A STUDY OF PYRROLES IN BIOLOGICAL MATERIALS.

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I. A COLORIMETRIC METHOD FOR THE DETERMINATION OF PYRROLE

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by

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A Thesis

Presented to the Faculty of Graduate Studies and Research of McGill University, Montreal, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

September, 1938.

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GENERAL INTRODUCTION

In the gradual unravelling of the complexities of biological materials, it has become apparent that the pyrrole group of chemical compounds plays a prominent role in the production of plant and animal organisms. This family of compounds embraces all those molecules which contain a ring built up of four carbon atoms and a nitrogen atom (I).

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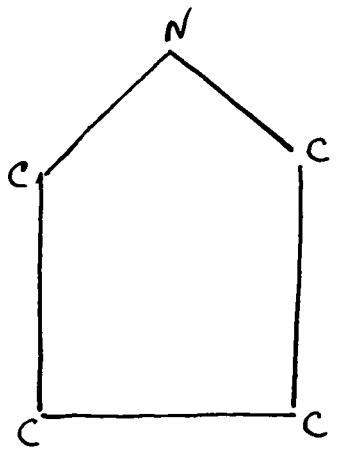

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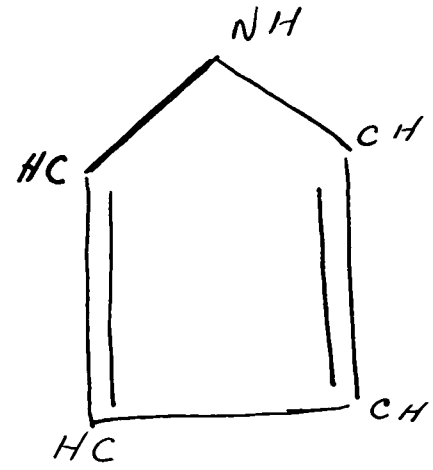


The presence of this ring has been established in a series of important vegetable bases, which had previously been regarded solely as derivatives of the six-membered ring pyridine; namely nicotine, hygrine, cuskhygrine, atropine, hyoscyamine, cocaine, tropacocaine, and others. Further, the reduced pyrrole derivative proline (pyrrolidine-2-carboxylic acid) is a hydrolytic product of nearly all proteins. Willstätter and other investigators have demonstrated that haemoglobin and chlorophyll are pyrrole derivatives, revealing an interesting relationship between the colouring matter of blood and of leaves.

The above examples provide sufficient illustration of the importance of pyrrole derivatives.

Pyrrole itself, the parent substance of this class, is found in coal-tar and bone-tar, and is also present among the distillation products of bituminous shale. Baeyer was the first to advance the formula (II) now generally accepted for pyrrole.

Pyrrole is a colorless liquid which turns brown on exposure to the air and smells somewhat like chloroform. It boils at 130° to 131°C under 761 mm. and has a specific gravity of 0.9752 at 12.5°C. It is miscible with most organic liquids, but is only slightly soluble in water.

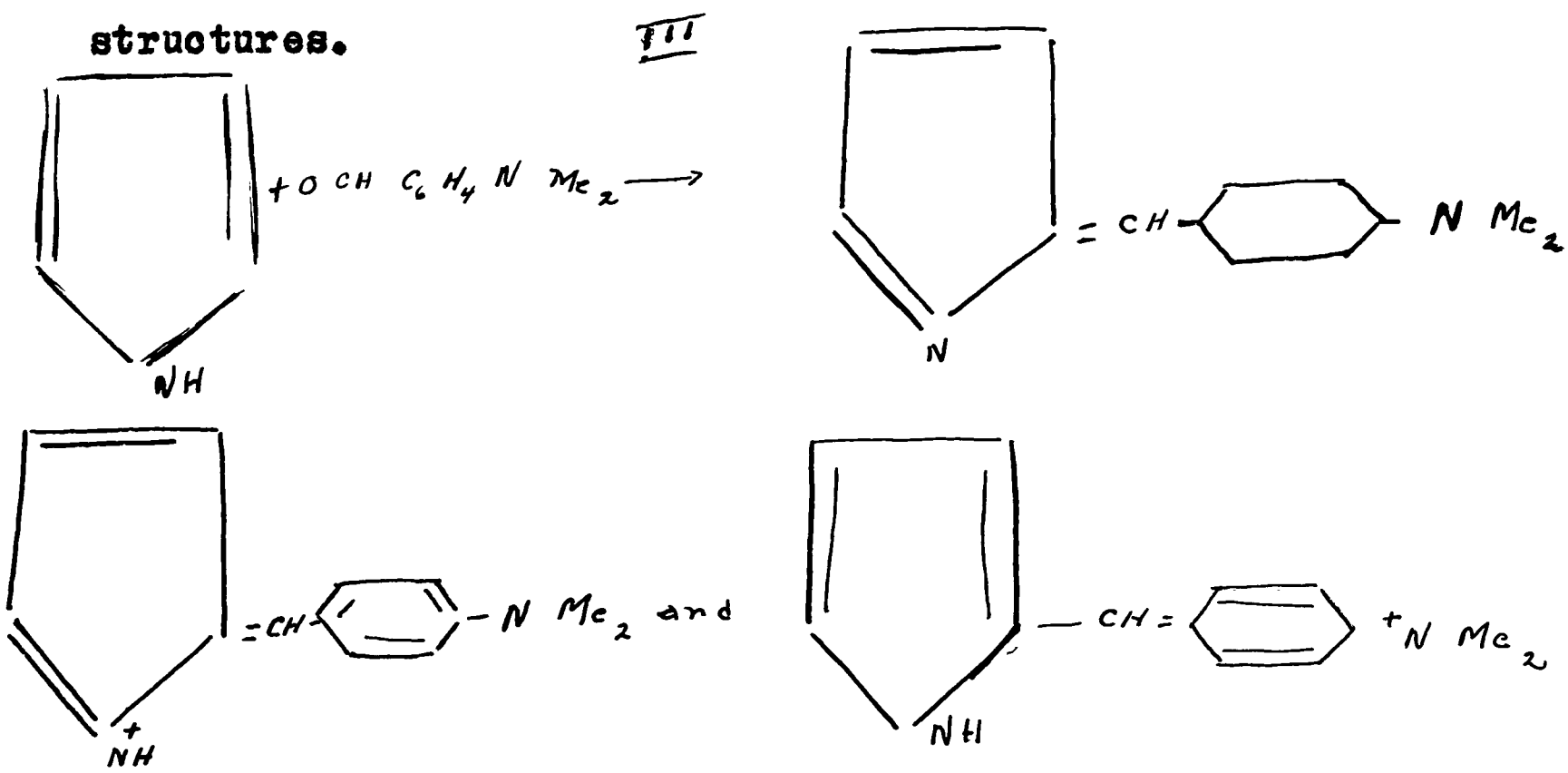
The formula for pyrrole would suggest that it is a secondary base, but its basic properties are extremely weak. They are to some extent concealed by the fact that pyrrole and most of its homologues are readily converted by acids into complicated red polymers, the so-called pyrrole-red.

Pyrroles relative to benzene are distinctly aromatic compounds for they possess the nuclear stability and diminished unsaturation of such compounds. However, unlike thiophene, which resembles benzene to a remarkable degree, pyrrole resembles phenol more than it does benzene.

The homologues of pyrrole show the typical properties of pyrrol itself, but are in general less

reactive and more stable. Those pyrroles which contain a free hydrogen atom in the α position produce an intense red colour in the cold with Ehrlich's reagent, which is a solution of p-dimethylamino-benzaldehyde in dilute hydrochloric acid. This aldehyde condenses with the pyrrole in the α position to give a pyrrolenine compound (III). The red colour is attributed to the salt of this condensation product. The cation of this salt is similar to that of a triphenylmethane dye in that two structures can be written, in one of which the pyrrole ring is quinonoid and in the other the benzene ring is quinonoid. There is no difference in the structures except in the distribution of the electrons, and we find the same intense absorption due to the resonance between the two structures.

(III)



Pyrroles with only a β-position free and tetra-substituted pyrroles scarcely give the reaction, even on heating, unless an easily eliminated group such as

carbethoxyl or carboxyl is present in the ∝ position.

It is not within the scope of this thesis to review the vast body of literature concerning the chemistry of pyrrole and its numerous derivatives. The brief summary of some of the more important facts about pyrrole and its homologues, as given above, will suffice to indicate the importance of this compound in nature. It is evident that any advance in our knowledge concerning pyrroles in biological materials would doubtless be of great assistance in the solving of many of the larger unsolved problems in bio-chemistry.

For many years qualitative colour tests have been known for pyrrole, but there appears to be a lack of quantitative methods for the detection of minute quantities of pyrrole in biological materials. It was deemed important to have quantitative methods available for the determination of pyrrole and therefore the preliminary part of the investigation described in this thesis had to do with the evolution of such methods for the quantitative estimation of small quantities of pyrrole.

It has been known for some time that pyrrole could be produced from biological materials by the process of dry distillation. In particular, certain proteins were capable of furnishing pyrrole by this distillation method and this fact seemed to lend some support to

Troensegaard's (1920) pyrrole hypothesis concerning the structure of proteins. In this thesis, observations are reported which fail to lend support to Troensegaard's theory of protein structure.

Certain investigators have reported the presence of pyrrole in protein hydrolysates produced by enzymic digestion. Attempts were made here to verify this, but with the methods available it was found impossible to detect free pyrrole in protein hydrolysates.

For many years it has been known that gelatin produced pyrrole on dry distillation, but the reason for this phenomenon had not been discovered. Experiments conducted in this investigation indicate that the amino acids proline and hydroxy-proline are responsible for the production of pyrrole on the dry distillation of gelatin.

The hydrolysates of nearly all proteins contain proline, and the hydrolytic products of a few proteins contain hydroxy-proline. Methods have been published by various investigators for the determination of these two amino acids, but they are laborious and time-consuming. An examination of the literature revealed the lack of rapid and quantitative methods for the estimation of these two important pyrrole derivatives. In the course of the work described in this thesis, colorimetric methods have been evolved for the quantitative determination of these amino acids in protein hydrolysates in the presence of the other decomposition products.

HISTORICAL

The Production of Pyrrole from Biological Materials

Early in the 19th century pyrrole was discovered in coal-tar by Runge (1834), but it was not until some years later that Anderson (1858) isolated it in a pure state from the products of distillation of bone, the so-called bone oil. This is still the chief source of pyrrole and certain of its homologues.

Schützenberger and Bourgeois (1876) prepared pyrrole by heating albumin and these same workers also obtained it by heating sheep's wool at 150°C with baryta water.

Weidel and Ciamician(1880) collected pyrrole and its homologues from the dry distillation of gelatin. Maly (1885) at a later date also observed that pyrrole could be liberated from proteins by dry distillation. Bernheimer (1881) obtained pyrrole from coffee and in 1909 Lehmann (1909) reported its presence in tobacco smoke.

Recently Visco (1930) found that heating certain amino acids with fused soda produced heterocyclic substances which gave the pyrrole reaction. The amino acids of the aromatic series did not undergo this transformation.

Later Fromm (1935) dry distilled gelatin and determined the amount of pyrrole in the distillate by the use of a step photometer. He condensed pyrrole with

isatin in acid solution to obtain pyrrole-blue, and from measurements of the intensity of this colour he calculated the percentage of pyrrole in the sample of gelatin.

Fromm (1937) has applied his method to the determination of pyrroles in tobacco smoke.

From an examination of the hydrolysis products of gluten, Pieroni (1932) concluded that pyrroles were present as shown by their reaction with p-bromophenyl-azoxycarboxamide and with the diazonium salt of anthranilic acid. He found that dry distillation left a graphitic residue which was pyrrolic in character and that the distillate contained pyrroles as well as pyridine.

Roncato (1933) reported the presence of pyrrole nuclei in the tryptic hydrolysate of gliadin. He found that when corn meal or gliadin was subjected to peptic digestion alone, or to tryptic digestion preceded by peptic digestion, a colorless or slightly yellow hydrolysate was obtained. On the other hand, the hydrolysate from tryptic digestion which was not preceded by peptic digestion turned brownish at the end of the digestion and days later gradually turned black. Tyrosinase was not detected in the gliadin. A positive test for pyrrole nuclei was obtained when the tryptic hydrolysate was treated with certain diazonium salts. It is interesting to note that he was unable to secure a positive test for the pyrrole nuclei in the case of the hydrolysates of other proteins. Experiments were carried out by the author in which

gelatin and gliadin were digested by trypsin, pepsin and papain and in every case the hydrolysate failed to give a positive test for pyrrole with Ehrlich's reagent or with isatin in the presence of acid. Roncato (1933) also reported the use of p-bromophenylazoxyformamide for the detection of the pyrrole nuclei in the tryptic hydrolysate of gliadin.

Some years ago Troensegaard (1920) advanced a theory that the protein molecule is composed chiefly of heterocyclic compounds, and he has made various attempts to prove it by demonstrating the presence of pyrrole substances in gliadin and gelatin. To avoid the decomposition of the pyrrole compounds he did not use solvents which contained water, and in addition he stabilized the pyrrole groups by introducing an acid radical.

Bernardi and Schwarz (1932) failed to obtain even traces of crystalline pyrrolic compounds which were mentioned by Troensegaard in his papers. This failure to duplicate Troensegaard 's work was attributed by these investigators to the omission of potassium hydroxide and the sodium reduction treatments. Troensegaard found that much higher yields of pyrrolic compounds were obtained by treatment with potassium hydroxide and methyl alcohol. Bernardi and Schwarz concluded that the presence of pyrroles in the acetylated products from proteins is due to secondary reactions which vary with the experimental conditions.

Qualitative and Quantitative Detection of Pyrrole

The pine-splinter test. The name pyrrole is derived from a Greek word which means "fiery red," because a pine shaving moistened with hydrochloric acid develops a red colour when exposed to pyrrole vapour. This reaction is also shown by many derivatives of pyrrole. Recently Reichstein (1932) has used the pine-splinter test for furan compounds. He has observed that mono-substituted furans give a green colour but that the highly methylated furans give a red colour similar to that given by pyrrole compounds. The test for the pyrrole ring with Ehrlich's reagent was also given by highly methylated furans.

Ehrlich's Reaction. Ehrlich (1901) observed that when p-dimethylamino-benzaldehyde was added to certain pathological urine an intense red colour was produced. This reaction applies to all compounds which have a free hydrogen atom in the α position. Tetra-substituted pyrroles fail to give the test except where we have a labile group such as an ester or carboxyl group on the carbon atom in the α position. This reaction was used by Fischer and Meyer-Betz (1911) as a qualitative test for pyrrole. Later Salkowski (1920) found that when a 1:4000 pyrrole solution is treated with a 2% solution of p-dimethylamino-benzaldehyde in normal hydrochloric acid the somewhat cloudy mixture becomes rose-coloured, changes to violet on heating and finally clears. If the solution was first warmed, he observed that the violet colour appeared at

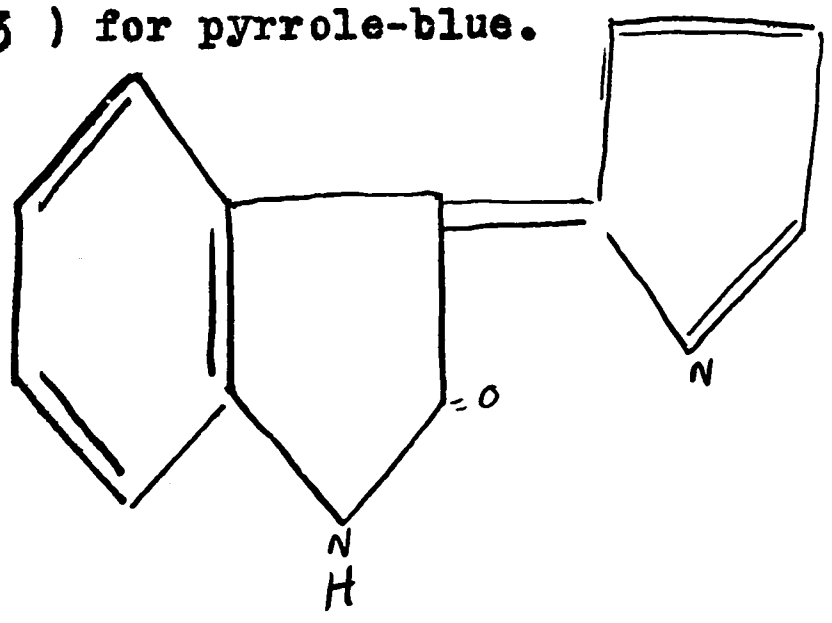
once. It was also discovered that the colour might be intensified by warming with a few drops of hydrochloric acid. It was found to be bleached by an excess of the fuming acid. Similar reactions were given by an indole solution 1:10,000.

Urk (1929) published a paper on the use of p-dimethylamino-benzaldehyde as a reagent for organic drugs. A large number (135) of organic substances, mostly medicinal products, were tested for colour reactions with this reagent. He observed that pyrrole and indole derivatives react with this reagent but pyridine derivatives do not.

Pyrrole-Blue. Meyer (1883) found that when pyrrole was condensed with isatin a blue colour was produced. This colour reaction was used by Ciamician and Silber (1884) and by Meyer and Stadler (1884) as a qualitative test for pyrrole. Apparently it was not used as a quantitative method for the determination of pyrrole until Fromm (1935) devised his method.

The following formula has been suggested by Pratesi (1933) for pyrrole-blue.

IV



In the general problem of determining the composition of pyrrole-blue Pratesi found that the N-methyl, N-phenyl, N-ethyl and N-acetylpyrroles do not give any blue product. He observed that 2.3-dimethylpyrrole and 3-methyl-4-ethyl pyrrole both gave blue products, $C_{14}H_{12}ON_2$ and $C_{15}H_{14}ON_2$ respectively with the elimination of water. However, he failed to secure a blue colour with 2.5-dimethyl pyrrole but 2.4-dimethyl-3-ethylpyrrole gave a blue product which has the formula $C_{16}H_{16}ON_2$.

Recently Steinkopf and Wilhelm (1937) have determined the molecular weight of pyrrole-blue. They condensed pyrrole with ethyl isatin-1-acetate and obtained ethyl pyrrole-blue acetate. This blue product had a melting point of 350°C and according to molecular weight determinations in various solvents has the formula $C_{33}H_{28}O_6N_4$. They concluded that the constitution assigned to pyrrole-blue by Pratesi (1933) cannot be correct. They also pointed out that other proposed formulae fail to account for its intense blue colour.

Fromm (1935) found that pyridine, pyrroline and pyrrolidine gave no colour with isatin under the conditions described by him. This investigator (1937) also showed that tryptophane and indole do not give colour reactions with isatin.

Using a step-photometer Fromm (1937) prepared standard curves for the condensation products of isatin with 2.3-dimethyl pyrrole, with kryptopyrrole and with opsopyrrole.

Selenious Acid. Montignie (1932) developed a colour test for pyrrole and indole compounds. He found that a deep violet colour was produced when an aqueous pyrrole solution is warmed with 8 to 10 drops of a 10% selenious acid solution and 1 ml. of concentrated nitric acid. It is believed that the colour produced in the above test is due to the oxidation of selenious acid to selenic by nitric acid and that the selenic acid in turn oxidizes the pyrrole into two coloured substances. One of these compounds is red and is insoluble in chloroform, whereas the other is blue and is very soluble in chloroform. According to Montignie, 0.00004 grams of pyrrole may be detected by this test.

Levine's Colour Reaction. Levine and Richman (1933) investigated the reaction of antimony trichloride with compounds containing five membered mono-heterocyclic rings. They found that antimony trichloride in chloroform gives characteristic colour reactions with pyrrole, thiophene, furfuran and with more complex compounds containing pyrrole or furfuran configurations. It was observed that the colour reaction is intensified and often modified by the addition of acetic anhydride.

