A STUDY OF UNKNOWN SULFUR CONTAINING COMPONENTS OF PROTEINS

by

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ABSTRACT

A review is made of the biochemistry of sulfur and of imidazoles. The results obtained by various workers on the fractional analysis of sulfur in proteins have been compiled and the possibility that a portion of the protein sulfur at present unaccounted for in certain proteins may be ascribed to the presence of thiolimidazoles has been critically examined. The study suggests that many of the cystine determinations recorded in the literature may be high due to the interference of thiolimidazole breakdown products.

A review of the chemistry of the thiolimidazoles is presented and the origin of the recent revival of interest in these compounds discussed.

A method has been developed whereby it is possible to separate added ergothioneine from those fractions of a protein hydrolysate that interfere with the Hunter diazo test. It has also been found that this betaine is destroyed by boiling with hydrochloric acid and with many other hydrolyzing agents in the presence of protein breakdown products. In an attempt to find a suitable hydrolyzing agent which would ensure the stability of the thiolimidazole ring many methods were tried but thus far without complete success. Until such a procedure is evolved no final conclusion can be reached as to the presence or absence of the thiolimidazole ring in the protein molecule.

ACKNOWLEDGEMENT

I wish to express my gratitude to Dr. B. A. Eagles under whose guidance this work has been carried out for the assistance and advice that made it possible.

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I Historical

1. A Review of the Biochemistry of Sulfur

Folk Lore

The importance of the role of sulfur in the functioning of living things has been recognized since the time of the early Greeks when Hercules was said to have used sulfur in cleansing the stables of Augea. Sulfur springs in various parts of the world have maintained reputations of great healing powers since the time of our earliest records and sulfur has been used since time immemorial in a variety of folk recipes as an internal medicament. (1) Possibly the most famous of these recipes is the sulfur and molassas "spring tonic". In all these sulfur containing pharmaceuticals the active principle is a very small fraction reduced to the -2 or -4 valence. The value of the sulfur containing medicaments is probably very much over estimated by the populace but pharmaceutical texts recognize

them as vascular stimulants and nervous sedatives when used externally and as laxatives internally. (2)

Alchemistry

The first extensive experimental work on the biochemistry of sulfur was carried out by the iatrochemists of the renaissance. Each alchemist had his own interpretation of the confusing literature on the significance of his "elements" but finally in the early sixteenth century Paracelsus put his concept of the importance of sulfur in an understandable form. He considered the "hypostatical principles", mercury, sulfur, and salt to be parallel to the Greek elements, fire, air, and earth. These three elements made up all things including the seven metals. Sulfur imparted to the substances in which it occurred the property of inflamability and bound together the mercury and salt in the same manner as the soul of man united the body and spirit. Other alchemists considered sulfur identical with the quintessence. (3) Although none of these alchemical ideas of sulfur biochemistry has been accepted by modern science, the fact that these mysticists considered sulfur of importance raised it to a place of prominence in the minds of scientific men.

Early Quantitative

When, in the nineteenth century men finally began to study the chemistry of living things extensively on a carefully reasoned basis, they found that they could make no progress until they had broken the living tissue down into simpler units. The first methods used in doing this, such as ashing and

treatment with strong acids and oxidizing agents, were very drastic and resulted usually in oxidizing all the sulfur to the sulfate. In this form the total sulfur was determined but nothing was learned of the numerous different forms in which it might occur. Some thought that they had enough knowledge from this work to write a general formula for proteins thus, $(C_{40}H_{62}O_{12}N_{10})_xS_yP_z$. Gradually however less drastic methods of analysis were developed which revealed that several different forms of sulfur resulted from the same treatment. Sulfur was thus shown to occur in living tissues linked in a variety of ways. Much has now been done in this analytical phase of sulfur biochemistry, but it is still not complete for sulfur containing products are still often obtained from natural products by treatments which would not yield these specific fractions from any of the sulfur containing units now known to occur.

Sulfates

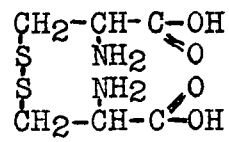
The commonest form of sulfur in the soil is the inorganic sulfate ion and in this form it also occurs in living cells to a limited extent. The role played by plants in reducing nitrates of the soil in order to yield nitrogen in a form available to animals is generally appreciated but mention is seldom made of the fact that, as far as can be determined, animals are also dependent on plants for their total supply of reduced sulfur. (4) Inasmuch as sulfates cannot be reduced by animals any sulfur that becomes completely oxidized by the body processes cannot be reclaimed but must be excreted in this

form and therefore 75-95% of the sulfur excreted is in the form of sulfate. Sulfates, like nitrates, are present in animal tissues in limited amounts and have but a limited effect on the osmotic concentration. The greatest use of sulfates by animals is for the purpose of detoxifying aromatic and heterocyclic hydroxyl groups. They are combined with these groups as etheral sulfates and excreted thus in the urine. There are a few other compounds of biological importance which contain oxidized sulfur but they will be discussed individually.

Cystine

In 1810 Wollaston (5) isolated a substance from bladder cysts which proved to be the first natural sulfur containing organic compound studied and also the first amino acid. (6) He gave it the name cystic oxide and this name has since been modified to cystine. In spite of the lability of the sulfur in this compound, neither Wollaston nor any one else noticed it until 1837 and thus it was the first of a long series of compounds continuing to extend even to the present time, in which one sulfur atom was at first mistaken for two atoms of oxygen. When the correct empirical formula was first worked out by Thaulow (7) very little was known of organic structures or even of atomic weights. Thaulow's formula $C_6H_{12}N_2O_4S_2$ was correct only because his errors in taking the atomic weight of oxygen as eight and in not recognizing the double nature of this molecule compensated each other. Possible structural formulae for cystine were bandied about all through the course of the various radical and type theories

that were successively proposed during the nineteenth century but it was not until 1903 that Erlenmeyer (8) finally established by synthesis the structure now accepted.



Cystine

During this period of nearly a century, over which work was being done to establish the structural formula of cystine, others were studying its occurrence. All the reactions indicated that much of the sulfur in proteins occured as cystine but no one had been able to isolate it from this source. One attempt after another failed because of the failure to observe various simple technical procedures which were later found to be essential for its isolation and it was only after twelve other amino acids had been isolated that, in 1899, Mörner finally succeeded in obtaining cystine from proteins. For a long period cystine and the closely related cysteine were the only forms of sulfur known to occur in proteins and therefore many of the analyses done in this period were misleading. It was found however that cystine is present at least in small amounts in nearly all proteins except the protamines. The keratins have a very large percentage of cystine and it is believed that it has an important function in this protein in protecting the animal from ultra-violet light. Tanning is more a thickening of the skin than an increase in the pigment content and the added protection would come from the increased amount of cystine-containing keratin present. Cystine also

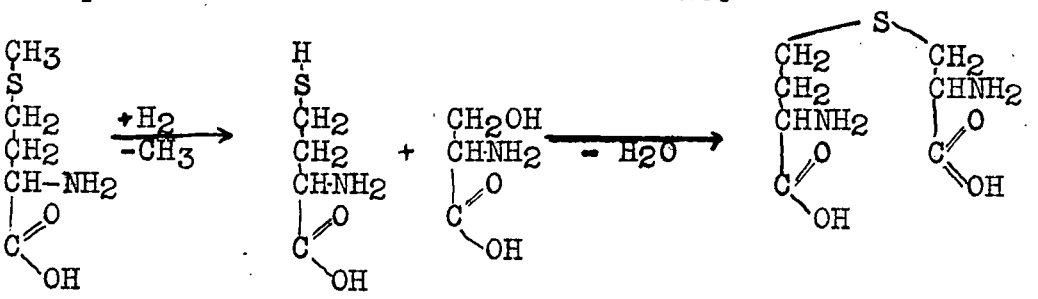
seems to have another more dynamic function in the body. Experiments done on the effect of iodoacetate on enzymes indicate that -SH groups are essential to enzyme action and cysteine is the only amino acid containing this group that is known to occur in proteins. Cystine has been shown to fill much of but not the entire need of an organism for sulfur and the ability of most animals to synthesize cystine from other forms of reduced sulfur emphasises its importance.

Methionine

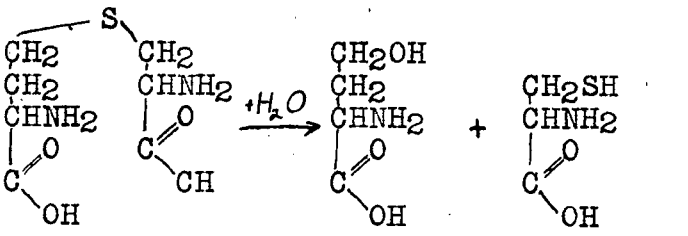
Even before Mörner succeeded in isolating cystine from proteins it was recognized that they contained more than one form of sulfur. As early as 1846 Fleitmann (10) observed that only a fraction of the total protein sulfur is alkali labile. This fraction he called unoxidized sulfur in contrast to the remainder which he mistakenly termed oxidized. In 1898 Shultz (11) was able to gather together the results of several workers who had made comparative determinations of different sulfur fractions. From these results Shultz tried unsuccessfully to find a constant relationship between the total sulfur and the alkali labile fraction. Another form of sulfur, α-thio-lactic acid, which seemed to have been derived from something other than cystine was isolated by Suter, Friedmann and Fränkel (12) from several proteins. A review has been given by Johnson (13) of the papers, published up till 1911, concerning these various sulfur linkages. Most, but not all, of these sulfur reactions, unexplained at the time, have since been accounted for by the presence of

methionine.

It was the fact that cystine does not supply all the sulfur requirements of certain microorganisms that led to the discovery of methionine by Mueller (14). Meuller, who was engaged in studies concerned with the nitrogen and activator requirements of streptococci, found that there was a substance in casein hydrolysates necessary for their growth that was not included in some other hydrolysates. In 1922 he succeeded in isolating from casein a factor which was named methionine. Although it turned out that this amino acid was not an essential factor for the growth of the streptococci, it proved to be necessary for most species of animals. Methionine can be converted in the body to cysteine by the following reactions; the process however is not reversible.



Methionine — Homocysteine — Serine — Cystethione



Homoserine — Cysteine

The various intermediate products of these reactions have been

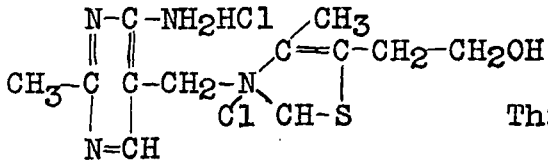
isolated from natural sources. The analytical data available shows that in general proteins contain a larger fraction of methionine than cystine and the former accounts for the majority of sulfur in foodstuffs.

Mention should also be made of the role played by methionine in transmethylation. The body would appear to be incapable of synthesizing methyl groups and is, therefore, dependent on the relatively few compounds in the diet which contain these groups in a labile form. Du Vigneaud and co-workers (15) have shown by tests with deuturium that the methyl group of methionine is used in synthesizing choline and creatine and it is probably also necessary for the synthesis of adrenalin, anserine and other methylated products. It has been found (16, 17) that betaine may act as a source of methyl groups in the rat and it is possible that ergothioneine and the other betaines, stachydrine, trimethylhistidine, hypaphorine, and trigonelline in the various locations in which they are found may perform similar functions.

Thiamin

Thiamin, another essential metabolite containing sulfur, is a substance of paramount importance. Lack of thiamin in the diet interferes with many metabolic functions ultimately resulting in beri-beri. Eijkmann was one of the first to work with this disease finding that pigeons could contact it and therefore be used as an experimental animal for its study. Other workers established the fact that the disease was due to a deficiency of some substance and in 1898

Jansen and Donath (18) succeeded in isolating the vitamin in crystaline form. It was the first vitamin to be obtained in pure form but it was not synthesized until nearly forty years later (19).



Thiamin hydrochloride

One of the chief functions of thiamin is concerned with the synthesis of cocarboxylase, thiamin pyrophosphate, and the prosthetic groups of many carboxylases. These compounds of the vitamin are necessary for the breakdown of pyruvic acid and hence for the process of glycolysis. However, a lack of thiamin is evidenced particularly in the nerves as polyneuritis. This seems to be a direct effect on the neurones, not a secondary result of a generally disrupted metabolism. A theory has been proposed (20) that the deficiency disrupts the following series of reactions, preventing the synthesis of acetylcholine.

$$\text{ATP} + \text{Thiamin} \longrightarrow \text{Adenylic acid} + \text{cocarboxylase}$$

$$2CH_3\text{-}C(=O)\text{-}C(=O)\text{-}OH + O_2 \xrightarrow[\text{cocarboxylase}]{\text{carboxylase}} 2CH_3\text{-}C(=O)\text{-}OH + 2CO_2$$

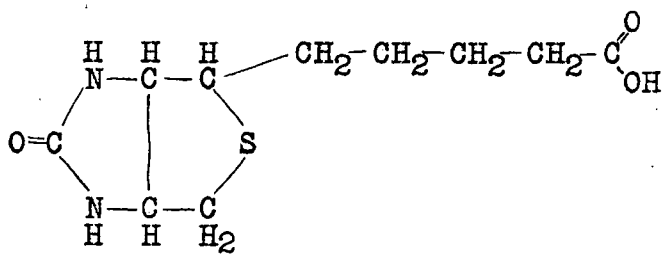
$$CH_3\text{-}C(=O)\text{-}OH + CH_3\text{-}CH_2\text{-}N(CH_3)_3OH \longrightarrow CH_3\text{-}CH_2\text{-}O\text{-}C(=O)\text{-}CH_3 \quad (N(CH_3)_3)$$

Acetylcholine

Biotin

The third and only other form of sulfur known to be essential in the diet is the vitamin biotin. Work leading to

the discovery of this vitamin progressed along three separate channels and hence it has been variously designated as biotin, coenzyme R, and vitamin H. Working with the charcoal adsorbable fraction of bios, the bios II B of Miller (21), Kogl and Tonnis (22) succeeded in 1935 in getting a minute amount of a crystaline product which contained most of the activity of the original adsorbate. This they called biotin but there was too little of it to make a full study possible. Meanwhile Allison, Hoover and Burk (23), obtained an extract, coenzyme R, which was active in promoting the growth of Rhizobium trifolii, a legume nodule organism. Also the work of Boas (24), on the effect of a diet containing much raw egg-white led to the discovery of an anti-egg-white injury factor, vitamin H. Biotin and coenzyme R were shown to be identical in 1939 and in 1940 Szent-Györgyi (25) demonstrated that vitamin H had similar properties. The vitamin was synthesized in 1943 (26) by a long and difficult process and the structure was shown to be:

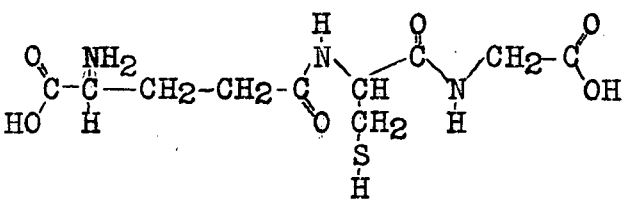


As yet little is known about the metabolism of biotin. Szent-Györgyi found that the vitamin must be supplied to practically all species of animals and micro-organisms that he studied. In certain instances the amounts required were so infinitesmal that a deficiency could only be demonstrated by feeding raw egg-white. Native egg white has a small amount of

a protein, avidin, which combines with biotin to prevent its assimilation. Owing to the wide range of substances in which biotin occurs, the minute amount usually required, its occurrence in the bound state in which it is available to only a few organisms, and due to the fact that the intestinal flora seem to be able to synthesize it, it is difficult to determine the exact biotin requirements of living organisms. Rapidly growing tissues such as embryos and tumors contain a large amount of biotin and several attempts have been made though unsuccessfully to prevent or cure cancers by reducing the biotin intake.

