THE PLACE OF HISTIDINE IN THE PROTEIN MOLECULE

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The physiological importance of histidine and its derivative, histamine, is so great that the question of the position of this amino acid in the protein molecule and the manner in which it is united to the other amino acids is one to which an answer is urgently desired. Is the carboxyl group of the acid free? Or is its amino group free? Or are both of them substituted by other amino acids?

As regards the physiological importance of this heterocyclic amino acid, it will be recalled, that proteins which produce anaphylaxis usually, if not always, have histidine in their molecule. And proteins which lack histidine do not usually produce anaphylaxis. In fact, there is on record only a single observation in which a protein or its decomposition products have been reported as possessing the power of sensitization when

these protein products lacked histidine. All other observers have been unable to produce anaphylactic shock with proteins free from histidine. Whether, therefore, histidine be an absolute necessity for sensitization or not, there can hardly be a doubt that its presence in the protein molecule greatly increases its power of sensitization. Histidine is thus brought into relation with the phenomena of immunity, of defense against disease, and with the pathology of all the sensitization or allergy diseases.

Observations by Professor Vaughan on the toxic products formed from proteins by alkaline alcoholysis point also to this amino acid as an essential constituent of such toxic products. The toxic protein derivatives he obtained from the bodies of bacteria, from egg albumin and other proteins all gave a very strong color reaction indicating the presence of the imidazole ring in this toxin. Histidine, as is well known, is the principal amino acid, and often the only amino acid, which contains this ring in proteins. The reaction indicates, therefore, the presence of histidine in Vaughan's material. The composition of this toxic material has never been adequately studied and explained altho, as will appear in the historical

part, some very interesting work has been done upon it.

Some years ago Mr. Charles G. Merrell, President of the William S. Merrell Chemical Company of Cincinnati, becoming greatly interested in the so-called non-specific protein therapy so widely used at that time abroad, and now also in this country, desired to have investigated the way proteins foreign to the body acted, when introduced parenterally, in other words other than by the digestive system, into the animal economy. It is well known that such proteins or their split products may produce under these circumstances a variety of symptoms, such as fever, leucocytosis, erythrocytosis and so on, and their injection is sometimes followed by a marked amelioration of pathological symptoms. Mr. Merrell gave, on behalf of the William S. Merrell Company, a sum of money annually to the Biochemical Department of the University of Cincinnati, to form a fellowship which is called the Merrell Fellowship.

In the course of these investigations in this laboratory it was found by Dr. C. A. Mills (29) that a complex protein derived from calves' lung, containing no histidine, would not sensitize guinea pigs. This directed attention to histidine as the essential element in sensitization phenomena and suggested that it might

be a necessary constituent in proteins or their decomposition products which had a therapeutic or physiological action. This idea was borne out by other facts in the literature.

Accordingly some four or five years ago, Dr. M. Isaacs (22), while he was Merrell Fellow, started to prepare Vaughan's toxic protein at the suggestion of Professor Mathews. He made the discovery, in the course of his observations, that histidine, or something giving a very powerful imidazol reaction, was derived from proteins by alkaline alcoholysis in the first few minutes of the lysis and before the molecule had been extensively digested by the alkali.

This suggested that histidine might be a terminal amino acid, or one which for some other reason was most easily split off from the protein molecule. Professor Mathews gave me the problem of discovering whether this was actually histidine which was thus quickly split out of the molecule; whether it was the only amino acid set free at this time or whether some others accompanied it; whether the histidine had its carboxyl group free or combined, by determining whether the histidine thus set free had its full optical activity or had been racemized. If the carboxyl group was free and histidine

terminal it was expected that the histidine thus set free would be optically active; if the carboxyl was combined or substituted it was expected that the histidine would be racemized. I was also to see whether all proteins yielded histidine easily or only some of them.

The results showed that histidine is indeed split off from several proteins very early in the alcoholysis by sodium alcoholate; that the histidine thus set free is often accompanied by tyrosin and leucine, suggesting that the two or three are in close association in the molecule; that the histidine thus set free is but a part of the total histidine in the molecule; that the histidine first split off by alkaline alcoholysis is practically of full optical activity, whereas that set free by subsequent acid hydrolysis of the unhydrolysed residue of the alkaline alcoholysis is to a considerable degree racemized, altho not entirely so. These facts indicate, therefore, that a part of the histidine in the proteins studied occupies either a terminal or peripheral position in the molecule with its carboxyl group free. This fact may explain the great ease with which histidine, or histamine is split

off from the protein molecule and why these substances and particularly histamine appears so regularly in extracts of glandular and other organs even when these extracts are prepared by very weak acid. It is probable, also, that if any amines are formed by decarboxylation in the proteins histamine will be one of those formed most easily.

I wish to express here my gratitude to the William S. Merrell Company for the generous fellowship they have provided to make this work possible, and to Professor A. P. Mathews for suggesting and guiding this problem.

Historical

The toxicity of proteins or their cleavage products has been variously explained. As Underhill (47) says "almost every study along this line points to a different amino acid as the cause of the 'peptone' effect". Much of the earlier work on the physiological action of protein derivatives was carried through with proteoses prepared from Witte's peptone. Schmidt - Mulheim (39), in trying to discover the fate of peptone in the blood, (it being assumed at that time that peptones were absorbed as such from the alimentary canal)

found that the intravenous injection of Witte's peptone caused in dogs striking physiological effects. The most noted among these were (1) an inhibitory action on the coagulation of the blood, and (2) a rapid and marked reduction in blood pressure. The suggestion was made that the toxic body exists preformed in the animal tissues, and passes unchanged to the proteoses either by say of fibrin or pepsin. Chittenden, Mendel, and Henderson (1) found that proteoses made by using papain, or superheated water or acid were quite as toxic as those made from Witte's peptone.

