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ON UROBILIN. PART I. THE UNITY OF UROBILIN.

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I. Historical Sketch.

The earliest practical outcome of the application of the spectroscope to the study of the colouring matters of urine was the discovery by Jaffé[1], in 1868, of the substance which yields the well-known absorption band between the *b* and F lines of the solar spectrum. Jaffé detected the presence of this pigment both in normal and morbid urines, and showed that it exists in them in part ready-formed, and in part as a precursor, or compound, of negative spectroscopic properties, which readily becomes converted into the band yielding pigment on standing, or on the addition of an acid. To this substance Jaffé assigned the name of urobilin, believing it to be derived from the pigment of bile, and subsequent investigations have converted this belief into a practical certainty. He quickly recognised the fact that urobilin is only one of several colouring matters of urine, and that it is not that to which its yellow colour is mainly due.

The ninth volume (that for 1871) of the *Centralblatt für die medicinische Wissenschaften*, the periodical in which Jaffé first announced his discovery, contains three short communications which must be ranked among the most important contributions yet made to our knowledge of this, the hitherto most extensively studied of the urinary pigments.

[1] *Centralblatt f. die med. Wissenschaften*, VI. p. 243, 1868, and *Virchow's Archiv*, XLVII. p. 405, 1869.

In the first of these Vanlair and Masius[1] showed that the fæces contain a pigment closely allied to the urobilin of urine, for which they proposed the kindred name of stercobilin. In the second[2] Jaffé claimed stercobilin as not merely allied to, but identical with the urobilin of urine. In the third Maly[3] described the formation, by the action of sodium amalgam upon bilirubin, of a substance possessing the characteristic properties of urobilin. Maly[4] subsequently determined the ultimate composition of his artificial product, to which, as it contained more hydrogen and less carbon than bilirubin, he gave the name of hydrobilirubin, and assigned to it the formula $C_{32}H_{40}N_4O_7$. Maly believed hydrobilirubin to be identical with the urobilin of Jaffé, but this view has been questioned by several later observers (Le Nobel[5], Disqué[6], MacMunn[7], Eichholz[8]), who have maintained that Maly's product is either an impure substance, or represents an intermediate stage in the reduction of bilirubin to urobilin.

Hoppe-Seyler[9] next showed that a pigment possessing similar properties may be obtained by the reduction of hæmatin with tin and hydrochloric acid, and Nencki and Sieber[10] have obtained a like product by the reduction of hæmatoporphyrin. Here again, although the pigments obtained resemble urobilin very closely, their identity has been questioned on various grounds.

The subject of the artificial production of urobilin-like pigments is greatly complicated by the remarkable fact that products bearing an equally close resemblance to the urinary pigment have been obtained by Stokvis[11] from bilirubin, and by MacMunn[12] from hæmatin, by the employment of oxidizing in place of reducing agents.

More recently F. Müller[13] has made the important observation that intestinal micro-organisms possess the power of converting bilirubin into a pigment which he believes to be identical with urinary urobilin. Gerhardt[14], Adolf Schmidt[15], Beck[16], and Esser[17] have obtained

[1] *Centralb. f. d. med. Wissensch.* IX. p. 369. 1871. [2] *Ibid.* p. 465. [3] *Ibid.* p. 849.
[4] *Ann. der Chemie u. Pharmacie*, CLXIII. p. 77. 1872.
[5] *Arch. f. d. ges. Physiol.* XL. p. 501. 1887.
[6] *Zeitsch. f. physiol. Chemie*, II. p. 259. 1878–9. [7] This *Journal*, X. p. 71. 1889.
[8] *Ibid.* XIV. p. 326. 1893. [9] *Ber. deutsch. chem. Gesellschft.* VII. p. 1065. 1874.
[10] *Arch. f. exper. Pathol. u. Pharmakol.* XXIV. p. 430. 1888.
[11] *Centralb. f. d. med. Wissensch.* XI. p. 211. 1873.
[12] *Proc. Roy. Soc.* XXXI. p. 226. 1880.
[13] *Schlesische Gesellsch. f. vaterland. Kultur.* Jan. 1892.
[14] *Ueber Hydrobilirubin.* Diss. Berlin, 1889.
[15] *Verhandlungen des xiii Cong. f. inn. Med.* p. 320.
[16] *Wiener klin. Wochenschr.* VIII. p. 617. 1895. [17] *Dissert.* Bonn, 1896.

similar results, and there appear to be strong reasons for thinking that this is the ordinary mode of formation of urobilin in the animal body, although the possibility that hæmoglobin may, under certain circumstances, be directly converted into urobilin, without passing through the stage of bilirubin, cannot yet be excluded. That urobilin occurs in excess in the urine when extravasations of blood are being absorbed, and also when an unusually active hæmolysis is in progress must be regarded as an established fact.

Of the many observers who have contributed to our knowledge of urobilin the majority have devoted special attention to the mode of origin of the pigment and the clinical significance of its presence in the urine in excessive amount, whilst others, who constitute a much smaller group, have chiefly studied its chemical and spectroscopic properties.

We do not propose to enter here upon any discussion of the rival theories of urobilin formation which have been so ably advocated by F. Müller, Riva, Hayem, and many others, as these theories lie outside the province of the present investigation. MacMunn maintains that the urobilin of normal urine, although exhibiting an absorption band between *b* and F, and yielding a green fluorescence with zinc chloride and ammonia, is a distinct substance from that which yields the conspicuous band seen with many morbid specimens, and believes that whereas the latter is a reduction product of the bile and blood pigments, the former is an oxidation product. He bases the distinction chiefly upon the presence of certain extra spectroscopic bands, and on differences in the character of the essential urobilin band. Eichholz and Adolf Jolles[1] have more recently drawn a similar distinction between normal and pathological urobilin, but their descriptions of the two pigments do not accord with each other, or with those of MacMunn.

In this, the first, instalment of our paper we propose to describe a series of observations and experiments directed towards the following objects—

1. The extraction of urobilin from urine in as pure a condition as possible by processes free from the objections which may be urged against those hitherto employed.

2. The study of the properties of the pigment so extracted.

3. The comparison of the urobilin of normal and morbid urines with that obtained from other sources within the body.

[1] *Archiv f. d. ges. Physiol.* LXI. p. 628. 1895.

In a second part we hope to discuss the nature and properties of the so-called chromogen of urobilin; the properties of the various artificial products resembling or identical with urobilin; and the ultimate composition of the urinary pigment.

In the course of our work we have been fortunate enough to light upon a new spectroscopic property of the pigment to which we attach very great importance, as affording evidence of the identity of the various products which yield it, and we may state at the outset, that our observations have convinced us of the unity of urobilin as it is obtained from the various human excreta and secreta in which it occurs.

II. The Separation of Urobilin from Urine.

A. *Methods hitherto employed.*

Jaffé's original process for the separation of urinary urobilin involved a preliminary precipitation by means of zinc chloride and ammonia. He was led to the use of these reagents by a chance observation made in the course of a determination of creatinin by Neubauer's process, in which the filtrate from the creatinin-zinc chloride precipitate was noticed to be highly fluorescent. It was found, in other and later observations, that urines possessing in a marked degree the special optical properties which Jaffé had already learnt to associate with the presence of urobilin, were just those which exhibited in the most definite manner this fluorescence with zinc chloride and ammonia.

Thus by a fortunate chance Jaffé was enabled at once to establish the most important reaction of the pigment, and to secure a process for its isolation; for whilst the fluorescence was seen to be due to a compound of urobilin remaining in solution, it was found that, by accurately adjusting the proportions between the zinc chloride and ammonia added, much of the pigment could be precipitated. The historic process based upon this observation is well known, but it will be convenient to give here a translation of Jaffé's own account of it:

"The urine is treated with a not too small excess of ammonia, and filtered from the precipitated phosphates. To the filtrate a concentrated alcoholic solution of zinc chloride is added until no further precipitate is produced. If the filtrate still contains much of the pigment the remainder may be thrown down by the further addition of ammonia.

"The voluminous zinc precipitates have usually a fine red or red-brown colour; they are carefully washed with cold, and afterwards with hot water, until the filtrate gives no reaction for chlorides; they are then boiled up with alcohol, and finally thoroughly dried at a low temperature. The dry powdered mass is now dissolved in aqueous ammonia, which leaves but a very slight insoluble residue. The solution, which is loaded with pigment, is precipitated by lead acetate.

"The lead precipitate, which has usually a well-marked red colour, is washed with cold water (but not for too long a time, else an appreciable quantity of pigment goes again into solution), dried and extracted with alcohol containing sulphuric acid. The solution is mixed with about half its volume of chloroform, and is then shaken with a large excess of distilled water.

"When the chloroform solution has separated and become clear, it is removed by means of a separating funnel and washed once or twice with distilled water. The washing must be performed as quickly as possible, and not too much water must be used, or considerable quantities of the pigment will go into the washings[1]. As soon as the latter begin to be coloured the chloroform is removed and distilled off."

