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I hereby recommend that the thesis prepared under my supervision by Elmer G. Gerwe *entitled* The Autoxidizability of Pure Cysteine and the Action of Cyanides. *be accepted as fulfilling this part of the requirements for the degree of* Doctor of Philosophy

Approved by: A. P. Mathews

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THE AUTOXIDIZABILITY OF PURE CYSTEINE AND THE ACTION OF CYANIDES

A dissertation submitted in partial
fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

to the Graduate School of the
University of Cincinnati

1930

by

Elmer G. Gerwe
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THE AUTOXIDIZABILITY OF PURE CYSTEINE AND THE ACTION OF CYANIDES

by

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Introduction

Within the past thirty years many investigations have been carried out on the chemical processes of cellular respiration. It has been shown by Professor Mathews that the life of tissue and cells in general depends upon a stage of partial oxidation, and that only partially oxidized protoplasm exhibits the structure properties and chemical characteristics of living matter. If a cell be deprived of this state, then it loses all of the fundamental properties of life:- it ceases to grow, it no longer conducts an impulse, the synthesis of essential constituents necessary for cellular repair ceases, respiration stops and heat is no longer produced at a normal rate.

The fact that oxidations are continuously taking place within living matter which in itself is strongly reducing, points to the existence of one or more well regulated systems of oxidizing-reducing substances which

maintain and control this state of partial oxidation.

Now in aerobic forms this state of partial oxidation may be secured by contact of the protoplasm with atmospheric oxygen; in anaerobic forms, however, it is secured by contact with substances within the cell which are capable of existing in both an oxidized and a reduced form. Moreover, these cell systems must be easily reversible in order to maintain an oxidation-reduction equilibrium in the cell.

It is known that a number of substances exist in protoplasm which may serve such purpose, among which are sugars, aldehydes, ketones and sulfur compounds. The oxidation of these substances is catalyzed by iron and in addition often by oxidases and peroxidases which doubtless play important roles in respiratory and metabolic processes.

The possible role of iron in respiration was long ago perceived by physiologists and biochemists and has been especially emphasized by Dr. Warburg and his students among others. Its ubiquitous occurrence indicates its importance. Iron is found in inorganic state in all cells of both plants and animals; and it forms a part of hemoglobin, the oxygen carrier of blood. Besides, iron is a powerful accelerator of those oxidations which are due to hydrogen peroxide and is a catalyst of the spontaneous oxidations in air of the sulfur compounds. That there is a distinct interaction between iron and sulfur is evident from investigations in

inorganic chemistry which show that rusting of iron is largely due to the sulfur content in the iron. Steel freed from sulfur by the newer processes of smelting is more nearly rustless than steel not so free from sulfur.

The occurrence of the sulphydryl compounds in tissue.

The part which sulfur plays in metabolic processes has been excellently reviewed by Lewis,(2). It is a constituent of several important substances such as insulin and the alpha and beta pituitary hormones. It is also present in many proteins ranging in quantity from none at all in the protamines to about 6% in human hair (3,4). Cystine discovered by Wollaston (5) in 1810 in a urinary calculus, and isolated as a product of tryptic digestion of fibrin by Kulz (6) in 1890, and by Emmerling (7) in 1894 from the hydrolysis products of horn, is undoubtedly the more common sulfur containing constituent in the protein molecule, altho other thioamino acids are known to exist.

It is still uncertain whether the disulfide, cystine, is exclusively present or whether its reduction product, cysteine, may also exist in the protein molecule. The readiness with which cysteine, when neutral, oxidizes to cystine as well as the solubility of cysteine, might explain why no cysteine has ever been isolated as such from the hydrolytic products of proteins, even tho it existed in the protein molecule.

If, for example, during the preparation of the cysteine polypeptide, glutathione, one does not avoid excessive heating and exposure to oxygen, the oxidized form usually results altho it is known to exist in tissue almost wholly in the reduced form.

Opinion as to the actual existence of cysteine as such in the protein molecule is divided. That it existed in tissue proteins and in protein free filtrates was shown by Arnold (8) on the basis of the violet color it produces with sodium nitroprusside in a feebly alkaline medium (9, 10,11). This color reaction, the importance of which was earlier pointed out by Buffa (12), Heffter (10) and Mathews and Walker is characteristic of substances which contain the sulphydryl (SH) group, e.g., cysteine, thiolactic and thioglycollic acids, ethyl mercaptan, etc. Arnold (9) reported a positive nitroprusside reaction with the crystalline lens of the eye and the peptic digest of egg white. Goldschmidt (13) also reports the presence of cysteine in the lens of the eye and found that the amount decreases with age. Sullivan (14) reports its presence in the tissues of pigeons receiving a diet of rice plus vitamin; on a rice diet alone, however, the cysteine disappears. Abderhalden and Wertheimer (15) describe the presence in all cells of either cysteine or cysteine containing substances.

With the discovery of Hopkins (16) of glutathione, a tripeptide which contains cysteine, the attention of students

of protein chemistry was again focused upon the possible occurrence of cysteine in proteins. Hopkins demonstrated the presence of glutathione both in animal and plant tissue or cells and he attributed the nitroprusside reaction in such tissue to it. It is obvious that if cysteine be not present as such but as the disulfide in native proteins, it may easily be formed in them as a result of relatively simple reactions.

In conclusion the presence of cysteine in the protein molecule must be regarded as probable but not proven, as Lewis (2) points out, until cysteine shall have been actually isolated from the products of protein hydrolysis.

There is no doubt, however, that cysteine alone or in peptide form does exist as such in tissue and in the animal organism. It may be as Lewis suggests that the first step in the catabolism of cystine in the body is its reduction to cysteine, a reaction readily brought about in vitro. Baumann and Preusse (17)and Jaffe (18) observed that the feeding of monohalogen benzene derivatives to dogs induced a synthesis of acetylated cysteine conjugates of the benzene halides (mercapturic acids) which were excreted in the urine. Later investigations have confirmed this result, and have shown that on minimum protein diets this synthesis does not occur (19), unless cystine is fed or injected subcutaneously at the same time (20). The occurrence of taurine, amino ethyl sulfonic acid, in bile as a conjugated

taurocholic acid, together with its close chemical relationship to cysteine from which it may be prepared in vitro (21), is but another indication of the normal formation of cysteine from cystine in the animal organism. There are a number of other reasons for believing cysteine to be a precursor of taurine (22).

While this evidence points undoubtedly to the possibility that cystine may be converted into cysteine in the body, it cannot be interpreted that this conversion is a necessary step in cystine catabolism. Lewis and McGinty (23), however, have supplied evidence of this nature. When the deaminization of cystine is prevented by its conjugation with phenylisocyanate, the resulting phenyl uramino cystine when administered to rabbits is excreted in part in the urine as phenyl uramino cysteine. It was found that dibenzoyl cystine was also excreted in part as dibenzoyl cysteine (24). The reduction may, however, have been due to bacterial action in the bowel. Rose, Shiple and Sherwin (25) have confirmed these results and have shown that the conversion of cystine to cysteine is a reversible one. The evidence just reviewed appears to the authors to justify the conclusion that the first step in the catabolism of cystine is its reduction to cysteine.

The discovery of glutathione clinched the proof that cysteine, occurring in peptide combination with other

amino acids could exist in the reduced state in the body. Tunnicliffe (26), developing a method of estimating glutathione in tissues, concluded that in the tissues of normal animals glutathione is present chiefly in the reduced form. It was also found that glutathione sulfur in tissue extracted with 10 per cent trichloracetic acid, accounted for the greater part of the neutral sulfur. Thompson and Voegtlin (27) also investigated the glutathione content of acid extracts of liver, brain, kidney and muscle of the rat, making use of the colorimetric method of Sullivan (28) for the estimation of cystine, cysteine and glutathione. These extracts were found by them to contain neither free cysteine nor cystine but only glutathione. However, making use of the same reaction, Sullivan detected the presence of cysteine in the tissues of polyneuritic pigeons fed on a diet supplemental with vitamin.

Altho the results of Tunnicliffe (29) and those of Uyei (30) definitely indicate that glutathione accounts for practically all of the non-protein sulfur of animal tissues, it is not unlikely that their method of analysis include other sulfur compounds which are now known also to exist in tissue. In 1921 Mueller (31) isolated from the products of hydrolysis of casein a sulfur containing amino acid to which he assigned the formula $C_5H_{11}SNO_2$. More recently Barger and Coyne (32) have synthesized the substance

$CH_3.S.CH_2.CH_2CH(NH_2).COOH$, gamma methyl thio alpha amino butyric acid, which they call methionine. It was shown to be identical to Mueller's amino acid. The recent discovery of ergothionene in blood may also necessitate a revision of the conclusions drawn above. Since many of these sulfur compounds behave on oxidation in a similar manner by changing into the disulfide form,any of the usual chemical methods such as iodine titration applied to the estimation of the reduced compound, measures the reduced form of most or all of these thiol compounds, hence the error in concluding that all of the sulfur present exists in the form of one certain sulphydryl compound.

