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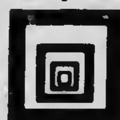

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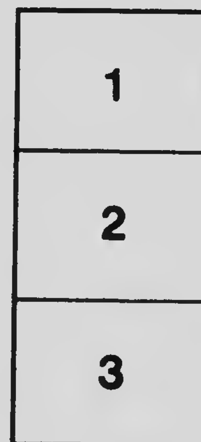

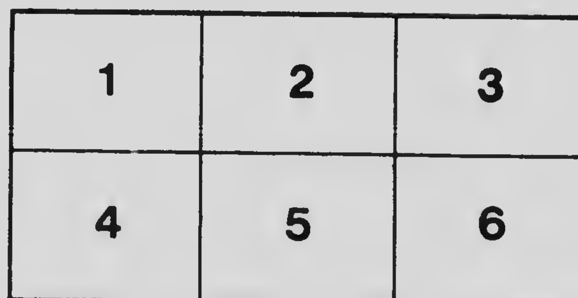

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No. 7: A SPECTROSCOPIC EXAMINATION OF THE COLOUR REACTIONS OF CERTAIN INDOL DERIVATIVES AND OF THE URINE OF DOGS AFTER THEIR ADMINISTRATION, BY ANNIE HOMER

No. 8: A METHOD FOR THE ESTIMATION OF THE TRYPTOPHANE CONTENTS OF PROTEINS, INVOLVING THE USE OF BARYTA AS A HYDROLYZING AGENT, BY ANNIE HOMER

No. 9: THE RELATION BETWEEN THE ADMINISTRATION OF TRYPTOPHANE TO DOGS AND THE ELIMINATION OF KYNURENIC ACID IN THEIR URINE, BY ANNIE HOMER

(REPRINTED FROM THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOL XXII.)

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A SPECTROSCOPIC EXAMINATION OF THE COLOR REACTIONS OF CERTAIN INDOL DERIVATIVES AND OF THE URINE OF DOGS AFTER THEIR ADMINISTRATION.

By ANNIE HOMER.

(From the Lister Institute, London, and the Departments of Biochemistry and Pathological Chemistry, University of Toronto.)

(Received for publication, July 7, 1915.)

A study of the literature of the subject of urinary pigments shows that the presence of a variety of chromogens has been detected in normal and pathological urines amongst which may be mentioned: urochrom, urobilin, urohematoporphyrin, urohematin, urocyanin, indirubin, indigotin, urorosein, uroerythrin, purpurin, skatol red, etc.

There is some difference of opinion as to whether the above mentioned substances represent separate pigments, or whether some of the color reactions were due to the presence of the same chromogen in an impure condition, or even to a mixture of two or more commonly occurring chromogens. It will be seen that there has been much discussion as to whether the indigotin of animals and of plants were one and the same substance; whether the indirubin of Rosin is not identical with the uroerythrin of Simon, with the urorubihematin of Harley, with the purpurin of Golding Bird, with the uromelanin of Thudichum, and with the urorosein of Sieber; whether the skatol red of Brieger and urorosein, uroerythrin, and purpurin are not the same substance; whether urobilin is a mixture of pigments, etc.

It is quite possible that a pigment noticed and named by one observer was in reality the same as that noted by another as a new substance under the special conditions of his experiments.

With a complex fluid such as urine in which there may be several chromogens present the question as to the possible identification of a pigment by its absorption spectrum is open to criticism un-

less precautions are taken to ensure the elimination of the other chromogens present in the fluid. A little experience with spectroscopic analysis shows that the position of the absorption bands of a substance in solution is often affected by the solvent and by the reaction of the solution. Moreover, if there are two or more substances in solution each of which has a characteristic absorption spectrum then the absorption bands of the one will be affected by the presence of the other in the solution and *vice versa*.

From considerations such as these it is obvious that, unless steps be taken to separate the pigment under observation from others in solution, varying results will be obtained and some already known chromogen will be described as an apparently new pigment precursor in the urine.

In many cases of the description and identification of pigments in the urine no exact measurements of the color reaction have been recorded; this in itself leads to confusion as neither the naked eye nor a spectroscope with the Fraunhoffer lines as the only scale indication can be used with any degree of accuracy. Moreover, many of the observations recorded have not been made under comparable conditions; this again leads to the production of contradictory results.

It was ascertained that there was a certain amount of fragmentary date as to the color reactions of some indol derivatives; in a few cases measurements had been made of the pigments obtained from the chromogens in the urine after the administration of certain indol substances and speculations as to the possibility of the precursors of certain pathological pigments being indol derivatives had been put forward.

As I was fortunate enough to have in my possession specimens of several indol compounds closely related to tryptophane, it was thought of interest and perhaps of service to make a systematic spectroscopic study of the color reactions of these compounds and of the urine of dogs to which these substances had been administered whether by mouth or by subcutaneous injection; such a study might demonstrate the connection between certain urinary pigments and indol derivatives.

It was also possible that a comparative study of the absorption spectra thus mapped out would furnish indirect qualitative evidence as to whether the absorption of tryptophane from the ali-

mentary canal normally involves its preliminary decomposition by intestinal bacteria, the quantitative aspect of which problem is being carried out in conjunction with Dr. Hopkins of Cambridge, England.

The substances investigated were: indol, skatol, indolaldehyde, indolcarboxylic acid, indolacetic, indolaceturic, and indolpropionic acids, isatin, tryptophane, and some of the condensation products of tryptophane with aldehydo- and keto-compounds.

The method of procedure was as follows.

1. *For the Substances Themselves.*—Dilute aqueous solutions or suspensions of the substances were treated with concentrated hydrochloric acid and, if necessary, with a few drops of a dilute solution of an oxidizing agent such as potassium nitrite, ferric chloride, hydrogen peroxide, or calcium hypochlorite. In most cases it was necessary to warm the reacting liquids for the production of the color reaction; in some the reaction took place at room temperature. The concentration of acid required for the reactions varied considerably; in each case the temperature of the reaction and the degree of acidity were adjusted so as to ensure the production of the full color reaction and to make the absorption bands as pronounced and as sharp as possible.

In most cases the pigments were extracted with an organic solvent and the extract was well washed with dilute acid; in others it was found advisable to examine the acid solutions directly

It was noticed with all these indol compounds that an excess of the oxidizing agent transformed the color of the solutions to a yellowish or brown shade; also that the pigments in solution in organic or inorganic solvents required the presence of acidic ions for their colors to be evident; alkalis converted the color of the solutions from purple or red to brown.

2. *For the Urines under Investigation.*—The samples of the urines of dogs to which the above mentioned substances had been administered by mouth or by subcutaneous injection were subjected to the treatment used for the substances themselves. It was necessary to add acid to the extent of one-half to one-third of the volume of urine taken, and it was noticed that a greater amount of oxidizing agent was required than for the aqueous solutions of the substances themselves; this was to be expected.

The dogs were put on a diet on which they were not excreting the chromogen of indican. The normal urine of every dog used was tested for the presence of the chromogens of indican, urobilin, and other pigments.

It was found that the dogs employed excreted no indoxyl compounds and no urobilin on diets of bread and milk. Even after the administration of comparatively large doses of some of the above mentioned indol compounds there was no urobilin detected in the urine.

In the case of pathological human urines it was found necessary to remove urobilin by means of lead acetate solution before proceeding to an examination for indol pigments. If indican were present, the urine, after treatment with nitrite or other oxidizing agent, was repeatedly extracted with chloroform to remove all traces of indigo pigments before attempting to make an examination of the amyl alcohol-soluble pigments. After the complete removal of the chloroform-soluble substances the urine was extracted with amyl alcohol and the extract well washed with dilute acid before being examined spectroscopically.

a. A Spectroscopic Examination of the Color Reactions of Various Indol Derivatives.

An examination of Table I shows that of the simple indol compounds all except indolpropionic acid gave characteristic absorption spectra: none of them gave any indication of the production of indigo by the oxidation methods employed.

Indolaldehyde gave a color reaction with acid alone; the addition of an oxidizing agent did not affect the position of the absorption bands. The position of these bands measured with the instruments at my disposal agreed with those given by Ellinger (Table III).

It will be seen that indolaldehyde, skatol, and tryptophane have an absorption band in common, *viz:* λ549–536. Indolacetic acid has a corresponding band but the position is slightly shifted (λ552–539).

Indolacetic acid, indolaceturic acid, and tryptophane show signs of a band λ505–485 which is the characteristic absorption band of the indolcarboxylic acid pigment. It looks as though

in the oxidation of these substances there is a tendency to produce indolcarboxylic acid or some product in common with that formed from the latter substance.

b. A Spectroscopic Examination of the Urine of Dogs after the Administration of Various Indol Derivatives.

An examination of Table II shows that the administration of indol and indolaldehyde was followed by a vivid indican reaction which could be detected for three days after doses of 0.1 gram of these substances.

After the administration of skatol the dog urine exhibited a well marked cuior reaction and the absorption band agreed with that given by Brieger and by Porcher and Hervieux for skatol red (Table III). On the second day after the dose, whether given by mouth or cubcutaneously, the urine gave a well marked indican reaction.

The administration of indolcarboxylic acid was followed by the elimination of the substance in the unchanged state, a fact evidenced by the color reaction of the urine.[1]

Laidlaw and Ewins[2] have shown that the administration of indolacetic acid is followed by the elimination of indolaceturic acid. The accompanying comparison (Tables I and II) of the color reactions of the urine after the administration of indolacetic acid with the color reactions of indolaceturic acid itself lends support to the quantitative experiments of these observers. It was noticed, however, that if the urines were allowed to become "stale" then the color reactions of the urine were those of indolacetic and not of indolaceturic acid.

Peculiar interest is attached to the chromogen present in the urine after the administration of indolpropionic acid. The acid itself does not give any characteristic color reaction with an oxidizing agent, but administration of the substance to a dog is followed by the elimination of a chromogen which is very sensitive to the merest trace of acid.

The color reaction was given by the urine on addition of acid and being allowed to stand at room temperature in contact with

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[1] The excreted indolcarboxylic acid was identified by qualitative and quantitative analyses (unpublished observations).

[2] Ewins, A., and Laidlaw, P. P., *Biochem. Jour.*, 1913, vii, 18.

air but the absorption spectrum exhibited under these conditions is not the same as that given by the urine after oxidation with nitrite or with ferric chloride and acid.

The production of a cherry-red color from the urine suggested the presence of the chromogen of urorosein, but the spectroscopic examination revealed the presence of a substance different from the chromogen of urorosein.

From Table II it will be seen that incomplete oxidation of the chromogen under consideration gives rise to the bands λ557–537 and λ521–496 whereas after complete oxidation three bands were measured, *viz.*, λ587–561, λ546–533, and λ499–485 of which the band λ499–485 was the best marked. The wave length of the third band suggests that of urobilin (Garrod and Hopkins λ505–485) and the question arose as to whether this band was an inherent property of the pigment or whether it indicated the presence of urobilin, a factor which might well be introduced on account of the reaction of the animal to this substance.[3]

It was found that the unknown chromogen like urobilin was precipitated by lead acetate solution and by saturation of the urine with ammonium sulphate. The precipitated chromogen (Garrod and Hopkins' method for the separation of urobilin was used) was carefully tested for the presence of urobilin; the ether-chloroform solution showed a faint, ill defined band at about λ490. An alcoholic solution of the chromogen made alkaline with ammonia and treated with zinc chloride did not give a fluorescent solution. It was obvious that there could not have been more than a trace of urobilin present in the urine.

The precipitated chromogen when treated with acid and an oxidizing agent was converted into the colored substance having the three well marked bands described above.

From these observations it is evident that the band λ499–485 is an inherent property of the pigment and is not due to the presence of urobilin.

The production of this pigment is of interest as it probably represents some profound change in the molecule of indolpropionic acid during its sojourn in the animal body. The chemical

[3] The account of the chemical and physiological reaction of the animal body to this substance will be given in a later paper by Dr. Hopkins and the author.

aspect of this change is one of the problems which Dr. Hopkins and the writer of this paper are attempting to solve.

Administration of tryptophane, whether in small or large amounts, and also of its condensation products with formaldehyde, with glyoxylic acid, and with pyruvic acid, was not followed by the elimination of any corresponding chromogen in the unrine there was no indication of the chromogens of indican or of any other indol pigment, of urobilin, or of any unchanged tryptophane in the urine.

The spectroscopic evidence is in favor of the statement that normally the metabolism of tryptophane in the dog does not require the preliminary formation of the substances known to be the products of bacterial action on tryptophane. In the feeding experiments under consideration as much as 10 grams of tryptophane, both in the solid form and in solution were given in single doses to a dog of 23½ pounds' weight. In this way the organs of the animal were overloaded with the substance and the chances were that any of the above mentioned bacterial products, if formed from tryptophane normally, would be present under these conditions in sufficiently large amounts to be detected in the urine by their particular color reactions as it had been observed that after the administration of the various indol derivatives the color reaction, even in the case of small doses, could be detected in the urine excreted during the next thirty-six hours or more.

In certain pathological conditions or by the adoption of some special diet whereby the flora of the intestine is altered, the metabolism of tryptophane is often affected so that there is production of indol compounds leading to the elimination of the chromogens of indigo and of indol pigments in the urine.

The pigments of clinical interest in the urine which seem to have a close connection with indol compounds are *urorosein* and *indican.*

The urorosein reaction is characteristic of the urines of patients suffering from a variety of diseases such as chlorosis, pulmonary diseases, osteo-malacia, nephritis, typhoid, carcinoma of the esophagus, ulcer of the stomach, anacidity, perityphlitis, and diabetes. The production of the chromogen of this pigment from such a variety of diseases is probably due to disturbances in the intestinal tract, but at the same time it must not be forgotten that the detection of a

chromogen by the production of a rose-red color is somewhat unreliable unless the position of the absorption band is measured in each case. It has been my own experience with several pathological urines sent to me as exhibiting the urorosein reaction that, if the color reaction were carefully carried out so as to get the maximum effect, the spectroscopic examination revealed the presence of indican and not of urorosein.