Glutathione

The tri-peptide, glutathione or glutamyl-cystenyl-glycine, is another sulfur containing compound that has been found in a very wide range of tissues and cells.



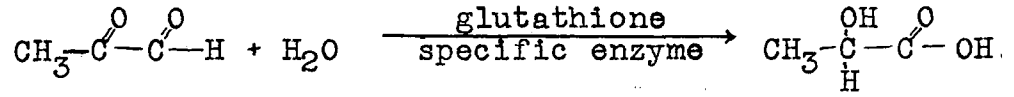
Glutathione (Reduced Form)

Glutathione may be reversibly oxidized by removing the hydrogen atoms from the sulphur to form disulfide:

$$2GSH \rightleftharpoons 2H + GS\text{-}SG$$

This occurs in neutral solution at the rather low potential of -0.233 indicating that the compound might play a part in the transfer of hydrogen near the carbohydrate end of glycolysis. However, though reactions between ascorbic acid and glutathione have been noted in vitro no one has yet found evidence to establish the role of glutathione in any specific hydrogen

transfer reaction. The only proven function of glutathione in living cells is as a coenzyme for the reaction forming lactic acid from methyl glyoxal.



Because this one function does not seem to account for the presence of such relatively large and universal quantities as occur in living things it is generally assumed that glutathione does act as a hydrogen carrier. There is for example 34-37mg/100ml in blood, 60mg/100ml in yeast and 70mg/100ml in liver. It seems probable that organisms use the peptide glutathione rather than cysteine as a hydrogen carrier because of its much greater solubility. Glutathione was first discovered by Hopkins in 1921 (27). At first he considered it to be glutamyl-cysteine but because of evidence presented by Hunter and Eagles (28), Hopkins (29) made another study and revised his original formula.

There are, as well as the six widely distributed sulfur compounds to which reference has already been made, a number of others which are less common and presumably less important to living organisms.

Taurine

Taurine like glycine may occur conjugated with cholic acid in the bile salts. It also occurs in very high concentrations in the muscle tissue of certain invertebrates where it may act by an effect on the osmotic concentration. Presumably, taurine is formed from cysteine by decarboxylation and oxidation of the sulfur.

$NH_2-CH_2-CH_2-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-OH$ Taurine

Mucin

The glycoprotein mucin may have two different types of sulfur containing prosthetic groups, chondroitin sulfuric acid or mucoitin sulfuric acid. The former is composed of a molecule of sulfuric acid, with one of amino-galactose and one of gluconic acid. Mucoitin sulfuric acid is the corresponding compound with glucose substituted for galactose. Mucin is important in maintaining a moist smooth surface on the mucous membranes; free chondroitin sulfuric acid is used pharmacologically in the treatment of migraine.

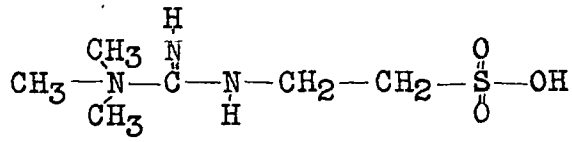
Heparin

Heparin is another sulfur containing substance in which sulfuric acid is combined with a carbohydrate. It is produced by the liver in small amounts and inhibits the conversion of prothrombin into thrombin. Its action is different from that of dicoumerol which prevents the formation of prothrombin. Both of these drugs are used medicinally as anticoagulants, heparin having the prompter action but requiring hyperdermic injection. In mucin the sulfuric acid apparently acts only as a link between the protein and the carbohydrate but in heparin it seems to play a more active part for the activity of the heparin is directly proportional to the percentage sulfur (30).

Asterubin

Asterubin is another oxidized sulfur compound,

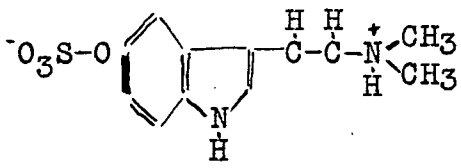
isolated from starfish and identified by Akermann in 1935 (31). Nothing is known of its function.



Asterubin

Toad Poisons

Some of the toad poisons contain sulfur but it is believed that these sulfate groups are not essential to their action. The formula of bufothionine, the commonest of these poisons, is as follows:



Bufothionine

Sulfones

Lefevre and Rangier (32) found that 7% of the sulfur of deproteinated blood serum is in the sulfonic form but no study seems to have been made as to the exact nature of this fraction.

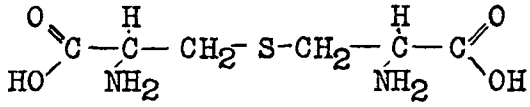
Sulfatides

Sulfatides constitute another naturally occurring group of compounds containing oxidized sulfur. Sulfate occurs in certain of the cerebrosides and phospho-lipids of the brain (33). Possibly sulfate replaces the phosphate in these compounds.

Lanthionine

As well as the two accepted amino acids, cystine and methionine to which reference has been made, there are three

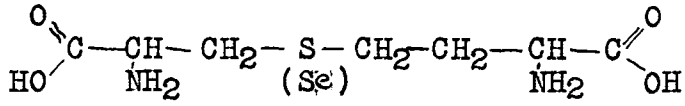
other sulfur containing compounds that have been isolated from proteinateous materials and therefore considered to be possible constituents of the protein molecule. Of these the best known is lanthionine which may be isolated from hair after hydrolysis with sodium carbonate. Whether or not lanthionine is formed during the hydrolysis from cysteine is a matter of debate; it is probably of biological significance, however, for it would likewise be formed in the intestinal tract.



Lanthionine

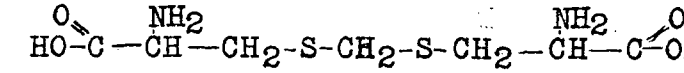
A Thio Ether

A compound very similar to lanthionine but consisting of a mixed ether of cysteine and homocysteine has been isolated by Horn and Jones (34) from the weed Astragalus vulgaris. This compound is of particular interest because it occurs with the isomorphous compound containing selenium when the weed is grown on seleniferous soil.



Djenkolic Acid

Djenkolic acid, isolated from the djenkol nut by Van Veen and Hyman (35) is another reduced sulfur compound that is thought to occur in proteins. As far as has been determined it has not yet been isolated from any other source. The formula given for it is as below.

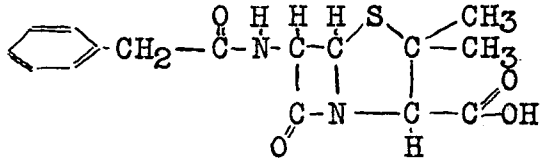


Djenkolic acid

Urochrome

Urochrome is an impure fraction of the urine which contains the pigment urobilin and a polypeptide with a rather high content of sulfur. It must, therefore, be considered in studying the forms of sulfur occuring in the urine.

Penicillin



G-penicillin

The antibiotic, penicillin, contains a mixture of several active compounds with different side chains but they all contain sulfur in a thiasine ring. The action of penicillin in inhibiting the growth of bacteria is not yet understood nor is it known whether the sulfur is essential for the antibiotic effect. Synthesis of G-penicillin was recently effected through the combined efforts of several laboratories both British and American and its structure is now definitely established (36).

Thio-sugar

A thio sugar believed to be a thio-methyl-ribose has been isolated from yeast by Mandell and Dunham (37) and further studied by Suzuki (38) and by Levene and Sobotka (39). It is known that the sugar occurs in yeast linked to adenine but the position of the different groups has not been completely elucidated yet and nothing is known of its specific function.

Odours and Flavours

Some of the sharp flavors and odors of natural

substances are due to sulfur containing substances. The compound chiefly responsible for the flavor of mustard is allyl-isothiocyanate:

$CH_2=CH-CH_2-N=C=S$

A somewhat similar compound gives the distinctive odour and flavour to garlic. It is allyl-disulfide:

$CH_2=CH-CH_2-S-S-CH_2-CH=CH_2$

One of the distinctive components of asparagus is methyl mercaptan. This compound may be absorbed from the gastro-intestinal tract and excreted by the kidneys within fifteen minutes of its ingestion.

Although the odour of methyl mercaptan is not unpleasant, that of its homologue, butyl mercaptan, is extremely so and this forms the principle component of the skunk's odour.

Thiocyanates

A simple compound containing reduced sulfur that occurs widely distributed in variable amounts in all body fluids is thiocyanate. It may enter the body in the diet but most of it would appear to be formed endogenously, possibly as a detoxification product of cyanides (40). It is sometimes used medically in the treatment of hypertension but has no known function in the quantities in which it is normally present in the body. However, it is possible that thiocyanates are used in the biological synthesis of certain natural sulfur compounds such as ergothioneine for in the laboratory reactions between it and a number of other organic compounds occur very easily.

The twenty-five sulfur compounds described above together with ergothioneine (see section I, 3) comprise most of the forms in which sulfur is known to occur commonly in plants and animals but do not by any means account for all the reactions that have been observed to occur between natural sulfur containing substances and various laboratory reagents. It is true that several of the compounds that have been listed are apparently of very limited importance but certain of them play a very active role in metabolism and it is probable that some of the as yet unknown compounds of sulfur will also be found to be of great importance.

Synthetic Drugs

In addition to the naturally occurring sulfur compounds a number of synthetic substances have been found to possess a physiological action that has proved valuable in medicine. By far the most important class of these compounds is that of the sulfonamides. A full discussion of these compounds is beyond the scope of this report but it is interesting to note that, although there are several different theories on their exact mode of action, most of the theories agree in assuming that the sulfonamides interfere with the function of some normal metabolite, that is the sulfonamide group is similar in structure to but different in function from the substance taking part in normal metabolism.

Thiourea, thiouracil and certain derivatives of these compounds are used in the treatment of thyrotoxicosis. This action will be more fully discussed in a later section of

this report.

Sulfonmethane and sulfonnethylmethane are both used as hypnotics and in some cases as antispasmodics. They are usually known by their trade names, sulfonal and trional.

Sulfur Bacteria

Although only a brief mention can be made here of the Thiobacilli and Thiobacteriales, the review of the biochemistry of sulfur would not be complete if nothing were said of them. Various species of these remarkable bacteria are able to oxidize hydrogen sulfide, sulfur or thiosulfates and with the energy so obtained they can reduce carbon-dioxide. In a good many species light is required for this process. These bacteria synthesize the reduced carbon into all the organic compounds needed to build their protoplasm including the vitamins, thiamin, biotin, nictotinic acid, pantothenic acid, pyridoxine and riboflavin (41).

2. A Review of the Biochemistry of the Imidazoles

The imidazoles form one of the most important classes of natural heterocyclic compounds for this ring occurs as an integral part of the purine ring as well as independantly in a number of commonly occurring compounds.

Purines

Only a very brief discussion of the purines is possible here. With the pyrimidines they occur attached to the pentoses in nucleic acids and in this form probably play a

key role in the controlling mechanism of cells. It has been shown that nucleic acids which presumably can vary only in the positions of the linkages and the bases contained therein are the controlling factor in the production of a specific type of capsule by pneumococci (42). Purines also occur in the prosthetic groups and co-enzymes of a large number of enzyme systems and here again play a very active part in cell metabolism. There is some evidence for believing that the purines are synthesized by the body from the simple imidazole ring of histidine for excretion of the purine breakdown product, allantoin, by the rat ceases if the diet is lacking in histidine.

Histidine

The most important of the simple imidazole compounds is the amino acid histidine. Histidine was simultaneously isolated in 1896 by Kossel (43) from sturine and by Hedin (44) from a mixture of proteins. It is βimidazole α amino-proprionic acid and structurally it is closely related to arginine though it has now been established that these are not inter-changeable in mammalian metabolism.

Histidine is essential in the diet of the young growing animal but it seems that it can be synthesized to some extent and the need for it in the adult probably varies with the species. It occurs in almost all proteins but in particularly high concentration in histones, hemoglobin and in most protamines. The metabolic path of histidine is but poorly

understood.