It is very likely that regardless in what compound the sulfur exists, whether it be cystine, cysteine, either form of glutathione, Mueller's amino acid, ergothionene or de Rey Pailhade's philothion, the essential feature is that it exists in sulphydryl groups. On oxidation of the compounds two molecules combine thru the disulfide linkage, S - S.

Altho some of the evidence points to the existence of free cysteine in tissue, most of it points to the occurrence there of reduced glutathione. This substance, however, acts in nearly all respects like cysteine in oxidation, differing mainly in the fact that the disulfide form is soluble.

The sulfide-disulfide equilibrium in cellular oxidation.

The cystine-cysteine system is the simplest and the most widely studied of the several thiol systems. That this may be playing an important part in cellular respiration was suggested by Heffter (10) and in 1907 and independently by Professor Mathews in 1908 (33) who showed that cysteine oxidation is remarkably comparable to that taking place within the cells in the following particulars:

(1) Cysteine oxidation takes place with rapidity only very near to the neutral point, i.e., at a pH of 7.0 to 8.0 which is the reaction of most forms of protoplasm. It is checked particularly by acids which also check cell respiration.

(2) It is greatly hastened by the presence of some iron and copper compounds, these being also cell catalysts of respiration.

(3) It is inhibited or greatly reduced by small amounts of cyanide and some other poisons which depress living respiration.

There were certain points of difference, however, between this reaction and cell respiration. These differences were:

(1) The cysteine oxidation was not checked by anaesthetics, which do reduce respiration (Personal communication by Professor Mathews).

(2) Cysteine oxidation did not lead to the simultaneous oxidation of amino acids, sugars and other substances which are not self oxidizable but which undergo oxidation in living matter. It was shown by Hopkins that glutathione also failed to oxidize these substances at a pH reconcilable to that existing in tissues. These facts show that the oxidation is not accompanied by the liberation of active oxygen.

Struck by the first of these facts and by the results of his own experiments on the stimulating action of iron salts on cell respiration Warburg (34,35,36) has advanced the theory that the intracellular iron represents the so-called oxygen carrier of the respiration enzyme. "In respiring cells molecular oxygen reacts with bivalent iron, whereby iron in a higher state of oxidation is formed. The oxidized iron reacts with the organic substance and is again reduced to bivalent iron,......... molecular oxygen never reacts directly with the organic substance." He considered introcellular iron as the universal biological oxidation catalyst, and toxic substances such as arsenious acid, hydrogen sulfide and cyanides he considered as specific poisons to

intracellular iron.

He adduced the following facts to support his theory:

(a) The addition of iron salts to a suspension of sea urchin (Arbacacia) eggs in sea water increases their respiratory rate.

(b) The amount of intracellular iron as determined by direct analysis of the eggs suffices to carry the oxygen which disappears during respiration.

(c) Substances, such as arsenious acid, hydrogen sulfide and cyanides inhibit respiration and combine with iron.

As a result of further investigations Warburg (37) and Sakuma, and Sakuma (38) alone conclude that the oxidation of cysteine is really a catalysis by iron, since their preparation which was made with special care so as to be practically free from iron oxidized only about one per cent as fast as the oxidations of other preparations described in the literature. Rates as low as 0.77 cmm. O_2 per hour were recorded by Sakuma for a 16.3 mg. sample of his purest cysteine hydrochloride when dissolved in water to which a sufficient amount of ammonia had been added so that the final 10 cc. of solution had a pH of 7.6 . From this they conclude that since at least 99 per cent of the

oxidation is nothing more than catalysis by iron contamination, it was probable that all of it was due to iron.

Warburg assumes that the oxidation of cysteine takes place thru the formation of a very reactive Fe-cysteine complex which is autoxidizable. On the addition of cyanide, however, a complex consisting of cyanide-Fe-cysteine is formed in its stead which is unreactive.

Abderhalden and Wertheimer (39) attacked this general theory of Warburg. They attached great significance to the small residual oxidation which Warburg and Sakuma found to occur even in their purest samples of cysteine. Working with glass apparatus in place of the quartz used by Sakuma, which was difficult to obtain at that time, these investigators (40) prepared samples of cysteine which they believed to be free from iron and which oxidized at a slow rate. Since Tunnicliffe (41) reported that cystine accelerated the oxidation of cysteine probably thru the formation of a catalytically reactive cystine-cysteine complex, they concluded that the substance responsible for the small residual uptake of the pure samples was cystine, which they believed contaminated their samples as well as those of Warburg and Sakuma. Altho the addition of cyanides greatly inhibited the oxidation rates of their sample they believed that the inhibition was due not to a formation of an inactive Fe-cysteine-cyanide complex but

rather to the removal of traces of the active cystine which they assumed their sample to contain. This reasoning was based on the fact that cyanide, as was shown by Mauthner (42) in 1912 and later by Abderhalden and Wertheimer, reduces cystine to cysteine, the conversion of which was detected by means of the sodium nitroprusside reaction.

The Problem.

While there is now a general agreement that iron is an important catalyst of the spontaneous oxidation of cysteine itself and of the cysteine peptides such as glutathione, and it is certain that both iron and cysteine compounds exist in many if not all forms of living matter, it is still uncertain whether cysteine is capable of self oxidation from the air in the absence of iron. Thus Warburg maintains that spontaneous oxidation is impossible without the iron, while Abderhalden believes cysteine itself will oxidize at a slow rate provided some cystine be present. The question is of particular importance also in interpreting the nature of the action of cyanides on living matter. These substances universally depress respiration. If all respiration depends upon iron, then cyanides may act as suggested by Warburg by combining with iron to make a non-ionic form and thus inhibit respiration. If, however, self oxidation can take place in the absence of iron then

some other explanation of the action of cyanides than that suggested by Warburg must be looked for.

That certainly some self-oxidations are not dependent upon iron is indicated not only by the unlikelihood of atmospheric oxygen combining only with a single element, iron, but also by the fact that while cyanides <u>depress</u> respiration they do not entirely inhibit it.

The universal presence of iron in cells of all kinds, and its well known power of catalysing a great variety of oxidations clearly indicates its importance in cellular respiration. Professor John Uri Lloyd suggested that it would be well to study more closely the respiratory activity of iron, and to discover the mechanism of its action. At his request I began the investigation of the relation of iron to the self oxidation of cysteine under the direction of Professor A. P. Mathews.

In view of the several conflicting theories which attempt to explain the oxidation of cysteine in terms of iron catalysis and the inhibitory action of cyanides in terms of the formation of a cysteine-Fe-cyanide complex, or the removal a catalytically active cystine-cysteine complex, the following questions presented themselves:

1. Is cysteine autoxidizable when completely freed from iron?

2. Will cyanides completely inhibit the oxidation of such material?
3. What is the action of cyanides on cysteine oxidation catalyzed by iron?
4. Is there any action of cyanide on cysteine?
5. Does cystine free from iron catalyze cysteine oxidation?
6. Will iron in the form of complex cyanides influence oxidation?

Method of oxidation measurement.

The oxidation rates were determined with the blood-gas microrespirometer of Barcroft and Roberts. The apparatus consists of two glass pear-shaped receptacles, approximately equal in size and attached to a U tube manometer which contains clove oil of a specific gravity of about 1.032, chosen so that 760 mm. of mercury corresponds to 10,000 mm. of clove oil. Since the oil is roughly 1/13 as dense as the mercury, obviously, this type is 13 times as sensitive as the usual mercury manometer.

The apparatus is shaken mechanically in a water bath which holds a constant temperature during the period of experimentation, moreover, the operations were carried out in a well insulated room in which the temperature remained practically constant over long periods of time.

It was standardized according to the methods described in the original papers of Barcroft (43) and others, (44,45) the following constants having been determined:

(1) Cross sectional area, S, of the manometer tubes 1.566 sq. mm.

(2) Capacity, V, of bottles plus additional volume due to stem and manometer tubes above the level at 20 m m.,

Bottle A = 40.012 c. c.

Bottle B = 38.556 c c.

In order to greatly simplify calculations the volumes of both systems must be equal, and the difference is made up by adding to Bottle A a paraffin sphere molded so that the volume is equal to the difference of the volumes of system A and B, namely, 1.456 c. c.

When oxygen is absorbed in either of the bottles, the negative pressure resulting is indicated by the difference of the levels of the manometric fluid in the two limbs. The volume of gas absorbed, $V(x-y)$, is calculated from the following equation:

$$V(x-y) = 2r(V/P + S)$$

where,

V = Volume in c m m. of either system, namely, 38.556 c. m. m.

$2r$ = Difference in level of manometer fluid.

S = Cross sectional area of manometer tube (1.566 sq. m m.)

P = Atmospheric pressure in m m. of clove oil.

Preparation of cystine.

The method employed is that described by Baumann (46) and Friedmann (47) with various modifications in the final step of the process to insure freedom from iron. The starting point was l-cystine prepared from human hair according to Morrow (48). Special precautions were taken to remove from the hair, as completely as possible, any metal objects such as wire or hairpins.