Pick and Spiro (35) believed that the physiological action of the proteoses was not a property of pure proteoses, but was caused by an adhering toxic substance which they called "peptozyme". They thought that it was present in the animal tissues as a "peptozymogen". To support their conclusions they claimed that edestin and casein did not yield poisonour proteoses under any circumstances, and that hydrolysis by alkali or tryptic digestion failed to form toxic products. They were able to destroy the poisonous properties of their digestion mixtures by boiling with 50 per cent alcohol which had been made alkaline with sodium hydroxide.

Underhill (1903) (45) states that Pick and Spiro failed to separate the proteoses in their experiments and did not know whether the material which they injected contained any noticeable amount of proteoses. He pointed out the hydrolytic action of sodium hydroxide on proteoses, inferring that the loss of toxicity when proteoses were treated with alkaline alcohol was due to their destruction. He obtained toxic proteoses by digesting crystallized vegetable proteins with enzymes, or by acids and superheated water, to demonstrate that poisonous proteoses are formed from pure protein. He injected the undigested proteins to show that they contained no adhering toxic impurity.

Popielski (36), continuing the work with alcoholic extracts of commercial "peptones" and of the separated proteoses, regarded the toxic portion of proteoses as a distinct compound to which he gave the name "vasodilatin". It was characterized by its solubility in alcohol, its protein nature, and a physiological action suggestive of β-iminazolylethylamine. Though the toxicity of alcoholic extracts of proteose preparations had been clearly shown, this method of distinguishing the nature of the toxic substance fails, as certain of the proteoses are soluble in alcohol.

Pfaundler (32) isolated from the products of the peptic digestion of albumin an alcohol soluble product that was precipitable by mercuric sulphate but not by alkaloidal reagents, and which dissociated into leucine and a di-amino-acid probably histidine, on being treated with boiling hydrochloric acid. He says that study of these products will give the most important information regarding the chemistry of albumins.

Vaughan and his coworkers (50, 51) have greatly increased the interest in toxic protein products. Most of Vaughan's preparations were made with the toxins obtained by alkaline alcoholic hydrolysis of proteins. The toxin or toxins thus secured are often referred to as "Vaughan's Crude Soluble Poison".

Vaughan's method follows:-

To a weighed amount of protein 15 to 25 times its weight of absolute alcohol containing 2 per cent sodium hydroxide is added, a reflux condenser is attahced and the flask with its contents is heated on a water bath for one hour. Then the undissolved part of the protein is freed from the solution by filtration, and is again placed in the flask to repeat the process with a fresh mixture of alkali and alcohol. The protein is treated thus three times, the filtrates combined and

neutralized with hydrochloric acid. The precipitate which contains both sodium chloride and part of the base is removed by filtration and the filtrate evaporated in vacuo at 40° to dryness. This is redissolved in absolute alcohol, filtered to remove sodium chloride, and again evaporated to dryness at 40° C. The gummy residue is Vaughan's "Crude Soluble Poison", and is highly toxic. So small an amount as 8 to 50 mgm. of the poison is lethal to guinea pigs when given intraperitoneally. However, preparations made with stronger alkali-alcohol solutions are not nearly so toxic.

Vaughan obtained the poison from egg-white, casein, serum albumin, edestin, Witte's peptone, and the cellular substance of many types of bacteria. Pryer (37) failed to find histamine or choline derivatives in the poison. In the hope of finding some amino acid that could be held responsible he injected intraperitoneally leucine, glycocoll, alanine, histidine, glutamic acid, aspartic acid, tyrosine, phenylalanine, and tryptophane with negative results. He then administered a mixture of alanine, phenylalanine, glycocoll, leucine, tryptophane, glutamic acid, histidine, tyrosine, uric acid, and glucose with like success. Koessler and Hanke have since shown that in vitro the colon bacillus will

convert 50 per cent of the histidine in such a mixture to histamine.

Underhill and Hjort (46) realized that Vaughan's product in its dry form may contain as much as ten per cent HCl. They concluded that Vaughan's crude soluble poison less the acid it contained was no more toxic than acid alcohol extracts of proteoses.

Heyde (20) believes that methyl guanidine and guanidine produce all the effects characteristic of proteoses and he believed that his experiments showed that guanidine and methyl guanidine were responsible for proteose intoxication. These compounds may arise from arginine; but this type of cleavage of arginine does not ordinarily occur during the course of proteolysis produced by enzymes or acids. Alkali decomposes arginine into ornithine and urea, and urea into ammonia. None of these are toxic except the ammonia. If guanidine and methyl guanidine are responsible for proteose intoxication the protamines should be particularly toxic on account of the large amount of arginine in them.

Knaffl-Lenz (23) proposed that the toxicity of proteoses was due to the tryptophane present in the molecule of injected proteose. The proposal was based on experiments with zein in which no tryptophane has been

found, and gliadin which contains very little. However, Szumowski (41), Underhill (45), and Wells and Osborne (53) found that zeoses gave typical anaphylactic reactions. Underhill and Hendrix (47) attribute Knaffl-Lenz' failure to obtain the characteristic effects with his preparation to the relatively small amount of proteoses in his digestion mixtures.

In 1910, the extraordinary physiological properties of β-imidazolylethylamine, or histamine as it is popularly called, were discovered independently by Barger and Dale (6) and by Kutscher (27) as one of the active principles of ergot. The action of this base was studied by Barger and Dale (5) and Dale and Laidlaw (15). They suggested it might be a possible factor in proteose intoxication. These workers (5) obtained histamine from the mucosa of the small intestine of the ox, and assigned to it, at least in part, the depressor action of secretin. Isolating, from the intestinal contents of the guinea pig, a bacillus of the colon-typhoid group capable of converting histidine into histamine, Mellanby and Twort (28) decided that histamine is not a normal product of the intestinal mucosa but rather a product of the bacterial decomposition of proteins containing histidine. Then Berthelot and Bertrand (7) isolated from the human intestine an organism that

had all the chemical and morphological characteristics of Friedlander's pneumo-bacillus and in addition decarboxylated histidine to histamine.