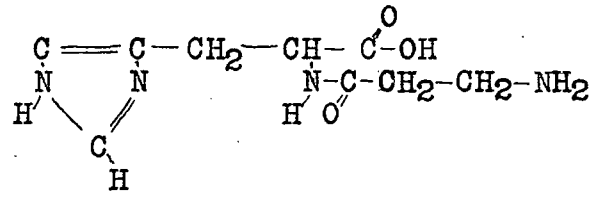
It may lead through the removal of urea to glutamic acid and hence to glycogenesis or through the removal of ammonia to urocanic acid but in normal metabolism neither of these products seem to be formed in sufficient quantity to account for an appreciable portion of the total histidine metabolized.

Histamine

Histamine, the decarbozylation product of histidine, is a compound of very great medical importance. It occurs naturally in ergot and as a putrifaction product in meat and feces. That formed by bacterial action in the intestine may be absorbed into the blood stream but it is usually detoxified at a sufficient rate to prevent noticeable effects. If, however, histamine is injected hyperdermically it causes contraction of smooth muscle, stimulation of the salivary, gastric, pancreatic and lacrimal glands and a fall in blood pressure due to increased permeability of the capillaries; all these are symptoms characteristic of anaphylactic shock, asthma and allergy. The similarity of the symptoms of these different conditions which may be brought about by any of a wide variety of different substances has been explained by considering them all as antigen-antibody reactions. The theory most generally accepted at present states that, in cases where only a slight immunity is built up, most of the antibody formed will be held to the cell surfaces. If antigen is now

introduced into the blood stream it will not be neutralized till it reaches the cell surface where the reaction causes the cell to release the histamine into the blood. Thus it is always histamine that causes the observed effects. Evidence for this theory comes from the fact that compounds which will react with histamine or which will cause the development of antibodies for histamine are effective in the treatment of allergy and anaphylactic shock. In smaller concentrations histamine may be a normal hormone stimulating certain glands to secrete and the removal of it from the blood stream, may therefore, cause serious complications.

Carnosine and Anserine

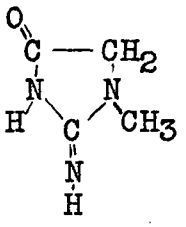


Carnosine

Carnosine and anserine are two compounds containing histidine that occur very widely in vertebrate muscle tissue. The distribution of carnosine in particular has been extensively studied by Cifford (45) and Hunter (46). Hunter has also developed a colorimetric method for its determination based on Knoop's diazo reaction (47). Practically nothing is known of the function of either carnosine or anserine although they are present in significant and constant quantities. The only clue that we have at present is that they are both strong buffers. Carnosine is βalanyl-histidine, a dipeptide of histidine linked to the rather rare β amino acid. Anserine is

similar but has a methyl group in the number three position, that is on one of the nitrogens of the ring. This is in contrast to ergothioneine which is methylated on the amino nitrogen.

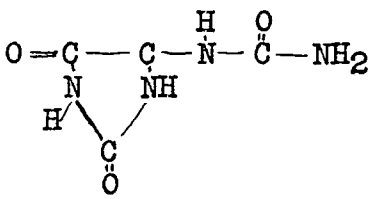
Creatinine



Creatinine is not generally considered to be related to histidine because metabolically it is formed by the dehydration of creatine which in turn is synthesized from glycine, arginine and methionine. Creatinine does, however, contain the imidazole ring with keto, imino and methyl groups attached. Thus it forms an intermediate in what is a possible path of formation of imidazoles from guanidine compounds or visa versa.

Allantoin

Another imidazole occurring in the urine of certain animals is allantoin, a di-keto substituted imidazole ring

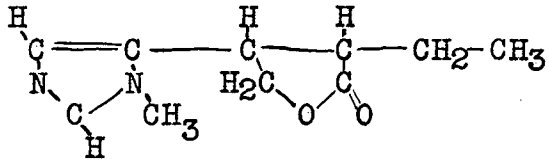


with a urea side chain. It is thus a substituted hydantoin. Allantoin has a pronounced stimulating effect on the growth of tissues and this is doubtless the function of the large amounts found in plant and animal embryos. It has also been

found that allantoin will greatly hasten the healing of many wounds and it has been used extensively, particularly in the treatment of war wounds. Reference has already been made to the possibility of a metabolic relationship between histidine, allantoin and the purines.

Other hydantoin derrivatives have found a pharmacological use in the treatment of epilepsy and chorea. Their beneficial action in these diseases reflects their similarity to the barbiturates.

Pilocarpine

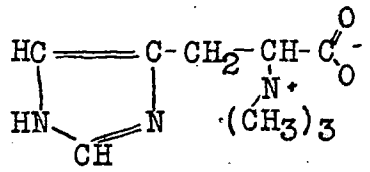


Still another imidazole of importance in pharmacology is pilocarpine, a natural alkaloid found in the leaves of a South American shrub. In pharmacy it is one of the most important of the drugs used to stimulate the parasympathetic nervous system being used in the treatment of glaucoma, bronchitis, and skin disorders. The demethylated compound pilocarpidine occurs with pilocarpine in the same shrub and has a similar but weaker physiological action.

Biotin

The biochemistry of biotin has been discussed with the other sulfur containing compounds but it may be pointed out that it also contains a substituted imidazole ring. In biotin there is an oxygen atom in the 2 position, the position corresponding to that occupied by the sulfur of ergothioneine.

Trimethyl Histidine



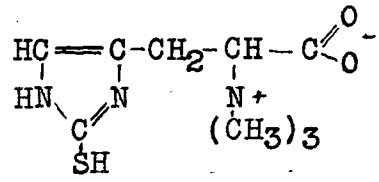
Trimethyl histidine

Trimethyl-histidine is a betaine that has been isolated from certain edible mushrooms. In spite of its limited distribution it is worthy of note because it is a possible intermediate in the hypothetical synthesis of ergothioneine from histidine.

3. A Review of the Chemistry of Thiolimidazoles

Ergothioneine from Ergot

The history of the thiolimidazoles is in some ways similar to that of cystine. Ergothioneine was early isolated from a non-protein source and in spite of many efforts to isolate it or a related compound from proteins and in spite of many indications of the presence of a thioliminazole in proteins no one has yet succeeded in proving any of these compounds to be a natural constituent of proteins. C. Tanret (48) was the first to isolate a natural thiolimidazole when he extracted ergothioneine from ergot of rye in 1909. He studied the physical and chemical properties of the compound and worked out the empirical formula, $C_9H_{14}N_3O_2S$. In further studies Barger and Ewins (49) gave good evidence for the following structural formula.



Although there was never much doubt of the validity of this formula it was only proved by synthesis during the past year by Winegard (50). Harrington and his co-workers (51, 52) had previously succeeded in synthesizing thiolhistidine by two different methods but were unable to methylate this compound to form the betaine, ergothioneine. Barger and Ewins based their structure on the following evidence. Heating ergothineine with concentrated alkali splits off trimethylamine leaving an unsaturated acid; this shows that it is a betaine. This compound, if oxidized with nitric acid to remove the sulfhydryl group and reduced with sodium to saturate the double bond, yields β(4imidazole) proprionic acid which has been synthesized. Barger and Ewin assumed that the assumed that the sulfur was attached to carbon 2 of the ring because its lability in the presence of ferric chloride and stability in alkali is like that of known compounds so constituted. This was later confirmed by the synthesis of thiolhistidine.

In these two papers, the one by Tanret and the other by Barger and Ewins there is a fairly complete study of the most important reactions of ergothioneine. It is precipitated by silver and mercury salts, by phospho-tungstic acid, and basic lead acetate but not by neutral lead acetate, picric acid or tannic acid. Iodine also causes a precipitation but by oxidizing the ergothioneine to a disulfide. It is soluble

in water and dilute alcohol and slightly so in pyridine but insoluble in absolute alcohol and other organic solvents.

Barger and Ewins also stated that ergothioneine has no marked physiological action though they did not give their evidence. Though this may be correct, at the present time the statement is subject to some doubt and for some time it did discourage further work on the compound.

Ergothioneine from Blood

In 1925, Bulmer, Eagles and Hunter (53) found that they obtained consistently lower results in determining the uric acid in the blood by precipitation of the uric acid with silver lactate according to the method of Folin and Wu (54) than they did using the direct method of Benedict (55). They also found that the reducing substance which was interfering with the direct method occurred in high concentration in the corpuscles. The unknown substance was found to be present in the blood of the rabbit, dog, cat, ox, and guinea pig.

Immediately after the publication of these results Benedict (56) made a defense of the direct method on the ground that it was much simpler than the indirect method and that the results agreed closely in most cases. He stated that he had long recognized the presence of another reducing substance in blood and that he with his associates had been working on the isolation of it for some time. A paper published in 1926 by Benedict, Newton and Behre (57) announced that a substance which they called thiasine had been isolated from blood by Mrs. Dugdale in 1921 and that they had now confirmed this as

the substance which interfered with the direct uric acid determination. They gave physical constants for the compound and postulated the empirical formula as, $C_{12}H_{20}N_4O_3S$.

Meanwhile, in 1925, Hunter and Eagles (58) had independently isolated the interfering substance and they gave the empirical formula, $C_6H_{11}N_2O_3$ and a rather different set of physical constants. For example the melting point was given as 224-225 C for this compound while the melting point of thiasine was given as 162-163. Both groups found that the respective compounds occurred almost entirely in the corpuscles and hence would little effect the uric acid determination.

In a later paper Hunter and Eagles (59) named the substance that they had isolated, sympectothion (firmly bound sulfur) and submitted the revised formula $C_{18}H_{30}N_6S_2O_4$ which is two molecules of ergothioneine plus one of water. However before this had been published Newton, Benedict and Dakin submitted evidence on December 6, 1926 (60) to show that thiasine was identical with ergothioneine and on December 23 Eagles and Johnson (61) showed that sympectothion was fundamentally the same as both the above compounds. The differences in the physical constants of thiasine and sympectothion had arisen because Benedict and his co-workers had isolated the hydrochloride while Hunter and Eagles had isolated the free base. In the above paper Newton et al. suggested that the name ergothioneine be shortened to thioneine but possibly due to confusion with thionine, 7aminophenothiazine, this name has not been generally accepted.

Isolation

Since these three original methods of isolating ergothioneine were developed, several modifications have appeared in the literature. The first was developed by Eagles in 1928 (62) and was based primarily on a combination of some of the reactions used by Tanret (48) with those of Hunter and Eagles (58). Ergot was extracted with alcohol, the fats and alkaloids removed and the extract was clarified with barium hydroxide. The ergothioneine was successively precipitated with basic lead acetate, mercuric chloride, lead acetate and sodium hydroxide and phosphotungstic acid. It was finally isolated as the free base by crystalization from absolute alcohol. A yield of 0.65% of pure ergothioneine was obtained from the ergot.

In 1929 Hopkins (63) developed a method for the isolation of glutathione using cuprous oxide and thus brought into prominence the relatively specific reaction between thiol groups and cuprous ions. By applying the use of this reagent to the precipitation of ergothioneine from blood Williamson and Meldrum (64) were able to shorten the procedure considerably. The proteins and some of the other interfering substances are removed with dilute acid and "dialized iron". The glutathione is then oxidized by aeration and the ergothioneine precipitated as the cuprous salt.

Pirie (65) modified this method somewhat so as to make it applicable to ergot which is richer in ergothioneine than is blood but which has more interfering substances.

With this method Pirie got a yield of 0.18% ergothioneine. It must be pointed out, however that while these methods have been generally compared on the basis of the percentage yield, it has recently been shown by Lawson and Rimington (66) and by Hunter et al. (67) that there is considerable variation in the normal ergothioneine content of ergot and hence this basis of comparison is not reliable.

Recently Hunter and co-workers (68) has combined the copper precipitation with certain reactions used by Hunter and Eagles in the original isolation of ergothioneine from blood and has obtained a 0.26% yield of ergothioneine from ergot. In this method the ergot is extracted with water, much of the impurity removed with uranium acetate, and the ergothioneine precipitated with cuprous oxide. It is then freed of the copper with hydrogen sulfide, treated with charcoal and crystalized from absolute alcohol.

Experiments

Nutrition

After the identity of the ergothioneine in the blood had been established, Eagles and his co-workers proceeded to study the possible functions of it in the body. First Eagles and Cox (69) proved that ergothioneine could not replace histidine in the diet and this work has since been supplemented by Neuberger and Webster (70) who showed that even thiolhistidine cannot take the place of histidine. Further evidence of

the distinct metabolsim of the thiolimidazoles has been furnished by Dale, Gaddum and Broom (71) who found that thiolhistamine has only one two-thousandth the pressor effect of histamine on the rat and less than one fifty-thousandth the effect on the cat. The effect of thiolhistamine on the rabbit blood sugar was not detectable. If the body has not the mechanism to remove the sulfur from thiolimidazoles it seems unlikely that it could add sulfur to the imidazole ring and therefore the metabolism of thiolimidazoles is most likely quite distinct from that of histidine. If it is of a distinct origin the thiolimidazole might come from the diet or might be synthesized in the animal body, possibly from thiocyanate and ornithine. A certain amount of circumstantial evidence has been gathered which favors the theory of exogenous origin.

Some of the most important evidence for the exogenous origin of ergothioneine is in the paper by Eagles and Vars who carried out a study of the conditions governing the ergothioneine content of pigs blood. The content of the blood of grain fattened pigs from a Toronto abattoir had been found to be very high in comparison with that of garbage fed pigs from the Springside Farm, New Haven. This led them to a series of dietary experiments which showed that while there was practically no ergothioneine in the blood of pigs on a garbage or casein diet there was a very considerable amount when they were on a diet of grasses, corn or other grains.

The Hunter diazo test (72) which will be described in a later part of this report was known at this time and Eagles and Vars (73) applied it directly to a sample of zein hydrolyzed with sulfuric acid. The results indicated the presence of a thiolimidazole though they found that the interference of histidine prevented the drawing of definite conclusions. This report will later demonstrate that histidine is not the only interfering substance. On the basis of this work Eagles and Vars postulated the presence in certain proteins of a thiolimidazole which would most probably occur as the amino acid thiolhistidine. The amino acid might be methylated in the animal body. This is the origin of the thesis that has been the basis of this report.

