. of boiling absolute ether in order to remove any remaining phthalic acid, filtered at the pump and dried in a vacuum desiccator over sulphuric acid. M.p. 279-280° (uncorr.). The yield was 8 g. (81% of the theory based on the α -methylthiolphthalamido-ethylmalonic acid employed, or 58% of the theory based on the α -methylthiol- β -chloroethane employed.

Analysis: 5.115 mg. of sample gave 7.590 mg. CO₂ and 3.36 mg. H₂O.

Found: 40.47% C, 7.29% H; Calculated for C₅H₁₁O₂NS
40.23% C, 7.43% H.

11.934 mg. of sample gave 18.645 mg. BaSO₄.

Found: 21.46% S; calculated for C₅H₁₁O₂NS 21.50% S.

Methionine methyl ester hydrochloride.

2 G. of methionine were suspended in 125 c.c. of absolute methyl alcohol, the suspension cooled in an ice-salt mixture, and dry HCl passed in for 2 hours. The alcohol and HCl were then distilled off in vacuo. The residue was taken up in acetone from which it crystallised after one day in a vacuum desiccator. The ester hydrochloride was filtered off and washed with a small quantity of absolute ether. The yield was 1.9 g. (71% of the theory) of/

of white needle-like crystals. M.p. 135° (uncorr.)

Analysis:

5.41 mg. sample gave 3.881 AgCl.

Found 17.74% Cl; calculated for $C_6H_{11}O_2NSCl$ 17.59
Cl

III /

III. METABOLISM EXPERIMENTS WITH METHIONINE

- (a) Feeding experiments with methionine and a description of a characteristic syndrome of cystine deficiency in the albino rat.

Since methionine is the only sulphur amino acid except cystine that has been isolated from proteins and since cystine is an "essential amino acid" for the normal growth of young rats as was shown conclusively by Osborne and Mendel (1915), Abderhalden (1915-16), Sherman and co-workers (1925 etc.), it seemed interesting to determine whether methionine could be interchangeable with cystine in the diet of growing young rats as Sherman and Woods (1925) have shown that there is, between certain limits, an arithmetical proportion between the amount of cystine added to a cystine-deficient diet and the growth increase in response to this addition. In parallel experiments with graded additions of casein, a protein which a deficiency in cystine is the limiting amino acid deficiency, to the same basal diet gave correspondingly graded responses in the growth rate. They believed that this growth response of the added casein may be interpreted as a measure of the cystine or "cystine plus nutritionally equivalent sulphur-containing radicles" in the casein molecule, and thus interpreted/

interpreted their results would indicate that casein contains not less than 1.3% nor more than 2.5% of cystine or of cystine equivalents or that approximately $\frac{3}{8}$ to $\frac{3}{4}$ of the total S in casein is in the form of cystine or of cystine equivalents and from isolation methods and quantitative chemical analysis the cystine content of casein has been found to be from 0.2% to 0.3%.

The animals used in our experiments were albino rats of the Wistar strain from the "A" stock of a colony belonging to Dr B.P. Wiesner of the Department of Animal Genetics, Edinburgh University. This is a colony which had been inbred by brother-sister mating without special selection for about three years (1935). The animals used were from 50-65 g. in weight and from 8-10 weeks of age. The sex ratio was kept as equal as possible and in our experiments litter mates were paired against litter mates.

The cystine deficient diet used was that of Sherman and Merrill (1925) because it is more stable to deterioration than the classical diet of Osborne and Mendel and allowed a sufficient supply to be mixed in one batch for the duration of the experiment which/

which is in our mind an important factor for homogeneity. The exact composition of our diet was as follows:

Corn starch (Brown and Polson, nitrogen free)	81.85%
Whole milk (Dutch, dried full cream)	16.31%
Sodium chloride (Griffin and Tatlock, A.R.)	1.64%
Ferric ammonium citrate (B.P.)	<u>0.16%</u>
	<u>99.96%</u>

Cod liver oil (Parke Davis, standardised) 4 drops daily

Marmite 75 mg. daily

The cod liver oil supplied an adequate supply of vitamins A and D and the B fraction was supplied by marmite. As vitamin C is not necessary for growth in the rat it was not supplied.

Galvanised iron wire cages with false bottoms were used and cleaned daily with hot water and soap, and fortnightly washed in 10% lysol solution. The temperature of the rat-house was kept at 65-70°F. Both food and animals were weighed on a chemical balance accurate to 0.10 g. The food was weighed in the dry state and then made into a dough, to minimise scattering, by the addition of water into the individual glass feeding cups.

The/

The animals were weighed in a small tared wire cage. These weighings were carried every five days throughout the experiment and were done just before feeding, i.e. 24 hours after the last supply of food. This was found to be very important as weighing at other times gave results which might vary for the same animal, within a period of 24 hours, over as much as 4-6 g. This is a fact which is not sufficiently stressed in the literature of feeding experiments. Water was given ad lib. by the usual glass tube stoppered test tube method.

Our experimental technique varied from any previously published feeding experiments in the following important points: all animals after becoming accustomed to the change from their stock diet (Wiesner and Sheard, 1935) to the basal diet were kept for a period of 25 days on the basal diet. This preliminary control period gives a direct comparison of growth periods after the addition of the deficient substance to the diet. Thus each animal is his own control as well as being controlled by animals kept on the basal diet for the duration of the experiment. This factor, we believe, makes possible the use of a smaller number of animals for a significant experiment. By first feeding our young/

young animals ad lib. of the basal diet we found that they were consuming an average of 8 g. per day. This figure we found is above that of the literature, for example, the experiments of Westerman and Rose (1927) using a diet very similar to our own found that the average daily food consumption over a period of 100 days was 5.7 g. on the basal diet and 7.2 g. on basal plus cystine. The food consumption of our animals was remarkably constant and of the twenty-one animals in a representative experiment (see Table I) only one animal (female 3129) did not consume her 8 g. per day. From the above figures we reasoned that 8 g. per day of our diet would supply an adequate quantity of food. This constant feeding of 8 g. of basal diet per day constituted a restricted diet and took in no account of appetite fluctuation above this amount, yet we believed that a positive result, i.e. a marked increase of growth after the addition of cystine or methionine to the basal diet, would be vastly more significant than an experiment in which daily food consumption varied from animal to animal.