Mercury Compounds of Pyrrole. It was observed by Fischer and Müller (1925) that pyrrole derivatives of the general formula

$$(Pyrrole)_2 - Hg - (HgCl_2)_4$$

are readily obtained in crystal form by treatment of the pyrrole solution in acetic acid with 4% mercuric chloride. Such compounds are decomposed with the regeneration of the original pyrrole when the alcoholic or aqueous suspension is treated with hydrogen sulphide.

An Unidentified Base Among the Hydrolytic Products of Gelatin

Van Slyke and Hiller (1921) and Van Slyke and Robson (1925) have reported the presence in gelatin of a new amino acid which is precipitable by phosphotungstic acid. On the basis of the elementary analysis of the copper salt, a positive test for a pyrrole group, and the ratio of amino to total nitrogen, the product was considered as being probably dihydroxy-pyrrole-alanine. According to the criteria which have been suggested by Vickery and Schmidt (1931) the evidence brought out by Van SIyke and his coworkers is not sufficient to consider this substance as one of the accepted amino acids. Furthermore, Emerson and Schmidt (1934) attempted to isolate dihydroxy-pyrrole-alanine from gelatin hydrolysates without success. In the conclusion of their paper they pointed out that the empirical formula of dihydroxy-pyrrole-alanine differs from that of glycyl-hydroxyproline only by having two more hydrogen atoms. On hydrolysis there should be no increase in amino nitrogen. It is not im-

possible that this peptide may have been mistaken for an apparently new amino acid.

Morse's Qualitative Test for Hydroxy-Proline

Recently Morse (1933) proposed a new colour reaction for hydroxy-proline and suggested that it would be useful in distinguishing the scleroproteins. The test consists of heating gently a mixture of hydroxy-proline, a few drops of a secondary alcohol and 0.5 gm. of sodium peroxide in a test tube. When the contents of the tube are nearly dry it is cooled thoroughly under running water and then 2 ml. of normal hydrochloric acid is added to the material in the tube. Finally the tube is placed in a boiling water bath to develop the characteristic colour, an amber rose. Morse found that of the various amino acids obtained by Dakin (1920) from gelatin, only hydroxy-proline gave a positive test with his procedure as described above. He believed that the hydroxyl group is necessary because proline fails to give the reaction.

Colorimetric Methods of Determining Proline and Hydroxy-Proline

Lang (1933) described a micro-method for the determination of proline and hydroxy-proline. The protein hydrolysate under consideration was treated with sodium hypochlorite. This converted proline into pyrrol-

ine and hydroxy-proline into hydroxy-pyrroline. Steam distillation removed the pyrroline and hydroxy-pyrroline from the reaction mixture and the total amount of these two compounds present in a certain fraction of the distillate was determined by means of the colour reaction with Ehrlich's reagent. Another fraction of the distillate was taken for the determination of hydroxy-pyrroline by condensation with isatin in dilute sulphuric acid. According to Lang the difference between the two determinations gave the amount of pyrroline present in the distillate.

Waldschmidt-Leitz and Akabori (1934) maintained that Lang's method was based on a misapprehension. They stated that hydroxy-proline when treated with sodium hypochlorite gives pyrrole in 80% yield and not hydroxy-pyrroline. Moreover, they claimed that proline does not react with hypochlorite. These investigators applied Lang's method to the detection of hydroxy-proline in protein hydrolysates. They were unable to detect hydroxy-proline in casein and clupein by this method but gelatin gave 9.4 and 8.9%.

EXPERIMENTAL

I. A COLORIMETRIC METHOD FOR THE DETERMINATION OF PYRROLE

Modification of Fromm's Method for the Determination of Pyrrole

An examination of the literature revealed that there were apparently only two quantitative colorimetric reactions for pyrrole, namely, Ehrlich's p-dimethylamino-benzaldehyde reaction and the formation of pyrrole-blue by condensing pyrrole with isatin in acid solution. Since it was known that Ehrlich's reagent reacts with so many organic compounds it was considered best to study the reaction of isatin with pyrrole in the presence of acid.

A study of Fromm's (1935) method for the determination of pyrrole suggested several possible modifications and the first part of this investigation is an attempt to improve this method. A single-cell type of photo-electric colorimeter equipped with light filters, as described by Evelyn (1936), was utilized to measure the intensity of the blue colour which is produced when isatin is condensed with pyrrole in the presence of acid.

Fromm (1935) dissolved freshly distilled pyrrole in 0.5% hydrochloric acid and to aliquots of the pyrrole solution he added 2 ml. of 6N hydrochloric acid and diluted to 10 ml. He then added 1 ml. of a solution of 0.05% isatin in acetic acid and heated this mixture for

10 minutes on a boiling water bath. At the end of that time the solution was cooled to room temperature and the intensity of the colour measured on a step-photometer.

It was pointed out by Fromm (1935) that when the pyrrole was dissolved in 0.5% hydrochloric acid there was a distinct loss of pyrrole after the solution had stood for only two days. Thus, he found that after standing for one day the pyrrole content of a solution of freshly distilled pyrrole in 0.5% hydrochloric acid was 80.3% of the original, and at the end of three days it had decreased to 67.3%. The author found that 0.5% acetic acid was a much better solvent for pyrrole from the standpoint of keeping quality. This was observed both qualitatively and quantitatively. In a few days a solution of pyrrole in 0.5% hydrochloric acid becomes light yellow in colour, whereas when 0.5% acetic acid is used as the solvent the solution is clear at the end of two months. The pyrrole content of a solution of pyrrole in 0.5% acetic acid was determined at intervals for seventy days and decreased only 1.97% at the end of that time.

Experiments were performed to show the effect of the concentration of hydrochloric acid on the formation of pyrrole-blue. The intensity of the colour was measured by the photoelectric colorimeter, using a light filter of a wave length of 660 millimicrons, and the data obtained are given in Table I.

Table I

The Effect of Hydrochloric Acid on the Pyrrole-Isatin Colour

Hydrochloric acid (Normality)	Galvanometer Reading (660 Filter)
0.10	89.00
0.25	75.00
0.50	65.00
1.00	58.00
1.45	52.00
1.70	47.00
2.00	42.00
2.50	37.00
3.00	34.00
3.50	31.00
3.75	30.00
4.15	29.00
4.50	28.00

Examination of these data reveals that the concentration of hydrochloric acid has a marked effect on the production of pyrrole-blue. It is to be noted that a high reading on the galvanometer denotes a low color intensity, whereas a low reading signifies a high color intensity. It was decided to use 6 N hydrochloric acid and this is obtained in practice by the addition of exactly 1 ml. of concentrated hydrochloric acid (12 N) to the one ml. of pyrrole solution under consideration.

By experiment it was found that 0.2 ml. of 0.5% isatin in glacial acetic acid was the most satisfactory amount of this reagent to add to the reaction mixture. More of this reagent might increase the intensity of the pyrrole-blue but it would give too high a reading for the blank determination on the galvanometer.

Fromm (1935) brought about condensation of the isatin with pyrrole by heating on a boiling water bath for 10 minutes. It was found that under the conditions used here this heating could be eliminated and the maximum intensity of colour was developed by standing at room temperature for five minutes. This fact was established by the following experiment. Four 1 ml. aliquots of a freshly prepared pyrrole solution in 0.5% acetic acid were placed in 10 ml. glass-stoppered graduated cylinders. To each of these was added 0.2 ml. of 0.05% isatin and 1 ml. of concentrated hydrochloric acid. They were allowed to stand at room temperature for 5, 10, 15 and 20 minutes, respectively, before dilution with 95% alcohol. The observations are recorded in Table II.

Table II

The Effect of Time of Standing on the Pyrrole-Isatin Colour

Time of Standing (minutes)	Galvanometer Reading (660 filter)
5	31.25
10	32.50
15	32.00
20	32.50

After the reaction mixture has stood for 5 minutes it is diluted to 10 ml., mixed thoroughly and placed in a colorimeter tube. It was found by experiment that

dilution with 95% ethyl alcohol produces a more intense blue colour than dilution with water.

It was also observed that the blue colour was quite stable after dilution with alcohol. Even after two hours from the time of adding the alcohol the intensity of the colour as measured by the colorimeter was almost the same as when it was first produced.

Finally, Fromm's (1935) method for the determination of pyrrole was modified to the following: transfer 1 ml. of the pyrrole solution, containing 5 to 30 micrograms of pyrrole per ml., to a 10 ml. glass-stoppered graduated cylinder and add 0.2 ml. of 0.05% solution of isatin in glacial acetic acid and 1 ml. of concentrated hydrochloric acid. After five minutes dilute to 10 ml. with 95% alcohol. The pyrrole-blue colour is measured with an Evelyn photoelectric colorimeter, using a 660 light filter.

The above procedure was applied to several pyrrole derivatives which were available. Indole, 2-carbethoxy-pyrrole and 1.2-dicarbethoxy pyrrolidine gave a negative test, whereas 1.carbethoxypyrrole gave pyrrole-blue colour.

Preparation of the Calibration Curve for the Isatin-Pyrrole--Blue Colour

0.3063 gram of freshly distilled pyrrole was diluted to 2 litres with 0.5% acetic acid. Aliquots of this solution were diluted so as to give a number of pyrrole solutions ranging in concentration from 8 to 46 micrograms

of pyrrole per ml. In all cases sufficient glacial acetic acid was added before dilution with water to make a 10% acetic acid solution. The pyrrole-blue colours were measured according to the above procedure. The data obtained are recorded in Table III. From these data a calibration curve was prepared, which is shown in Figure I.

To compensate for any colour due to reagents, a blank determination was performed using 1 ml. of distilled water instead of 1 ml. of pyrrole solution, and the usual method was followed. The blank was placed in the colorimeter and the rheostat was so adjusted that the glavanometer showed a reading of 100, the 660 filter being used. On removal of the blank the galvanometer reading was found to be 62. The galvanometer was always adjusted to this reading before measurements of the pyrrole-blue colour intensity were made. This procedure was used to avoid preparation of a blank at too frequent intervals.

Examination of Table III shows that the isatin-pyrrole reaction conforms to Beer 's law. This law can be expressed mathematically as follows:-

$$\frac{2-\log G}{C} = K$$

where C is the concentration, G is the galvanometer reading (tables of 2-log G are provided with the instrument), and K is a constant. By calculation the mean value of K was found to be 0.0321 $\pm$ 0.0010, with a maximum variation of 0.0031. From the straight line relation between pyrrole concentration and 2-log G, as shown in Figure I, it is evident that K is constant over the

range plotted.

Table III

To Show that Beer's Law Applies to the Pyrrole-Blue Colour

C Concentration of Pyrrole (γ per ml.)	G Galvanometer Reading (660 Filter)	L $L = 2-\text{Log } G$	K $\frac{2-\text{Log } G}{C} = K$
7.66	60.00	0.2218	0.0290
11.49	44.00	0.3570	0.0311
15.32	32.00	0.4950	0.0323
19.15	22.50	0.6480	0.0338
22.97	17.75	0.7510	0.0327
26.80	13.00	0.8860	0.0331
30.64	10.00	1.0000	0.0326
34.47	7.50	1.1260	0.0327
38.30	6.00	1.2220	0.0319
42.13	4.00	1.3980	0.0332
45.96	3.75	1.4260	0.0310
Mean Value of K = 0.0321 ± 0.0010			

When the intensity of pyrrole-blue colour is determined by the deflection shown on the galvanometer, the concentration of pyrrole may be obtained by reference to the calibration curve as shown in Figure I, or, since the reaction conforms to Beer's Law, it may be calculated from the following relation:

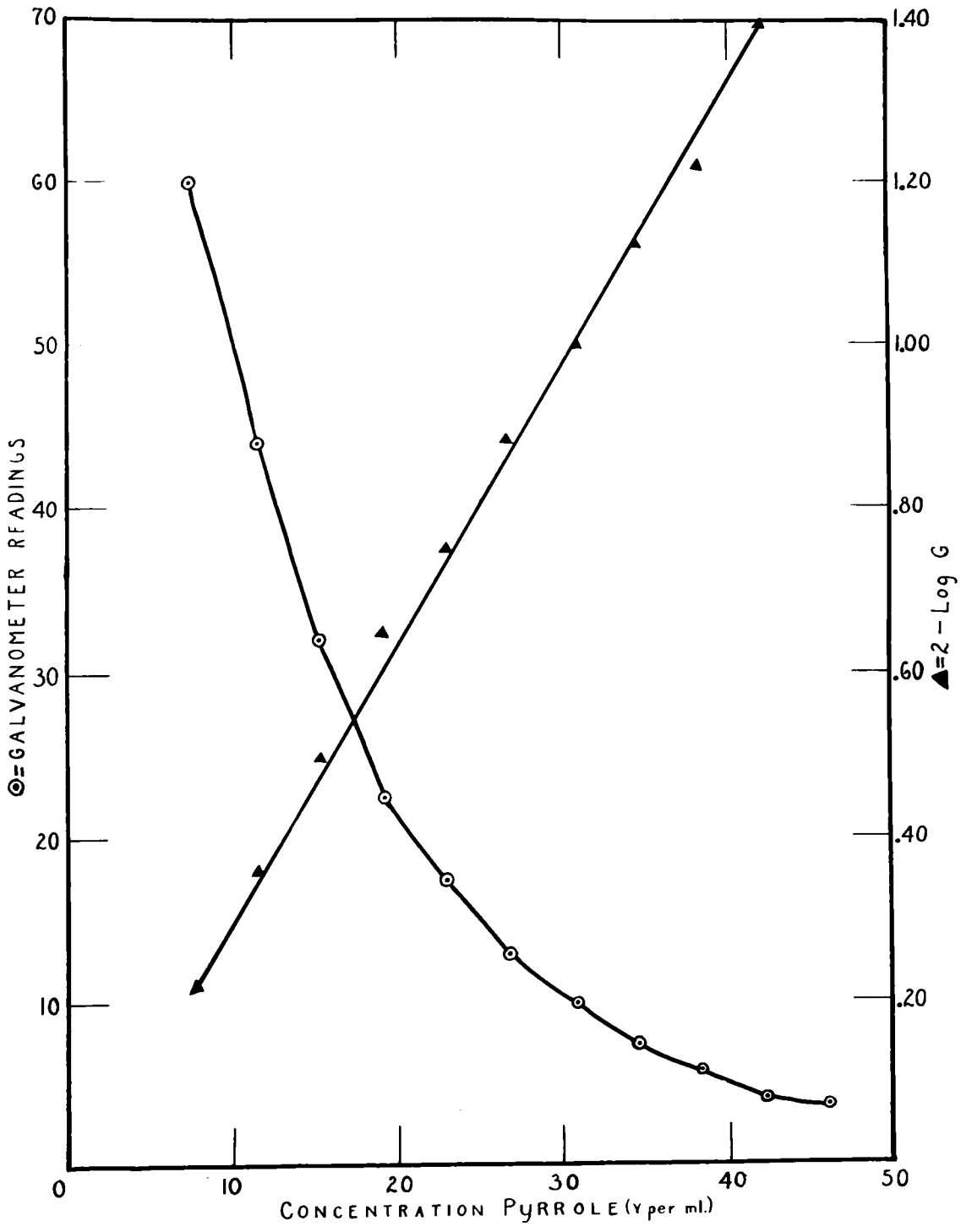
$$C = \frac{2-\log G}{K}$$

The Determination of Small Amounts of Pyrrole by Precipitation with Mercuric Chloride

In the search for pyrrole in biological materials it was found necessary to have available various

Figure 1

Calibration Curve for Pyrrole



means of extracting small quantities of pyrrole from certain mixtures. For example, when a protein hydrolysate or a distillate from the dry distillation of a protein was under consideration, it was found that coloured impurities often interfered with the accurate colorimetric determination of pyrrole. Pyrrole can be precipitated from solution by mercuric chloride, and this fact has been utilized in the quantitative estimation of pyrrole.

Fromm (1935) reported the determination of quantities as small as 1.41 mg. of pyrrole per ml. by means of precipitation with mercuric chloride. He washed the precipitate with alcohol several times and dissolved it by warming with 1 ml. of a solution of 5% sodium cyanide. The solution was made up to 100 ml. and the colorimetry performed on an aliquot.

A study has been made of the use of mercuric chloride as a precipitating agent for pyrrole from solution, and it has been shown that very small quantities of pyrrole may be determined by this method.

Experiments were next performed to study the effect of various washing agents. Thus, 1 ml. of a pyrrole solution was placed in each of six centrifuge tubes and 2 ml. of a saturated mercuric chloride solution was added to each of them. They were allowed to stand for 15 minutes and then centrifuged, after which the supernatant liquid was poured off carefully.

Table IV

To Show the Effects of Various Washing Agents on the Pyrrole-Mercuric Chloride Precipitate

Experiment No.	Washing Agent Used	Galvanometer Reading (620 filter)	Loss of Pyrrole %
1	No washing	70.00	0.0
2	Distilled water	72.00	7.7
3	Ethyl alcohol	97.00	85.0
4	Ethyl alcohol (95%) + mercuric chloride (1%)	80.00	38.5
5	Methyl alcohol (pure)	96.00	80.0
6	Methyl alcohol + mercuric chloride (1%)	91.00	70.0

Each precipitate was treated twice with 5 ml. portions of different washing agents. The precipitates were in every case dissolved by warming on a boiling water bath with 1 ml. of a 5% solution of sodium cyanide. They were then cooled and made up to 10 ml. with distilled water. 1 ml. was taken for the isatin colour reaction. The data obtained are shown in Table IV.

It is evident that washing with alcohol is unsatisfactory as 85% of the pyrrole was lost when 95% ethyl alcohol was used and 80% when pure methyl alcohol was the washing liquid. When a 1 % solution of mercuric chloride in ethyl alcohol was used the loss of pyrrole

was much less, namely 38.5%, but with a 1% solution of mercuric chloride in methyl alcohol the loss was 70%. It is difficult to see how Fromm (1935) was able to secure such a high recovery of pyrrole by this precipitation method when he used alcohol as the washing agent. He reported as high as 98.5% recovery of pyrrole.

Experiments were carried out in which the pyrrole-mercuric chloride complex was decomposed with hydrogen sulphide. This had the advantage, also, of removing the mercury from solution as the sulphide. However, the use of hydrogen sulphide to dissolve the precipitate did not lend itself to quantitative recovery of pyrrole. It was found that 0.5% hydrochloric acid decomposed the complex, and it was used instead of the sodium cyanide for that purpose.

After much preliminary work the following method was adopted:- 5 ml. of pyrrole solution was placed in a 15 ml. graduated centrifuge tube and to it was added 5 ml. of a phosphate buffer solution (pH 6.3). Then 5 ml. of a saturated aqueous solution of mercuric chloride was introduced into the tube with vigorous stirring. The mixture was allowed to stand for one hour, and at the end of that time it was centrifuged for 10 minutes. The precipitate was washed twice with 10 ml. portions of distilled water. After each washing the supernatant liquid was removed carefully by suction. The precipitate was dissolved in 15 ml. of 0.5% hydrochloric acid and made

up to 50 ml. with distilled water. 1 ml. was used for the isatin colour reaction.

Thus, 5 ml. of a pyrrole solution containing 0.1586 mg. of pyrrole per ml. was treated according to the above procedure, and 96.47% of the pyrrole was recovered.

The Determination of Pyrrole by means of Ether Extraction

It was also deemed advisable to have a method available for the extraction of pyrrole by some solvent such as ether. Many experiments were done before a quantitative method was evolved for the extraction of small quantities of pyrrole from an aqueous solution by ether.

The first attempts consisted of extracting 10 ml. of pyrrole solution in 0.5% acetic acid three times with 10 ml. portions of ether. The combined extracts were dropped slowly onto 10 ml. of 5% acetic acid in a micro-Kjeldahl flask which was placed in a beaker of warm water (50-60°C). When all the ether had been evaporated off, the contents of the flask were made up to 100 ml., and 1 ml. was taken for the isatin colour reaction. In experiments carried out according to the above procedure the recovery of pyrrole was about 65%.

