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Title: Experimental and clinical studies in the physiology and pathology of carbohydrate and protein metabolism: Analytical methods

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ANALYTICAL METHODS.

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ANALYTICAL METHODS.

Introduction.

On the following pages I shall describe the methods for determining quantitatively the various nitrogenous and and other constituents of the urine.. They have been, excepting the Kjeldahl method, nearly all introduced by Folin and his co-workers. The methods described will be those for the estimation of the total nitrogen, urea-nitrogen, ammonia-nitrogen, uric acid nitrogen, creatinin- and creatin-nitrogen, acidity, and ammonia+amino acids, as well as the various inorganic constituents of the urine. Besides this I shall also give the latest methods for the estimation of the sugar in the blood and blood plasma, as well as for the estimation of the glycogen content of the liver.

I shall also present some researches which go to show that certain of the acetone bodies interfere with the creatinin estimation, and that the addition of chloroform to the urine (as a preservative) causes uric acid to separate out and so interferes with the estimation of that substance.

I shall conclude by mentioning some new methods introduced by Folin, which are better adapted for clinical use than the foregoing and discuss the advisability of the introduction of these more up-to-date methods for clinical use.

THE ESTIMATION OF THE TOTAL NITROGEN IN THE URINE.

THE ESTIMATION OF THE TOTAL NITROGEN IN THE URINE

The introduction of a number of new methods in physiological chemistry has helped to give us more correct and precise conceptions of the various chemical physiological problems. Of these there is perhaps none which has become so generally useful and has given us more new results than the Kjeldahl method for the estimation of the total nitrogen not only in urines but in all kinds of tissues and substances. Various modifications of details have been put forward (some by Kjeldahl himself), since his method was first introduced (1883) to the scientific world; but the principle on which it is based is still the same.

In principle the method consists in heating the urine or substance to be analysed with an excess of concentrated sulphuric acid for about 3 hours or more, the organic matter is broken up, the carbon containing part being oxidised (water and carbon dioxide being formed, Dafert.) and the nitrogen part is reduced, forming ammonia. Part of the sulphuric acid is required for oxidation purposes, with the result that fumes of sulphur dioxide are given off.

Folin (1907) holds that the splitting off, of ammonia is in part an hydrolysis, and that there are /

are amines formed, which later are distilled over with the ammonia.

In 1885 Wilfarth showed that the presence of certain metallic oxides act as catalysers to the reaction. The best were mercuric oxide, copper oxide or sulphate. A definite amount of the oxide had to be present (as much as will dissolve in the acid) in order to get the maximum effect. The use of mercury or its oxide has one great disadvantage, for if it is allowed to stand in presence of the concentrated acid after the combustion, and even if the solution is diluted down, a precipitate of mercuric amidosulphate if formed after some time. This precipitate is not readily dissolved even on boiling, and a special procedure has to be adopted.

According to Malfatti (1903), Folin (1904), and Salkowski (1908), the presence of water is necessary for the reaction, as they hold that the process is at first one of hydrolysis. They therefore recommend the addition of some water. The presence of water, however, lowers the temperature of the liquid, the decomposition takes longer and is also not so complete, for amino acids (such as lysin etc.) are decomposed only with difficulty. In such cases potassium sulphate is a useful catalyser. It acts mainly by raising the temperature of the solution.

Arnold (1886) showed that if several catalysers /

catalysers are used the reaction is more complete. Gunning (1889) pointed out that the addition of potassium sulphate (by helping to raise the temperature) to another catalyser such as a mercury or a copper salt accelerates the reaction. A combination of both the Arnold and Gunning method has been shown to be necessary in certain special cases (Arnold and Wedemeyer.)

When potassium sulphate is added it should not be at the beginning of the heating, or one is apt to lose some of the ammonia owing to the raised temperature (Koefoed); therefore, where necessary, it is advisable to add it near the end of the reaction.

Modifications have also been brought forward in connection with the apparatus, and with the distilling over; but these are far too numerous to be detailed here.

The method I employed for urines was as follows:-

5c.c. of urine were measured with a pipette and put into a long necked flask (Kjeldahl flask) of about 500 c.c. capacity. To this 10c.c. of concentrated (nitrogen free) sulphuric acid were added, also a crystal of pure copper sulphate, about the size of a bean. The flask and its contents were put on a wire gauze and heat applied. The combustion has to be /

be carried out in a fume-chamber, or if a large lead pipe is taken with a number of holes in the side for the introduction of the mouths of the flasks, the one end of the lead pipe is closed and the other end opens into some ventilating shaft. With a good draught there is no risk of any fumes getting into the room. By this means a large amount of space is saved, and a large number of flasks can be heated at one time (according to the length of the pipe and the number of holes in it).

The contents of the flask on heating at first become dark, owing to the charring of the carbon present in the urine. This, however, disappears as the process proceeds, until finally the solution is colourless. After this the solution must still be heated for 20-30 minutes. The contents are then allowed to cool, and distilled water is added, till the flask is about half full. The condenser for the distillation is now got ready. (I used a large copper condenser with 8 leading off tubes going through it, so that I could distil over the contents of 8 flasks at one time).