As was mentioned the whole supply of food for the experiment was mixed in one batch. To a quarter of this l-cystine (from wool) was added in the/

the proportion of 2 g. per kilo (0.2%), while to a second quarter dl-methionine (synthetic) was added in the proportion of 2.5 g. per kilo (same amount as cystine based on sulphur content). The above percentage of cystine was based on the experiments of Sherman and Woods (1925) whereby using a very similar diet plus 0.2% cystine they found that young albino rats of their own stock grew at the rate of 28.6 g. per 6 weeks, or 0.68 g. per day, this rate giving a sufficient margin over the basal growth rate to allow for experimental differences. The percentage of methionine, as mentioned, was based on the sulphur content of the cystine portion, our reasoning being that this would probably give a fairer comparison of the two A-A than equimolar quantities.

In our first experiment 24 animals of the Wiesner colony were used, observing the aforesaid experimental conditions; at the end of the 6th week on the basal diet the rats began to die off in a very strange manner. They had eaten their full quota of food, 8 g. per day, up to 2-4 days before their death and were gaining weight at the rate of 2.5 g. (average) per 5 day weighing period. The only/

only symptoms noted at the time were a general sluggishness of movement, and a rather general cyanosis of the feet, ears and nose. On post-mortem examination haemorrhagic spots were noted on the livers and there were no signs of pneumonia. At the end of the 53rd day the rest of this series were killed off. (These animals are not accounted in the summary of the results of these experiments). Believing that there was some infectious disease among the stock all of the cages were disinfected and the place of keeping removed to another building. Another experiment, using 24 animals of the same stock was begun, and after 30 days on the basal diet they were divided into three groups of eight; one group put on the basal diet plus cystine, one group on the basal diet plus methionine, and one group kept on the basal diet.

On the 10th, 11th and 12th days after this division into groups three animals of the basal fed or control rats were dead and showing the same symptoms as the animals of the previous experiment. At this point we were in a rather troubled position as it looked as if our experiment would be uncontrolled if these deaths continued. It was then decided to try the feeding of a 100 mg. suspension/

suspension of cystine in water to one of these animals on the day before its expected death to see if this had any possible effect, reasoning on the basis that none of the cystine or methionine fed rats had died. Rat (male 3030, control group, Table I) on the 13th day consumed only 6.5 g. of food and his movements were extremely sluggish and on the 14th day his condition was worse and only 3 g. of food was eaten. On this night the animal was given a suspension of 100 mg. of cystine in water by means of a tube passed into the throat, and no food was given for 16 hours. At the end of this period the improvement in the animal's appearance was remarkable. He was then given 8 g. of cystine containing diet of which he consumed 6 g. and on the following day he consumed 8 g. of the same diet and was then placed back on the basal diet,^{of} which he continued to eat his full quota. Rat (male 3129, control group) was similarly affected on the 16th day, although he had consumed his entire quota of food up to this day. After the administration by mouth of 100 mg. of cystine his improvement was also remarkable. Two feedings of cystine containing food were given to this animal and/

and he too was replaced on the basal diet. This phenomenon seemed very strange considering the fact of all the previous experiments of Osborne and Mendel, Lewis (1926, 1927), Sherman and Rose on cystine deficient diets, in which no such phenomenon was reported and in which their animals were maintained in health for well over 100 days. The only explanation we have to offer is that these people were using a different strain of animals in which this condition does not appear on a cystine deficient diet. If any of the above experimenters have used the Wistar strain of rat the fact is not mentioned and a search has been made of the literature.

The results of this experiment are summarised in Table I. The average increase in weight per rat over a period of 25 days was as follows:
Controls 16 g.; cystine 27 g.; methionine 22.5 g.
During the preceding control period of 25 days the three groups increased by 12.5 g., 12.1 g., and 13.6 g. per rat respectively. It appears that d-l-methionine added to the Sherman-Merrill diet, can produce increased growth in rats and in this respect it is almost as efficient as cystine. As was reported/

reported in the appended paper other experiments were planned and some were in progress. We decided, however, at this point to use another strain of rats as we believed that really critical experimentation could not be performed with a strain of rats showing the above described effects on a cystine deficient diet. The only colony of known origin available to us was the Glaxo colony of A.L. Bacharach, also originally of the Wistar strain. We obtained 24 rats of this colony, but on feeding these animals on the Sherman-Merrill diet they too were affected in the same manner as the Wiesner stock but in a smaller percentage, as will be discussed later. These experiments were therefore abandoned and an investigation was begun to determine the facts of this phenomenon which we believed to be of great importance.

At the end of the 25th day period of feeding the cystine and methionine in the above experiment these animals were again put on the control diet and after only 11 days one of these animals was affected (methionine fed male 3129). He was fed 125 mg. of methionine in water solution but after two days died. Next (male cystine fed rat (I) 2943) was affected and on treatment with cystine recovered.

In/

In all four rats (those marked * , Table I) of the methionine fed rats were affected and none recovered on treatment with methionine. In all five (those marked † , Table I) of the previously fed cystine rats were affected and four of these animals were recovered by 100 mg. doses of cystine plus 2 feeding of cystine-containing food. The average time of recurrence of the affection after being replaced on the basal diet was ten days. Two of the above rats were recovered again and two failed to recover. The most remarkable of these was rat (female 3075, cystine group). This rat looked quite normal at the night feeding and the next afternoon it was noticed that it had not consumed its food and was extremely sluggish, in fact almost moribund. 100 Mg. of cystine suspension in water was given by tube; the throat was completely paralysed instead of the usual semi-paralysis. The treatment was repeated with 50 mg. of cystine on the next day as the animal was still unable to swallow food. The next day, after this second dose there was a noticeable improvement in its condition, although it had eaten nothing for 3 days. On the fourth day the animal was able to eat 4 gm. of cystine-containing food, the/

the next day was able to consume 6 g. and on the following day the full 8 g. The animal was kept on cystine food for a further 5 days and at the end of this period seemed almost as normal as her mates, except for a slight spinal curvature which persisted. After this 5 day period the animal was replaced on the basal diet and after 17 days died untreated with the typical symptoms.

Two groups of six animals each, three of each group being Wiesner stock and three Glaxo stock, were placed on the cystine plus basal diet and methionine plus basal diet for 90 days and no deaths occurred in any of these animals.

Both the Wiesner and Glaxo stocks were put on the Sherman-Merrill diet to test with further numbers the genuineness of our previous observations. These animals when affected were treated with cystine, methionine, sodium thiosulphate and glycine, with the results as shown in Table II.