An increase in the amount of pyrrole recovered was observed when the solution was extracted 5 times with

5 ml. portions of ether and when suction was used to assist the evaporation of the ether. However, the recovery was still far from quantitative, and it was not until the idea of placing a trap containing acetic acid between the flask containing the ether and the suction line that a high percentage of pyrrole was recovered. The importance of using a trap in this determination is evident from a study of Table V. When a trap was used 94.0% pyrrole was recovered, whereas when it was omitted only 27.0% pyrrole could be measured in the solution after the ether had been evaporated off.

Table V

To Show that a Trap is Necessary during the Evaporation of Ether in the Determination of Pyrrole

Description of experiment	Galvanometer Reading (660 filter)	Pyrrole Recovered (γ per ml.)	Recovery per cent
No extraction	32.75	15.11	100.00
Ether extraction with trap	35.00	14.21	94.00
Ether extraction without trap	74.00	4.08	27.00

This method was used to a considerable extent in this investigation, and whenever it has been employed the above procedure has been adhered to.

II. THE FORMATION OF PYRROLE FROM PROTEINS

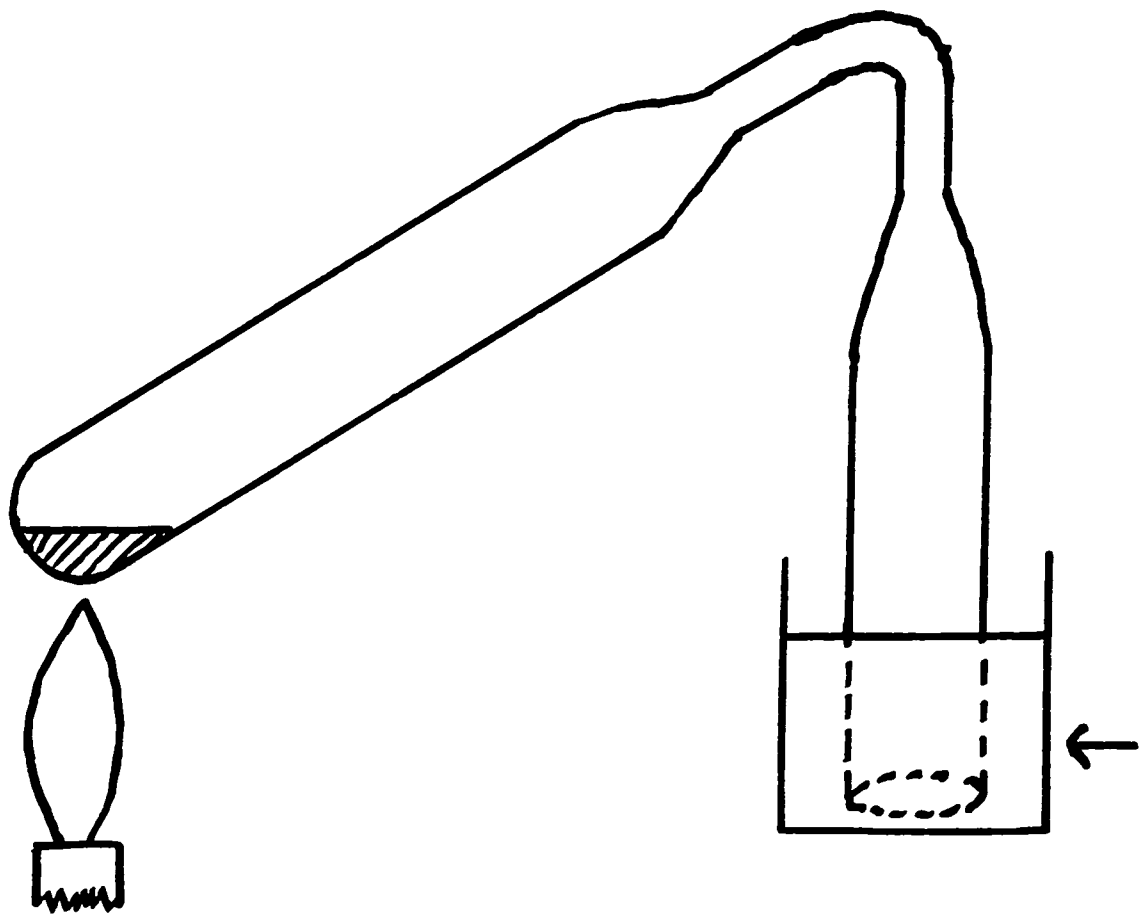
The Determination of Pyrrole in Proteins and other Biological Materials by Dry Distillation

In Gelatin. It has long been known that proteins yield pyrrole on dry distillation, and since gelatin is readily available in a relatively high degree of purity, it was selected for use when commencing studies on the dry distillation of proteins.

After a few preliminary experiments, the following procedure was adopted:- A weighed sample (e.g. 0.1 gm.) of powdered gelatin was introduced into a soft glass test-tube (5/8 x 6 ins) and the tube, after being softened, was drawn out and bent at an angle of about 45° as shown in Figure II. The tube was held by a clamp so that its mouth was below the surface of an acetic acid solution (10 ml. of 5%) contained in a 30 ml. beaker. The test tube was heated gently at first and then the amount of heat applied to the tube was gradually increased to redness. Heating was continued until no more vapour distilled over. The delivery tube was severed just above the shoulder and used as a funnel when the contents of the beaker were transferred to a 100 ml. volumetric flask. The solution was diluted with water to the mark, thoroughly mixed, and 1 ml. was taken for the colour reaction with isatin. The intensity of the pyrrole-blue was measured by means of the photoelectric colorimeter, using the 660 light filter.

Figure II

Distillation Apparatus



In all cases a blank determination was made as already described. The solution for the blank consisted of 1 ml. of the gelatin distillate to which had been added 0.2 ml. of glacial acetic acid and 1 ml. of conc. hydrochloric acid.

Examination of the data in Table VI shows that constant results can be obtained by this method. The concentration of pyrrole was calculated from Beer's law equation using the mean value of 0.0321 for K as obtained in the preparation of the calibration curve. The mean value of 9 determinations was 1.201 ± 0.075 with a maximum deviation of 0.145.

The gelatin distillate obtained by dry distillation contained coloured impurities and various devices were tried to remove them before determining the pyrrole. It was found that aluminium oxide did not adsorb the coloured impurities in the distillate and charcoal (Norite) also failed to adsorb them. Lloyd's reagent, however, adsorbed the colour and did not adsorb pyrrole from solution. Thus 5 ml. of a pyrrole solution was diluted to 50 ml. with 0.5% acetic acid and three 10 ml. portions were placed in centrifuge tubes. Various amounts of Lloyd's reagent were added to two of the tubes, and the contents were stirred vigorously for 5 minutes. They were then centrifuged for 10 minutes, and 1 ml. was taken from each tube for the isatin colour reaction. The observations are recorded in Table VII,

and it is evident that Lloyd 's reagent does not adsorb pyrrole to any appreciable extent.

Table VI

Determination of Pyrrole in Gelatin Distillates

Weight of Sample (gms.)	Galvanometer Reading (660 filter)	Concentration of Pyrrole (γ per ml.)	Yield Per Cent
0.100	43.00	11.43	1.143
0.100	45.50	10.66	1.066
0.100	42.00	11.74	1.174
0.100	43.00	11.43	1.143
0.100	40.25	12.30	1.230
0.100	37.00	13.46	1.346
0.100	37.50	13.27	1.327
0.200	16.00	24.80	1.240
0.300	8.00	34.17	1.139
	Mean Yield (9 determinations)		1.201 ± 0.075

Table VII

Data Obtained from Adsorption Experiments with Lloyd's Reagent

Lloyd's Reagent (gms)	Galvanometer Readings (660 filter)	Concentration of Pyrrole after use of reagent (γ per ml.)	Recovery Per Cent
0.0	33.50	14.80	100.00
0.2	34.00	14.61	98.73
0.3	35.25	14.11	95.37

Dry Distillation of Some Other Proteins. It has been known for some years that proteins yield pyrrole on dry distillation, but no quantitative experiments were performed, as far as the author is aware, until Fromm (1935) determined the concentration of pyrrole in the product obtained by the dry distillation of gelatin. The method here described for the dry distillation of gelatin has been applied to a number of proteins and the data obtained are shown in Table VIII. The yields obtained are low, but the results indicate quite definitely that these proteins contain some substance or substances which yield pyrrole on dry distillation.

Table VIII

Determination of Pyrrole in Some Other Proteins

Protein	Weight of Sample (gm.)	Volume of Distillate (ml.)	Galvanometer Reading (660 filter)	Concentration of pyrrole in distillate (γ per ml)	Yield of Pyrrole Per Cent
Casein	0.21	50	66.00	6.00	0.14
Gluten	0.58	100	46.50	10.34	0.18
Gliadin	0.23	50	47.50	10.06	0.22
Glutenin	0.43	50	36.00	14.00	0.16
Egg Albumin	0.20	50	72.00	5.00	0.13
Lactalbumin	0.52	50	81.50	3.25	0.03
Edestin	0.10	25	83.00	2.52	0.06

Dry Distillation of Chlorophyll. A solution of pure chlorophyll (Schering-Kahlbaum A.G. Berlin) was evaporated to dryness on a steam bath and 0.0776 gm. of dry chlorophyll was dry distilled in the usual manner into 10 ml. of 5% acetic acid. The distillate was filtered and diluted to 50 ml. with water. The distillate was clear and no oil was observed on it. One ml. was taken for the isatin reaction. No pyrrole-blue colour was observed. This was an interesting observation and indicated that the dry distillation method could not be used to determine the total amount of pyrrole nuclei present in plant materials. However, the dry distillation method is still of value as an aid in determining what materials contain proteins capable of producing pyrrole by such treatment. The following experiment illustrates this point.

Dry Distillation of Corn Leaves. A small amount of pulverized corn leaves was dry distilled in the usual manner. A positive test for pyrrole was observed, so it was concluded that the protein material in the corn leaves was probably responsible for the production of pyrrole.

Dry Distillation of Ammonium Mucate. The following experiment was performed to ascertain the reliability of the author's dry distillation method as compared with similar procedures of other investigators. 0.5 gm. of mucic acid (Kahlbaum) was dissolved in a few ml. of concentrated ammonium hydroxide and the solution was evaporated to dry-

ness on a water bath. A white powder was obtained, and 0.1348 grams of this material was dry distilled in the usual manner. A yield of 37.15% pyrrole was obtained, which compared favourably with values reported by McElvain and Bollinger (1929) who obtained 37 to 40 per cent yields of pyrrole from the dry distillation of ammonium mucate.

A Search for Pyrrole in the Enzymic Hydrolysates of Certain Proteins

Pieroni (1932) and Roncato (1933) reported the presence of pyrroles in the hydrolysis products of glutenin and gliadin, and Troensegaard (1920) had previously brought forward evidence to support his view that the protein molecule is composed chiefly of heterocyclic compounds, largely of the pyrrole type. Other investigators have failed to substantiate the work of the above-mentioned investigators, and hence the pyrrole hypothesis concerning the structure of proteins has received little support during recent years. However, proteins are usually hydrolysed by acids or bases and these compounds are known to be capable of destroying the pyrrole molecule. This might be an explanation of the absence of pyrrole in the protein hydrolysates.

Roncato (1933) used enzymes in his work and under the conditions of his experiment he obtained a positive test for pyrrole nuclei in the tryptic hydrolysate of

gliadin. However, he was unable to secure a positive test for pyrrole nuclei in the case of other proteins, and therefore it is hardly correct to maintain that he definitely established the presence of pyrrole nuclei in the enzymic hydrolysates of proteins.

In an attempt to substantiate Roncato's work, it was decided to subject a number of proteins to enzymic hydrolysis, and the isatin colour reaction was used to ascertain whether free pyrrole was produced during the course of the digestion. Details of these experiments are described below.

Tryptic Digestion of Gelatin. To 1 gm. of powdered gelatin in a 125 ml. Erlenmeyer flask was added 0.25 gm. of U.S.P. trypsin and 25 ml. of a 0.4% solution of sodium carbonate, together with a few drops of toluene which served as a preservative. The mixture was incubated at 38°C. After 6 hours from the time of making up the digest, 1 ml. was filtered and the isatin test tried on it. The test was negative. At the end of one, two, three, and four days the colour reaction for pyrrole was made on 1 ml. of the filtered hydrolysate, and in every case it was negative.

After 5 days, the digest was filtered and extracted three times with an equal volume of ether. The combined extracts were added to 10 ml. of 0.5% acetic acid in a 100 ml. volumetric flask and the ether distilled off under diminished pressure at a temperature of 30-40°C. A

trap containing 10 ml. of glacial acetic acid was placed in the suction line between the volumetric flask and the suction pump. When all the ether had been removed, the contents of the trap were added to the volumetric flask, and then 10 ml. of a saturated solution of mercuric chloride was added with vigorous stirring. The mixture was allowed to stand for two hours and at the end of that time no precipitate was observed. This was additional evidence that in this experiment no pyrrole was produced by the action of trypsin on gelatin.

Peptic Digestion of Gelatin. To 1 gm. of powdered gelatin dissolved in 25 ml. of distilled water was added 0.1 gm. of pepsin dissolved in 25 ml. of 0.1 N hydrochloric acid. This mixture was digested at 38°C for four days, and 1 ml. filtered off each day for the colour test for pyrrole. In all cases a negative result was obtained. At the end of the fourth day the digest was filtered and 10 ml. of saturated mercuric chloride was added to the filtrate. No precipitate was observed which supported the negative findings with isatin.

Peptic Digestion of Crude Gluten. To 1 gm. of pulverized crude gluten was added 0.1 gm. of pepsin dissolved in 60 ml. of 0.1 N hydrochloric acid and this mixture was placed in an incubation cabinet at 38°C. for 6 days. The isatin test was performed on 1 ml. of the fil-

tered hydrolysate on the second, third and sixth days, and in every case a negative result was obtained.

Digestion of Gelatin with Papain. To 1 gm. of gelatin dissolved in 50 ml. of water was added 0.2 gm. of papain and the solution was saturated with hydrogen sulphide to activate the papain. The solution was kept at 38°C for 3 days. Tests made with isatin on the filtered hydrolysate every day during the period of digestion showed the absence of pyrrole.

At the end of the third day, 15 ml. of the filtrate was extracted three times with 15 ml. portions of ether. The combined extracts were treated in the usual way, and 1 ml. of the resulting solution failed to give the pyrrole test with isatin.

Tryptic Digestion of Flour. A mixture consisting of 20 gm. flour, 0.5 gm. trypsin, and 100 ml. of 0.4% solution of sodium carbonate was placed in the incubation cabinet at 38°C for three days. 1 ml. of the filtered hydrolysate was treated with isatin every day during the period of digestion, and negative results were obtained in all cases.

Tryptic Digestion of Crude Gliadin. To a mixture of 0.365 gm. of crude gliadin and 25 ml. of a phosphate buffer solution of pH 8.1 was added 0.5 gm. of trypsin.

After shaking the mixture it was placed in the incubator at 38°C for eighteen days. At the end of the first, fourth, seventh, and eighteenth day, 1 ml. of the digest was filtered and the colour test for pyrrole with isatin performed. In every case it was negative.

Digestion of Gelatin with Papain at a pH of 4.9.

A mixture consisting of 1 gm. of powdered gelatin, 0.4 gm. of papain, 50 ml. of a phosphate buffer solution of a pH of 5.6, and 5 drops of toluene was saturated with hydrogen sulphide. After this treatment, the pH of the solution was determined by a Hellige pH meter and found to be 4.9. The digest was then placed in the incubation cabinet at 38°C for seventeen days. After three, six, and seventeen days 1 ml. of the filtered hydrolysate was tested for pyrrole in the usual way and in all cases a negative result was obtained.

Digestion of Gelatin with Papain at a pH of 6.3.

To 1 gm. of powdered gelatin was added 0.4 gms. of papain and 50 ml. of phosphate buffer solution (pH of 7.1). To this mixture was added 5 drops of toluene, as a preservative, but in this experiment no hydrogen sulphide was introduced. The pH as determined by the Hellige meter was found to be 6.3. The digest was kept at a temperature of 38°C for seventeen days, and tests performed on filtered portions of the hydrolysate at various intervals of time during this period failed to reveal the presence of pyrrole.

Tryptic Digestion of Gelatin at a pH of 8.1.

A mixture of 60 ml. of 0.4% sodium carbonate, 2 gm. of gelatin, 0.5 gm. of trypsin, 5 drops of toluene and 50 ml. of phosphate buffer (pH of 8.7) was prepared, and the pH of this mixture as determined by the Hellige meter was found to be 8.1. It was then digested at 38°C for 3 days. At the end of that time the digest was filtered and 1 ml. of the clear light yellow filtrate failed to give a colour reaction with isatin.

An excess of trichloracetic acid was added to 5 ml. of the filtrate and no precipitate was observed. This indicated that the gelatin had been completely hydrolysed.

5 ml. of the filtrate was acidified with glacial acetic acid and then treated with an equal volume of saturated mercuric chloride. No precipitate was observed, which substantiated the negative result obtained with isatin.

To ascertain whether the substance which produced pyrrole on dry distillation was still present in the hydrolysate, the following experiment was performed. The filtrate from the above experiment was distilled under diminished pressure at 40-50°C. When the frothing in the distilling flask prevented further reduction in volume, the residue was placed in an evaporating dish and evaporated to dryness in a vacuum oven at 50-60°. A light brown sticky residue was obtained. 0.1017 gm. of this material

was dry distilled in a test tube in the usual manner, and the distillate was made up to 100 ml. with water. A reading of 41.0 was observed on the galvanometer, using the 660 light filter, and reference to the calibration curve in Figure I showed that 12 micrograms of pyrrole were presend in 1 ml. of the distillate. By calculation it was found that the total amount of pyrrole in the distillate was 1.2 mg. or 1.18% of the sample. Nine dry distillation experiments with powdered gelatin gave a mean value of 1.201% and so the above experiment indicated conclusively that the pyrrologenic material was not destroyed during the tryptic digestion of gelatin.

The Determination of Pyrrole in a Sodium Hydroxide Hydrolysate of Gelatin

It is evident from the above experiment that the pyrrole producing substance is not destroyed during the tryptic digestion of gelatin. To discover whether sodium hydroxide hydrolysis would destroy the pyrrologenic material the following experiment was performed.

A 5 gm. sample of gelatin was hydrolysed with 10% sodium hydroxide and the hydrolysate was neutralized with conc. hydrochloric acid. The tyrosine was filtered off and the filtrate concentrated under diminished pressure to a small volume. The concentrated solution was evaporated to dryness on a water bath. The residue weighed 10.8

grams and 0.7256 gm. of this was dry-distilled in the usual way and the distillate was made up to 100 ml. The galvanometer reading observed was 16.00, using the 660 light filter, and from the calibration curve it was found that the distillate contained 2.4 mg. of pyrrole. Calculations showed that the total residue was capable of producing 35.72 mg. on dry distillation and hence the yield of pyrrole was only 0.71% after hydrolysis with sodium hydroxide as compared with 1.201% for unhydrolysed gelatin.

<u>An Attempt to Detect Pyrrole during the Course of Hydrolysis of Gelatin with Sodium Hydroxide</u>

From the above it is evident that a fraction of the pyrrole producing substance was lost during hydrolysis with sodium hydroxide. There was the possibility that part of it might have been converted into free pyrrole during the course of hydrolysis, and that the free pyrrole so formed might in time be destroyed by the sodium hydroxide. To test this belief the following experiment was conducted.

A 5 gm. sample of gelatin was refluxed with 100 ml. 10% sodium hydroxide for 1 hour. At the end of that time 1 ml. of the solution was taken for the isatin colour test. No pyrrole-blue was observed.