Nencki and Sieber,[4] Rosin,[5] and Garrod and Hopkins[6] have made careful measurements of the absorption band exhibited by urorosein so that its detection, provided the investigator possesses a spectroscope fitted with a scale, should be a simple matter.

Herter[7] investigating the case of a child with a peculiar intestinal flora whose urine exhibited a marked urorosein reaction isolated indolacetic acid as the precursor of the pigment. But Laidlaw and Ewins[3] have shown that absorption of indolacetic acid from the intestine was followed by the elimination of indolaceturic acid in the urine. Noticing the color of the urine they suggested that indolaceturic acid was the chromogen of the pigment but they did not make measurements of the absorption band exhibited by the pigment derived from indolaceturic acid or by urorosein urines.

The work described in this paper had been started some time previous to the publication of Laidlaw and Ewins' paper; these observers very kindly furnished me with a colorless sample of crystalline indolaceturic acid and so enabled me to continue my spectroscopic work without having to isolate this substance from the urine of dogs to which I had administered indolacetic acid for other purposes.

It will be seen from the accompanying tables that indolacetic acid treated with an acid and an oxidizing agent gave a characteristic absorption band λ552–539; indolaceturic acid under the same conditions gave a well marked band λ579–539. Of these two the mean position for the indolaceturic pigment agrees with that given by Nencki, Rosin, and Garrod and Hopkins for urorosein urines.

Laidlaw and Ewins' observations on the fate of indolacetic acid in the organism together with the spectroscopic evidence adduced in this paper make it unlikely that indolacetic acid is the

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[4] Nencki, M., and Sieber, N., *Jour. f. prakt. Chem.*, 1882, cxxxiv, 333.
[5] Rosin, H. R., *Arch. f. path. Anat. u. Physiol.*, 1891, cxxiii, 519.
[6] Garrod, A. E., and Hopkins, G., *Jour. Physiol.*, 1896, xx, 112.
[7] Herter, C. A., *Jour. Biol. Chem*,. 1908. ccxxxix, 253.

chromogen of urorosein but at the same t[illegible] the fact that Herter actually isolated indolacetic acid as the [illegible]hromogen has to be accounted for.

In order to throw some light on the subject I attempted to isolate the chromogen from human urines exhibiting the urorosein reaction. There were many difficulties in the way, amongst others the fact that so many of the urines sent to me did not yield the urorosein but the indican reaction and that in many cases (especially of chlorosis) the reaction was so slight that there was little hope of isolating a crystalline product: a fraction of a milligram of the pure substance gives an intense color reaction.

There was sent to me a sample of urine from a patient at Guy's Hospital, London, suffering from achlorhydria gastrica. In the first sample sent there was indication of the presence of indolaceturic acid (absorption band λ573–540). The urine, evaporated to half its bulk *in vacuo*, was treated according to the directions given by Laidlaw and Ewins as follows: The urine after saturation with ammonium sulphate was acidified, extracted with ether, and the ethereal layer washed with ammonium sulphate solution and then with water. The acid was shaken out of the ether with sodium carbonate solution which was withdrawn, acidified, and again saturated with ammonium sulphate and extracted with ether. The ether extract was washed as before. After the removal of the ether by evaporation a small amount of a gummy oil was left. However, this would not form a picrate in the manner described by Laidlaw and Ewins.

The gummy residue gave an intense glyoxylic reaction and on treatment with acid and an oxidizing agent gave an intense color λ553–533 which proved to be that of indolacetic acid and not of indolaceturic acid and also not that of the original urine.

It was obvious that during the concentration and subsequent treatment the chromogen in the urine had been hydrolyzed to indolacetic acid.

Further samples were sent from the same case. However, a spectroscopic examination after the removal of urobilin revealed the presence of indolacetic and not indolaceturic acids. The urine was extracted as before and an oily magma of crystals was obtained; these crystals were identified as hippuric acid. The oily magma was therefore dissolved in 5 per cent. sulphuric acid and precipi-

tated with mercury reagent. After the decomposition of the mercury precipitate in the usual way the filtrate from the sulphide was freed from sulphuretted hydrogen and from acid, and extracted with ether. The ethereal solution was well washed and after the evaporation of the ether there remained a small amount of an oily substance which slowly began to crystallize. The crystalline residue was too small in amount to be recrystallized for melting point determinations, but spectroscopic examination of the crystals indicated that the substance was indolacetic acid.

In another case of urine supposed to be exhibiting the urorosein reaction it was observed that the absorption band was λ533–524; this corresponds to neither indolacetic nor indolaceturic acid pigments and was probably due to the presence of a mixture of pigments, although to the eye the color was that of urorosein.

Moreover, it was found that with samples of the urine of dogs after the administration of indolacetic acid to them, if the urorosein test were made with the freshly excreted urine the reactions were those of indolaceturic acid, but if the urine were allowed to become "stale" the color reaction changed to that of indolacetic acid. Further, if crystalline indolaceturic acid were added to normal urine which was then allowed to stand for some days, the color reaction changed to that of indolacetic acid.

From the work of Laidlaw and Ewins and from the evidence adduced in this paper it is obvious that the chromogen of urorosein is indolaceturic acid; from the above observations it is possible that on standing the indolaceturic acid urines may undergo bacterial decomposition with formation of indolacetic acid and glycine and, further, that even starting with a urine giving the reaction for indolaceturic acid, attempts to isolate the latter substance when present in moderately small amounts may fail on account of its hydrolysis into indolacetic acid.

From considerations such as these it is easy to understand the isolation of indolacetic acid and not of indolaceturic acid by Herter as the chromogen of urorosein. Had he measured the position of the absorption band of the pigment derived from indolacetic acid he would have seen that it did not agree with that given by Rosin and others for the absorption band of urorosein.

It is thus evident that the urorosein reaction is due to the presence of indolaceturic acid in the urine which (from the obser-

vations of Laidlaw and Ewins) has been formed from indolacetic acid absorbed from the intestine. Indolacetic acid may therefore be regarded as the precursor of the pigment urorosein in the metabolic functions of the body but indolaceturic acid is the actual chromogen of the pigment: the failure to realize this point may lead to confusion in the examination of urines with a "rose-red" color reaction.

In connection with a study of the urorosein reaction it seemed necessary to consider the question of the identity of ***skatol red*** and ***urorosein***.

Brieger[8] isolated skatol from the products obtained by the distillation of feces and showed that after administration of this substance to a dog the urine contained a chromogen which on oxidation gave rise to a pigment which he called *skatol red*. The work of Brieger, Mester,[9] and others has shown that the chromogen of skatol red is skatoxyl sulphate. The only evidence of the occurrence of skatoxyl compounds in human urines is that furnished by Otto[10] who has shown that in the early stages of diabetes mellitus indoxyl compounds could be isolated from the urine whereas in the later phases of the disease, when gastric symptoms occurred, skatoxyl compounds were excreted.

In view of the more recent work on the color reactions of the urine of patients with disordered digestive functions and of the capacity of substances, even when analytically pure, to adsorb chromogenic materials this work of Otto calls for repetition, especially as he identified the compound isolated by him by analysis of the nitrogen and sulphuric acid constituents only; analysis of the carbon and hydrogen content was also necessary in a case in which a decision was being made between compounds whose composition and molecular weights so nearly approximated.

But whether skatoxyl compounds are to be found in human urines or not, the fact remains that skatol after administration by mouth or by subcutaneous injection is eliminated as skatoxyl sulphate, and that this substance on treatment with an acid and an oxidizing agent is transformed into a pigment having an absorption band –577–550 (Brieger). The position of this band is the same as that observed for urorosein. Brieger considered it different from indirubin but the same as the pigments urorubin, urorosein, uroerythrin, and purpurin, a view shared by Porcher and Hervieux[11] but not held by Rössler[12] and Maillard.[13]

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[8] Brieger, L., *Ztschr. f. physiol. Chem.*, 1880, iv, 414.

[9] Mester, B., *ibid.*, 1888, xii, 130.

[10] Otto, J. G., *Arch. f. d. ges. Physiol.*, 1884, xxxiii, 607.

[11] Porcher, C., and Hervieux, C., *Compt. rend. Acad. d. sc.*, 1904, cxxxviii, 1725.

[12] Rössler, C., *Centralbl. f. inn. Med.*, 1901, xxii, 847.

[13] Maillard, L. C., *Ztschr. f. physoil. Chem.*, 1905, xlvi, 515.

Maillard considered that the skatol red of Brieger was not a pure pigment but was contaminated with indirubin and, further, that the skatoxyl eliminated in the urine does not account for the whole of the skatol injected; he suggests that some may have been converted into an indoxyl compound. Porcher and Hervieux were opposed to Maillard's views and considered that skatol becomes entirely converted into skatoxyl: they emphatically upheld their views as to the identity of skatol red and the above mentioned pigments.

The work of MacMunn and of Garrod has demonstrated that urorosein and uroerythrin are different substances (Table III).

From the work of Laidlaw and Ewins, of Herter, and from the evidence adduced in this research, it seems clear that the precursor of the urorosein pigment is indolaceturic acid. On the other hand it has been clearly demonstrated by Brieger, Otto, and Mester that the chromogen of skatol red is skatoxyl. But the evidence as to the occurrence of skatoxyl in human urines is by no means established whereas the urorosein reaction has been commonly noted and its connection with indolacetic and indolaceturic acids demonstrated.

It is thus obvious that under suitable conditions there can be isolated from the urine two distinct chromogens which produce the same color reaction. Some explanation of this phenomenon must be found.

It is possible that the same oxidation product may have been formed from these two chromogens, although the fact that the absorption spectrum of the indolacetic acid pigment is different from that of the indolaceturic acid pigment does not favor this hypothesis.

It is known that the character of an absorption spectrum depends on the atomic and molecular vibrations of the substance and that the actual position of the bands is influenced by the size, character, and weighting effect of the side chains of the ringed compounds: thus, the position of the band of the indolaceturic acid pigment is nearer the red end of the spectrum than that of the indolacetic acid pigment, and the latter nearer the red than the indolcarboxylic acid pigment. There is very little difference between the weighting of the molecules of indolaceturic acid and skatoxyl sulphate, and it is conceivable that the weighting of their respective oxidation products (which presumably belong to the same type of compound) is such that there is similarity of their absorption spectra both as regards character and position of absorption bands; but even so, until we know the type of reaction this color reaction involves it is with extreme diffidence that suggestions of this kind are brought forward.

At the same time it must be remembered that even though there is a certain

similarity between the colors produced in the two cases absolute identity or a difference cannot be claimed until the colored substances themselves thus formed have been isolated and identified; for the present purposes such a course is not expedient.

However, an examination of the pigments formed from indolaceturic acid and from skatoxyl urines indicated that although at first the evidence seemed to point to their identity it was shown later that skatol red apparently consists of two pigments.

The two chromogens of the pigments whose identity was under consideration were not precipitated to any appreciable extent by the addition of lead acetate solution to the urine; they were also incompletely precipitated by saturation of the urine with ammonium sulphate.

The pigments were insoluble in chloroform, ether, and xylol but were readily soluble in ethyl and amyl alcohols.

The *indolaceturic pigment* is insoluble in chloroform to which has been added one-third its bulk of alcohol, but if the chromogen be dissolved in ethyl alcohol and oxidized with nitrite in the presence of the required amount of acid and then shaken with chloroform, the latter solvent takes up the pigment to a slight extent. The absorption band of the pigment in the chloroform-alcohol layer shows a shift in position (λ580–545). On washing the chloroform-alcohol extract with water the color is removed from the chloroform to the aqueous-alcohol layer. Reacidification of the liquids does not restore the color to the chloroform layer; this observation is contrary to Garrod and Hopkins' experience.[8]

On the other hand if a small amount of amyl alcohol were added to the chloroform the indolaceturic pigment was readily absorbed and could not be washed away with water. The solution showed a well marked band λ580–540.

The *skatol red* urines were treated with lead acetate solution and the excess of lead was removed. The clear filtrate was then oxidized in the usual way.

It was found that the skatol red pigment was insoluble in chloroform but on shaking the urine to which a small amount of ethyl alcohol had been added, there was a separation of the color into two component parts, the one soluble in the chloroform-ethyl alcohol layer as a bluish purple almost blue color and the other

remaining in the aqueous acid layer as a rose-red color. After the removal of the chloroform-alcohol-soluble pigment the acid layer was extracted with amyl alcohol.

The bluish purple chloroform-alcohol-soluble pigment showed a diffuse band λ585–560 with sharp edges; the rose-red amyl alcohol extract showed a diffuse band λ560–530 and a band λ505–480.

Mixture of the chloroform-ethyl alcohol and the amyl alcohol extracts of the components of skatol red gave a band λ572–545 and in this case the band λ505–485 was hardly discernible.

It was found that an amyl alcohol-chloroform extract of the Pigment obtained from the skatol red urines previously treated with lead acetate solution showed a slight shift of the band (λ575–545) but the character of the band was not that of the chloroform-ethyl-alcohol component (above): it was ill defined and diffuse. It is clear therefore that the latter extract contains a separate pigment and the position of the band λ585–560 can not be ascribed to the shifting effect of the chloroform on the position of the absorption band of skatol red.

It is evident therefore that the pigment known as skatol red can be resolved into two component parts; such a separation could not be accomplished with the indolaceturic pigment.

A further point of dissimilarity was the comparative stability of skatol red both in acid-aqueous and in acid-amyl-alcohol solutions whereas the characteristic color of urorosein urines and of the pigment from the isolated indolaceturic acid fades very rapidly, even from concentrated solutions in acid-amyl-alcohol.