Abel and Kubota (2) held the view that histamine is present wherever living protoplasm exists, or at least whenever it is killed. They believed that histamine appeared whenever a true protein was split by enzymes, acid or other hydrolyzing agents. They said, "Histamine is the plain muscle stimulating and depressor constituent of the posterior lobe of the pituitary gland". Hanke and Koessler (18) were unable to find histamine in fresh glands and explain the positive findings of Abel and Kubota to putrefactive changes in the commercial product investigated by them. Dale and Dudley (14) believed that if histamine occurs at all in the pituitary gland, it is in such small traces that chemical identification is impracticable.

Later, Roca (38) obtained evidence for the presence of histamine in fresh glands. Quite recently Best, Dale, Dudley and Thorpe (8) have announced the isolation of histamine from a number of fresh animal tissues, lung tissue especially giving relatively large amounts. However, at present, the evidence for Abel and Kubota (2) for the occurrence of histamine in

proteins is considered unsatisfactory and the negative results of Hanke and Koessler (18) are given greater credence. Hanke and Koessler have found, however, a substance pharmacologically similar to histamine that is split from casein by acid hydrolysis. But it may be pertinent to quote them: "To expect a similarity in physiological action from compounds having a similar chemical structure is logical and justified by evidence; but to conclude from a similar pharmacodynamic action to the identity of chemical structure or the invariable presence of one and the same substance is a perilous undertaking."

Arai (3) has made an interesting suggestion that may in time explain many of the phenomena that Abel and Kubota have explained in terms of histamine. Since histidine becomes active pharmacologically upon removal of its carboxyl group, it appeared to Arai that compounds of amino acids involving the carboxyl group should exhibit amine-like effects. He found that the chlorhydrate of the methyl ester of histidine, possessed an effect on blood pressure and guinea pig's uterus similar, though less powerful, to histamine. Proteins are considered to consist of combinations of amino acids coupled in groups of the acid-amide type. Arai suggests that an ester-like coupling between carboxyl and hydroxyl

groups is not impossible. In view of his experimental results, amino acids, which in themselves are physiologically inert, could become toxic by their manner of linkage together.

It has not been shown, as far as I know, whether or not histamine can be formed in the absence of bacteria by the enzymatic activity of organ cells alone. Neuberg and Gottschalk (30) have shown that liver and muscle tissue contain a carboxylase that possesses the capacity of decarboxylating pyruvic acid. Its existence suggests that other amino acids may be involved, in which case the production of amines from amino acids may occur in the animal cell.

The question arises why should an imidazole grouping in protein be particularly labile and why should the organism manufacture histamine. According to Hanke and Koessler (19), decarboxylase activity is a protective mechanism and is resorted to, by the microorganism, when the accumulation of H-ions within the bacterial protoplasm is incompatible with life processes. According to this conception, histamine is a reaction buffer.

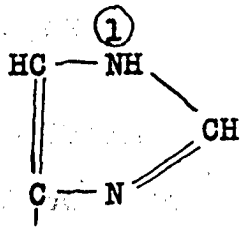
That so few of the imidazole complexes broken off so easily from living protoplasm have been identified

has been unfortunate particularly in consideration of the important position of histidine as a constituent of protoplasm. Nearly 30 per cent of the extractive nitrogen of skeletal muscle is due to carnosine, the β-alanyl derivative of histidine. Hemoglobin contains about 9 per cent histidine. Recently, Tadokoro, Abe, and Watanabe (42) have shown that the histidine content of female muscle proteins is from 20 to over 100 per cent greater than those of the male in a large variety of species. In 1926 Newton, Benedict, and Dakin (31) identified a sulfur compound obtained from blood corpuscles, which had been variously named as "sympectothion" and "thiosine", with ergothioneine, a base isolated by Tanret in 1909 and chemically identified as the betaine of thiolhistidine by Barger and Ewins (4) two years later.

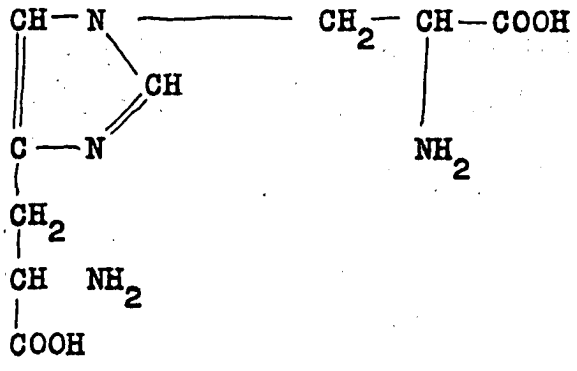
Theoretical considerations concerning position of histidine in the protein molecule.

Although histidine appears to be more abundant than any other imidazole compound, there are others that may be found important. We have already mentioned histamine and carnosine which contains histidine. The purines may be considered as condensation products of

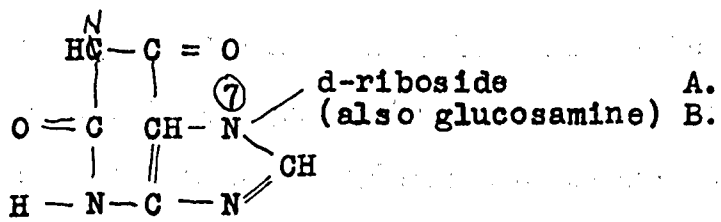
the imidazole ring with the pyrimidine. Containing a nuclear cyclic structure like that of the imidazoles although not true imidazoles are the hydantoins, creatinine, and the glycocyamidines. The most important color reaction for the detection of imidazoles is that given by an alkaline solution of p-phenyl diazonium sulfonate. But no color would result if the imide hydrogen,



position one, of the imidazole ring, is replaced by some less easily mobile radical. Thus, substituted derivatives of this type in protein split products would not be detected or estimated although they are true imidazoles. Abderhalden (1) has prepared a compound of this type composed of histidine and β-hydroxy-α-amino-propionic acid (serine) that is far more labile than the histidine-serine dipeptide



If such a compound could be demonstrated in proteins, which has not been the case as far as I know, it might be one explanation for the appearance of unracemized histidine in alkaline protein hydrolysates. Analogous systems exist in the xanthing series, that is, with combinations in position 7.