At the end of 2.5 hours, 10 ml. of the hydrolysate was extracted three times with 5 ml. portions of ether. The combined extracts together with 10 ml. of 5%

acetic acid were placed in a flask and using a trap containing 10 ml. of 5% acetic acid, the ether was distilled off under reduced pressure at 30-40°C. The contents of the trap and flask were made up to 25 ml. and the isatin colour test was performed on 1 ml. It was negative.

After refluxing for 6.5 hours the hydrolysate failed to give a biuret test, and 10 ml. was extracted three times with 10 ml. portions of ether. The ether was distilled off in the usual manner and the residue made up to 50 ml. Again the colour test for pyrrole with isatin was negative, and, furthermore, the addition of saturated mercuric chloride to the solution gave no precipitate.

Steam Distillation of Pyrrole and Gelatin Solutions.

The failure to detect pyrrole in the above experiment suggested that it might have been destroyed by the hot sodium hydroxide as fast as it was produced from the pyrrologenic material. Pyrrole is volatile with steam but it was not known whether it could be steam distilled from concentrated alkaline solution. If it were possible to recover pyrrole quantitatively from concentrated alkaline solutions by steam distillation, one might be able to detect it in a gelatin hydrolysate with sodium hydroxide. With this idea in mind, the following experiments were performed.

To 0.1040 gm. of freshly distilled pyrrole was added an aqueous solution of 0.5% acetic acid, to a volume of 2 litres. This solution contained 52 micrograms of

pyrrole per ml. An aliquot of 25 ml. of this solution was diluted to 100 ml. with 0.5% acetic acid and 1 ml. was used for the colour reaction with isatin. The galvanometer reading is recorded in Table IX. The above experiment was repeated except that the pyrrole solution was made up with water and glacial acetic acid so that a solution of pyrrole in 10% acetic acid resulted. Exactly the same reading was observed on the galvanometer as in the case of the solution of pyrrole in 0.5% acetic acid, and thus all the solutions of pyrrole used in the subsequent experiments were made up to volume with 0.5% acetic acid.

Another 25 ml. aliquot of the original pyrrole solution was made up to 100 ml. with 0.5% acetic acid and steam distilled. The distillate was collected in a 250 ml. suction flask to which was attached a trap containing 10 ml. of 5% acetic acid. Steam distillation was conducted for 30 minutes and then the contents of the trap and flask were made up to 100 ml. with water. The usual colour reaction was performed and it was found that the galvanometer reading was exactly the same as in the case of the solution which had not been steam distilled.

To find out whether the trap was necessary, the above experiment was repeated except that the suction flask and trap were replaced by a 100 ml. volumetric flask. Again the galvanometer reading was almost exactly the same as in the previous experiments.

It was evident from these experiments that pyrrole was not destroyed by steam distillation, and, in fact, that it could be recovered quantitatively from an acetic acid solution. To ascertain whether pyrrole could be steam distilled from alkaline solutions the following experiments were performed.

To 25 ml. of the original pyrrole solution was added 25 ml. of 20% sodium hydroxide and the volume was made up to 100 ml. with water. The same apparatus and procedure were used as in the steam distillation experiments described above. Steam distillation was carried on for 30 minutes and the distillate was made up to 100 ml. A measurement of the intensity of the pyrrole-blue colour obtained by the usual procedure showed that there was no loss of pyrrole. Similar steam distillations of pyrrole from 10% and 20% solutions of sodium hydroxide were performed with only a slight loss of pyrrole. The data obtained are shown in Table IX.

It was evident from the above experiments that pyrrole could be steam distilled from alkaline solutions, and so if pyrrole were liberated from gelatin during hydrolysis with concentrated sodium hydroxide, it might be obtained by steam distillation. To discover whether such was the case the following experiment was performed.

A 10 gm. sample of gelatin was dissolved in 100 ml. of 20 % sodium hydroxide and steam distilled in exactly the same way as was described above. The distillation

was conducted for five hours and seven consecutive 100 ml. fractions of the distillate were collected. 1 ml. of each of these distillates was treated with isatin in the usual way and in every case no pyrrole was detected. Hydrolysis of the protein was complete at the end of 2.5 hours as indicated by a negative biuret test at the end of that time. This experiment proved conclusively that free pyrrole was not produced during the alkaline hydrolysis of gelatin.

Table IX

Steam Distillation of Pyrrole Solutions

Experiment	Galvanometer Reading (660 Filter)	Concentration of Pyrrole in distillate (γ per ml.)	Recovery Per cent
No steam distillation. Pyrrole dissolved in 0.5% acetic acid.	40.00	12.4	100
No steam distillation Pyrrole dissolved in 10% acetic acid.	40.00	12.4	100
Steam distilled from 0.5% acetic acid. Trap used on receiving flask.	40.00	12.4	100
Steam distilled from 0.5% acetic acid. No trap used on receiving flask.	40.00	12.4	100
Steam distilled from 5% sodium hydroxide.	40.00	12.4	100
Steam distilled from 10% sodium hydroxide.	40.50	12.2	98.4
Steam distilled from 20% sodium hydroxide	41.50	11.9	96.00

A Comparison of Acid and Alkaline Hydrolysis of Gelatin

It has already been demonstrated that pyrrole can be determined in the distillates obtained by the dry distillation of the hydrolysates from the tryptic and alkaline hydrolysis of gelatin. To ascertain whether the pyrrologenic substance was destroyed during acid hydrolysis, and if not, to compare the yields obtained by acid hydrolysis with those obtained with sodium hydroxide, the following experiments were conducted.

Hydrochloric Acid Hydrolysis. To 1 gm. of gelatin in a 500 Kjeldahl flask was added 25 ml. of hydrochloric acid (5 ml. conc. hydrochloric acid and 20 ml. water). The flask was fitted with an air condenser and it was placed on a steam bath for 48 hours. At the end of that time the hydrolysate was neutralised with 20% sodium hydroxide, filtered, and the filtrate was made up to 100 ml. with water. 2 ml. of this solution gave a negative biuret test which indicated that hydrolysis was complete. 50 ml. of this solution was evaporated on a steam bath to dryness and 2.3355 gm. of dry residue was obtained.

A 0.3546 gm. portion of this residue was dry distilled in the usual way and made up to 50 ml. with water. The galvanometer reading and the calculations obtained from this observation are recorded in Table X. Examination of this table shows that the pyrrologenic material in gelatin only suffered a slight loss during hydrolysis with hydrochloric acid.

Sulphuric Acid Hydrolysis. A 1 gm. sample of gelatin and 25 ml. of sulphuric acid (5 ml. conc. sulphuric acid to 20 ml. water) were placed in a 500 ml. Kjeldahl flask. The flask was fitted with an air condenser and it was heated on a steam bath for 48 hours. At the end of that time the hydrolysate gave a negative biuret test, indicating that hydrolysis was complete. The hydrolysate was neutralized with 20% sodium hydroxide, filtered and diluted to 100 ml. with water. 25 ml. of this solution was evaporated to dryness on a steam bath and 3.8443 gms. of dry residue was obtained.

1.3314 gm. of this residue was dry distilled in the usual manner and made up to 50 ml. The galvanometer reading and the calculations obtained from this observation are recorded in Table X, and these data show that the pyrrologenic substance was not destroyed to any appreciable extent by hydrolysis with sulphuric acid.

Hydrolysis with 10% Sodium Hydroxide. To 1 gm. of gelatin in a 500 ml. Kjeldahl flask was added 25 ml. of 10% sodium hydroxide. The mixture was refluxed for 24 hours on a steam bath. At the end of that time the hydrolysate gave a negative biuret test. It was neutralized with conc. hydrochloric acid, filtered and diluted to 100 ml. with water. 25 ml. of this solution was evaporated to dryness on a steam bath and 1.4057 gm. of dry residue was obtained.

0.3862 gm. of this residue was dry distilled and

made up to 50 ml. with water. The data obtained are recorded in Table X.

Hydrolysis with 20% Sodium Hydroxide. The above experiment was repeated except that 20% sodium hydroxide was used instead of 10% sodium hydroxide. The data secured are recorded in Table X.

Table X

Comparison of Acid and Alkaline Hydrolysis of Gelatin

Hydrolysing Agent	Galvanometer Reading (660 Filter)	Pyrrole obtained from 1 gm. gelatin (mgs.)	Pyrrole Per cent
Hydrochloric Acid	32.00	10.09	1.01
Sulphuric Acid	21.00	11.55	1.16
Sodium Hydroxide (10%)	45.50	8.01	0.80
Sodium Hydroxide (20%)	45.00	6.80	0.68

An examination of Table X reveals that pyrrole can be produced by the dry distillation of gelatin hydrolysates obtained by both acid and alkaline hydrolysis. Acid appears to destroy less of the pyrrole producing substance than does the alkali, and therefore in the next experiment hydrochloric acid was used as the hydrolysing agent.

The Fractionation of a Gelatin Hydrolysate

To discover the nature of the pyrrologenic material which is present in a gelatin hydrolysate, the following experiments were conducted. Preliminary experiments were performed on a gelatin hydrolysate obtained by tryptic digestion and these suggested the method of fractionation about to be described.

To 500 gm. of silver leaf gelatin in a 3 litre Pyrex Florence flask was added 2 litres of hydrochloric acid containing 480 ml. of concentrated hydrochloric acid per litre of solution. This mixture was refluxed on a steam bath for 20 hours. To increase the rate of hydrolysis the flask was removed from the steam bath and placed on a sand bath. The hydrolysate was then boiled gently for 48 hours.

At the end of that time the hydrolysate was concentrated under diminished pressure to a thick oil to remove as much hydrochloric acid as possible. It was next diluted with water and an aliquot was treated with 20% sodium hydroxide until the solution was distinctly alkaline to litmus. The ammonia was distilled off under reduced pressure, and the solution remaining in the distilling flask was treated with finely powdered basic lead acetate until a slight excess of lead was present in the solution. The precipitate was filtered off under suction using a Büchner funnel, and the excess lead was removed from the filtrate by the addition of sulphuric acid. The lead sulphate was filtered off and solid barium hydroxide was added to the filtrate until the solution was distinctly alkaline. The mixture was then

filtered and distillation was performed under diminished pressure on a boiling water bath to decrease the volume of the filtrate. To remove the barium, concentrated sulphuric acid was added a little at a time until a few millilitres of the filtered solution failed to give a precipitate with dilute sulphuric acid. After filtration, the solution was diluted to 1200 ml.

A 10 ml. aliquot of this solution was exactly neutralized with sodium hydroxide and evaporated to dryness on a steam bath. 1.8623 gm. of residue was obtained and 0.1412 gm. of this was dry distilled and made up to 50 ml. with water. The galvanometer reading, using the 660 filter, was 29.50, and from the calibration curve it was found that the concentration of pyrrole was 16 micrograms per ml. Calculations based on these observations showed that each ml. of the above 1200 ml. solution produced 1.055 mg. of pyrrole on dry distillation.

A 100 ml. aliquot of this 1200 ml. solution was acidified with sulphuric acid until the solution contained 5% sulphuric acid by weight. Phosphotungstic acid (20%) dissolved in 5% sulphuric acid was added to the solution until complete precipitation had been accomplished. After standing in the ice box for three days the precipitate was filtered off and washed with small portions of a 2.5% solution of phosphotungstic acid dissolved in 5% sulphuric acid. 100 ml. of wash solution was used altogether.

The precipitate was then transferred to a large mortar and hot saturated barium hydroxide was added until the mixture was pink to phenolphthalein. Additional hot barium hydroxide was added until there was no further precipitation of barium phosphotungstate. The precipitate was filtered off, using a Büchner funnel. Sulphuric acid was than added to the filtrate to remove excess barium ions and the barium sulphate was removed by filtration. The filtrate was concentrated under diminished pressure on a boiling water bath and finally made up to 500 ml. with water.

A 10 ml. portion of this solution was evaporated to dryness and 0.0458 gm. of residue was obtained. 0.0096 gm. of this residue was dry distilled into 10 ml. of 5% acetic acid. The galvanometer reading, using the 660 light filter, was 75.5, and from the calibration curve it was found that 1 ml. of the distillate contained 4.25 micrograms of pyrrole. By calculation it was evident that there was a total of 10.14 mgs. of pyrrole in the phosphotungstic acid precipitate.

The filtrate and combined washings from the phosphotungstic acid precipitate had a volume of 365 ml. 10 ml. of this solution was exactly neutralized with sodium hydroxide and evaporated to dryness. 1.2374 gm. of dry residue was obtained and 0.5387 gm. of it was dry distilled and the distillate made up to 100 ml. The galvanometer reading, using the 660 filter, was 45.00, and thus 1 ml. of the distillate contained 11.0 micrograms of pyrrole. By calculation

it was found that the total filtrate contained 92.22 milligrams. The data obtained in this fractionation of a gelatin hydrolysate are given in Table XI and it is evident from the data given therein that the filtrate from the phosphotungstic acid precipitation contained the bulk of the pyrrologenic material.

Table XI

Fractionation of a Gelatin Hydrolysate

500 gm. of gelatin (silver leaf) hydrolysed with hydrochloric acid. Hydrolysate freed from hydrochloric acid and ammonia.

Basic lead acetate

Precipitate (no pyrrole)

Filtrate

An aliquot (105.5 mg. pyrrole) precipitated with phosphotungstic acid

Filtrate (92.22 mg. pyrrole)

Precipitate (10.14 mg. pyrrole)

Alcoholic Extraction of Dry Residue from a Gelatin Hydrolysate

It was evident from the above experiment that phosphotungstic acid was unsatisfactory as a precipitating agent for the pyrrole producing substances. An attempt was therefore made to extract the pyrrologenic materials with alcohol from the dry residue obtained by evaporation of the

of the filtrate from the basic lead acetate treatment. The details of this experiment are described below.

A 1 gm. portion of the above residue, containing 5.794 mg. of pyrrole, was thoroughly mixed with 10 gm. of pure sea-sand. This mixture was placed in the porous cup of a Soxhlet apparatus and extracted continuously with 100 ml. of hot absolute alcohol for 5 hours. At the end of that time the alcohol extract was placed in the ice-box over night.

The porous cup was washed with warm water and the washings were evaporated to dryness. 0.2450 gm. of residue was obtained from this aqueous extract. 0.0802 gm. of this residue was dry distilled into 10 ml. of 5% acetic acid and 1 ml. of the distillate was found to contain 7.75 micrograms of pyrrole. Calculations showed that the fraction which was insoluble in hot alcohol contained a total of 0.2368 mg. of pyrrole.

The next morning a white precipitate was observed in the alcoholic filtrate. This was filtered off and washed twice with absolute alcohol. Unfortunately this fraction which was insoluble in cold alcohol was lost.

The filtrate and washings from the above experiment were evaporated to dryness on a steam bath and 0.6343 gm. of dry material was obtained. To make certain that no carbohydrate material was present which might be responsible for the formation of pyrrole on dry distillation, an improved Molisch test (Foulger 1931) was performed on a small amount of the above

residue. The test was negative, whereas with gelatin a slight pink colour was obtained, signifying the presence of a small amount of carbohydrate material. It would hardly be expected that any carbohydrate material would escape the basic lead acetate treatment, and it was only as a precaution that Molisch's test was performed on the alcoholic extract.

A 0.0945 gm. sample of the alcoholic extract was dry distilled and the distillate made up to 50 ml. It was found that 1 ml. of distillate contained 12.75 micrograms of pyrrole, and calculation showed that the total alcoholic extract, namely 0.6343 gm. contained 4.279 mgs. of pyrrole. This indicated that most of the pyrrologenic material was in the alcohol soluble fraction.

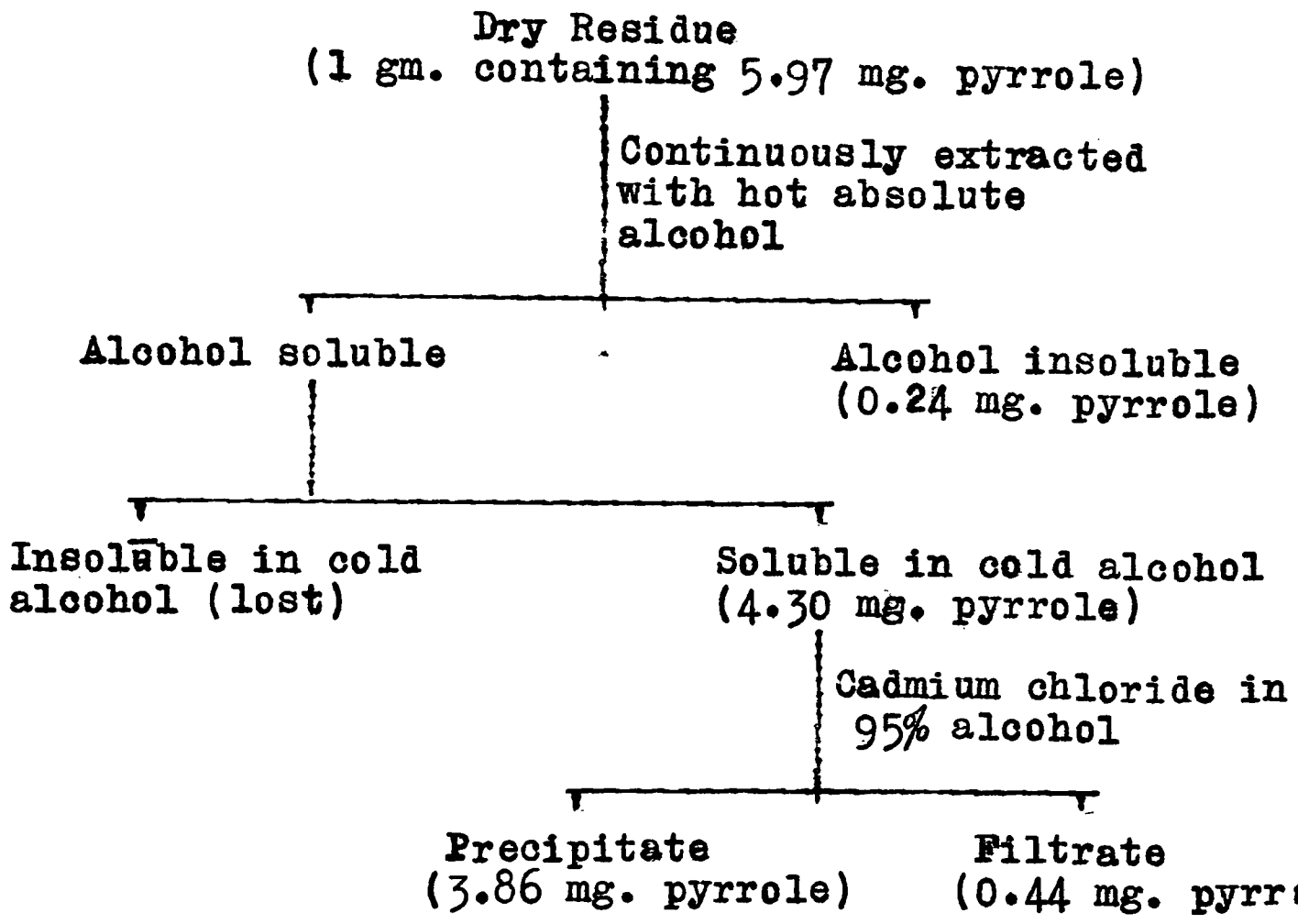
The observation made in the above experiment at once suggested that proline and hydroxy-proline were responsible for the formation of pyrrole from gelatin on dry distillation. Proline is precipitated by cadmium chloride and hence the following experiment was performed to find out whether the alcoholic extract contained proline.

