Graduation Thesis

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On some colouring matters of Urine, and their Pathological Significance.

On Some Colouring Matters of Urine and their Pathological Significance-

By J Arnold Armitage M.B. Chn

The colouring matters of urine have engaged the attention of Physiological Chemists since the time when Proust (Annales de Chimie. Alte Folge XXXVI Band) attempted to Isolate a special colouring matter from this fluid. The results obtained have been so contradictory that we possessed very little certain Knowledge till quite recent years, when the Spectroscope was pressed into the Service of the Physiological Chemist, and furnished him with a means of analysis infinitely superior to any other - By its means it is easy to prove that what were lely supposed to be colouring matters, are in reality decomposition products of pigments, and of their chromogens, of which several are present in both normal and pathological

urine -
Nevertheless it is interesting in the light of recent investigation to shortly review what has been done by the older chemists.
Proust in the paper mentioned above, describes how he isolated a substance, which was obtained by evaporating the urine to the consistence of a syrup and treating the residue with Sulphuric acid. By this treatment he obtained a brown, foul smelling, resinous substance. He believed that the bitter taste and the smell of urine were due to this substance, but he evidently did not think that this existed as such in urine, since he assumed that it arose from an unknown body, which he could not detect.
Duvernoy (Chemisch-Medicinische Untersuchungen über den menschlichen Urin Stuttgart 1835) assumed that the darkening in colour of urine was dependent on acidity, and

shewed that mineral acid caused such darkening –

Scharling (Ann. de Chemie Bd xiii) subjected the urine to a freezing process, and allowed ether to stand on the frozen urine for 24 hours, then poured the ether off and evaporated it. The residue was washed with water in order to remove urea and other substances, the washing being repeated until finally a brown, oily mass remained behind; this was dissolved in caustic potash solution, precipitated with Sulphuric acid and again dissolved in ether. The substance so obtained was named "Omichmyloxyd" by Scharling, and this he supposed to be the colouring matter of urine. But other observers found the substance impure, as it contained traces of hippuric acid.

Heller (Von Urophaein Hellers Archiv Neue Folge Bd I S3 1852) succeeded in procuring from urine

a yellow body soluble in water and alcohol, which he named "Uroxanthin". This he supposed to be the yellow colouring matter of urine, probably of the nature of a chromogen, and capable of being split up by acids into two colouring matters, a blue one which he named "Uroglaucin" and a red one Urrhodin. Heller also described another colouring matter "Urophaein" whose amount might be approximately measured by the amount of black colouration which arose when the urine was ~~tested~~ treated with sulphuric acid.

Schunck (Philosoph Magaz (4) Vol XIV p 288) by treating the urine with acetate of lead and other subsequent treatment, which it is unnecessary to describe, isolated approximately a yellow body which he supposed to be identical with plant indigo. Schunck supposed that Heller's uroxanthin was a similar body,

and maintained that all its decomposition products were identical with those of plant indigo.
Jaffé (Pflügers Archiv f. d. ges. Physiologie Bd iii s 448) and Hoppe-Seyler (Archiv f Path Anat Bd xxvii) then shewed that Heller's uroglaucin and Schunck's brune decomposition product possessed the properties of plant indigo-blue. Hoppe-Seyler however, has since shewn that animal indican and plant indican are not identical.
Scherer also endeavoured to isolate the urinary colouring matter by treating urine with hydrochloric acid and extracting with alcohol.
Dr George Harley (Ueber Urohematin und seine Verbindungen mit animalischem Harze Verhandl der phys-med Gesellschaft Würzburg) then attempted to prove that what he calls Urohematin was the colouring matter of urine. But his process was quite sufficient to

destroy any colouring matter present as we now know in the light of more recent investigation - Thudichum (Urochrome, the colouring matter of urine Hastings Prize Essay British Medical Journal Nov 1864) then professed to have finally settled this complex problem by the isolation of his urochrome, which was obtained by a variety of methods - This urochrome, he supposed, by oxidation under the influence of air passed into a red modification corresponding to the matter hitherto described as Uroerythrine, the colouring matter of pink urates. This supposition has since been proved to be erroneous. By decomposition under the influence of acids and time, or of acids and heat, urochrome as well as its red modification uroerythrine according to Thudichum yield three insoluble decomposition products namely "Uropittine" the fallow resin of Proust before mentioned, a

resinous acid, corresponding with the Omichmyoxyd of Scharling called by Thudichum "Omicholic acid", and thirdly "Uromelanine" the particular black matter of Proust. In addition to this he found several volatile bodies, amongst them perhaps a neutral essential oil of peculiar properties and several acids, and advanced a supposition that a fixed soluble product of decomposition might remain in the mother liquor — The existence of these substances is, however, according to the best authorities, very doubtful. (Cf Hoppe-Seyler's Physiologische Chemie and Handbuch der Physiologisch- und Pathologischen Chemischen Analyse 5th ed.)

Rabuteau (Gazette medicale de Paris 1875 p 337) treated the urine with hydrochloric acid and amyl-alcohol and got a pigment into solution which he called Uroerythrin, and he supposed that it arose from

the decomposition of Urochrome. This however, as stated above has been already disproved.

It is unnecessary to refer at length to the observations of Kunkel Massen and Dagrove.

Neucki and Sieber (Journal f. prakt. Chemie Bd xxvi S333) observed a colouring matter in urine which they isolated by treating the urine with hydrochloric acid and shaking out with amyl-alcohol, this they have named Urrosëin.