In 1935 Potter and Franke (74) carried out an expanded series of dietary experiments using rats as the experimental animal. The very striking differences that they obtained in the ergothioneine content of the blood with different diets are summarized in Table I. It is interesting to note that they found considerable difference between two samples of the same grain. Potter and Franke conclude from these results that the ergothioneine of the blood is entirely exogenous but do not venture to suggest in what form it may occur.

Table I. Ergothioneine content of blood of rats

Diet	Minimum mg/100ml	Maximum mg/100ml	Mean mg/100ml	Number of tests
Corn[1]	0.69	1.23	0.93	27
Wheat	0.12	0.66	0.43	57
Oats 1	1.18	1.30	1.22	7
2	0.46	0.87	0.59	7
Barley	0.15	0.15	0.15	9
Potato	0.08	0.08	0.08	7
Stock[2]	0.98	1.28	1.17	6
Low Thioneine[3]	0.08	0.08	0.08	12

1. The grain diets contained as well as 82% of the grain, 10% casein, 3% lard, 2% cod liver oil, 2% dried yeast and 1% McCollum's salt mixture.

2. Stock diet contained 25% wheat, 25% corn, 20% oats, 10% fish meal, 5% wheat germ, 5% alfalfa, 5% peas, 3% cod liver oil, 1% NaCl, 1% $CaCO_3$.

3. Low thioneine diet contained 45% sucrose, 20% casein, 10% yeast, 10% corn starch, 10% butter, 3% salts, 2% cod liver oil.

Physiology

Because ergothioneine had now been shown to be a normal constituent of mammalian blood, it was felt that the statement of Barger and Ewins that ergothioneine had no physiological action should be verified and more fully studied.

Tainter (75) did this by testing the action of ergothioneine injected intravenously on the blood pressure, pulse rate, bilary secretion, smooth muscle contraction and blood sugar level using both the rabbit and cat. He found no effect in any case.

A few years later Trabucchi (76) carried out another series of tests using various experimental animals. Apparently Trabucchi was not acquainted with the work of Tainter. He also found ergothioneine without action on the uterine muscle of the rabbit but noted an action on the frog's heart. Bathed in a 0.1% solution of ergothioneine in Ringer's solution the heart beat faster while a 1% solution caused a rapid decrease in the amplitude of the beat and caused it to stop in diastole. Trabucchi also studied the effect of ergothioneine on pigeons with beri-beri because an early incorrect formula for thiamin gave it a structure rather closely related to and possibly derived from ergothioneine (77). He found that 2.5mg of ergothioneine intramuscularly daily raised the temperature of the bird one degree, prevented further decrease in weight and steadied the walking but did not delay death. Finally, Trabucchi found that ergothioneine, like cysteine and glutathione, acts as an antidote in cyanide poisoning. There was a theory that cyanide acted by poisoning the glutathione and cysteine-cystine reduction-oxidation systems but Trabucchi said that if so ergothioneine must also act in a similar manner. The evidence presented by Pirie (78) to show that both thiolhistidine and ergothioneine are

catalysts of the oxidation of glutathione and cysteine, might help explain this effect. Thus, while Tainter, on the basis of his work, agreed with Barger and Ewins, Trabucchi concluded that ergothioneine has several physiological actions.

Braun, Mason and Brown (79) investigated the possibility that ergothioneine might act in place of insulin but got negative results. The fact that neither thiol-histidine nor ergothioneine occurs in insulin had already been established by du Vigneaud, Sifferd and Millar (80) using the bromine oxidation method of Zahnd and Clark (81).

Pathology

Several workers have found the ergothioneine content of the blood and urine to vary with disease. Salt (82) found 3-12mg/100ml of red blood corpuscles in normal and nephritic persons but found it to be from 7-15mg/100ml in fifteen diabetic cases. Sulivan and Hess (83) found 90mg per liter of ergothioneine in normal urine and noticed that with different diseases it might be either higher or lower, being high in both cancer and arthritis.

This relation between ergothioneine and cancer has been further studied by the late Herbert Winegard and his associates but, as far as is known their results have not yet been published (52). A study of the diazo reaction of the blood was made by Nakayama (84) and he arrived at the conclusion that ergothioneine was probably the chief cause of the reaction. In diseases where he found a strong diazo

reaction in the blood he found a weak one in the urine. In all this work on pathology each figure is based on about fifteen individual tests but it is doubtful if the diets were properly controlled. If these results are significant and the concentration of ergothioneine in the blood and urine does vary on a constant diet then it must either be synthesized or retained to a varying degree. Nakayama's work makes the latter theory seem more probable.

Anti-thyroid Effect

What may prove to be a very important physiological function of ergothioneine was first demonstrated by Lawson and Rimmington (66) in 1947. The discovery in 1943 by Mackenzie and Mackenzie (85) that thiourea has a goitrogenic action led to the investigation of many related sulfur compounds in an effort to find something more active and less toxic. Astwood and his co-workers (86, 87, 88) tested a long series of compounds finding several which were active, the least toxic of which, propyl-thiouracil, is now widely used. Even this substance may, however, cause agranulocytosis and other toxic effects. Lawson and Rimington noticed that all the active compounds contained a carbon atom linked to two nitrogens and a sulfur atom and they suggested that the compound ergothioneine which contains this group and occurs in the blood naturally without toxic effects might be useful in clinical treatments.

Lawson and Rimington therefore proceeded to test the efficiency of ergothioneine as a thyroid inhibitor. They

found that their results varied when they used the rat growth method of determining its activity but were more consistent when they measured the iodine content of the thyroid gland of rats administered ergothioneine by subcutaneous injection. The latter method was based on the theory that these drugs act by preventing the accumulation of iodine in the thyroid gland. Their results, given in Table II, indicate that the activity of ergothioneine is comparable to that of thiouracil. In as much as variable quantities of ergothioneine of the same order as those administered are normally present in the blood these workers found it difficult to properly control their tests and suggest that some of the variations encountered by them may have been due to this difficulty.

Table II. Effect of Ergothioneine on the Iodine of the Thyroid Gland

Dosage mg/Kg body wt.	Iodine content in g/100mg of wet thyroid			
	Ergothioneine		Thiouracil	
	Mean	Deviation	Mean	Deviation
0.2	20.0	4.03	19.6	1.49
0.5	13.5	4.11	8.0	2.40
0.75	21.0	4.00	4.65	2.25
2.0	5.0	1.76	6.4	1.20
Control	58.2 ± 3.48 g/100mg of wet thyroid			

Astwood and Stanley (89) carried this work further

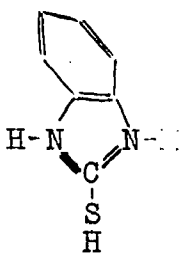
by testing the clinical effectiveness of ergothioneine, both as a therapeutic agent in thyrotoxicosis and as a possible cause of simple goiter. Measuring the activity of the ergothioneine by its effect on the rate of uptake of radio-active iodine by the thyroid gland, they found no change of rate in five normal persons on the administration of 50 to 400 mg of ergothioneine orally or intravenously. Persons injected with mercaptothiazole or methyl thiouracil, on the other hand showed a sharp break in the rate of uptake and leveling of the radio-activity of the thyroid gland on administration of the drugs.

Owing to the failure of these experiments on humans, Astwood and Stanley tried to repeat the work of Lawson and Rimington on rats but could find no change in the weight or iodine content of the thyroids of rats treated with ergothioneine whether it was administered in the food or by injection. They also tried feeding an impure concentrate of ergothioneine to see if the effect observed by Lawson and Rimington could have been due to some impurity in their ergothioneine but again with negative results.

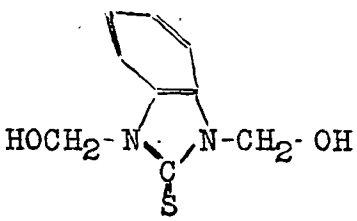
Lawson and Rimington (90) had proceeded at the same time to use ergothioneine clinically in the treatment of two cases of human thyrotoxicosis but got no response in either case. They suggest the possibility that this failure and those of Astwood and Stanley may have been due to oxidation of the ergothioneine to the disulfide.

Latner and Mowbray (91) have made a study of the ergothioneine content of the blood of six thyrotoxicosis patients when fasting and found it below normal in four cases and a low normal in the other two. They suggest that the difficulties of Astwood and Stanley may be due to the fact that ergothioneine does not seem to be bound by serum protein as is thiouracil and hence it could be more easily excreted by the kidney.

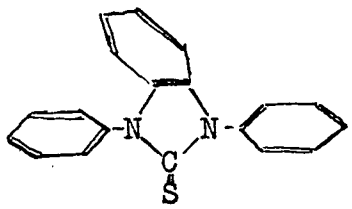
More indirect support for the thesis that ergothioneine has goitrogenic properties comes from the work of Monti and Venturi (92) who synthesized and tested three thiol-benzimidazoles finding them of comparable activity to methyl thio-uracil. If these three compounds are all active it seems probable that substitution of betaine for the benzene ring would not affect the active grouping to a great extent.



Thio-Benzimidazole



1,3 di(hydroxymethyl)-thiobenzimidazole



1,3 - di-phenyl-thiobenzimidazole

Warner-Jauregg and Koch (93) have carried out studies on the goitrogenic effect of several natural products. Among the active compounds found was a heat labile compound from white cabbage. They postulate that this is a benzyl-thiourea but have not isolated it and therefore it is possible that it may be related to the thiol-imidazoles.

Analysis

Several methods and modifications thereof have been developed for the detection and determination of thiolimidazoles. The first and still the most generally useful method is an adaptation of the diazo reaction which was published by Hunter in 1928 (72). This test gives a yellow color with thiolimidazoles which changes to a purple red on the addition of concentrated alkali. Tyrosine when treated in this way gives a pink color very similar to that of the thiolimidazoles and histidine gives a yellow color which may obscure the desired test and make quantitative estimation of thiolimidazole difficult. The method has been slightly modified recently to increase its accuracy with the modern photoelectric colorimeter (94). Also another modification has been proposed by Latner (95) for use in the determination of ergothioneine in blood.

In 1929 Behre and Benedict (96) brought out another method based on the original method of isolation of ergothioneine used by Benedict, Newton and Behre (56). This method is based on the precipitation of uric acid and ergothioneine with silver lactate and the separation of these two substances by first dissolving the uric acid with acid sodium chloride and subsequently the ergothioneine with sodium cyanide. The ergothioneine is then determined colorimetrically by the color developed with Folin and Wu's tungstic acid reagent (54). This test is, however, considered rather less specific than that developed by Hunter.

A quantitative study of the reactions of the various forms of sulfur present in proteins has been carried out by Zahnd and Clarke (97) and Blumenthal and Clarke (81).[1] This study included an investigation of the effect of oxidation by bromine and it was found that only that sulfur in $\rangle C=S$, $\overset{O}{\rangle} C-SH$, and $\overset{-N}{\rangle} C-SH$ linkages was labile in this reagent. This sulfur includes that of the thiolimidazoles but not of any other commonly occurring sulfur compound. Bromine oxidation has been used by Du Vigneaud, Sifferd and Miller (80) to prove that there is no thiolhistidine in insullin but the presence of a bromine labile sulfur fraction has not yet been established as proof of the presence of thiolimidazoles.

Other methods of analysis which as yet do not seem to have been used for the determination of thiolimidazoles but which might prove useful in this field of work are the oxidative procedure of Kitamura and Masuka (98) and the colorimetric method of Grote. The technique of Kitamura and Masuka utilizes the oxidation of sulfur by means of hydrogen peroxide. In their study on the influence of H_2O_2 on various sulfur compounds they found that, whereas RCH_2SH and thiophene groups were stable in the presence of this reagent, substances containing the following are quantitatively oxidized: $-N=\dot{C}-SH$, $O=\dot{C}-SH$, $S=\dot{C}-SH$, $=N-\dot{C}=S$, $-O-\dot{C}=S$, $-N=\dot{C}-S-\dot{C}=N-$, and $\overset{C}{\underset{C}{\diagdown\diagup}}S=S$.

Grote (99) developed an interesting method of differentiating sulfur compounds, using the product resulting

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1 See also section II.

from the action of light on sodium nitroprusside. This reagent which may also be made by treating nitroprusside with bromine, gives a green color passing through turquoise, blue and purple to crimson with >C=S, -N=S, or >S=S groups. The reaction is sensitive to 1ppm of thiourea. The reagent also gives a transient blue color with >C-SH groups and with >C-S-S-C< groups on reduction. The results obtained in using this reagent with ergothioneine are discussed in the experimental section of this report.