The symptoms of this phenomenon are summarised as follows:

1. The animal's appetite generally checks off the day before any marked physical change is noticeable.

- 2./

2. In the early stages of the affection there is a marked curvature of the spine which grows worse as the disease progresses and improves as treatment is given.

3. The animal walks very high in contrast to the usual "belly walk" of the normal rat.

4. There are usually clear signs of icterus.

5. There is a slight cyanosis of the feet, ears and nose in the early stages of the affection, growing more severe as the affection progresses.

6. In the early stages there is a semi-paralysis of the throat and in the last stages there is a complete paralysis of the throat.

7. In all of the animals examined either after death or by killing all of the livers were definitely haemorrhagic.

Summary /

Summary.

In separate batches of young Wistar strain albino rats from two different sources kept on the Sherman-Merrill cystine-deficient diet, a large proportion developed a characteristic syndrome and when untreated died. Cystine or methionine prevented this. Once the symptoms had definitely appeared, cystine brought about recovery in most cases, but methionine was without effect. Males were markedly more susceptible than females.

It is suggested that the long deprivation of cystine and methionine shows a difference in the readiness with which these amino acids can be utilised, but this difference may be connected with the liver damage, since it is probable that the formation of the common metabolite takes place there.

Table I.

Controls

Sex	Litter	1st period (control)	2nd period	Increase in 2nd period
M	3030	12.5 g.	13 g.	0.5 g.
F	3030	12.0	16	4.0
F	3129	16.5	19	2.5
M	3129	12.5	16.5	4.0
F	2943	9.0	15.5	6.5
Mean =		12.5	16.0	3.5

Cystine

F	3030	10.0	22.5	12.5
F	†3075	13.0	25.0	12.0
F	†3129	13.0	27.5	14.5
M	†2943(1)	9.0	28.0	19.0
M	†2943(2)	15.50	30.5	15.0
F	3044	10.0	26.5	16.5
M	†3044	15.0	29.5	14.5
F	3030	11.0	26.0	15.0
Mean =		12.1	27.0	14.9

Methionine

F	3030	14.5	22.5	8.0
M	3129*	15.5	21.0	5.5
F	3044*	15.0	20.5	5.5
F	3129	13.5	25.5	12.0
F	2943	12.5	20.0	7.5
M	3044*	9.5	23.0	13.5
F	3044	13.0	25.0	12.0
M	3030*	15.0	22.0	7.0
Mean =		13.6	22.5	8.9

Percentage of litters affected = 50.0
 No. of litters affected = 7
 Percentage of females affected = 25.0
 Actual number of animals affected = 27
 Percentage of animals affected = 50.0

Table II.

WIESNER'S STOCK.

Total number of animals used	=	92
Sex ratio	=	$\frac{50 \text{ males}}{42 \text{ females}}$
No. of males affected	=	44
Percentage of males affected	=	91.6
No. of females affected	=	20
Percentage of females affected	=	47.6
Total number of animals affected	=	64
Percentage of animals affected	=	71.1
25 animals untreated	-	no recoveries
13 animals treated with methionine	-	no recoveries
6 animals treated with $\text{Na}_2\text{S}_2\text{O}_3$	-	no recoveries
5 animals treated with glycine	-	no recoveries
15 animals treated with cystine	-	11 recoveries or 73% recovery
2 animals treated with glutathione	-	2 recoveries

BACHARACH'S GLAXO RATS.

Total number of animals used	=	48
Sex ratio	=	$\frac{24 \text{ males}}{24 \text{ females}}$
No. of males affected	=	20
Percentage of males affected	=	83.3
No. of females affected	=	7
Percentage of females affected	=	29.1
Total number of animals affected	=	27
Percentage of animals affected	=	56.2

Table II (contd.)

10 animals untreated	- no recoveries
4 animals treated with methionine	- no recoveries
3 animals treated with $\text{Na}_2\text{S}_2\text{O}_3$	- no recoveries
10 animals treated with cystine	- 7 recoveries or 70% recovery

(b) An Apparatus and Method for the Determination of the Respiratory Quotient of Small Animals.

The usual gas analytical methods for the determination of the respiratory quotients of small animals, such as the rat, are unsatisfactory if the quotients to be measured are not absolutely basal and approximately constant. It was for the above reason that this apparatus was devised for the measurements of the quotients of the albino rats suffering from the cystine deficiency syndrome previously described.

Benedict and MacLeod (1929) gave a complete review of the methods of the study of the respiratory exchanges of small animals. In this review the work of 53 investigators is cited. The method of Wesson (1927) approaches closest to our own in being a closed circuit type and having an accurate chemical measurement of carbon dioxide, but his oxygen determination method is laborious and requires constant attention throughout the experiment.

Our apparatus is of the closed circuit type. The circulation of the air is carried out by means of the pump (see Fig. I, 1, 2, 3). This pump was devised by Mr N.E. Condon of the Department of Pharmacology of this University and is extremely efficient.

Fig. I.