A known quantity of $n/10$ acid is measured into an Erlenmeyer flask or a wide necked bottle. The delivery tube from the condenser must dip into the dilute acid (to which a few drops of rosolic acid, as indicator, have been added). A sufficient amount of /

of concentrated NaOH is added to the contents of the flask, to make them definitely alkaline; having done so the cork (rubber) of the safety bulb must at once be fixed into the neck of the flask, or otherwise some of the ammonia, which is liberated on the addition of the caustic soda is apt to be lost. Heat is now applied and the ammonia + water distilled over into the collecting flask. If by any means insufficient acid has been placed in the receiving flask, as soon as the acid is neutralised, the colour of the liquid changes from yellow to red, and more $\frac{N}{10}$ acid has to be added. When all the ammonia has been distilled over, the acid solution is titrated with $\frac{N}{10}$ NaOH, and the amount of acid neutralised by the ammonia estimated. The number of ccs. of $\frac{N}{10}$ acid multiplied by 1·4 gives the amount of nitrogen in mgms. in the amount of urine examined.

THE ESTIMATION

of

UREA IN THE URINE .

ESTIMATION of UREA in the URINE

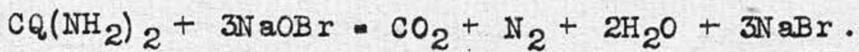
The methods for the estimation of urea may be divided into two groups, namely,

(a) in which there is an hydrolysis of the urea, and the ammonia that is formed estimated;

(b) in which the urea is decomposed by alkaline hypobromite into carbon dioxide and nitrogen, and the nitrogen estimated.

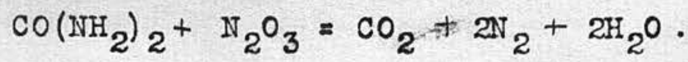
The latter method is simpler than the hydrolysis method, and more rapidly carried out. It is, therefore, clinically much in vogue. Its accuracy is, however, not very great, and variations occur in spite of the numerous corrections that have been adopted.

The reaction on which this method is based is as follows :-



$$CQ(NH_2)_2 + 3NaOBr = CO_2 + N_2 + 2H_2O + 3NaBr.$$

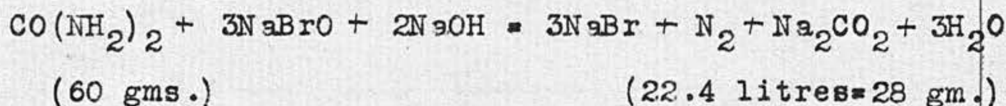
Here the sodium hypobromite acts on the urea, breaking it up to ~~to~~ form carbon dioxide, nitrogen, water and sodium bromide. This reaction is similar to that of nitrous acid on urea :-



$$CO(NH_2)_2 + N_2O_3 = CO_2 + 2N_2 + 2H_2O.$$

The carbon dioxide which is formed by the action of sodium hypobromite on urea, is taken up by the alkali present in the solution (which is made up of a solution of Bromine and one of caustic soda), and the nitrogen is liberated and estimated. The estimation

is best carried out in Duprés ureometer. Clinically, however, a modified ureometer is used, such as that of Doremus (also known as Southall's or Thursfield's Ureometer), in which certain corrections are supposed to have been made, and graduated so that one can estimate the amount of urea directly :-



$$CO(NH_2)_2 + 3NaBrO + 2NaOH = 3NaBr + N_2 + Na_2CO_2 + 3H_2O$$

(60 gms.) (22.4 litres = 28 gm.)

Therefore 1 gm. of urea theoretically should evolve 373 cc. of nitrogen; in practice it is found only to evolve 354 cc. If this difference were constant, then quite good results would be obtained on correction. But it has been found that in this method the nitrogen deficit varies, and that various other nitrogen containing substances in the urine, such as ammonia, uric acid, creatinin, creatin, allantoin etc., may give off nitrogen. The estimations by this method are, therefore, quite uncertain and so of no value. A criticism of the method is fully gone into by Mörner.

Of the various methods used, in which the urea is hydrolysed, the two most important are the Mörner and Sjöquist and the Folin method.

(1) Mörner and Sjöquist Method :-

5 cc. of the urine are pipetted into a large flask or beaker, then 5 cc. of a baryta mixture (a solution of saturated barium chloride, to which is added 5% barium hydroxide), are added as well as

Mehu, Fauconnier, Hufner, Ostwald, Luther, Knop, Esbach.

100 cc. of a mixture of two parts of alcohol and one of ether. The flask or beaker is now closed and left to stand for 24 hours. The alcohol and ether mixture is then filtered off, and the residue washed with fresh alcohol-ether mixture. The different filtrates are collected and the alcohol and ether allowed to evaporate at about a temperature of 50°C, but must not exceed 60°C (preferably in a vacuum dish.) Some powdered magnesium oxide is added, when the contents of the flask have shrunk to about 30-40 cc., also some distilled water (20 cc.) and the mixture gently boiled to drive off the ammonia. The contents of the flask are then transferred to a Kjeldahl flask and combusted with concentrated sulphuric acid; the remaining part of the process is just like an ordinary nitrogen estimation by the Kjeldahl method.

In this method all nitrogen containing substances, such as ammonia, uric acid, purine bases, oxyproteins etc. are removed. It can also be used when even large quantities of sugar are present. In that case Mörner (1903) recommends the addition of 1.5-2 gms. of powdered barium hydroxide to the 5 cc. of urine; and the mixture is thoroughly shaken, before adding any of the other reagents.