It is very important according to Hoffmann (49) at no step during the process, should the preparation be made alkaline, for hair treated with hot 1% sodium carbonate for even a short period of time (1 - 4 hours) cannot be used as a source of cystine. Even though only about 25% of the total sulphur has been removed by such treatment, a change has been brought about, presumably in the cystine molecule, which prevents the formation of cystine crystals during the regular method of preparation.

The carbon which was used in decolorizing was carefully digested with concentrated hydrochloric acid to remove iron and the phosphates of calcium and magnesium which if allowed to remain would precipitate along with the cystine at its isoelectric point and then washed free from HCl with distilled water.

The cystine was obtained on recrystallization as the typical hexagonal plates and possessed a specific rotation

of α_D -209°. E. Fischer and Susuki gave -222° as the specific rotation of 1-cystine while Sukuma (38) reports a value of -216°. The specific rotation was determined according to the method which was standardized by Andrews (50) who studied the effect of the concentration, pH, temperature, etc., on optical activity. He states that the values given for cystine which vary from -200 degrees, as reported by Gaskell (51), to values above -250 degrees as obtained by Rothera (52) are due either to varying degrees of racemization or to conditions under which the determinations were made.

During the preparation of cystine it was found that cystine hydrochloride is markedly insoluble in concentrated hydrochloric acid and can be readily purified from iron by precipitating the cystine as the salt, by saturating its solution with dry hydrochloric acid gas. This precipitation is carried out in a quartz Erlenmeyer flask surrounded by a freezing mixture of salt and ice. At saturation of the gas the hydrochloride precipitates in prismatic needles which are removed by filtration through a Buchner funnel fitted with acid washed filter paper. The crystals are then washed by pouring over them small amounts of pure concentrated hydrochloric acid. The mother liquor, which still contained some cystine, was intensely yellow and when tested with KCNS gave a strong test for iron.

As a matter of fact, the yellow color of the acid solution may serve as an indicator of the relative freedom of the preparation from iron. Yoe (53) states that the color due to iron is of maximum intensity when the concentration of hydrochloric acid is about 28%. I have found that iron in as small amounts as 1 part in a million imparts a distinct color to a 28 per cent acid solution.

A portion of the cystine was recrystallized many times to remove all traces of iron and was preserved for special use to be described later. The rest of the preparation was recrystallized only a few times in the manner described above which was sufficient to remove most of the iron; further purification was unnecessary since more iron is added later as an impurity in the tin used for the reduction.

Reduction of cystine to cysteine and the preparation of iron free cysteine.

If a reducing substance could be found which could be prepared in a state of great purity and which would reduce cystine quantitatively the preparation of an iron free cysteine would be comparatively simple.

An attempt was made to reduce cystine with sulphur dioxide but no reduction was noted even after a treatment of several hours. This confirms observations of Andrews

who found no reduction to take place even after 24 hours as determined by polarimetric measurements. However, about 65% reduction is brought about by the use of sodium sulfite plus sodium carbonate. Of a number of various methods used in an attempt to reduce cystine the same author concludes that the use of metallic tin and acid is the only efficient one.

In view of the fact that KCN has been shown to reduce cystine to cysteine and that it is employed in Sullivan's naphthaquinone test (29) for cysteine, a reduction by purified HCN was attempted. But it was found that the cystine was converted to cysteine only to a limited extent; in addition the solution soon became colored and on concentrating, a resinous precipitate formed which possibly consisted of a polymerized cyanide decomposition product, the removal of which was extremely difficult.

Since it was possible to purify cystine hydrochloride from iron by the gas saturation method, it occurred to me that possibly cysteine could be purified in the same manner. After unsuccessful attempts to reduce cystine quantitatively by the methods mentioned above, the cystine was finally reduced by tin and hydrochloric acid in the usual way. After the reduction and the subsequent removal of the tin by hydrogen sulfide the solution was evaporated to dryness. The crude cysteine hydrochloride thus prepared contained a marked amount of iron.

The following steps of purification were carried out exclusively in fused quartz vessels, since the use of glass, and especially that of the softer varieties, is liable to cause considerable contamination with iron. The quartz vessels were repeatedly boiled out with strong hydrochloric acid, and were proved to be iron free by the method described below.

Water was distilled from a quartz distilling flask into a quartz Erlenmeyer cooled by a stream of water. Pure concentrated hydrochloric acid was then boiled in the distilling flask, the side tube of the latter being placed just above the level of the water in the Erlenmeyer. The distillation was continued until the resulting acid solution in the receiver was 6 N was determined by titration. 50 c c. of the acid was then evaporated to dryness in a quartz evaporating dish over an aluminum sand bath carefully protected from dust by an especially constructed glass hood. The evaporation was carried out in the presence of a small crystal of pure $KClO_3$, the purpose of which was to oxidize any ferrous iron to the ferric state. To the residue was then added 1 c c. of the distilled acid and 1 c c. of pure distilled water. This acid solution was carefully washed about the sides of the vessel and then poured into a clear acid washed test tube, to which was added 1 c c. of a 10% solution of specially

purified KCNS. The tube was then viewed over a perfectly white background and compared with one containing 3 c c. of the reagents. The final concentration of the acid was 2N at which the test is most sensitive according to Friedenthal and Lachs (54). An absence of color indicates that the 50 c c. of acid solution contained less than 1/10,000,000 gm. of iron at which the limit of sensitivity is reached. The water, acid and ammonia solutions were freshly distilled in quartz in the manner described above and stored in 500 c c. quartz flasks equipped with quartz ground stoppers, the tops of which were kept at all times covered with cellophane to insure freedom from dust.

A sample of the crude cysteine hydrochloride was put aside for later study and the remainder was dissolved in a quartz Erlenmeyer in just enough pure water to bring it into complete solution on heating. Iron free carbon was added and the solution decolorized until no trace of color persisted. All filtrations were carried out in a carefully washed Buchner funnel fitted with acid washed filter papers. Pure hydrochloric acid gas was then led thru the solution contained in the quartz Erlenmeyer which was surrounded by a freezing mixture. The cysteine hydrochloride precipitated in much the same manner as did the cystine salt, in clean prismatic needles. When no further precipitation took place the crystals were sucked dry from

the mother liquor and were washed with pure hydrochloric acid. This process was repeated until 1 c.c. of the mother liquor no longer gave a test for iron. This degree of purity was reached after the 12th recrystallization. After the 18th crystallization a 1 gm. sample of the dry cysteine hydrochloride ignited in a quartz crucible and tested for iron gave not the slightest trace of color, indicating that it contained less than 1/10,000,000 gm. of iron. Four more crystallizations were carried out, after which a 2 gm. sample of the cysteine hydrochloride ignited and tested gave no trace of color. It certainly contained less than 1/10,000,000 gram of iron and was presumably iron free. The sample on ignition left no visible residue.

The recrystallizations involved great loss, for the cysteine hydrochloride is many times more soluble in concentrated hydrochloric acid than is the corresponding cystine salt. From an original amount of 175 gms. of the crude cysteine hydrochloride, but 7.4 gms. of the highly purified salt remained.

Physical and chemical tests on the Cysteine Hydrochloride.

A. Test for iron.

(See the section on the preparation of iron free cysteine HCl.) Since the smallest amount of iron which can be detected by the KCNS method described above is .000,000,1 gm. and since the 2 gm. sample of cysteine HCl contained less than this amount, then 20 mgms. of the salt, which is the amount used in the oxidation measurements, contain less than 1/1,000,000,000 gm. iron. Any copper which might have been present would presumably have been removed by this method as completely as was the iron.

B. Titration with iodate solution.

Samples of the pure preparation were titrated according to Okuda's method (55) with standard potassium iodate solution. A 41.5 mg. sample of the hydrochloride, which contained by theory 31.9 mg. of cysteine required 14.8 c c. of the iodate solution. This, as determined by the following equation,

$$\frac{.01 \times \text{required volume of } KIO_3}{4.65} = \text{cysteine (gms.)},$$

corresponds to 31.8 mg. of cysteine. A 40.4 mg. sample containing 31.1 mg. cysteine by theory required 14.4 c c. of the iodate solution. This amount corresponds to 31.0 mg. of cysteine. The found values thus represent 99.7% of

the theoretical.

Harrison (56) and Sakuma (38) noted that their samples of the solid cysteine hydrochloride required considerably more than the theoretical amount when titrated with iodate solution, which indicated the presence of some free cysteine in addition to the hydrochloride. This was evidently absent from my preparation. The samples of cysteine hydrochloride which were used in this investigation were kept in dessicators charged with $CaCl_2$ and in which was placed a vessel containing several cubic centimeters of concentrated hydrochloric acid which prevented the loss of HCl from the cysteine salt. The results of spontaneous oxidation measurements also indicated that all of the cysteine existed in the salt form.