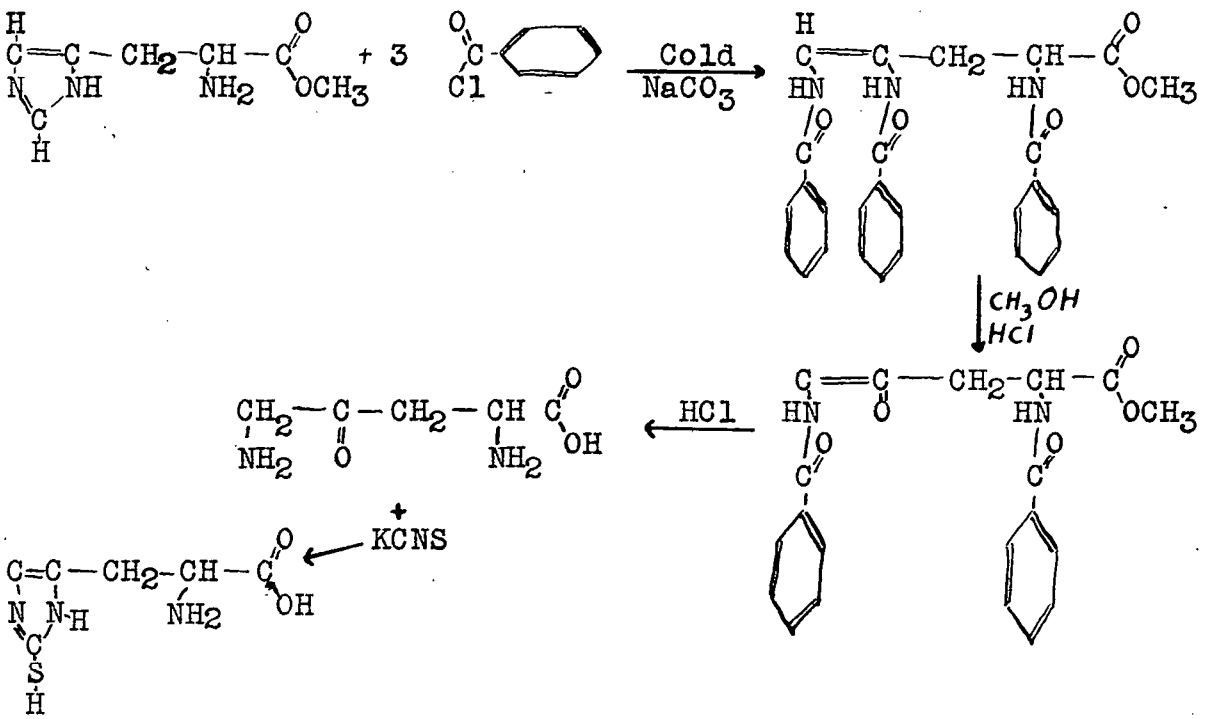
Synthesis

It has been pointed out that Barger and Ewins (49) demonstrated the structure of ergothioneine by means of its reactions but did not confirm this structure by synthesis. When it was discovered that ergothioneine was a normal constituent of the blood it became desirable to synthesize both to provide material for experimentation and to prove the structure. Several attempts were made but no one succeeded until November 1948 when Winegard (50) finally developed a method. The details of his proceedure do not seem to have been published as yet and it is only known that the synthesis involves the use of diazomethane.

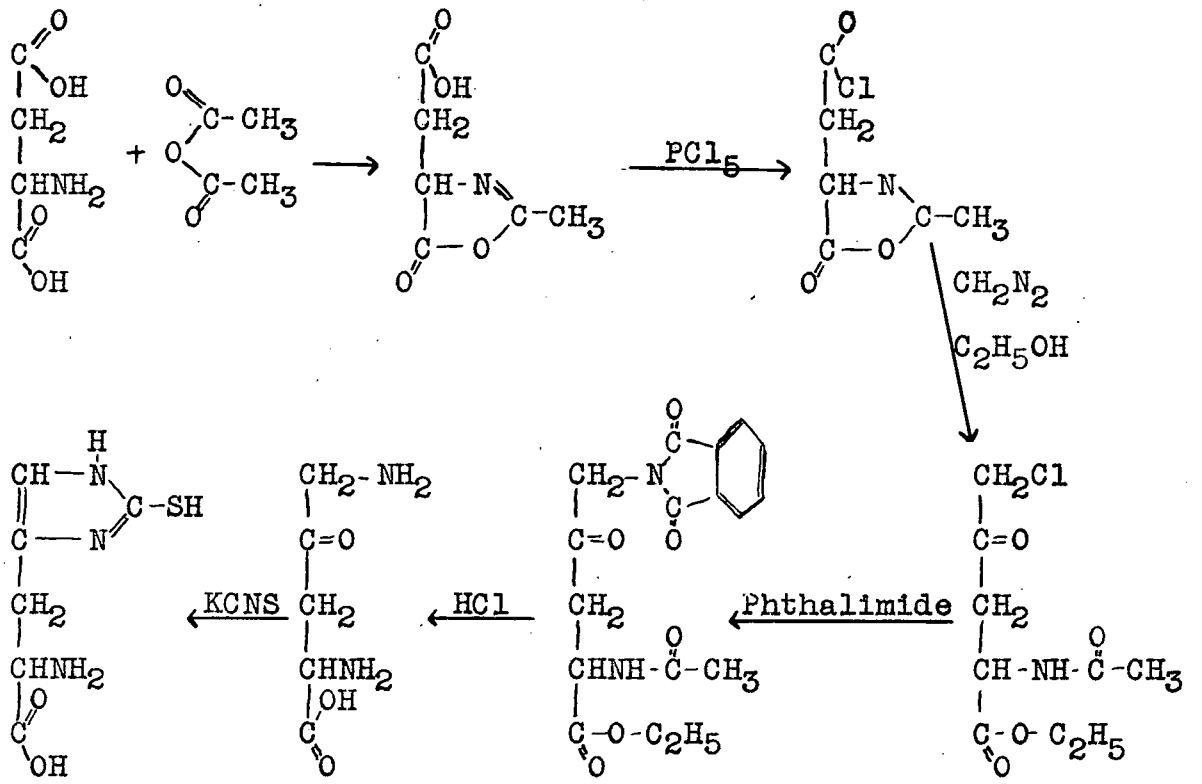
Other thiolimidazoles had, however been synthesized many years before this. The first was thiolhistamine synthesized by Pyman (71) from dibenzamidoketo butane. The steps of the synthesis are parallel to those used shortly afterward by Ashley and Harrington (51) in the synthesis of thiolhistidine. It was Pyman who first developed the method

method of synthesizing the thiolimidazole ring by the use of thiocyanate, the method which has been used by all subsequent proceedures.

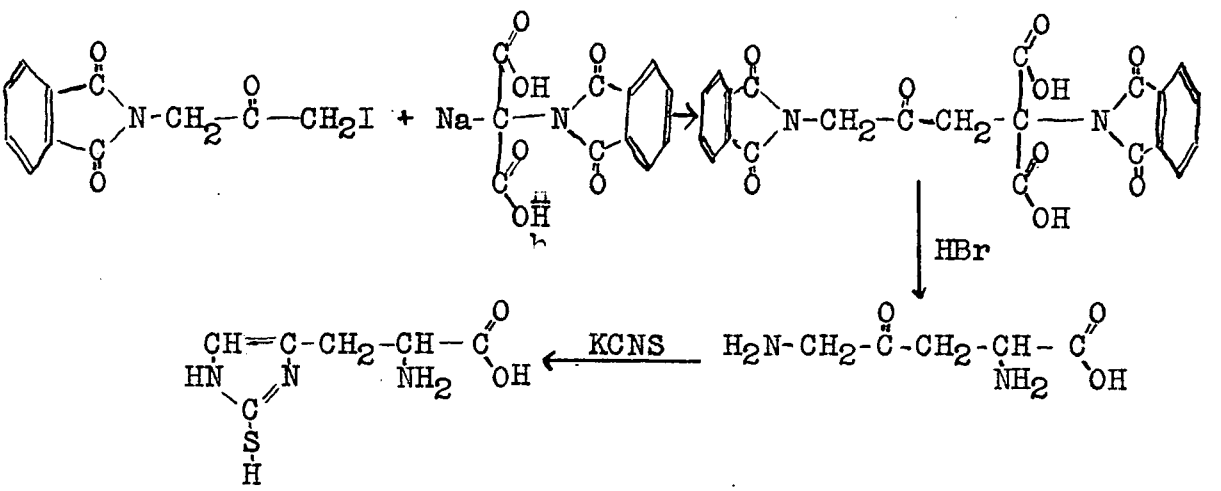
To synthesize thiolhistidine Ashley and Harrinton started with histidine methyl ester and benzoylated it according to the method of Windaus, Dörries and Jenson (100) breaking the imidazole ring. One benzamido group is eliminated to give a ketone and the ring reassembled with thiocyanate to give thiolhistidine.



Harrinton and Overoff (52) tried unsuccessfully to form ergothioneine by methylating thiolhistidine but incidentally developed another method to synthesize the amino acid starting with aspartic acid.



Still another synthesis of thiolhistidine was worked out by Dey (101) using *phthalo-ω-iodo-* acetonylimide and malonic ester.



II. Consideration of Problem

It has been pointed out that the discovery of methionine made possible the explanation of previously recognized reactions of proteins and accounted for a large fraction of the protein sulfur that was known to be different from that of cystine or cysteine. It is likewise of importance that in the light of the literature a reinvestigation of the relationship between the total sulfur of proteins and the sulfur accounted for by the known amino acids be undertaken to see if there exists a fraction of the total sulfur that might occur as a thiolimidazole. The unexplained reactions of protein sulfur must also be studied to obtain an estimate of the amount and nature of the unknown fractions.

The evidence in favor of the hypothesis that thiolimidazoles occur in proteins come from the feeding experiments and the study made by Clarke et al. (81, 97). Sullivan and Hess (103) have found that normal human urine contains about 90 mg/liter as measured by colorimetric analysis. If it is assumed that this ergothioneine is entirely exogenous then an average daily intake of ergothioneine would be at least 135 mg. and the food must contain on the average in the neighborhood of 0.01% ergothioneine. If this were concentrated in the protein it would be in a concentration of about 0.15%.

The work of Zahnd and Clarke and of Blumenthal

and Clarke[1] indicates an even higher concentration of thiolimidazoles in proteins. Proteins, protein hydrolysates and fractions thereof have been treated with alkaline plubite, nitric acid and with bromine to yield different fractions of the total sulfur present. Of particular interest is the fraction found to be oxidizable by bromine, a reagent which will oxidize the sulfur of ergothioneine or that occurring in the $>C=S$ $^{O}\!\gtrless\!C-SH$ or $^{-N}\!\gtrless\!C-SH$ linkages but not that of cystine or methionine. If the bromine oxidizable sulfur in proteins occurs as a thiolimidazole it is difficult to reconcile the fact that Blumenthal and Clarke find as much of this fraction in the hydrochloric acid hydrolysates as in the whole protein with the work of Lefevre and Rangier (32) who found that thiolimidazoles are destroyed by this treatment. It has been found in this laboratory that ergothioneine does not give a positive Hunter test after boiling with hydrochloric acid[2] but it is possible that the breakdown products are still oxidizable by bromine. From the results of their work Blumenthal and Clarke conclude that there is also another unknown form of sulfur in proteins which is labile in alkaline plumbite but not oxidizable by nitric acid. Some of the results obtained by Blumenthal and Clarke are given in Table III. It will be noticed that the bromine oxidizable sulfur content of the albuminoids is particularly high but that it also occurs to some extent in all the other proteins studied.

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1 See also section I, 3, "Isolation".
2 See section III, "Separation of ergothioneine from amino acids".

Table III Sulfur in Proteins from Blumenthal and Clarke

Protein	Total S	Preexisting Sulfate S	Corr. SO_4 S by Br_2	Corr. SO_4 S by HNO_3	Alkali labile S
Gelatin 1	0.20	0.06	0.01	0.03	0.03
Gelatin 2	1.10	0.99	0.00	0.04	0.02
Blood alb.	1.10	0.07	0.05	0.83	0.62
Lactalbumin	1.22	0.00	0.02	0.81	0.65
Egg white	1.60	0.03	0.04	0.68	0.43
Zein	0.52	0.16	0.02	0.25	0.22
Casein	0.63	0.00	0.01	0.09	0.09
Wool	3.06	0.00	0.21	3.06	3.07
Horn	3.60	0.09	0.26	3.51	3.59

Some of the results obtained from sulfur and amino acid analyses of proteins by various workers using several different methods are compiled in Table IVA. For convenience the results found for each protein have been averaged although it is realized that figures thus obtained may be misleading. The proteins were doubtless of variable purity, some of the results have been corrected for the addition of water during hydrolysis and those of Block and Bolling have been calculated on a common basis of sixteen percent nitrogen in the proteins.

One of the most striking facts shown by table is the variability of the values found particularly for cystine and cysteine and for sulfur itself. The average figures for

sulfur in zein account for twenty-two percent more sulfur reported as known amino acids than is found in the total sulfur but on the other hand if the figure given by Baernstein for the total sulfur is correct there is more than 20% of the sulfur not accounted for. The general picture shows only a small fraction of the total sulfur unaccounted for in most of the proteins considered and because most of these figures were calculated from percentages of the amino acids on the assumption that no correction had been made for the water of hydrolysis, this small fraction may be insignificant. Pre-existing sulfate in the proteins may also give rise to a part of the sulfur unaccounted for. This sulfate is usually not detectable but the few figures available for it, show that in some protein samples it may comprise up to twenty percent of the total sulfur. Where one sample of a protein has been analyzed for all known fractions and total sulfur, the results are usually in good agreement and this has led Baernstein (104) and Brand (105) to discount the possibility of unknown sulfur containing units being present. However, the dietary and bromine oxidation experiments indicate the presence in certain proteins of as yet unaccounted for forms of sulfur. It is possible that a portion of this sulfur exists as thiolimidazole. Possible explanations of the discrepancies which exist in the figures obtained by the respective groups of workers employing different techniques to assay the nature and amounts of the various forms of unknown sulfur from proteins may be offered.