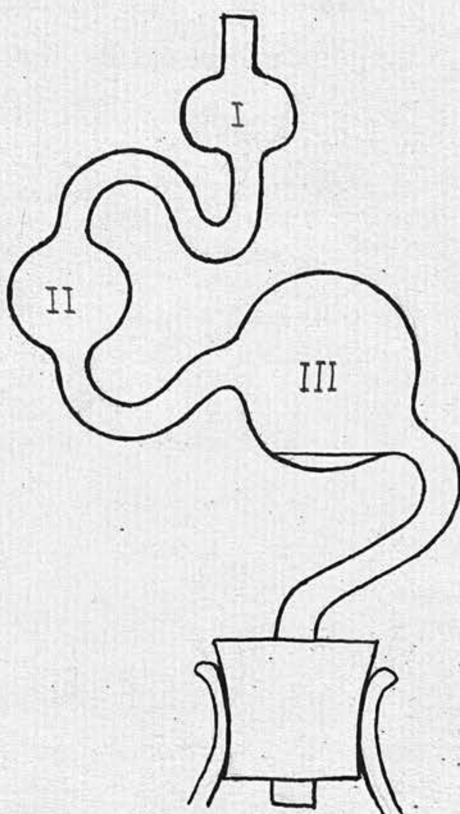
In this method the creatinin and hippuric acid are left along with the urea, and are estimated with the above method. This can be obviated by

hydrolysing the concentrated solution after the ammonia is driven off.

K. Spiro has also introduced a modification in which by first removing the free ammonia by Folin's method; he gets the amount of ammonia and then proceeds with the Mörner-Sjöquist method as above.

(2) Folin's Method depends upon the fact that crystalline magnesium chloride ($MgCl_2$, $6H_2O$) melts in its water of crystallisation at about 115-120°C, and boils at about 160°C. Such a solution has the property of quantitatively hydrolysing urea in about half an hour. Hydrochloric acid is added to prevent the escape of the ammonia, and ammonium chloride is formed.

5 cc. of urine are placed in an Erlenmeyer flask of about 250 cc. capacity, to this are added 6 cc. pure concentrated hydrochloric acid, 20 gms. of crystalline magnesium chloride (this always contains a certain amount of ammonia and, therefore, control estimations should be made). A piece of paraffin about the size of a bean (this is to prevent foaming), and a few drops of alizarin red as indicator are added.



The flask is now closed by a safety tube (Cathcart's modified form was used by myself), in which some dilute hydrochloric acid has been put, so as to lie in III. This safety tube serves the purpose of condensing the hot vapour to a certain extent, and so prevents a too great loss of water and and hydrochloric acid. As the lower bulb of the tube fills the liquid begins to overflow drop by drop, and when the boiling has been properly regulated each time a drop falls back into the flask, with its hot contents, it produces a "bump". The flask and contents are now placed on a sand bath and heat applied.
When the magnesium chloride has melted and begins to boil, the process must be carefully watched, till the drops of liquid which fall back from the safety bulb

begin to produce a very audible bump. When this occurs the flame is now so regulated, that there is a bump every quarter or half of a minute. The boiling must now be continued for 1.5-3 hours, according to the amount of urea present. The liquid must not be allowed to overheat too much, or otherwise some of the nitrogen-containing substances, such as Creatinin or uric acid, are also partially decomposed. If all the hydrochloric acid has been driven off, which is indicated by the liquid turning red, by simply tilting the flask and safety bulb, some of the dilute acid in the latter will flow into the flask; and if this by any chance should be insufficient, more dilute hydrochloric acid can be poured into the upper end of the bulb.

After the heating has been finished, the contents of the flask are poured out into a distillation flask and water added. Strong caustic soda is added to make the solution alkaline, and the ammonia which has been set free from the ammonium chloride is distilled over, just as in the Kjeldahl process.

In the above process, only urea and ammonia are estimated. The ammonia estimation has, therefore, to be carried out on a separate specimen of urine, and subtracted from the result obtained from the above method.

The results obtained compare favourably with those from the Mörner Method. This method can,

however, not be used when sugar is present, because when sugar and urea are heated together they combine and form (ureids) very stable condensation products (Schoorl). If sugar is present then the urine must either be first fermented, and then Folin's method carried out, or the Folin method combined with the Mörner-Sjöquist method, as mentioned before.

The commonest mistake apt to be made with Folin's method, is that the urine has not been heated sufficient, and the results are too low. Another point is that plenty distilled water should be added to the solution prior to distillation, (I always used a 800 cc. Kjeldahl flask) for the distillation very often takes a long time. The ammonia is removed with difficulty. (Folin recommends that the distillation be carried out till the contents of the flask are nearly dry). The reason for this apparent retention of ammonia, according to Folin, is that owing to the absence of water the hydrolysis is not a complete one, and the cyanate which is formed is only broken upon on distilling.

The distillate which has been collected must first be boiled and cooled before titrating with $\frac{N}{10}$ NaOH, for there is always carbon dioxide distilled over, and this must be driven off by boiling.

The disadvantage of this method for clinical purposes is the time it requires as well as the almost

constant supervision. Folin has, therefore, introduced a simpler method, which has the advantage of being comparatively easily carried out, does not take very long, and requires no elaborate apparatus. A description is given on p. 54.

THE ESTIMATION

of

AMMONIA IN THE URINE.