Moreover, it was ascertained that after the administration of indolacetic acid to dogs there was no trace of indican in the urine. In the case of skatol given to a dog who was on a diet on which he was excreting no indoxyl compounds, no indican could be detected in the first day's urine but the chloroform extract was reddish purple in color and showed an absorption band λ535–560; this was probably the indirubin referred to by Maillard as contaminating Brieger's skatol red. On the second day the urine showed evidence of the formation of indoxyl compounds (positive indican reaction) and on this day the skatol red reaction was much less vivid than on the first day. Both the indican and skatol red reactions were faint on the third day.

It is possible that the bulk of the skatol is eliminated as skatoxyl sulphate while a small amount is converted into indoxyl sulphate. In the first day's urine, in the presence of excess of skatoxyl, the indoxyl combines with part of the skatoxyl to form a compound of the indirubin type while in the second day's urine, the skatoxyl compound being present in a much less amount, the indoxyl follows the usual path of indigotin formation.

From these considerations it is probable that skatol red and urorosein, although they possess similar absorption spectra are not identical. The experimental evidence shows that the skatol red of the urine is not a simple pigment but a mixture of pigments which, when present together, mutually affect each other's absorption so as to produce an absorption band corresponding to that of urorosein.

The indican reaction is exhibited by the urine of dogs excreted for several days following after the administration of indol and indolaldehyde. After the administration of skatol the indican reaction is not evidenced until the second day. In the case of indol and indolaldehyde the color reaction is very intense and persists for some time after their administration (Table II). The question as to the production of indican in the urine is one of interest in view of these observations.

The chromogen of indican is a sulpho- or gluco-compound of indoxyl and is to be found normally in the urine of herbivora and in pathological human urines. While Ellinger considers that the indoxyl compounds of the urine owe their origin solely to the bacterial production of indol from tryptophane in the intestine. Rosenfeld states that under certain conditions there appear, in the urine of rabbits, indoxyl compounds which can only have been produced from the breakdown of the tissues and not from the intestinal decomposition of tryptophane. Ellinger, criticizing Rosenfeld's experimental evidence,[14] states emphatically that the indican of the urine comes solely from indol formed in the intestine, a view also held by Salkowski, Underhill, Porcher, and others.[15]

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[14] Blumenthal, F., and Rosenfeld, F., [illegible] *nn.*, 1903, xxvii, 46. Ellinger, A., *Ztschr. f. physiol. Chem.*, 1903, xxxix, [illegible] Ellinger, A., and Gentzen, M. *Beitr. z. chem. Phys. u. Path.*, 1904, iv, 171. Also, Rosenfeld, F., *ibid.*, 1904, v, 83.

[15] Underhill, F. P., *Am. Jour. Physiol.*, 1903–04, x, p. xxvii. Salkowski E., *Ztschr. f. physiol. Chem.*, 1904, xlii, 213.

The indican reaction seemed to be a feature of many of the pathological urines sent to me. In some cases the reaction was slight; in others, especially in the urine of patients suffering from pernicious anemia and cirrhosis of the liver, the reactions were most vivid.

The experimental data which I am slowly accumulating point to the liver as being the seat of the decomposition of tryptophane. It is quite feasible that in pathological cases such as those cited above, the presence of indoxyl compounds in the urine is not of necessity due to the bacterial disturbances in the intestinal tract but may owe its origin to the formation of indolaldehyde in the liver as a result of the failure of the disordered liver cells to deal normally with tryptophane.

SUMMARY.

A study of the absorption spectra of the colored substances obtained by the action of certain reagents on indol derivatives has been made and correlated with those derived from the chromogens eliminated in the urine of dogs to which these indol derivatives had been administered. The results show that:

1. Of the various indol derivatives administered, indol, skatol, indolaldehyde, indolcarboxylic and indolacetic acids undergo little change in their passage through the animal body. Apparently with indolpropionic acid there are more deep seated changes taking place.

2. Tryptophane under normal circumstances is apparently not broken down into any of the above ,mentioned substances before absorption from the alimentary canal.

3. The *urorosein* reaction is due to the presence of indolaceturic acid in the urine which has been produced from indolacetic acid in the intestine, the latter probably being formed as the result of bacterial action on tryptophane. Under some conditions the urorosein reaction is changed to that of indolacetic acid.

4. The pigment known as *skatol red* has the same absorption spectrum as urorosein and some observers consider them to be identical. The evidence adduced in this research shows that they are different. Skatol red consists of a mixture of two pigments which can be isolated by the use of suitable solvents; further, the

stability and solubility of the mixed pigments are different from those of urorosein.

5. The indican reaction is given markedly by the urine of dogs within a short time after the administration of indol and indolaldehyde and persists for some days. This color reaction is not given until the second day after the administration of skatol.

No indican could be detected in the urine after the administration of other indol derivatives.

6. The presence of indoxyl compounds in pathological human urines may be due to: (*a*) disorders of the liver whereby the liver cells may be incapable of dealing with tryptophane beyond the stage of indolaldehyde, or (*b*) the absorption from the intestinal tract of indol or of indolaldehyde produced from tryptophane by the action of intestinal bacteria.

TABLE I.

Spectroscopic Examination of the Color Reactions of Indol Derivatives.

Substance.	Treatment.	Solvent for pigment.	Absorption bands.
Indol	HCl and KNO_3	Amyl alcohol	λ528.5–490 (concentrat- solution). λ528.5–505 (dilute solution).
Skatol	HCl and KNO_3	Amyl alcohol $CHCl_3$	λ546.5–533.5 and a diffuse ill defined band λ494–505. λ546.5–533.5 and a diffuse ill defined band λ494–505.
Indolaldehyde	HCl (concentrated) HCl and KNO_3 HCl and $FeCl_3$	HCl aqueous HCl aqueous HCl aqueous Amyl alcohol	λ549–536.5 and a strong band λ495–480. λ549–533.5 and λ494.5–480. (Dilute solution) λ546–527.5 and λ494.5–475 (well marked). λ549–533.5 and λ499.5–475.
Indolcarboxylic acid	HCl and KNO_3	Amyl alcohol	λ505–484.
Indolacetic acid	HCl and KNO_3 HCl and $FeCl_3$ HCl and KNO_3	HCl aqueous Amyl alcohol Amyl alcohol Amyl alcohol	λ552–539 (faint) and λ505–490 (faint). λ557–539 and λ505–492. λ552–531 and λ505–492. λ552–531 and λ505–492.
Indolaceturic acid	HCl and KNO_3 HCl and H_2O_2 HCl and $FeCl_3$	Amyl alcohol Amyl alcohol Amyl alcohol	λ579–539 (strong) and λ502–485 (very faint) λ579–539 and λ502–492. λ579–539 and λ502–492.
Tryptophane	Slow oxidation with $FeCl_3$ in presence of air and acid HCl and KNO_3 HCl and $FeCl_3$	$CHCl_3$ Amyl alcohol Amyl alcohol	λ570–471 (faint). Ill defined bands λ552–534 and λ505–485. λ549–531 and λ502–485.

TABLE I—*Concluded.*

Substance.	Treatment.	Solvent for pigment.	Absorption bands.
Condensation products of tryptophane with:			
1. formaldehyde	HCl (concentrated)	Acid aqueous	Glyoxylic color reaction.
	HCl and KNO_3	Amyl alcohol	Yellowish brown solutions.
	HCl and $FeCl_3$	Amyl alcohol	
	HCl and H_2O_2	Amyl alcohol and also $CHCl_3$	No bands.
2. glyoxylic acid	HCl and KNO_3	Amyl alcohol	Yellowish brown solutions.
	HCl and $FeCl_3$	Amyl alcohol	
	HCl and H_2O_2	Amyl alcohol and also $CHCl_3$	No bands.
3. pyruvic acid	HCl and KNO_3	Amyl alcohol	Yellowish brown solutions.
	HCl and $FeCl_3$	Amyl alcohol	
	HCl and H_2O_2	Amyl alcohol and also $CHCl_3$	No bands.
Isatin	HCl and KNO_2	Amyl alcohol	Brown solution. No bands.
	HCl and $CaOCl_2$	$CHCl_3$	No bands.

TABLE II.

*Spectroscopic Examination of the Urine of dogs after the Administration of Various Indol Derivatives.**

Substance administered	Treatment of urine.	Solvent for pigment.	Absorption bands.
Indol (by mouth)	HCl and $CaOCl_2$	CHCl	λ627–598 (indican).
(by injection)	HCl and $CaOCl_2$ HCl and KNO_3	CHCl Amyl alcohol	λ627–598 (indican). No other pigment could be detected even three days after the dose.
Saktol† (by mouth and by injection)	HCl and $CaOCl_2$ HCl and $FeCl_3$	$CHCl_3$ $CHCl_3$ $CHCl_3$	No indican first day. Purple color λ560–535 first day. λ627–598 second day. Indican.
Skatol† (by mouth and by injection)	KNO_3 and HCl	Amyl alcohol	λ572–545. More dilute 565–546.
Component 1		*Chloroform-alcohol* solution: sharp band λ585–560; solution almost blue in color.	
Component 2		*Amyl alcohol* solution: diffuse and ill defined band λ560–530 and another λ505–480; this latter is not due to the presence of urobilin. Amyl alcohol solution almost rose-red in color.	
Chloroform-amyl alcohol solution of mixture of 1 and 2 shows band λ575–545.			
Indolaldehyde (by mouth)	HCl and $CaOCl_2$ HCl and $FeCl_3$ HCl and KNO_3	$CHCl_3$ $CHCl_3$ Amyl alcohol after removal of pigments soluble in $CHCl_3$	λ627–598 (well marked). λ627–598. None.
Indolcarboxylic acid (by mouth)	HCl and $CaOCl_2$ HCl and $FeCl_3$ HCl and KNO_3	$CHCl_3$ $CHCl_3$ Amyl alcohol	None (no indican). None. λ505–484.

TABLE II—*Continued*

Substance administered.	Treatment of urine.	Solvent for pigment.	Absorption bands.
Indolacetic acid (by mouth and by injection)	HCl and $CaOCl_2$ HCl and $FeCl_3$	$CHCl_3$	None (no indican).
Fresh urine	HCl and KNO_3 HCl and H_2O_2 HCl and $FeCl_3$	Amyl alcohol Amyl alcohol Amyl alcohol	λ570–542 and λ505–490. λ570–542 and λ505–490. λ570–542 and λ505–490.
Stale urine	HCl and KNO_3 HCl and H_2O_2 HCl and $FeCl_3$	Amyl alcohol	λ550–533 and λ505–490.
Normal urine to which had been added:			
1. indolacetic acid	HCl and KNO_3	Amyl alcohol	λ555–533 and λ505–490.
2. indolaceturic acid	HCl and KNO_3	Amyl alcohol	λ570–542 and λ505–490.
3 indolaceturic acid, and allowed to stand for some time	HCl and KNO_3	Amyl alcohol	λ552–534 and λ505–490.
Indolpropionic acid (by mouth and by injection)	Acidified urine allowed to stand in contact with air	Amyl alcohol	λ521–469 (very marked) and λ557–537.
	Kynurenic acid precipitated from urine (Capaldi) colored red	Ether or ethyl alcohol extract of kynurenic acid precipitate	λ527–500.
	HCl and $CaOCl_2$ HCl and $FeCl_3$	$CHCl^3$	None except in one case when after injection on the third day there was a band λ625–598 (indican)
	HCl and KNO_3 HCl and H_2O_2 HCl and $FeCl_3$	Amyl alcohol Amyl alcohol Amyl alcohol	Three well marked bands. λ587–561, λ546–533, and λ499–485 (best marked).
Tryptophane (by mouth and by injection)	HCl and $CaOCl_2$	$CHCl_3$	None (no indican).
	HCl and $FeCl_3$	$CHCl_3$	None.
	HCl and KNO_3 HCl and H_2O_2 HCl and $FeCl_3$	Amyl alcohol Amyl alcohol Amyl alcohol	None; ill defined brown color.

TABLE II—*Concluded.*

Substance administered.	Treatment of urine.	Solvent for pigment.	Absorption bands.
Formaldehyde, glyoxylic acid, and pyruvic acid condensation products with tryptophane (by mouth)	HCl and $CaOCl_2$ HCl and $FeCl_3$ HCl and KNO_3	$CHCl_3$ Amyl alcohol	None. None; ill defined brown color.
Isatin (by mouth)	HCl and $CaOCl_2$ HCl and KNO_3	$CHCl_3$ Amyl alcohol	None. None; ill defined reddish brown color.

* Normal dog urine, with the diet chosen, gave negative tests for urobilin, indican, and other pigments with characteristic absorption spectra. Rabbit urine was taken and tested for indican and the band measured.

The absorption band of indican extended from λ624–598.

†The skatol pigment was fractionally extracted and was separated into two components, the one soluble in chloroform-alcohol and the other in amy alcohol.

TABLE III a.

Absorption Bands of Pigments Derived from Indol Derivatives Previously Described by other Observers.

Substance.	Observer.	Absorption bands.
Indolaldehyde (with HCl)	Ellinger	λ540–530 and λ490–480.
Indolacetic acid	Salkowski	λ550–530.

TABLE III b.

Absorption Bands of the Pigments Obtained from Reactions with Urine Previously Described by other Observers.

Substance.	Observer.	Absorption bands.
Indican	Bouma De Negri	λ630–590. λ620–559.
Indirubin	Bouma De Negri Rosin	λ562–527. λ580–535. λ589–474 (maximum 578).
Uroerythrin	MacMunn Garrod	λ546–481. In presence of H_2SO_4 λ586–552; in presence of HCl λ608–517. In alcoholic solution λ546-520 and λ506–481.
Urohematoporphyrin	Garrod	In acid solution: λ597–587; λ576–570; λ557-541.
Urorosein	Garrod and Hopkins Rosin Nencki	λ561–540; in $CHCl_3$ λ582.5–550. λ538 (maximum). λ557 (maximum).
Skatol red	Brieger Porcher and Hervieux	λ577–550. λ577–550.

TABLE IV.