Lately P Ploss has published some observations on the colouring matter of urine. (Ueber einen neuen Krystallinischen Harnbestandtheil Zeits. f. physiol. Chemie Bd VI S504) and (Ueber einige Chromogene des Harns und deren Derivate Ibid Bd viii S85) Ploss shews that when normal urine is treated with hydrochloric acid 5 to 10 per cent for from 10 to 20 minutes, a darkening is produced, and

on agitation with Ether, or Chloroform a more or less violet red extract is produced, which consists of indigo blue and another red colouring matter. It seems that the latter is identical with indirubin and that it arises from the same mother substance as ~~the latter~~ indirubin — Mac Munn had described the Spectroscopic appearances accompanying the reaction before the publication of Ploss's paper. (Proceedings Royal Society No 226 1883) The urine, after the Chloroform or ether is separated off, has a brown colour, and it yields to amyl alcohol the colouring matter, which, after the evaporation of the amyl alcohol, is a black brown colour, and which, on washing with warm water to remove soluble salts, gives to the water a yellow colour. This yellow colour was found to be due to the presence of Urobilin. The residue then dried forms brittle black-brown

leaflets soluble in hydrochloric acid and caustic soda, but insoluble in most solvents. Ploss named this Urochromine, a name which as I said above, was applied by Thudichum to one of the decomposition products of urochrome.

Jaffé showed (Arch. f. path. Anat. Bd X LVII S 405) that normal urine frequently contains a yellow body with peculiar reaction which he named "Urobilin"; he demonstrated that this body can be recognised in febrile urine directly with the Spectroscope and that a body of the same optical character could be got from bile by treatment with hydrochloric acid etc.

Maly (Ann d. Chem-u-Pharm Bd 161 S 368 und Bd 163 S 77) then found that by the action of Sodium amalgam on bilirubin suspended in water a body which he named "Hydrobilirubin" was produced - This he assumed to be identical with Jaffés Urobilin..

Vaulair and Massius (Centralb. f. d. med. Wiss 1871 No 24) and Maly (Do No 54) then examined the colouring matter of faeces and showed its relationship to Jaffés urobilin - Hoppe-Seyler also found that a similar body arose from haematin by the action of Tin and Hydrochloric acid.

Mac Munn (Proceedings Royal Society No 208 1880) showed that there were three colouring matters in urine which previous observers had confused together and took for Urobilin namely: (1) The urobilin of normal urine (2) The urobilin of febrile and pathological urine. (3) Urohaematin which he subsequently named "Urohaematoporphyrin". He traced these pigments back to their source, prepared them from bile and blood pigments and showed how to differentiate one from the other - Subsequently (Journal of Physiology vol VI No 122)

he showed that febrile urobilin and the Stercobilin of Vanlair and Masius were closely connected and finally summed up in the following words — "Putting all the facts obtained together, it would appear that the Stercobilin, resulting from the putrefactive processes in the intestine and accompanied by imperfectly changed biliary pigments, is taken up by the branches of the portal vein and carried into the liver, where it is probably again changed by the action of a ferment into a chromogen; a portion of this chromogen gets into the blood and is excreted in the urine as a chromogen. A portion may escape in the condition of biliary urobilin and appear in the urine in a further oxidised condition, or owing to disturbance of circulation in the liver a large portion of unchanged biliary urobilin may appear in the urine. Besides this,

the urine under normal conditions may contain a pigment which has no biliary origin, and which may be derived entirely from haematin, while in certain diseased states a reduction product of haematin, having no connection with bilirubin or biliverdin, and closely related to haematoporphyrin, may appear in the urine, and to a great extent, if not entirely, may replace urobilin" Some of these conclusions have lately received an independent confirmation at the hands of Le Nobel of Leyden who (Ueber die Einwirkung von Reduction mitteln auf Haematin und das Verkommen der Reductions producte in pathologischem Harne Archiv f d ges Physiol Bd xxx Bonn 1887) has also detected Urohaematoporphyrin in pathological urine and prepared it according to Mac Munn's method. This urohaematoporphyrin then appears to be the pigment which Hoppe-Seyler

took for urobilin.
This brief review of the literature of urinary chromatology would be incomplete without some reference to the work of Udránszky (Zeits. physiol Chem Bd xi S 537-560 and Bd xii S 33-36) This observer has shown that the following conclusions may be drawn from the work at present done in this line of investigation. (1) By the action of oxidising agents, indigo blue and other indigo compounds e.g. urdinurin, can be obtained from normal urine. (2) In most cases urobilin, which is identical with hydrobilirubin (a statement which I hope to show is erroneous) is also present. (3) In addition to these, pigments are obtained by boiling the urine with mineral acids, and are probably derived from the splitting up of certain chromogens in the urine by these strong reagents. One of these is urmelanine.

Adriansky directed his attention to the last class of substances and found that they were due to the decomposition of the reducing substance of normal urine, and that their amount was related to the reducing power of the urine. "The aldoxyl compounds seem to have only a small influence on their formation – It is assumed too that the normal colour of urine is due to the change of carbohydrates into ~~pigmentary~~ humous substances, which has commenced inside the body." B Mester has recently concluded (Zeits phys'iol Chemie Bd xii S130-144) that urorubin, urorosein, uroerythrin, purpurin etc are different names for the Skatol pigment, but this statement cannot be accepted, as well marked differences exist between these pigments, and the author above named has not observed the spectroscopic appearances of the

Skatol pigment.

It would be hopeless within the limits of a single paper to attempt to reinvestigate all the colouring matters which have been described; so I shall confine my attention to those pigments which have the greatest interest for the pathologist; namely those traceable back to bile and blood colouring matters. The following investigations were carried out in Dr Mac Munn's private laboratory and under his supervision. I am indebted to him for kindly checking my results, and considering his well known reputation as an eminent Spectroscopist, they may therefore be considered accurate.