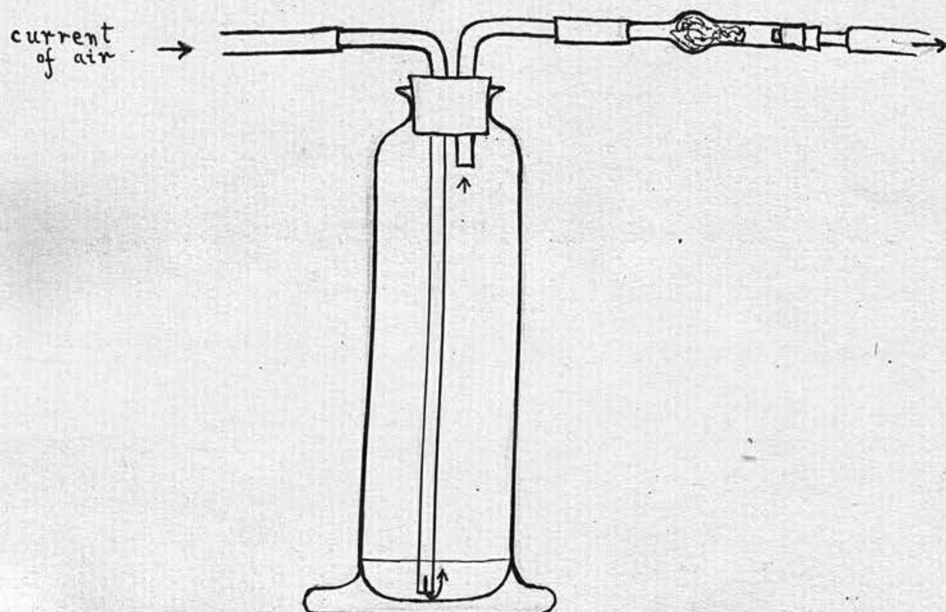
AMMONIA - ESTIMATION in the URINE

The methods for the estimation of ammonia in the urine depend upon the addition of an alkali which sets all the ammonia free, but does not act upon any of the other nitrogen containing substances of the urine. For this purpose the use of the strong alkalis is excluded, as they readily act on the urea. Magnesium oxide is also of no use, for, though it does not decompose urea it acts upon some of the other substances with the formation of ammonia (Krüger u. Reich). Though calcium and barium hydrate only set the preformed ammonia free, yet when albumin is present in the urine they act on it and decompose part of it with the formation of ammonia. Folin, therefore, uses sodium carbonate in his method, which can also be applied to albumin-containing urines. (in this case the addition of common salt is an improvement.)

Ammonia is not readily set free from triple phosphates by means of sodium carbonate, (Matthew, Gies and Steel) therefore, whenever there is a tendency to the fomation of triple phosphates and where they have already been formed, special measures have to be taken to set the ammonia free, such as decomposing the triple phosphates by the addition of salts of barium or of lead.

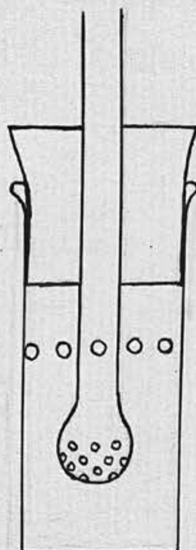
The ammonia estimation used in this research was that of Folin. It is both an easy as well as an accurate method. The only complaint brought against it is, that in some laboratories there is not a sufficiently strong water pressure to propel the air current.

The urine (25 cc. or more) is placed in a tall narrow cylinder, 1 gm. of anhydrous sodium carbonate, and some toluol (to form a thin layer on the top of the urine, and prevent foaming) are added.



The cylinder is closed by a cork with a long entrance tube (when air is to be driven through) dipping into the urine, and a short outlet tube which is connected to a calcium chloride tube, filled with cotton wool to absorb any moisture; this leads to a flask or a broad bottle, fitted with a cork with a long entrance tube and a short exit tube. The entrance tube is a

tube closed at one end, and into this blown out end small holes are made with a patinum needle.



On the stem of this entrance tube is a rubber cork on which is fixed a glass cylinder with large holes but at a higher level than the small holes. Into this bottle is put the necessary quantity of $\frac{N}{10}$ H_2SO_4 to absorb the ammonia, and water is added till the level of the liquid is above the large holes of the cylinder; the ~~air~~ air with the ammonia which has passed through the chloride tube and down the long entrance tube, comes out through the small holes and passes through the dilute sulphuric acid within the cylinder and then passes through the larger holes in the cylinder and into the dilute acid outside the cylinder. The air current, therefore, passes through two different portions of the sulphuric acid solution, and so the retention of all the ammonia is ensured. The air current is passed through the system for 2-3 hours

according to its strength. The acid solution is then taken and titrated with $\frac{N}{10}$ NaOH to see how much $\frac{N}{10}$ H_2SO_4 has been neutralised by the ammonia. The result multiplied by 1.4 gives the amount of nitrogen in milligrammes.

Another method for the estimation of ammonia has been introduced by Malfatti. This method is described on pp.31. The values got for the ammonia by this method are higher than those got with Folin's method, owing to the amino-acids also being estimated. But for clinical purposes this method is excellent when one considers the ease with which it is worked, and the short time in which it can be carried out (about one minute).

THE ESTIMATION

of

CREATININ AND CREATIN .