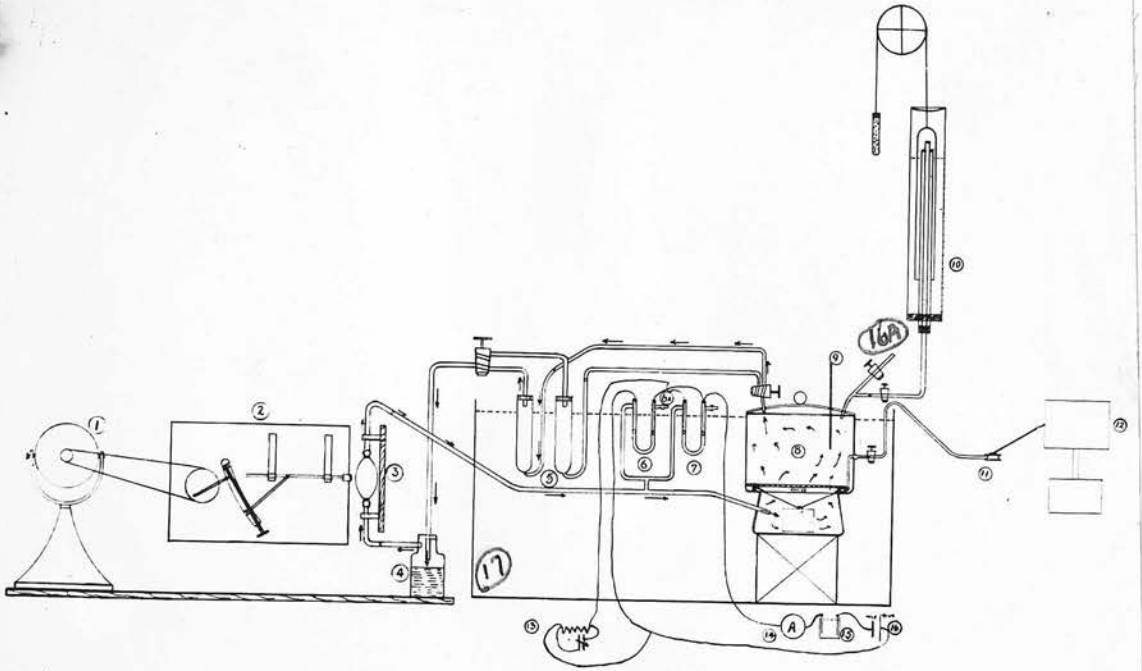
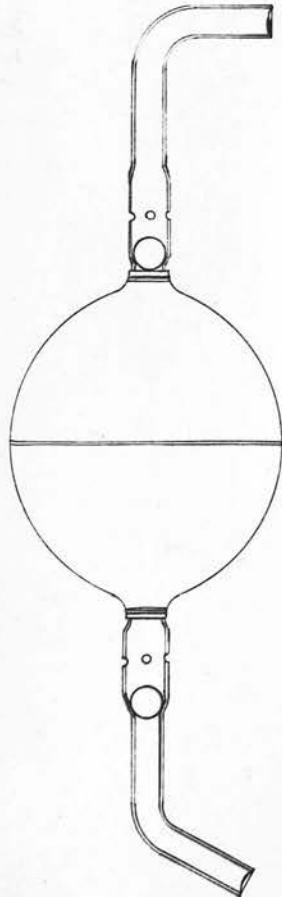


Fig. II.



The piston (2) is the variable stroke piston of the well known Dixon type. The pump itself (3) is a hollow rubber ball (a thick strong atomiser bulb) fitted as shown in Fig. II with outlet and inlet valves, which are made of glass, and the balls, made of light steel, are ground in air tight. This pump is absolutely air tight and one rubber ball lasts as long as three months in constant use. By means of the variable stroke piston the rate of the circulation of the air can be altered. The direction of the air is shown by the arrows on Fig. I.

The carbon dioxide expired by the animal in the chamber (Fig. I, 8) is removed from the air by it passing through one of the tubes (Fig. I, 5) which contains a known quantity of approximately normal sodium hydroxide; the air is then passed over concentrated sulphuric acid (Fig. I, 4) to remove excess moisture gathered from the sodium hydroxide solution and then passed through the pump back into the animal chamber. The carbon dioxide that is removed is replaced by an equal volume of oxygen from the electrolysis of a solution of sodium hydroxide in the U-tubes (Fig. I, 6, 7), fitted with calcium chloride tubes (Fig. I, 6A).

Electrolysis/

Electrolysis only takes place when a volume of gas, i.e. carbon dioxide, is removed from the system and alters the pressure of the system; otherwise the level of the sodium hydroxide solution is automatically depressed below the level of the anodes and the circuit is broken. In parallel with the above electrodes are two copper electrodes immersed in a solution of copper sulphate (Fig. I, 15) as in accordance with Faraday's second law of electrolysis: "The same quantity of electricity sets free the same number of equivalents of substances at the electrodes", that is, for every grammolecule of oxygen set free at the anodes in (6) (7) of Fig. I, two gram atoms of copper are deposited on the cathode in (15) of Fig. I. At the end of a determination the pressure of the system is tested by a gas burette attached to the opening (16A) (Fig. I), and it is determined whether there is a positive or negative pressure for the correction of final oxygen consumption. This correction was found to be very small in relation to the volume of the system. The copper cathode was then dried and weighed and the weight subtracted from its previous weight and the oxygen/

oxygen consumption of the animal calculated as shown below. The reason for the use of the two U-tubes (Fig. I, 6, 7) instead of one large tube for the electrolysis of the sodium hydroxide solution is that slighter variations in pressure make and break the circuit. The circuit is shown complete in Fig. I; (16) is a 230 volt supply of direct current, (15) is a variable resistance to give an amperage of approximately 2 amperes on the ammeter (14). The U-tubes (6, 7) are shown enlarged in Fig. III.

The movements of the animal in the chamber (8) (Fig. I) are recorded on the kymograph (12) by the rubber tambour (11). The wire platform shown in the chamber (8) is connected to the tin cone for the disposal of excreta. This tin cone is suspended rigidly at two points, and a third rests on a rubber diaphragm. When the animal moves a change of pressure is transmitted to the rubber tambour (11) which is recorded on the drum of the kymograph. Our quotients were measured only in the periods in which the animal showed relatively little or no movement.

Fig. III //

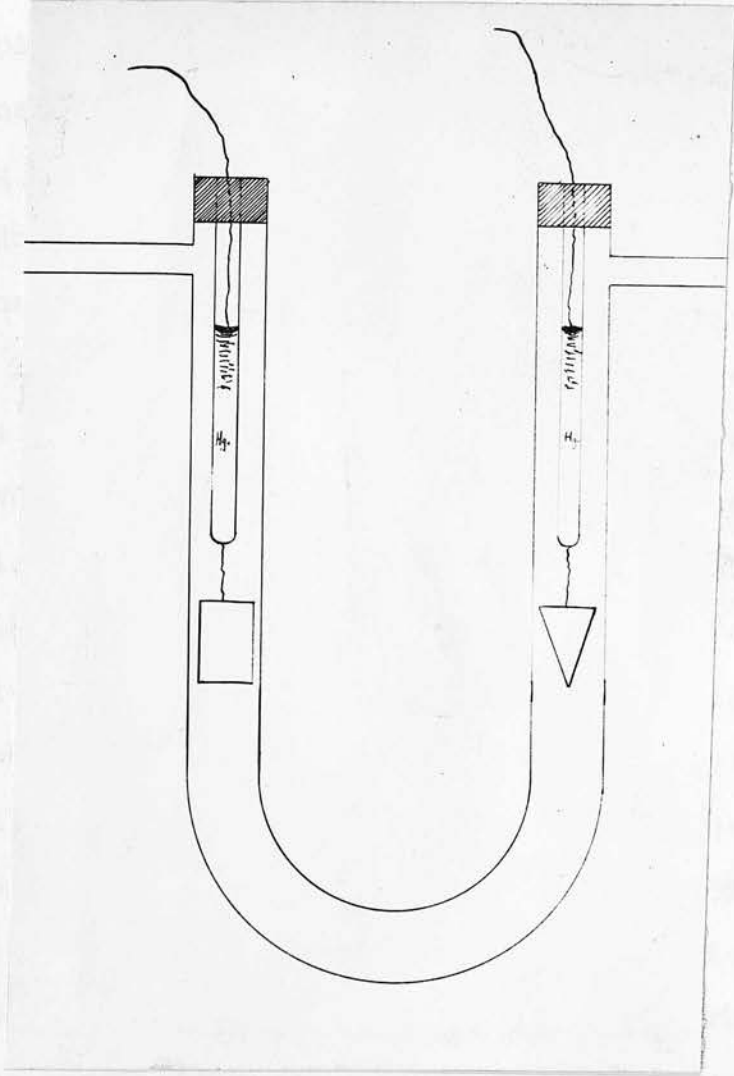


Fig. 3.