C. The optical activity of cysteine.

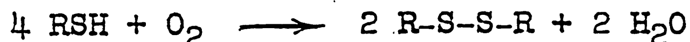
In a paper dealing with this subject, Andrews (57) states that the solid cysteine hydrochloride oxidizes very readily and that any attempts to prepare it without oxidation required the most elaborate precautions to exclude oxygen. He observed that even in the presence of hydrochloric acid the oxidation slowly took place. No such difficulty was experienced by the author, whose preparations were highly purified from iron. Possibly the presence of iron in his preparation might account for the discrepancy between my results and those noted by Andrews in this

particular, or possibly he had some free cysteine in his hydrochloride.

A sample of the pure cysteine hydrochloride which possessed an original specific rotation of $\alpha_D^{26^\circ}$ + 6.16° and which was kept in a dessicator charged with $CaCl_2$ and HCl exhibited a value of + 6.05° when measured a year later. Even exposure of samples of the same specimen for a period of 10 hours to ultra violet light, which, it was believed, might hasten the oxidation, failed to change this value. These results indicate, contrary to views expressed by Andrews, that the pure cysteine hydrochloride is extremely stable and in the form of its salt requires no precautions to exclude O_2 from its presence.

D. Oxidation measurements.

A 20 mg. sample of cysteine HCl in oxidizing completely to cystine according to the following equation:



$$4\ RSH + O_2 \longrightarrow 2\ R{-}S{-}S{-}R + 2\ H_2O$$

will require under standard conditions .000,507 gm. or 710.3 cmm. of O_2. Under the conditions of the experiment, which was carried out at 23° C. and a pressure of 749.6 mm. mercury, the requirement is 780.2 cmm. O_2. The difference in the manometric level of the Barcroft apparatus, after a period of continuous shaking for 67 hours after which O_2 was no longer taken up, was 155 mm. According to the formula, the volume of O_2 absorbed equals:-

$$v(x-y) = 2\ r\ (V/p + S)$$
$$= 155\ (33420.36/9744.8 + 1.566)$$
$$= 774.23 \text{ cmm.}$$

which is 99.3% of the theoretical value. This result and those of the previous tests show that the sample of cysteine hydrochloride is extremely pure.

Rate of oxidation of purified cysteine.

A series of experiments was carried out to determine the rate of oxidation of the pure cysteine hydorchloride. As had been noted by other investigators, the rate of oxygen uptake when determined on equal amounts of the same sample of cysteine hydrochloride and when run under identical conditions varies quite widely altho within definite limits. The cause or causes of this variability has been undetermined, but may be due to slight variation in the pH of the solutions used, altho this was adjusted as carefully as possible by colorimetric methods.

The rate of shaking, regulated by a small electric motor attached to the shaking apparatus, was adjusted so as to give 190 to 210 complete oscillations per minute . In a previous experiment it had been found that altho the rate of oxygen uptake when the system is at rest is considerably slower than when shaken, yet it is rather independent of the rate of shaking within wide limits. Even

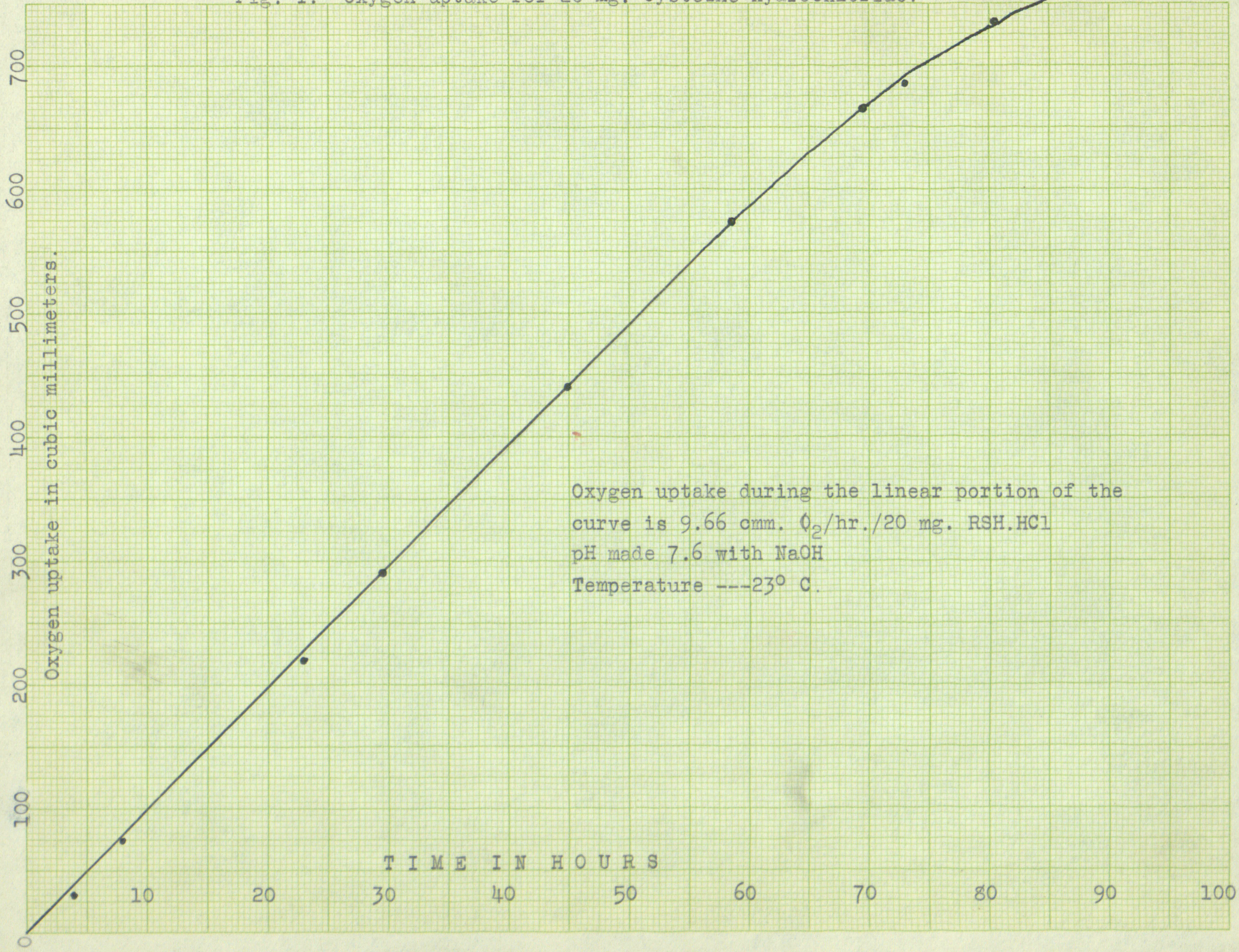
at slow shaking rates it seems that there is always sufficient oxygen introduced into the solution when the cysteine is iron free for the oxidation which is taking place. The limiting factors in this oxidation seem to be cysteine, the pH and any iron which may be added, rather than the speed of solution of the oxygen. This is shown in Figure I, where the velocity of oxygen uptake of pure cysteine is approximately linear over a considerable period and only towards the extreme end of the oxidation, when the amount of unoxidized cysteine remaining is considerably diminished, does the rate appreciably decrease.

(Insert Figure I)

20 mgs. of cysteine hydrochloride on oxidation under laboratory conditions require approximately 780 cmm. O_2, and since the 33 cc. of air, held in the bulb, additional to the 5 cc. of cysteine solution, contains approximately 6,600 cmm. of O_2, it is evident that there is present nearly 9 times as much oxygen as is required to oxidize the cysteine.

Samples of the water and ammonia solutions used in these experiments were evaporated in 500 cc. quantities in quartz dishes, and the theoretical residues (for there was no evident residue) tested in the usual manner (thiocyanate) for iron. In no case was the slightest trace of color observed, which would indicate that the 5 cc. of ammonia

Fig. I. Oxygen uptake for 20 mg. Cysteine hydrochloride.



solution used in neutralizing the cysteine HCl in the oxidation determinations contains no appreciable amounts of iron and certainly something less than 1/1,000,000,000 gm. of iron; the total amount of iron present then due to the 20 mg. cysteine hydrochloride and the ammonia solution in any one experiment is certainly less than 2/1,000,000,000 gm. and there was no indication of any iron at all being present.

The amount of alkali solution required to bring 20 mg. cysteine hydrochloride to a pH of 7.6, as determined by a separate titration, was added to the hydrochloride in the Barcroft apparatus together with sufficient water to make a total volume of of 5 cc. To the compensatory bulb was added 5 cc. of ammonia solution of a pH of 7.6 . The pH was checked both at the beginning and at the end of each experiment by means of Clark and Lub's standard indicators.

The apparatus was then shaken with the stop cocks open in the water bath for 1/2 hour in order to allow the temperature of the solution in the bulbs to reach that of the bath. The shaking was then stopped and the apparatus was brought to an exact vertical position so that the manometer fluid reached the same level in both tubes. The stop cocks were then closed, the shaking begun and the barometric pressure noted. Readings were taken every hour or two, when ever convenient during runs extending over long periods. Three minutes were allowed after the

shaking periods for an equilibrium to be reached before readings were taken.