THE ESTIMATION OF CREATININ AND CREATIN

Jaffe (1886) discovered that when picric acid is added to a sample of urine, and to the mixture caustic soda in excess, there results a ruby red colour which attains its maximum intensity in about two minutes, and which, according to Jaffe, remains unaltered for hours. This reaction he held was due to the formation of creatinin picrate, but this according to Chapman is not so. Chapman recently showed that the red colouration is due to the reduction of the picric acid in alkaline solution to a mixture of amino-dinitro-phenol (picramic acid) and diamino-nitro-phenol, the alkaline salts of which are deeply coloured. The same colouration is produced by numerous reducing agents, such as nascent hydrogen, hydroxylamine, acetone, aldehyde, ammonium sulphide, etc.

Colour measurements showed the red colouration to be due to both the monamino- and the diamino-phenol, and that the solution of the sodium salt of picramic acid could not be used for matching purposes. Since the colouration is due to a somewhat complex reducing action, it is clear that the conditions under which the test is carried out must be fairly closely defined if accurate results are to be obtained. In the cold, acetone gives a similar reaction but is not so intense.

Folin in 1901 introduced a colorimetric method for the estimation of creatinin, using Jaffe's reaction as a basis.

The method is carried out as follows:-

10cc. of the urine are pipetted into a 500cc, flask (or a 250cc, flask if the amount of creatinin is small), 15cc. of a saturated watery solution of picric acid, and 5cc. of a 10% caustic soda solution. The mixture is shaken and allowed to stand for 5 minutes. Distilled water is added up to the 500cc. mark (or 250cc. mark) and the whole thoroughly mixed, This solution is then compared to a semi-normal potassium bichromate solution, in the Duboscq colorimeter. The column of the standard solution stands at 8·1mm. on the vernier.

To estimate the creatin, 10cc. of the urine are heated with 5cc. normal hydrochloric acid for 3 hours on a water-bath to convert the creatin into creatinin, and then having neutralised the urine with normal caustic soda (5cc.) the method employed is as indicated with creatinin.

To get the amount of creatin in a urine, the amount of preformed creatinin is subtracted from the amount of creatinin creatin (now converted into creatinin).

Full details as regards the method, apparatus used, and the various factors which influence the readings are given in my thesis presented for the Ellis

Prize in 1910 - "The Excretion of Creatin and Creatinin." I shall briefly mention some of them, as well as give a more detailed description of work carried out by me on the disturbing influence of acetone and its allies on the creatin and creatinin estimations, as it is of some importance in connection with the estimation of creatin and creatinin in diabetes and in other conditions of acetonuria.

The temperature of the reacting fluids must be constant, (Folin, Hoogenhuyze and Verploegh, and Mellanby) in order to get constant + comparable results. I, therefore, always kept my solutions in a copper thermostat. The temperature which I found most convenient was about 18ºC. Time is not quite so important a factor (Mellanby), although 5 minutes is the standard time for the reacting solution to stand before being diluted with water, but a slight extension of time is not unsafe.

The depth of the columns at which the reading should be carried out is between 5mm. and 13mm. (Folin). Personally, I find that readings between 4mm. and 12mm. give the best results. The best light for the colorimeter readings is the diffused light of a fairly bright day. Artificial lights are not good, the only one of any use at all is that got from the Nernst light.

The effect of acetone, diacetic acid and β-oxybutyric acid in a urine, on the creatinin readings, requires a somewhat fuller discussion, since these substances

are present in a number of pathological conditions and especially in diabetes. Therefore, any work on the creatinin excretion in any such conditions must take those substances into consideration.

Jaffe, in his original paper (1886) on the colour reaction, states that acetone shows only a faint reddish-yellow colour after the addition of picric acid and caustic soda solutions.

Folin (1904) also states that acetone, acetoacetic acid and aceto-acetic ether disturb the reaction, but does not say in what manner these substances interfere with the creatinin and creatin estimations.

Klercker in his paper states that it is impossible to take readings if acetone be present, and he therefore advises the heating of the urine to drive the acetone away. This, however, is not advisable for there is then the risk of getting some of the creatin converted into creatinin

V. Hoogenhuyze and Verploegh (1908) state in their paper that the colour reaction which is got with acetone appears more quickly and disappears also more rapidly than the colour reaction obtained with creatinin. They state that if a sufficient number of successive readings be taken no error will arise in colorimetric work.

I have investigated this subject very carefully, using various urines to which different known quantities of acetone, aceto-acetic acid and ether, and β-oxybutyric acid were added. These urines were treated in the same way as described above for the estimations of creatinin and

TABLE I

Normal Urine, 10cc.		Same urine + B-Oxy-butyric acid (about 1%)	
Creatinin	Creatin + Creatinin	Creatinin	Creatin + Creatinin
7·8	7·8	7·85	7·75
7·9	7·8	7·9	7·6
8·0	7·8	7·8	7·8
7·85	8·0	7·8	7·8
7·9	8·0	7·85	7·85
7·8	7·9	7·9	7·7
Av. 7·87	7·8	Av. 7·85	Av. 7·74
	Av. 7·87		

Same urine + acetone (2 per cent).

Time in minutes	Creatinin	Creatin + Creatinin
2-	6·0	7·0
	6·2	7·0
		6·9
4-	6·75	7·3
	6·85	7·3
6-	7·5	7·65
	7·4	7·8
8-	8·1	7·7
	7·9	7·9
	7·9	8·1
	7·9	7·85
	7·85	7·9
10-	7·9	7·85
	8·1	7·85
	7·84	7·9
	7·85	

creatin. In the case of the urines to which acetone had been added, I followed out the advice of v. Hoogenhuyze and Verploegh, and did a number of estimations at different time intervals, in order to see whether the readings eventually become constant, i.e. before the colour due to the creatinin begins to fade.