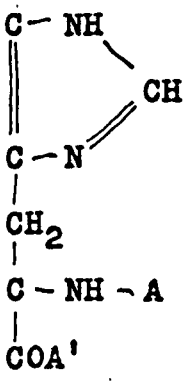


A. Xanthine + d-riboside xanthosine.

B. Xanthine + glucosamine amino glucoside derivative of histidine.

Kossel and Weiss (25) have studied the iminazole group of the histidine-containing protamines, sturine and percine. Comparison of the basicity of the whole protamine molecule with the relative proportions of the basic units gave no reliable evidence with sturine, since the basicity of the iminazole group is very slight.

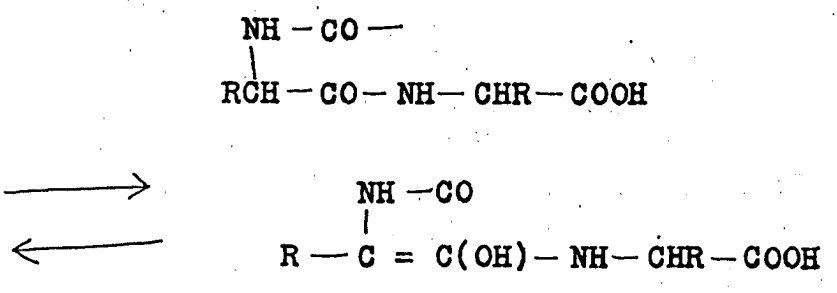
If both the carboxyl and amino groups of histidine enter in a peptide linking, (A, A'), the structural formula for the protamine is as follows:-



The position of the iminazole nucleus in the protamine molecule is suggested by experiments of Pauly (33) who studied its coupling with diazo compounds and its combining power with iodine. He found that if the hydrogen atom of the imino group is replaced by an acyl group, the diazo coupling does not take place. He showed that the histidine combined in the sturine molecule coupled with diazo compounds in the same way as free histidine showing the NH group to be free. Hirayama (21) found that the iminazole imino-group lost its hydrogen by introducing a benzene-sulphonic or naphthalenesulphonic group. Gerngross showed that the compound formed by acylating a histidine ester on the imino group of the iminazole nucleus was easily decomposed. Pauly (34) studying the iodo-derivatives of histidine and iminazole and estimating their absorption of iodine found that the absorption of iodine by sturine as great as that of the histidine derivatives

in which the iminazole group is free. So, in sturine, it is believed that there is no peptide linkage through the imino group of the iminazole nucleus and that this nucleus is in the free state.

This paper presents evidence that horse hemoglobin, edestin, casein, and Witte's peptone contain histidine that is very easily split off from the protein molecule by alkali, and that it is in such combination that its carboxyl group is free, according to Dakin's theory (12, 13). He and Kossel (26) showed that treatment of proteins with alkali leads to a progressive diminution of the optical rotatory power of their solutions. This Dakin attributes to an internal racemization or emolization of the protein molecule analogous to that which he had earlier shown occurs in the hydantoins (11). The reaction may be expressed



Alkali shifts the equilibrium towards the right. The central carbon atom in place of being attached to four different groups is on the right of the equation attached to three groups, to one of them by a double bond,

and any optical activity it possessed due to asymmetry must have been lost. Of the amino acid groups present in any peptide complex only the one containing the

CO CH group could exhibit keto-enol tautomerism and hence racemization; the terminal amino acid group containing a free carboxyl group would be unchanged. Since racemization does not occur with alkali in dipeptides in which one amino group is free

$$R-\underset{\displaystyle NH_2}{\underset{|}{CH}}-CO-NH-CHR-COOH$$

it is concluded that the conditions necessary for racemization require the attachment of other groups to both amino and carboxyl radicals. Hence, the amino acids which are optically active after alkali hydrolysis either occupy the terminal position in the peptide chain or are rapidly liberated in the free state and so escape racemization.

Experimental

(a) Is histidine split off by early alkaline alcoholysis?

The first experiments were directed to discover whether histidine was split off from proteins by alkaline alcoholysis, how rapidly it was split off and whether it was the only amino acid set free or was accompanied by

others. To solve this problem the following experiments were tried:-

1. Lysis of proteins by alcohol.

To 200 grams of dried red horse blood corpuscles in a six liter flask were added 3,500 c.c. of 95 per cent alcohol. The horse blood corpuscles had been washed four times with 1.5 per cent sodium chloride solution to free them from plasma proteins and then spread on a glass plate to be dried by an air current of about 35° C. This mixture was boiled under a reflux condenser for forty hours, the mixture being constantly sitrred with a mercury sealed stirrer. The experiment was repeated with edestin and Witte's peptone. No histidine or any other amino acid could be split off from the protein by the action of alcohol.

At a pressure of twenty-five pounds per square inch no histidine split off from blood cells when boiled with alcohol in the autoclave.