It is of interest to note that this, the earliest described process for the separation of urobilin, surpasses all others since described in the purity of the product which it is capable of yielding. We feel sure that no one, who becomes acquainted with the properties of pure urobilin, can doubt, after reading Jaffé's description of the substance which he prepared, that he was dealing with urobilin in a state of purity.

Esoff[2] has indeed stated that from residues left by the chloroform in the final stage of Jaffé's process he was able to extract with ether a reddish substance, distinct from urobilin, leaving behind the latter in purer condition as a brown amorphous mass. Our own experience leads us to believe that the reddish substance thus extracted was urobilin itself. After it has been heated upon the water bath the pigment does not go into ether with great readiness, and on extracting dried residues with this solvent solutions may be obtained, which have a pink colour, because very weak. It is a fact which we emphasize

[1] As long as the liquids are acid, the urobilin tends to remain in the chloroform, but the first washing waters tend to remove the acid, and as the later ones become more and more nearly neutral, they are increasingly apt to remove the pigment.

[2] *Archiv f. d. ges. Physiol.* XII. p. 50. 1876.

elsewhere, and one well known to Jaffé, that very dilute solutions of urobilin are distinctly pink in colour, and we can assert at any rate that in repeating Esoff's observation, the pinkish ethereal extracts sometimes obtained have always shown a band at F proportionate to the amount of colour present.

There are many difficulties connected with the zinc chloride separation, some of which are referred to below, but we venture to repeat our conviction that Jaffé obtained an excellent product by its means, and that his descriptions, so far as they go, apply exactly to the properties of the pure pigment.

Ten years after Jaffé's discovery of the pigment, Méhu[1] made the important observation that urobilin could be precipitated from urine, to which a little sulphuric acid had been added, by saturation with crystals of ammonium sulphate. From the precipitate thus produced he isolated the pigment by extraction with absolute alcohol, rendered alkaline with ammonia, the alcoholic extract being afterwards evaporated to dryness at a low temperature.

This method has since been employed by many observers, not only for the separation of urobilin, but also for the estimation of its quantity. Minor modifications have sometimes been introduced in the final stages[2], but an efficient fractionation of the product has never been secured.

We shall have occasion, immediately, to discuss this process further, and it is also dealt with in a later section of this paper. To avoid repetition we will here only state our belief that the ammonium sulphate separation, although a most valuable one, has never yet been so used as to yield a product even approximately pure. We may note in this place the important observation of Eichholz that the chromogen of urobilin is also precipitated by ammonium sulphate, and may be obtained from urines in which it is present by using Méhu's method without preliminary acidification with sulphuric acid.

The only other methods of importance which have been used for the separation of urobilin are based upon precipitation with the acetates of lead. MacMunn precipitates the urine with both acetates, and treats the mixed precipitates with alcohol containing sulphuric acid. The solution filtered from lead sulphate is largely diluted with water, and shaken with chloroform. The latter is then evaporated to dryness, and the residue redissolved in alcohol or a second time in chloroform.

[1] *Bull. de l'Acad. de Méd.* [2] VII. p. 671. 1878.

[2] Cf. G. Hoppe-Seyler. *Virchow's Archiv*, CXXIV. p. 30.

Adolf Jolles precipitates with the basic acetate only, decomposes the lead precipitate in accordance with MacMunn's method, and purifies the product by repeated solution in alcohol.

Lead precipitation is a process with so little selective power for individual urinary pigments (most urines yielding practically colourless filtrates after treatment with the mixed acetates) that it is not surprising if the precipitates obtained should yield to solvents such as acid alcohol and chloroform—which as regards the majority of animal pigments are themselves not selective—extracts which contain a medley of pigments.

It is remarkable that whilst the first aim of the chemist in other departments is to secure efficient fractionation of mixed products into chemical individuals; in the domain of pigments, where the control of chemical analysis is frequently difficult or impossible of application, the question of fractionation is so often neglected.

The method of Jaffé is free from such objections, but he himself admits that by its means, even when large quantities of highly pigmented urines were employed, only very trifling "geringfügigen" amounts of urobilin were obtainable[1]. This has certainly been our experience of the process. We have found, that the conditions requisite for precipitation are difficult to control, especially in working on a large scale, and even when the original precipitation with zinc chloride and ammonia is approximately complete the product is frittered away in the subsequent stages. It therefore became necessary, before any more complete examination of the pigment could be made, and especially before ultimate analyses could be attempted, that some other line of separation should be devised.

B. *New methods of extracting urobilin from urine.*

In this section are detailed the principles of two methods which we have found satisfactory. Both are primarily based upon the separation by ammonium sulphate, but each involves important modification of Méhu's original method. It should be premised that they are described as methods by which pure urobilin may be obtained, and *not* as processes for its estimation.

(*a*) *A Precipitation method.* The precipitate obtained when urine is saturated with ammonium sulphate is mainly composed of ammonium urate. The sulphate will, in fact, precipitate uric acid in the form of ammonium urate as completely as the chloride, although

[1] Jaffé. *Loc. cit.* p. 416.

more slowly and in a less pure condition. With the urate are associated not only urobilin and its chromogen, but other pigments and chromogens also[1]. Even when the urine is first feebly acidified, as in Méhu's process, the precipitate is still largely composed of ammonium urate; the chromogens are decomposed, but other pigments are precipitated in addition to urobilin. Investigation shows that some at least of these pigments depend for their precipitation upon association with the urates and are not thrown down when these latter are absent, whereas for urobilin itself ammonium sulphate is a true precipitant. The behaviour of ammonium chloride is strikingly different, for urobilin is not precipitated at all by excess of this salt. When large quantities of the pigment are present some may be carried down mechanically with the urates, but there is no true precipitation. If therefore the urates be first thrown down by saturation with ammonium chloride and filtered off, and if crystals of the sulphate be added to the filtrate, a precipitate of urobilin is obtained on standing, which is much purer than the product of Méhu's process.

It should next be noted that water, although by no means an active solvent of urobilin, takes up this substance much more readily than it does most of the other urinary pigments. If therefore the precipitate yielded by ammonium sulphate, after previous removal of the urates, be dried upon the filter-paper, and then extracted with distilled water (a large proportionate bulk of the solvent being necessary), the latter takes up the urobilin, and effects a further fractionation of the product.

The aqueous solution is now again saturated with ammonium sulphate and the precipitate will consist of urobilin not very far from a state of purity. Such precipitation with the salt and re-solution in water may of course be repeated as often as is thought necessary; the final precipitate is dried and the pigment is extracted with absolute alcohol. When plenty of material is available the final stage should be carried out as follows. The product of the second or third precipitation with ammonium sulphate is dissolved, no longer in pure water, but in dilute ammonia. Whereas a large bulk of neutral water was requisite for solution, in the alkali the urobilin is very freely soluble, and a quantity only just sufficient to dissolve the precipitate is employed, by which means a very concentrated solution is obtained.

[1] Cf. Méhu, *Jour. Pharm.* [5]. VII. 122; and Michailoff, *Jour. Chem. Soc.* LIV. 880.

If now the solution be acidified with a minimal excess of sulphuric acid the urobilin is to a great extent precipitated as an amorphous brown-red powder. This is a step of great value in purification, provided that the urates have been efficiently removed at the first stage.

The precipitate obtained on acidification is best separated by the centrifuge (as it may filter badly). It is washed with a small quantity of a saturated solution of ammonium sulphate, again centrifuged, dried, and finally separated from adherent ammonium sulphate by solution and re-solution in absolute alcohol.

(*b*) *An Extraction process.* When a urine rich in urobilin is shaken with certain organic solvents, and especially with chloroform, it yields to the latter a certain proportion of its pigment. The amount is increased if the urine be first rendered just acid with sulphuric acid, and we have found that the proportion of the pigment going into chloroform is very greatly increased if the urine be saturated with ammonium sulphate in addition to being acidified. The transference now becomes approximately complete, and a urine comparatively poor in urobilin will yield its pigment to chloroform quite satisfactorily.

But in this case also it is of great advantage first to remove the urates by saturation with ammonium chloride, the sulphate being added to the acidified filtrate as before. The removal of urates is in this case valuable for the following reasons:—The urates carry down with them small quantities of certain pigments and chromogens which might otherwise accompany the urobilin into the organic solvent. More important still is the fact that urates when present begin to precipitate almost immediately when saturation with ammonium sulphate is carried out, and they carry with them part of the urobilin, which is no longer available for extraction by the solvent. When, on the other hand, urates are absent the precipitation of the urobilin as such is much slower. Saturation with ammonium sulphate at once diminishes the power of the liquid to hold its urobilin, but actual precipitation is slow to begin.

By these three steps therefore, viz., removal of urates, acidification with sulphuric acid, and, finally, saturation with ammonium sulphate, the urine is brought into a condition to yield up its urobilin with the greatest readiness, and except in cases in which very large amounts of the pigment are present all three steps are necessary before very much of it is taken up by any available solvent.