*Pigments Exhibited by a Few of the Pathological Urines Submitted for Investigation.**

Case.	Urobilin.	Indican.	Other pigments after treatment with KNO_2 and HCl.
Chlorosis 1	Yes	Yes	λ573–546 (urorosein).
2	Yes	Yes	None.
3	Yes	Very marked	Ill defined band λ524–504.
Pernicious anemia	Yes	Vivid reaction	None.
Pernicious anemia	Yes	Vivid reaction (no oxidizing reagent required)	None.
Cirrhosis of the liver	Yes	Vivid reaction	Ill defined band in the green.
Achlorhydrin gastrica 1	Yes	No	λ573–540 (urorosein).
An attempt to isolate the chromogen resulted in the separation of a substance having an absorption			λ552–533 (indolacetic pigment).
Achlorhydrin gastrica 2	Yes	No	λ552–533 (indolacetic pigment).
Achlorhydrin gastrica 2 Isolated crystalline residue			λ552–534 (indolacetic pigment).
Achlorhydrin gastrica 3	Yes	No	λ533–524 (? a mixture of pigments).

* If the urines gave a marked urobilin reaction they were treated with lead acetate before being tested for the presence of indol pigments. If the urines contained the chromogen of indican, after oxidation with nitrate and acid, they were repeatedly extracted with chloroform to remove this pigment before being extracted with amyl alcohol.

Reprinted from The Journal of Biological Chemistry, Vol. XXII, No. 2, 1915.

A METHOD FOR THE ESTIMATION OF THE TRYPTOPHANE CONTENT OF PROTEINS, INVOLVING THE USE OF BARYTA AS A HYDROLYZING AGENT.

By ANNIE HOMER.

(From the Lister Institute, London, and the Departments of Biochemistry and Pathological Chemistry, University of Toronto.)

(Received for publication, July 9, 1915.)

INTRODUCTION.

A. Historical.

At the time that the work described in this paper was started there were very few data as to the amounts of tryptophane present in proteins, and for the lack of such data their tryptophane content was usually represented as positive or negative.

Hopkins and Cole,[1] the first investigators to isolate tryptophane from a protein digest, weighed the substance in the crystalline form and found that 1.5 per cent. of tryptophane could be obtained from pure casein. Osborne and Harris[2] made use of Hopkins and Cole's glyoxylic reaction to find the relative amounts of tryptophane present in various vegetable proteins. Their observations were of a qualitative rather than a quantitative nature.

Levene and Rouiller[3] suggested that for the estimation of the tryptophane content of proteins, the digestion mixtures, made 5 per cent acid, be treated with mercury sulphate reagent; the mercury sulphate precipitate containing tryptophane be decomposed in the way described by Hopkins and Cole; and the tryptophane in solution be estimated by direct titration with bromine water in the presence of amyl alcohol. The disappearance of the characteristic purple color was taken by them as an indication of the end of the reaction. They published data with regard to the estimation of tryptophane in presence of tyrosine

[1] Hopkins, F. G., and Cole, S. W., *Jour. Physiol.*, 1901–02, xxvii, 418.
[2] Osborne, T. B., and Harris, I. F., *Ztschr. f. analyt. Chem.*, 1904, xliii, 376.
[3] Levene, P. A., and Rouiller, C. A., *Jour. Biol. Chem.*, 1906–07, ii, 481.

and cystine; made suggestions as to the method of procedure should these two substances be present in the digest; and stated their intention of publishing data as to the tryptophane content of proteins; but nothing further has been published by them on this subject.

Fasal[4] precipitated the tryptophane in the protein digest by means of mercury sulphate reagent and after decomposition of the mercury sulphate precipitate, estimated the amount of tryptophane in the solution by means of Hopkins and Cole's glyoxylic reaction. Using the same method he also estimated the percentage of tryptophane in malignant tumors.[5] He published the following figures for the percentage of tryptophane in various proteins: Hammarsten's casein 0.65 per cent.; crude lactalbumin 3.07 per cent.; and edestin 0.38 per cent.

Sanders and May[6] subjected proteins to pancreatic digestion and then inoculated the digest with fecal bacteria and colorimetrically estimated the indol thus produced. Their method assumes that the tryptophane is completely converted into indol, but such is not necessarily the case; a certain proportion of skatol and other derivatives of indol will always be simultaneously formed.

Herzfeld[7] has taken the tryptic digest of proteins and has colorimetrically estimated the tryptophane present in the digest by means of *p*-dimethylaminobenzaldehyde. He compared the color produced by the interaction of this reagent and tryptophane with the color of a solution of ammoniacal copper sulphate previously expressed in terms of a solution of pure tryptophane similarly treated with *p*-dimethylaminobenzaldehyde.

The writer of this paper adopting Herzfeld's directions has investigated the color reactions of various indol derivatives with this reagent, and has ascertained that the production of a blue color is not specific to tryptophane. A similar blue color is produced by indolacetic, indolaceturic, and indolpropionic acids and by skatol: indolaldehyde and indolcarboxylic acid give a greenish blue color while indol under the same conditions gives a red color ultimately changing to purple. Under the conditions given by Herzfeld in another paper[8] the red color of indol with this reagent does not change.

Herzfeld has estimated the tryptophane content of about 20 proteins and amongst others he quotes: casein 0.51 per cent.; edestin 0.58 per cent.; lactalbumin 0.91 per cent.; Witte's peptone 1.25 per cent.

It will be noticed that these numbers for casein are lower than those given by Fasal and by Hopkins and Cole. Herzfeld's low results may be due to the fact that he worked with small quantities of the respective proteins (0.1 gm.) and that he continued the digestion for twenty-four hours only, and further that he made use of a color reaction not specific to tryptophane. Hopkins and Cole showed that the maximum yield of tryptophane was obtained after seven

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[4] Fasal, H., *Biochem. Ztschr.*, 1912, xliv, 392.
[5] Fasal, *ibid.*, 1913, lv. 88.
[6] Sanders, J. A., and May, C. E., *Biochem. Bull.*, 1912–13, ii, 373.
[7] Herzfeld, E., *Biochem. Ztschr.*, 1913, lvi, 259.
[8] Herzfeld, *loc. cit.*, 82.

to ten day's digestion, a fact recognized and appreciated by Plimmer and Eaves[9] in their estimation of tyrosine in protein digests. The result given by Fasal for casein is much lower than that given by Hopkins and Cole who actually isolated tryptophane in the crystalline form. It is a matter of common experience to obtain a 1 per cent. yield of tryptophane from commercial casein. Fasal's numbers were based on colorimetric reactions involving the use of glyoxylic acid. From the author's observations on the factors affecting the color reaction in question[10] it is conceivable that the discrepancy between the figures quoted by Fasal and by Hopkins and Cole was due to the interference of other substances of an indol nature thrown down by the precipitating reagent.

Finally Elizabeth Kurchin,[11] using Herzfeld's method, has estimated the tryptophane content of normal and pathological kidneys.

B. *Method Adopted in the Present Investigation.*

It is a matter of common experience that tryptophane can not be isolated in any appreciable extent from the products formed during an acid hydrolysis of proteins and for this reason in most of the previously described methods the protein has been hydrolyzed by trypsin. As the use of trypsin is accompanied by many disadvantages it seemed worth while to ascertain whether any other hydrolyzing agent could be used with any degree of satisfaction: it was found that baryta answered the purpose.

In the method for the estimation of the tryptophane content of proteins which is advocated in this paper the protein is hydrolyzed with baryta water. After the removal of the baryta with sulphuric acid the protein digest, made 5 per cent acid with sulphuric acid, is precipitated with mercury sulphate reagent. The precipitate is decomposed and the filtrate from the mercury sulphide is freed from substances likely to absorb bromine. The tryptophane content of the final solution thus obtained is estimated by titration with solutions both of nascent and of molecular bromine previously standardized against solutions of pure recrystallized tryptophane.

The determinations of the percentage of tryptophane in casein by the method described in this paper were made in the spring and autumn of 1913; the publication of the results has been delayed

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[9] Plimmer, R. H. A., and Eaves, E. C., *Biochem. Jour.*, 1913, vii, 297.

[10] Homer, A., *ibid.*, 1913, vii, 101.

[11] Kurchin, E., *Biochem. Ztschr.*, 1914, lxv, 451.

as it was desired to ascertain whether indol derivatives likely to be formed in the digest would be precipitated by the mercury reagent, and if so to what extent their formation would affect the determinations. It was also desired to make various quantitative experiments on the action of acids and alkalis on tryptophane.

The estimation of the tryptophane content of casein by a method involving the use of baryta as the hydrolyzing agent, of mercury sulphate as the precipitant for the tryptophane liberated during the hydrolysis, and of bromine for the final estimation of the tryptophane in solution, therefore resolved itself into the following considerations:

I. The absorption of bromine by tryptophane in aqueous solution.

II. A study of the extent to which substances other than tryptophane are precipitated by mercury sulphate reagent and of the bromine capacity of the substances thus precipitated which are likely to appear in the final solution for estimation.

III. The estimation of the tryptophane content of the hydrolysis mixtures involving: (*a*) The treatment of the alkaline digests so as finally to obtain for bromination a solution containing the tryptophane and as free as possible from other substances likely to absorb bromine. (*b*) The estimation of the tryptophane content of the final solutions by a determination of the ratio of their absorption of bromine to that of a solution of pure recrystallized tryptophane under the same experimental conditions.

IV. The action of acids and alkalis on tryptophane.

Section I. The Estimation of the Bromine Absorption of Tryptophane in Aqueous Solution.

The following solutions were required for the estimations:

A. Solutions of tryptophane prepared by dissolving known weights of the substance in known volumes of water.

B. A saturated solution of bromine in water. This solution was standardized (every time used) against potassium iodide and a standard solution of thiosulphate.

C. Solutions of nascent bromine prepared by the interaction (in presence of acid) of aliquot parts of the following: a solution

of sodium bromate containing 15.1 grams per liter and a solution of sodium bromide containing 51.5 grams per liter.

10 cc. of each of these solutions were measured into stoppered bottles and 5 cc. of concentrated hydrochloric acid were added. After standing for some time there was added an excess of a solution of potassium iodide, and the liberated iodine was titrated with a standard solution of thiosulphate. The bromine value of the mixture of bromide and bromate was thus made.

D. A standard solution of thiosulphate ($\frac{N}{10}$): a 10 per cent. solution ol potassium iodide, and a freshly prepared solution of starch.

Method oj Procedure.—Accurately weighed amounts of tryptophane were dissolved in known volumes of distilled water and aliquot parts of the solution were measured into stoppered flasks and treated with a known excess of an aqueous solution of bromine, or with a known amount of nascent bromine liberated by the addition of 5 cc. of concentrated hydrochloric acid to the mixture of the previously measured volumes of sodium bromate, bromide, and tryptophane solutions. The flasks were tightly corked and left at room temperature for fixed periods of time. Excess of a 10 per cent. solution of potassium iodide was then added, and the flasks were recorked and allowed to stand for at least fifteen minutes. The iodine, representing the excess of bromine, was estimated by titration with standard thiosulphate solution. The difference between the amount of bromine originally added to the solution and the unabsorbed bromine finally titrated represents the bromine absorbed by the substance.

In these determinations the use of a thermostat is to be recommended as it was found that the bromine absorption of solutions of pure tryptophane at room temperature during a given period of time was greater in summer than in winter seasons of the year (England); further, to ensure constant values for the absorption of bromine it is necessary to have considerable excess of bromine and to adhere rigidly to a definite time for the reaction to take place; a variation in the time of the reaction is accompanied by slight variations in the absorption of bromine by a given weight of the substance.

It has been stated by Plimmer and Eaves that under the conditions employed by them, one molecule of tryptophane absorbs

about six atoms of bromine. My results (Table I) indicate a greater absorption; one molecule of tryptophane absorbs about eight atoms of bromine, although there are indications that its bromine capacity is higher still. In the initial stages there is rapid absorption of bromine but the rate of absorption soon falls off and, as would be expected, complete saturation does not take place for some time. It will also be seen that the bromine absorption with molecular bromine is slightly higher than with nascent bromine.

From these results it is obvious that the absorption of bromine by tryptophane in aqueous solution takes place in stages and that in the complete saturation of the substance with bromine the time and temperature of the reaction must be taken into account.

Section II. The Use of Mercury Sulphate Reagent as a Precipitant and the Extent to Which the Substances Other than Tryptophane Precipitated Therweith Will Absorb Bromine.

The mercury sulphate reagent[12] according to Hopkins and Cole precipitates tryptophane, cystine, tyrosine to a certain extent, polypeptides and other incompletely hydrolyzed products. Of these, tryptophane, tyrosine, and polypeptides will absorb bromine: there is conflicting evidence as to whether cystine absorbs bromine or not. Levene and Rouiller[3] consider that it does, whereas Plimmer and Eaves,[9] in view of the work of Knoop[13] on the absorption of bromine by amino-acids in digests, do not regard cystine as a factor to be considered in the bromination of protein digests. However, cystine, although precipitated by the mercury reagent, can not interfere because it is destroyed by the alkaline hydrolysis. To make quite sure of this point 300 cc. of the solutions as finally prepared for estimation with bromine were treated with zinc and hydrochloric acid, and the reduced liquid was tested for the presence of cystine by means of the sodium nitroprusside and the ferric chloride color reactions. Negative results were obtained, thus showing that after a baryta hydrolysis

[12] 10 per cent. mercury sulphate in 5 per cent. sulphuric acid.

[13] Knoop, F., *Beitr. z. chem. Phys. u. Path.*, 1908, xi, 356.

TABLE I.

The Absorption of Bromine by Tryptophane in Aqueous Solution.