0.5398 gm. of the alcoholic extract was dissolved in 60 ml. of absolute alcohol and a solution of cadmium chloride dissolved in 95% alcohol was added until no further precipitation occurred. The precipitate was filtered off and washed with a small amount of absolute alcohol. The filtrate was evaporated to dryness on a steam bath and the dry residue dissolved in water. A small amount of material

was insoluble in water and was removed by filtration. Silver sulphate was added to the filtrate to remove the chloride as silver chloride and when this precipitate was removed the filtrate was treated with hydrogen sulphide to precipitate the silver and cadmium ions as the sulphides. The sulphides were filtered off and the filtrate was neutralized with sodium hydroxide and evaporated to dryness on a steam bath. 0.4813 gm. of residue was obtained and 0.1442 gm. of this was dry distilled into 10 ml. of 5% acetic acid. It was found that 1 ml. of the distillate contained 13.25 micrograms of pyrrole and calculations showed that the total residue was capable of producing only 0.442 mg. of pyrrole on dry distillation. This experiment indicated that most of the pyrrologenic materials were precipitated by the cadmium chloride and so it was concluded that proline and hydroxyproline were likely responsible for the production of pyrrole when a gelatin hydrolysate was dry distilled. The data obtained in these experiments are shown in Table XII.

Table XII

Alcoholic Extraction of Dry Residue from Gelatin Hydrolysate



The Dry Distillation of Some Amino Acids

The experiments on the fractionation of a gelatin hydrolysate suggested that the formation of pyrrole on the dry distillation of gelatin or gelatin hydrolysates was due to the oxidation of proline and hydroxy-proline to pyrrole. To find out whether other amino acids might also product pyrrole on dry distillation, the following experiments were performed.

As many amino acids as were available were dry distilled in the usual manner into 10 ml. 5% acetic acid. The colour reaction with isatin was performed on 1 ml. of the distillate and the observations are recorded in Table

XIII. Proline and hydroxy-proline were the only amino acids which gave a positive test for pyrrole. Of the other amino acids which were examined, only arginine gave a trace of colour with isatin.

There was the possibility that the ammonium salt of an amino acid might be more readily converted into pyrrole than the amino acid itself, under the conditions of the dry distillation procedure. To investigate this the ammonium salts of a few amino acids were prepared and dry distilled in the usual manner. In all cases the isatin test for pyrrole was negative. The salts used in this experiment are listed in Table XIII.

Table XIII

Dry Distillation of Some Amino Acids

Compound	Pyrrole-Isatin Reaction
Proline	Positive
Hydroxy-Proline	Positive
Arginine	Trace
Glutamic Acid	Negative
Glycine	"
Tryptophan	"
Histidine	"
Lysine	"
Aspartic Acid	"
Alanine	"
Leucine	"
Tyrosine	"
Serine	"
Valine	"
Phenylalanine	"
Cystine	"
Ammonium salt of	
Arginine	Negative
Glutamic Acid	"
Glycine	"

The Effect of Oxidizing Agents on the Formation of Pyrrole

It has been demonstrated that proline and hydroxy-proline were responsible for the production of pyrrole when gelatin was dry distilled. This phenomenon suggested a possible method for the determination of the total amount of these two amino acids when present in protein hydrolysates. In the previous experiments on the dry distillation of proteins it was observed that the yields of pyrrole were quite low, and with the hope of discovering an oxidizing agent which would increase the yield of pyrrole the following experiments were performed.

Proline and Sulphur. To 0.0139 gm. of proline was added a small amount of sulphur and this mixture was heated in a test tube in the usual way. The distillate was collected in 10 ml. of 5% acetic acid and 1 ml. was taken for the isatin reaction. The yield of pyrrole obtained was approximately one-half that obtained when no sulphur was used.

Hydroxy-Proline and Selenium. To 0.0112 gm. of hydroxy-proline was added a small amount of selenium, and this mixture was dry distilled. The distillate was made up to 100 ml. and a pyrrole determination was made in the usual way. A slight increase in the yield of pyrrole was observed but it was not sufficient to warrant the use of selenium as an oxidizing agent.

Gelatin and Chloride of Lime. Some solid chloride of lime was added to 0.0327 gm. of gelatin and dry distillation was performed as usual. There was no appreciable increase in the amount of pyrrole found in the distillate.

Gelatin Hydrolysate and Ferric Chloride. A solution of a gelatin hydrolysate which had been through the lead acetate treatment was used in this experiment and in some of the subsequent ones. It was made up so that 1 ml. produced exactly 1.0 mg. of pyrrole on dry distillation in a test-tube with 5 gm. of pure sea-sand. The usual procedure was followed and the distillate was made up to 100 ml.

1 ml. of this hydrolysate was added to 5 gm. of pure sea-sand and 0.1 ml. of 50% ferric chloride in a test tube. Dry distillation was carried out and the pyrrole content was determined in the usual way. No appreciable increase in the amount of pyrrole was observed.

Gelatin Hydrolysate and Sodium Hypochlorite.

(a) In the cold. 5 ml. of a solution of sodium hypochlorite was added to 1 ml. of the hydrolysate and the mixture was stored in the ice-box for 2 hours. Then 2 ml. of glacial acetic acid was added and the solution was made up to a volume of 100 ml. of water. 1 ml. of this solution was treated with isatin in the usual manner but no pyrrole-blue was observed. This fact suggested that the conditions of this experiment were not suitable for the production of pyrrole.

It was felt that perhaps insufficient hypochlorite had been used in the above experiment and so an experiment was performed in which the quantity of hypochlorite was doubled. Again a negative result was obtained.

(b) Steam Distillation. It was possible that pyrrole was formed in the above experiments but that it was destroyed by standing with an excess of sodium hypochlorite. In an attempt to discover if such were the case the reaction mixture was steam distilled and it was hoped that this procedure would drive over the pyrrole as rapidly as it might be produced. The details of this experiment are given below.

To 1 ml. of the hydrolysate was added 10 ml. of the sodium hypochlorite solution and the solution was diluted to 100 ml. with water. This mixture was subjected to steam distillation and 100 ml. of the distillate was collected. No pyrrole could be detected in the distillate.

(c) Dry Distillation. To 1 ml. of the hydrolysate was added 0.2 ml. of the sodium hypochlorite solution and 5 gm. of sand. Dry distillation was performed, and it was found that there was an increase in the yield of pyrrole in the distillate. However, the increase was not great enough to warrant the use of sodium hypochlorite in this procedure.

Gelatin Hydrolysate and Hydrogen Peroxide. A mixture of 1 ml. of the hydrolysate and 1 ml. of 6% hydrogen

peroxide was dry distilled with 10 gm. of sand and in this case a loss of pyrrole was observed.

Gelatin Hydrolysate and Alkaline Permanganate

An excess of potassium permanganate dissolved in 20% sodium hydroxide was added to 1 ml. of hydrolysate and the resulting mixture was steam distilled. 100 ml. of distillate was collected and the usual colour reaction failed to detect pyrrole in the distillate.

Gelatin Hydrolysate and Sodium Peroxide. The oxidizing agents used in the above experiments did not prove satisfactory as a measure of increasing the yield of pyrrole from a gelatin hydrolysate. However, it was found that sodium peroxide did increase to a marked degree the quantity of pyrrole obtained on the dry distillation of a gelatin hydrolysate. To determine the most effective concentration of the peroxide, a series of experiments was performed with a gelatin hydrolysate and different amounts of sodium peroxide. The data obtained are recorded in Table XIV.

It was evident from these observations that 0.5 gm. of the peroxide produced the largest amount of pyrrole. When this quantity of sodium peroxide was used, the yield of pyrrole was increased three fold as compared with the amount obtained without the use of this oxidizing agent.

In all these experiments, 1 ml. of hydrolysate and 5 gm. of sand were dry distilled with various amounts

of the peroxide and the distillate was diluted to 100 ml. except in the case of experiment 6 where the distillate was diluted to 200 ml.

In addition to increasing the yield of pyrrole the use of sodium peroxide gave a clear distillate which is a distinct advantage in colorimetric determinations.

Table XIV

The Effect of Sodium Peroxide on the Production of Pyrrole from a Gelatin Hydrolysate

Experiment No.	Weight of Sodium Peroxide (gms)	Galvanometer Reading (660 Filter)	Quantity of Pyrrole obtained from 1 ml. of Hydrolysate (mgs)	Increase in Yield Per Cent
1	0.00	48.50	1.00	0
2	0.25	24.00	1.85	85
3	0.50	12.00	2.80	180
4	0.50	10.00	3.00	200
5	0.50	11.00	2.90	190
6 *	1.00	48.00	2.05	105

* Distillate diluted to 200 ml.

Gelatin and Sodium Peroxide. The above experiments proved definitely that sodium peroxide increased the yield of pyrrole from a gelatin hydrolysate. To find whether the peroxide would also increase the yield from dry gelatin, the following experiments were carried out.

0.1160 gm. of dry gelatin was dry distilled in a test tube with 1 gm. of sodium peroxide. A clear filtrate was obtained, which contained 1.375 mg. of pyrrole and calculations showed that this represented a yield of 1.19%. It is to be noted that no sand or water was used in this experiment.

By decreasing the concentration of sodium peroxide to 0.5 gm, an increase in the yield of pyrrole was observed. Thus, when 0.137 gm. of gelatin was dry distilled with 0.5 gm. of sodium peroxide and 5 gm. of sand, a yield of 1.74% of pyrrole was obtained. The amount of pyrrole in the distillate was increased still more by wetting the mixture with 1 ml. of water before distilling. The details of this experiment are described below.

A mixture of 0.5 gm. of sodium peroxide, 5 gm. of sand, 1 ml. of water and 0.1025 gm. of gelatin was placed in a test tube and dry distillation was performed in the usual manner. 2.15% of pyrrole was found in the distillate.

The data obtained in these experiments are recorded in Table XV.

Table XV

The Effect of Sodium Peroxide on the Production of Pyrrole from Gelatin

Weight of Gelatin (gms)	Weight of Sodium Peroxide (gms)	Volume of Distillate (ml.)	Galvanometer Reading (660 Filter)	Total Concentration of Pyrrole (mgs)	Pyrrole Per Cent
0.1000	0.0	100	42.00	1.17	1.17
0.1160	1.0	250	68.00	1.38	1.19
0.1037	0.5	100	25.00	1.80	1.74
*0.1025	0.5	200	46.00	2.20	2.15

* Wetted with 1 ml. of water before distilling.

An examination of Table XV shows that the yield of pyrrole from the dry distillation of gelatin can almost be doubled by the use of sodium peroxide.

Attempts to Determine the Temperature at which Pyrrole is Produced from a Gelatin Hydrolysate

A serious criticism of the dry distillation method is that the temperature of the reaction may vary considerably from one experiment to the next. This variability of temperature may seriously affect the constancy of the yields of pyrrole obtained, and so experiments were commenced to find the temperature at which the maximum amount of pyrrole could be secured from a given amount of gelatin hydrolysate. A few of these experiments are described below.

A mixture of 1 ml. of gelatin hydrolysate, which produced exactly 1.0 mg. of pyrrole on dry distillation, 1 gm. sodium peroxide and 20 ml. of water was placed on a steam bath and steam was passed through the reaction flask until 100 ml. of distillate had been collected. 1 ml. of this distillate failed to give the test for pyrrole with isatin.

The above experiment was repeated, using 1 ml. of the hydrolysate, 0.5 gm. of sodium peroxide and 5 ml. of water. Again no pyrrole was detected in the distillate.

Then 1 ml. of the hydrolysate, 0.5 gm. of sodium peroxide and 1 ml. of water were placed in a 100 ml. Kjeldahl flask and the flask was heated to dryness with a micro-burner, as the steam was passed through it. 100 ml. of distillate was collected and 1 ml. taken for the isatin reaction. In this case 2.25 mg. of pyrrole was found to be present in the distillate.

The above experiment was repeated except that the flask was not heated to dryness. This time 1 ml. of the distillate gave a negative test for pyrrole with isatin.

To increase the boiling point of the mixture in the reaction flask, pulverized potassium sulphate was introduced, and to prevent cracking of the flask during heating, superheated steam was used in the subsequent experiments.

Thus, 1 gm. of sodium peroxide was added to 1 ml. of the hydrolysate, 5 gm. of potassium sulphate and 2 ml. of water in a 100 ml. Kjeldahl flask. The flask was heated to dryness with a micro-burner and then superheated steam was passed through until 100 ml. of distillate was collected. 2.6 mg. of pyrrole was found in the distillate.

The above experiment was repeated and 2.5 mg.of pyrrole obtained. This indicated that the results could be duplicated when this procedure was used.

These experiments proved definitely that pyrrole was not produced by the action of sodium peroxide on a gelatin hydrolysate at the temperature of steam. In those cases where it was observed in the distillate, the reaction flask had been heated to dryness instead of being kept at 100°C.

In the next experiments the reaction flask was heated above 100°C by various means and any pyrrole produced was swept over by superheated steam.

Thus 1 ml. of hydrolysate was added to 1 gm. of sodium peroxide, 5 gm. of pulverized potassium sulphate, and 2 ml. of water in a Pyrex Florence flask. The flask was placed in an oil bath and superheated steam was passed through it as the temperature of the bath was gradually increased. 100 ml. portions of distillate were collected and 1 ml. was taken for the isatin test. The first distillate was collected between 100°C and 150°C, and it was found to contain no pyrrole. The next fraction was col-

lected between 150° and 200°C, and it also contained no pyrrole. The last distillate was collected between 200°C and 250°C, and it likewise failed to give a positive test for pyrrole with isatin.

The oil bath was replaced by a glycerine bath and the above experiment repeated. Distillates were collected at various temperature intervals up to 284°C, and in every case no pyrrole was detected.

In order to secure a higher uniform temperature a 220 volt, 350 watt Cenco heater was used in the next experiment. 1 ml. of the hydrolysate, 2 ml. of water, 5 gm. potassium sulphate, and 1 gm. sodium peroxide were introduced into a 100 ml. Kjeldahl flask and the flask placed on a sand bath. The sand bath was heated by the electric heater and superheated steam was passed through the flask as in the previous experiments. The temperature was observed by means of a thermometer placed in the sand. 100 ml. of distillate was collected between certain temperature ranges and the pyrrole determined in the usual manner. The first distillate was collected between 280° and 300°C, and it contained 0.775 mg. pyrrole. The second distillate was collected between 300° and 320°C, and contained 1.0 mg. pyrrole. Thus the total amount of pyrrole obtained in this experiment was 1.775 mg.

In the next experiment, the sand bath was replaced by an air bath and a temperature of 350°C was reached. The distillate between 290°C and 350°C was

collected and made up to 100 ml. It contained 1.53 mg. pyrrole.

Several other similar experiments were performed but the maximum yield of pyrrole obtained by such methods was always less than that secured by the dry distillation of a gelatin hydrolysate with sodium peroxide in a test tube.

Thus, 1 ml. of the hydrolysate used in these experiments produced 3.0 mg. of pyrrole when dry distilled with 0.5 gm. of sodium peroxide in a test tube, whereas 1.8 mg. of pyrrole was the largest amount secured when an electric heater was employed. These experiments, however, indicated that a temperature of at least 300°C was necessary to produce pyrrole from a gelatin hydrolysate with sodium peroxide.

The Effect of Copper Sulphate on the Oxidation of Proline and Hydroxy-Proline with Sodium Peroxide

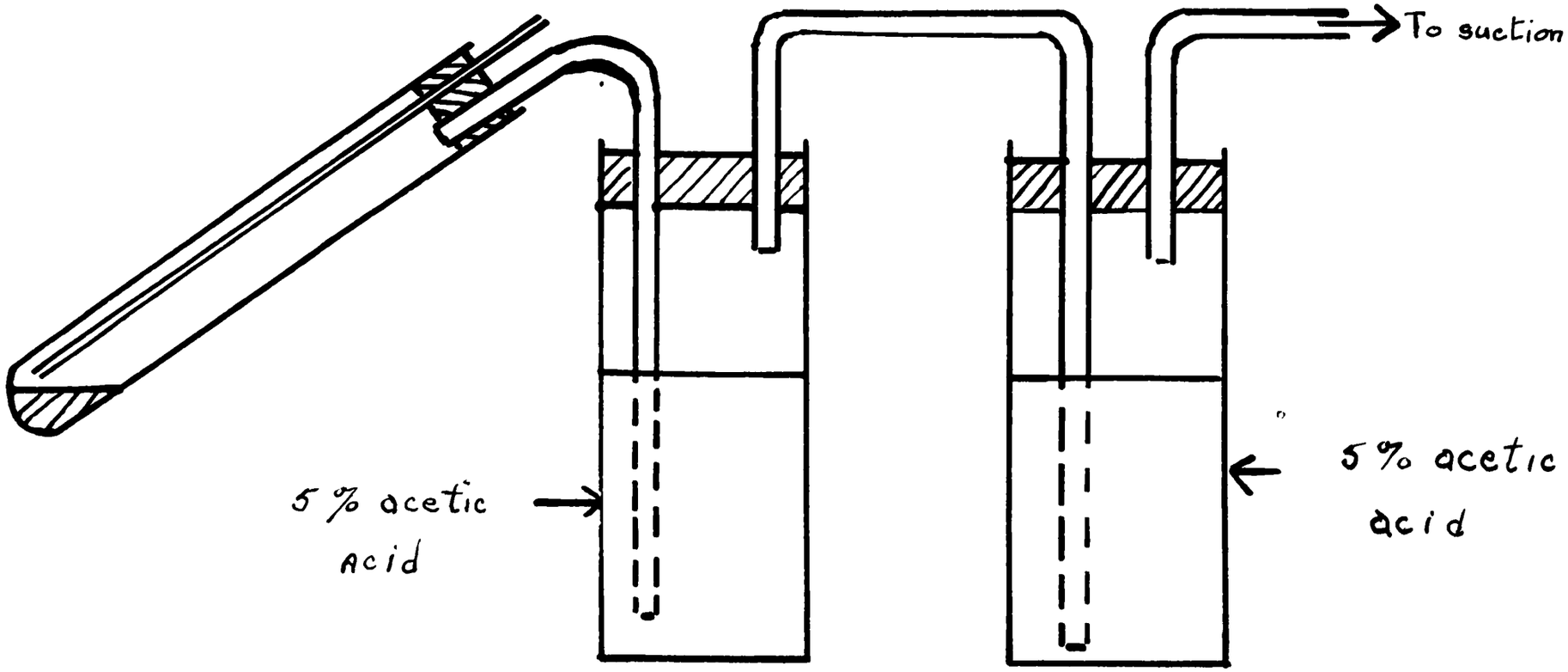
Various methods of obtaining pyrrole by the distillation of a gelatin hydrolysate have been described but in all cases the yields did not exceed those secured by dry distillation from a test tube. For that reason it was decided to utilize the latter method in studying the effect of copper sulphate upon the oxidation of proline and hydroxy-proline by sodium peroxide at high temperatures. It had been observed in some preliminary experiments that copper sulphate greatly enhanced the oxidizing power of sodium peroxide and in order to study this effect on the production

of pyrrole from gelatin the following experiments were performed.

A mixture of 1 ml. of a gelatin hydrolysate, 0.5 gm. of sodium peroxide and 5 gm. of sand was dry distilled and the distillate was made up to 100 ml. The pyrrole content was determined in the usual way and 1.70 mg. of pyrrole was obtained in the distillate. The above experiment was repeated except that 2 drops of a 10% solution of copper sulphate were added. The pyrrole content of the distillate was increased to 2.28 mg.

It was decided at this point in the investigation to improve upon the dry distillation method, and after considerable preliminary work the apparatus shown in Figure III was adopted and used in the subsequent dry distillation experiments. The sample and reagents were placed in a Pyrex test tube (25 x 150 mm) with rim, and air was drawn slowly through this tube by suction, during the heating with a Bunsen burner. Thus the pyrrole was swept over as fast as it was formed into two 50 ml. test tubes containing 20 ml. of 5% acetic acid. When no more vapour distilled over the heating was discontinued and the apparatus was cooled. The combined contents of the receiving tubes were then made up to 100 ml. with water, and the pyrrole determined in the usual manner. This procedure was followed in the experiments described below.

