Isolation of Histidine from Whale Blood using 3,4-Dichlorobenzene Sulfonic Acid

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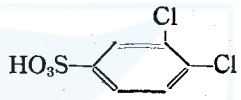
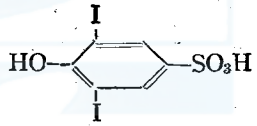
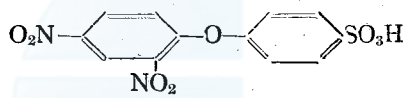
Isolation[1] of histidine from hydrolyzed product of proteins used silver or mercury salts method, and a sublimate method in industry. However, the latter necessitates the use of hydrogen sulfide gas, a large amount of mercuric chloride, and results in a variety of yield on histidine according to the condition of precipitation, as well as the process being very complicated.

Stein and Bergmann[2),3)], in 1938-1940, made a wide ressearch to find aromatic sulfonic acids which would form insoluble salts with amino acids and found three kinds which made insoluble salts with histidine. These acids were:

3,4-Dichlorobenzene sulfonic acid 

2,6-Diiodophenol-4-sulfonic acid 

0-(2,4-Dinitrophenyl)-p-phenol sulfonic acid 

Vickery[4)], in 1941, employed one of the salts, 3,4-dichlorobenzene sulfonic acid (hereafter designated 3,4-D), and obtained good results in isolating histidine from the hydrolyzed solution of horse red blood cells. Further, in 1942, he applied 3,4-D method for the quantitative estimation of histidine.[5)]

The features of histidine isolation by 3,4-D are as follows:

1) Preparation of 3,4-D is very easy and 3,4-D is the specific precipitation agent for histidine.

2) Histidine precipitates gradually as a disulfonate from acid solution whereas precipitation as silver or mercury salts occurs from neutural to weak alkaline solutions.

3) Isolation of the disulfonate of histidine is complete and easy since it is crystalline and can easily be purified by discoloration and recrystallization. The crystals also have far higher decomposition point (275-280°C) than 3,4-D salts of other amino acides (generally m.p. around 200°C) which makes it easy to prove.

4) Although the process is not quantitative, it is much easier than the silver or mercury salt method.

Tawara[6] separated histidine from dried whale blood powder. The authors examined isolation of histidine from whale and cattle blood by the use of 3,4-D and the results were compared with that of Tawara's.

It should be noted that the dried and powdered whale blood contains far larger amounts of moisuture and salts than those of cattle and horse blood, so that a correction must be made of the values. If this crude whale blood powder is hydrolyzed and 3,4-D used, isolation does not proceed smoothly due to obstruction by salts, resulting in the decrease in the amount of histidine disulfonate, so that the yield cannot be made better by adjusting the content of moisture and salts.

In order to remove salts from whale blood powder prior to hydrolysis, a pretreatment is made by adding water to the crude blood powder, stirring well and filtering. The residue is washed well with water and air-dried. This blood powder (hereafter designated as the pretreated blood powder) contains almost no salts so that, when the hydrolyzed solution is concentrated, only the hydrochlorides of glutamic acid precipitates out. The yield of histidine also increases remarkably, being better than that obtained from cattle blood carried out as a control. On the other hand, isolation of glutamic acid hydrochloride is very difficult from untreated whale blood powder owing to occlusion of sodium hydrochloride.

Addition of water to cattle blood powder results in its swelling which makes it hard to wash with water. This elimination of washing with water allows some inclusion of salts. The blood cells used by Vickery was washed well with water so that the yield in final product is very high. It can be assumed, therefore, that the content of salt in blood powder controls the formation of histidine disulfonate when using 3,4-D method.

Monosulfonate of leucine is obtained in a considerable amount from the mother liquor after separating histidine disulfonate.

Recently, Takagi, Suzuki and Asahina[7], used acid clay in the hydrolyzed solution of cattle blood to adsorb basic amino acids, then eluted this by weak alkali. This alkaline solution was electrolytically dialyzed, then 3,4-D added, and histidine was isolated with a good yield as its disulfonate. As was explained in their report, majority of 3,4-D can be recovered as a barium salt.