(Insert Table I)

The results of a typical experiment which are given in Table I supplied the values for Figure I. The NaOH solution used in this experiment to neutralize the cysteine hydrochloride contained a small trace of iron and accounts for the increased rate of oxidation as compared to those of experiments in which pure ammonia solution had been used. Because of the great amount of time required for an oxidation to go to completion and since the rate is linear over a very long period, the rates given for the other experiments in this series were determined over 12 to 24 hour periods. The results of this series are given in Table II and it will be noted that the rates of oxygen uptake are expressed in terms of cmm. O_2 used by 10 mg. cysteine hydrochloride per hour, in order that they may be comparable to the results of other investigators which are summarized in Table III.

(Insert Table II)

(Insert Table III)

It is seen in Table III that when the uptake rates obtained by Sakuma are calculated in terms of oxygen uptake/per hour/per 10 mg. cysteine hydrochloride, the

Table I

Oxidation of Cysteine HCl

20 mg. Cysteine Hydrochloride in NaOH solution in Bulb B.

pH 7.4

Temperature = 22.0 to 22.6° C.

Barometric Pressure 749 mm. Hg or 9737 mm. clove oil.

Shaking rate 180 oscillations per minute.

Time	Manometer tubes in mm.		B – A	Total O_2 Uptake
Hours elapsed from beginning	Readings			
	A	B		
0.0	124.0	124.0	0.0	0.0 cmm.
4.0	121.0	127.0	6.0	30.04 cmm.
8.0	116.5	131.5	15.0	75.09 cmm.
23.0	102.0	146.0	44.0	220.26 cmm.
29.5	95.0	153.0	58.0	290.35 cmm.
45.0	80.0	168.0	88.0	440.52 cmm.
56.0	69.0	179.0	110.0	550.66 cmm.
58.5	66.5	181.5	115.0	575.69 cmm.
69.5	57.5	190.5	133.0	665.80 cmm.
73.0	55.7	192.3	136.5	683.32 cmm.
80.5	50.5	197.5	147.0	735.88 cmm.
93.0	47.0	201.0	154.0	770.92 cmm.
100.0	46.5	201.5	155.0	775.92 cmm.

(Table I continued)

The oxygen uptake, V(x-y) is calculated from the equation

$$v(x-y) = 2\ r\ (v/p + S)$$

$$v(x-y) = 2\ r\ (33495/9737 + 1.566)$$

$$= 2\ r\ (5.006)$$

For example after 45 hours the difference in pressure, 2 r, equaled 88 mm. Hence the oxygen absorbed was equal to 88 x 5.006 or 440.52 cmm. After 100 hours the total uptake is 776 cmm. O_2 which represents 99.3 per cent of the yield possible and a rate of 4.83 cmm. uptake of oxygen per hour per 10 mg. sample of cysteine hydrochloride.

Table II

Rate of O_2 Uptake of Pure Iron Free Cysteine Hydrochloride Neutralized by Iron Free Ammonia.

No.	Hours Run	pH	Temp. in Degrees Centigrade	O_2 Uptake/hr. per 10 mg. RSH.HCl
2	24.0	7.4	23.0 - 23.4	2.4 cmm.
3	20.5	7.6	24.0 - 25.5	3.2 cmm.
4	21.0	7.4	22.5	1.8 cmm.
5	12.0	7.5	22.8 - 23.0	2.1 cmm.
6	8.0	7.4	23.0	1.6 cmm.
7	51.0	7.5	22.0 - 23.0	1.9 cmm.
8	24.0	7.5	22.5 - 23.0	2.3 cmm.
9	8.0	7.6	22.0 - 23.0	.9 cmm.
10	22.0	7.4	24.0 - 24.5	2.0 cmm.
11	21.0	7.5	23.0	2.5 cmm.
12	12.0	7.4	24.0	3.6 cmm.

Average Uptake = 2.21 cmm. O_2/hr./10 mg. RSH.HCl.

Shaking Rate = 180 to 200 oscillations/minute

20 mg. sample of cysteine hydrochloride used in each experiment.

Table III

Rates of Oxidation of Cystein

as Obtained by Other Investigators.

Preparations	Mgs. of sample	pH	O_2 Uptake in cmm./hr.	O_2 Uptake in cmm. per 10 mg. RSH.HCl	Investigator
Cysteine HCl	2000.0	7.6	7000.0	35.0	Mathews + Walker
Cysteine HCl	9.1	7.6	114.0	125.0	Dixon and Tunnicliffe
Crude RSH.HCl	10.0	8.0	380.0	380.0	Harrison
Crude RSH.HCl*	16.0	6.8	191.0	120.0	Sakuma
Crude RSH.HCl*	16.0	9.2	370.0	231.0	Sakuma
Impure RSH I *	16.0	6.8	13.9	8.7	Sakuma
Impure RSH I *	16.0	7.6	15.6	9.8	Sakuma
Pure RSH II	24.8	7.7	3.66	1.47	Sakuma
Pure RSH II	42.3	717	3.35	.79	Sakuma Average 1.22
Pure RSH II	16.3	7.7	.77	.47	Sakuma cmm. O_2/hr.
Pure RSH II	66.8	7.7	21.70	3.25	Sakuma per 10 mg.
Pure RSH II	22.6	7.7	6.10	2.30	Sakuma sample
Pure RSH II	24.8	7.7	3.7	1.5	Sakuma
Pure RSH II	42.3	7.7	3.4	..8	Sakuma
Pure RSH A	10.0	7.7	6.22	6.22	Harrison
Pure RSH B	10.0	7.7	2.49	2.49	Harrison
Pure RSH B	12.0	7.7	4.21	3.50	Harrison

(Table III continued)

Preparations	Mgs. of sample	pH	O_2 Uptake in cmm./hr.	O_2 Uptake in cmm. per 10 mg. RSH.HCl	Investigator	
Pure RSH C	9.0	7.3	3.32	3.69	Harrison	Average = 3.47 cm. O_2/hr./per 10 mg. sample
Pure RSH C	10.0	7.6	2.32	2.32	Harrison	
Pure RSH C	10.0	7.7	4.60	4.60	Harrison	
Pure RSH C	10.0	7.7	2.80	2.80	Harrison	
Pure RSH C	10.0	7.8	5.00	5.00	Harrison	
Pure RSH C	13.5	8.0	4.80	3.55	Harrison	
Pure RSH C	36.0	8.0	17.50	2.32	Harrison	
	10.0	7.7	6.60	6.60	Harrison	
	12.0	7.7	4.20	3.50	Harrison	
Harrison rates	10.0	7.7	2.80	2.80	Harrison	
before addition	13.5	8.0	4.80	3.55	Harrison	
of iron	10.0	8.0	7.20	7.20	Harrison	

Temperature in all cases was 20.0° except samples marked with * in which case the temperature was 37.5° .

average of the oxidation rates of his purest sample is 1.22 cmm. O_2/hr. while the average of Harrison's rates calculated in a similar manner is 3.47 cmm. O_2/hr. These values compare favorably with the average uptake which I obtained. The variability of the oxidation rates carried out under similar conditions is evident in Sakuma's results where uptake rates as low as .47 cmm. O_2/hr. and as high as 3.25 cmm. O_2/hr. were obtained, as well as in my own and the observations of others. It cannot be attributed to varying amounts of iron since I used throughout the same sample. The exact reason for the variability is still uncertain.

Since a 2 gm. sample of the cysteine hydrochloride which I prepared when tested in the usual manner contained something less than .0001 mg. Fe (which is the smallest amount which can be detected by the test) it is evident that my sample is at least 20 times more pure than that of Harrison in which he detected .0001 mg. in a .1 gm. sample of cysteine hydrochloride. Sakuma gives no estimation of the amount of iron present in his preparation but in consideration of the extremely slow rates of oxygen uptake there is no doubt that his cysteine hydrochloride was free from iron.

The effect of iron on cysteine oxidation.

It was found by Sakuma that amounts of iron as small as 1/10,000,000 gm. produce a measurable action. His results are summarized in the following table. All of the figures have been recalculated in terms of oxidation rates per hour per 10 mg. sample of cysteine HCl.

(Insert Table IV)

If we assume 1.96 cmm. O_2/hr. to be the oxidation rate of pure cysteine HCl, then

.00050 mg.Fe increases rate by 10.4 cmm.O_2 or 2.1 cmm.O_2/.00010mg.Fe
.00020 mg.Fe increases rate by 4.0 cmm.O_2 or 2.0 cmm.O_2/.00010mg.Fe
.00017 mg.Fe increases rate by 6.5 cmm.O_2 or 3.8 cmm.O_2/.00010mg.Fe
.00010 mg.Fe increases rate by 2.5 cmm.O_2 or 2.5 cmm.O_2/.00010mg.Fe

which averages 2.6 cmm. O_2/hr. increase per .00010 mg. Fe.