Figure III



A mixture of 1 ml. of gelatin hydrolysate, 0.5 gm. sodium peroxide, and 5 gm. of sand was heated in this new apparatus, and 1.15 mg. of pyrrole was determined in the distillate.

The above experiment was repeated using 1 ml. of the hydrolysate, 0.5 gm. of sodium peroxide and 0.1 ml. of 10% copper sulphate. The yield of pyrrole was found to be 2.85 mg. This experiment was repeated and almost exactly the same value for pyrrole was obtained, showing that the results would be duplicated.

The data obtained in these experiments are shown in Table XVI and it is evident from an examination of this table that the addition of copper sulphate increased the yield of pyrrole to a marked degree.

Table XVI

The Effect of Copper Sulphate on the Production of Pyrrole from a Gelatin Hydrolysate

Description of Experiment	Amount of 10% $CuSO_4$ added	Galvanometer Reading (660 filter)	Pyrrole in Distillate (mgs.)
Dry Distillation (old method)	none	27.50	1.70
Dry Distillation (old method)	2 drops	17.50	2.28
Dry Distillation (new method)	none	43.00	1.15
Dry Distillation (new method)	0.1 ml.	11.50	2.85
Dry Distillation (new method)	0.1 ml.	11.25	2.88

The next experiments were performed with gelatin and some interesting data were obtained. Thus 0.1 gm. of gelatin and 1 ml. of water on distillation gave 0.93% pyrrole, whereas 0.1 gm. gelatin when distilled with 1 ml. of water and 0.5 gm. of sodium peroxide produced 2.65% pyrrole. However, when a mixture of 0.1 gm. of gelatin, 1 ml. of water, 0.5 gm. of sodium peroxide and 0.1 ml. of a 10 % solution of copper sulphate was distilled the yield of pyrrole decreased to 2.00%.

These observations suggested that it would be interesting to know the effect of copper sulphate on the oxidation of proline and hydroxy-proline and so the following experiments were performed.

To 2 ml. of a solution of hydroxy-proline containing 10 mg. was added 0.1 ml. of 10% copper sulphate and 0.5 gm. of sodium peroxide. This mixture was dry distilled and 3.25% pyrrole was obtained. The above experiment was repeated omitting the copper sulphate and in this case 7.25% pyrrole was obtained.

A mixture of 10 mg. of proline, 2 ml. water, 0.1 ml. of 10% copper sulphate and 0.5 gm. of sodium peroxide was dry distilled and a yield of 8.44% of pyrrole was secured.

When the above experiment was repeated, omitting the copper sulphate, no pyrrole could be detected in the distillate.

The data obtained in these experiments with gelatin, proline and hydroxy-proline are given in Table XVII.

Table XVII

The Effect of Copper Sulphate on the Oxidation of Proline and Hydroxy-Proline

Sample	Volume of Water used	Weight of Sodium Peroxide used	Volume of 10% $CuSO_4$ used	Pyrrole Obtained Per Cent
0.1 gm. gelatin	1 ml.	nil	nil	0.93
0.1 gm. gelatin	1 ml.	0.5 gm.	nil	2.65
0.1 gm. gelatin	1 ml.	0.5 gm.	1 ml.	2.00
10 mg. hydroxy-proline	2 ml.	0.5 gm.	nil	7.25
10 mg. hydroxy-proline	2 ml.	0.5 gm.	1 ml.	3.25
10 mg. proline	2 ml.	0.5 gm.	nil	nil
10 mg. proline	2 ml.	0.5 gm.	1 ml.	8.44

These experiments suggested that the hydroxy-proline and proline molecules differ greatly in their susceptibility to oxidation. It would appear that in the case of hydroxy-proline the oxidation was too vigorous when copper sulphate was used with sodium peroxide and so the reaction was carried too far, resulting in a loss of a fraction of the pyrrole. Proline, on the other hand, is apparently more difficult to oxidize because when the copper sulphate was omitted the sodium peroxide alone was not capable of bringing about oxidation to pyrrole.

III. COLORIMETRIC METHODS FOR THE DETERMINATION OF PROLINE AND HYDROXY-PROLINE IN PROTEINS

A Colorimetric Method for the Determination of Hydroxy-Proline

It has been demonstrated that proline and hydroxy-proline were responsible for the production of pyrrole when gelatin was dry distilled. TO increase the yield under such conditions a number of oxidizing agents were examined and it was found that sodium peroxide and copper sulphate were excellent reagents for this purpose. Furthermore it was discovered that there was a marked difference in the susceptibility of proline and hydroxy-proline to oxidation. These facts suggested the possibility of evolving colorimetric methods for the quantitative determination of these two amino acids.

An examination of the literature revealed that few colour reactions were available for the detection of proline and hydroxy-proline. Morse (1933) proposed a new colour reaction for hydroxy-proline but he made no attempt to develop it into a quantitative method. Lang (1933) described a micro-method for the determination of these two amino acids. The protein hydrolysate under consideration was treated with sodium hypochlorite and Lang assumed that this reagent converted proline into pyrroline and hydroxy-proline into hydroxy-pyrroline. This method was criticised by Waldschmidt-Leitz and Akabori (1934) who maintained that Lang's method was based on a misapprehension. They

stated that hydroxy-proline when treated with hypochlorite gives pyrrole in 80% yield and not hydroxy-pyrroline. Moreover, they claimed that proline does not react with hypochlorite. These investigators applied Lang's method to the determination of hydroxy-proline in protein hydrolysates. They were unable to detect hydroxy-proline in casein and clupein by this method and their values for gelatin, namely 9.4 and 8.9%, were quite low as compared with 14.1% obtained by Dakin (1920) using extraction with butyl alcohol.

As far as could be ascertained, the above constituted the only known colorimetric method for the determination of hydroxy-proline. Morse's reaction was found to be very unsatisfactory, even as a qualitative test. However, he used sodium peroxide as an oxidising agent and this furnished a meagre starting point in the search for a colour reaction to distinguish hydroxy-proline from proline or other amino acids. With this background preliminary experiments were commenced to evolve a quantitative colorimetric method for the determination of hydroxy-proline in protein hydrolysates.

Many attempts were made to obtain a colour when solid sodium peroxide was added to a solution of hydroxy-proline but no success was achieved until a solution of copper sulphate was introduced into the reaction mixture. When this reagent was used a red colour was obtained and

after many experiments had been performed the following tentative procedure was adopted.

Tentative Procedure. To 1 ml. of a solution containing 5 mg. of hydroxy-proline was added 0.25 gm. of sodium peroxide and 1 ml. of a solution of 0.02 M copper sulphate. When effervescence had ceased, the mixture was neutralized with 2 N hydrochloric acid, using phenolphthalein as an indicator and then an excess of hydrochloric was added to bring the solution to an approximate concentration of 0.1 N. The solution was placed in a boiling water bath for a few minutes and a stable red colour developed.

The Effect of Copper Sulphate on the Production of the Colour. No colour was produced without the use of copper sulphate and proline gave no colour with or without copper even on heating for a prolonged period in a boiling water bath. If hydrogen sulphide was used to remove the copper before heating, it was found that the colour failed to develop. It was also observed that the addition of copper sulphate to the reaction mixture after acidifying with hydrochloric acid gave no colour.

At first the colour was developed by heating the solution with dilute hydrochloric acid but experiments demonstrated that condensation with isatin gave a more satisfactory colour.

When the copper sulphate was replaced by 1 ml. of ferric sulphate, nickelous sulphate or manganous sulph-

ate no colour was developed. The use of cobaltous sulphate produced a trace of colour.

Since the copper sulphate appeared to play a paramount role in the reaction under consideration it was decided to investigate the effect of various concentrations of this reagent upon the intensity of the colour. The data obtained are shown in Table XVIII, and from an examination of these data it is evident that under the conditions used a solution of 0.04 M copper sulphate brought about the maximum intensity of colour.

Table XVIII

The Effect of the Concentration of Copper Sulphate on the Colour Intensity

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Procedure: 1 ml. of a gelatin hydrolysate, 1 ml. of copper sulphate and 0.2 gm. of sodium peroxide, cooled and neutralized with 2 N hydrochloric acid. Made up to 10 ml.
Colour Reaction: 1 ml. of solution, 1 ml. of 0.05% isatin in glacial acetic acid and 1 ml. of 2 N hydrochloric acid were warmed for 1 minute, cooled and made up to 10 ml. with water.

Concentration of Copper Sulphate (molar)	Galvanometer Readings (520 Filter)
nil	nil
0.001	nil
0.02	21.75
0.03	8.00
0.04	7.00
0.05	8.25
0.075	9.75
0.10	19.00

The Use of Solid Sodium Peroxide

The next variable in the reaction under consideration to be examined was the use of solid sodium peroxide as the oxidizing agent. It was observed that the method of adding this reagent had a marked effect on the intensity of the colour produced. This fact was made evident by the values obtained in the following experiments.

A 0.2 gm. sample of powdered sodium peroxide was added quickly to a mixture of 1 ml. of a gelatin hydrolysate and 1 ml. of a 0.04 M solution of copper sulphate contained in a 50 ml. Erlenmeyer flask. The flask was rotated until all effervescence had ceased and then it was cooled in running water. The solution was neutralized with 2 N hydrochloric acid,and a slight excess of acid was added. It was made up to 10 ml.,and the colour was developed in the usual manner. A measurement of the intensity of the colour was made on the colorimeter, and a value of 28.50 was obtained, using the 520 filter.

The above experiment was repeated, and a reading of 23.00 was obtained. This indicated that the results could not be duplicated when this procedure was used. The effect of adding the sodium peroxide slowly was next observed, and it was found that a more intense colour was produced as indicated by a galvanometer reading of 13.50. When this experiment was repeated a value of 13.75 was obtained. This showed that the results could be readily duplicated under these conditions.

However, when the sodium peroxide was added still more slowly than in the above experiments, a reading of 9.50 was observed on the galvanometer. Experiments such as these suggested that the addition of solid sodium peroxide was unsatisfactory and so in the next determinations the sodium peroxide was added in solution.

The Use of Sodium Peroxide Solutions. Solutions of sodium peroxide were prepared, varying in concentration from 0.2% to 2% and these were used to determine the concentration of sodium peroxide which gave the maximum intensity of colour in the reaction under consideration. The data obtained are shown in Table XIX, and it is evident that 5 ml. of a 1% solution of sodium peroxide was sufficient to produce the maximum intensity of colour. An examination of Table XIX also reveals that the values can be readily duplicated under these conditions.

Table XIX

The Effect of Various Concentrations of Sodium Peroxide Solutions

Procedure: To 1 ml. of a gelatin hydrolysate was added 1 ml. of 0.04 M copper sulphate and 5 ml. of a sodium peroxide solution, and the usual procedure was followed for the development of the colour

Concentration of Sodium Peroxide used Per cent	Galvanometer Reading (520 Filter)
0.2	78.00
0.5	58.50
1.0	32.50
1.0	32.25
2.0	33.00

The Use of Hydrogen Peroxide and Sodium Hydroxide as the Oxidizing Agent. It was demonstrated in the above experiments that a 1% solution of sodium peroxide was satisfactory but it was believed that a solution of hydrogen peroxide and sodium hydroxide would be as effective and more convenient. By calculation it was found that 1 ml. of a 6% solution of hydrogen peroxide and 1 ml. of 10% sodium hydroxide would be equivalent to 5 ml. of a 1% solution of sodium peroxide. To make certain that these amounts of hydrogen peroxide and sodium hydroxide were sufficient experiments were conducted, varying the concentration of these reagents, and the data obtained are given in Table XX. An examination of this table reveals that 1 ml. of 6% hydrogen peroxide and 1 ml. of 10% sodium hydroxide are adequate and that the values can be readily duplicated.

Table XX

The Effect of Varying the Amounts of Sodium Hydroxide and Hydrogen Peroxide

Amount of 10% Sodium Hydroxide used (ml.)	Amount of 6% hydrogen peroxide used (ml.)	Galvanometer Reading (520 filter)
1.0	1.0	27.00
2.0	2.0	27.50
1.0	0.5	27.50

The Effect of Temperature and Time of Heating

Experiments were next performed in which the red colour was developed at 80°C and 100°C, and the effect of the time of heating at these temperatures was observed. These data are recorded in Table XXI.

Since a slightly more intense colour was obtained by heating at 100°C for only 1 minute it was decided to develop the red colour by heating the solution in a boiling water bath. An experiment was next performed to observe the effect of the time of heating at 100°C, and the data are recorded in Table XXI.

Table XXI
The Effect of Time of Heating on the Intensity of the Red Colour

Time (minutes)	Temperature (°C)	Galvanometer Reading (520 filter)
1	80	63.5
2	80	36.5
3	80	26.0
1	100	23.00
2	100	14.00
3	100	13.00
4	100	13.00
5	100	13.00

From this data it was evident that the maximum intensity of colour was developed in 3 minutes and so this time of heating was used in all subsequent experiments.

The Effect of the Hydrochloric Acid Concentration.

To discover the effect of the concentration of hydrochloric acid on the production of the red colour, a number of experiments were performed using the acid in varying strengths. The usual procedure was followed. Data secured in these experiments are shown in Table XXII, and it is evident that 2N acid is almost as effective as 12 N and so in all subsequent determinations 1 ml. of this concentration of hydrochloric acid was used in the development of the colour.

Table XXII

The Effect of Hydrochloric Acid on the Intensity of the Red Colour

Hydrochloric Acid Concentration (normality)	Galvanometer Reading (520 Filter)
12	39.00
6	37.00
2.4	39.00
2.0	41.50

The Effect of Isatin on the Colour Intensity.

It had been found by experiment that 1 ml. of 0.05% isatin in glacial acetic acid produced the maximum intensity in the development of the red colour. However, the value for the blank with this concentration of isatin was 76.00, and this was considered to be too high for the blank reading. Furthermore, when the intensity of red colour was

measured using all the available filters, the observations obtained suggested that two colours were being measured instead of one. These facts made it necessary to reduce the concentration of isatin used in the development of the colour, and so experiments were conducted to find the concentration of isatin which would produce the maximum intensity of colour and at the same time give a low reading in a blank determination. The data obtained from these experiments are recorded in Table XXIII.

Table XXIII

The Effect of Isatin Concentration on the Red Colour

Amount of 0.05% Isatin used (ml.)	Galvanometer Readings (520 filter)	
	For Red Colour	For Blank
0.05	49.50	69.00
0.10	47.00	71.00
0.15	44.50	72.00
0.20	35.50	69.00
0.30	36.00	70.00

Examination of Table, XXIII shows that the maximum intensity of colour was secured when 0.20 ml. of 0.05% isatin was used and the blank reading was the same as that obtained with much lower concentrations of this reagent.

It was considered advantageous to use 1 ml. of an isatin solution rather than 0.2 ml., and so 1 ml. of a 0.01% aqueous solution of isatin was used in all subsequent

experiments. Experience has shown that a fresh solution of the reagent should be prepared every few days. If this precaution is not observed, low values for hydroxy-proline will be obtained.

The Time of Heating the Reaction Mixture. In the preliminary experiments the mixture of hydroxy-proline, copper sulphate and sodium peroxide was stirred until effervescence ceased and then cooled before neutralization with hydrochloric acid. It was observed that the results could be more readily duplicated if the reaction mixture was allowed to stand for exactly 5 minutes from the time of preparation, shaken occasionally, and then placed on a boiling water bath for another five minutes. At the end of that time it was cooled in running water and neutralized with hydrochloric acid. To make certain that five minutes was sufficient time for heating on the water bath, an experiment was performed in which the reaction mixture was heated for ten minutes. It was found that almost exactly the same result was obtained as when the time of heating was five minutes. When the heating was omitted and the reaction mixture placed in the ice-box for ten minutes, it was found that no colour was developed. These experiments indicated that this heating on the water bath was necessary and that five minutes was an adequate time.

A Further Study of the Effect of Copper Sulphate

At the commencement of this investigation it was found that 1 ml. of 0.04 M copper sulphate produced the maximum colour under the conditions used at that time. Subsequently the procedure had been altered considerably, and so it was decided to study again the effect of the copper sulphate concentration upon the production of the red colour. 1 ml. of a gelatin hydrolysate was the source of the hydroxy-proline and the usual procedure was followed.

The data obtained are given in Table XXIV, and an examination of this table shows that a solution of 0.01 M copper sulphate produced the maximum intensity of colour, and so in all subsequent experiments, this concentration of copper sulphate was used.

Table XXIV

The Effect of Concentration of Copper Sulphate on the Intensity of the Red Colour

Concentration of Copper Sulphate (molar)	Galvanometer Reading (520 filter) (average of 2 determinations)
0.001	49.00
0.005	47.75
0.010	37.50
0.010	37.00
0.020	46.75
0.050	42.00
0.100	42.88

Stability of the Red Colour. The stability of the red colour was determined by measuring its intensity at various intervals of time, and the data obtained are given in Table XXV. These values show that this colour is quite stable.

Table XXV

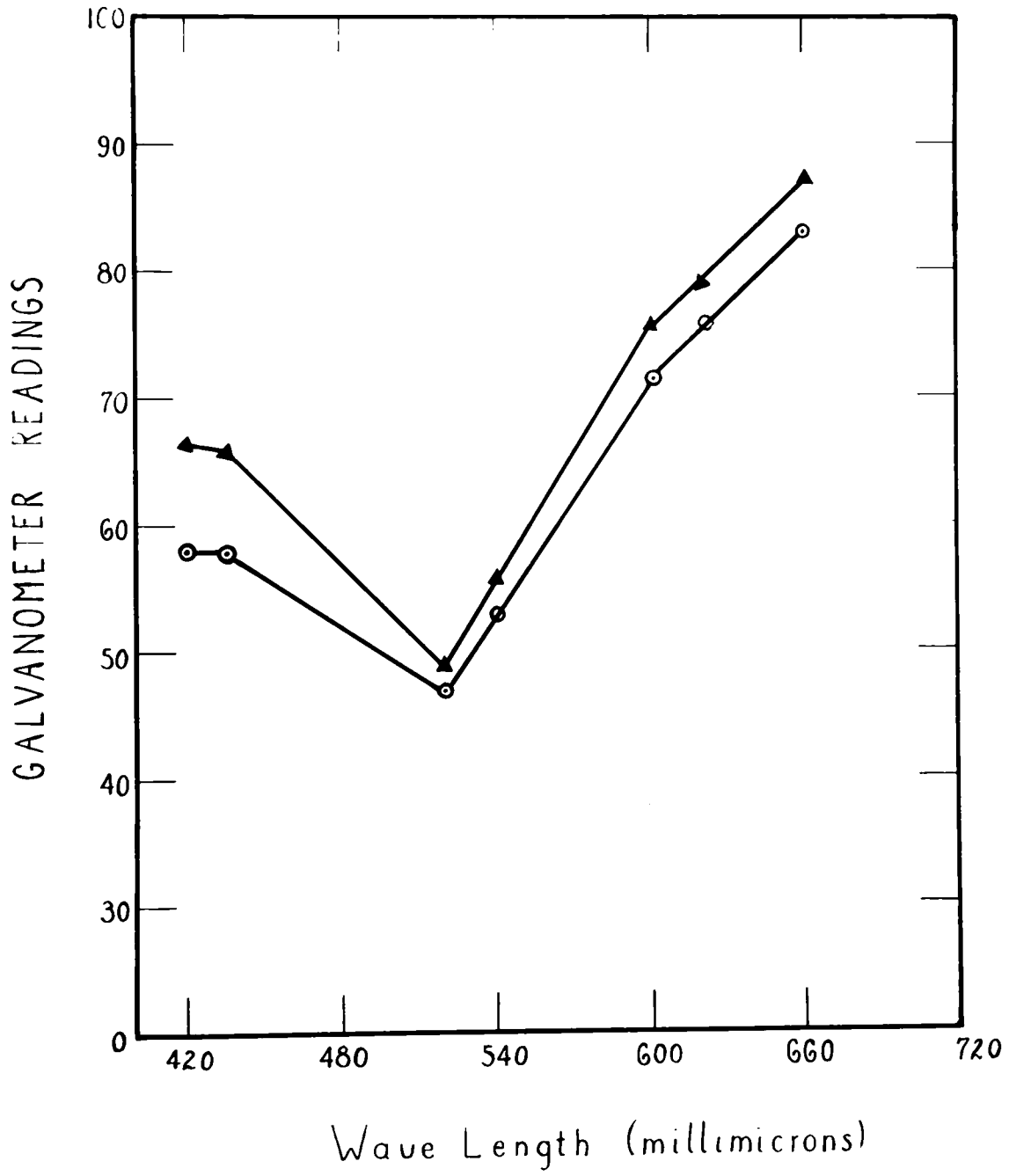
Stability of the Red Colour

Time (minutes)	Galvanometer Reading (520 Filter)
0	29.00
15	29.25
30	29.50
60	30.00

Preparation of Approximate Absorption Curves for the Red Colour. The question arose as to whether the red colour obtained from pure hydroxy-proline by this method was identical with that secured from a gelatin hydrolysate. The only available means to settle this problem was to prepare approximate absorption curves by the use of a series of filters, each transmitting narrow portions of the visible spectrum. The data observed are recorded in Table XXVI, and the curves are shown in Figure IV. An inspection of Figure IV suggests that the red colour obtained from a gelatin hydrolysate was identical with that developed from pure hydroxy-proline.