The instruments I used were a Browning's Microspectroscope, with a tube fixed to the side of the prism tube containing a

photographed scale illuminated by a small mirror and which is capable of being so focussed by a small lens, that on looking into the instrument the spectrum can be seen accurately divided into 100 equal parts — By means of this scale readings can be taken at once — In addition to this, I also used in all my observations a One prism chemical spectroscope likewise provided with a photographed scale and having a much wider dispersion than the Microspectroscope. The cells for holding the solutions to be examined were merely flat bottomed tubes fitted into thin slabs of wood. These are Dr Mac Munns invention and are a distinct improvement on Mr Sorby's cells, the cement on which mixed with the solutions and vitiated the results, and in addition the cells themselves were continually coming off the glass plates to

which they were fixed, when solvents such as alcohol, ether and Chloroform were put into them - Dr Mac Munn's tubes also have the great advantage of only admitting rays which come through the bottom of the tube, the wood in which they are set shutting off all extraneous light -

Having obtained readings from both Spectroscopes in the manner described, I next proceeded to reduce these to wave lengths - My reason for doing this was the well known fact that the relative distances between the Fraunhofer lines depend upon the material of which the prism is composed, So that it is necessary to obtain a standard to which all prismatic Spectra may be referred, for no two arbitrary scales will give the same reading - Such a Standard is found in the Diffraction Spectrum, where deviation is simply proportional

to wave length, and therefore the distance between two colours represents the difference of their wave lengths - I subjoin the following table which is taken from Deschanel's Natural Philosophy, ft 1029 and which gives the approximate distances between fixed lines in certain prismatic spectra, and in the standard diffraction Spectrum. The distance between B and G being in each case taken as 1000

	Flint glass L=60°	Bisulphide of Carbon L=60	Diffraction or difference of wave length
B to D	220	194	381
D to E	214	206	243
E to F	192	190	160
F to G	374	410	216
	1000	1000	1000

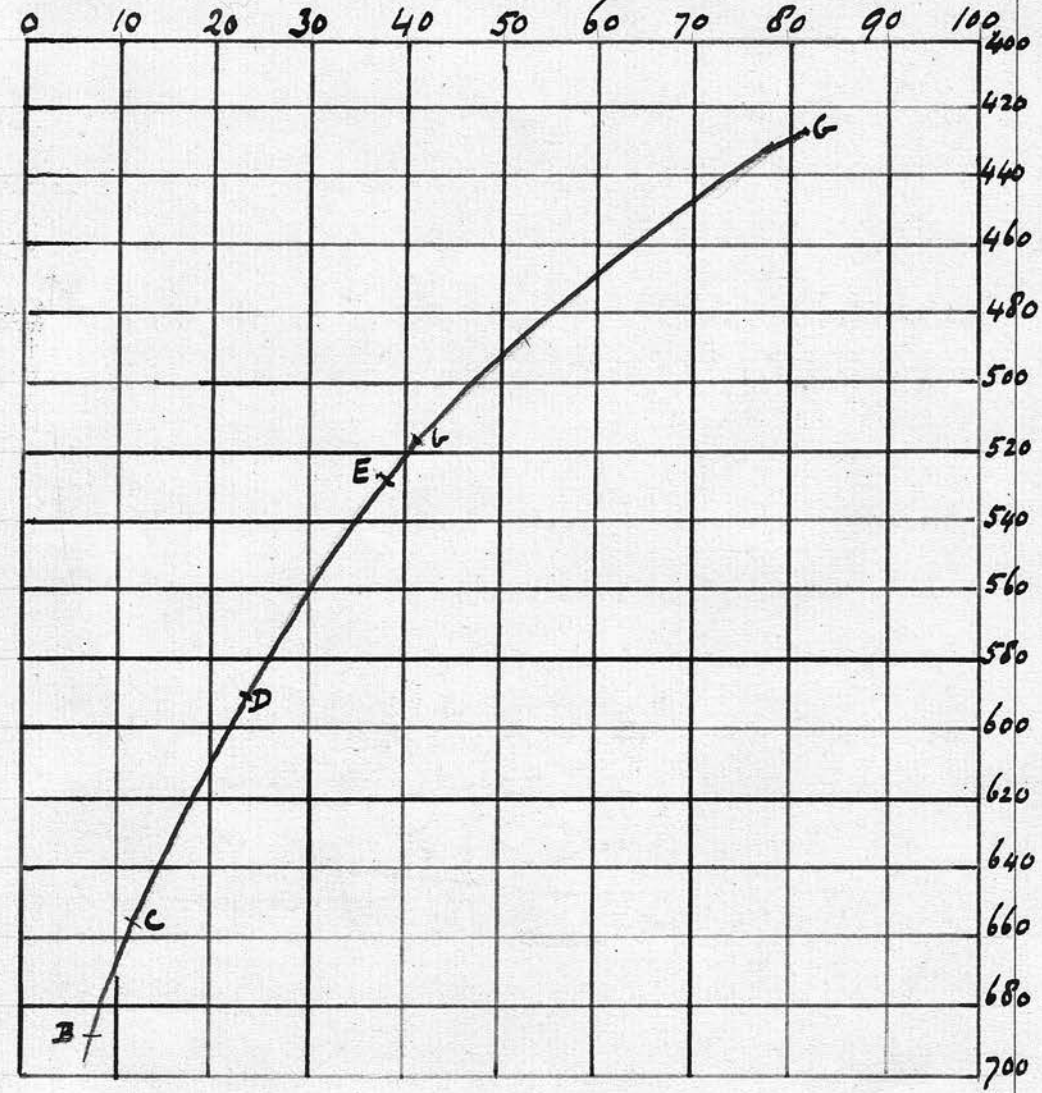
Angstrom has calculated the wave lengths of light in terms of which 10^{10} make a metre, hence called tenth metres, and the following

are the wave lengths of some of the Fraunhofer lines

A	7604
a	7185
B	6867
C	6562
D	5892
E	5269
b	5172
F	4861
G	4307
H_1	3968
H_2	3933

The last figure in each of these numbers may be neglected and we then get the wave lengths in millionths of a millimetre.