2. Alkaline alcoholysis of proteins in absolute alcohol; preparation of "Vaughan's crude protein toxin".

To 250 grams of red blood corpuscles in a six liter flask were added 70 grams of NaOH dissolved in 3,500 c.c. of absolute alcohol. A reflux condenser was attached and the flask with its contents heated on a water bath for one hour. The undissolved fraction was

removed by filtration, and boiled with a fresh mixture of alkali and alcohol for three hours and again filtered. Both filtrates were neutralized with hydrochloric acid. The precipitates of sodium chloride were removed by filtration and the filtrates evaporated, in vacuo, at 40° C. to dryness. These were extracted by absolute alcohol, filtered to remove sodium chloride and the extract evaporated to dryness. The fractions correspond to "Vaughan's Crude Soluble Poison", and although somewhat darker in color, had the characteristics of this substance as described by Vaughan and later by Underhill and his students.

In the hope of finding the nature of the imidazole complexes in the mixture, 10 grams of the poison were dissolved in 200 c.c. of 20 per cent NaOH. This alkaline liquid was ~~dissolved~~ extracted by shaking with twice its volume of amyl alcohol. If histamine and methyl imidazol were present in this fraction they could not be demonstrated in the residue left after the evaporation of the amyl alcohol extract. Nor could methyl imidazol be separated from the alkaline solution of the crude poison by steam distillation. From the alkaline aqueous liquid no histidine, imidazol propionic or acetic acids could be separated by precipitation with phosphotungstic acid.

Another portion of the "toxin" was hydrolyzed with 37 per cent hydrochloric acid for five hours.

Histidine, tyrosine, and leucine were separated from the hydrolysate by the method of Hanke and Koessler (14). 100 grams of the "toxin" were mixed with 500 c.c. of 37 per cent hydrochloric acid in a 1,000 c.c. long necked, round bottomed Pyrex flask; the mixture was hydrolyzed by boiling for five hours, under a reflux condenser over an electrically heated sand bath. The hydrochloric acid was removed by distillation in vacuo, at 60° C., from the same flask. The residue was dried at 100° C. for 2 hours, and then dissolved in 500 c.c. of distilled water. Powdered calcium oxide was then added until the reddish brown precipitate began to change to a gray color due to the presence of undissolved lime. This precipitated both ferric hydroxide and humin; 250 c.c. of alcohol were added to the filtrate and the mixture distilled at 40° C. in vacuo until 400 c.c. of distillate were obtained. The brownish fluid syrup was transferred to a Buchner funnel and was washed on the filter with 1,000 c.c. of a hot saturated solution of calcium hydroxide. The filtrate thus obtained contained the amino acids as calcium salts.

From these tyrosine, leucine, and histidine were separated according to the methods of Hanke and Koessler. About 0.8 gram of tyrosine was obtained which was identified by its melting point (310°); its fine feathery

crystals and Millon's reaction. About 0.5 gram of leucine was separated and melted at the metling point of leucine, namely 290° C. Two grams of histidine dichloride were obtained and identified by the melting at 247° with gaseous decomposition; by its crystalline form; and by its content of chlorine. The chloride ion from .247 gram of the substance was completely precipitated by 21.74 c.c. of 0.1 N $AgNO_3$. The theoretical quantity needed for 100 per cent histidine dichloride is 21.68 c.c.

The optical activity of these three amino acids was determined with a Hilger three shadow polariscope, through a four decimeter tube. The polariscope was fitted with a spectroscopic attachment for providing homogeneous light and the rotation was determined of the green line of mercury. The source of light was a Cooper-Hewitt mercury lamp.

The tyrosine and leucine thus obtained from "Vaughan's toxin" were found to be optically inactive. They had evidently been racemized by the alkaline treatment. A 2.046 per cent solution of histidine dichloride in 1 3/4 per cent hydrochloric acid gave a rotation of +0.146° at 20° C. Then $\left[\alpha\right]_{Hg}^{20} = +1.78°$, in place of +7.68° the specific rotatory power of optically active histidine dichloride. There was, hence,

76.7 per cent racemization, since the rotatory power was only 28.8 per cent of the theoretical. See Table 2.

This experiment indicated that about 1/4 of the histidine, but noen of the tyrosine or leucine had a free carboxyl group and was presumably terminal.

3. Alkaline alcoholysis of proteins in 95 per cent alcohol.

In this experiment 95 per cent alcohol was used in place of absolute alcohol used in the preceding. This makes considerable difference in the result.

To 250 grams of red blood corpuscles in a six liter flask were added 70 grams of NaOH dissolved in 3,500 c.c. of 95 per cent alcohol. A reflux condenser was attached and the flask with its contents brought to boiling in one hour. The mixture was kept thoroughly agitated by a mercury sealed, electrically driven stirrer. Separations were made as in the previous experiment in the preparation of "Vaughan's crude protein toxin". From this material small amounts (somewhat less than .5 gram) of inactive tyrosine and leucine were separated; and 0.8 gram of histidine dichloride was obtained. A 2.118 per cent solution of the histidine dichloride thus obtained and measured under the same conditions as the preceding experiment, gave a rotation

of +0.591°, instead of the value calculated for active histidine dichloride of +0.647°. This showed an optical activity 91.4 per cent of the theoretical.

This experiment confirmed the preceding but indicated a larger proportion of the histidine recovered was terminal.

Having shown that the histidine thus obtained from corpuscles was in part and sometimes in chief part optically active, I proceeded to study the character of the histidine obtained from purified proteins. While most of the histidine in the preceding experiments undoubtedly came from the hemoglobin there was a possibility that a part came from other proteins present in the corpuscles. This might account for the fact that some of the histidine was racemized.

(b) Is histidine split off from crystalline hemoglobin?

Preparation of crystalline hemoglobin.