If as the figures incidate, the increase in oxidation rate is proportional to the amount of iron added, and if we should attribute the residual oxidation to the presence of iron in the sample, then from a simple algebraic ratio,

$$2.6 \text{ cmm. } O_2 : .00010 \text{ mg. Fe} :: 1.22 \text{ cmm. } O_2 : X \text{ mg. Fe}$$

$$X = .000047 \text{ mg. Fe present;}$$

the amount present in 10 mg. cysteine HCl is calculated to be .000047 mg. Fe. A 1 gm. sample of cysteine HCl would then contain .0047 mg. Fe an amount which might easily have been detected on igniting a 1 gm. sample and testing with

Table IV.

Effect of Iron on Cysteine Oxidation (Sakuma).

	Rate with no iron	Iron added	Rate after Fe addition
Sakuma	3.25 cmm. O_2	.00050 mg.	12.4 cmm. O_2
Sakuma	.80 cmm. O_2	.00020 mg.	6.0 cmm. O_2
Sakuma	2.30 cmm. O_2	.00017 mg.	8.5 cmm. O_2
Sakuma	1.50 cmm. O_2	.00010 mg.	4.5 cmm. O_2

Average = 1.96 cmm. O_2 without addition of iron.

KCNS solution. He does not cite any tests of his cysteine for iron.

Harrison's results, when calculated in terms of uptake per 10 mg. sample of cysteine HCl per hour, are summarized in the same manner. The value taken for the oxidation rate of his purest sample is the average rate expressed in Table III.

(Insert Table V)

In this case if we assume the oxidation rate of his pure cysteine HCl to be 3.47 cmm. O_2/hr./10 mg. RSH. HCl then,

.0016 mg. Fe increases rate by 97.5 cmm.O_2 or 6.1 cmm.O_2/.0001 mg.Fe
.0012 mg. Fe increases rate by 71.5 cmm.O_2 or 6.0 cmm.O_2/.0001 mg.Fe
.0008 mg. Fe increases rate by 50.5 cmm.O_2 or 6.3 cmm.O_2/.0001 mg.Fe
.0004 mg. Fe increases rate by 25.5 cmm.O_2 or 6.4 cmm.O_2/.0001 mg.Fe
which averages 6.2 cmm. O_2/hr. increase per .0001 mg. Fe.

He has shown that the oxidation rate is nearly directly proportional to the amount of iron added, hence, if the residual oxidation is assumed to be due to the iron present as impurity, then there should be present .000056 mg. Fe/10 mg. RSH. HCl as determined by the following equation.

$$6.2 \text{ cmm. } O_2 : .0001 \text{ mg. Fe} :: 3.47 \text{ cmm. } O_2 : X \text{ mg. Fe}$$

$$X = .000056 \text{ mg. Fe}$$

Then .1 gm. RSH. HCl should contain .00056 mg. Fe, but he found that .1 gm. of his sample of RSH HCl contained about .0001 mg. Fe and certainly less than .0002 mg. Fe. From

Table V.

Effect of Iron on Cysteine Oxidation (Harrison).

Rate with no iron	Iron added	Rate after iron addition
6.6 cmm. O_2	.00010 mg. Fe+++	15.60 cmm. O_2
3.5 cmm. O_2	.00020 mg. Fe+++	16.50 cmm. O_2
2.8 cmm. O_2	.00010 mg. Fe++	12.80 cmm. O_2
-	.00160 mg. Fe+++	101.00 cmm. O_2
-	.00120 mg. Fe+++	75.00 cmm. O_2
-	.00080 mg. Fe+++	54.00 cmm. O_2
-	.00040 mg. Fe+++	29.00 cmm. O_2

Average rate when no iron is added = 3.47 cmm. O_2/hr. (Table III).

this he concluded that the residual oxidation did not appear to be wholly due to the presence of iron.

In order to determine the relation between the residual oxidation of the cysteine hydrochloride, prepared by the author which was shown to be exceedingly pure, and the oxidation due to added iron, a series of experiments was carried out in which varying amounts of iron were added as $FeCl_3$. The results are given in the following table.

(Insert Table VI)

(Insert Figure II)

Figure II shows the effect of adding increasing amounts of iron as ferric chloride on the velocity of oxygen uptake of pure cysteine. The increase in velocity is seen to be directly proportional to the quantity of iron added. The initial velocity of pure cysteine was 2.2 (see Table II) cmm. O_2 per hour/10 mg. sample and if this residual uptake were assumed to be due to a trace of iron remaining in the preparation, then, the rate of uptake calculated from values obtained from added iron requires the presence of .000,044 mg. iron as calculated from the following equation,

$$2.2 \text{ cmm. } O_2 : X \text{ mg. Fe} :: 5.2 \text{ cmm. } O_2 : .0001 \text{ mg. Fe}$$

$$X = .000{,}044 \text{ mg. Fe.}$$

On actual test, however, 20 mg. of the cysteine

Table VI

Oxidation of Cysteine Plus Various Amounts of Fe.

Amount of iron added	O_2 Uptake	Increase Uptake per .0001 mg. Fe
.0010 mg. Fe	55.2 cmm.	5.3
.0008 mg. Fe	45.5 cmm.	5.2
.0006 mg. Fe	33.8 cmm.	5.3
.0004 mg. Fe	23.6 cmm.	5.1
.0002 mg. Fe	13.0 cmm.	5.4
.0001 mg. Fe	7.2 cmm.	5.0
No iron added	2.2 cmm.	

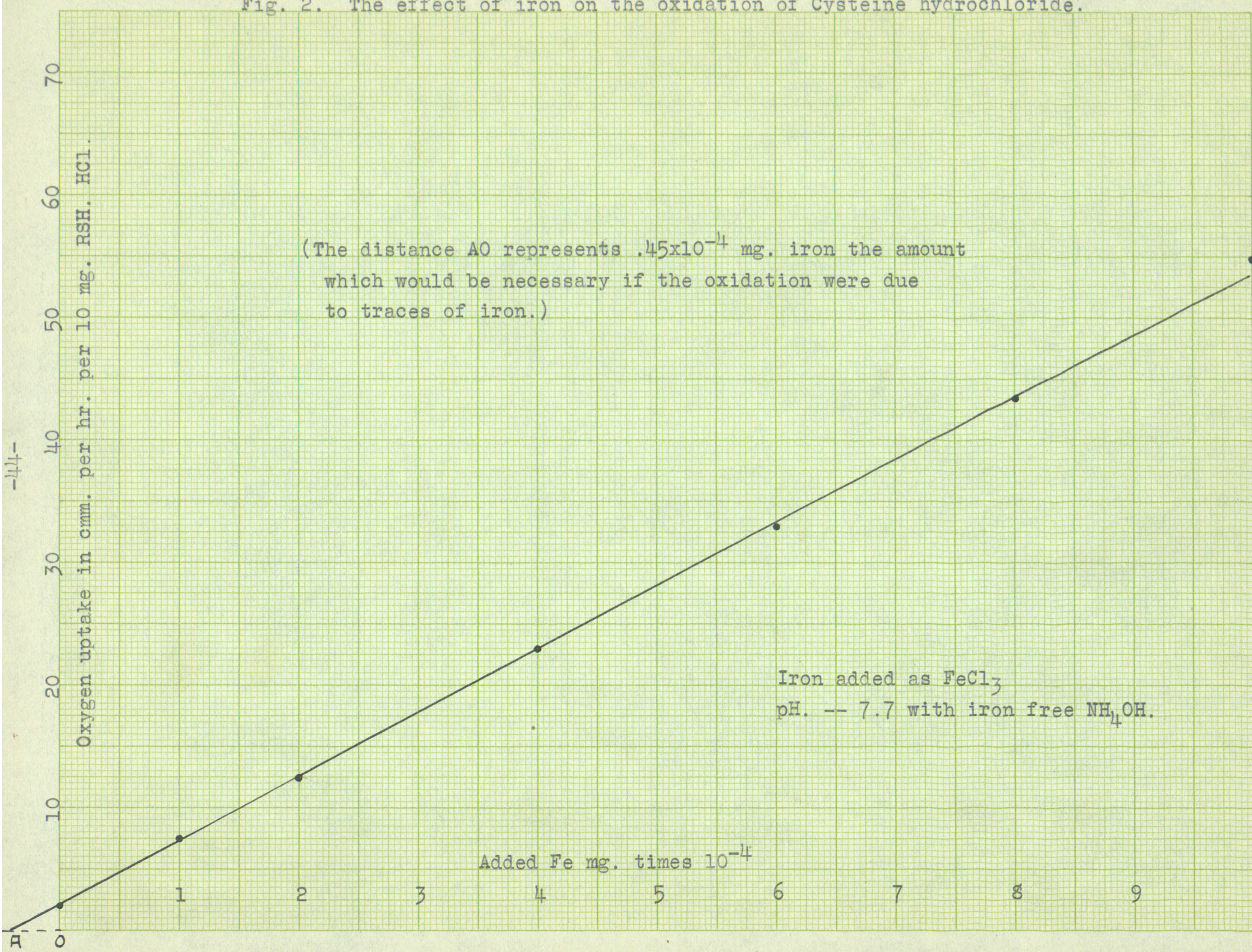


Fig. 2. The effect of iron on the oxidation of Cysteine hydrochloride.

hydrochloride plus the 5 cc. of alkali solution contains but .000,002 mg. of Fe instead of the .000,044 mg. required from a consideration of the iron oxidation graph given in Figure II.