Figure 1V

Approximate Absorption Curves



Legend

▲ -Hydroxy-Proline

⊙ -Gelatin Hydrolysate

Table XXVI

Data for the Preparation of Absorption Curves for the Red Colour

Wave Length of Filter (millimicrons)	Galvanometer Readings	
	Hydroxy-Proline	Gelatin Hydrolysate
420	66.50	58.00
440	66.00	58.00
520	49.00	47.00
540	56.00	53.00
600	76.00	71.50
620	79.00	76.00
660	87.50	83.00

It is also evident, from an examination of Figure IV, that the peak of absorption occurs at 520 millimicrons. Accordingly, the 520 filter, standard for the instrument, was always used for this determination.

Preparation of the Calibration Curve. Some preliminary experiments had indicated that the reaction as evolved would likely serve as a quantitative colorimetric method for determination for hydroxy-proline in protein hydrolysates. Accordingly, the following experiments were performed to secure data for a calibration curve.

A 100 mg. sample of pure hydroxy-proline (Eastman Kodak) was dissolved in water, and the solution was made up to 50 ml. It was then poured into an accurate burette, and various amounts were transferred to 10 ml.

volumetric flasks. These flasks were made up carefully with water to the 10 ml. mark, and after being thoroughly mixed, 1 ml. was taken for the colorimetric determination.

The procedure used was as follows: 1 ml. of the hydroxy-proline solution was placed in a 50 ml. Erlenmeyer flask, and to it 1 ml. portions of copper sulphate (0.1 M), sodium hydroxide (10%), and hydrogen peroxide (6%) were added in the order mentioned. This mixture was allowed to stand for exactly five minutes, with shaking at frequent intervals. It was then placed on a boiling water bath for five minutes, cooled, and neutralized by the addition of 1.5 ml. of 2N hydrochloric acid. The solution was next made up with water to exactly 10 ml. in a 10 ml. graduated glass-stoppered cylinder. After thorough mixing, 1 ml. was taken from the cylinder with a pipette and placed in another dry 10 ml. graduated, glass-stoppered cylinder. 1 ml. of a freshly prepared aqueous solution of 0.01% isatin and 1 ml. of 2N hydrochloric acid were added to this 1 ml. portion of the solution, and the mixture was placed in a boiling water bath for exactly three minutes. The cylinder was first cooled in air for five minutes, and then cooling was continued in running water until the solution had attained the temperature of the laboratory. Water was added to the 10 ml. mark, and after being thoroughly mixed the red solution was placed in a colorimeter tube. The intensity of the colour was measured at once in the colorimeter, using the 520 light filter. The data obtained are

given in Table XXVII and from these data a calibration curve was prepared, which is shown in Figure V.

Table XXVII

To Show that Beer's Law Applies to the Red Colour Developed in the Determination of Hydroxy-Proline

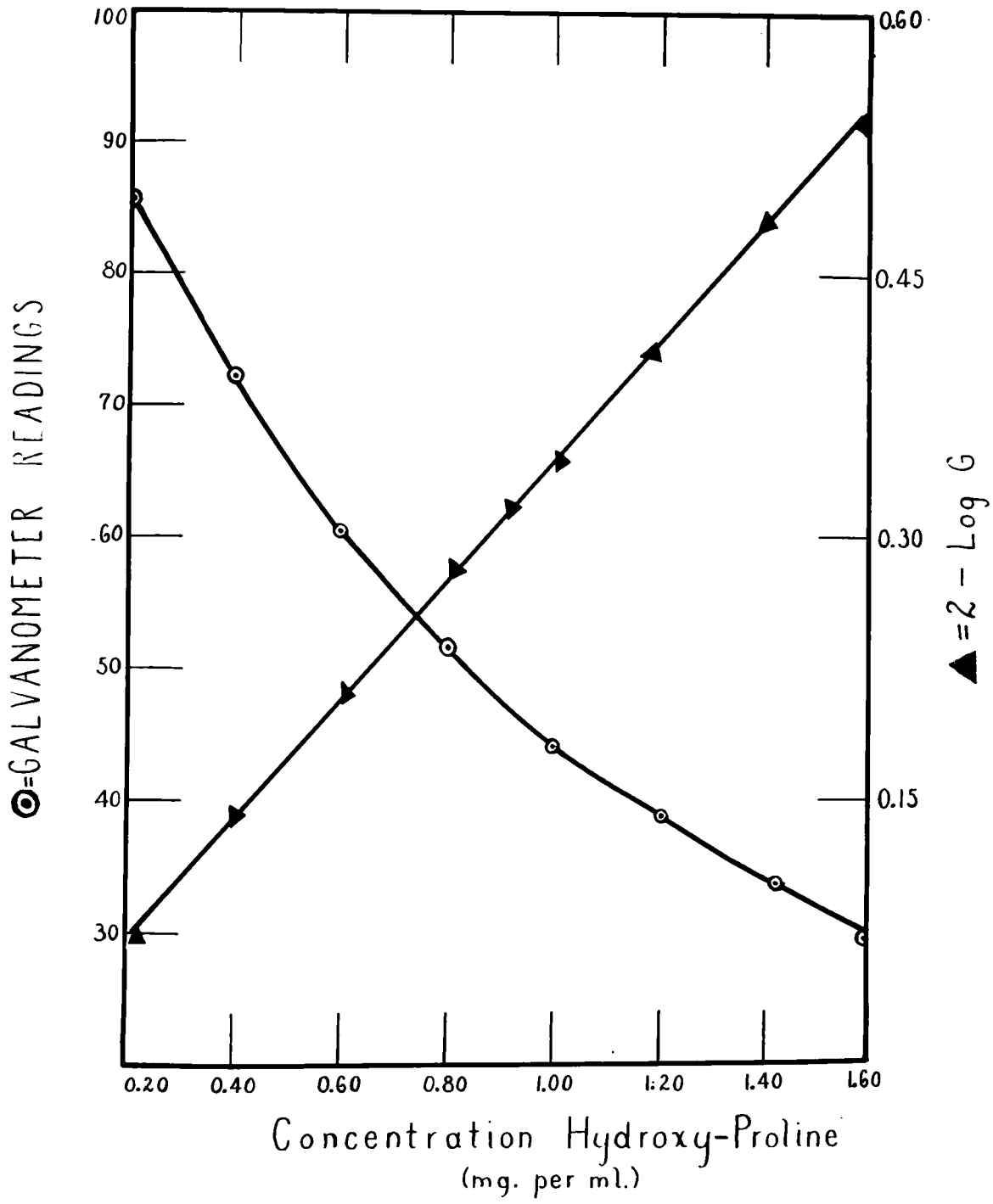
C Concentration of Hydroxy-Proline (mg. per ml.)	G Galvanometer Reading (520 filter) (average of 2 determinations)	L $L = 2-\log G$	K $K = \frac{2-\log G}{C}$
0.20	85.37	0.0687	0.3435
0.40	72.00	0.1427	0.3568
0.60	60.69	0.2168	0.3613
0.80	51.63	0.2870	0.3588
0.90	47.44	0.3230	0.3589
1.00	45.25	0.3440	0.3440
1.20	40.00	0.3980	0.3317
1.40	32.63	0.4860	0.3471
1.60	29.38	0.5320	0.3325

Mean Value of K = 0.3483 ± 0.0095

Examination of Table XXVII shows that the red colour developed in these determinations conforms to Beer's Law. By calculation the mean value of K was found to be 0.3483 ± 0.0095, with a maximum variation of 0.0166. From the straight line relation between hydroxy-proline concentration and 2-log G, as shown in Figure V, it is evident that K is a constant over the range plotted.

Figure V

Calibration Curve for Hydroxy-Proline



Application of the Method to Protein Hydrolysates.

To discover whether this method could be applied to the determination of hydroxy-proline in protein hydrolysates, a number of determinations were performed on gelatin hydrolysates. Various methods of hydrolysis were used, and comparable results were obtained. A few of these experiments are described below.

A 0.5 gm. sample of gelatin was heated with 10 ml. of 3N hydrochloric acid in an autoclave for 6 hours at a temperature of 150°C, and a pressure of 170 pounds. At the end of that time the hydrolysate was neutralized with sodium hydroxide to a phenolphthalein end-point, filtered and made up to 50 ml. with water. 1 ml. of this hydrolysate was taken for a determination, and the data obtained are recorded in Table XXVIII.The moisture content of the gelatin used in the experiments was found to be 10.17%, and the percentage of hydroxy-proline was always calculated on the dry basis. In this experiment, 14.53% of hydroxy-proline was found in the hydrolysate.

The above experiment was repeated, except that 10 ml. of concentrated hydrochloric acid was used instead of 10 ml. of 3N hydrochloric acid for hydrolysis. In this case, 13.69% of hydroxy-proline was found, indicating that a small amount of the amino acid was lost under these conditions.

A number of other similar determinations on gelatin were performed and the data from a few of these are

recorded in Table XXVIII.

It was found that saturated barium hydroxide was more satisfactory for the hydrolysis of proteins for this purpose than the other methods used, as the hydrolysates obtained by this method were almost free of coloured impurities. Gelatin hydrolysates were almost water clear and those from casein were light yellow in colour. A typical experiment using barium hydroxide is described below.

A 0.5 gm. sample of gelatin was heated with 20 ml. of saturated barium hydroxide in an autoclave at 120°C for six hours. At the end of that time, 5 ml. of 10% sulphuric acid was added to the hydrolysate, and after cooling the mixture was filtered and made up to volume. The 1 ml. portion which was taken for the hydroxy-proline determination was neutralized with a few drops of 10% sodium hydroxide, using phenolphthalein as an indicator, before the reagents were added. A total of 66.24 milligrams of hydroxy-proline was found in this hydrolysate, and calculation showed that this represented 14.75% of the sample.

The mean value of the results shown in Table XXVIII was found to be 14.03. This is in close agreement with the values obtained by Dakin (1920), 14.1%, and by Bergmann (1935), 14.4%.

Table XXVIII

The Determination of Hydroxy-Proline in Gelatin Hydrolysates

Method of Hydrolysis	Volume of Hydrolysate (ml.)	Galvanometer Reading (520 filter) (av. of 2 determinations)	Hydroxy-Proline (mg. per ml. of hydrolysate)	Hydroxy-Proline Per Cent (dry basis)
10 ml. 3N HCl, 6 hrs. 150°C	50	35.13	1.305	14.53
10 ml. conc. HCl, 6 hrs., 150°C	50	37.25	1.230	13.69
10 ml. 3N HCl, 5 hrs. 150°C.	50	35.63	1.288	14.34
10 ml. 3N HCl, 3 hrs. 150°C	50	36.00	1.275	14.19
10 ml. 3N HCl, 6 hrs. 150°C	50	34.75	1.318	14.67
20 ml. sat. $Ba(OH)_2$, 6 hrs., 120°C	115*	39.75	0.0576	14.75
15 ml. sat. $Ba(OH)_2$ 3 hrs., 150°C	50	39.00	1.174	13.07
Mean value of Hydroxy-Proline (%)			14.03	

* 2 ml. of this hydrolysate used for determination of hydroxy-proline.

To secure some information regarding the purity of the gelatin used in these experiments, analyses for nitrogen were made, using a micro-Kjeldahl method. The average of two determinations gave a value of 17.46% on the dry basis. This agrees closely with the value of 17.89% given by Mitchell and Hamilton (1929) and indicates that the sample of gelatin is reasonably pure.

Several attempts were made, without success, to determine hydroxy-proline in casein, lactalbumin, egg albumin, edestin, and blood albumin, and so it was evident that the method as employed was not suitable for the determination of very minute quantities of hydroxy-proline. but it could probably be modified for this purpose.

A Colorimetric Method for the Determination of Proline in Proteins

An examination of the literature revealed that apparently Lang's (1933) method was the only available colorimetric one for the determination of proline in protein hydrolysates. According to Waldschmidt-Leitz and Akabori (1934) his method is based on a misapprehension, and they claimed that it was only suitable for the determination of hydroxy-proline. Lang evidently did not recognize that proline and hydroxy-proline differ greatly in their susceptibility to oxidation. This difference was observed several times during the course of this investigation, and it must be considered in any method in which these compounds are both subjected to the same oxidizing agent as in Lang's (1933) method.

The Use of Sodium Peroxide and Copper Sulphate For the Oxidation of Proline.

It was found when proline was dry distilled with sodium peroxide and copper sulphate that pyrrole was present in the distillate. This experiment suggested that these reagents might be used to oxidize proline to pyrrole or some derivative of pyrrole which would give a colour with isatin or Ehrlich's reagent. With this idea in mind the following experiment was performed.

A mixture consisting of 1 ml. of proline containing 2 mg., 0.5 gm. sodium peroxide and 0.1 ml. of 10% cop-

per sulphate was heated in a test tube over a Bunsen burner for 5 minutes. The tube was cooled, and the solution was neutralized with hydrochloric acid, and the volume made up to 10 ml. 1 ml. of this solution was warmed on a boiling water bath for 1 minute with 0.2 ml. of a 2% solution of Ehrlich's reagent, dissolved in 95% alcohol. A red colour was observed. The isatin test for pyrrole was made on 1 ml. of this same solution, but no pyrrole-blue was observed. In addition, the procedure used for the determination of hydroxy-proline was applied to this solution but no colour was developed.

To discover whether hydroxy-proline would also react in a similar manner, the following experiment was performed. To 1 ml. of a hydroxy-proline solution containing 2 mg. was added 0.5 gm. sodium peroxide and 1 ml. of 10% copper sulphate. This mixture was placed in a test tube and heated on a boiling water bath for five minutes. It was then cooled and acidified with 1 ml. of concentrated hydrochloric acid. The solution was made up to 10 ml. with water, and 1 ml. was warmed with 0.2 ml. of 2% Ehrlich's reagent, and 1 ml. of 2N hydrochloric acid. A red colour was produced under these conditions.

<u>The Oxidation of Proline with a Mixture of Copper Sulphate, Sodium Hydroxide and Hydrogen Peroxide.</u>

To ascertain whether proline would be oxidized under less drastic conditions than those used in the above experiments, the following procedure was followed.

To 1 ml. of a proline solution containing 2 mg. was added 1 ml. of a 0.01 M solution of copper sulphate, 1 ml. of a 10% solution of sodium hydroxide and 1 ml. of a 6% solution of hydrogen peroxide. This mixture was subjected to the same treatment as was used in the determination of hydroxy-proline. The final solution was made up to 10 ml. and 1 ml. was used for the colour reaction with Ehrlich's reagent. A red colour similar to that observed in the above experiments was produced.

The above experiment was repeated except that a solution of hydroxy-proline was used instead of proline. A red colour was again developed with Ehrlich's reagent, which appeared to be the same shade of colour as was produced from proline under the same conditions.

A Suggested Method for the Determination of Proline in Protein Hydrolysates which also Contain Hydroxy-Proline

These preliminary experiments suggested the possibility of evolving a method for the determination of proline in protein hydrolysates which also contained hydroxy-proline.

The proposed procedure was as follows:-

(1) Determine the relative intensity of the red colour which is developed by the use of Ehrlich's reagent from the same amount of proline and hydroxy-proline under identical conditions. It was found that 1 mg. of hydroxy-proline produced the same intensity of colour as 10 mg. of proline.

(2) Determine the hydroxy-proline content of the hydrolysate by the colorimetric method already described.

(3) Measure the intensity of the red colour obtained from 1 ml. of the hydrolysate when Ehrlich's reagent is used, and from a calibration curve, prepared by subjecting various concentrations of proline to the procedure described in the above experiments and developing the colour with Ehrlich's reagent, the combined proline and hydroxy-proline content of the hydrolysate would be obtained in terms of mg. of proline.

(4) Convert the value obtained in (2) into equivalents of proline and subtract this from the value secured in (4). This difference would be an expression of the mg. of proline in 1 ml. of the hydrolysate.

To illustrate this method, the following example will be discussed. Suppose a hydrolysate contained 2 mg. of hydroxy-proline and 1 mg. of proline. By following the procedure outlined, in (3) we secure a value of 21 mg. of proline per ml. The value obtained in (2) could be 2 mg. of hydroxy-proline, and this would be equivalent to 20 mg. of proline. Subtraction of 20 mg. from 21 mg. gives us a value of 1 mg. per ml. for proline.

This is admittedly a rather involved procedure but it seemed to be the only way to determine proline colorimetrically when hydroxy-proline was present. Of course, in hydrolysates which contained no hydroxy-pro-

line, the method would be a straightforward one. With this idea in mind, a considerable amount of experimental work was performed. Some of these experiments are described in detail below.

The Effect of Temperature on the Intensity of the Colour Produced with Ehrlich's Reagent

To study the effect of temperature on the production of colour with Ehrlich's reagent, the following experiment was carried out.

A mixture of 5 ml. of 0.01 M copper sulphate solution, 5 ml. of 10% sodium hydroxide, and 5 ml. of 6% hydrogen peroxide was added to 5 ml. of a gelatin hydrolysate. The solution was allowed to stand for 5 minutes, then placed on a boiling water bath for the same length of time. It was next cooled in running water and neutralized with 3 N hydrochloric acid. The final solution was made up to a volume of 50 ml. with water. Six 1 ml. portions of this solution were treated with 0.2 ml. of 2% Ehrlich's reagent, and 1 ml. of 2N hydrochloric acid, and they were heated for 3 minutes at different temperatures. At the end of that time, they were cooled in running water and made up to 10 ml. with water. The intensity of the colour was measured by the colorimeter, using 520 and 540 filters. The data secured are shown in Table XXIX.

Table XXIX

The Effect of Temperature on Colour Produced with Ehrlich's Reagent

Temperature °C	Galvanometer Readings	
	520 Filter	540 Filter
50	81.50	80.00
60	68.00	67.00
70	49.00	50.00
80	25.50	27.50
90	18.00	19.00
100	19.50	20.50

Examination of Table XXIX shows that the maximum intensity of colour was developed at 90°C, and that there was a very slight decrease in the intensity at 100°C. Since it is difficult to maintain a constant temperature of 90°C, it was decided to use 100°C in the development of the colour. This temperature was used in all subsequent experiments to develop the red colour with Ehrlich's reagent.

It was discovered that dilution with 95% ethyl alcohol to the 10 ml. mark, after the colour had been produced, gave a more intense colour than when the dilution was performed with water. Thus, in one experiment, dilution with alcohol gave a reading on the galvanometer of 18.00, whereas dilution with water only gave a reading of 29.00. For this reason, alcohol was used in all subsequent experiments when diluting to the 10 ml. mark.