The temperature of the chamber varied although immersed in the constant temperature water-bath (17) and this was measured by the thermometer (9). The spirometer (Fig. I, 10) was used for testing the tightness of the apparatus for leaks before introducing an animal into the chamber (8) and for the introduction of carbon dioxide into the apparatus for the checking of its efficiency.

The carbon dioxide absorbed in the tubes (Fig. I, 5) was determined by the method of Winkler (see Treadwell, 1924). These tubes were used singly, that is, one tube was used during the initial or equilibrium period which was always done on every animal previous to a proper quotient determination. The method of Winkler is in short as follows: the alkali and washings were transferred from the tube into an Erlenmeyer flask and an excess of 10% barium chloride solution added, for the precipitation of the sodium carbonate as insoluble barium carbonate, and the remaining alkali in solution is titrated with standardised hydrochloric acid in the presence of phenolphthalein.

Calculations /

Calculations:

Example.

Rat I. N. Male, weight 162 g., starved 18 hours.
Barometer 747.4 mm. Temperature of rat chamber 25.6°C.
Carbon dioxide value of 1.014 N HCl, 1c.c. = 11.35 c.c.

CO₂ at 0°C. and 760 mm.

Titration value of NaOH solution, 25 c.c. = 24.64 c.c.

HCl solution.

Time of experiment after equilibrium = 1 hour.

CO₂ = 14.64 c.c. 1.014 N HCl.

11.35 x 14.64 = 166.1 c.c. CO₂ at 0°C. and 760 mm.

expired by the rat I.N during
the experimental period of 1 hour.

First weight of the electrode = 14.7600 g.

Final do. do. = 15.9875 g.

1.2275 g. = copper deposited in 1 hour.

O₂ correction = 6.5 c.c. at 747.4 mm. and 25.6°C.

do. = 5.7 c.c. at 760 mm. and 0°C.

32 g. O₂ = 127.14 g. Cu

Therefore x g. O₂ = 1.2275 g. Cu

x = 0.3089 g. O₂

32 g. O₂ at 0°C. and 760 mm. = 22,400 c.c. O₂

and 0.3089 g. O₂ at 0°C. and 760 mm. = ~~432.5 c.c. O~~

= 216.2 O₂

Total/

Total O₂ consumption = 216.2 + 5.7 = 221.9 c.c.

at 0°C. and 760 mm.

R.Q. = 0.75.

For the checking of the efficiency of the apparatus all modifications of the alcohol lamp method were found unsuitable as the smallest lamp that would burn continuously was far too large for the capacity of the apparatus. The passing of ether over a hot platinum wire was also found to be unsatisfactory as the ether formed an explosive mixture with the air in the chamber. The only methods left open to us were therefore indirect methods. The method used was as follows: carbon dioxide was introduced into the spirometer (Fig. I, 10) and a measured amount introduced into the apparatus and the pump set into action and at the end of 30 minute periods the determinations of carbon dioxide and oxygen carried out in the usual manner. The error of our apparatus measured in this manner was from 1-2 per cent. The carbon dioxide determination was also tested separately by cutting out the oxygen supply and allowing the carbon dioxide to replace the volume of carbon dioxide/

dioxide removed and the error was the same as the above. The oxygen determination was also tested separately by cutting the U-tubes (Fig. I, 6, 7) out of the system and generating oxygen and collecting it in a gas burette and weighing the copper cathode over this period and the error was of the same order as the above.

(c) The Respiratory Quotients of Albino Rats on Normal and Cystine Deficient Diets.

From the results of Benedict et al (1930) and Wesson (1927) one can see the difficulties of comparing the fasting respiratory quotients obtained in two different laboratories. Benedict (1930) states: "The respiratory quotient of the rat after 24 hours fasting is so near 0.72 on the average, that one may with confidence measure the carbon dioxide production or the oxygen consumption only, assume a respiratory quotient of 0.72 and compute the total metabolism with a minimum error." Wesson (1927) using an apparatus which Benedict says "leaves no/

no possibility for criticism", obtained the following quotients on normal rats: 0.85 after 24 hours fasting, 0.78 after 48 hours fasting and 0.74 after 72 hours fasting. From the above it is also clear that any comparison of fasting quotients of rats on a normal diet with rats on a deficient diet must be exceedingly difficult and the order of difference between two relatively small groups would need to be tremendous to be of real significance. Nevertheless as our experimental animals obviously varied so much from our normal animals we decided to determine their respiratory quotients as a possible clue to the cystine deficiency syndrome previously described.

The results on normal animals presented in Table III are more in agreement with Wesson (1927) than with those of Benedict (1930) as his rats after 5 hours fasting rarely gave an R.Q. above 0.75. The respiratory quotients of seven normal rats, fasting from 24 to 36 hours, were determined before our oxygen determination method was perfected by using the spirometer (Fig. I, 10) as a method of measuring oxygen consumption and an average R.Q. of 0.79 was obtained. The time of fasting was cut down in the case of the normal animals as after 16-18 hours their/

their restless movement in the animal chamber made it difficult to get a sufficient period of relative inactivity for a proper determination. In the experimental animals the restless tendency on prolonged fasting was not nearly so troublesome.