Having now got the wave lengths of the principal Fraunhofer lines, the wave lengths corresponding to the edges, or centre of any band can be easily calculated by graphical interpolation, as the accompanying diagram, which is taken from Mac Munn The Spectroscope in Medicine



p 33 will shew - The value of the Fraunhofer lines on the scale of the Spectroscope was observed and their value in wave lengths obtained from Angstrom's table. They were then marked in their proper position on the scale, and a curve drawn through them as uniformly as possible - In mapping a band,

its reading on the scale is taken, then we find its position on the curve opposite which its wave length is marked on the right hand edge. Curves of this description were drawn for both Spectroscopes which I used, on paper ruled in square inches and tenths, and on a much larger scale than in the diagram I have given -

The first point to which I directed my attention was

The Relationship of Thudichum's urochrome to Normal urobilin

It seemed a priori probable that Thudichum's urochrome was related to normal urobilin as Thudichum Says (Further Researches on Bile-Pigments and its compounds Journal Chemical Society May 1875) "Urochrome ... Shows a narrow faint band in acid mixture, none in neutral or alkaline mixture" However he states in his book "Pathology of the Urine," published

two years later "As pure urochrome does not shew any specific absorption before the Spectroscope when strongly acidified, it is not the chromogen of urobilin, and is not derived from it" It may be as well here to state that Maly (Annal der Chemie Bd 163 ~~see~~ 60) found that Urochrome contains Urobilin - Whether that be the case or no I found no urobilin in the substance which I prepared after Thudichums directions, although I feel bound to say that I failed to prepare it by some of the methods which Thudichum recommends.

Experiment I

1200 c c normal urine were precipitated with neutral and basic acetate of lead and filtered; the precipitate was decomposed with dilute sulphuric acid 1 in 7 and filtered, the filtrate had a reddish yellow colour which, on standing, changed to a

brownish-red. This solution on being examined with the Spectroscope showed a feeble band in the orange, and another in green while the violet end of the Spectrum was strongly absorbed; the band of urobilin was also visible, and had the appearance of that of normal urobilin - Phosphomolybdic acid was then added to the filtrate and a bulky precipitate fell, of a yellowish colour, with a tinge of grey. This was filtered off and the blue green filtrate showed only a doubtful urobilin band. The precipitate was washed with water repeatedly, but it was found to be impure and could not be got into solution and was found to contain urobilin - Hence another method had to be adopted.

Experiment 2

1000 C C normal urine was treated with 3 grammes of Barium hydrate, then a saturated

solution of Barium acetate was added - It was then filtered - To the filtrate neutral lead acetate and ammonia were added and the solution filtered. The precipitate was then washed with water, and treated in a mortar with dilute sulphuric acid 1 in 7, until a little of the filtrate showed a precipitate with Baric chloride, insoluble in hydrochloric acid - The resulting solution when this point was reached, was of a deep orange colour. On being examined before the spectroscope it showed a faint trace of a band before D, another one faint and ill defined in green, and a distinct violet band - It was then treated with Barium carbonate and Barium hydrate until the acid was neutralised; a stream of Carbon dioxide was then passed through it, and it was again filtered. The filtrate was

yellow, and still showed a faint urobilin band. It was now precip-
-itated with a solution of Mercuric acetate and filtered, the precipitate was washed with cold and hot water, then decomposed with Sulphuretted Hydrogen, the filtrate was yellowish although pale. It was then evaporated on the water bath and I obtained a brownish yellow amorphous substance insoluble in Chloroform, in absolute alcohol, in ether, in rectified Spirit, but freely soluble in water, forming a reddish-yellow solution by Gaslight, absorbing the violet end of the Spectrum strongly - It showed no urobilin band neither alone nor after hydrochloric acid had been added, and on testing for urobilin by other methods none was detected - Hence then Thudichum's urochrome is not connected with normal urobilin, and it does not give

any specific absorption in the Spectrum.

Normal Urobilin.

Two distinct colouring matters, namely Normal urobilin and Pathological urobilin, have been confused according to Mac Munn, under the one name Urobilin. I therefore determined to investigate them both, to see whether they were really distinct pigments or not. To come to an accurate conclusion it was necessary for me to isolate the urobilin from normal urine, and also that from pathological urine, and then to compare the two resulting substances and to note their behaviour when treated with the same reagents -

Even in the urine itself however a striking difference can be observed on examining the two Specimens with the Spectroscope. Pathological urine shows a much darker band at F than normal

urine. Another point worthy of notice is this: that sometimes the band of normal urobilin may be seen in fresh urine, whereas after it has stood some time the band may become invisible - This is doubtless due to the presence of some reducing substance in the urine, of which the existence has now been definitely proved.

Experiment

1050 c c of normal urine were treated with neutral and basic acetate of lead till the supernatant fluid had lost all its colouring matter - It was then filtered - The precipitate was then decomposed by rectified spirit acidulated with sulphuric acid, and the extract filtered - The resulting filtrate appeared to be of a yellow-brown colour by gas light. On examining it with the spectroscope, it showed an ill-defined band at F with

indistinct washed out edges, quite different from the band of febrile or pathological urobilin. This band read approximately from wave length 503 to wave length 481. Even in the deepest layer of fluid this band did not appear black, as is the case with the band of pathological urobilin —

The solution was now agitated with chloroform in a separating funnel, the chloroform separated off and filtered. The chloroform solution was yellow in a thin layer and an orange-yellow in a deep layer. It was allowed to evaporate at the temperature of the air, and became darker on exposure to the air. It left a brown amorphous residue which was soluble in alcohol, ether, bisulphide of carbon, chloroform and acidulated water.

A solution in alcohol was examined with the spectroscope, and in deep layer transmitted red and a

portion of green; some feeble bands were seen in the red end of the spectrum, not identical with those of a similar solution of pathological urobilin. In a thin layer a band was seen at F, reading from wave-length 504 to wave length 477. The solution was of a fine red colour in deep layer by gas light, but not so red by daylight.

The alcoholic solution was then treated with Ammonia and filtered. The red colour of the solution was then noticed to be less distinct. Examined with the spectroscope, although a feeble band was just discernible in the red end of the spectrum, yet no bands like those seen in a similar solution of pathological urobilin could be observed. The band at F disappeared and was not replaced by another nearer the red end of the spectrum.