The Effect of Time of Heating on the Production of Colour

Experiments were next performed to study the effect of the time of heating in the water bath on the production of the colour. A gelatin hydrolysate was used and the usual procedure followed. The data obtained are recorded in Table XXX, and it is evident that heating for two minutes produced the maximum intensity of colour.

Table XXX

The Effect of Time of Heating on Production of Colour

Time (minutes)	Galvanometer Reading (520 filter)
0.5	64.00
1.0	39.00
1.5	38.00
2.0	37.50
3.0	41.00
5.0	42.00

The Effect of Various Concentrations of Ehrlich's Reagent on the Production of Colour

Experiments were next conducted, using the same procedure as above, to ascertain the effect of the concentration of Ehrlich's reagent on the production of the colour. The data obtained showed that 1 ml. of 2% Ehrlich's reagent produced the most intense colour, and so this concentration of the reagent was used in all subsequent experiments.

The Effect of Various Concentrations of Hydrochloric Acid on the Production of the Red Colour

To discover the effect of various concentrations of hydrochloric acid upon the production of the red colour, experiments were performed using different strengths of this reagent. The data obtained are given in Table XXXI. These observations showed that 2 N hydrochloric acid was the most effective, and so that concentration of acid was utilized in all the subsequent experiments.

Table XXXI

The Effect of Various Concentrations of Hydrochloric Acid

Normality of Hydrochloric acid	Galvanometer Reading (540 Filter)
0.5	63.00
1.0	43.00
1.5	40.00
2.0	40.00
3.0	48.50
12.0	86.00

Attempts to Remove the Oxidation Product of Proline and Hydroxy-Proline by Steam Distillation

It was considered that it would be a distinct advantage to be able to remove by steam distillation the compound which produced the red colour with Ehrlich's reagent. The reason for attempting to do this was that it was feared the reagent might possibly react with other substances in a protein hydrolysate besides the oxidation product of proline or hydroxy-proline. With this aim in mind, the following experiments were performed.

To a 1 ml. solution of hydroxy-proline containing 1 mg. was added 1 ml. of 0.01 M copper sulphate, 1 ml. of sodium hydroxide (10%), and 1 ml. of hydrogen peroxide (6%). This mixture was allowed to stand for 5 minutes and then steam distilled until 50 ml. of distillate was obtained. 1 ml. aliquots were taken for the tests with Ehrlich's reagent, and with isatin. In both cases no colour developed.

The above experiment was repeated, using 5 ml. of proline containing 4.0 mg., and 5 ml. of each of the reagents. The mixture was steam distilled until 25 ml. of distillate was obtained. 1 ml. portions were used for the tests with Ehrlich's reagent, and with isatin. In both cases the results were negative.

An examination of the liquid in the distilling flask showed that it gave a positive colour reaction with Ehrlich's reagent, but no colour could be developed with

isatin. This indicated, rather definitely, that the oxidation product formed under the conditions of the above procedure could not be removed by steam distillation.

An Attempt to Extract the Oxidation Product with Ether

An attempt was next made to extract the oxidation product with ether. 1 ml. of a gelatin hydrolysate was treated with the usual reagents, and the resulting mixture was extracted several times with 5 ml. portions of ether. The ether was evaporated off, in the manner as already described, and the residue was made up to 10 ml. 1 ml. of this solution failed to give a colour with Ehrlich's reagent.

The Use of Lead Dioxide to Oxidize Proline and Hydroxy-Proline

At this point in the investigation, it was decided to search for other means of oxidizing proline and hydroxy-proline with the hope of securing oxidation products which would be volatile in steam. After examining a number of oxidizing agents for this purpose, it was finally discovered that lead dioxide was capable of oxidizing both proline and hydroxy-proline to products which gave a red colour with Ehrlich's reagent. Furthermore, these oxidation products were also volatile in steam. A typical experiment which illustrates these facts is described below.

To 1 gm. of lead dioxide was added 2 ml. of proline containing 8 mg., and the mixture was steam distilled until 50 ml. of distillate was collected. 1 ml. was taken for

the colour reaction with Ehrlich's reagent, and a red colour was observed which gave a reading of 52.75 on the galvanometer when the 540 filter was used.

A number of similar experiments were conducted with solutions of proline and hydroxy-proline, but the results could not be duplicated when the above procedure was used. However, it was discovered that if the proline or hydroxy-proline solutions were first refluxed with lead dioxide for a definite time and then steam distilled, the values could be duplicated. It was found that 100 ml. of distillate must be collected to secure a complete recovery of the volatile oxidation product, and so in all subsequent steam distillation experiments this volume of distillate was collected.

The Effect of the Time of Refluxing on the Production of the Red Colour

Experiments were next performed to discover the effect of the time of refluxing on the production of the red colour. The procedure used in these experiments was as follows:- 5 ml. of a hydroxy-proline solution was added to 1 gm. of lead dioxide in a 50 ml. Erlenmeyer flask and the flask was fitted with an air condenser. The flask was placed in a boiling water bath and heated for various intervals of time. At the end of the time of heating, it was cooled in running water, and the contents were filtered. The condenser was washed with 25 ml. of water and the washings were added to the filtrate. The residue on the filter

paper was washed with water until the volume of the filtrate was 50 ml. 1 ml. of this solution was used for the development of the red colour by the usual procedure. The data obtained are shown in Table XXXII. After an examination of these data, it was decided to use 30 minutes as the time of refluxing in all subsequent experiments of this kind.

Table XXXII

The Effect of Time of Refluxing

Time of Refluxing (minutes)	Galvanometer Reading (540 Filter)
5	58.00
10	42.00
15	43.00
30	31.50
60	29.00

The Effect of Various Quantities of Lead Dioxide on the Colour Intensity

Similar experiments were conducted to discover the effect of various quantities of lead dioxide upon the reaction under consideration, and the data secured are shown in Table XXXIII. An examination of this table shows that 1 gm. of the oxidizing agent was adequate for the production of the maximum intensity of colour.

Table XXXIII

The Effect of Lead Dioxide Concentration

Amount of Lead Dioxide (gms.)	Galvanometer Reading (540 Filter)
0.2	45.50
0.5	41.50
1.0	27.00
1.5	26.00
2.0	28.50
3.0	29.00
5.0	43.25

Procedure Adopted, using Lead Dioxide and Steam Distillation

After many preliminary experiments had been performed, the following procedure was adopted:- 5 ml. of proline solution and 1 gm. of lead dioxide were refluxed for 30 minutes on a boiling water bath. The mixture was then cooled in running water and the condenser washed with 25 ml. of water. The lead dioxide was filtered off and washed with 25 ml. of water. The filtrate was collected in a steam distillation reaction flask and then it was steam distilled until 100 ml. of distillate was collected. 1 ml. of the distillate wasused for the colour reaction with Ehrlich's reagent.

Attempts to Determine Proline in Gelatin Hydrolysates

Using the above procedure calibration curves were prepared for proline and hydroxy-proline and many unsuccessful attempts were made to determine proline in gelatin hydrolysates by the method proposed on page 94 for the determination of proline in hydrolysates which also contain hydroxy-proline. In nearly all cases the values for proline were considerably lower than those given in the literature and so this proposed procedure had to be discarded and efforts were next made to evolve a method for the determination of proline in protein hydrolysates which were known to be almost free of hydroxy-proline.

The Effect of Phosphate Buffer Solutions on the Colour Production

A large number of preliminary experiments had indicated that the pH of the reaction mixture had a marked effect on the oxidation of proline and hydroxy-proline by lead dioxide. To discover what the effect of phosphate buffers would be on the reaction under consideration the following experiments were carried out.

Two ml. aliquots of an egg albumin hydrolysate were refluxed with 10 ml. of various phosphate buffers and 1 gm. of lead dioxide for 30 minutes. The usual procedure was employed except that a slightly different procedure was used in the development of the colour. The colour reaction used here consisted of heating 5 ml. of the final solution with 1 ml. of 4% Ehrlich's reagent and

1 ml. of 6 N hydrochloric acid on a boiling water bath for 1 minute. The data secured are recorded in Table XXXIV, and an examination of this table reveals that the phosphate buffer with a pH of 8.7 was the most effective, and so it was used in all subsequent experiments.

Table XXXIV

The Effect of Phosphate Buffer Solutions

pH of Buffer	Galvanometer Reading (540 Filter)
water (no buffer)	66.00
7.1	43.00
4.7	54.00
8.7	39.50

Colour Procedure Adopted

In the above experiment, 5 ml. of the final solution was used instead of 1 ml. for the development of the colour. To make the procedure even more sensitive, 10 ml. were henceforth taken for the colour reaction. Of course that meant that the variables in the colour reaction had to be studied again, with a view to securing those conditions which would produce the maximum intensity of colour. Experiments were performed to discover these conditions and finally the following colour procedure was adopted.

10 ml. of the final solution was heated with 1 ml. of 4% Ehrlich's solution in 95% alcohol and 1 ml. of 2 N hydrochloric acid for 1 minute on a boiling water bath. The solution was cooled for 5 minutes in air and then in running water. It was then poured into a colorimeter tube and the intensity of the colour was measured.

It was found that steam distillation appeared to destroy a fraction of the oxidation product and so that part of the procedure was discarded.

Procedure Adopted Using Lead Dioxide and Omitting Steam Distillation

After many preliminary experiments had been performed, it was decided to adopt the following procedure:

The volume of the solution under consideration was diluted to exactly 10 ml. with water and added to 10 ml. of phosphate buffer (pH of 8.7) and 1 gm. of lead dioxide. This mixture was refluxed for 30 minutes, cooled and filtered. The residue on the filter paper was washed with 25 ml. of water and the filtrate made up to a definite volume. 10 ml. was taken for the colour reaction.

Preparation of a Calibration Curve

To secure data for a calibration curve the following experiment was performed. 1 ml. of proline (5 mg.) was refluxed with the usual reagents, and the method as described above was followed. The filtrate was made up to 100 ml, and the colour reaction was performed on aliquots of this

solution. The data obtained are recorded in Table XXXV.

Table XXXV

To Show that Beer's Law Applies to the Red Colour Developed in the Proline Procedure

C Concentration of Proline (mg./100 ml.)	G Galvanometer Reading (520 Filter)	L $L = 2-\log G$	K $K = \frac{2-\log G}{C}$
1	87.50	0.0580	0.0580
2	76.00	0.1192	0.0596
3	66.00	0.1805	0.0602
4	59.00	0.2291	0.0573
5	54.50	0.2636	0.0527

Mean value of K = 0.0578 ± 0.0020

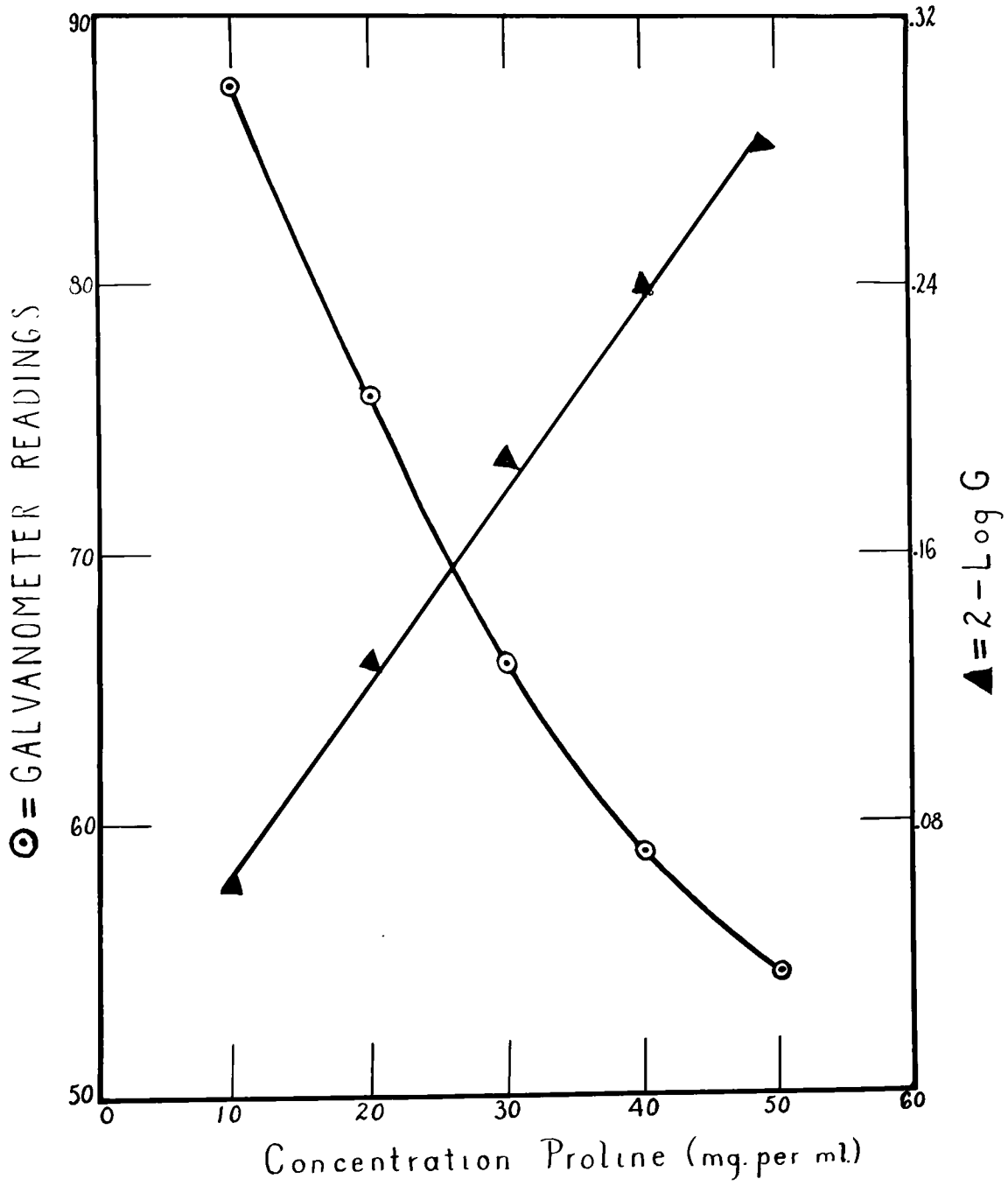
Examination of Table XXXV shows that the colour reaction used in the above procedure conforms to Beer's law. By calculation the mean value of K was found to be 0.0578 ± 0.0020, with a maximum variation of 0.0051. From the straight line relation between proline concentration and 2-log G, as shown in Figure VI, it is evident that K is a constant over the range plotted.

Application of this Method to Determination of Proline in Casein

To test the usefulness of this method the following experiment was performed. 2 gm. of casein was introduced into a 50 ml. Erlenmeyer flask and 40 ml. of saturated barium hydroxide was added to it. The flask was placed in

Figure V1

Calibration Curve for Proline



an autoclave for 3 hours at a temperature of 150°C, and a pressure of 70 lbs. At the end of that time 10 ml. of 10% sulphuric acid (10 ml. conc. sulphuric acid diluted to 100 ml. with water) was added to the flask and after being cooled, the mixture was filtered. The residue was washed with distilled water until exactly 100 ml. of filtrate were collected.

A 5 ml. aliquot of this filtrate was neutralized with 1 N sodium hydroxide to a phenolphthalein end-point, and the usual procedure was followed, except that the filtrate was made up to 200 ml. and 5 ml. of this was diluted to 10 ml. with water for the colour reaction. The average of two determinations gave a value of 78.50 for the galvanometer reading, using the 520 filter, and calculations using Beer's Law equation showed that 5 ml. of the casein hydrolysate contained 7.272 mg. of proline, or 145.44 mg. of the amino acid was contained in the sample used. The moisture content of the casein was 8.41%, and using this fact, further calculations gave a value of 7.94% for the proline content of this sample of casein.

The value of proline in casein obtained by this colorimetric procedure is in close agreement with that reported by Dakin (1918) of 8.0%, when he extracted proline from casein by his butyl alcohol method.

SUMMARY AND CONCLUSIONS

1. Fromm's quantitative method for the determination of small quantities of pyrrole has been adapted to be used with an Evelyn photoelectric colorimeter.

2. The blue colour produced by the condensation of pyrrole with isatin in the presence of hydrochloric acid was shown to conform with Beer's law.

3. As an aid in the quantitative determination of minute quantities of pyrrole in biological materials, mercuric chloride was used as a precipitating agent.

4. Ether extraction of pyrrole was also found to be amenable for the determination of small quantities of pyrrole.

5. Pyrrole was determined quantitatively in proteins and other biological materials by a dry distillation procedure. 1.20% was obtained from gelatin but the values secured for other proteins were considerably lower. Chlorophyll failed to produce pyrrole on dry distillation but it was obtained from corn leaves.

6. A search was made for pyrrole in the enzymic hydrolysates of certain proteins. No pyrrole was detected in these with isatin.

7. An attempt was made to detect pyrrole during the course of the hydrolysis of gelatin with sodium hydroxide. The means at our disposal failed to detect pyrrole.

8. It was found that pyrrole could be recovered quantitatively from alkaline solutions by steam distillation, but no pyrrole was detected in the distillate obtained when a mixture of gelatin and 20% sodium hydroxide was steam distilled.

9. A gelatin hydrolysate was fractionated to find what substance was responsible for the production of pyrrole on dry distillation. Most of the pyrrologenic material was found to be in the alcohol-soluble fraction.

10. Dry distillation of a number of amino acids was performed and only proline and hydroxy-proline gave pyrrole.

11. A study was made of the effect of a number of oxidizing agents on the yield of pyrrole by dry distillation. Sodium peroxide increased the yield to a marked extent.

12. Attempts to determine the temperature at which pyrrole is produced from a gelatin hydrolysate were made, and a temperature of at least 300°C was found to be necessary.

13. The effect of copper sulphate on the production of pyrrole from proline and hydroxy-proline on dry distillation with sodium peroxide was observed. Copper sulphate decreased the yield of pyrrole from hydroxy-proline, but its presence was essential for the production of pyrrole from proline. No pyrrole was obtained from the latter unless a copper sulphate solution was added before distillation.

14. A quantitative colorimetric method for the determination of hydroxy-proline has been evolved, and a number of determinations were performed on gelatin hydrolysates obtained by various methods of hydrolysis in an autoclave. The mean value of these determinations was 14.03% which is in close agreement with Dakin's value of 14.1%.

15. A colorimetric method was also developed for the quantitative determination of proline in protein hydrolysates which contain very little or no hydroxy-proline. Applying this method to casein, a value of 7.94% was obtained, which is in good agreement with Dakin's value of 8.00%.

As a result of this investigation, methods have been developed for the quantitative determination of small quantities of pyrrole in biological materials. The phenomenon of the production of pyrrole by dry distillation of proteins has been investigated, and it has been shown that in the case of gelatin the formation of pyrrole is due to the conversion of proline and hydroxy-proline into this compound. As far as could be ascertained with the means at our disposal, protein hydrolysates did not contain pyrrole and so it was concluded that hydrolysis of proteins did not liberate free pyrrole.

A quantitative colorimetric method has been made available for the determination of hydroxy-proline in protein hydrolysates in the presence of the other decomposition products. As yet it has only been applied to gelatin and does not appear to be suitable for minute quantities of hydroxy-proline as found in such proteins as casein. However, it is probable that this method can be modified to determine very small amounts of this amino acid. A quantitative colorimetric method was also evolved for the determination of proline in those proteins which contain little or no hydroxy-proline. This method was only applied to casein, but it could probably be applied to similar proteins.

ACKNOWLEDGMENT

The author wishes to express his sincere thanks to Dr. W. D. McFarlane under whose supervision this work was carried out, for his continued interest and helpful suggestions during the course of these investigations.

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