To extract the urobilin we employ a mixture of chloroform (1 part)

and ether (2 parts), shaking the urine after it has been treated in the manner just described with about its own bulk of the ether chloroform mixture, in a large separating funnel.

The mixture of ether and chloroform does not extract the urobilin so readily as does chloroform alone, but we strongly recommend it as a solvent because it is more selective in its action. If the ether chloroform extract obtained as above be separated from the subjacent saturated urine, and shaken with distilled water, the urobilin is immediately re-transferred to the water. To obtain a ready transfer a trace of alkali should be added, to neutralise any acid which may have passed into the organic solvent from the acidified urine.

If the solution be neutral the water takes up the whole of the urobilin immediately and completely. The ether-chloroform may remain somewhat pigmented, but will no longer show the urobilin band. The aqueous solution being now again saturated with ammonium sulphate, and slightly acidified, once more yields its pigment to ether chloroform.

It should be noted how selective is this method of separation. The pigment leaves the urine or the aqueous solution mainly on account of the quite special effect of ammonium sulphate saturation, and in the absence of this it goes back into water. Any substance which accompanies it into the organic solvent (for reasons independent of the influence of ammonium sulphate) is very unlikely to follow it back into pure water. If however the pigment is to be taken a second time into ether chloroform, as is necessary if the full advantages of the selective influence are to be obtained, the following course is desirable. The aqueous solution obtained at the first stage must be deprived of any ether held by it, before the crystals of ammonium sulphate are again added, since a subsidiary effect of the salt is to cause a rapid separation of the dissolved ether, which, rising to the surface, carries with it much of the pigment, and deposits it upon the surface of the liquid or upon the sides of the vessel.

The ether must not be driven off on the water bath, because urobilin is oxidized with remarkable rapidity when aqueous solutions containing a little ether are thus evaporated. A current of air drawn through the liquid by means of a filter pump will effectually remove the ether in the course of an hour or so, and after such treatment ammonium sulphate may be added and the liquid again shaken with ether-chloroform.

If the pigment has thus been taken a second time into ether

chloroform it may next be removed by shaking, no longer with pure water, but with water rendered distinctly alkaline with ammonia. Great concentration of the product is thus obtained (for the pigment will entirely leave the organic solvent for less than one-twentieth of its bulk of alkaline water[1]). To complete the separation the alkaline solution is acidified and shaken with pure chloroform, and its solution in the latter is filtered and evaporated to dryness. As a final step, to make sure of the removal of all traces of ammonium sulphate, the residue may be taken up in absolute alcohol, the alcoholic solution being again filtered and evaporated.

In the separation by ether chloroform there is some tendency to loss of pigment, because after shaking the saturated aqueous solution with the organic solvent a certain amount of urobilin is apt to be separated at the junction of the liquids. It must indeed be understood that neither of the methods here described is easily made available for quantitative estimation.

In all physical and chemical properties described in this, the first, part of our paper the products obtained by the two methods exactly agree, and they also agree with Jaffé's description of the pigment in all the details to which his observations extended. These methods give, however, a much better yield than does the zinc chloride process, and, although the description of them is necessarily somewhat lengthy, will be found much easier to carry out.

In the second part of this paper we hope to give analyses of products obtained by the two lines of separation here described, respectively.

In concluding this section it will be well to emphasize the necessity of working with urines which contain a sufficiency of the pigment. Cases should be chosen in which the urine as passed shows a well-marked urobilin band.

By working on a large scale we have, indeed, been able to obtain by the ether chloroform process enough urobilin from normal urines for the purposes of this paper, but normal urine contains as a rule such an infinitesimal quantity of urobilin (a fact recognised by Jaffé, but sometimes lost sight of since) that it is at all times a most inconvenient source of the pigment for purposes of investigation.

[1] Disqué has previously shown that urobilin may be removed from chloroform by shaking with a dilute solution of sodium hydrate.

III. The Properties of Urobilin.

(*a*) *Properties of Solutions.* A specimen of urobilin such as is obtained by evaporating to dryness an ammoniacal solution, and dissolving the residue in alcohol, has a brown colour when concentrated; when more dilute becomes brownish yellow, and when very dilute has a dull pink tint. The solution exhibits a green fluorescence, apart from the addition of any reagent (conf. Jaffé and Eichholz). When prepared in the above manner the solution has a feebly acid reaction and shows the absorption band of acid urobilin, but a strictly neutral solution has a somewhat brighter fluorescence, and shows the alkaline band.

On the addition of a drop of an acid, either mineral or organic, the liquid assumes a more orange colour, and the fluorescence disappears entirely.

The acid solution has a warm brown tint when concentrated, becomes bright yellow when more dilute, and when very dilute has, like the original liquid, a dull pink tint. At the yellow stage the colour closely resembles that of normal urine, but at the degree of concentration required for the production of this tint the characteristic absorption band is seen with great intensity, whereas a solution so dilute as to show a band comparable with the urobilin band sometimes seen in normal urine is almost colourless, exhibiting merely a faint pinkish hue.

The true yellow pigment of the urine on the other hand, as isolated by the process described by one of us, yields no absorption band, and retains its yellow colour, however great the degree to which its solutions are diluted[1].

If the solution of urobilin be free from other pigments or pigment yielding substances, no extra bands are developed on the addition of an acid, and as will be shown later any specimen that develops a pink or red colour, under such treatment, must be regarded as impure.

This series of tints, brown, yellow and pink, is common to all the compounds of urobilin, which exhibit, for the most part, the single absorption band, more or less altered in position and character. The pink tint is apparently connected with the extent to which the blue and violet rays penetrate the solution, and the further the absorption band is shifted towards red the sooner is the pink tint developed on dilution.

[1] A. E. Garrod. *Proc. Royal Soc.* LV. p. 394. 1894.

Even in ordinary acid solutions the pink tint persists to a higher degree of concentration in chloroform than in alcohol, and to a higher still in bromoform, and with these latter solvents there is a corresponding displacement of the band towards red.

Position of the redward border of the band of acid urobilin in various solvents, with solutions of equal concentration.

Alcohol	λ 5080	N.B. The displacement is not observed
Chloroform	λ 5130	unless the chloroform or bromoform
Bromoform	λ 5170	be free from alcohol.

In very concentrated acid solutions of urobilin the absorption band is completely lost in general absorption of the more refrangible rays. As dilution proceeds the absorption is seen to be sharply bounded by the redward border of the band; next the blue and violet rays begin to penetrate, and the broad composite band, with its region of complete blackness towards red, and a dark shading extending towards violet, becomes clearly visible. Further dilution enfeebles and ultimately removes the shading, whilst the violet end of the spectrum becomes clear to its utmost limits. The darker portion of the band next undergoes some shrinkage in both directions, its borders become hazy and ill defined, and when a certain stage is reached no further contraction takes place, the band although maintaining a constant breadth becoming fainter and fainter until it ultimately disappears.

The following table illustrates the above changes, each successive solution having been twice as dilute as the preceding one (see also Plate I. *A* 1—6).

Effect of dilution upon the band of urobilin in acidified alcoholic solution.

	Darker portion of band	Shaded portion
1.	λ 5200	Complete absorption of blue and violet.
2.	λ 5130	λ 4590 Uniformly dark.
3.	λ 5110—λ 4810	λ 4810—λ 4590
4.	λ 5080—λ 4825	
5.	λ 5080—λ 4860	
6.	λ 5060—λ 4860	Edges of band hazy.
7.	λ 5060—λ 4860	Band very faint.

The band above described is the only one shown by pure solutions

of urobilin, and we may add that Professor Gamgee, who has kindly examined some of our specimens, reports that he has found no absorption band in the ultra violet spectrum.

Properties of Solid Urobilin. Solid urobilin is amorphous, and not deliquescent. When heated it emits a slight peculiar odour. It varies in colour according to the method of preparation, the precipitate obtained by saturating an aqueous solution with ammonium sulphate being brown, whereas the residue left after the evaporation of an alcoholic solution is reddish brown, and that got by precipitation with acids from alkaline watery solutions is bright red.

We know of no liquid in which urobilin is insoluble, with the exception of water completely saturated with ammonium sulphate. Alcohol and chloroform dissolve it very readily, ether and acetic ether much less readily.

In water it is sparingly soluble, and still less so when a small quantity of acid is added, but it is freely dissolved by water rendered alkaline with ammonia, potash or soda.

A new spectroscopic property of Urobilin. The E band. Jaffé and several later investigators observed the fact that urobilin is much more soluble in alkaline than in acid water, and that by acidifying a concentrated aqueous solution a partial precipitation of the pigment is brought about. However no one of those who mention this fact seems to have observed the remarkable spectroscopic phenomena which accompany this precipitation.

When, to a concentrated solution of nearly pure urobilin in water rendered alkaline with sodium or potassium hydrate, sufficient sulphuric or hydrochloric acid is added to render the liquid faintly acid, a slight turbidity is observed, due to the formation of an impalpable precipitate. If now the turbid liquid be examined with the spectroscope there is seen, in addition to the band of acid urobilin, a sharply defined, narrow band, enclosing, and almost bisected by, the Fraunhofer line E, and which is connected with the urobilin band by a faint shading. (This narrow extra band will be spoken of in future as the E band.) (Plate I. *B.* Fig. 1.)