In Table I , I give the results got in each case with the colorimeter. On examining this Table it is seen that β-Oxybutyric acid has no effect on the colour reaction. Acetone on the other hand shows a definite interference with the readings as compared to the normal controls. At first it lowers the readings (i.e. increased values for the creatin), which, as the acetone reaction diminishes, gradually rise until they become constant, and here one gets the ordinary creatinin readings. The latter, of course, after some time (15 minutes or more) also begin to fade. The effect of acetone, when present in the urine would tend to give increased values for the creatinin, and lower values for the creatin. For in the latter case the acetone is often driven away already or is present in smaller amount, and thus the readings would, therefore, be more those of the creatinin creatin (as creatinin, if creatin is present).

In Table II are given readings of urines treated with aceto-acetic ether and acid. It will be seen that aceto-acetic acid and ether in the quantities given in the Table interfere with the quantitative estimation of creatinin far more pronouncedly than acetone.

The creatinin readings are diminished (i.e. the

TABLE II

Normal Urine	Same urine + aceto-acetic-ether (·5%)		Normal urine	Same urine +aceto-acetic acid (1%)
Creatinin	Creatinin	Creatinin + Creatin	Creatinin	Creatinin
8·35	7·5	8·2	10·25	10·1
8·2	7·5	8·3	10·2	10·2
8·25	7·5	8·4	10·2	10·15
8·3	7·55	8·25	10·2	10·1
8·25	7·5	8·3	10·15	10·2
Av. 8·27	Av. 7·51	Av. 8·29	10·25	10·15
			Av. 10·21	10·1
				Av. 10·14

Normal Urine		Same urine + aceto-acetix ac. (·1%)	
Creatinin	Creatinin + Creatin	Creatinin	Creatinin + Creatin
7·9	7·85	6·8	7·85
7·95	7·9	6·8	7·75
7·85	7·9	6·75	7·8
7·85	7·9	6·75	7·85
7·95	7·85	6·8	7·8
Av. 7·88	Av. 7·88	Av. 6·78	7·85
			Av. 7·81

value for the amount of creatinin is increased) just as with acetone, but the effect of the former does not pass off, as in the case of acetone. The readings for the creatin are the same as those of the normal urine, Therefore the results show that diacetic acid interferes with the creatinin estimation in such a way as to give the creatinin higher values than it really has. The diacetic acid does not, however, interfere with the actual readings for creatin, and so the values got for the latter are diminished, owing to the increased values of the creatinin, and so the values for creatinin in such a urine would be too high and those for the creatin too low.

THE ESTIMATION OF URIC ACID.

ESTIMATION of URIC ACID.

Uric acid forms an ammonium salt which is insoluble in concentrated ammonium salt solution. Using this as a basis Hopkins worked out a method for the estimation of uric acid. This was modified by Wörner.

A definite improvement of this method was made by Folin and Shaffer; not only did their method give more exact results, but the time required was also shortened.

For their method the following solutions are required:-

Solution 1.

Ammonium sulphate	500gms.
Uranium acetate (powdered)	5gms.
10% acetic acid	60cc.
Distilled water	650cc.

Solution 2.

10% Ammonium sulphate solution.

Solution 3.

$\frac{N}{10}$ Potassium permanganate.

150cc. of the urine are measured into a tall narrow vessel, and 37·5cc. of the uranium solution added. A granular precipitate forms, which gradually settles. It usually takes half to one hour to settle. 150cc. of the supermatant liquid are pipetted off and put into a beaker. To this, 5cc. of strong ammonia are added, and the beaker covered with a watch-glass and put aside for 24 hours, during which time

a precipitate of ammonium urate is formed. The supernatant liquid is now filtered off and the precipitate washed with 10% ammonium sulphate until the filtrate is quite clear from chlorides. The filter is then taken, opened out and the precipitate washed back into the beaker with hot water. The quantity of water added should amount to about 100cc. The precipitate in the beaker is now dissolved by the addition of 15cc. of concentrated sulphuric acid, and it is titrated with $\frac{N}{20}$ pot. permanganate, until a permanent red colour is produced. Each cc. of the pot. permanganate solution is equal to 3·75mgm. of uric acid. To the result obtained one has to add 3mgm., due to the solubility of the ammonium urate.

The temperature of the solution when titrating should be about 60°C.. Also whilst adding the permanganate solution, the beaker and contents must either be shaken or stirred with a glass rod. The first rosy-red colour got is the end point.

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§2

HOW CHLOROFORM AFFECTS THE URIC ACID ESTIMATIONS, WHEN ADDED TO THE URINE AS A PRESERVATIVE.

Whilst carrying out some experiments to study the effect of altitude on the protein metabolism, I noticed that the the presence of chloroform (added to the urine as a preservative) very definitely affected the uric acid estimations. I shall therefore briefly describe my results of those experiments and indicate in which manner the chloroform interferes with the estimation of the uric acid.

The diet was creatin- as well as purin-free. Controls were carried out here in Edinburgh, whilst the altitude experiments were made in the Engadine in Switzerland.

No striking effect on the protein metabolism can be observed in these results. There is no increase or decrease in the creatinin and no definite creatinuria was noted. The results got by Hoogenhuyze and Verploegh regarding the creatinin excretion at varying altitudes lead them to the conclusion that the increase of creatinin at the greater height was due to the want of oxygen: this high altitude greatly exceeds that of the Engadine, it may be noted. Löwy found an increased excretion of the amino-acids at a high altitude: Jacques and Stähelin, and also Wendt found a definite nitrogen retention after continued residence under similar conditions of altitude.