Table IVA Distribution of Sulfur in Proteins

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted for % of protein	S unaccounted for % of sulfur
Egg Albumin	1[1]	0.51%	1.09%	1.75%	0.15%	8.6%
	2[2]	0.60	0.98	1.60	0.02	1.2
	3[3]	0.46	1.13	1.60	0.01	0.6
	4[3]	0.47	1.12	1.85	0.26	14.1
	5[3]	0.40	1.40			
	6[4]	0.35	0.99			
	7[5]	0.53	1.11			
	8 a[6]	0.24				
	b	0.27				
	c	0.27				
	9[7]	0.26				
	10[7]	0.26				
	11 a[8]	0.63				
	b	0.64				
	12[9]		1.36			
	13[9]		1.20			
	14[10]			2.0		
	15[10]			1.66		
	16[10]			1.71		
	17[10]			1.18		
	18[11]			1.60		
	19[12] a		1.12			
	b		0.87			

Table IVA Continued

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted for % of protein	S unaccounted for % of sulfur
Egg Albumen Cont'd	20[24]		0.82			
	21[25] a		0.96			
	b		0.97			
	c		0.95			
	Average	6.42	1.06	1.60	0.12	7.4
Casein	1[1]	0.09	0.73	0.75	-0.07	-9.3
	2[2]	0.18	0.72	0.80	-0.10	-12.5
	3[2]	0.15	1.70	0.83	-0.02	-2.4
	4[3]	0.08	0.87			
	5[4]	0.07	0.73			
	6[5]	0.08	0.79			
	7[7]	0.08				
	8[8]	0.08				
	9[8]	0.08				
	10[9] a		0.68			
	b		0.64			
	11[10]			1.2		
	12[11]			0.63		
	13[12]	0.09	0.69	0.78	0.0	0.0
	14[14]	0.06				
	15[15]	0.08				
	16[16]	0.08				
	17[18]	0.07				

Table IVA Continued

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted for % of protein	S unaccounted for % of sulfur
Casein Cont'd	18[19]	0.07				
	19[20]	0.18				
	20[21]	0.09				
	21[22]	0.08				
	22[24]		0.63			
	22[25] a		0.58			
	b		0.58			
	c		0.58			
	Average	0.09	0.68	0.83	0.06	7.2
Lact-albumin	1[2]	1.01	0.56	1.56	-0.01	-0.6
	2[3]	0.83	0.60	1.42	-0.01	-0.7
	3[4]	1.15	0.55			
	4[5]	0.91	0.57			
	5[6] a	0.56				
	b	0.65				
	c	1.02				
	6[8] a	0.86				
	b	0.86				
	7[9] a		0.52			
	b		0.50			
	8[11]			1.22		
	9[12]	0.82	0.60	1.42	0.0	0.0
	10[14]	0.68				

Table IVA Continued

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted for % of Protein	S unaccounted for % of Sulfur
Lact-albumin Cont'd	11^{16}	0.61				
	12^{17}	0.65				
	13^{18}	1.06				
	14^{20}	1.00				
	15^{21}	0.70				
	16^{23}a	0.81				
	b	0.81				
	c	0.81				
	d	0.82				
	17^{25}a		0.38			
	b		0.38			
	c		0.36			
	Average	0.82	0.50	1.40	0.08	5.5
Wool	1^{1}	3.8	0.13	3.9	0.0	0.0
	2^{4}	3.5				
	3^{6}a	3.4				
	b	3.4				
	c	3.8				
	4^{8}a	2.7				
	b	2.7				
	5^{10}			3.06		
	6^{14}	2.6				
	Average	3.2	0.13	3.5	0.2	5.8

Table IVA Continued

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted for % of protein	S unaccounted for % of sulfur
Gelatin	1[1]	0.05	0.24	0.5	0.2	40.0
	2[2]		0.21			
	3[4]	0.05	0.21			
	4[5]	0.043	0.21			
	5[12]a		0.19			
	b		0.13			
	6[25]b		0.17			
	c		0.18			
	Average	0.048	0.19	0.5	0.25	50.0
Edestin	1[1]	0.40	0.53	1.2	0.3	25.0
	2[2]	0.48	0.44	0.99	0.07	7.0
	3[4]	0.27	0.45			
	4[6]a	0.31				
	b	0.34				
	c	0.34				
	5[7]	0.36				
	6[7]	0.33				
	7[7]	0.37				
	8[9]a	0.37				
	b	0.47				
	9[13]			0.85		
	10[14]	0.33				
	11[15]	0.36				

Table IVA Continued

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted for % of protein	S unaccounted for % of sulfur
Edestin Cont'd	12[16]	0.32				
	13[17]	0.35				
	14[18]	0.20				
	15[19]	0.26				
	16[20]	0.48				
	17[21]	0.32				
	18[22]	0.32				
	19[25] a		0.48			
	b		0.48			
	c		0.48			
	Average	0.34	0.48	1.01	0.19	19.
Zein	1[1]	0.24	0.51	0.36	-0.39	-108.
	2[2]		0.47			
	3[2]	0.50	0.42	0.93	0.01	1.1
	4[4]	0.21	0.49			
	5[5]	0.27	0.62			
	6[7]	0.28				
	7[9] a		0.54			
	b		0.50			
	8[9] a		0.48			
	b		0.47			
	9	0.24				
	10	0.27				

Table IVA Continued

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted. for % of protein	S unaccounted. for % of sulfur
Zein Cont'd	11	0.22				
	12	0.13				
	13	0.23				
	14	0.42				
	15		0.45			
	16 a		0.36			
	b		0.36			
	c		0.32			
	Average	0.27	0.46	0.60	-0.13	-22.
Serum albumin	1[1]	1.7	0.28	1.7	-0.3	-18.
	2[4]	1.63				
	3[6]a	1.52				
	b	1.52				
	c	1.62				
	4[7]	1.62				
	5[10]			1.89		
	6[10]			1.75		
	7[11]			1.10		
	8[12]	1.74	0.18	1.94	0.02	1.0
	9[12]	1.68	0.28	1.96	0.00	0.0
	Average	1.63	0.25	1.72	-0.16	-9.3

Table IVA Continued

Protein	Sample	Cystine & Cysteine S	Methionine S	Total S	S unaccounted for % of protein	S unaccounted for % of sulfur
Gliadin	1[1]	0.69	0.5	1.2	0.0	0.0
	2[2]	0.26	0.43	0.99	0.30	30.0
	3[4]		0.45			
	4[7]	0.58				
	5[8]a	0.60				
	b	0.61				
	6[14]	0.55				
	7[15]	0.58				
	8[16]	0.58				
	9[17]	0.65				
	10[18]	0.62				
	11[19]	0.38				
	12[20]	0.73				
	13[24]		0.57			
	Average	0.56	0.46	1.1	0.1	9.1

Footnotes to Table IVA.

1. From Block, R. J. and Bolling, D., The Determination of the Amino Acids, Minneapolis, Burgess Publishing Co.,1940.
   Cystine-cysteine by phospho 18 tungstic acid, colorimetrically.
   Methionine by homocysteine method.
   Sulfur by Burgess-Parr sulfur bomb.
2. From Baernstein, H. D., J. Biol. Chem., 97, 663-8 and 669-74 (1931).
   Cystine-cysteine by Baernstein's gasometric method.
   Methionine by Baernstein's volatile iodide method.
   Sulfur by peroxide fusion in Parr bomb.
3. Baernstein, H. D., J. Biol. Chem., 115, 25 (1936).
   Methods not available. Hydrolysis by hydro-iodic acid.
4. Cohn, E. J., Ergeb. Physiol., 33, 781 (1931).
   Methods not available.
5. Figures selected from the literature by Dr. B. A. Eagles.
6. Sullivan, M. X. and Hess, W. C., U. S. Pub. Health Reports, suppl. 86, (1930).
   a. Sullivan test.
   b. Okuda test (iodometric).
   c. Folin - Looney test.
7. Folin, O. and Marenzi, J. Biol. Chem., 83, 103-108 (1929).
   Method a modification of that of Folin and Looney.
8. Graff, Maculla and Graff, J. Biol. Chem., 121, 81-86 (1937).
   Method a modification of that of Vickery and White.

9. Baernstein, H. D., J. Biol. Chem., 115, 31 (1936).

a. Volatile iodide method.

b. Homocysteine method.

10. Shultz, F. M., Z. physiol. Chem., 25, 16-25 (1898).

A compilation from several early workers. Methods not available.

11. Blumenthal, D. and Clarke, H. T., J. Biol. Chem., 110, 343 (1935).

Method not given.

12. Brand, E., Ann. N.Y. Acad. Sc., 47, 187-228 (1946).

Cystine-cysteine by photometric iodine method.

Methionine by homocysteine method.

Sulfur by method of Pregl (Saschek).

13. Osborne, T. B. and Campbell, J. Am. Chem. Soc., 18, 609 (1896).

Method not available.

14. Vickery, H. B. and White, A., J. Biol. Chem., 99, 701 (1933).

Original method involving isolation of cysteine with cuprous oxide.

15. Loc. cit., Method of Folin and Marenzi.

16. Loc. cit., Method of Sullivan.

17. Loc. cit., Method of Okuda.

18. Loc. cit., Method of Folin and Looney.

19. Loc. cit., Method of Jones, Gersdorff and Moeller.

20. Loc. cit., Baernstein's gasometric method.

21. Vassell, B., J. Biol. Chem., 140, 323 (1941).
Original method involving a color developed with p-amino-dimethyl-aniline.

22. Loc. cit., Polarographic method.

23. Kassell and Brand, J. Biol. Chem., 125, 115 (1938).
a. Photometric method.
b. Microphotometric method.
c. Sullivan method.
d. Baernstein method.

24. Csonka, F. A., and Denton, C. A., J. Biol. Chem., 163, 329 (1946).
Method a modification of that of McCarthy and Sullivan.

25. Horn, M. J., Jones, D. B., and Blum, A. E., J. Biol. Chem., 166, 313 and 321 (1946).
a. Modified method of McCarthy and Sullivan with acid hydrolysis.
b. Modified method of McCarthy and Sullivan with papain hydrolysis.
c. Microbiological assay.

Table IVB

Deviations from the Mean of Cystine-Cysteine Results by Various Methods

Method	Egg albumin	Casein	Lactalbumin	Wool	Edestin	Zein	Serum albumin	Gliadin	Average
	Deviation from mean in % of sulfur of protein								
Vickery and White		−.03	−.15	−.7	−.02	−.03		−.02	−.05
Folin and Marenzi	−.16 −.16	−.01 −.01			+.02 −.01 +.03 +.01	+.00 +.01	−.01	+.02 +.01	−.02
Sullivan	−.18	−.01	−.26 −.02 −.22	+.2	−.03 −.03	−.05	−.11	+.00	−.08
Okuda	−.15		−.17 −.18	+.2	+.00 +.00		−.11	+.07	−.06
Folin and Looney	−.15	−.02	+.20 +.22	+.6	+.00 −.14	−.14	−.01	+.04	+.01
Jones, Gersdorf and Moeller		−.02			−.09	−.05		−.18	−.08
Baernstein's gasometric	+.18	+.06 +.09 +.08	−.01 +.00 +.17		+.14 +.12	+.23 +.14		−.30 .15	+.08
Vassell		+.00	−.13		−.01				−.05
Polarograph		−.01			−.01				−.01
Block and Bolling	+.09	+.00		+.6	+.06	−.03	+.07	+.13	+.05
Graff Kjeldahl	+.21	−.01	+.04	−.5				+.04	+.05
Graff $BaSO_4$	+.22	−.01	+.04	−.5				+.05	+.05
Baernstein	−.02 +.04 +.05	−.01	+.01				+.11		+.01
Photometric		+.00	−.02				+.11 +.05		+.03

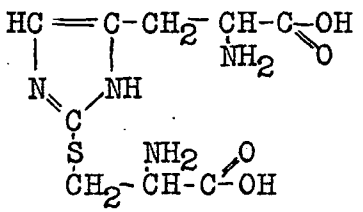
It has been found that the thiolimidazole ring is destroyed by the usual methods of hydrolysis. The product of this breakdown has not as yet been identified but it is possible that it could react both with bromine and behave as one of the other sulfur containing amino acids in certain analytical methods. The fact that the accuracy of some of these methods was judged by the degree to which they accounted for all the sulfur of proteins lends plausibility to this theory (106, 107). If the breakdown products of thiolimidazoles are measured with the known amino acids it seems most likely that it is the determinations of cysteine that are at fault for more bromine oxidizable sulfur is found in the albuminoids than could be present in the methionine fraction of these proteins and the results obtained in the determination of cysteine by different methods are much more more variable than in methionine determinations. In one method Graff, Maculla and Graff (108) have determined cysteine by isolating it from the hydrolysates with Cu_2O and measuring both the nitrogen and the sulfur contents. This method would measure any unsplit thiolhistidine or ergothioneine present in the hydrolysate with the cysteine but if they were included there would be a discrepancy between the values as determined by nitrogen and by sulfur. The results of Graff et al. show no significant discrepancy but Shultz and Vars (109) have pointed out that all results obtained by this method do not agree so closely. By the barium sulfate method they found hepatic protein to contain 1-2% cysteine nitrogen but Graff

and Barth found it to have 4.65% by Kjeldahl determinations. This difference in the findings could be explained if a large fraction of thiolhistidine were present.

In Table IVA are listed the results of cystine-cysteine determinations by sixteen different methods. Most of these methods are based on the determination of the reducing power of the -SH group and would probably not differentiate cysteine sulfhydryl groups from those of thiolimidazole breakdown products. The deviations from the mean of the results obtained by these different methods are compared in Table IVB. If only certain methods do not measure sulfur from the thiolimidazoles these would be expected to show a variable but consistently negative deviation. In the case of those methods for which enough figures are available to make statistical treatment applicable this is true of the methods developed by Vickery and White, by Jones, Gersdorff and Moeller and, except for the result from wool, of Sullivan's procedure. A modification of the Vickery and White method by Graff et al. shows results higher than the average and it, therefore, seems probable that the deviation shown by the original method arises from factors other than the differences in the sulfhydryl groups. Similarly the Jones, Gersdorff and Moeller method is a modification of the one developed by Folin and Looney and taken together the two methods do not show a consistent deviation. The method of Sullivan, on the other hand, is based on a very specific color reaction with cysteine and the low results obtained by

it may be of considerable significance. Unfortunately there has been no microbiological method for the assay of cystine or cysteine developed as yet (110) but it would be interesting to see if results obtained by this method agree with those given by Sullivan's method. It is possible that it has been interference from thiolimidazoles that has thus far prevented the formulation of a microbiological method.

A third possible explanation of the differences between the sulfur content of proteins and their indicated content of amino acids and thiolimidazoles is that cysteine and histidine might occur linked as a thio ether. On hydrolysis by the usual laboratory methods the thio ether might yield cysteine and a derivative of histidine but it is possible that it would give serine and thiolhistidine by enzymatic hydrolysis. If this be true it is difficult, to explain the nature of the bromine oxidizable fraction observed by Zahnd and Clarke.