Weight of tryptophane in volume of solution taken.	Time of reaction	Weight of bromine added.	Weight of bromine residual.	Bromine absorbed by 1 gm. of tryptophane.	Bromine absorbed by 1 gm. molecular weight of tryptophane.
	min.	gm.	gm.	gm.	gm.
Mean results.					
Room temperature (summer).					
May					
0.0648	30	0.4901 (nascent)	0.2770	3.29	671
June					
0.0311	30	0.4884 (nascent)	0.3859	3.31	673
August					
0.0311	30	0 4884 (nascent)	0.3848	3.33	679
October					
0.0311	30	0.4884 (nascent)	0.3858	3.30	673
May					
0.0648	30	0.4130 (molecular)	0.1903	3.43	700
June					
0.0467	30	0.1935 (molecular)	0.0391	3.30	673
July					
0.0648	30	0.4136 (molecular)	0.1909	3.41	696
October					
0.0311	30	0.1290 (molecular)	0.0261	3.38	690
Room temperature (winter).					
0.02255	30	0.2301 (nascent)	0.1619	3.02	616
0.02255	45	0.2301 "	0.1588	3.16	645
0.02255	60	0.2301 "	0.1577	3.21	654
0.02255	2 hrs.	0.2301 "	0.1572	3.23	659
0.02255	4 hrs.	0.2301 "	0.1546	3.35	683
Temperature of reaction 17°C.					
0.0624	30	0.4602 "	0.2732	3.00	608
0.04992	2 hrs.	0.2301 "	0.0711	3.19	650
0.04721	4 hrs.	0.4683 "	0.3122	3.31	675
0.04721	7 hrs.	0.4683 "	0.3085	3.40	693
0.04721	8 hrs.	0.2301 "	0.0723	3.34	681
Temperature of reaction 17°C.					
0.0500	30	0.4720 "	0.3213	3.00	608
0.0500	1 hr.	0.4720 "	0.3136	3.17	647
0.0500	2 hrs.	0.4720 "	0.3116	3.21	655
0.0500	4 hrs.	0.4720 "	0.3037	3.36	685
0.0500	6 hrs.	0.4720 "	0.2985	3.47	708
0.0500	8 hrs.	0.4720 "	0.2990	3.46	706

of proteins and subsequent precipitation with mercury sulphate reagent there is no cystine in the precipitate.

It was found that solutions of indol, skatol, indolaldehyde, indolcarboxylic, indolacetic, indolpropionic acids, tryptophane and the various additive compounds of tryptophane[14] when dissolved in 5 per cent. sulphuric acid were all precipitated by mercury sulphate reagent. Further, it was found that all these compounds would absorb bromine under the conditions employed for the estimation of tryptophane.

In the case of tryptophane it has been shown (Table I) that the bromination is affected by conditions of time and temperature The same is probably true for the absorption of bromine by other indol derivatives, but unfortunately, owing to scarcity of material, it was impossible to do similar time experiments with regard to their absorption of bromine: their bromine absorption after half an hour's reaction was investigated, as this was the time chosen for the bromination of the solutions obtained from the protein digests.

The solubility of indol, skatol, indolaldehyde, and indolcarboxylic acid in water is so slight that it was impossible to get reliable data as to their bromine capacity under the conditions adopted for the bromination of the final solutions obtained from the protein digests (Section III). Bromination of weighed amounts of the solids themselves did not yield concordant results.

Solutions of indol and skatol in absolute alcohol were brominated: the bromine absorption of a volume of alcohol corresponding to that used in the titrations of indol and skatol was estimated and deducted from the value for the bromine absorption of the solutions: again no concordant results could be obtained.

In another series of experiments the indol and skatol were dissolved in a small volume of absolute alcohol (25 cc.) and the solution was diluted with water to 100 cc. Aliquot parts of these solutions were treated with bromine and a correction was introduced for the bromine absorption of the alcohol. It is with diffidence that the results are published: the author does not consider that much reliance can be placed in them, they merely serve to show the order of the bromine absorption of these substances.

The following results were obtained:

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[14] Homer, *loc. cit.*, 117.

TABLE II.

The Absorption of Bromine by Aqueous-Alcoholic Solutions of Indol and Skatol.

Weight of substance in solution.	Weight of bromine added.	Weight of residual bromine.	Weight of bromine used by volume of solvent taken.	Weight of bromine absorbed by	
				1 gm.	1 gm. molecular weight.
				of substance.	
Mean results.	(Nascent)				
gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Indol					
0.0364	0.4683	0.2651	0.0745	3.54	414
0 0402	0.4620	0.2965	0.0745	3.44	402
Skatol					
0.03045	0.4683	0.3128	0.0745	2.66	348

The bromine absorption of indolacetic and indolpropionic acid in aqueous solution was also measured and the results have been embodied in Table III.

TABLE III.

The Absorption of Bromine by Indolacetic and Indolpropionic Acid in Aqueous Solution.

Weight in substance of volume of solution taken.	Weight of bromine added.	Weight of residual bromine.	Bromine absorbed by	
			1 gm.	1 gm. molecular weight.
			of substance.	
Mean results.				
Time of reaction 30 min.				
gm.	*gm.*	*gm.*	*gm.*	*gm.*
Indolacetic acid				
0.0402	0.4856	0.3483	3.40	595
0.0400	0.3793	0 2402	3.43	600
Indolpropionic acid				
0.0400	0.4856	0.3408	3.62*	674
0.0401	0.4683	0.3358	3.30	62[illegible]
0.0227	0.2295	0.1583	3.13	591

* The high value in this set of determinations was probably due to the fact that a small amount of sodium carbonate solution had been added to dissolve the acid.

Section III. The Baryta Hydrolysis of Casein and the Estimation of the Tryptophane Liberated Therein.

a. The Preparation from the Hydrolysis Mixture of the Solution for Estimation with Bromine.

100 grams of casein were treated with 350 grams of barium hydrate in 2.5 liters of water, and the mixture was heated on a water bath for periods varying from twenty to one hundred and twenty hours. The baryta was removed by slight excess of sulphuric acid and the liquid filtered. The filtrate was made 5 per cent. acid with sulphuric acid, treated with an excess of mercury sulphate reagent and allowed to stand for forty-eight hours. The mercury sulphate precipitate may contain tyrosine, crystine various incompletely digested protein substances, and other indol derivatives as well as tryptophane.

The tryptophane was separated from the other substances present in the mercury sulphate precipitate and likely to absorb bromine by the following process: The precipitate, after having been freed from tyrosine by repeated washing with 5 per cent. sulphuric acid until the washings no longer gave Millon's test, was suspended in 2 per cent. sulphuric acid and decomposed with sulphuretted hydrogen. The filtrate from the mercury sulphide was freed from sulphuretted hydrogen and while still acid (approximately 2 per cent.) was treated with a solution of phosphotungstic acid with avoidance of excess. After standing, the precipitate of polypeptides, etc., if any, was filtered off; the excess of phosphotungstic acid was removed by means of baryta, and the latter by means of sulphuric acid. The liquid was again tested with Millon's reagent, and if there were any indications of tyrosine the precipitation with the mercury sulphate reagent was repeated. In the experiments under consideration it was found unnecessary to reprecipitate.

Cystine, although precipitable by the mercury reagent, can not interfere because it is destroyed by the alkaline hydrolysis.

In view of these observations (Section II) with regard to the precipitation of indol derivatives by the reagent it was necessary to make tests for the presence of indol derivatives other than tryptophane in the solutions for bromination.

The following four color tests are in vogue for the detection of indol derivatives in solution:

1. *The glyoxylic reaction* originally employed as a specific test for tryptophane, is given by all indol derivatives although the actual shade of color produced varies considerably.[10]

2. *The purple color reaction with bromine water* is specific to tryptophane and, under certain conditions, for its additive compounds. The other indol derivatives absorb bromine but do not give any characteristic color reaction with this reagent.

3. *Sodium nitrite and hydrochloric acid* give color reductions with all indol derivatives. Indol, skatol, indolaldehyde, indolcarboxylic acid, indolacetic, indolaceturic acids treated with th[illegible] reagents give color reactions which are characterized by [illegible]ned absorption bands, whereas indolpropionic acid gives [illegible]teristic color under the same conditions.[15]

[illegible]ndol derivatives treated with Ehrlich's *p-dimethylaminobenzaldehyde* according to the directions given by Herzfeld yield characteristic color reactions. Indol gives a characteristic red color, and tryptophane, as shown by Herzfeld, gives a beautiful blue color. However in the present research it was ascertained that a similar blue color is given by indolacetic, indolaceturic, and indolpropionic acids and by skatol: indolaldehyde and indolcarboxylic acid give a greenish blue color.

The original protein digests and also the solutions finally obtained after the decomposition of the mercury sulphate precipitate were therefore tested for the presence of indol, skatol, indolaldehyde, indolcarboxylic and indolacetic acids, and for tryptophane by the color tests given above. Indolpropionic acid can not be detected in presence of tryptophane except by extraction of the solution with ether, in which tryptophane is insoluble, removal of the solvent, and conversion of the residue, if any, into the nitroso-derivative.

If indol, skatol, etc., are present in a digest, presumably they must have been formed from tryptophane during the process of hydrolysis, and they will be precipitated by the mercury reagent together with tryptophane. Owing to the slight solubility of indol, skatol, indolaldehyde, and indolcarboxylic acid there can

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[15] Homer, *loc. cit.*

be, at the most, but traces of these substances in the final solutions for estimation with bromine: as regards indolacetic and indolpropionic acids their absorption of bromine is so close to that of tryptophane (page 377) that for the small amounts present they will not appreciably affect the estimation: that is to say, they will influence the bromine absorption to the same extent as though they had been present as the parent substance, tryptophane.

As a matter of experience in fourteen hydrolyses with baryta there were three only of the final solutions which gave any indication of the presence of indol derivatives other than tryptophane. Of these, two indicated traces of indol, and one traces of indolacetic acid. Moreover, that there could not have been any appreciable conversion of tryptophane into derivatives not appearing in the final solutions is shown by the results obtained from the hydrolyses of the same sample of casein for varying periods of time (page 381) and from the results of the hydrolyses of pure tryptophane.

b. Estimation of the Tryptophane Content of the Solutions obtained after the Decomposition of the Mercury Sulphate Precipitates, etc.

An aliquot part (50 cc.) of the solution for estimation was treated with excess of nascent or molecular bromine of known strength and the unabsorbed bromine estimated by means of potassium iodide and standard thiosulphate solution. The bromination was carried out in well stoppered bottles fitted with ground glass stoppers or with tightly fitting waxed corks. The bromine absorption of the solutions was compared with that of a freshly prepared solution of pure recrystallized tryptophane treated with the brominating solutions under comparable conditions of time and temperature; from these observations the tryptophane content of the hydrolysis mixtures could be calculated.

It was found that concordant results could be obtained if the conditions as indicated above (page 373) were kept constant during each series of experiments.

Samples of commercial casein obtained from three different firms were hydrolyzed with baryta and the hydrolysis mixture was

treated in the manner described above to ascertain (α) the tryptophane content of the protein by the method under investigation and to see how it compared with the values given by other observers; (β) the length of time of hydrolysis for a maximum yield of tryptophane; and (γ) to what extent further hydrolysis would lead to a diminution of tryptophane in the final solution.

The results of the determinations have been embodied in Table IV and in each case the mean results of a series of observations have been given.

TABLE IV.

The Tryptophane Content of Commercial Casein as Estimated by the Method Described in This Paper.

Sample.	Weight of casein taken.	Time of hydrolysis.	Volume of final solution.	Bromine absorbed by final solution. (a) Nascent.	Bromine absorbed by final solution. (b) Molecular.	Bromine absorbed by 1 gm. tryptophane under the same conditions.	Tryptophane content of 100 gm. casein.
	gm.	hrs.	cc.	gm.	gm.	gm.	gm.
A	200	40	500	10.45		3.30	1.59
B (1)	100	20	1000	3.24		3.29	0.99
					3.64	3.44	1.06
(2)	100	40	1000	3.41		3.29	1.04
					3.85	3.44	1.1
(3)	100	60	1000	3.41		3.29	1.04
					3.75	3.44	1.09
(4)	100	80	1000	3.41		3.29	1.04
					3.88	3.44	1.13
C (1)	100	120	1000	4.07		3.30	1.24
(2)	100	120	1000	4.07		3.30	1.24

An examination of the results given in Table IV shows that except for Sample A the percentages of tryptophane indicated are all lower than those given by Hopkins and Cole but higher than those given by Fasal and by Herzfeld. Hopkins and Cole obtained 1.5 grams of tryptophane from 100 grams of Hammarsten's pure casein; but to the best of my knowledge their average yield from commercial casein was not much more than 1 per cent.

From the determinations made with Sample B it appears that

the length of time suitable for a complete separation of tryptophane from casein is about forty hours.

That there could not have been any appreciable loss of tryptophane due to the formation of indol derivatives or other compounds not present in the final solution is evidenced first by the results obtained from the hydrolysis of the same sample (Sample B) for varying periods of time and secondly, by the fact that duplicate hydrolyses of Sample C conducted for the same periods of time and treated separately have given concordant results.

The solutions for estimation with bromine were all tested for the presence of indol derivatives (possibly precipitated by the reagent): where detected they were present as traces only.

The advantages for the estimation of the tryptophane content of proteins are:

1. The ease with which the hydrolysis can be conducted.

2. The fact that the tryptophane does not tend to disappear during the hydrolysis.

3. The values obtained for casein are nearer those obtained by Hopkins and Cole, who actually isolated and weighed the tryptophane, than those given by investigators using colorimetric methods. In any case the values obtained in this research, in view of the possible formation of decomposition products from tryptophane and their subsequent loss in the processes employed, are doubtless too low rather than too high.

4. The method is proving of the utmost service in a series of experiments in which the action of tissue enzymes on tryptophane is being studied.

From the consideration of the question of the possible destruction of tryptophane by prolongation of the time of digestion of the protein with baryta I was led to a consideration of the action of alkalis and acids on pure tryptophane.

Section IV. The Action of Acids and Alkalis on Pure Tryptophane.

a. The Action of Acids on Tryptophane.

It is a matter of common experience that tryptophane can not be estimated in any appreciable quantity from the products formed during an acid hydrolysis of tryptophane.