In our experimental animals, as in our normals, the respiratory quotients on fasting are far above those reported by Benedict (1930). To obtain an R.Q. of 0.72 required a fast of 36 hours and in two animals R.Q. of 0.74 and 0.77 were obtained after 36 hour fasts. As in the case of Wesson (1927) we obtained respiratory quotients of over 1, which of course indicates that carbohydrate is being converted into fat. This is at least to say strange in an animal that has been fasting as long as 8 hours. The Sherman-Merrill diet used by us contains approximately 3.5% milk fats. Animal IV.A. (Table III) is the only complete picture of the syndrome, i.e. the only animal in which we were able to show quotients both before and after recovery. This animal was affected on November 2nd and on November 4th was extremely ill, in fact almost moribund, and as shown gave the remarkably low quotient of 0.55 after 24 hours without food. This animal made a rapid recovery after two doses of 100 mg. of cystine/

cystine and was put on cystine-containing food for one week. We do not believe that this low respiratory quotient together with the extremely low oxygen consumption and carbon dioxide production, is specific for the syndrome of cystine deficiency as Benedict and MacLeod (1929) had three instances of rats which had been studied for one or more days, apparently in good condition, and then for some reason, died shortly after the experiments. Under these conditions a pronounced fall in the metabolism was noted and they say that carbon dioxide production fell from one third to one fifth of normal, which is comparable with our finding. This finding is very interesting, however, in that it shows how very low the metabolism of these animals suffering from the syndrome may fall and yet the animal may recover on treatment.

From Table III one may see that there is some difference in oxygen consumption between the normal and experimental animals. The experimental animals have a greater oxygen consumption per gram of rat per minute, but considering the relatively small groups any conclusion would be very doubtful.

Comparisons/

Comparisons between oxygen consumptions between other investigators' animals and our own were not productive due to differences in size of animals and other experimental conditions, notably temperature of the experimental chamber, as Benedict et al (1930) have shown that the oxygen consumption of female rats at 16°C. approximately doubles their consumption shown at 26°C.

Table III.

No. of animals	Date of expt.	Sex	Body wt. in gm.	Minimum hrs starved	R/Q	Oxygen consumpt. per rat per min. in c.c.	c.c. O ₂ per gram of rat per min.	Temp. of rat chamber	c.c. CO ₂ output per rat per min.	c.c. CO ₂ per gm. of rat per min.
<u>NORMALS</u>										
I N	June 23	M	161	16	0.80	4.6	.0285	26°C.	3.65	.02270
II N	27	M	156.5	12	0.89	4.23	.0270	25.7°C.	3.96	.02531
III N	29	F	149.5	18	0.73	3.84	.02568	25.6°C.	2.83	.01894
IV N	July 1	F	144.5	16	0.84	2.97	.02058	25.4°C.	2.51	.0174
V N	5	F	143.0	16	0.80	4.29	.03004	26.4°C.	3.44	.02409
<u>EXPERIMENTAL</u>										
II X	June 6	M	94.5	8	1.02	2.05	.02173	25°C.	2.096	.02218
II X	June 7	M	96.5	8	1.29	1.53	.0159	25°C.	2.16	.02238
II X	9	M	96.5	12	0.90	2.66	.02765	25.3°C.	2.415	.02502
II X	13	M	98.0	14	0.80	2.79	.02796	25°C.	2.24	.02289
II X	16	M	98.0	12	0.83	2.73	.02789	25.4°C.	2.288	.02335
II X	19	M	93.0	30	0.787	2.88	.03102	25.0°C.	2.27	.02441
III X	22	F	98.5	30	0.770	3.22	.03277	25.2°C.	2.49	.02533
III X	22	F	98.5	31	0.78	3.42	.03496	25.2°C.	2.69	.02737
I A	26	F	89.0	24	0.79	2.83	.03186	25°C.	2.23	.02514
I A	July 6	F	91.5	12	0.92	2.31	.02532	25.4°C.	2.153	.02354
I A	11	F	92.5	12	0.89	2.00	.02183	25.2°C.	1.81	.01958
II A	13	M	81.5	18	0.76	2.31	.02838	25.1°C.	1.76	.02166
III A	27	F	74.0	14	0.79	2.05	.0278	25°C.	1.63	.02205
III A	Aug. 1	F	77.0	18	0.74	2.50	.03253	25°C.	1.86	.02422
IV X	4	F	90.0	not starved	1.00	2.89	.03285	25.5°C. 25.1°C.	2.86 1.84	.03187 .02234

No. of animals	Date of expt.	Sex	Body wt. in gm.	Minimum hrs starved	R/Q	Oxygen consumpt. per rat per min in c.c.	c.c. O ₂ per gram of rat per min.	Temp. of rat chamber	c.c. CO ₂ output per rat per min.	c.c. CO ₂ per gm. of rat per min.
IV X	Aug. 9	F	82.5	36	0.72	2.54	.03089	25.1°C.	1.84	.02234
V X	Sept. 7	F	81.0	12	0.93	2.18	.0270	25.6°C.	2.04	.02529
V X	13	F	85.0	2	1.05	2.31	.02726	25.2°C.	2.43	.02866
V X	14	F	85.0	12	0.81	2.64	.03106	25.2°C.	2.15	.02530
V X	15	F	85.5	12	0.81	2.55	.02985	25°C.	2.08	.02434
V X	16	F	86.0	18	0.78	2.52	.02937	25.2°C.	1.98	.02305
IV A	Oct. 2	M	83.0	12	0.83	2.06	.02492	25°C.	1.72	.02075
IV A	4	M	79.0	36	0.74	2.10	.02666	25°C.	1.57	.01989
IV A	5	M	79.0	20	0.77	2.53	.03212	25°C.	1.96	.02493
IV A	30	M	82.0	2	1.09	2.47	.03015	25°C.	2.67	.03258
IV A	Nov. 2	M	78.0	6	0.97	2.25	.02894	25°C.	2.20	.02821
IV A	4	M	69.0	24	0.55	1.12	.01626	25°C.	0.619	.008963
IV A	14	M	76.0	12	0.80	2.08	.02742	25°C.	1.63	.02152
V A	Oct. 6	F	74.5	12	0.91	2.28	.03062	25.3°C.	2.09	.02807
V A	9	F	79.0	6	0.95	2.43	.03081	25°C.	2.33	.02956
V A	11	F	74.0	24	0.78	2.26	.03057	25°C.	1.78	.02408
V A	22	F	84.0	not starved	1.11	2.29	.02726	25°C.	2.55	.03046
VI A	26	F	74.0	36	0.77	2.28	.03091	25°C.	1.76	.02390

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APPENDIX

The following is a list of the names of the persons who were present at the meeting held on the 15th of the month of the year 1941.

I. Members of the Executive Committee

II. APPENDIX.

III. Members

I. Members of the Executive Committee

On the 15th of the month of the year 1941, a meeting was held at the residence of the Secretary of the Executive Committee. The meeting was held in the presence of the following persons: [The names of the persons present are listed here, but they are extremely faint and difficult to read.]