Hypothetical thio ether

III. Experimental

Preparation of ergothioneine

In order to carry out the proposed search for thiolimidazoles it was felt that the first requisite was a

supply of ergothioneine and thiolhistidine for use in controls. Seventy milligrams of ergothioneine prepared by Dr. B. A. Eagles in 1928 were available. Recrystalization of this material from alcohol showed that it had suffered relatively little decomposition since originally prepared but it was, nevertheless, felt that a larger supply was desirable.

Twelve hundred grams of ergot were obtained through the generosity of the Upjohn Company of Kalamazoo, Michigan. A preliminary test of the ergothioneine content of this ergot was made by extracting a finely ground sample with boiling water, clarifying the filtrate with neutral lead acetate and applying the Hunter test. In comparison with some of the ergothioneine prepared by Eagles this test indicated only 1 mg of ergothioneine per gram of ergot. In this test the extraction may have been incomplete, the clarification with neutral lead acetate less satisfactory than the uranium acetate used by Hunter (67) in his method of determining ergothioneine in ergot and the visual comparison inaccurate. However, it does seem that this sample had a rather lower ergothioneine content than those from which Pirie (65) was able to extract 1.8 mg/g and Hunter et al. (68) 2.6 mg/g. During the course of this work a paper by Lawson and Rimington (66) has been published which describes even greater differences in the ergothioneine content of ergot from different sources and more recently Hunter has found values for the ergothioneine content of ergot on Alberta rye varying

from 0.184 to 0.474% (67).

Nine hundred grams of the ergot were treated according to Pirie's method (66). Only a small amount of white precipitate formed on the addition of cuprous oxide and all subsequent steps gave correspondingly low but typical yields. However, the final product of about 40 mg. was insoluble in 3:4 acetone:water and gave an orange color by the Hunter test (94) such as might be given by a mixture of histidine and ergothioneine. Evidently only a small amount of ergothioneine was extracted from the ergot and much of the sulfur was removed from it during purification. These difficulties may have been caused by the fact that the procedure was several times interrupted and the complete isolation carried out over a period of several weeks.

The remaining three hundred grams were extracted according to the method of Eagles (62). Again small but typical precipitates of the ergothioneine fraction were obtained at each step. This time, again, a small yield, 53 mg, was obtained but this substance gave only a color typical of histidine by the Hunter test. It is thought that impurities formed in the alcohol during drying with sodium may have oxidized the ergothioneine.

Synthesis of thiolhistidine

Attempts to synthesize thiolhistidine have thus far been as unsuccessful as those to isolate ergothioneine. The method of Ashley and Harrington (51) was chosen as the most suitable. In the first attempt the reactants exploded

with considerable violence during benzoylation. This may have been due to carbon dioxide released by the formation of acid or to a small rise in temperature after most of the ice had melted. In subsequent runs the reaction vessel was periodically opened although excessive pressure was never noticed.

In the second attempt to synthesize thiolhistidine a good yield was obtained from the benzolyation but that of methyl $\alpha\delta$-dibenzamido-γ-ketoval-erate was very small. Failure here appears to have been due to the fact that the reaction was not carried out in anhydrous conditions.

One more effort has been made to date to carry out this synthesis. This time yields were obtained as indicated by Ashley and Harrington in all except the final step when no thiolhistidine crystalized out. The Hunter test when applied to the impure solution of products showed only the yellow color typical of histidine and it is considered possible that the reactants may have been contaminated with iron which would catalyze the removal of the sulfhydryl groups.

Initial work with protein hydrolysates

There are no records available of any attempt to isolate thiolimidazoles from proteins by methods parallel to those used for the isolation of these compounds from other sources. This, the simplest method of approach, was, therefore, the first studied in this work. Unfortunately a test for ergothioneine could not be made at the termination of each step due to interference from amino acids and humus. The

first tests, made on zein, human hair and finger nails, hydrolyzed with sulfuric acid and clarified with charcoal, gave apparently positive colors with the Hunter test but no purple precipitate. A control using pure ergothioneine, however, showed that when small quantities were used the ergothioneine was almost quantitatively removed by the charcoal (Darco). It may be that another brand of charcoal could be found which would be capable of adsorbing the coloured impurities without destruction of ergothioneine but this has not as yet been investigated.

A test on egg white, hydrolyzed with sulfuric acid in the presence of titanous chloride and treated according to Pirie's (65) method for the isolation of ergothioneine from ergot, gave a strong red-purple color by the Hunter test but again no purple precipitate. However, when tested again a week later this product gave a definitely negative test and no fraction of a sample of egg white divided into albumin, globulin, mucin and protein free filtrate gave a positive test. These results, baffling at the time, may have been due to the interference of tyrosine as discussed in a following section.

Following these preliminary familiarization tests a series of tests was made in an effort to determine which proteins would be the most satisfactory for a detailed study. The method used was again chiefly based on the earlier methods for isolating ergothioneine. The protein was hydrolyzed with hydrochloric acid and titanous chloride, a method

successfully used by Sullivan and Hess (103) in the isolation of cystine. The hydrolysate was neutralized, clarified with basic lead acetate and the thiolimidazole precipitated with cuprous oxide as in Pirie's procedure. The precipitate was treated with hydrogen sulfide and a Hunter test carried out on the copper free filtrate. The protein containing substances tested and the results of the color reaction obtained are given in Table V.

Table V Hunter Diazo Reaction on a Protein Hydrolysate Fraction

Substance Tested	Result	Substance Tested	Result
Zein[1]	Positive	Cabbage	Negative
Corn Glutinin	Doubtful	Peccan	Doubtful
Alc. & H_2O[2] soluble corn	Probable	Filberts	Negative
Bad Corn (whole)[3]	Negative	Brazil Nut	Negative
Wheat Glutinin	Negative	Ox Hoof	Probable
Wheat Gliadin	Possible	Ox Horn	Negative
Oat Gliadin	Negative	Ox Lung	Possible
Hordein	Negative	Ox Brain	Positive
Pea Albumin	Negative	Ox Fibrin	Negative
Pea (whole)	Possible		

1 Prepared according to method of Mason and Palmer (111).

2 The filtrate from the precipitation of the alcohol solution of zein in water. This might contain any free ergothioneine of the corn.

3 Moldy and discolored kernels which were present in the corn used.

No odor of trimethylamine was noticed in any case and no purple precipitates were obtained although obscuring precipitates of humus were often present. It was not definitely known if these were essential characteristics of a positive test under these conditions. By the time the tests listed in the table had been completed work carried out concurrently on improving the method of isolation revealed that these results were probably only a measure of the tyrosine left in solution after neutralization and the project was abandoned.

Revision of the method

The objective of this second line of endeavour was to test and modify each step of the above procedure so as to make accurate and reliable results possible. Difficulty was experienced because it was impossible to detect anything but inordinately high concentrations of ergothioneine until after the fractionation was complete. Therefore, if two steps of the procedure were at fault it might not be possible to detect improvement in one unless the other were simultaneously corrected. In each test a series of dilutions were made, usually 1/1, 1/10, 1/100, and 1/1000 in order to dilute out interfering substances. Because there was most evidence from dietary experiments for the presence of thiolimidazoles in zein this protein was used in the following tests.

Sullivan and Hess (103) have found that the addition of titanous chloride to a protein during hydrolysis shortens the time required for hydrolysis and decreases the destruction

of cystine. In the initial work in this study this reducing compound was used and it was now considered desireable to investigate its specific effect on thiolimidazoles.

The first tests gave negative colors with the hydrolysates of zein both with and without titanium while repetition of the tests gave weakly positive results in both cases. None of these results was as strong as those given by the initial tests with zein but there was no evidence of any improvement due to the titanium and it was, therefore, omitted from subsequent hydrolyses.

Barger and Ewins (49) have shown that the sulfur of thiolimidazoles is oxidized and removed in the presence of ferric ions. Because the iron reduced in this reaction would be reoxidized by air it was feared that small amounts of iron if present in a thiolimidazole solution would catalyze the oxidation of the sulfur. In work performed thus far care had been taken to avoid any possible contamination with iron but it was deemed desirable to investigate the necessity of this precaution. The first test again gave negative results both when the protein was hydrolyzed in distilled reagents and when it was hydrolyzed in 0.03% ferric chloride. However, when 6.5% ferric chloride was boiled with a hydrolysate that was known to give a strong red diazo test, the resultant product gave a distinctly negative colour. This was considered to support the hypothesis that the substance being measured was a thiolimidazole. Precautions were continued for the avoidance of all possible contamination with iron.

A series of tests were made in an effort to determine the best conditions for hydrolysis. The results of these tests are summarized in Table VI according to the intensity of the color formed but because of interference by impurities and the variability of the effect of dilution the real significance of these results is difficult to assess. The results do not fall into any definite pattern although some of the strongest color tests were obtained by hydrolysing with hydrochloric acid. Hydrolysis with pepsin (Difco.), papain (Difco.), and pancreatin (Nutritional Biochemicals) were tried but none of the preparations digested the zein completely and the results were inconclusive but definitely not as strong as those with hydrochloric acid.

In many of these tests a transient pink color was obtained at the interface on adding alkali. It is not known whether this was caused by decomposition of the coloured compound in the strong alkali or by dilution of it beyond the limit of sensitivity.

It will be noticed that relatively low results were obtained in series VI of Table VI where the alcohol and water soluble fraction of the corn was used. This fraction, the filtrate obtained on the removal of zein after treating the alcohol extract of corn with water, is that in which any free ergothioneine from the corn would probably appear. The color obtained in this series was distinct and relatively free of interference. In fractionating the corn a large volume of this solution was obtained so that, although it

Table VI

Effect of Conditions of Hydrolysis on the Intensity of the Color Given by the Hunter Test

Temperature °C	100		125				140	150	165	210	20
Time hrs.	1.5	5.0	2.5	6.0	16.	28.	18.	2.5	2.0	0.17	0.0
Series	Expressed as equivalent amount of ergothioneine in per cent of zein.										
I	0.13	0.08	0.13	0.17	-	0.08	0.4	0.2	0.13	0.04	
II			0.13 0.08	0.13 0.08	0.17 0.08						
III					?						
IV			0.04	0.04	0.32						
V			0.08	0.2							
VI			0.08		0.13						0.08
VII			0.0	0.0	?						

Key to Series

I. 3g of zein in 15ml of 20% HCl.
II. 3g of zein in 15ml of 20% H_2SO_4.
III. 3g of zein in 15ml of 40% H_2SO_4.
IV. 3g of zein in 15ml of 50% formic acid, 7% HCl.
V. 3g of zein in 15ml of 20% NaOH.
VI. 8ml of alcohol and water soluble fraction of corn with 7ml of 37% HCl.
VII. 3g of residue from resolution of zein in alcohol hydrolyzed with 20% HCl.

gave a relatively weak colour compared to that from an equal weight of zein, it must have contained a greater amount of the colour giving compound than the zein prepared from the same amount of corn. If this compound in the alcohol and water soluble fraction were ergothioneine it would amount to 0.07% of the total weight of the corn. However, concentration of this solution did not cause a proportional increase in the intensity of the reaction nor did hydrolysis with hydrochloric acid destroy it as it was later found to destroy ergothioneine. When the zein was re-purified by four successive precipitations from alcohol, the colour developed by a hydrolysate prepared from the purified product was not appreciably weakened. Work was, therefore, continued with the protein but it is hoped also to investigate further the nature of the colour giving substance in the soluble fraction.

Tests were made using different combinations of the precipitating agents that had been found of value in the isolation of ergothioneine from ergot and blood. Several combinations of baryta, phosphotungstic acid, basic lead acetate, mercuric chloride and cuprous oxide were tried but in no case was there less interference or a stronger colour than that in the tests performed with the method originally outlined.

During this study it became clear that the method employed for removing metallic salts with hydrogen sulfide was a possible source of destruction of thiolimidazoles. After precipitation of ergothioneine with copper, the

sensitivity of the Hunter test was greatly reduced if the copper was not completely removed. However filtration of the copper sulfide was often difficult due to its colloidal nature and much ergothioneine was destroyed when the process was prolonged. Ten different substitutes were used for the gas in separating the ergothioneine and copper but only cupferron was found to give complete removal of the copper without destruction of the ergothioneine or interference with the Hunter diazo test.

Interference by Amino Acids

Early in the course of this study an investigation had been made of possible sources of interference with the Hunter test. It was found that histidine gave a yellow color and tyrosine a pink color closely resembling that given by thiolimidazoles in shade and sensitivity. An attempt was made to distinguish these colors spectroscopically but there were no distinct absorption bands. A spectrophotometer might serve to differenciate the colours when present in combinations but it would not be suitable for routine work.

Interference due to histidine was easily reconizable and was only serious in a qualitative test when it was sufficiently intense to obscure a weak thiolimidazole reaction. The colour reaction for tyrosine was sensitive to about 0.003 mg/ml and would be definitely positive if all the tyrosine of zein were present in the hydrolysate. The hydrolysate was, therefore, always carefully neutralized and filtered after standing a few hours and it was thought to contain only

the 0.41 mg/ml soluble in water. This amount could not account for the reactions observed when the hydrolysate was diluted with one thousand parts of water.