When the liquid is run through a filter-paper the precipitate is in part removed, but for its complete removal many filtrations, and perhaps some dilution with water will be required. As the precipitate is removed the E band fades and ultimately disappears from the filtrate, the ordinary urobilin band alone remaining. The precipitate upon the filter-paper has a bright red colour and shows the E band when placed

before the spectroscope, and a narrower and less intense urobilin band than the original liquid.

The precipitate is readily dissolved off the paper by alcohol, and yields a pure yellow solution which shows the single band of acid urobilin, all trace of the E band having now disappeared.

It is evident from its complete disappearance when the precipitate is dissolved in alcohol that the extra band is not due to any impurity, but to a peculiar physical state of the solid urobilin thus precipitated, and no such band is seen on examining solid urobilin obtained by other means, as for example by evaporation or by precipitation with ammonium sulphate. Under the microscope the red precipitate is seen to consist of minute amorphous granules.

Precipitation of urobilin from alkaline aqueous solution by means of an acid.

	The E band	Band between *b* and F
1. Spectrum of the liquid with the precipitate in suspension.	λ 5350—λ 5220	λ 5080—λ 4550
2. Spectrum of the precipitate upon the filter-paper.	λ 5350—λ 5220	λ 5080—λ 4770
3. Spectrum of an alcoholic solution of the precipitate.	absent	λ 5080—λ 4550

As might be expected from what has gone before this reaction is a somewhat delicate one, and various disturbing factors, some of them unknown, may interfere with the observation of the E band.

In the first place, it is desirable that the specimen of urobilin used in the experiment should be freshly prepared and as pure as possible, and especially that it should contain no impurity precipitable by acids, for the presence of such impurities may entirely prevent the development of the band.

Secondly, it is important that the pigment should not have undergone any of the modifications to which, as will presently be seen, it is liable.

Thirdly, an excess of acid destroys the E band: and lastly, when the liquid is warmed the suspended precipitate dissolves and the E band does not reappear on cooling.

For the above reasons it does not by any means follow that the reaction will be obtained at the first attempt with any given specimen of urobilin.

Some observers have stated that in faintly alkaline solutions the bands of alkaline and acid urobilin may be simultaneously observed, but, since the darker portions of these two bands actually overlap, this can only amount to an extension of the urobilin band towards red, a phenomenon which is frequently seen in nearly neutral specimens. The E band, on the other hand, is much nearer red, and differs entirely in its characters from that of alkaline urobilin, with which it cannot be confused. Moreover it is never seen in faintly alkaline solutions. Seeing that the spectroscopic recognition of urobilin has hitherto rested upon the identification of a single absorption band, it is evident that the appearance of this more complex spectrum affords very strong evidence indeed that the substances which yield it are identical, and whilst we do not attach any very great weight to the failure to obtain the E band with any given specimen, we cannot but regard a positive result as affording an evidence of identity practically amounting to proof.

As will presently be seen we have obtained and measured the E band spectrum when working with urobilin obtained from normal urine, from morbid urines, from fæces, and from the bile removed from the gall-bladder *post mortem.*

The instability of Urobilin. Urobilin must be regarded as a decidedly unstable substance, although its solutions may be heated with hydrochloric or sulphuric acid without undergoing change. Nitric acid removes the colour of its solutions in a short time, and entirely abolishes the absorption band. Peroxide of hydrogen has a similar but much slower action. As has been shown by several observers, reducing agents also decolorize the solutions and remove the band, but here both colour and band gradually return on exposure to the air.

Urobilin is also liable to undergo modification, and some of the modified forms of the pigment will be described later.

Metallic compounds of Urobilin. Urobilin appears to form compounds with metals some of which show no absorption band in solution, whereas others present a single band differing in position and character from that of the uncombined pigment.

The behaviour of urobilin with ammonia is somewhat peculiar, and if an ammoniacal compound is formed it must be much less stable than the corresponding compound of hæmatoporphyrin, for organic acids are able to restore the acid spectrum, and even by merely evaporating an ammoniacal solution to dryness a residue is obtained which, when dissolved in alcohol, shows the ordinary urobilin band.

On the other hand, neither evaporation to dryness nor addition of an organic acid suffices to decompose the ammonia compound of hæmatoporphyrin, and this difference affords a ready means of ascertaining whether one is dealing with a mixture of these two pigments. If a mixed solution of urobilin and hæmatoporphyrin be rendered alkaline with ammonia and afterwards acidified with acetic acid, the acid liquid will show, in superposition, the spectra of acid urobilin and of alkaline hæmatoporphyrin.

On the addition of ammonia to a solution of urobilin the colour changes to canary yellow, and no absorption band is seen unless the solution be very concentrated. (For an exception to this rule see Section V.) If the original liquid was fluorescent the fluorescence promptly disappears.

Jaffé, whose description of the properties of urobilin is, as has been already mentioned, in complete accord with our observations, noticed and clearly described this change of tint.

As Jaffé further observed, the effect of sodium or potassium hydrate is different. As a rule the canary yellow tint is seen at first, but quickly changes to orange-yellow if the solution be concentrated, or to pink if it be more dilute, and at the same time a well-defined band appears situated nearer to the red than that of acid urobilin. This band preserves the composite character, consisting, like the original band, of two portions, a dark part towards red and a shading extending into the blue (Plate I. *B.* fig. 4). Here as with the ammoniacal solution acetic acid restores the colour and band of acid urobilin.

The zinc compound, as obtained by the addition of zinc chloride and ammonia to a solution of urobilin, is well known on account of the brilliant green fluorescence which it exhibits. When fairly dilute the solution has a rich pink tint by transmitted light, which is replaced by the usual orange yellow as concentration proceeds. The band although rather more pronounced is almost identical in position with that of the sodium and potassium compounds, and has similar characters. (Plate I. *B.* fig. 5.)

On the addition to urobilin solutions of cadmium chloride and ammonia no fluorescence is produced, as Jaffé remarked, and no absorption band is yielded by the pale yellow liquid.

The calcium compound shows a feeble green fluorescence, is yellow in solution and shows no absorption band.

The mercury compound, which is obtained by the addition of a solution of mercuric chloride to a neutral or faintly alkaline solution of

urobilin, is characterised by its beautiful pink colour, which persists to a high degree of concentration, but is ultimately replaced by a yellow tint. This compound has been studied by Adolf Schmidt[1], who recommends the application of a solution of mercuric chloride to the intestinal walls and contents as a test for urobilin in them. When the pigment is present a pink colour is developed. The band of mercury urobilin is nearer to the red than those of the compounds previously described. Its borders are less defined, but it more or less retains the dual character so frequently described. (*B.* fig. 6.)

Zawadski[2] obtained a pink product by adding calomel to a faintly alkaline (soda) solution of artificially prepared urobilin, and the calomel was blackened. We have repeated his experiment with urobilin from urine and have obtained the rich pink colour which he describes, the product being certainly mercury urobilin, as was shown by the character of its absorption band and by the action of acids upon it.

Zawadski describes this reaction as the conversion of urobilin into urorosein, but apart from what has been already said it is impossible that urorosein should thus be formed in an alkaline liquid, seeing that its solutions are promptly decolorized by alkalies.

Silver urobilin is also a pink product, which may be obtained by the addition of silver nitrate to an alkaline solution of urobilin. Its solutions show no distinct band, but merely a broad and very ill-defined shading extending approximately from $\lambda\,5290 - \lambda\,4860$.

Fluorescence. One of the most interesting properties of urobilin is the tendency of its solutions to fluoresce. As we have seen neutral solutions of the pure pigment possess this property, but the brilliancy of the fluorescence varies greatly in different specimens, as might be expected when we consider how readily it is abolished by the presence of small amounts of acid or alkali.

The feeble fluorescence of the calcium salt has also been referred to, but the phenomenon is seen at its best in the solutions of the zinc compound.

In studying fluorescence in such solutions it is necessary to be on one's guard against mistaking for true fluorescence a sort of pseudo-fluorescence due to the dichroic character of urobilin solutions, we refer to the pink and yellow tints which they exhibit in different depths or concentrations.

Adolf Schmidt has described a feeble yellow fluorescence of the

[1] *Loc. cit.*

[2] *Archiv f. exp. Path. u. Pharmakol.* XXVIII. p. 450. 1891.

pink mercurial solutions, but it has seemed to us that we are here dealing with an example of the pseudo fluorescence or dichroism in question. Undoubtedly a yellow light is seen in the pink liquid, but this we believe to be due to the fact that the light, reflected from the further surface of the bottle, having traversed a double thickness of the solution has acquired a yellow tint; and it can be shown that solutions which present this appearance appear yellow instead of pink when examined in double depth by transmitted light.