APPENDIX.

This section is intended as a short review of the latest work on methionine. It is proposed to review this work under the following headings:

- I. Isolation and quantitative determination in proteins.
- II. Physical data and derivatives.
- III. Metabolism.

-
- I. Isolation and quantitative determination in proteins.

Abderhalden and Heyns (1932) by the use of their fractionation method isolated from 5 kg. of oxhorn 1 g. of an impure sample of l-methionine. They state that the sulphur content was 3% under that required by the theory and that the impurity was glutamic acid and that after purification the yield was very small. Their substance began to brown/

brown at 255° and melted at 275-277°. $[\alpha]_D^{20}$

(5% water solution) = -7.3°.

Du Vigneaud and Meyer (1932) isolated l-methionine by the enzymatic hydrolysis of commercial casein, using a commercial preparation of pancreatin as a source of the enzyme trypsin. As previously mentioned, these authors overlooked the paper of J.H. Mueller (1922) and therefore state that Mueller gave no details of the isolation of l-methionine from the commercial enzyme digest "Aminoids". The reason that these authors selected trypsin as the enzymatic hydrolysing agent is a statement in Mitchell and Hamilton (1929) that trypsin splits off tyrosine from the protein molecule without simultaneously splitting off phenylalanine and proline and therefore if trypsin also splits off methionine the difficult problem of contamination with phenylalanine would be solved. Trypsin did split off methionine which was isolated by means of a slight modification of Mueller's method after first removing tyrosine and tryptophane by the method of Cox and King (1930). The yield of methionine was 1 to 2 g. per kilo of casein used.

Pirie/

Pirie (1932) described a method of isolation of l-methionine by which he was able to obtain from casein a yield of 1%-1.4%. His method in short is as follows: A partial separation of the protein hydrolysate by extraction with butyl alcohol in strongly acid solution; he found that methionine is appreciably soluble in the alcohol in strongly acid solution. (Before the above alcohol extraction he recommends precipitation by lead acetate to avoid the formation of a semi-solid foam). Instead of distilling the butyl alcohol off in the usual manner he extracted the alcoholic mixture with 10% sodium hydroxide solution. The filtrates from the sodium hydroxide extractions are evaporated in vacuo and brought to pH 5 with hydrochloric acid. This solution is precipitated by mercuric acetate in 3% acetic acid, the precipitate filtered off, triturated with water and refiltered. The precipitate is suspended in water and extracted first with N/3 barium hydroxide and secondly with N/7 barium hydroxide. The barium ions were removed from the extracts and the extracts evaporated in vacuo. Methionine was isolated from these concentrated extracts by mercuric chloride in the usual manner.

Pirie/

Pirie (1933) modified the above isolation method by using an enzymatic hydrolysate and eliminating the butyl alcohol extraction. The precipitating agent used was a mixture of mercuric acetate and phosphotungstic acid which he declared was more efficient than the mercuric acetate used alone, although he stated that phosphotungstic acid "does not itself precipitate methionine either from pure solution or from an amino acid mixture". He also states that the mercury-phosphotungstic precipitate is more easily decomposed by barium hydroxide. The filtrate from the extraction by barium hydroxide is evaporated down on the water bath and is precipitated by alcohol. This precipitate is dissolved in water and is precipitated by mercuric chloride in the usual manner. From 1 kg. of casein 9-10 g. of pure l-methionine was obtained.

Baernstein (1932) described a method of quantitative determination of methionine in proteins based on the determination of the volatile iodide liberated when proteins are treated with HI (hydroiodic acid). This method is based on the assumption that methionine is probably the only amino acid in the protein molecule that is methylated. Eleven other amino acids were treated with hydroiodic acid with/

with negative results. Although this method is obviously open to criticism the results on analysis of proteins are of significance. The procedure is based on the method of Pollak and Spitzer (1922) for the determination of methylthiol group except that a volumetric determination is made of the silver remaining in the filtrate from the silver iodide precipitate instead of determining directly the amount of silver iodide formed. The methionine content of 32 purified proteins was determined, the lowest being Arachin, 0.54%, and the highest being Ovalbumin, 4.57%. Three samples of casein had the following methionine content: 3.53%, 3.36% and 3.25%. In general the animal proteins have a higher content of methionine than the vegetable proteins.

Baernstein (1934) modified his method as follows: A mixture of glacial acetic acid, potassium acetate and bromine is used to absorb the methyl iodide instead of a solution of silver nitrate after the method of Vieböck and Schwappach (see Clark 1932). The methyl iodide is converted to iodate and iodine liberated therefrom by potassium iodide and sulphuric acid is titrated by thiosulphate. This method is considered to be more accurate than the silver/

silver titration and also much simpler. With this modification of the method ovalbumin gave 5.24% methionine and a sample of casein 3.5% methionine.

J. Barritt (1934) using the method of Baernstein (1932) determined the methionine content of various wools. The percentage of methionine found based on dry weight varied from 0.44% to 0.67% in various types of wool, the lowest being Welsh mountain sheep wool and the highest being camels' hair. This work shows that the amounts present do not substantially affect previous work on the relation between the total and cystine sulphur in wool which indicated that practically all of the sulphur in wool could be accounted for as cystine. The fact that methionine occurs widely in foodstuffs and grasses is stressed and it may play an important role in the ultimate synthesis of wool and hair proteins.

Hill and Robson (1934) describe a method of isolating methionine together with tyrosine and leucine by means of "salting-out" from the hydrochloric acid hydrolysate of casein. Tyrosine separates out at pH 2.4 (on addition of 40% sodium hydroxide solution) and at pH 6.0 a mixture of leucine and methionine separates. Methionine is separated from leucine by precipitation with mercuric acetate in/

in 3% acetic acid solution and further purified by the usual mercuric chloride precipitation. No yields are given but it is stated that the yields "compare very favourable" with those in the literature.

Tomiyama and Hanada (1934) using the method of Odake (1925) isolated methionine from the following proteins (yields based on dry and ash-free protein): muscle protein of sardine 0.52%; muscle protein of whale 0.37%; protein of silk-worm pupa 0.43%; protein of Soya bean 0.08%, casein 0.41%. Judging from the percentage of methionine isolated from casein all of the above results must be very low.

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II. Physical Data and Derivatives.