The following experiments demonstrate this clearly:

1. 100 grams of casein were heated with 150 cc. of a 25 per cent. solution of sulphuric acid for periods of 1, 2, 4, 8, 12, and 20 hours. Baryta was used to remove the acid and after filtration of the barium sulphate the filtrate was made 5 per cent acid with sulphuric acid and treated with mercury sulphate reagent. After washing the precipitate free from tyrosine it was found that in each case there was little more than a trace of precipitate left on the filters, at any rate not sufficient for estimation.

Thus at no period of the digestion was tryptophane present in any appreciable quantity.

In order to ascertain whether the poor yield of tryptophane in an acid hydrolysis is due to the direct action of acid on the substance as soon as it is liberated from the protein molecule the following experiments were made with pure tryptophane.

2. 25 grams of pure tryptophane dissolved in 150 cc. of a 25 per cent. solution of sulphuric acid were heated on a sand bath for forty-two hours. The acid was removed by baryta and after filtration of the barium sulphate, removal of the last trace of baryta or of acid, the liquid was evaporated to crystallizing point and 12.5 grams of tryptophane were recovered. The recovery of so much tryptophane showed that the decomposing action of the acid had been slight.

In two other experiments 5.5 and 10 grams of tryptophane, to each of which had been added 1 gram of ferric sulphate, were heated on sand baths with 100 cc. of a 25 per cent solution of sulphuric acid for twenty-one and twenty-four hours respectively. The reacting liquids became deeply pigmented and in neither case was it possible to recover any tryptophane nor was any indol derivative isolated from the gummy residues although they both gave a marked glyoxylic color reaction.

3. Small weighed amounts of pure tryptophane were treated (*a*) with 25 per cent. sulphuric acid alone, (*b*) with 25 per cent. sulphuric acid in presence of a small amount of ferric sulphate, and (*c*) with the acid in presence of small amounts of copper sulphate. At the end of the time allowed for the reaction the liquids were made slightly alkaline with baryta, filtered, the precipitates well washed, and the filtrate after the removal of the baryta was made up to a certain bulk (Solution A).

An aliquot part of Solution A was made 5 per cent. acid and after filtration from barium sulphate was precipitated with mercury sulphate reagent. The mercury sulphate precipitate was treated in the usual way (page 378) and the resulting neutral solution made up to a known volume (Solution B) aliquot parts of which were treated with nascent bromine.

The other portion of Solution A was made slightly alkaline with baryta and steam distilled. Both distillate and residue were tested colorimetrically for the presence of indol derivatives; the tests were negative.

In some cases the bromine absorption of both Solutions A and B was estimated with a view to ascertaining whether after the hydrolysis of tryptophane with acid there were present in the digest substances which absorbed bromine but which were not precipitated by the mercury sulphate reagent.

An examination of Table V shows that the bromine capacity of Solution A when estimated directly is considerably higher than can be accounted for by the tryptophane precipitated as the mercury sulphate compound and estimated in Solution B. It is thus obvious that there are formed during an acid hydrolysis of tryptophane substances which absorb bromine but which are not precipitated by the mercury sulphate reagent.

It is also evident that acid alone has a slow action on pure tryptophane whereas in the presence of an oxygen carrier there is considerable decomposition of the substance: for the same period of time the effect of a salt of iron is somewhat more marked than that of a salt of copper. The rate of decomposition, however, does not provide a simple explanation of the disappearance of tryptophane in acid digests.

Some observers consider that during an acid hydrolysis of tryptophane an internal anhydride is formed, but from the evidence adduced in this paper (Section II) it is clear that so long as the indol ring remains intact, it should be precipitated by mercury sulphate reagent and should absorb bromine.

The results recorded above demonstrate the presence of substances in the hydrolysis mixture which absorb bromine but which are not precipitated by the mercury sulphate reagent. It may be that the tryptophane newly split off from the protein molecule is very susceptible to chemical reaction, and it is con-

TABLE V.

The Action of 25 per cent. Sulphuric Acid on Pure Tryptophane.

Kind of metallic salt added.	Weight of tryptophane taken.	Weight of metallic salt added.	Time of hydrolysis.	Bromine absorption of Solution A measured directly.	Bromine absorption of the tryptophane content of Solution A (calculated from the absorption of bromine by Solution B).	Bromine absorbed by 1 gm. of tryptophane under the same conditions.	Tryptophane content of Solution A.	Loss of tryptophane during the hydrolysis.
	gm.	*gm.*	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent.*
(a) —	2	—	20	6.38	6.58	3.30	1.99	0.5
(b) $Fe_2(SO_4)_3$	1.5	0.15	17	4.70	3.78	3.30	1.14	23
	2	0.2	40	4.84	3.84	3.30	1.16	42
"	1	1	40		0.74	3.00	0.25	75
(c) $CuSO_4$	1	1	40		1.05	3.00	0.35	65

Solution A was the hydrolysis mixture after the removal of acid and metallic salt.

Solution B was prepared from the decomposition of the mercury sulphate precipitate thrown down from an aliquot part of Solution A and represents the tryptophane content of Solution A.

ceivable that during an acid hydrolysis of protein some product of hydrolysis acts as an oxygen carrier, or it may be that the other amino-acids serve as catalysers and induce the destruction of the tryptophane as quickly as it is split off from the complex molecule of the protein.

b. The action of Baryta on Pure Tryptophane.

Before the completion of this research Herzfeld[8] had published the results of his observations on the action of alkalis on proteins and on tryptophane itself. He hydrolyzed small quantities of protein and of tryptophane itself with different alkaline solutions. After distilling the hydrolysis mixtures, he estimated the indol

in the distillate. His results showed that there is slight production of indol from the action of water, more from the action of 0.5 per cent. sodium carbonate solution, and a considerable amount from that of a 9 per cent. solution of caustic soda in presence of copper sulphate. He states that the indol in the distillates from the baryta hydrolysis is even less than from the hydrolysis with 0.5 -- cent. sodium carbonate.

experiments on the action of baryta on tryptophane and on tryptophane in presence of ferric salts recorded in this paper had been done before the appearance of Herzfeld's paper. Since then I have also tried the action of baryta on tryptophane in presence of copper sulphate. The results of my experiments confirm the observation of Herzfeld, although he estimated the indol production, whereas I estimated the tryptophane content of the hydrolysis mixtures. It will be seen that after forty hours' hydrolysis there has been practically no loss of tryptophane, and even in the presence of salts of iron and copper the loss was not very marked.

As in previous cases the hydrolysis mixtures after being freed from baryta and from copper or iron were made up to a known volume (Solution A). An aliquot part of Solution A was precipitated with mercury sulphate reagent and the remainder was used for colorimetric tests.

The mercury sulphate precipitate was decomposed in the usual way and the filtrate made up to a known volume, Solution B. Part of Solution B was estimated for its tryptophane content by means of solutions of nascent bromine: the remainder was used for colorimetric tests.

Of the final solutions, that obtained from the hydrolysis with baryta in presence of copper was the only one which showed any indication of indol, but even so the intensity of the color reaction revealed the presence of traces only.

In other experiments the hydrolysis mixtures were not used for precipitation with the mercury reagent but were made slightly alkaline with baryta and distilled; the distillates were extracted with xylol and tested with *p*-dimethylaminobenzaldehyde; there was a distinct indol coloration but the intensity of color given by the xylol extract of the distillate obtained from one-half the total bulk of the hydrolysis mixture was not as great as that given

by 0.005 gram of pure indol dissolved in xylol and treated with the reagent in the same way. It was also noticed that if the baryta were completely removed from the hydrolysis mixture and the latter then made alkaline with sodium carbonate and distilled, the intensity of the color reaction of the xylol extract of the distillate was much greater than in the previous case. This observation is also in confirmation of Herzfeld's statements.

It is obvious that at the temperature at which the hydrolysis of tryptophane with baryta was conducted, there was little production of indol. Colorimetric tests failed to reveal the presence of other indol derivatives in the hydrolysis mixtures or in the final solutions for estimation with bromine.

TABLE VI.

The Action of Baryta on Tryptophane.

Kind of metallic salt added.	Weight of tryptophane taken.	Weight of metallic salt added.	Time of hydrolysis.	Bromine absorption of Solution A calculated from that of Solution B.	Bromine absorption of 1 gm. tryptophane under the same conditions.	Tryptophane content of hydrplysis mixture	Loss of tryptophane during hydrolysis.
	gm.	*gm.*	*hrs.*	*gm.*	*gm.*	*gm.*	*per cent.*
—	t	—	20	3.48	3.30	t.06	—
—	0.5	—	40	1.50	3.00	0.50	—
$Fe_2(SO)_3$	t	1	40	2.53	3.00	0.84	t6
$CuSO_4$	0.5	1	40	1.42	3.00	0 4t	18

Solution A was the hydrolysis mixture after the removal of baryta and metallic salt.

Solution B was prepared from the decomposition of the mercury sulphate precipitate thrown down from an aliquot part of Solution A and represents the tryptophane content of Solution A.

From the results indicated in Table VI it is obvious that there is practically no loss of tryptophane during the hydrolysis with baryta even for forty hours. If the hydrolysis was conducted in the presence of a relatively large amount of metallic salts there was less than 20 per cent. loss during the forty hours' hydrolysis.

SUMMARY.

The baryta hydrolysis of proteins, the precipitation of the tryptophane from the hydrolysis mixtures with mercury sulphate reagent, and the subsequent bromination of the tryptophane in solution after the decomposition of the mercury sulphate precipitate is advocated as a method for the estimation of the tryptophane content of proteins for the following reasons:

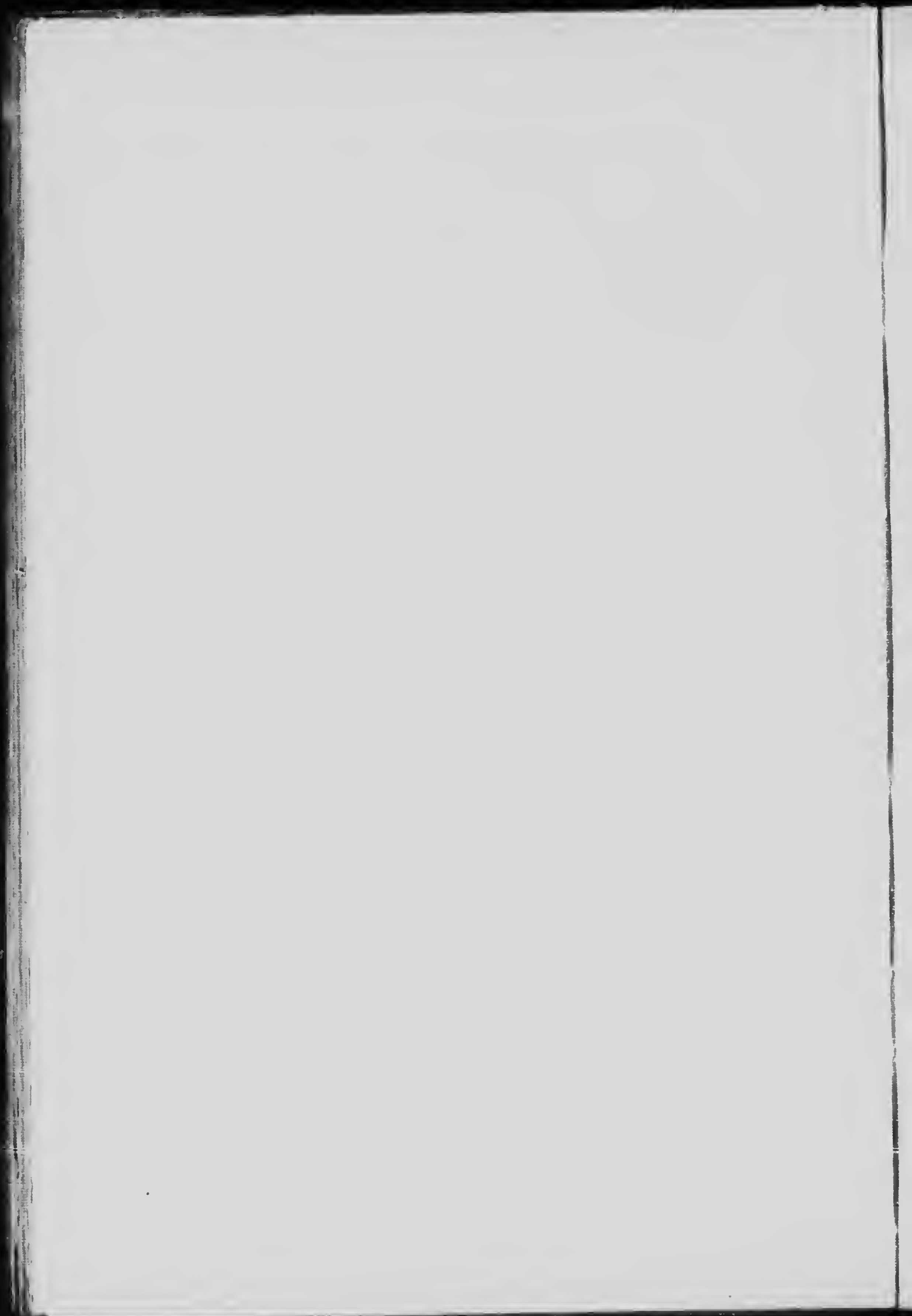
1. The ease with which the hydrolysis can be conducted.
2. The separation of the tryptophane from the other substances precipitated by the reagent is simplified by the fact that cystine is destroyed during an alkaline hydrolysis.
3. Prolongation of the time of hydrolysis does not lead to a destruction of the tryptophane split off from the protein complex.
4. The results obtained by this method for the tryptophane content of casein more nearly approximate those of Hopkins and Cole who actually isolated the tryptophane in a crystalline form than do those of other observers who have based their results on colorimetric methods.
5. The precipitation of tryptophane in solution and the estimation of the tryptophane content of the filtrate from the decomposition of the mercury compound by means of its bromine absorption are proving of great service and reliability in experiments now under progress in which the action of various tissue enzymes on tryptophane is being measured.