Even in the zinc solutions the brilliancy of the fluorescence varies greatly. It is to some extent dependent upon their purity; for extraneous pigments tend to arrest the fluorescent rays from the deeper parts of the liquid. For the same reason turbidity of the liquid has a very great effect in impairing the fluorescence. We have not found that colourless neutral salts in solution have any inhibitive effect as long as they do not produce turbidity. As might be expected the fluorescence is most brilliant with concentrated solutions, but it remains clearly visible until a high degree of dilution is reached.

The character of the illumination, in respect of the presence of actinic rays, and the manner of its incidence upon the liquid, have a most important influence.

The amount of alkali present has a profound influence upon the fluorescence of the zinc compound, and excess of ammonia impairs and ultimately destroys it, whilst the pink tint of the liquid is replaced by a pale yellow, and the absorption band at the same time disappears. The appearances suggest, indeed, that the ammonia has replaced the zinc in the compound. The addition of acetic acid destroys alike the fluorescence and the tint.

The usual tint of the fluorescence of urobilin solutions is pale green, but a red tint is sometimes observed, of which it will be necessary to speak later in connexion with the modifications of the pigment.

IV. Are there Two kinds of Urinary Urobilin?

The point has now been reached when it will be necessary to discuss the views of those observers who maintain that two distinct kinds of urobilin occur in urine, a normal and a pathological, and it may be pointed out at the outset that no two of them assign the same properties to the pathological pigment.

The normal and pathological Urobilins of MacMunn.

MacMunn[1] was the earliest exponent of the above view, and his statements, on a subject so closely associated with his name, call for a somewhat detailed examination. He bases the distinction between normal and pathological urobilin upon, (1) differences in the breadth and character of the essential band of urobilin, (2) differences in fluorescent power, (3) differences in colour of the solid pigments and their solutions, (4) the presence of certain extra bands in the spectra of the pathological pigment, and (5) the disappearance of the absorption band of normal urobilin on the addition of an alkali.

It will be well to examine these several differences in turn.

1. MacMunn describes the band of normal urobilin as narrower and less sharply defined than that of pathological, and as wanting the dark shading extending towards the violet. It has been already pointed out that these are exactly the modifications which are produced by dilution of a solution of the pigment, and are those which we might expect to observe if, as is certainly the case, the amount of urobilin in normal urines be very minute compared to that present in many morbid specimens.

2. Dilution has a potent influence in reducing the fluorescent power of solutions of urobilin, and a similar explanation will therefore account for the feebler fluorescence observed with solutions of the normal pigment.

3 and 4. The method employed by MacMunn, viz. precipitation by the neutral and tribasic acetates of lead, extraction by alcohol and sulphuric acid, and shaking the extract with chloroform, after the addition of water, has the great disadvantage of exercising no selective action among the contained pigments, various colouring matters and pigment-yielding substances being carried down; and on extraction with acidified alcohol a solution of mixed pigments is obtained. Among these may be hæmatoporphyrin, urorosein, uroerythrin, and, when much sulphuric acid is employed, the crimson product of the action of that acid upon uroerythrin[2]. There is also present the essential yellow pigment of urine, which shows no absorption band, and the products of its decomposition by the acid. To this list should almost certainly be added some unknown pigmentary substances.

[1] For most recent exposition see This *Journal*, x. p. 71. 1889.

[2] This *Journal*, xvii. p. 446. 1895.

From such solutions chloroform extracts urobilin, hæmatoporphyrin, and urorosein if present, the band of urorosein overlapping and broadening the second band of acid hæmatoporphyrin. It is therefore obviously impossible to regard the products obtained by this method as approaching to purity, but the extracts obtained from normal urine, from which several of the above pigments may be absent, or present in mere traces, are much purer than those from morbid urines.

When working with the above method we have obtained results agreeing closely with MacMunn's descriptions, and we differ from him rather as to the interpretation to be put upon the observed phenomena than as to the phenomena themselves. On the other hand, the results obtained have not in our hands exhibited such uniformity as might be expected if pathological urobilin were a distinct and well-defined pigment, even when all specimens were excluded in which the urobilin band was not exceptionally broad and sharply defined.

A study of MacMunn's published measurements strongly suggests, as one of us has already pointed out[1], that hæmatoporphyrin present as an impurity was largely responsible for the extra bands assigned by him to pathological urobilin; but a further study of preparations made by the lead method showed that such contamination does not explain all the observed phenomena. We have seen the bands described by MacMunn as developed by the addition of ammonia and zinc chloride in specimens which have shown no hæmatoporphyrin bands in the acid state.

We must acknowledge our inability to assign these latter bands to any recognised impurity, for we have not obtained them with solutions of any of the known urinary pigments, but seeing that MacMunn has himself observed a similar zinc spectrum when examining extracts from normal urine[2], the presence of these bands cannot be regarded as affording a distinction between the normal and pathological pigments. On the other hand, we are convinced that the extra bands and the red colour of morbid specimens are due to the presence of impurities for the following reasons:

1. When the specimens are kept for a time the extra bands tend to disappear, the urobilin band alone remaining.

2. The extra bands are not seen with specimens of urobilin extracted from morbid urines by the ammonium sulphate process, either in its simpler form, or as modified by us.

[1] F. G. Hopkins. *Guy's Hosp. Reports*, L. p. 363. 1893.

[2] This *Journal*, x. p. 96. 1889.

3. When an extract obtained by treating the lead precipitate with acidified alcohol, and showing the extra bands, is diluted with several times its bulk of water and filtered, the red impurities are almost completely retained upon the filter-paper, whereas the filtrate has a pure orange or yellow colour; and by saturation of the filtrate with ammonium sulphate, and solution of the precipitate so formed in alcohol, a nearly pure solution of urobilin is obtained, which shows the essential band with great intensity, but no supplementary bands.

4. The original precipitate formed on the addition of water yields when treated with alcohol a pinkish red solution, which shows the extra bands, and sometimes a fairly pronounced urobilin band, but sometimes hardly any. Under any circumstances the great bulk of the urobilin has obviously passed on into the aqueous filtrate.

5. As to the disappearance of the band of normal urobilin on the addition of an alkali, and the mere displacement of that of pathological urobilin by similar treatment, we can only state that in our hands specimens from normal and morbid urines have alike shown the displaced band when rendered alkaline with sodium or potassium hydrate, whereas with ammonia the urobilin from both sources, unless modified, has lost its band, a faint band being only seen with very concentrated solutions. On the other hand, the appearance of a distinct alkaline band in ammoniacal solutions is one of the leading characteristics of the modified form of urobilin most frequently met with.

On the above grounds we believe that we are justified in attributing the differences which MacMunn describes as separating pathological from normal urobilin, in so far as they relate to the character of the band at F, to the far smaller amount of urobilin present in normal urines than in many morbid specimens: and, in so far as they relate to extra bands, to the far more abundant presence of certain unknown pigmentary impurities in the extracts obtained from morbid urines.

The normal and pathological Urobilins of Eichholz.

Eichholz[1], working with the ammonium sulphate method, has obtained results different indeed from those of MacMunn, but which lead him also to the conclusion that there are two distinct kinds of urobilin met with in normal and morbid urines respectively.

When employing the method made use of by Eichholz, viz. saturation of the urine with ammonium sulphate, without previous acidifica-

[1] This *Journal*, XIV. p. 326. 1893.

tion, drying of the precipitate upon the filter and extraction with alcohol, we have obtained results practically identical with his. Normal urines so treated yielded weak solutions of urobilin like those obtained by our process, but from very many morbid urines solutions were obtained which showed a much more intense urobilin band, and which, on the addition of hydrochloric acid gradually acquired a red or pink colour, and showed, in addition to the urobilin band, a shading extending towards the red, and terminating in a comparatively narrow band in green. The fact that the extra band agreed in position and breadth with that of urorosein, which also produces a shading of the whole of the green part of the spectrum, suggested that the observed phenomena might be due to the presence of urorosein as an impurity.

Urorosein is one of the pink pigments which is produced from colourless chromogens present in urine by the action of mineral acids. It was first described by Nencki and Sieber[1] and has been made the subject of a somewhat exhaustive study by Rosin[2], who was enabled to isolate its chromogen in the form of colourless crystals from the urine of horses, in which it is abundantly present. It is greedily extracted from its solutions by amylic alcohol, and is stated by Nencki and Sieber, and by Rosin also, to be insoluble in chloroform, or at any rate to be incapable of extraction by chloroform from the urine in which it is developed. This latter fact, which we can completely confirm, seemed at first sight opposed to the view that this pigment is responsible for the extra band in Eichholz's pathological specimens, seeing that the substance which yields the extra band goes into chloroform with the urobilin. We found, however, that in the presence of alcohol chloroform takes up urorosein readily, and that the alcohol may be washed out of the chloroform, leaving the pigment in that solvent. Under such conditions, *i.e.* in alcohol-free chloroform, the band shows a displacement towards red, just as the extra band of the Eichholz product does under similar circumstances.