Emerson, Kirls and Schmidt (1931) studied the apparent dissociation constants of dl-methionine. Estimations of the hydrogen ion activity of the amino acid in solutions were carried out at 25°C. with the aid of the quinhydrone and hydrogen electrodes after the method (1930). For methionine the following values for apparent constants at 25°C. were calculated:

$$K'_a = 6.17 \times 10^{-10}$$

$$pK'_a = 9.21, \quad K'_b = 1.91 \times 10^{-12}, \quad pK'_b = 11.72$$

$$\text{and } pI = 5.74.$$

These authors say: "The dissociation constants of methionine are characteristic of the monoamino-carboxylin amino acids. The value of K'_a (apparent acid dissociation constant), is, however, slightly greater than that for most of the members of this group of amino acids". This higher value of K'_a is thought to be due to the presence in methionine of the methylthiol group.

Dalton and Schmidt (1935) studied the solubility of d-l-methionine in water from 0°-62°C. and in all made 36 determinations. They found that S_{100}/S_0

(The/

(The ratio of solubility at 100°C. to solubility at 0°C. (all values in g. per 1000 g. of water)), was 9.68. These authors also studied the density of solutions of d-l-methionine at 25°C. and the partial molal volumes of solute. The heat of solution of d-l-methionine was calculated and observed by means of direct calorimetric measurements, with the following results:

$$\Delta H \text{ (calculated)} = 4240, \Delta H \text{ (observed)} = 4100.$$

Butz and Du Vigneaud (1932) found that methionine on being heated with 18/N sulphuric acid gave a positive test with the Folin-Morensi cystine reagent and that this colour was 2/5 as much as cystine itself gave. This non-volatile cystine reacting substance was isolated and found to have the formula $C_8H_{16}O_4N_2S_2$. By the Van Slyke method the nitrogen was demonstrated to all in the form of amino nitrogen and since the compound dissolved in both acids and bases and the ninhydrin reaction was positive it was decided that this compound was a disulphide amino acid. From the above facts and from a consideration of the possible decomposition products/

products of methionine these authors drew the conclusion that this compound was bis (γ -amino- γ -carboxypropyl) disulphide, the next higher symmetrical homologue of cystine. This compound was given the name "homocystine".

Du Vigneaud, Dyer and Harmon (1933) gave the proof of the structure of "homocystine" by converting it to methionine by reduction in liquid ammonia with sodium to homocystine and this compound is methylated in the liquid ammonia solution by the addition of methyl iodide.

Baernstein (1934) isolated from the hydriodic acid digest from 1 g. d-l-methionine a white crystalline compound which he believed to be the thiolactone of homocystine hydriodide. The analysis agreed very closely with the formula C_4H_8ONSI but no other proof of structure is given except the statement that this compound gives a positive nitroprusside reaction after 15 seconds which he believes indicates the opening of the thiolactone ring.

Du Vigneaud, Dyer, Jones and Patterson (1934) synthesised "homomethionine" by first synthesising "pentocystine" (bis-delta-amino-delta-carboxybutyl disulphide) by the general method of Sørensen and then reducing "pentocystine" in liquid ammonia by sodium/

sodium and methylation of the "pentocystine" by methyl iodide to form "homomethionine" (delta-methylthiol- α -aminovaleric acid) which is the next higher homologue of methionine.

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III. Metabolism.

It has been observed by Jackson and Block (1931), Weichselbaum and Stewart (1932), Jackson and Block (1932, 1933) that d-l-methionine, d- and l-methionine and l-formylmethionine are able to supplement cystine for growth promotion in young albino rats on a cystine deficient diet (d-formylmethionine is not able to supplement cystine).

Du Vigneaud, Dyer and Harmon (1933) and Dyer and Du Vigneaud (1935) have shown that d-l-homocystine and d- and l-homocystine are also able to supplement cystine for growth promotion in young albino rats on cystine deficient diets, and this promotion of growth is as effective as the addition of cystine or methionine to the diet. This equal action of growth promotion by d- and l-homocystine is not surprising when one considers the configuration relationship of these optically active isomerides to naturally occurring methionine as shown by Du Vigneaud and Patterson (1935).

Homomethionine and pentocystine (Dyer and du Vigneaud, 1935) and S-methylcystine (Jackson and Block, 1932) were shown to have no growth-promoting action/

action on young albino rats on cystine deficient diets, thus showing that the above mentioned growth promotion of cystine, methionine and homocystine is not a function of a single group in the molecule, but is probably connected to the structure of the molecule as a whole.

Further relationship between methionine and cystine has been shown in the studies of White and Lewis (1932) and White and Jackson (1933) on the administration of monobrombenzene to dogs and white rats respectively. These workers showed that the administration of d-l-methionine to animals receiving monobrombenzene gave the same effect as administration of l-cystine, i.e. an increase in the excretion of organic sulphur and a decrease in the excretion of nitrogen.

The oxidation of methionine in the animal body has been studied by Pirie (1932), White and Lewis (1932), Stekol and Schmidt (1933), Stekol (1935), Virtue and Lewis (1934) and du Vigneaud, Loring and Craft (1934). These workers all agree that the sulphur of methionine, like that of cystine, is easily and almost completely oxidised to sulphates in the animal body. Several of the above workers found/

found that the urines of their animals gave a positive cyanide-nitroprusside reaction. (This reaction is specific for disulphides). Although Virtue and Lewis (1934) did not attempt the isolation of the small amount of substance in their urines responsible for this reaction, they made the following tentative hypothesis: "that a primary reaction in the metabolism of methionine is demethylation to yield the next higher homologue of cysteine (homocysteine) and that two molecules of this compound by oxidation form homocystine which is excreted in small amounts in the urine." Further support of the above hypothesis is given by the work of Pirie (1934) in which he showed that the fluid from slices of rats' livers and solutions of methionine incubated together give a definite nitroprusside reaction that it did not give before incubation. Real evidence for this demethylation hypothesis will, of course, have to await the actual isolation of the substance or substances giving this positive nitroprusside reaction.

Vars (1933) showed that d-l-methionine can be utilised for the production of glucose in the phlorhizinized dog. He also obtained a positive test for a disulphide in the urine of his dog using, however/

however, Grote's test instead of the nitroprusside test. He found the Sullivan reaction negative in his urines.

A discussion of cystinuria probably does not come under the scope of this appendix but the findings of Brand, Cahill and Harris (1935) that the methionine of the diet and not the cystine is the source of the cystine excreted in cystinuria are of such great importance that the mention of this work is perhaps not out of place.

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