Acids used for hydrolysis were hydrochloric and sulfuric acids but the yield of histidine disulfonate was not so good when 30-35% sulfuric acid was used.

The method of isolation and the results are given in Tables I and II.

Table I. Method of Isolation

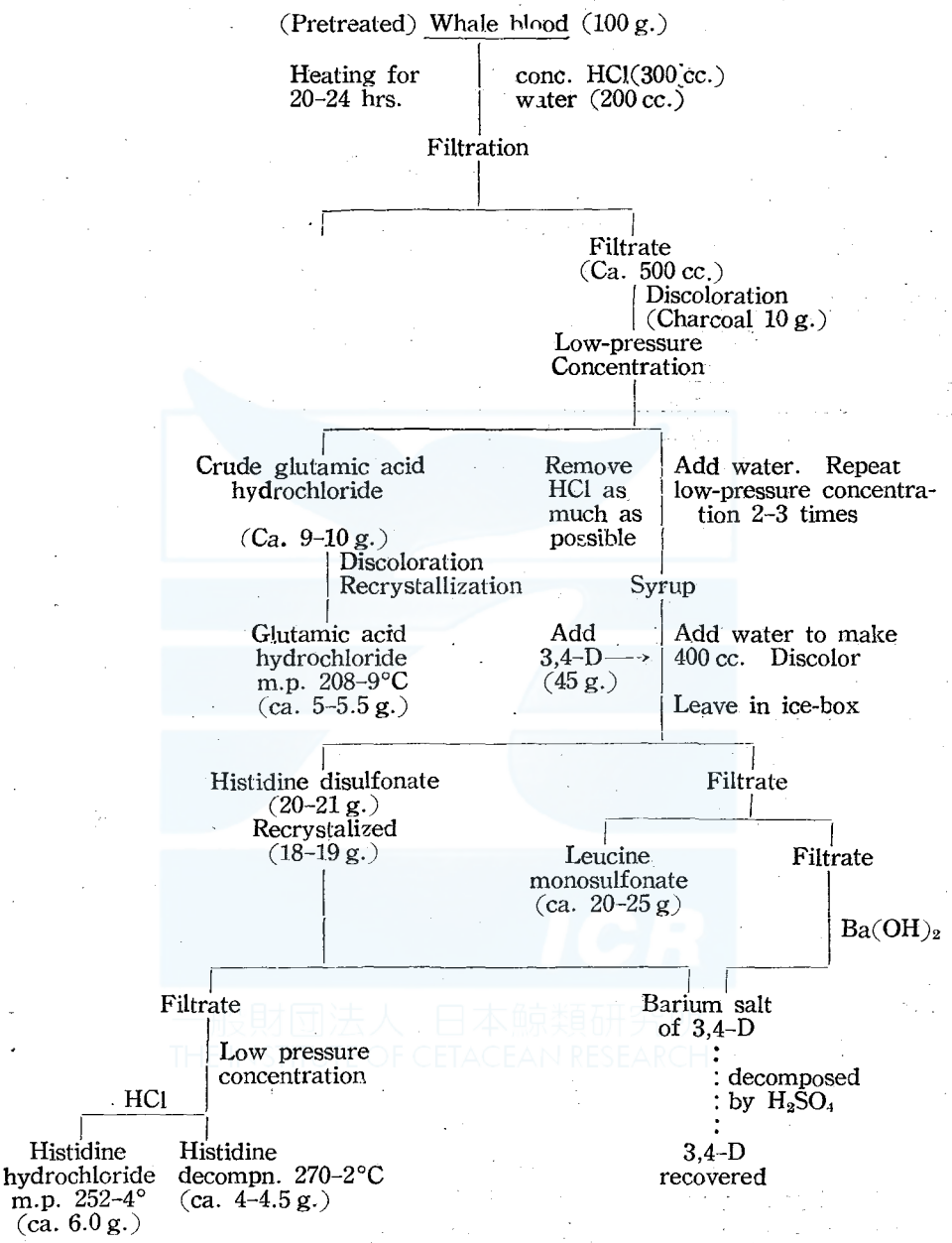


Table II. Amount of Histidine Disulfonate isolated from 100 g. (moisture corrected) of Various Blood Powder (salts uncorrected)