It was found, moreover, that when a urine rich in the chromogen of urorosein was saturated with ammonium sulphate the chromogen was to a large extent precipitated, and that in the alcoholic solution of the precipitate thus obtained, from a specimen of urine poor in urobilin, there was developed, on the addition of hydrochloric acid, a pink colour, and a spectrum like that of the pathological urobilin of Eichholz, save that the urobilin band was narrow and very faint.

[1] *Journ. f. prakt. Chemie* [2] XXVI. p. 333. 1882.
[2] *Deutsche med. Wochenschrift.* XIX. p. 51. 1893.

Solutions of urorosein are decolorized by alkalies and regain their pink colour promptly on reacidification, and in this way it was possible to explain the change of colour to pale yellow on the addition of ammonia to the red solutions obtained by Eichholz's method.

Lastly, just as in the Eichholz specimens the pink colour is *gradually* developed on the addition of hydrochloric acid, so in urine, urorosein is *gradually* formed from its chromogen on the addition of hydrochloric acid.

Taking the above facts into consideration, viz., the gradual appearance of the pink colour and band on the addition of an acid, the characters of the band and shading, the disappearance of all indications of the presence of the impurity when the solutions are rendered alkaline, coupled with the observed facts that the chromogen of urorosein is readily precipitated by saturation with ammonium sulphate, and that the pigment is readily taken up from an alcoholic solution by chloroform, we were led to the conclusion that our original surmise was correct, and that the pathological urobilin of Eichholz is a mixture of urobilin with urorosein.

We found, moreover, that the chromogen of urorosein is precipitated by ammonium sulphate sooner than urobilin is, so that on adding ammonium sulphate to the cold urine until turbidity was produced, filtration, and complete saturation of the filtrate with the aid of heat, it was possible to obtain from this second precipitate a solution of urobilin almost free from this impurity.

The frequent development of the red or pink tint in the specimens is not opposed to the above view, but is in accordance with the observation of Rosin that the chromogen of urorosein is a very common constituent of morbid urines, and is sometimes present in traces in normal urines also.

It is very probable that other red impurities produced by acids, but with less characteristic spectroscopic features, may also be present, and in some instances we have obtained indications that this was the case.

It should be mentioned that the alcoholic extracts obtained by Eichholz's method sometimes exhibit a shading and extra band towards red, even before an acid is added. This is easily shown by measurement and otherwise, to be due to the presence of uroerythrin, precipitated upon the urate sediment which ammonium sulphate throws down, and the bands of hæmatoporphyrin are also occasionally seen more or less distinctly.

Spectrum of an Eichholz extract from a specimen of urine rich in urorosein and poor in urobilin	Same more dilute	Spectrum of extract from the urine of a typhlitis patient	Spectrum of urorosein in alcohol-free chloroform
Band of urorosein λ 5700—5400 shading	λ 5610—5400[1] shading	λ 5600—5400[2] shading	λ 5825—5520 shading of green
Band of urobilin λ 5060—4860	λ 5060—4860	λ 5080—4770	Band displaced towards red

The physiological and pathological Urobilins of Jolles.

In a recent paper Adolf Jolles[3] gives his adhesion to the view that two distinct forms of urobilin occur in urine, a physiological and a pathological, which only agree in yielding the absorption band near F., and a green fluorescence with zinc chloride and ammonia. Like MacMunn, Jolles regards the normal pigment as an oxidation product of bile pigment, and believes the pathological to be a reduction product, identical with Maly's hydrobilirubin.

It may be noted that Jolles describes pathological urobilin as a red pigment, whereas we have found that when pure it acquires no red colour, even under the influence of mineral acids.

In order to regard the physiological pigment of Jolles as a chemical individual it is necessary to assume that basic lead acetate precipitation exercises a highly selective action, throwing down the urobilin alone from normal urine, for the subsequent purification by alcohol cannot exert any such action; but, although the precipitation of the pigments by this salt is far less complete than when both acetates are used, we cannot look upon this proceeding as having any such selective power. We believe that the ill-defined character of the urobilin band seen in extracts from normal urine, which Jolles, like MacMunn, regards as characteristic of normal urobilin, to be simply due to the fact that these brown extracts contain mere traces of urobilin, and owe their colour almost exclusively to other pigmentary substances carried down by the basic acetate of lead.

Jolles draws this further distinction between the two forms of

[1] On dilution the urorosein band shrinks from the red end.

[2] Eichholz places the extra band between λ5510—5290, and his readings of the urobilin band show a corresponding difference from the above. Seeing that his reading of the band of urobilin-zinc shows a similar displacement, we can only attribute the discrepancies to differences of scale.

[3] *Loc. cit.*

urobilin which he describes, that whereas the normal pigment loses alike its absorption band and power of fluorescing with zinc chloride and ammonia, when treated with nitric acid, or an alcoholic solution of iodine and mercuric chloride, the pathological pigment is unaffected by these reagents.

We have found however that solutions of urobilin obtained by our processes from morbid urines very rich in that pigment, and showing an absorption band of great breadth and intensity, were decolorized by nitric acid as surely, although more slowly, than the more dilute solutions obtained from normal urines, the liquid becoming perfectly colourless. The urobilin band disappears and the power of fluorescing with zinc chloride and ammonia is at the same time destroyed. We have not, indeed, met with any kind of urobilin which is not thus destroyed by nitric acid, and this applies equally to the natural products and to that formed by the action of sodium amalgam upon bilirubin.

It is not however upon such negative evidence alone that we base our contention that the urobilin of normal urine is identical with that which is so far more abundantly present under a variety of morbid conditions, but rather upon the direct comparison of the products obtained by the ether-chloroform process from these two sources.

The minuteness of the amount of urobilin present in normal urines renders it somewhat difficult to prepare satisfactory specimens from them, but by working with large quantities of urine we have obtained concentrated solutions of normal urobilin.

Such solutions, when acid, showed a broad, sharply-defined absorption band between *b* and F and a dark shading extending from it towards the violet; and, on the addition of alkalies, and of zinc chloride and ammonia, specimens were obtained which agreed both in colour and in optical properties with those prepared from morbid urines.

Moreover on partial precipitation of a concentrated, alkaline, watery solution of normal urobilin by acids the E band spectrum was observed as distinctly as with any pathological specimen.

In the face of such evidences of identity we maintain that stronger evidence than any as yet brought forward is necessary, in order to carry conviction that the urobilins of normal and of morbid urines are essentially distinct pigments.

On the other hand, urobilin is undoubtedly apt to undergo modifications, which produce differences both of optical and chemical

properties, but as such modifications are met with in special frequency, although by no means exclusively, in specimens extracted from fæces, it will be convenient to speak of them more fully in the following section of the paper, which is devoted to the description of fæcal urobilin.

V. Fæcal Urobilin.

For the extraction of urobilin from fæces we have employed the following method:—The fæcal material is broken up and is allowed to soak in rectified spirit, which acquires an orange or even a red colour. This plan has the great advantage over extraction by water that the alcoholic extract is readily filtered off and is moreover free from some of the pigment-yielding impurities which are present in the watery extract. The filtrate is next diluted with twice its bulk of water, the addition of which causes a turbidity which cannot be completely removed even by repeated filtration. The diluted liquid sometimes exhibits a well-marked green fluorescence. Some acetic acid is added and on standing for a short time the colour becomes deeper and an intense urobilin band usually develops. On shaking with chloroform the urobilin is taken up from the acid liquid, and when the chloroform extract has been separated and evaporated to dryness the urobilin may be again dissolved in an excess of water acidified with acetic acid.

For the purification of the product the aqueous solution is saturated with ammonium sulphate, and the urobilin is extracted with ether chloroform in the manner already described. The extraction with ether chloroform may be repeated if necessary. By such a process we have obtained from fæces specimens of urobilin absolutely resembling those extracted from urine; such solutions are not reddened by mineral acids, and both in their colour and in the position and character of the absorption band as well as in the absence of any extra bands are indistinguishable from the urinary ones. On the other hand the product will very frequently be found to be somewhat modified, and it will be best to describe the typical and modified forms separately.

1. *Typical fæcal urobilin.* The specimens of the first variety exhibit in acid solution the characteristic band of acid urobilin, consisting of a darker portion and of a deep shading extending towards the violet. When moderately concentrated the liquid has a yellow colour, and becomes pink on dilution. Towards solvents the product behaves just like urinary urobilin.

Ammonia changes the colour of the solutions to canary yellow and

no band is seen, or only a faint one in concentration. The addition of zinc chloride brings out a green fluorescence quite as brilliant as that of the urinary product when similarly treated. A careful comparison both by apposition and by measurements of the spectra of solutions of equal strengths of the ammonia, zinc, sodium and potassium compounds of the two was also made, and absolute uniformity was found to run through the entire series, the resemblance extending to the tint of the specimens as well as their spectra.

Lastly, fæcal urobilin was found to be partly precipitated from an alkaline aqueous solution by the addition of an acid, and the slightly turbid liquid having the red precipitate in suspension showed the narrow band embracing the E line just as did the urinary specimens.