Crystalline hemoglobin was prepared in the following easy and direct method dependent on the electrodialysis of red blood cells. This excellent procedure has been described by Stadie and Ross (47) using apparatus similar to the three compartment electrodialysis cells of Foster and Schmidt (48). In this

work I made the following modifications in the apparatus and procedure of Stadie and Ross. Cellophane was used as a membrane instead of the parchment paper and linen coated with gelatin which had been heated with formaldehyde used by Stadie and Ross. The cellophane membrane is easily obtained in various thicknesses and it is cleaner and freer from flaws than parchment membranes. With cellophane also there is a low electro-endosmotic transport of water and thus less flooding of the cathode compartment during electrolysis. However, some difficulty was encountered in inserting and holding it in the ordinary three compartment cell; also leaks often occurred between the membrane and the rubber gaskets.

An improved three compartment cell was constructed as follows (see Figures 1, 2 and 3):

Insert Figures 1,2,3.

A box 10 x 6 and 3/8 x 7 and 1/2 inches, inside dimensions, was constructed of galvanized sheet metal about 3/32 of an inch thick. The upper edges were doubled to strengthen the box. The entire inside was covered with a coat of some insulating material. The most convenient is that used to seal acid type storage batteries and may be obtained at any storage battery shop. It is a substance easily melted and spread over

the metal surface. The cell is then tested for any breaks in this material by testing its resistance to a direct current of 110 volts when one electrode is connected with the metal and the other placed in tap water within the cell. If no appreciable leaks are found the construction of cell is proceeded with.

A rubber composition battery cell 2 and 1/2 x 6 and 1/4 x 7 and 3/8 inches, outside dimensions, is altered to form the middle dialyzing chamber. These cells can be taken from discarded, acid type storage batteries that have wooden frames or bought separately. They are not contained in batteries that have a molded case. From the two largest sides pieces 5 x 6 and 1/2 inches are cut out. These can be cut without cracking with a knife if the cell is warmed over a gas flame. The ridges in the bottom of the cell are also removed. The outside of the remainder of the cell is covered with battery sealing mixture. The cell is wrapped with a single piece of cellophane 8 x 17 inches which allows for folding and sealing the bottom much as a package is sealed. Light pressure will cause the cellophane to stick to the sealing mixture. At all points of contact with the inner cell, the cellophane is covered with Duco or water proof varnish to protect the

membrane from absorbing water and drawing away from the cell.

The apparatus is now assembled as a three compartment cell, two side electrode compartments and the middle dialyzing compartment. Leaks between the two end compartments are stopped with sealing compound. By using a larger or smaller cell and by shifting it one way or the other the size of any of the three compartments can be easily changed.

Graphite electrodes, 8 x 5 x 1/4 inches are placed in each end compartment about 3/4 inche from the cellophane membrane on either side and supported on two glass rods running longitudinally on top of the cell, and passing through small holes in the electrodes. Direct current from a 110 volt line is led into the cell through binding posts bolted to the carbon plates.

The cell is cooled by passing water through glass coils placed between the electrodes and the membranes in the end compartments. These glass coils are not needed for hemoglobin, the tap water running directly over the electrodes in this case. About 1/2 inch from the top in each electrode compartment are placed 2 and 3/8 inch glass tubes as overflow outlets. These are stopped when the material in either electrode compartment is kept.

To prepare crystalline hemoglobin horse blood corpuscles are washed by settling and decanting four times with 1.5 per cent sodium chloride solution. The cells are then laked with about one-seventh their volume of toluene, and centrifuged. The upper layers contain toluene, red cell ghosts, white cells, and cell globulins. The lowest layer contains hemoglobin. Of this about 1,250 c.c. are poured into the center compartment of the electrolytic cell. A stream of tap water is allowed to run through the cathode and anode compartments, and out through the overflow outlets. The direct current of 110 volts is turned on. To prevent the gathering of an amorphous layer of hemoglobin on the membrane toward the cathode by cataphoresis, the masses as they accumulate must be stirred frequently during the first hour of dialysis. In about two hours there is a considerable formation of crystals. Usually about 80 grams of dried hemoglobin, or 250 grams of moist hemoglobin may be separated after two hours. The crystals are centrifuged, the supernatant liquid poured off, and the upper surface of the packed crystals washed with ice cold distilled water. These crystals are dried on a glass plate by a current of air at about 35° C.

250 grams of dry, crystalline hemoglobin thus prepared gave about 1.5 grams of histidine dichloride when

hydrolyzed with 70 grams of sodium hydroxide in 3,500 c.c. 95 per cent alcohol for one hour, which is about the time necessary to bring the mixture to boiling. The histidine dichloride was separated as follows:

The sodium hydroxide was removed as sodium chloride. The residue from the evaporated solution was dissolved in water to make 1,250 c.c. or enough to fill the center compartment of the electrolytic cell. It was slightly acid toward litmus. This was subjected to electrical transport by a direct current of 110 volts. As soon as the current flowing through the cell reached 0.5 ampere, a 100 watt lamp was placed in series with the cell, and the electrolysis contained for 5 hours. The temperature was kept below 30° C. by circulating water through glass coils placed between the electrodes and the cellophane membranes. The solution in the center compartment was kept agitated by means of an electric stirrer. Carbon dioxide was bubbled slowly through the cathode solution to keep the alkalinity at a low level. The contents of the cathode compartment at the end of five hours (fraction A) were removed and replaced with distilled water, and the electrodialysis continued until the center compartment gave only a slight precipitate with phosphotungstic acid. The

The solution at the cathode (fraction B) contains most of the histidine. Fraction A contains only a small amount of histidine.