Zinc Chloride was next added to the alcoholic solution already treated

with Ammonia - The resulting solution assumed a yellowish colour by daylight, but still had a reddish tint by gaslight - The fluid fluoresced green; but the fluorescence was much less distinct than is usually the case in similar solutions of pathological urobilin, stercobilin, and hydrobilirubin -
On being examined with the Spectroscope a feeble band appeared in the red from about wave length 650 to wave length 625, and another at D, whose edge towards the red, was much less shaded than its edge towards the violet; its darker part read from wave-length 587 to wave length 571 - The band at F was replaced by another nearer the red, narrower than the original one, and this was not continuous with a lighter shading stretching towards the violet, as is the case in pathological urobilin when similarly treated -

The appearance of the above mentioned bands under the action of Ammonia and Zinc chloride points to a close relationship between normal & pathological urobilin; but the shading of the band at F is quite different. The alcoholic solution was then treated with Zinc chloride without the addition of Ammonia, and it then showed no green fluorescence whereas similarly treated solutions of pathological urobilin and Stercobilin do show a green fluorescence. The difference came out strongly when the respective solutions were examined by daylight-

Pathological or Febrile Urobilin

The urine was obtained from a case of Peritonitis and was of an orange colour - When examined with the Spectroscope it showed a dark band extending

from wave-length 502 to wave-length 475. The urine was precipitated with neutral and basic acetate of lead, filtered, the precipitate decomposed with alcohol acidulated with sulphuric acid and filtered, the filtrate agitated with chloroform in a separating funnel, the chloroform separated off and filtered. The chloroform solution had a deep red colour, whereas in the case of normal urobilin it was yellow. When examined in a deep layer with the spectroscope, the violet end of the spectrum was strongly absorbed and two bands appeared one before D, and one after D. The former read from wave-length 619 to wave length 591, and the latter, from wave-length 567½ to wave-length 547, with a shading extending from its edge nearest D towards the red.
In a thinner layer the band at F became visible and was observed

to be very broad, and extended from wave-length 517 to wave-length 466 – The band at ⅞ of normal urobilin, never shows such a great breadth. The edge of the band towards the red is much more abruptly shaded than the edge towards the violet – On diluting the solution sufficiently, the band was made to read from wave length 506, to wave length 483.

On evaporating the Chloroform a reddish brown amorphous residue was left which was soluble in alcohol, ether, bisulphide of carbon, Chloroform and acidulated water i.e the same solvents as normal urobilin.

I next added Ammonia to an alcoholic solution of this pathological urobilin and examined the result with the Spectroscope – Three bands became visible in a deep layer, which are not seen in a corresponding solution of

Normal urobilin - These bands corresponded very closely to those of Mac Munn's alkaline urohæmato-porphyrin, and are not seen in a corresponding solution of hydro-bilirubin, but they are found again in a similarly treated solution of Stercobilin. Hence there is a strong probability, of which this is one of the links in the chain of evidence, that this colouring matter is derived from hæmatin, and not from bile pigments - Of the three bands there is a feebler one in the red and two in the green. In wave-lengths they measure.

(1) From 625 to 615.
(2) The dark part from 581 to 560½
(3) From abt 542 to 521½

~~To the alcoholic solution~~

On examining a thin layer of the same solution the band at 7 was found to have disappeared -

To the alcoholic solution already treated with Ammonia I next added Zinc Chloride and a splendid green fluorescence was at once developed, and on examining the solution with the spectroscope a new band now appeared in the red, the second of the Ammonia bands was much the same as before, while the band at F of the original alcoholic solution is replaced by another, which has a very characteristic appearance, as that part of it which is nearest the red end is very dark, whilst its violet edge is continued into a shading which extends for its own breadth towards the violet. This appearance alone will distinguish pathological from normal urobilin.

The above mentioned bands read in wave lengths.

(1) From 653 to 622

(2) From 587 to 567.5

(3) A doubtful band
(4) Dark part from 517-495

By the action of Zinc Chloride alone a green fluorescence is obtained, and now two bands are seen at 7.

These facts are sufficient to prove my point, that normal and pathological urobilin are not identical.

Urohæmatoporphyrin

The third colouring matter which gives a band at 7, and which, as Mac Munn has shewn, has been included under the name urobilin, is Urohæmatoporphyrin. This has been found by Mac Munn in the urines of rheumatism, so called idiopathic pericarditis, Addison's disease, Hodgkin's disease, Cirrhosis of the liver, Croupous pneumonia, typhoid fever, measles and meningitis - Hoppe Seyler maintained that he had prepared Urobilin by the action of tin and

hydrochloric acid on haematin a haemoglobin, but Mac Munn shewed that it is not urobilin which is formed under these circumstances, but the present colouring matter Urohaematoporphyrin (Proc Royal Society No 208 1880 & Journal of Physiology vol vi Nos 1&2) Mac Munn at first named this pigment Urohaematin, but he subsequently altered it to Urohaematoporphyrin "since it is a kind of haematoporphyrin Le Nobel (Archiv f d ges Phys Bd xL 1887) has recently confirmed his observation although there are some points of detail not agreed upon by these observers, which however do not concern the subject treated in this paper - The fact is now however definitely proved that by the action of reducing agents, such as Zinc & Sulphuric acid, or Sodium amalgam on haematin a colouring matter is formed which is identical with a pigment

found in the urine in the above mentioned and probably in other diseases. Mac Munn has recently been engaged in reinvestigating this point and I have had an opportunity of examining some urines which were found to contain Urohaematoporphyrin. I shall however only describe the character of the pigment I obtained from the urine of a case of measles, taken on the 3rd day after the eruption appeared —

On adding to the urine a little mineral acid three bands came into view; a narrow one before D, another in green, and the third at F resembling the pathological urobilin band. These bands can not always be seen so readily, and it is therefore necessary to isolate the pigment in the same way as the Urobilin pigments were isolated — This I next proceeded to do —

The urine was precipitated with neutral and basic acetate of lead, filtered, the precipitate decomposed with alcohol acidulated with Sulphuric acid and filtered - The filtrate was of an orange colour and showed the Acid Urohæmatoporphyrin bands well marked - On reducing them to wave lengths they read:

(1) From 597 to 587
(2) From 555 to 542
(3) From 504 to 481

The solution was then agitated with Chloroform in a separating funnel, the Chloroform separated off and filtered and the filtrate examined with the spectroscope; when the same three bands were observed. On evaporating the Chloroform a residue of a dark brown colour was left soluble in Alcohol, ether bisulphide of carbon, Chloroform and acidulated water -

This was next dissolved in rectified spirit and the resulting solution

was brownish when examined in a white dish, but brownish yellow with transmitted light. On examining it with the Spectroscope a marked difference between this, and a rectified spirit extract of urobilin, either normal or pathological, was observed. There were no less than five bands in the Spectrum, which, when reduced to wave lengths read as follows:

(1) From 630 to 619?
(2) From 607.5 to 599?
(3) From 581 to 555
(4) From 538 to 520
(5) From 506 to 481

When Ammonia was added to this solution, the second band counting from the red disappeared, namely, that reading from wave-length 607.5 to wave-length 599?. The other bands were slightly altered in position; but the most noticeable change was this:

The band at 7 was replaced by

another nearer the red end.
This band approximately from wave length 511 to wave length 494.
In the case of normal urobilin the band at F disappears and may be replaced by a faint shading nearer red, which as a rule cannot be measured.
To the alcohol solution treated with Ammonia, I next added Zinc chloride, and a solution was obtained which showed a green fluorescence, not nearly so distinct however, as that obtained from similar solutions of pathological urobilin and hydrobilirubin.
On examining this Zinc Chloride & Ammonia solution with the Spectroscope certain bands appeared which are peculiar to Urohæmatoporphyrin
Their wave lengths read as follows:
(1) From 583 to 569
(2) From 547 to 529
(3) From 514 to 499

On adding Zinc chloride alone to the alcohol solution and examining with the spectroscope I found that the first two bands remained unchanged namely:

I From 583 to 569

II From 547 to 529

The 3rd band, that at F remained unchanged at first, but next day it was found to have shifted slightly nearer the red.

The Zinc chloride solution showed no green fluorescence, which is another point distinguishing this pigment from hydrobilirubin.

I have compared these solutions with those of Urohaematoporphyrin prepared by Dr Mac Munn by the action of Zinc and Sulphuric acid, and of Sodium amalgam on haematin, and I can find no difference between them. This will be seen by an inspection of the table of wave-lengths

measurements prepared by Dr Macmunn for the meeting of the Physiological Society at Oxford (17th of March 1888) This table I append to my paper for reference.

In preparing these pigments artificially from haematin it is noticeable that in an early stage of the reaction a band appears at 7, which as the reduction process goes on, gets gradually darker until the stage of Urohaematoporphyrin is reached. Continued reduction leads eventually to the formation of a yellow solution, which then shows only a band at 7 very like that of urobilin; while the bands of Urohaematoporphyrin before and after D have gone. If however, the solution be left exposed to the air these bands before and after D return - Le Nobel noticed this and supposed that the body indicated by the band

at 7 only, which he calls "Urobilinoidin" is changed by oxidation into Urohæmatoporphyrin. It would seem however that as in the case of Urobilin, this reduction product is merely the chromogen of Urohæmatoporphyrin, as Machmann assumes.

Having now investigated the pigments of Normal Urobilin, of Pathological Urobilin, and of Urohæmatoporphyrin I next turned my attention to two other colouring matters not contained in the Urine, but closely connected with the Urinary colouring matters, namely, those of Stercobilin obtained from human faeces, and of Hydrobilirubin obtained from human gall stones, and I hope to shew that a study of them bears directly on the subject treated in this paper.

Stercobilin

Vaulair and Massius were the first observers who examined

the colouring matter of faeces (Centralb. f. d. med. Wiss. 1871 Nr 24) by means of the spectroscope; and they named this colouring matter Stercobilin, and maintained its identity with Hydrobilirubin. Mac Munn subsequently (Journal of Physiology vol VI nos 1 & 2) investigated Stercobilin and came to the conclusion that "The position of the bands in an alcoholic solution with Zinc chloride and with Caustic soda, both of that close to I and at D, differs very slightly from that of those of a similar solution of febrile urobilin, and resembles closely that of the bands of a similarly treated solution of the urobilin of bile" He also found that the best method of isolating the pigment is to extract the faeces with acidulated rectified Spirit, and agitate with Chloroform - On evaporation of the Chloroform solution the stercobilin

is left behind in the form of a brown, amorphous residue, which is soluble in the solvents that dissolve febrile urobilin.

If Stercobilin be the source of febrile urobilin, its amount in urine would be an index of the activity of absorption from the intestine, and it could hardly be taken up in any quantity without its being accompanied by some pigmentaire like substances, as MacMunn has remarked.

It was this possibility which made me desirous of examining stercobilin and comparing it with the pigments previously described.

On extracting the faeces with rectified spirit acidulated with sulphuric acid, I obtained a deep red solution (by gaslight). On examining this with the Spectroscope in a deep layer, the violet end of the spectrum was strongly absorbed there was a faint band at D,

which, when reduced to wave-lengths, began feebly at λ625, became darker at λ610 and extended to about λ566 - But in a more dilute solution it may be said to extend from wave-length 610 to wave-length 573 - In a thin layer a band became detached at 7, bearing a close resemblance to that of pathological urobilin reading from about wave length 506 to wave-length 479. I then diluted the solution with water, and agitated it with chloroform in a separating funnel. The chloroform was separated off and filtered - The resulting solution was of an orange colour and when examined with the Spectroscope showed a black band at 7, besides a band before D, another just after D and a faint one in the green. The readings of these bands when reduced to wave lengths were as follows:

(1) From 612.5 to 591
(2) A shading from about 589 to 567.5
(3) A feeble band from about 550.5 to 529
(4) Dark part from 506 to 481

The chloroform solution was then evaporated, and the residue was of a brown colour—
It dissolved easily in rectified spirit, forming a reddish brown solution. On examining a deep layer of this solution spectroscopically, it only transmitted a slice of the red end of the spectrum. In a shallower layer a band appeared at D- In a thin layer a black band was seen at 7. These bands when reduced to wave-lengths read approximately as follows:
The band at D began to be shaded at about λ 617; got darker at λ 607.5 and ended about λ 589
The band at 7 in the thin layer extended in its darkest part from wave-length 504 to wave-length 479.