Here a further word of warning is necessary in connexion with this reaction, which is the more readily obtained the purer the urobilin employed. With fæcal urobilin it is not always very easy to obtain for two reasons. In the first place, the reaction is seriously interfered with if the alkaline aqueous solution contains, as the fæcal specimens unless very carefully prepared are rather apt to do, colourless impurities which are precipitated by the addition of an acid. When such are present the turbidity may prevent the spectrum being seen, and the colourless precipitate is apt to carry down the urobilin with it. Secondly, the modification which fæcal urobilin is so liable to undergo may lead to failure of the experiment.

The above properties appear to leave no doubt that Jaffé was right when he claimed that fæcal and urinary urobilin are identical, and, if this be so, it is obviously undesirable to apply two distinct names such as stercobilin and urobilin to the same substance according as it is extracted from the solid or liquid excreta. MacMunn too regards stercobilin as practically identical with pathological urobilin, a concession which involves the conclusion that the fæces of a healthy person may contain abundance of pathological urobilin whilst his urine contains only the normal variety.

2. *Modified fæcal urobilin.* As already mentioned, in a considerable number of instances the product obtained from fæces by the process above described will be found to be somewhat modified. We use the term modified advisedly, because the differences between the typical and modified pigments are much slighter than would justify their description as distinct substances, and how slight they are is shown by the fact that the modified product is readily converted into the typical form and *vice versâ*. Moreover we do not claim that the

modification to be here described is the only one to which the unstable urobilin is liable, but merely that it is that which is most clearly defined and most frequently met with. Other modifications of a somewhat similar character are not uncommonly obtained in working both with fæcal and with urinary urobilin, and they are for the most part characterised by a displacement and elaboration of the urobilin band.

The presence of the modified form of fæcal urobilin is usually first revealed by the fact that the precipitate obtained by saturation with ammonium sulphate is redder than it should be, and by the further fact that the alcoholic solution of the precipitate retains its pink colour to a considerably higher degree of concentration than does a typical solution.

The absorption band of acid or neutral solutions extends as far towards the red as the *b* line and when the solution is dilute is seen to be distinctly triple, consisting of two darker portions connected by a shading (Plate I. *B.* fig. 3).

Vanlair and Masius figured the band of stercobilin as extending as far as the *b* line, and we have little doubt that this observation of theirs was due to the fact that they were dealing with the modification under consideration.

On the addition of ammonia a further difference from the typical pigment becomes apparent. Instead of assuming a canary yellow colour the liquid remains pink, and shows a dark absorption band, like that of the sodium or potassium compound. This observation serves to throw light upon the somewhat uncertain behaviour of urobilin solutions when they are rendered alkaline with ammonia. On the further addition of zinc chloride the fluorescence is much feebler than with typical specimens and appears to have a red rather than a yellow substratum. The absorption band has the ordinary characters. On acidifying an alkaline aqueous solution the E band does not appear, unless, as sometimes happens, reconversion into the ordinary pigment is brought about in the process.

No definite directions can be given for the conversion of urobilin thus modified into the typical form. Sometimes when the specimen is quite freshly prepared mere addition of ammonia and reacidification with hydrochloric acid serves to bring about the change. With other specimens, with which this does not suffice, the conversion is produced by evaporation of a chloroform solution upon the water bath, and in some cases the change is very difficult to bring about.

In connexion with this subject some recent observations by Riva[1] are of much interest. This observer has succeeded in separating from some specimens of urobilin, both urinary and fæcal, a modification of the pigment which is characterised by yielding a red instead of a green fluorescence with zinc chloride and ammonia (such as MacMunn observed with urobilin freshly extracted from bile), and which when precipitated from an aqueous solution by these reagents is insoluble in neutral alcohol. This modification, like that above described, is readily converted into the ordinary form. It is difficult to be sure whether this modification is the same as that which we have described above, which agrees with it in yielding with zinc chloride and ammonia a precipitate insoluble in alcohol. It is obviously difficult to describe in words variations in the characters of fluorescence, and some doubt remains in our minds as to the exact interpretation to be put upon the epithets which Riva employs, such for example as a yellow-green, yellow-red, and yellow-red-green fluorescence (giallo-verde, giallo-rossa, giallo-rossa-verde).

We should add however that working with Riva's method, viz. by shaking chloroform solutions with water and precipitating the washed-out pigment from the aqueous solution by the cautious addition of zinc chloride and ammonia, we have obtained such results as he describes, and we have certainly seen a double band near E to which he alludes in the liquid rendered turbid by precipitation by these reagents.

Whether or no the modification above described should prove to be identical with Riva's "rubrobilina," we must assume that a pigment such as his which is so readily convertible into the ordinary form is, as he himself supposes, rather a modification of urobilin than a distinct substance.

Spectroscopic characters of modified Urobilin.

In acid alcoholic solution	Band triple, in dilute solutions	λ 5170—λ 5080 λ 5080—λ 5010 λ 5010—λ 4770	Shaded portion	
In ammoniacal solution		λ 5170—λ 4970 λ 4970—λ 4770	Dark portion Shaded portion	of band
With zinc chloride and ammonia		λ 5190—λ 4970 λ 4970—λ 4690	Dark portion Shaded portion	of band

[1] "Urobilina o Urobiline." *Gazetta Medica di Torino*, LXV. p. 741. 1894.

VI. The Urobilin of Bile.

It was shown, long ago, by Jaffé and by MacMunn[1] and the observation has been repeatedly confirmed, that a form of urobilin can frequently be extracted from bile removed from the gall-bladder *post mortem*, it therefore seemed important to examine the product obtained from this source also.

The method employed was as follows:—

The bile removed from the gall-bladder at an autopsy was treated with rectified spirit. The deep brown alcoholic extract obtained was then largely diluted with water and after acidification with acetic acid a 10% solution of barium chloride was added, which precipitated the bile pigment. The filtrate from this precipitate had a yellow colour and showed a dark urobilin band. It was next saturated with ammonium sulphate and extracted with ether chloroform. On shaking this extract with water a yellow solution of urobilin was obtained, which was again saturated with ammonium sulphate and shaken with chloroform, which took up the pigment readily. The urobilin left the chloroform for a dilute solution of sodium hydrate, and on rendering the concentrated solution, thus obtained, slightly acid with hydrochloric acid, a slight reddish turbidity was produced, and the E band spectrum was seen with great distinctness when the turbid liquid was examined with the spectroscope.

In addition to the strong evidence thus afforded of the identity of the urobilin obtained from bile with that derived from other sources, the solutions of the pigment so isolated exactly resembled in colour those of urinary and fæcal urobilin. On the addition of ammonia a canary yellow colour was produced, and with zinc chloride and ammonia a green fluorescence was obtained, whilst the zinc solution, and solutions rendered alkaline with sodium hydrate, showed the usual band shifted towards the red.

In the following table measurements of the various spectra of biliary urobilin are brought into direct comparison with those of urobilin obtained from normal and morbid urines and from fæces respectively. The trifling differences observed in the measurements are in part attributable to differences of concentration, and in part to such causes as variations in the degrees of acidity and alkalinity of the specimens examined from which the measurements were made.

[1] *Proc. Royal Soc.* xxxi. p. 29, 1880–1, and This *Journal*, x. p. 108. 1889.

Comparative Spectroscopic Readings from specimens of Urobilin from various sources.

Spectra of	Urobilin from Normal Urine	Urobilin from Morbid Urine	Urobilin from Fæces (typical)	Urobilin from Bile.
In alcoholic solution, acidified with hydrochloric acid	λ5080—λ4770 dark shading to λ4550	λ5080—λ4770 dark shading to λ4550	λ5080—λ4770 dark shading to λ4550	λ5080—λ4770 dark shading to λ4550
In alcoholic solution, with sodium hydrate	λ5200—λ4970 shading to λ4790	λ5200—λ4990 shading to λ4770	λ5200—λ4990 shading to λ4770	λ5190—λ4970 shading to λ4770
With zinc chloride and ammonia	λ5190—λ4970 shading to λ4770	λ5170—λ4950 shading to λ4770	λ5170—λ4950 shading to λ4770	λ5170—λ4950 shading to λ4770
The *E* band spectrum	λ5350—λ5220[1] λ5060—λ4620	λ5350—λ5220 λ5080—λ4550	λ5350—λ5220 λ5080—λ4550	λ5350—λ5220 λ5080—λ4550

[1] The solar E line = λ5290.

Conclusions.

The conclusions to be drawn from the evidence brought forward in the present paper may be briefly summed up as follows:—

1. With the exception of the method of Jaffé, which is difficult of application, the processes usually employed for the extraction of urobilin from urine are not calculated to yield a pure product.

2. Urobilin obtained from various human sources, *i.e.* from normal and morbid urines, from fæces and from gall removed from the gall-bladder *post-mortem*, is one and the same substance. Specimens from these several sources having, when pure, identical chemical and optical properties, and sharing in common the property of yielding the E band spectrum when partially precipitated from an aqueous alkaline solution by acidification.