This fact strongly points to the conclusion that pure cysteine is autoxidizable tho at a very slow rate. This conclusion is later confirmed by the results of experiments concerning the action of cyanides on cysteine oxidation in the presence and in the absence of iron.

The oxidation rates of pure cysteine as obtained by the various investigators are given in Table VII together with the increased rates brought about by the addition of .0001 mgm. Fe. It is of interest to note the amounts of iron which must be present in the purified substances if we assume that the residual oxidations are due to iron contaminations. These amounts are calculated from the graphs obtained when the oxygen uptake/per hour is plotted against the amount of iron added.

(Insert Table VII)

Harrison's results as well as those of the author indicate that the rate of oxidation of the pure cysteine HCl cannot be due to traces of iron, since such oxidation would require the presence of considerably more iron than was detected on analysis. In the case of the cysteine prepared by the author, such oxidation would require the

Table VII

Cysteine Preparation	Average O_2 Uptake/10 mg. Pure RSH.HCl	Increase O_2 Uptake per .0001 mg. Fe	Fe required in 10 mg. RSH.HCl to account of residual oxidation	Fe present as determined by KCNS
Sakuma	1.22	2.6	.000,047 mg.	-
Harrison	3.47	6.4	.000,054 mg.	.000,010 mg.
Gerwe	2.20	5.2	.000,044 mg.	less than .000,001 mg.

presence of more than 44 times as much iron as could possibly be present.

The action of cyanide on respiration and cysteine oxidation.

Claude Bernard (58) in 1857 investigated the physiological effect of cyanide and the succeeding contribution of Gaethgens (59) (1868) and Geppert (60) (1889) and many others indicate that in some way or other exposure of living organisms to adequate concentrations of cyanide is followed by a reduction in the O_2 consumption and CO_2 production. Cyanide is therefore considered as a respiratory poison. The fact that cyanide inhibits cellular respiration is believed to indicate that this poison interferes with the normal oxidation-reduction phenomena and it is for this reason that Warburg (34), Wieland (61), and Thunberg (62) have made extensive use of the cyanide reaction in order to support their theiories of biological oxidation-reduction. An exhaustive review of the pharmacology of cyanide was given by Hunt (63) in 1923.

Warburg believes that the cyanides inhibit respiration by combining with the iron which, in combination with cysteine, he believes to be the respiratory enzyme responsible for cellular oxidations. He concludes that intracellular iron combines in some unknown chemical way with the cyanide.

Voegtlin, opposing this theory, states that Warburg failed to perform the crucial experiment, i.e., he did not demonstrate that the inhibition of respiration produced by threshold concentrations of cyanide can be overcome by equivalent amounts of iron salts. This same author found the M.L.D. of cyanide required to cause the death of rats to be 2 cc. of a .1 molar NaCN. Previous injections of various iron salts failed to antagonize the action of cyanide. He did find, however, that either cystine, cysteine or glutathine did antagonize this action if the ratio of S : NaCN stood in the proportion of 5:1.

From these results he concludes that the inhibiting action of the cyanides on respiration is due not to a conversion of intracellular iron to an inactive complex substance but rather to a disturbance in the equilibrium between reduced and oxidized sulfur compounds as represented in the following,

$$R - S - S - R \rightleftharpoons 2R - SH$$

He believes as was suggested by Professor Mathews, that the toxic action of cyanides is due to a chemical reaction which takes place between the cyanide and the cystine sulfur of tissues. Making use of polarimetric measurements on a solution of cystine and varying amounts of cyanide, he found that in such solutions the cystine was reduced to cysteine as indicated by specific rotations of small negative values. Then on aeration the specific

rotation again reached a high negative figure which indicated complete reconversion to cystine. Hence he assumes that the reaction taking place is that of reduction of cystine to cysteine and the subsequent conversion to the oxidized form by aeration. The possibility that NaCN is oxidized to NaCNS is practically ruled out, according to his evidence, as such a change would involve the splitting off of S from the cystine. The aeration experiments indicate that the sulphur is not split off.

He believes that the cyanide is converted into the cyanate according to the following equation,

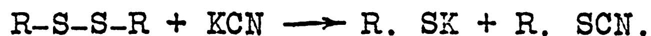
$$R\text{-}S\text{-}S\text{-}R + H_2O + NaCN \longrightarrow NaCNO + 2RSH,$$

which does not involve the participation of molecular O_2 and is therefore applicable to anaerobic oxidation.

However, much evidence is supplied by Bodansky and Levy (65) and others confirming much earlier work to show that cyanide and nitriles are converted into thiocyanate in the body. The former (66) has shown recently that in alkaline solutions cystine converts cyanide into the thiocyanate in vitro to the extent of as much as 60%. But in neutral solution the reaction occurs only to a slight extent. Testing such solutions by a special method he failed to detect the slightest amount of NaCNO. It was also shown that the oral administration of KCN produced a marked

increase of the thiocyanate content of the saliva, which he believes argues for the physiological conversion of cyanides into thiocyanate.

Using the procedure of Mauthner (41) he isolated from alkaline cyanide-cystine mixtures a substance which on analysis proved to be alpha amino beta sulfocyano-propionic acid. This result suggests that at least a portion of the thiocyanate is in organic combination as the compound mentioned above. This conversion is expressed thus,



$$R{-}S{-}S{-}R + KCN \longrightarrow R.\,SK + R.\,SCN.$$

thus confirming Pulewka and Winzer (67) who report that in the reaction between cystine and cyanide, a molecule of cysteine is formed for each molecule of cystine, as determined by iodometric titration.

Dixon and Elliot (68) attempting to determine the extent of cyanide inhibition on the oxygen uptake of various tissues found the average maximum inhibition reached to be about 60%. In no case was the respiration completely inhibited by cyanides but in some tissues and especially in yeast, the inhibition reached 90% of the normal rate of oxygen uptake. In the majority of cases M/1000 concentration of cyanide was sufficient to produce the maximum degree of inhibition obtainable.

From these results they conclude that the respiration of animal tissues is made up of two parts. One, accounting for about 2/3 of the total, is due to systems poisoned

by cyanides; the other one-third is due to systems which are stable to cyanides. The same authors believe that cyanide-stable systems such as the xanthine oxidase may contribute to the cyanide-stable part of respiration. However, xanthine oxidase cannot account for the whole of this part, since Morgan (69) has shown that it is absolutely absent from muscle, yet it is likely that there are a number of other systems of this type.

Altho it has been shown that cyanide inhibits cysteine oxidation in much the same manner as it does tissue oxidation, yet in no case, as far as is known by the author, has the inhibition been complete. Even tho it has been shown by Sakuma, Harrison and the author that pure cysteine oxidizes with extreme slowness when compared to that catalized by metals, yet there is no proof that cysteine is not autoxidizable.

Since cyanide is such a powerful inhibitor of iron catalysis it would certainly seem reasonable to believe that if the residual oxidation were due to iron, then cyanide should completely inhibit oxidation. If there are present in a 20 mg. sample of cysteine HCl about .00004 mg. Fe, as was shown in a previous section to be necessary to cause this residual oxidation if we are to assume that iron is the active substance, then if the solution contained cyanide to the extent of M/1000 concentration, there would

be present 1,000,000 molecules of cyanide for every molecule of iron.

Harrison, who obtained oxidation rates for his pure cysteine hydrochloride as low as 2.32 cmm. O_2/hr./10 mg. RSH.HCl, was able to inhibit oxidation only to the extent of a 1.86 cmm. O_2/hr. uptake in one case, and a 2.94 cmm. O_2/hr. uptake in another. In both experiments cyanide was present in M/1000 concentration.

(Insert Table VIII)

These results furnish definite proof that cyanides do not inhibit the oxidation of pure cysteine, contrary to results of Abderhalden and Wertheimer who prepared a pure cysteine the oxidation of which was still greatly inhibited by cyanides. The discrepancy between their results and mine is noteworthy. It is possible that their samples may have contained traces of iron as impurity. If so it would account for the inhibition these obtained.

That the oxygen uptake in my preparation could not be due to the oxidation of HCN to HCNO by atmospheric oxidation was ruled out by the results of a previous experiment in which it was shown that a pure M/100 HCN solution took up absolutely no measurable quantity of O_2 as measured by the Barcroft apparatus.

In order to show that the cyanide would inhibit the oxidation if it were due to iron, the following experiments

Table VIII

Effect of Cyanide on Oxidation of Cysteine.

pH	Temperature	Oxygen uptake of 10 mg. RSH.HCl in cmm. O_2/hr.	HCN added to make final concentration	Uptake after addition of cyanide
7.4	22. °	3.20 cmm. O_2	M/1000	2.80 cmm. O_2
7.6	22.5°	3.25 cmm. O_2	M/200	2.41 cmm. O_2
7.4	24.0°	2.80 cmm. O_2	M/25	3.00 cmm. O_2
7.6	23.0°	-	M/200	2.20 cmm. O_2
7.6	22.5°	-	M/100	2.30 cmm. O_2
7.4	24. °	-	M/1000	2.00 cmm. O_2
7.6	25. °	-	M/100	3.10 cmm. O_2

were performed which are summarized in Table IX. The up-takes after cyanide addition to the iron containing samples are just the same as in pure cysteine.