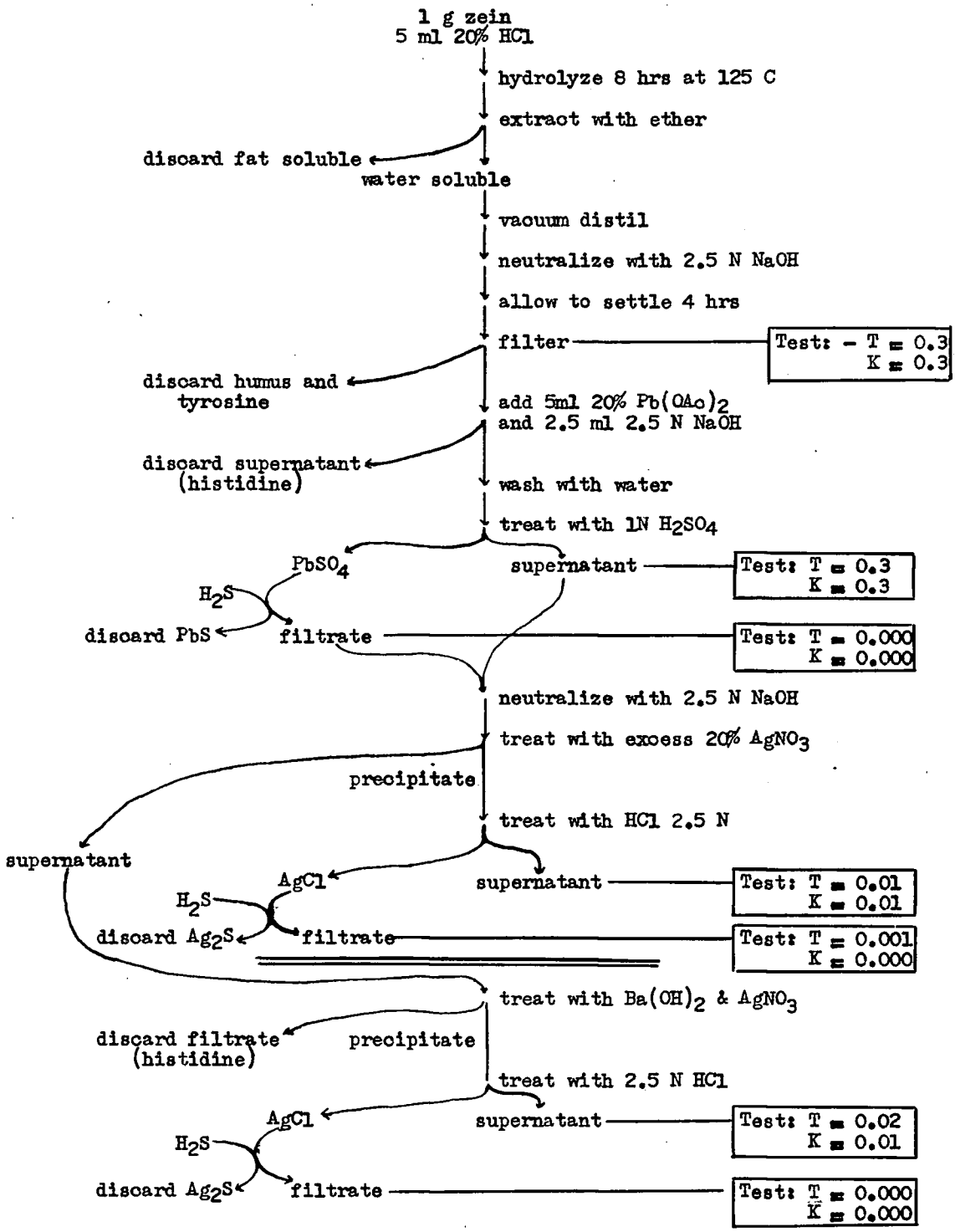
After the inconclusive results already outlined, a closer check of the effect of tyrosine was made at the suggestion of Dr. G. Hunter. Tests with Millon's reagent showed that tyrosine is not completely precipitated by neutralizing a protein hydrolysate but that a large part may remain in solution. Therefore, all the results obtained heretofore, except those from the work on the destructive effect of hydrogen sulfide where no hydrolysate was involved, may have been only a measure of the soluble tyrosine.

Separation of Thiolimidazoles from Hydrolysates

It was now necessary to devise a method for completely separating tyrosine and histidine from any thiolimidazole likely to be present. Basic lead acetate was used to precipitate either ergothioneine or thiolhistidine with out any histidine according to the method of Hunter and Raragosky (112). The thiolimidazoles were then freed with hydrogen sulfide and reprecipitated with silver (51, 53) or baryta and silver (113) leaving the tyrosine in solution. Several fractionations were made varying the order and pH of the precipitations. Tests were made at several stages in the fractionation and a control sample with ergothioneine added to the zein was run with the test. The procedure used and the results obtained in one of the most extensive of these tests is given in the accompanying chart. In no case was a positive

Chart I Fractionation of Protein Hydrolysate to Separate Thiolimidazoles from Tyrosine and Histidine

Results of Hunter test expressed as amount of ergothioneine to give an equivalent colour. Symbols: T = test hydrolysate, K = control hydrolysate with 5 mg of ergothioneine added before hydrolysis.



test for a thiolimidazole observed on completion of the fractionation. Tests performed on the control sample with added ergothioneine, at various points in the fractionation when they were positive, were not always as strong as those on the hydrolysate prepared without added ergothioneine.

Separation of Ergothioneine from Amino Acids

Because of the failure of these attempts to isolate any thiolimidazole from zein, even when it was added at the beginning, the reactions with pure ergothioneine and amino acids were studied. The rather startling discovery was now made that the conventional method of hydrolysis with hydrochloric acid completely destroys the thiolimidazole ring. A paper by Lefevre and Rangier (32) that recently became available gives evidence supporting this result. In contrast, sulfuric acid was found to give very little destruction.

Neutralization of the sulfuric acid was usually accomplished with barium hydroxide in order that no salts should be left in solution. However, it was found that barium sulfate co-precipitated eighty percent of the ergothioneine from the solutions used. Neutralization with 2.5 N sodium hydroxide was found to be better although about ten percent was still lost.

No difficulty was experienced in separating the ergothioneine from histidine with basic lead acetate but several methods were tried for the removal of tyrosine before a successful one was found. Lead will precipitate tyrosine from a more acid solution than that required for the

precipitation of ergothioneine but the separation could not be made quantitative. Both silver and baryta were tried as selective precipitants but no ergothioneine could be recovered from the precipitates. Finally it was found that mercuric sulfate, Hopkins-Cole reagent (114), satisfactorily removed ergothioneine from tyrosine. The ergothioneine was freed with hydrogen sulfide and the mercuric sulfide centrifuged off.

Further Studies of Hydrolysates

The methods developed in this work with mixtures of known amino acids were now applied to protein hydrolysates. When ergothioneine was added to the protein none could be isolated, but when it was added after hydrolysis most could be reclaimed. Thus it seems that this thiolimidazole is destroyed by sulfuric acid when protein breakdown products are present although in pure solution the destruction had been shown to be negligible.

A number of alternate methods of hydrolysis have been tested by fractionation according to the method outlined in Chart II and the results are listed in Table VII. At the present time no satisfactory method has been found although it seems that it is possible to isolate a small amount of thiolimidazole after hydrolysis with formic acid or combined enzymes. Work is being continued in this field.

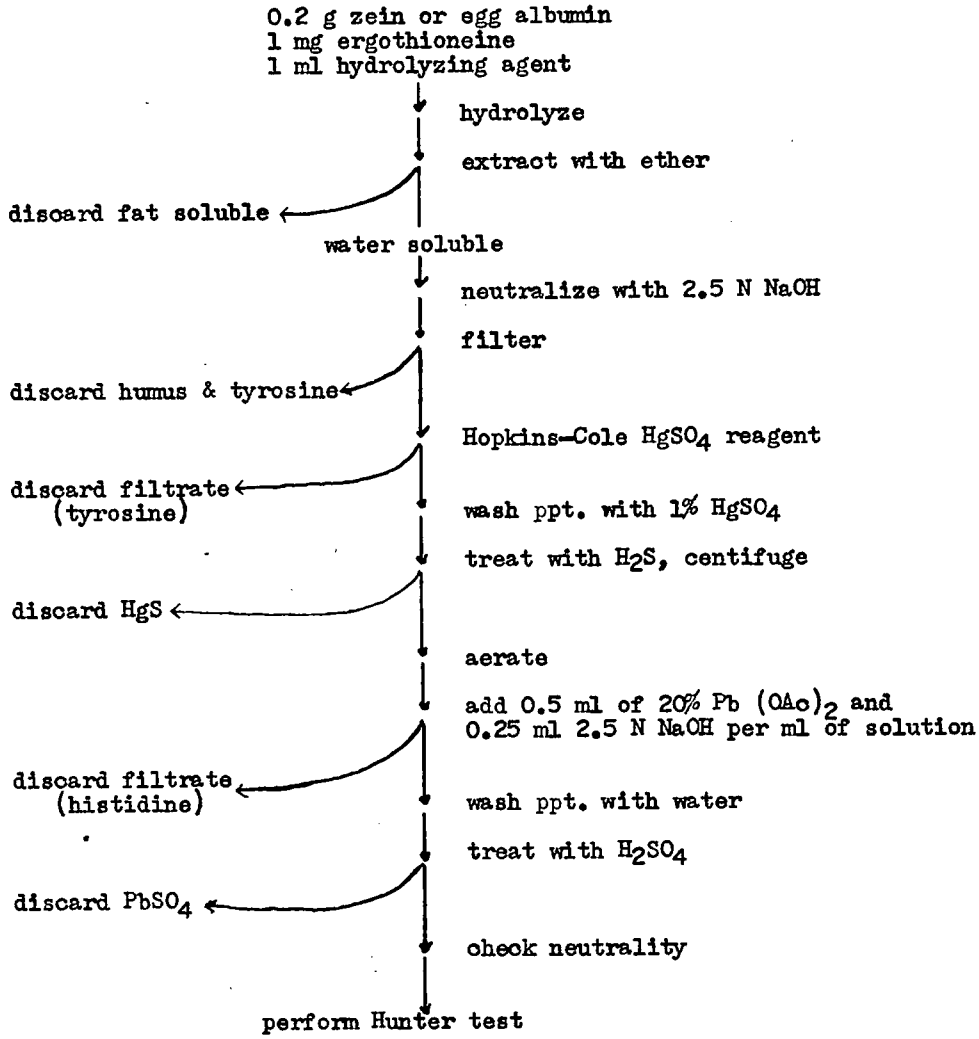
Table VII Methods of Hydrolysis

Temperature	175 C	165 C	125 C	40 C
Time	12 hrs	1.75 hrs	8 hrs	24 hrs
Agent	Expressed as the fraction of added ergothioneine reclaimed.			
NaOH 20%			0.0	
H_2SO_4 20%		0.0		
H_3PO_4 20%		0.0		
KOH 20%		0.0		
Trichloro acetic 70% with $SnSO_4$ 10%			0.0	
Trichloro acetic 70% with $Ti_2(SO_4)_3$			0.0	
Papain				0.0[1]
Trypsin				0.0
Pepsin				0.0
Pepsin & Trypsin				0.02[2]
Formic 50% with HCl 7%		0.01	0.01	
Formamide 90%	0.0			

1 A small but distinct purple precipitate formed in 24 hrs.

2 Hydrolyzed 24 hrs with pepsin at pH 2.0 and a further 24 hrs with trypsin at pH 8.0.

Chart II Method of Fractionation Currently Used

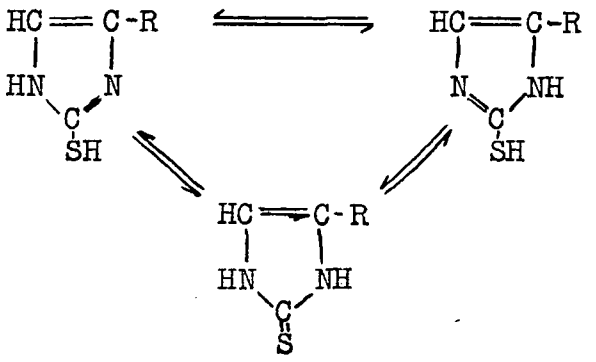


Grote's Reagent

In an effor to obtain a test which might serve to confirm the findings obtained when employing the diazo reaction developed by Hunter for thiolimidazoles, a study of the Grote reaction was undertaken. This test is said to give distinctive colours with both C-SH and C S groups.

In so far as time permitted, experiments carried out employing this test on thiolimidazoles were without result. No colour at all was observed. It is possible that further investigation may provide the proper conditions enabling this test to be satisfactory in this connection. It is, of course, possible, however, that under no circumstances is the reaction given by thiolimidazoles.

Imidazoles have been shown by Langley and Sexton (115) to have at least two tautameric forms and presumably thiolimidazoles, like thio-urea, would have three. These forms include both C-SH and C=S groups but the C-SH does not react with sodium nitoprusside and it may be that neither groups reacts with Grote's reagent.



IV Summary and Conclusions

A study of the results obtained by various workers on the fractional analysis of proteins suggests that many of the cystine determinations recorded in the literature may be high due to the interference of thiolimidazole breakdown

products.

Examination of the color given by the Hunter diazo test with ergothioneine by means of a simple grating spectoscope did not reveal any distinct absorption bands which could be used to characterize the color.

Some indication of the presence of free ergothioneine in corn has been found although other considerations make its occurrence seem unlikely. Time has not permitted a complete investigation of this subject as yet.

It has been shown that ergothioneine may disappear from a solution during purification employing charcoal as a decolorizing agent.

The method using hydrogen sulfide generally employed for separating metallic salts from combination with ergothioneine is another possible source of destruction of the betaine. Separation may be more satisfactorily accomplished when copper is removed by cupferron.

A survey has been made of the results obtained by the application of the Hunter reaction to fractionated hydrolysates of a number of proteins in an attempt to obtain figures on the distribution of thiolimidazoles in proteins. The results of this survey are, however, considered unreliable.

A method has been developed whereby ergothioneine may be separated from the factors in a protein hydrolysate which interfere with the Hunter diazo test (tyrosine and histidine) but it has not yet been possible to obtain a test for thiolimidazoles from a pure protein hydrolysate nor from

a hydrolysate to which ergothioneine has been added before hydrolysis.

Ergothioneine is destroyed by prolonged boiling in hydrochloric acid even when the reducing agent titanous chloride is present. The thiolimidazole ring is also broken by boiling in other hydrolyzing agents when protein breakdown products are present. This may explain the failure of earlier attempts to isolate thiolimidazoles from proteins.

Various techniques and procedures have been employed in an attempt to determine the presence of the thiolimidazole ring in proteins. It has not been possible as yet to devise a satisfactory technique for the determination of thiolimidazoles. During this study the influence of various interfering factors has been investigated.

Grote's reagent has been found to give no colour with ergothioneine under the conditions suggested for the differenciation of sulfur groups.

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