Kinds of Blood		Moisture (%)	Histidine disulfonate (g)	Remarks
Untreated Whale blood	No. 1	28.6	12.3	Contained chorides (as NaCl) 9.4% — Glutamic acid hydrochloride not isolated due to salt precipitation
	No. 2	28.6	13.3	
	No. 3	28.6	13.6	
Cattle blood	No. 1	12.0	14.5	
	No. 2	12.0	17.3	
Pretreated whale blood	No. 1	Hydrolyzed after corection	18.6	Glutamic acid hydrochloride isol'd
	No. 2		19.0	〃 not isolated
			25.7	
Horse blood	Blood cells	5.5	25.7	Taken from the data of Vickery[4] for comparison
	Technical hemo-globin	7.7	18.1	

(Note: The yield on histidine disulfonate does not differ much even when separation of glutamic acid hydrochloride is carried out as in No. 1 of pretreated whale blood. The amount of leucine monosulfonate decreases slightly).

EXPERIMENTAL

1) *Preparation of 3,4-Dichlorobenzen sulfonic acid*:

A mixture of 65 cc. of o-dichlorobenzen and 140 cc. of conc. H_2SO_4 (d=1.84) is heated in a flask in an oil bath at 185±5°C for 24 hours under air cooling. After cool, 700 cc. of water is added and discolored with activated charcoal. The discolored solution is concentrated to one-half of its volume on a water bath, cooled, and filtered through a glass filter. The residue on the filter is washed with conc. HCl, H_2SO_4 removed, suck and dried. In order to remove conc. HCl and water, this residue is placed in an evaporating dish, melted on a small flame and heated gradually to 130°C at which the heating is continued for a few minutes. After cooling and before solidification, the mass is stirred into small pieces and is kept in the dessicator since it is hygroscopic.

3,4-Dichlorobenzen sulfonic acid (3,4-D) herewith obtained contains a small amount of water and is a white, crystalline mass which melts at around 70°C. This can suitably be employed for the isolation of histidine.

2) *Hydrolysis of Whale Blood Powder*:

In order to examine the effect of salts, hydrolysis were carried out with crude whale blood powder, washed and unwashed with water.

(a) Untreated blood powder—Small pieces of crude whale blood powder is ground in a mortar, sieved to remove all occluded substances, and prepared into a fine powder.

Moisture—1 g. of untreated blood powder is dried at 100-110°C to con-

stant weight and the residual powder was found to weigh 0.714 g. Therefore, moisture is 28.6 %.

Chloride—Sufficient water is added to 10 g. of untreated blood powder to make 100 cc., filtered, and 1 cc. of the filtrate is titrated with 0.1 *N* $AgNO_3$ to determine the amount of chloride which, as NaCl, came out as 9.4%.

It follows, therefore, that the protein portion amounts to 62 %.

(b) Pretreated blood powder—The untreated blood powder is stirred into 5 times its volume of warm water of around 50°, filtered, washed well with water and dried. By this process, majority of haemoglobin remains in the residue while albumin and globulin dissolve into the aqueous solution together with salts and water-soluble proteins.

Untreated blood powder possesses a saline taste while such is not the case in treated blood in which the odor peculiar to whales has also disappeared.

Method of isolation using pretreated blood powder is given as follows:

A mixture of 100 g. of pretreated whale blood powder, 300 cc. of conc. HCl and 200 cc. of water is heated for 2–3 hours in a water bath, and then hydrolyzed by heating over an open flame for 20 hours with reflux attachment. After separating by filtration, the solution is discolored by a small amount of activated charcoal* and is concentrated under reduced pressure on a water bath of 50–60°C. The solution turns syrupy when reduced to one-third its original volume. When the solution becomes cool, the hydrochloride of glutamic acid precipitates out. If the untreated blood has been used, a large amount of salts precipitate out here. The syrup is left overnight in an ice-chamber after which it is filtered, the residue washed with a small amount of conc. HCl and dried. A viscous, crystalline mass of crude glutamic acid hydrochloride is obtained to the amount of 9–10 g. This is discolored and recrystastallized from hydrochloric acide to white crystals of m. p. 208–9°C. Yield, ca. 5–5.5 g.