3. The differences which have been described as existing between the several products are in part due to the influence of impurities in the specimens examined, and in part to the varying amounts of urobilin present in the specimens.

4. Urobilin is an unstable substance, and is liable to undergo certain modifications. These modified pigments have not the properties of the described pathological urobilins, and are capable of reconversion into the typical form.

PLATE I.

A.

Shows the effect of dilution upon the spectrum of urobilin in acidified alcoholic solutions.

Fig. 1. The specimen had a brown-orange colour.
2. Diluted to 4 volumes (yellow).
3. Diluted to 8 volumes (pale yellow).
4. Diluted to 16 volumes (dull pink).
5. Diluted to 32 volumes (very pale pink).
6. Diluted to 64 volumes (hardly appreciably tinted).

B.

Fig. 1. The E band spectrum, as yielded by the slightly turbid liquid having in suspension the precipitate formed by the acidification of a concentrated, alkaline, aqueous solution of urobilin.
2. Spectrum of urobilin in acid alcoholic solution.
3. Spectrum of modified urobilin in acid alcoholic solution.
4. Spectrum of urobilin in an alcoholic solution of sodium hydrate.
5. Spectrum of urobilin with zinc chloride and ammonia.
6. Spectrum of mercury-urobilin.

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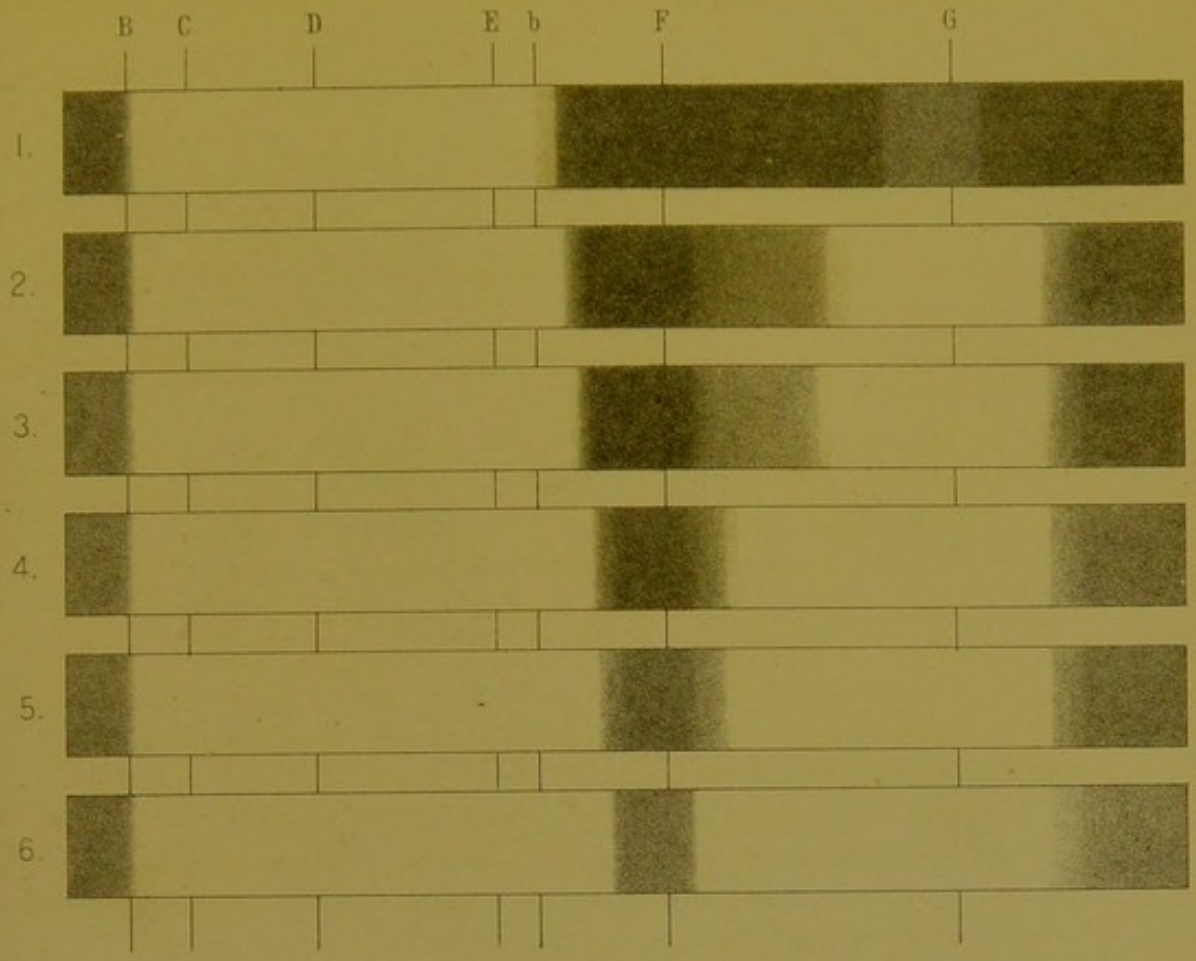
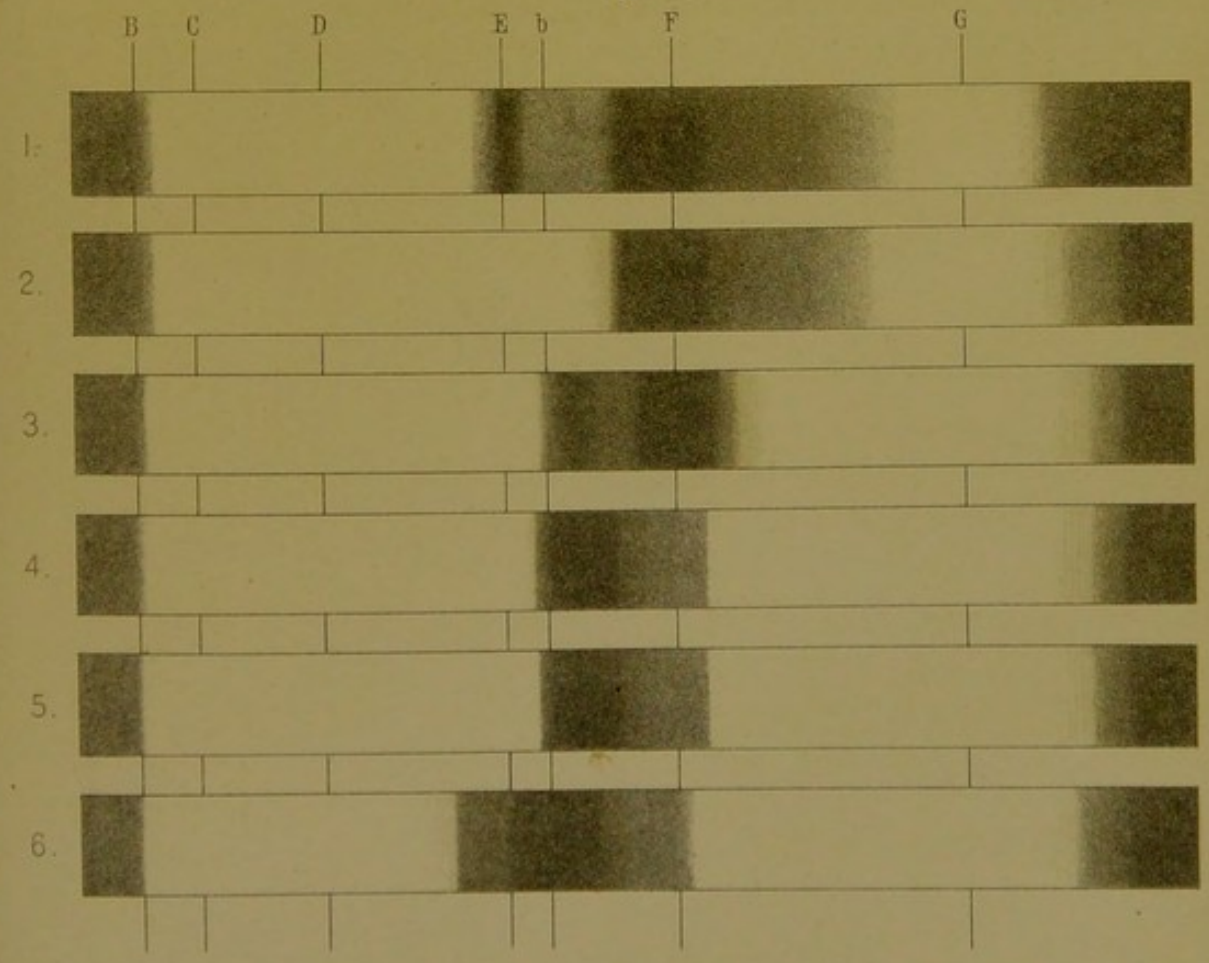
A



B



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