I next added Ammonia to this alcohol solution; and on examining the resulting solution with the Spectroscope, a series of bands appeared, which recalled to mind those which I saw in a similarly treated solution of urohaematoporphyrin but which were certainly not identical with those of a similarly treated solution of Hydrobilirubin -
These bands read in wave lengths approximately as follows:

1. From 636 to 619
2. From 589 to 564
3 From 549 to 526
4 From 517 to 494

I then added Zinc chloride to this ammonia alcohol solution of Stercobilin. The resulting solution showed a fine green fluorescence and on being examined spectroscopically Some bands appeared which, as in the last solution, were not identical in position with those of a similarly

treated solution of Hydrobilirubin. Reducing these bands to wave lengths they read as follows:

(1) The first band in red extended, including its shaded edges, from wave-length 656 to wave-length 619, and its darker part from wave-length 653 to wave-length 625

(2) An ill defined band, or shading from about wave-length 607.5 to wave-length 593 where it becomes darker, and extends to about wave-length 569

(3) From about wave-length 555 to wave-length 532

(4) Darkest part from wave-length 514 to wave length 496.

This last band shaded off gradually towards the violet; which is, as has been already shown, a characteristic of pathological urobilin.

When the alcohol solution was treated with Zinc chloride alone, a green fluorescence was obtained, and the band at F was affected

in the same manner as with Ammonia and Zinc chloride; that is, it was replaced by another band nearer the red, measuring the same as under the influence of these reagents.

These observations go to prove that Stercobilin is not identical with normal urobilin, nor with hydro-bilirubin, nor with Urohaematoporphyrin nor with Pathological urobilin; though it does closely resemble the last, but is not quite identical with it as the difference in the band at 7 when the two solutions are treated with Ammonia clearly proves.

Hydrobilirubin

It is quite evident from a perusal of the experiments of chemists on hydrobilirubin, that no pure substance has yet been obtained by the action of Sodium amalgam on bilirubin. Disque de Nobel and Machmann agree

in stating, that these are by-products formed, and Mac Munn has shown that one by-product is apparently identical with the Urobilin-like substance present in bile itself, which he has named "biliary urobilin," simply to distinguish it from allied pigments.

I prepared a solution of hydrobilirubin under Mac Munn's directions and with his assistance, and from the result can fully endorse his opinion that hydrobilirubin is not identical with any of the colouring matters I have previously described.

The bilirubin required for its preparation I obtained as follows: Several brown human gall stones were powdered in a mortar carefully dried, and the powder repeatedly extracted with ether, then boiled with water repeatedly, then extracted with dilute hydrochloric acid, and again washed with water, and finally dried in an air bath;

then when dried, it was extracted with ~~water~~ chloroform and boiled in the chloroform. The chloroform was evaporated on the water bath, the residue extracted with alcohol and with ether, again dissolved in chloroform, and absolute alcohol added to precipitate out the bilirubin. The bilirubin so obtained was not suspended in water as Maly directs, since its reduction in the state of suspension is not complete; but it was dissolved in water to which enough caustic soda was added to get it into solution; some rectified spirit being also added to prevent frothing. I then introduced it into a small flask provided with a perforated cork through which a glass tube reaching below the level of the ~~water~~ fluid was passed - Some freshly prepared sodium amalgam was also introduced into the flask. It was then heated on the water-bath

very gently from time to time, when the hydrogen began to come off slowly. At intervals the fluid was examined and was found to contain by-products, intermediate between hydrobilirubin and bilirubin, which will shortly be described elsewhere by Mac Munn. When the reduction was complete as evidenced by the pale yellow colour of the solution, the fluid was poured off the sodium amalgam and acidulated with hydrochloric acid in excess. The hydrochloric acid changed the colour of the solution to red, and on being examined spectroscopically the solution showed a band at D and another at F - I next diluted the solution with water and agitated it with chloroform in a separating funnel, when the chloroform became red in colour. The chloroform was separated off, filtered, and evaporated on the water bath - It left a brown,

a brown-red residue. Quite amorphous
It was soluble in absolute alcohol
and rectified spirit, in chloroform
. in ether, partially in benzene
partially in amyl alcohol, insoluble
in carbon bisulphide.
The rectified spirit extract was
deep red by gaslight, but more of
a red-brown colour by daylight.
On being examined with the spectroscope
it showed a band covering D,
and an intensely broad black
band at F, which gradually shaded
off towards violet. When reduced
to wave lengths, the band at D,
read from wave length 605 to wave-
-length 573. The band at F, in a
dilute solution read in its darkest
part from wave-length 501 to
wave-length 475.
On adding Ammonia to the
alcohol solution, and examining
with the spectroscope, a band was
seen in the red, and another at D,
while the band at F disappeared.

These bands when reduced to wave-lengths read as follows:
(1) From 647 to 630
(2) From 610 to 569
I next added Zinc chloride to the ammonia alcohol solution when a fine green fluorescence became developed, and before the spectroscope certain bands appeared which are peculiar to solutions of hydro-bilirubin, namely a dark band in the red, another at D, the band at F being replaced by a narrower band which shaded off gradually to the violet. When reduced to wave-lengths these bands read as follows.
(1) Including shadings of its edges from 647 to 612.5 and darker from 636 to 619.
(2) From about 599 to 566
(3) Dark from 514 to 494 with a shading of about its own breadth extending towards the violet end of the spectrum.