When Table is examined it will be seen that the

TABLE III

Date	Urine	Total -N	Urea -N	Ammonia -N	Creatinin -N	Creatin -N	Uric ac. -N	Notes.
	cc.	gms. %	gm. %	gm. %	gm. %	gm. %	gm. %	
1911								
5·9	1112	13·14	9·60 73	·73 5·6	·54 4·1	·01 ·08	·070 0·5	Expt. in Engadine but spec. examined in Edinburgh (5days after collection)
5·9	1174	12·43	9·76 72	·79 6·5	·56 4·5	·01 ·08	·070 0·5	
16·10	1100	11·45	–	·40 3·4	·59 5·0	·0[illegible]	·160 1·4	Expt. in Edinburgh. Urine examined at once
17·10	1000	11·37	–	·47 4·1	·56 4·9	0	·150 1·4	
1912								
19·6	1000	11·2	–	·58 5·2	·60 5·3	0	·170 1·5	Expt. in Edinburgh, urin examd at once.
20·6	1000	10·9	–	·52 4·8	·63 5·7	0	·190 1·7	
20·8	910	13·9	12·20 88	·53 3·8	·55 4·0	0	·220 1·5	Expt. in Engadine, spec. exam. there.
21·8	1010	12·9	10·60 82	·53 4·0	·56 4·3	trace	·190 1·4	Spec. also sent to Edinburgh, and examined.

The urine of the 20th. Aug. when exam. in Edinburgh contained - 0·057gm. Uric ac.-N.
The urine of the 21st. Aug. when exam. in Edinburgh contained- 0·047gm. ,, ,, ,,
showing a decided difference, due to the presence of the chloroform which was added as a precervative.

uric acid excretion was rather low when the experiment was carried out at the higher altitude. This was however discovered to be due to an accident of some practical interest. The specimens which were sent over to me from the Engadine had chloroform and thymol added to them as a preservative. I noticed that at the time of the arrival of the specimens there were uric acid crystals at the bottom of the flask. I did not think that the amount so deposited would account for the considerably smaller result got with the Engadine specimens than with those got here in Edinburgh. Deciding however to test the matter, I took various fresh specimens of urine and estimated the uric acid present, I then treated specimens of the same urines with several c.cs. of chloroform and thymol (solution of 5% thymol in chloroform) and allowed the urine to stand (after having shaken it) for varying periods. As a result I found that urine so treated, gradually deposited part of its uric acid, and so the the uric acid estimations gradually became smaller and smaller. The following example is similar to that obtained by a number of others, in this urine the amount of uric acid estimated as nitrogen was found to be 0·112gm. for the fresh specimen. The same urine when treated with chloroform and thymol and kept for a week and then the uric acid estimations again carried out, it was found that it now contained 0·043gm. of uric acid nitrogen.

That this was not due to fermentation was proven by the the fact that when ammonia and creatinin estimations were carried out (say 1-2 weeks after) then the result so obtained were not very much different from those originally got. Had

there been any fermentative decomposition, loss of ammonia and creatinin would have been recorded. This is also evident when TableIII is examined, and the analyses of the urines got at the Engadine compared with those of the urines got in Edinburgh; the results in both cases are similar, even though in the former case, the specimen was examined about 6 days after the urine was passed. That the uric acid excretion was the same in the Engadine as in Edinburgh, is seen in the second part of Table IIIwhere uric acid estimations were also carried out in the Engadine as well as in Edinburgh; here it will be seen that the estimations of the uric acid of the urine passed at the Engadine and examined there are similar to those of urine passed in Edinburgh before and after that period of residence in the Engadine.

These Investigations illustrate the necessity for using a fresh specimen of urine for uric acid estimation; or if that is not possible a preservative averting this complication must be employed.

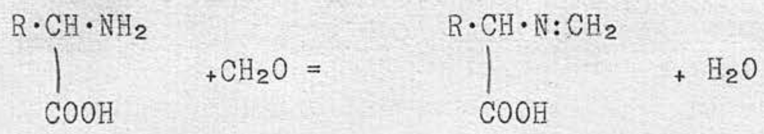
THE ESTIMATION OF ACIDITY, AMMONIA AND AMINO ACIDS.

THE ESTIMATION OF ACIDITY AND THE AMINO-ACIDS OF THE URINE.

The estimation of the acidity and the amino-acids of the urine were carried out on the same sample of urine and so I have put those two together.

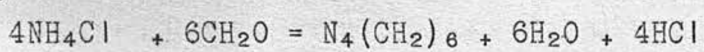
The acidity was carried out by Folin's method (1903); it gives the total acidity.