(Insert Table IX)

That the cyanide in solution is relatively stable for a fairly long period of time was shown in an experiment in which the original uptake was 1.8 cmm. O_2/hr./10 mg. RSH.HCl. Enought HCN solution was then added to make the final concentration M/1000 . The oxidation continued at approximately the same rate for ten hours, after which .001 mg. Fe was added and readings again taken. The added iron failed to increase the oxidation showing that the added iron was immediately converted into an iron-cyanide complex in which form it could not act catalytically.

In conclusion, pure cysteine oxidizes at approximately the same rate in the presence of varying amounts of cyanides as in its absence. Cyanides do not inhibit completely the oxidation even in the presence of iron and reduce the rate of uptake only to the extent of that catalyzed by iron. Cysteine, catalyzed by iron and oxidizing at a high rate, takes up oxygen only at a rate equal to that of cysteine alone after cyanide is added. From these experiments it is evident that cyanide acts by converting iron into a non catalytic form and exerts

Table IX

<u>Effect of Cyanides on Iron Catalysis</u>.

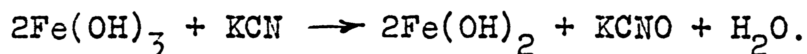
O_2 Uptake of 10 mg. RSH.HCl	Fe added as $FeCl_2$	O_2 Uptake	HCN added to make sol.	O_2 Uptake
2.5 cmm. O_2	.0010 mg.	57.0 cmm. O_2	M/100	1.9 cmm. O_2
3.2 cmm. O_2	.0010 mg.	50.3 cmm. O_2	M/100	3.0 cmm. O_2
1.9 cmm. O_2	.0005 mg.	21.2 cmm. O_2	M/1000	2.2 cmm. O_2

no influence on pure cysteine.

The action of cyanides on iron solutions.

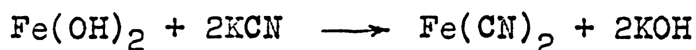
Since iron in a cysteine solution is so rapidly converted into a complex compound in which form it no longer exerts any catalytic effect, a study was made of the nature of the formation of a complex iron cyanide compound. When KCN is added to a ferrous ammonium sulfate solution a dirty blue precipitate forms which on standing settles to the bottom of the flask. This consists of ferrous hydroxide which partially oxidizes to the ferric state. The supernatant solution on neutralization and the subsequent addition of ferric chloride gives a heavy blue precipitate of the well known Prussian Blue, indicating the formation of $K_4Fe(CN)_6$. When ferric chloride is added to KCN there is formed a heavy brown precipitate consisting of ferric hydroxide. The yellow supernatant liquid when tested with a ferrous salt gives the typical Turnbull's blue which confirms the presence of $K_3Fe(CN)_6$. However, when a ferric salt instead is added to this same solution a heavy blue precipitate of Prussian Blue is formed. This means that in addition to $K_3Fe(CN)_6$ there is also $K_4Fe(CN)_6$ present. It is very improbable that KCN can reduce $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$. The formation of the $K_4Fe(CN)_6$ is no doubt due to an interaction between the cyanide and some $Fe(OH)_2$

formed as a result of a reduction of $Fe(OH)_3$ by KCN according to the following equation.

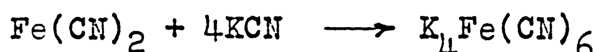


$$2Fe(OH)_3 + KCN \longrightarrow 2Fe(OH)_2 + KCNO + H_2O.$$

Then the $Fe(OH)_2$ combines with KCN to form $K_4Fe(CN)_6$ according to the reactions,



$$Fe(OH)_2 + 2KCN \longrightarrow Fe(CN)_2 + 2KOH$$



$$Fe(CN)_2 + 4KCN \longrightarrow K_4Fe(CN)_6$$

From the results of these reactions, which take place also at neutral reaction it is quite evident that this complex iron cyanide compound which Warburg assumes to be formed may in reality be either $K_4Fe(CN)_6$ or a combination of $K_4Fe(CN)_6$ and cysteine. If the iron is assumed to be bound up in this manner then it must be shown that iron added in the form of the complex cyanide does not catalyze cysteine oxidation.

When .001 mg. iron is added to cysteine in the form of either $K_3Fe(CN)_6$ or $K_4Fe(CN)_6$ the oxidation rate is only slightly greater than that of cysteine alone averaging 8.6 cmm. O_2/per hour whereas cysteine oxidizes at the average rate of 2.2 cmm. O_2/per hour. An equivalent amount of iron added in ionic form as $FeCl_3$ caused an increased rate of 50 - 60 cmm. O_2/hour. The slight increase over the oxidation of cysteine alone may be accounted for by the traces of iron which the complex cyanide contained.

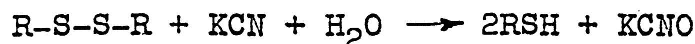
It is quite evident from these experiments that the iron in the complex cyanide cannot function as a catalyst.

If ferrous or ferric salt is added to a neutral cysteine solution containing KCN, $K_4Fe(CN)_6$ is formed as detected by the addition of a small amount of ferric salt the characteristic Prussian Blue being formed. If ferric salt is added to a neutral cysteine solution it is immediately reduced to the ferrous state. A consideration of the ease with which iron is converted into the complex cyanide on the addition of KCN both in the presence and absence of cysteine, points to the possibility that this reaction may represent the inhibiting action of cyanides on iron catalysis. The inability of $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ to act catalytically on the cysteine oxidation lends confirmation to this possibility.

The effect of cystine on the autoxidation of cysteine.

As had been mentioned in a previous section Abderhalden and Wertheimer (39) observed a definite inhibiting action of cyanides on the oxidation of their samples of cysteine·HCl which they believed to be iron free. They attributed this inhibiting effect to the action of cyanides on cystine rather than on any trace of iron which might have been present. Dixon and Tunnicliffe (41) claim that cystine greatly accelerates the oxidation of cysteine by

atmospheric oxygen, and that the maximum reactibility is reached when a ratio of 2.5 parts of the disulfide to one part of cysteine exists. Hence, Abderhalden and Wertheimer attribute the slight oxidizability of the cysteine to a contamination of cystine which they believed to be present in their samples. Since cyanide is able to reduce cystine to cysteine, the inhibition of the oxidation of cysteine by cyanide is due, according to them, to the removal of the catalytic cystine thus,



$$R\text{-}S\text{-}S\text{-}R + KCN + H_2O \longrightarrow 2RSH + KCNO$$

However no such action of cystine could be observed in my experiments. Pure iron free cystine hydrochloride when added to pure cysteine hydrochloride failed to increase the oxidation rates. An increased oxidation rate due to the addition of cystine could be explained only by assuming that the added cystine contained traces of iron. If cystine had such catalytic powers, then, in every experiment in which measurements were taken over long periods of time, one would observe a steady increase in oxidation rates as the conversion of cysteine to cystine proceeded, the rate reaching a maximum when 60% of the cysteine had been oxidized, then 2.5 parts of cystine would be present for every part of cysteine.

Instead of an increase in rate as would be the case if cystine were acting catalytically, one observes a

constant rate of oxidation and the uptake of the oxygen when plotted against time is a straight line over a considerable period of time as may be seen in Figure I, the rate decreasing only toward the very end of the experiment.

Summary

1. Cysteine hydrochloride has been prepared in such an exceedingly high state of purity that two grams of the substance when ignited and tested by the KCNS method contained no detectable amount of iron. The method detects .000,000,1 gm. Fe. Hence these two grams contained certainly less than this figure. We believe it to be essentially free from iron.
2. Such highly purified cysteine still possessed the power to oxidize at a considerable rate which was of the same order as that reported by Sakuma and by Harrison. Cysteine is hence autooxidizable. This is contrary to Warburg's conclusion.
3. The autoxidation of the cysteine is not inhibited by cyanides. This confirms Warburg's theory of the action of cyanides.
4. The addition of iron greatly accelerated the speed of oxidation, the rate of increase being directly proportional to the amount of iron added. The subsequent addition of KCN to this catalyzed oxidation reduced

the rate to that of cysteine alone.

5. The traces of iron, which may be present and still escape detection due to the limit of sensitivity of the test employed, are insufficient to account for the residual rate of oxygen uptake, which would require at least 44 times as much iron as the maximum amount which could possibly be present
6. Potassium cyanide readily converts ferric or ferrous chloride to the corresponding potassium iron cyanide compounds. Cyanides inhibit iron catalysis of cysteine oxidation by converting the iron into the ferrocyanide an iron cyanide complex which does not form ferrous ions.
7. Neither potassium ferrocyanide nor potassium ferricyanide is able to catalyze cysteine oxidation.
8. Cysteine oxidation is not catalyzed by the addition of cystine, provided the latter be free of iron.

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