A criticism of the method, one shared by the methods of Fasal and Herzfeld and others, can be made, *viz.*, that the reaction used in the final determinations is not specific to tryptophane but is shared by other members of the indol group. In this case the bromine absorption of other indol derivatives present in the solutions, presumably formed from tryptophane, would not effect the final results to any appreciable extent. However, as a matter of experience, it has been found that the final solutions do not contain more than traces of indol derivatives and, further, that the results obtained indicate that there is no appreciable formation of substances from tryptophane which do not appear in the final solutions for estimation with bromine.

From these considerations we see that, although the value obtained for the tryptophane content of a protein may be some-

what lower than the true value, yet it can be taken as the lowest value assignable to the protein under consideration. Such values, though only approximate, may be of the utmost service in experiments on nutrition and in the preparation of synthetic diets.

Finally a separate series of experiments has shown the greater resistance of tryptophane to the action of baryta than to the action of acids with or without the presence of oxygen carriers.



Reprinted from The Journal of Biological Chemistry, Vol. XXII, No. 2, 1915

THE RELATION BETWEEN THE ADMINISTRATION OF TRYPTOPHANE TO DOGS AND THE ELIMINATION OF KYNURENIC ACID IN THEIR URINE.

By ANNIE HOMER.

(From the Physiological Laboratories, University of Cambridge, the Physiological Laboratory, University College, London, the Lister Institute, London, and the Departments of Biochemistry and Pathological Chemistry, University of Toronto.)

(Received for publication, July 7, 1915.)

It is known from the work of Willcock and Hopkins[1] and from that of Osborne and Mendel[2] that tryptophane is an essential constituent of diet for the maintenance of the life of an animal and it is generally accepted that there is some close connection between tryptophane and blood pigment although up to the present this conjecture has not received the support of experimental evidence.

The only direct indication of the fate of this substance in the animal body has been furnished by Ellinger[3] who showed that in dogs and in rabbits the excretion of kynurenic acid was increased by the administration of tryptophane by the mouth or by subcutaneous injection: in the human being there was no excretion of kynurenic acid after the administration of tryptophane by the mouth.

The formation of kynurenic acid from tryptophane presents an interesting problem, as it involves the conversion of an indol into a quinoline compound. Kynurenic acid has hitherto been regarded as γ-hydroxy-β-carboxytryptophane,[4] and both Ellinger and Dakin have suggested possible ways of formation of the acid from tryptophane. Ellinger regards indol glyoxylic acid as an intermediate stage in the conversion whereas Dakin considers the formation of indolpyruvic acid as a prelude to the opening of the pyrrol ring thus:

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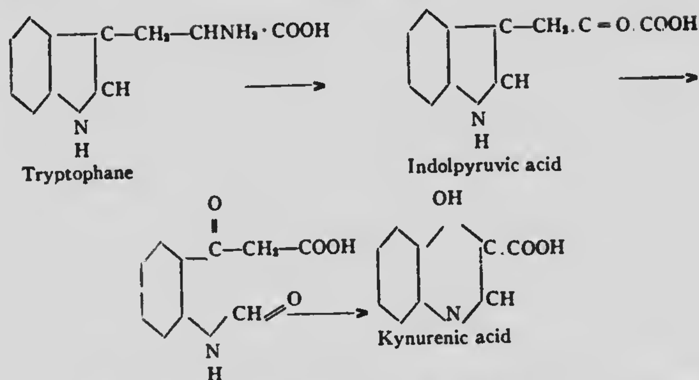
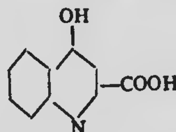
[1] Willcock, E. G., and Hopkins, F. G., *Jour. Physiol.*, 1906–07, xxxv, 88.
[2] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1914, xvii, 325.
[3] Ellinger, A., *Ztschr. f. physoil. Chem.*, 1904–05, xliii, 325.
[4] Camps, R., *ibid.*, 1901, xxxiii, 390.



But in view of the fact that kynurenic acid has been shown to be γ-hydroxy-α-carboxy quinoline[5]



these suggestions are no longer tenable. However, for the present the author is not prepared to offer any suggestion as to the method of formation of this compound from tryptophane.

Before proceeding to certain metabolism experiments, undertaken conjointly with Dr. Hopkins of Cambridge, England, in which it was desired to obtain some evidence of the way in which tryptophane becomes converted into kynurenic acid in the metabolic processes of the dog, it seemed advisable to ascertain whether the kynurenic acid eliminated represented a complete picture of the metabolism of tryptophane in the body or whether a certain amount of the tryptophane administered became "side-tracked" into kynurenic acid while the remainder was utilized in furnishing either the energy or the particular compounds necessary for the maintenance of the life of the individual.

With this object in view varying doses of tryptophane were administered to dogs of different sizes and ages and the output of

[5] Homer, A., *Jour. Biol. Chem.*, 1914, xvii, 509.

kynurenic acid in the urine was estimated by Capaldi's method[6] as follows:

The urine was treated with half its bulk of a 10 per cent. solution of barium chloride to which had been added 5 per cent. concentrated ammonia. After standing for half an hour the liquid was filtered and the filtrate evaporated to half the bulk of the original volume of urine taken for the estimation. The residual liquid was decomposed with a 4 per cent. solution of hydrochloric acid and allowed to stand for a period of sixteen to twenty-four hours. The crystalline precipitate was filtered, washed with dilute hydrochloric acid, dissolved in ammonia, and the excess of ammonia removed by evaporation on a water bath. The solution of kynurenic acid in ammonia was then treated with a 4 per cent. solution of hydrochloric acid and allowed to stand for six hours. The cyrstalline precipitate was filtered through a Gooch crucible, washed, dried at 110 C., and weighed.

In one series of determinations (Table I a) in which a dog was being fed on lean meat and in which, during the preliminary period the dog was excreting traces only of kynurenic acid, it was noticed, that, in the period after the removal of the tryptophane from the diet, there was apparently an appreciable amount of kynurenic acid being excreted. An examination of this supposed kynurenic acid showed that the substance was practically pure uric acid.

It was evident that the determination of kynurenic acid in the urine by Capaldi's method could not be relied upon in presence of uric acid. In order to get a true idea of the amount of kynurenic acid in the urine it was necessary therefore, either to modify Capaldi's method so as to eliminate the possible contamination of the precipitate with uric acid, or to make separate estimations of the uric acid content of the Capaldi precipitates, or to choose a diet for the dogs on which they would excrete an amount of uric acid insufficient to interfere with the estimations.

In one series of experiments (Table I b) in which a dog on a meat diet was given constant doses of tryptophane, the kynurenic acid (admixed with uric acid) was estimated by Capaldi's method (pptes. A) and the uric acid content of the precipitate was estimated as follows:

The kynurenic-uric acid precipitates (pptes. A) were dissolved in ammonia and the excess of ammonia was evaporated off on the water bath. The solution

[6] Capaldi, A., *Ztschr. f. physiol. Chem.*, 1897, xxiii, 92.

was made slightly alkaline with ammonia and saturated with Kahlbaum's pure recrystallized ammonium chloride. The precipitated ammonium urate was decomposed and ultimately weighed on a Gooch crucible as uric acid.

At the same time determinations of the total amount of uric acid present in the urine were made by Hopkins' method in order to ascertain whether there was any simple relationship between the amount of uric acid present in the urine and that carried down with the Capaldi precipitates: there was none.

In view of the interference of uric acid in the estimation of kynurenic acid by Capaldi's method and the labor involved in the subsequent estimations of the uric acid content of the mixed precipitates of kynurenic and uric acids, it seemed worth while to determine whether uric acid was excreted to any appreciable extent in the urine of dogs on a diet of a lower purine value.

Of all the observers who have studied the question of the origin of kynurenic acid in the metabolic processes of the dog, amongst whom may be mentioned Liebig, Schmidt, Schmeiderberg, Schultzen, Hauser, Voit and Reiderens, and Solomin, the last mentioned alone seems to have recognized that uric acid was excreted by dogs in any appreciable amount.

Solomin[7] made determinations of the relative amounts of kynurenic acid and uric acid excreted by dogs on a diet of 400 gm. of horse flesh, 250 cc. of milk, and 250 gm. of bread a day. He determined the total output of uric acid by the Salkowski-Ludwig method and the kynurenic acid by Capaldi's method. For a dog of about 9 kg. weight the average output of uric acid was 0.11 gm. and of kynurenic acid 0.30 gm. a day.

It will be seen (Tables I a and I b) that the dogs on a diet of 500 grams of meat per day were excreting somewhat larger amounts of uric acid than were excreted by the dogs in Solomin's experiments. Further, it was found (Tables IV a, b, and c) that traces only of uric acid were excreted by dogs on a diet of bread and milk, of bread and milk and a small amount of meat (25 to 50 grams) of dog biscuits alone, and on a starvation diet.

If a dog can be induced to take any of these diets with regularity then Capaldi's method can be used for the estimation of the kynurenic acid content of the urine. The results thus obtained will give a true representation of the acid present and will not be too high on account of its contamination with uric acid.

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[7]Solomin, P., *ibid.*, 1897, xxiii, 501.

Since the presence of uric acid interferes with the estimation of kynurenic acid it was necessary to ascertain whether the estimation of uric acid by (*a*) Hopkins' and (*b*) by Folin's methods could be relied on in presence of kynurenic acid.

(*a*) Mixtures of uric and kynurenic acids were taken. The mixture of acids was dissolved in the least possible quantity of sodium carbonate solution and the uric acid estimated by Hopkins' method.

Mixture of acids.		Uric acid precipitated as ammonium urate and estimated as uric acid by	
Weight of uric acid taken.	Weight of kynurenic acid taken.	1. Titration with $KMnO_4$ solution.	2. Weighing on a Gooch crucible.
gm.	*gm.*	*gm.*	*gm.*
0.1209	0.1232	0.1202	
0.2458	0.1506	0.2432	
0.3406	0.1256		0.3320
0.1980	0.1720		0.1920

There was no indication of the contamination of the final precipitates of uric acid with kynurenic acid.

(*b*) Mixtures of uric and kynurenic acids were taken and the uric acid was estimated by Folin's colorimetric method.

Three separate solutions of weighed amounts of pure uric and kynurenic acids were made by dissolving the mixed acids in a solution of lithium carbonate and making the volume up to 100 cc. Aliquot parts of these solutions (2 cc.) were treated with Folin's phosphotungstic reagent and estimated colorimetrically against a standard solution of pure uric acid treated in the same way.

Mixture of acids.		Weight of uric acid in total volume of solution estimated colorimetrically by Folin's method.
Weight of uric acid taken.	Weight of kynurenic acid taken.	
gm.	*gm.*	*gm.*
0.100	0.100	0.100
0.100	0.100	0.998
0.100	0.100	0.101

From these results it is obvious that the presence of kynurenic acid does not interfere with the estimation of uric acid by Hopkins' and by Folin's methods.

In the accompanying tables are given the results of the estimations of the amount of kynurenic acid eliminated in the urine of dogs after the administration of tryptophane. Tables I a, b, and c deal with the administration of repeated similar doses of tryptophane to dogs and puppies; Tables II a, b, and c with the administration of increasing doses of tryptophane to dogs and to puppies and a consideration of the output of total nitrogen during the tryptophane feeding; Table III, with the administration of large doses of tryptophane; Tables IV a, b, and c with the excretion of uric acids by dogs on various diets.

Before attempting to draw any conclusion as to the significance of the data given in these tables with regard to the possibility of the elimination of kynurenic acid being of the nature of a side issue, and not representing the main line of breakdown of tryptophane in the animal body, it was necessary to ascertain whether kynurenic acid, once formed, could be further dealt with by the body: in other words, does the kynurenic acid isolated from the urine represent the total amount produced from the tryptophane absorbed?

Hauser[6] administering kynurenic acid to dogs by the mouth and by subcutaneous injection was able to recover in one case, 36 per cent. and in another case, 56 per cent. of the tryptophane given. He found that after the administration of the substance to man none could be recovered from the urine. On the other hand Solomin[7] found that there was as much as 90 per cent. of the kynurenic acid administered by injection excreted in the unchanged form by dogs and 60 per cent. by rabbits. The amount of the substance recovered after administration by the mouth was much less for both animals. In the case of man none or only a very small percentage of the substance could be recovered after administration by the mouth.

It is possible that the samples of kynurenic acid used by Solomin, isolated by Capaldi's method from the urine of dogs kept on a high meat diet for some time, were contaminated with uric acid. The administration of a "mixed" product would lead to results showing a higher loss than had actually taken place. In Hauser's experiments with dogs a considerable loss was shown; here again the investigator may not have been dealing with a pure product.

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[6]Hauser, A., *Arch. f. exper. Path. u. Pharmakol*, 1895, xxxvi, 1.

With Hauser's results indicating a possible utilization of at least 50 per cent. of the kynurenic acid administered, it was necessary to ascertain whether, with pure kynurenic acid, the results would confirm those of Solomin or those of Hauser.

One of my dogs who had been on varying amounts of tryptophane was put on a diet on which he was excreting traces only of uric acid and no kynurenic acid. Doses of chemically pure kynurenic acid prepared according to the directions given in a previous paper (Homer)[5] were administered to the dog. The kynurenic acid content of the urine excreted during the thirty-six hours following the administration of the substance was estimated by Capaldi's method.

Weight of kynurenic acid administered.	Weight of kynurenic acid isolated from the urine by Capaldi's method.
gm.	gm.
0.690 (subcutaneously)	0.630
0.330 (by mouth)	0.266

These results support the view that the body of the dog does not utilize the kynurenic acid to any appreciable extent; whence it follows that, in the consideration of the experimental results recorded in the accompanying tables, the output of kynurenic acid can be taken as a true representation of the total amount of kynurenic acid formed by the body from the tryptophane administered to the animal.

A study of the accompanying tables shows that:

1. Kynurenic acid is not utilized by the body of the dog but is eliminated in the urine in an unchanged condition.

2. There is no simple relation between the amount of tryptophane administered and the kynurenic acid eliminated in the urine.