I next added Zinc chloride alone to the alcohol solution, when a fine green fluorescence was noticed. On examining it with the Spectroscope bands appeared practically identical with those obtained under the action of Zinc chloride and Ammonia. These results then prove that by the action of Zinc chloride and Ammonia hydrobilirubin can be shown to differ from any of the other pigments that I have described. Physiologists have hitherto relied too much upon the presence of a black band at F, and its replacement by another nearer the red end of the Spectrum under the influence of Zinc chloride and Ammonia, or Caustic Soda, and on the green fluorescence produced by Zinc chloride & Ammonia; but they have neglected the bands at the red end of the Spectrum, to which Mac Munn first called attention, and on which he has recently

insisted (Meeting of Physiological Society at Oxford March 1888).
de Nobel also has come to the conclusion by studying the spectrum produced by the action of Zinc chloride & Ammonia on alcohol solutions of hydrobilirubin, that this body is not identical with Jaffés urobilin. So that the contrary view which has been so long held, appears to be untenable.
These bands on which the decision turns, require a good spectroscope for their recognition.

Summary & Conclusion

In drawing this paper to a close I should state that I have prepared and examined most of the substances described more than once; but not finding any appreciable difference in my results, I have contented myself with describing one series of experiments. It remains for me to try and fathom

practical conclusions from the facts observed -

(1) Normal urobilin is not identical with pathological urobilin but can with care be prepared from any specimen of normal urine; hence its presence is not an indication of disease nor is the term "Urobilinuria" appropriate when applied to a disease of which pathological urobilin is an accompaniment, for, to quote Mac Munn "Every one suffers from urobilinuria."

(2) Pathological urobilin is not identical with Urohematoporphyrin, but is closely allied to Stercobilin though not identical with it. It is very possible that Stercobilin in passing from the portal into the hepatic vein, owing to pressure alterations of the hepatic circulation, such as vaso motor disturbance, may be accompanied by various ptomaines whose

presence in the general circulation would be indicated by the appearance of pathological urobilin in the urine. It is an undisputed fact that pathological urobilin appears under such circumstances.

(3) Hydrobilirubin is not identical with either normal or pathological urobilin - This is contrary to the teaching of many of the physiological text books - and it opens up the question as to whether there is sufficiently good ground for the statement that urobilin both normal and pathological are derived from Bile pigments directly - If we compare the Spectroscopic appearances of the solutions of the several pigments a general resemblance can be traced between Pathological urobilin Stercobilin and Urohaematoporphyrin while Hydrobilirubin stands by itself. This distinction is most apparent on examining the

Ammonia solutions - Now Urohæmato-
-porphyrin is almost undoubtedly
a derivative of hæmatin, and this
suggests the possibility that Pathological
urobilin & Stercobilin are also
directly derived from hæmatin,
and not from Bilirubin. Mac Munn
has recently shown in his Communication
to the Physiological Society at Oxford,
March 17 1888, that pigments very similar
to Stercobilin and pathological
urobilin can be artificially prepared
from hæmatin - whether this
is what actually takes place in
the body is another question, but
it would if true, explain the
fact that in cases of pelvic
hæmatocele or where there has
been absorption of large blood
extravasations we often find patho-
-logical urobilin in the urine in
considerable quantities.

(4) Urohæmatoporphyrin is almost
undoubtedly a reduction product
of hæmatin (Urobilin both normal

and pathological, being oxidation products in different stages of oxidation) and its occurrence in the urine is therefore of great pathological significance - Le Nobel affirms that he has prepared it by the action of acetone, or aldehyde on a solution of alkaline haematoporphyrin, and he makes the pregnant suggestion that perhaps it arises in the organism in the same way. It seems however whether this assumption be correct or not, to be present in urine under two conditions at least, (Mac Munn On Addison's disease and the Function of the Supra renal Bodies British Medical Journal Feb^{ry} 4th 1888) (1) When an excess of effete haemoglobin, or effete histohaematin is present in the circulation, the blood metabolising glands being healthy, but incapable of dealing with the excess of effete pigment.

or (2) when the amount of effete pigments may not be in excess, but the blood metabolising glands are diseased. e.g in Addison's disease; where the Suprarenal bodies are diseased also in Cirrhosis of the liver; where the secretory cells are encroached upon by new connective tissue, and consequently are incapable of metabolising the effete haemoglobin into biliary pigments.

It would appear that in the struggle between the oxidation and the reduction processes which is continually going on in the organism, the reduction process gets the upper hand and reduction products are formed which are taken up into the circulation, and excreted by the urine - The significance of the occurrence of Urobenditoporphyrin is very great, not indeed because the pigment in itself is a substance

dangerous to life, but because its presence indicates a reduction process and an abnormal one, which may lead to the contemporaneous formation of other substances (besides pigments) which may be of a toxic nature. Here however there is need of further investigation and it would be premature to say much at present-

A table of wave lengths of the bands of solutions of the above colouring matters will be found on the next page - It is a copy of the one which Mac Munn laid before the Physiological Society at Oxford March 17th 188-
It will be noticed that the band at F, measures practically the same in all the solutions. It is the other feeble bands which constitute the main differences between them -

Table of Wave-lengths

Normal Urobilin (alcohol)	Pathological Urobilin (alcohol)	Ster (a
	607.5 – 587 577 – 552	60
504 – 479	502 – 479	50
+ NH_4HO	+ NH_4HO	+ N
	625 – 615	63
	581 – 560	58
	542 – 521.5	54
0	0	51
+ NH_4HO + $ZnCl_2$	+ NH_4HO + $ZnCl_2$	+ NH_4
650 – 625	653 – 622	656
587 – 571	587 – 567.5	589
	?	555
514 – 496	517 – 494	514

ilirin (hol)	Urohæmatoporphyrin (alcohol)	Hydrobilirubin (alcohol)
- 577	630 - 619	605 - 573
	607·5 - 599	
	581 - 555	
	538 - 520	
- 479	506 - 481	501 - 475
H_4O	$+ NH_4HO$	$+ NH_4HO$
619	625 - 615	647 - 630
562	581 - 562	610 - 569
526	540 - 523	
- 494	511 - 494	0
$+ ZnCl_2$	$+ NH_4HO + ZnCl_2$	$+ NH_4HO + ZnCl_2$
- 619	?	647 - 612·3
569	587 - 569	599 - 566
532	549 - 529	
496	514 - 496	517 - 494