The amounts of reagents used in separating histidine are important and are based on the theoretical amount of histidine in the protein hydrolysate; in the case of 250 grams dried hemoglobin, about 20 grams of histidine. Fraction B was concentrated to about 400 c.c. and 50 c.c. of dilute (50 per cent by volume) sulfuric acid added. The histidine was then precipitated by adding 100 grams of mercuric sulfate dissolved in 1 liter of 5 per cent by volume sulfuric acid. (Mercuric sulfate is preferable to mercuric chloride and sodium carbonate because precipitates and solutions occupy smaller volumes and mercuric sulfate is a more specific precipitating agent for histidine.) The mixture is allowed to precipitate for two or three days in the ice box. The precipitate is filtered off and washed with 1 per cent mercuric sulfate in 5 per cent sulfuric acid. The precipitate is then suspended in 200 c.c. of water and 25 c.c. of 37 per cent HCl are added. The mercury is then removed with hydrogen sulfide, filtered and the excess of hydrogen sulfide driven off on the water bath. Sulfuric acid is then exactly

removed with $Ba(OH)_2$. The filtrate after removal of barium sulfate is concentrated to a thick syrup in vacuo. Concentrated hydrochloric acid in slight excess to the weight of the syrup is added. The histidine dichloride is allowed to crystallize out in the ice box for several days, and then removed by suction filtration and dried in vacuo over sulfuric acid.

The product is purified by dissolving in an equal weight of hot distilled water, and diluted with ten times its weight of 95 per cent ethyl alcohol. The mixture is heated to boiling and then filtered hot and allowed to crystallize in the ice box. The crystals are filtered off and dried in vacuo.

The optical activity of the histidine dichloride thus prepared from 1 hour alkaline hydrolysis at low temperature of crystalline hemoglobin prepared as just described was determined and found to be 98.5 per cent optically active. 1.789 per cent solution in a four decimeter tube at 20° C. gave a rotation +0.538°. Hence $\left[\alpha\right]_{Hg}^{20^\circ} = +7.42$. Theoretical +7.63 .

When the hydrolysis of 250 grams of crystalline hemoglobin with 70 grams NaOH in 3,500 c.c. 95 per cent alcohol was continued at boiling temperature for four hours more about 3.5 grams of histidine dichloride was

separated by the method just described. A 2.341 per cent solution of this histidine dichloride was found to give a reading of +0.693 which gave $\left[\alpha\right]_{Hg}^{20^\circ}$ +7.40 . Of this histidine 96.9 per cent was thus optically active. See Table 2.

(c) Is optically active histidine split off from other proteins?

Having shown that histidine came from crystalline hemoglobin very quickly on alkaline alcoholysis and that the histidine was not racemized, I then tried edestin, casein, and Witte's peptone.

At the end of 5 hours hydrolysis with 2 per cent NaOH in 95 per cent alcohol, edestin, casein, and Witte's peptone gave respectively about 1.0, 1.2 and 0.6 gram of histidine dichloride. Inactive tyrosine and leucine split off from casein and edestin, but could not be separated from Witte's peptone.

An inspection of Table 2 reveals that in the case of these three proteins the histidine dichlorides obtained by brief alkaline alcoholic hydrolysis were for the most part optically active, just as was that from hemoglobin and in fact were respectively 94.5 per cent; 82.3 per cent; and 87.5 per cent of the theoretical

activity. This again indicates the terminal situation of some histidine in these proteins.

(d) Analyses of proteins used.

In order to determine the quantity of histidine in the proteins used, the distribution of nitrogen in them was determined by Van Slyke's (49) method. The results are given in Table 1. Analyses were made on 5 gram samples of Witte's peptone and crystalline hemoglobin. The values given for edestin are those of Vickery and Leavenworth (50) and those for casein of Van Slyke (51).

Insert Tables 1 and 2.

An examination of this table reveals that Witte's peptone, such as was used in these experiments, contained only about 1.5 per cent of the total nitrogen as histidine nitrogen, which corresponds to 1 per cent of histidine in the molecule; that hemoglobin contains 13.22 per cent of the total nitrogen as histidine nitrogen, which corresponds to 8.5 per cent of histidine; and edestin 5.5 per cent and casein 6.2 per cent respectively of the total nitrogen, corresponding to about 3.8 per cent edestin and 4.1 per cent casein. Those figures for hemoglobin correspond closely with those

Table I.

5 grams hydrolyzed; made up to 250 c.c. 100 c.c. parts taken

	Witte's peptone		Hemoglobin		Edestin (50)	Casein (51)
	Grams N	% Total N	Grams N	% Total N	% Total N	% Total N
% N content of protein		12.55		16.95	18.69	15.62
Total N	0.2510		0.3390			
	0.2510		0.3390			
Total basic N	0.0646	25.74	0.1080	31.85		
	0.0650	25.92	0.1086	32.35		
Total basic amino N	0.0371	14.80	0.0580	17.11		
	0.0376	14.96	0.0591	17.43		
Arginine N	0.0329	13.12	0.0262	7.73		
	0.0336	13.40	0.0268	7.90	27.2	7.4
Cystine N	0.0025	1.00				
	0.0023	.92		0.00	0.6	0.2
Histidine N	0.0043	1.71	0.0456	13.44		
	0.0033	1.32	0.0441	13.00	5.5	6.2
Lysine N	0.0249	9.92	0.0369	10.88		
	0.0258	10.28	0.0377	11.12	3.8	10.3

Table II.

$[\alpha]^{20^\circ}_{\lambda 5461A}$ d-histidine dichloride = 7.63°.

250 grams protein in 3,500 c.c. alcohol.

*Proteins hydrolyzed for 5 hours with NaOH, then 10 hrs. with HCl.