If the tryptophane were completely changed into kynurenic acid the proportion should be: 1 gram of tryptophane: 0.95 gram of kynurenic acid.

The greatest amount of kynurenic acid excreted by any of the dogs was only 38 per cent. of the theoretical amount possible; the average was about 10 per cent. and in some cases it was as low as or even lower than 5 per cent. of the theoretical amount.

3. In each animal the excretion of kynurenic acid reached a maximum beyond which further increase in the dose of tryptophane was without effect.

The amount of tryptophane excreted by any one dog may depend upon the relative size of its liver. Thus a dog of 23½ pounds' weight excreted as much as 1.8 grams of kynurenic acid; a dog of 13½ pounds' weight excreted 0.64 gram of the acid; puppies of 6 pounds 14 ounces', and 4 pounds 5½ ounces' weight respectively excreted 0.3 and 0.13 gram of the acid on the same weights of tryptophane, etc.

4. The young animals have a greater capacity for the utilization of tryptophane than the fully grown dog has (Tables I c and II b).

5. There is no marked increase in the output of nitrogen during the tryptophane feeding period. The administration of the substance has therefore no marked effect on the general metabolic processes of the body (Table II c).

6. Even after flooding the digestive organs with a large dose of tryptophane, the corresponding amount of kynurenic acid was not eliminated: neither could any unchanged tryptophane be detected in the urine, nor could any other indol derivatives be detected in the urine by colorimetric methods (Table III).

SUMMARY.

The results of the investigation seem to indicate that:

1. Kynurenic acid apparently is not readily capable of further utilization in the body of the dog as all which is administered is excreted in the urine. It is therefore justifiable to assume that all the tryptophane converted into kynurenic acid in the body is excreted in the urine as such.

2. The production of kynurenic acid from tryptophane does not represent either the only or the main line of breakdown of tryptophane in the animal body; this is specially true of the young animal.

The evidence adduced in this paper seems to indicate that a certain amount of the tryptophane administered becomes "side-tracked" into kynurenic acid while the remainder is utilized in some direction not yet elucidated, but which may probably have

some intimate connection with the furnishing of the particular substance or substances necessary for the maintenance of life of the individual.

The work described in this paper was undertaken originally at the suggestion of Dr. Hopkins, Cambridge, England. The author wishes to express her appreciation of the help he has given and the interest he has shown during the progress of the investigation.

TABLE I a.

The Repeated Administration of Tryptophane.
Dog A. (Weight 13½ lbs.)

Date.	Diet.	Volume of urine.	Kynurenic acid in urine estimated by Capaldi's method.
		cc.	*gm.*
Mar. 31—Apr. 1	250 gm. lean meat	190	
Apr. 1- 2	" " " "	170	Traces.
2- 3	500 " " "	Not measured	
3- 4	500 gm. meat 3 gm. tryptophane	400	0.648
4- 5	" " " " " "	475	0.512
5- 6	" " " " " "	253	0.578
6- 7	" " " " " "	300	0.640
7- 8	" " " " " "	339	0.625
8- 9	" " " " " "	250	0.540
9-10	" " " " " "	350	0.518
10-11	" " " " " "	400	0.542
11-12	" " " " " "	310	0.535
12-13	500 gm. lean meat	350	0 1403
13-14	" " " "	440	0.0877
14-15	" " " "	500	0.2025
15-16	" " " "	Not estimated	
16-17	" " " "	370	0.1072
17-18	" " " "	290	0.0920
18-19	" " " "	390	Spoilt.
19-20	" " " "	360	0.0982

It was shown afterwards that the precipitates weighed as kynurenic acid between the dates April 12-20 were practically pure uric acid.

TABLE I b.

The Repeated Administration of Tryptophane to a Dog on a Meat Diet. Dog B. (Weight 23¼ lbs.) Estimation in the Urine (1) of the Kynurenic Acid by Capaldi's Method. (2) Estimation of the Uric Acid Contaminating the Capaldi Precipitates and Hence the True Value for the Kynurenic Aci[illegible] Output. (3) Estimation of the Uric Acid Content of the Urine by Hopkins' Method.

Date.	Diet.	Total volume of urine.	(1) Weight of *kynurenic acid* by Capaldi's method. *Pptes. A.* = kynurenic — uric acid.		(2) Kynurenic acid after deducting uric acid contaminating Pptes A.		(3) Total amount of *uric acid* in the urine estimated by Hopkins' method on 100 cc. of the urine.	
			(a) per cc.	(b) Total.	(a) per cc.	(b) Total.	(a) per cc.	(b) Total.
May		*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
21–22	500 gm. meat	288	0.000344	0.099	0.000277	0.080	0.000472	0.136
22–23	" "	380	0.000934	0.355	0.000724	0.275	0.000482	0.183
23–24	" "	340	0.00168	0.502	0.00130	0.441	0.000483	0.164
24–25	" "	544	0.00106	0.578	0.000901	0.491	0.000404	0.220
25–26	" "	464	0.00094	0.436	0.000728	0.338	0.000342	0.168
26–27	500 gm. meat and 5 gm. tryptophane	282	0.00218	0.707	0.00226	0.636	0.00056	0.155
27–28	" " " " "	349	0.00261	0.901	0.00300	0.832	0.000535	0.187
28–29	" " " " "	558	0.00349	1.946	0.00307	1.812	0.000571	0.319
29–30	" " " " "	322	0.00498	1.603	0.00487	1.573	0.000639	0.206
30–31	" " " " "	818	0.00221	1.804	0.00209	1.712	0.000309	0.253
June								
1–2	500 gm. meat	384	0.00081[illegible]	[illegible]	Spoilt		0.000560	0.215
2–3	" "	366	0.000752	[illegible].275	0.000511	0.187	0.000441	0.161
3–4	" "	316	0.000538	0.170	0.000332	0.105	0.00037[illegible]	0.117
4–5	" "	461	0.000455	0.210	0.000276	0.127	0.000324	0.149
5–6	" "	338	0.000462	0.150	0.000248	0.0838	0.000426	0.142
			Mixed precipitates A (Capaldi)		*True value for kynurenic acid.*			

TABLE I C.

The Administration of Tryptophane to Puppies from the Same Litter, Aged 2 Months. Dogs C and D.

Date.	Diet.	Dog C. Weight 4 lbs. 5½ oz.		Dog D. Weight 6 lbs. 14 oz.	
		Volume of urine.	Kynurenic acid excreted.	Volume of urine.	Kynurenic acid excreted.
July		cc.	gm.	cc.	gm.
22–23	Bread and milk		Traces		Traces.
23–24	" " "		"		"
24–25	" " "		"		"
25–26	Bread and milk and 3 gm. tryptophane	290	0.0885	204	0.170
26–27	" " " " " "	136	0.158	132	0.255
27–28	" " " " " "	152	0.148	160	0.283
28–29	" " " " " "	170	0.135	220	0.316
29–30	" " " " " "	166	0.131	202	0.292
30–31	" " " " " "	144	0.125	150	0.253
July 31–Aug. 1	" " " " " "		Traces		Traces.
Aug. 1–2	" " " " " "		Nil		Nil.

It was ascertained that on a diet of bread and milk there was no excretion of uric acid.

TABLE II a.

The Administration of Increasing Doses of Tryptophane to a Dog.
Dog B. (Weight 23½ lbs.)

Date.	Diet.	Volume of urine.	Uric acid excreted.	Kynurenic acid excreted.
		cc.		*gm.*
Nov.				
18–19	Bread and milk and 50 gm. meat		Traces	Traces
19–20	" " " " " " "		"	"
20–21	" " " " " " "	295	"	"
21–22	The same and 1 gm. tryptophane	305	"	0.0939
22–23	" " " " " "	None		
23–24	The same and 2 gm. tryptophane	560	"	0.156
24–25	" " " " " "	345	"	0.354
25–26	" " " 4 " "	None		
26–27	" " " 6 " "	180	"	0.224
27–28	" " " " " "	500	"	0.679
28–29	Dog died			

A post mortem examination of the dog showed considerable hemolysis of the liver and kidney.

TABLE II b.

The Administration of Increasing Doses of Tryptophane to a Puppy Aged 5 Months. Dog C. (Weight 16 lbs.)

Date.	Diet.	Volume of urine.	Uric acid excreted.	Kynurenic acid excreted.
		cc.		*gm.*
Oct.				
27–28	Bread 150 gm., milk 250 cc., meat 25 gm.		Nil.	Traces.
28–29	" " " " " " " " "		"	"
29–30	" " " " " " " " "		"	"
30–31	" " " " " " " "		"	"
Oct. 31 Nov. 1	The same and 1 gm. tryptophane	380	"	Spoilt
1– 2	" " " 2 " "	255	"	0[illegible]
2– 3	" " 4 " "	406	"	0.[illegible]
3– 4	" " " 6 " "	[illegible]	"	0[illegible]
4– 5	" " " 8 " "	2[illegible]	"	[illegible]
5– 6	Bread, milk, and meat	[illegible]	"	0[illegible]

TABLE II C.

Administration of Increasing Doses of Tryptophane. Estimation of the Total Output of Kynurenic Ac[illegible] of N[illegible]gen during the 24 hour Periods. Dog F [illegible] Weight 15 lbs.)

Date.	Diet.	Volume of urine.	Kynurenic acid excreted.	Total nitrogen (Kjeldahl).	Uric acid excreted
		cc.	*gm.*		
Feb. 27–Mar. 4	150 gm. dog biscuits and 25 gm. meat daily		Not estimated		Nil.
Mar. 4– 5	150 gm. biscuits and 25 gm. meat	132	0.0134	4.03	
5– 6	" " " " "	128	0.0110	3.56	
6– 7	" " " " "	218	0.100	5.21	"
7– 8	[illegible] and 1 g[illegible] [illegible]ane	188	0.0438	4.03	"
8– 9	" 2 "	149	0.1880	4 [illegible]	
9–10	" " 3	183	0.1994	4.1[illegible]	"
10–11	" " " 4 "	256	0.1736	4.05	"
11–12	" " " 5 "	[illegible]	0.4332	4.16	"
12–13	" " " 6 "	221	0.3038	5.20	"
13–14	" " " 7 "	1[illegible]	0.4120	4.46	"
14–15	150 gm. dog biscuits and 25 gm.[illegible]	[illegible]	[illegible]0048	4.58	"
15–16	" " " " "	[illegible]	[illegible]078	5.06	
16–17	" " " " "	[illegible]	0.0060	2.38	
17–18	" " " " "	[illegible]4	[illegible]0074	4.71	

There was no free tryptophane in the urine and no indication of the elimination of indol pigm[illegible].
1 gm. of kynurenic acid contains 0.074 gm. of nitrogen.
1 " " tryptophane " 0.134 " " "

TABLE III.

The Administration of Large Doses of Tryptophane to a Dog. Dog B. (Weight 23½ lbs.)

Date.	Diet.	Volume of urine.	Kynurenic acid excreted.
		cc.	*gm.*
Oct.			
13–14	Bread 250 gm., milk 250 cc., meat 50 gm.	540	Traces.
14–15	" " " " " " " "	342	"
15–16	The same and 10 gm. tryptophane (Only about ½ of food eaten.)	66	0.212
16–17	The same and 10 gm. tryptophane	685	1.256

A further dose of 10 gm. of tryptophane was given with the dog's food on Oct. 17 but by this time the dog refused to touch any more food in the cage whether with or without the addition of tryptophane.

Tryptophane has a very bitter taste and it was difficult to administer as much as 10 gm. at a time and successfully mask its bitterness.

TABLE IV a.

The Excretion of Uric Acid by a Dog on a Meat Diet. (Compare with Results for Dog B, Table I b.) Dog H.

Date.	Diet.	Volume of Urine.	Total output of uric acid for the 24 hours estimated by	
			Folin's method on 5 cc. urine.	Hopkin's method on 100 cc. urine.
		cc.	*gm.*	*gm.*
Apr.				
1– 2	Biscuits	150	Traces	
2– 3	"	235	"	
3– 4	1 lb. lean meat	167	0.0729	
4– 5	" " " "	264	0.131	
5– 6	" " " "	160	0.0929	
6– 7	" " " "	150	0.0622	
7– 8	" " " "	175	0.155	0.160
8– 9	" " " "	324	0.239	0.250
9–10	" " " "	232	0.130	
10–11	Biscuits	52	0.0150	
11–12	"	160	Traces	

TABLE IV b.

The Excretion of Uric Acid by Dogs on Diets with Low Purine Value.

Date.	Diet.	Uric acid excreted.	Kyourenic acid excreted.
Dog A on diet of bread and milk for some days previous.			
June			
13–14	Bread and milk	Traces	0.0148
14–15	" " "	"	0.0083
15–16	" " "	"	Traces.
Dog B on diet of bread and milk and small amount of meat for some days previous.			
Nov.			
18–19	Bread, milk, and 50 gm. meat	Traces	Traces.
19–20	" " " " " "	"	"
20–21	" " " " " "	"	"
Dog E on diet of dog biscuits for some days previous.			
Dec.			
16–17	150 gm. dog biscuits	Traces	Traces
17–18	" " " "	"	"
18–19	" " " "	"	"
Dog F on diet of dog biscuits and small amount of meat.			
Mar.			
4–5	150 gm. biscuits and 25 gm. meat	Traces	0.0134
5–6	" " " " " " "	"	0.0110
6–7	" " " " " " "	"	0.0110

TABLE IV c.

The Excretion of Uric Acid by a Dog Kept Without Food from Feb. 24 to Mar. 4. Dog G.

Date.	Volume of urine.	Total excretion of uric acid (estimated by Folin's method on 5 cc. of urine).
Feb.		
24–25	410	0.013
25–26	516	0.019
26–27	344	0.030
27–28	710	0.032
Feb. 28–Mar. 1	260	0.031
Mar.		
1–2	452	0.023
2–3	398	None.
3–4	276	None.

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