The filtrate obtained after separation of glutamic acid hydrochloride and the washings** from activated charcoal are brought together, sufficient water added to make the total amount about 300 cc, and is again concentrated under reduced pressure to a syrupy state. Addition of water and concentration under reduced

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* The pretreated whale blood powder No. 1 in Table II was discolored before concentration and No. 2 was not treated. When not discolored, the solution, during concentration, is very hard to bring down the volume, and the concentrated liquid is very dark in color. Glutamic acid hydrochloride cannot be separated from such a liquid.

** Activated charcoal used for discoloration is extracted twice with boiling water.

pressure is repeated again to remove as much HCl as possible. Water is then added to the syrup to bring the whole volume to 400 cc., and discolored until the solution becomes clear and yellow. The hot washings from activated charcoal is concentrated, added to the main syrup and the whole is again concentrated to about 400 cc. The pH of the solution becomes 1.0 to 2.0.

3) *Isolation of Histidine*:

To the hydrolyzed solution, 45 g. of 3,4-D is dissolved, a minute amount of histidine disulfonate crystals is added as a seed, and the whole is left in an ice-chamber for a few days. The disulfonate precipitates out in the bottom of the flask as a crystalline mass.

The crystals of histidine disulfonate are prismatic while monosulfonate of leucine is fine needles. The latter easily precipitates out by slight swirling of the hydrolyzed solution so that it must be decanted its supernatent liquid, quietly and carefully.

If the sulfonates of histidine and leucine percipitates out at the same time, the solution is heated to dissolve both crystals and then left for a few days in an ice-chamber so as to allow histidine disulfonate to precipite out. In general, histidine salt crystals precipitate out first, and separation of leucine monosulfonate is not difficult. Yield of crude leucine monosulfonate, 20-25 g.

The crude crystals of histidine disulfonate, decompn. 260-270°, is dissolved in water and discolored, and then recrystallized to white needle crystals of decompn. 275-280°C. The yield decreases by about 5-8 % when recrystallized.

To obtain histidine from its disulfonate, the latter is dissolved in eight times its volume of boiling water, cold, saturated solution of $Ba(OH)_2$ added to pH 7.2, and the barium salt of 3,4-D that separates out on cooling is filtered off. The barium salt is washed with water, the washings added to the mother liquid and the whole volume condensed to less than one-half of its volume under reduced pressure. In order to remove 3,4-D still present in the solution, the solution of $Ba(OH)_2$ is added until the pH is 8.5, and the small amount of barium salt that separates out is filtered off. Barium hydroxide is quantitatively removed by H_2SO_4, the solution brought to pH 7.2, and concentrated under reduced pressure by which free histidine separates out.

After removal of histidine, the filtrate is further concentrated and a small amount of histidine precipitates out upon addition of alcohol. These are recrystallized by dissolving in a small amount of water and adding alcohol. Histidine has a decomposition point of 270-2°C. Yield is about one-quarter of the disulfonate.

Histidine hydrochloride is obtained by the addition of equimolecular amount of HCl to the solution of pH 7.2 obtained after removal of 3,4-D by $Ba(OH)_2$

which brings the pH of the solution to 2.8. By concentration under reduced pressure, and allowing the whole to stand overnight after addition of alcohol, white crystals of histidine hydrochloride, m.p. 252–4°, can be obtained. These crystals did not show any depression of melting point when fused with histidine hydrochloride of Takeda Pharmaceutical Industries. Some histidine disulfonate can be recovered from the mother liquor by concentration and addition of an excess of 3,4-D. The yield of hydrochloride is about one-third of histidine disulfonate.

CONCLUSION

Isolation of histidine from whale blood powder using 3,4-dichlorobenzene sulfonic acid was carried out. Whale blood cotains a large amount of moisture and salts and the latter is responsible for the obstruction of isolation process. Therefore, the blood was pretreated to warm and cold water washings by which the salts were removed and a result approximately equal to those from cattle and horse blood was obtained. The hydrochlorides of glutamic acid can also be obtained easily from the pretreated blood whereas its separation is very difficult from untreated blood owing to the precipitation of salts.

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