Protein	Treatment				Per cent Histidine in polarimeter tube	Observed Rotation	$[\alpha]^{20^\circ}_{Hg}$	Per cent Optical Activity
	Per cent			Time hours				
	EtOH	NaOH	HCl					
Corpuscles	99.5	2		1	no histidine		recovered	
Corpuscles	99.5	2		5	no histidine		recovered	
Corpuscles	99.5			10	no histidine		recovered	
Corpuscles*	99.5	2	37	10	2.046	0.146	1.78	23.3
Hemoglobin	95	2		1	1.789	0.538	7.52	98.5
Hemoglobin	95	2		5	2.341	0.693	7.40	96.9
Corpuscles	95	2		1	2.118	0.591	6.98	91.4
Corpuscles*	95	2	37	10	1.642	0.108	1.64	21.4
Edestin	95	2		5	0.894	0.546	7.21	94.5
Edestin*	95	2	37	10	1.456	0.086	1.48	19.4
Witte's peptone	95	2		5	2.635	0.662	6.28	82.3
Witte's* peptone	95	2	37	10	2.398	0.213	2.22	29.1
Casein	95	2		5	1.824	0.487	6.68	87.5
Casein*	95	2	37	10	2.041	0.154	1.87	24.5

given by Van Slyke.

250 grams of hemoglobin contain approximately 21 grams of histidine. It will be noted that after one hour hydrolysis by sodium alcoholate 1.5 grams of histidine dichloride were recovered, which is approximately 7 per cent. After 4 hours continued boiling 3.5 grams of histidine dichloride were obtained, which is approximately 15 per cent of the total histidine present. Witte's peptone should have yielded 2.5 grams, but there was obtained only 0.6 gram of histidine dichloride or about 25 per cent of the total histidine; edestin yielded 1 gram in place of a total of 9.5 grams and casein 1.2 grams in place of about 10 grams.

These figures show that only a small proportion of the total histidine in the molecule ranging from 5 per cent - 25 per cent is split off in the non-racemized and active state by this alkaline hydrolysis.

This indicated that a larger part of the histidine was not terminal and should, therefore, have been racemized by the alkaline treatment. To prove that this was actually the case, other samples of these proteins were first treated by 2 per cent sodium hydrate in 95 per cent alcohol for five hours in the manner described. This split off part of the histidine and since other

amino acids and presumably racemized most of the other amino acids which still remained in peptide form. The hydrolysate was neutralized by adding concentrated hydrochloric acid and filtered to remove the sodium chloride. The alcohol was evaporated to dryness and the residue was then hydrolysed with concentrated hydrochloric acid for 10 hours. The histidine was separated from the hydrolysate by the methods already described using electrodialysis. Yields of histidine of approximately 80 per cent of the theoretical were thus obtained. These samples of histidine dichloride as will be seen from an inspection of Table 2 were only from 19.4 per cent to 29.1 per cent optically active.

Hence it is clear that most of the histidine has been racemized by the alkaline treatment and was accordingly substituted in its carboxyl group and was not terminal.

Conclusions.

1. A portion of the histidine in crystalline hemoglobin, in edestin, casein, and Witte's peptone is very quickly split off from the protein molecule by alcohol and sodium hydroxide.

2. The histidine fraction thus split off is not racemized and therefore probably occupies the terminal position in the molecule.

3. The appearance of inactive tyrosine and inactive leucine along with the active histidine suggests that these amino acids are closely associated in the molecule and in such a manner that the carboxyls of both tyrosin and leucine are substituted.

4. Subsequent acid hydrolysis of the hydrolysate resulting from the alkaline digestion of the proteins gives a larger yield of histidine that is largely racemized.

5. The quantities of optically active histidine obtained by alkaline hydrolysis alone suggests that most of the terminal histidine is split off early in the hydrolysis, but that the larger part of the histidine in the molecule is not terminal.

6. The terminal position of a part of the histidine in proteins is thus indicated and the fact that the carboxyl group in this portion is presumably free may account for the ease with which histamine is obtained in extracts of various organs; and may also be correlated with the toxic properties of some of the protein decomposition products.

7. Hydrolysis of blood corpuscles with sodium hydroxide in 95 per cent alcohol presents a better method for the preparation of histidine than any hitherto proposed, because of the large amount of histidine quickly split off from the protein in an optically active form and easily separated from the small amounts of other amino acids.

Summary.

1. An improved electrodialysis apparatus is described.

2. A quick method for the preparation of histidine is given.

3. A method for easily preparing considerable quantities of crystalline hemoglobin by electrodialysis of blood corpuscles is stated.

4. A portion of the histidine in crystalline hemoglobin, in edestin, casein, and Witte's peptone is quickly split off by alkaline alcoholysis and is optically active. It occupies hence probably a terminal position in the molecule with its carboxyl free. The quantitiy of histidine thus placed varies in the different proteins studied. Quantities of optically active histidine presumably terminal ranging from 5 - 20 per cent of the total histidine were actually recovered.

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Abstract

THE PLACE OF HISTIDINE IN THE PROTEIN MOLECULE

Conclusions.

1. A portion of the histidine in crystalline hemoglobin, in edestin, casein, and Witte's peptone is very quickly split off from the protein molecule by alcohol and sodium hydroxide.

2. The histidine fraction thus split off is not racemized and therefore probably occupies the terminal position in the molecule.

3. The appearance of inactive tyrosine and inactive leucine along with the active histidine suggests that these amino acids are closely associated in the molecule and in such a manner that the carboxyls of both tyrosin and leucine are substituted.

4. Subsequent acid hydrolysis of the hydrolysate resulting from the alkaline digestion of the proteins gives a larger yield of histidine that is largely racemized.

5. The quantities of optically active histidine obtained by alkaline hydrolysis alone suggests that most of the terminal histidine is split off early in

the hydrolysis, but that the larger part of the histidine in the molecule is not terminal.

6. The terminal position of a part of the histidine in proteins is thus indicated and the fact that the carboxyl group in this portion is presumably free may account for the ease with which histamine is obtained in extracts of various organs; and may also be correlated with the toxic properties of some of the protein decomposition products.

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