The estimation of the amino- acids is based upon the work of Sörensen - 1907 (who followed up the results of Schiff, that ammonium salts react with neutral formaldehyde to form hexamethylen tetramin), which showed that in an amino-acid solution, if formaldehyde be added, the amido group splits off water:-



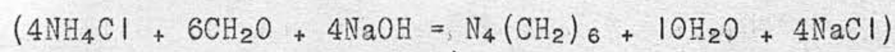
$$\begin{matrix} R \cdot CH \cdot NH_2 \\ | \\ COOH \end{matrix} + CH_2O = \begin{matrix} R \cdot CH \cdot N{:}CH_2 \\ | \\ COOH \end{matrix} + H_2O$$

A methylene combination takes place, and the neutral amino-acid becomes definitely acid and can now be titrated. Malfatti in 1908 brought forward a method for the estimation of ammonia. In this method he used the above reaction of ammonium salts and formaldehyde as a basis.



$$4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 6H_2O + 4HCl$$

and titrated the amount of acid set free.



$$(4NH_4Cl + 6CH_2O + 4NaOH = N_4(CH_2)_6 + 10H_2O + 4NaCl)$$

This method whem applied to urines gives higher results for ammonia than the Folin method (1902). This is

is owing to the presence, in the urine, of amino acids as well as ammonia. Therefore if the ammonia + amino acids be estimated by titration, and the ammonia separately by Folin·s method, by a process of subtraction the amount of amino acids will be arrived at..

The details of the method are as follows.- 25cc. of urine are taken, to this 15-20 gms of potassium oxalate (accurately neutral) are added, the mixture shaken for about a minute (there must still be a deposit of pot. oxalate on the bottom of the beaker), and whilst still cold titrated with $\frac{N}{10}$ NaOH, using phenolphthalein as indicator. Having got a faint rose tint one reads of the amount of cc. of $\frac{N}{10}$ NaOH used. The amount of NaOH used gives the total acidity of the urine. Then 5cc. of neutral formalin are added, the beaker and contents are again shaken and titrated with more NaOH, this time until a deep rose tint is got, and the reading thus obtained gives the number of cc. $\frac{N}{10}$ NaOH required to neutralise the acidity due to the formalin acting on the ammonium salts and amino acids. The number of cc. of NaOH used multiplied by 1·4 gives the amount of ammonia + amino acids expressed in terms of mgms. of nitrogen for the amount of urine used (25cc. in this case). By subtracting the amount of ammonia obtained by Folin·s method from that obtained by the previous method, one gets an approximate amount of the amino acids present in the sample of the urine examined.. Henriques and Sorensen in their method for the estimation of amino acids find out how much ammonia is present by the

Kruger and Reich method which however is not so correct as the that of Folin.

THE ESTIMATION OF

TOTAL, INORGANIC AND ETHEREAL SULPHATES.

ESTIMATION of the SULPHATES

Sulphur is excreted in two forms in the urine, (1) as loosely combined, unoxidised or neutral sulphur, mainly as constituents of cystine, taurine, hydrogen sulphide, oxyproteic acid, etc., (2) as oxidised or acid sulphur.

The oxidised sulphur is eliminated in the form of sulphuric acid, principally as salts of sodium, potassium, calcium and magnesium; a relatively small amount occurs as sulphuric acid in combination with such aromatic bodies as phenol, indole, skatol, etc. The sulphuric acid in combination with the Na, K, Ca, and Mg. is known as inorganic or preformed sulphuric acid, whereas the sulphuric acid combined with the aromatic substances is known as ethereal, or conjugated sulphuric acid.

Sulphates are estimated by the addition of barium chloride and so a precipitate of barium sulphate is formed. Folin (1906-7) has simplified the method of estimation of the sulphates. In order to get well-formed crystals of $BaSO_4$, the sulphate as well as the barium chloride solution must be diluted, and the latter added slowly. As the ethereal sulphates are not precipitated by the addition of barium chloride, the urine has first to be boiled with hydrochloric acid and then the barium chloride added, if the total sulphates are to be estimated.

<u>Procedure for Total Sulphates</u> :- 25cc of urine are measured into a 250cc.. Erlenmeyer flask, 20cc. of dilute (1 in 4) hydrochloric acid added, and the mixture gently boiled for 20-30 minutes. The mouth of the flask is covered with a watch glass to prevent too great loss of water. Cool flask in running water, dilute the contents to about 150cc. with distilled water. 10cc. of 5% barium chloride are now added, drop by drop (should take about 10 minutes) and without shaking the flask and contents, allow the mixture to stand for at least one hour, then shake and filter the solution through a Gooch filter with asbestos. The precipitate of $BaSO_4$ which has collected on the filter is now washed with cold water dried over a low flame, and then ignited. The Gooch crucible is best heated either standing on a platinum lid or in a larger crucible. Allow the crucible to cool in a dessicator, and when cold weigh. The weight of the crucible (which has been got previously to filtering) is now subtracted from the weight of the crucible + $BaSO_4$. This gives the amount of $BaSO_4$ in the 25cc. of urine. To get the weight of SO_4 which this represents, the following formula is used :-

Mol.wt.		wt.of		mol.wt.		
$BaSO_4$	:	$BaSO_4$ ppt.	:	SO_4	:	x (wt.of SO_4 in grammes)
i.e.231.7	:	(wt.found for specimen for the $BaSO_4$)	:	79.5	:	x

From the result obtained the amount is calculated for the 24 hours.

To get the amounts of of inorganic or ethereal sulphates the above method is somewhat modified.

(a) Inorganic SO_4 :-

25cc. of urine are taken in flask with 100cc. distilled water, and 10cc. hydrochloric acid, 10cc. of barium chloride (5%) are slowly added. From this point onwards the method follows out the above mentioned method.

(b) Ethereal SO_4 :-

125cc. of urine are taken and put into a flask, diluted with 75cc. of distilled water, acidified with 30cc. of dilute hydrochloric acid. To the cold solution are then added 10cc. of a 5% barium chloride solution drop by drop. Allow mixture to stand for an hour, shake and filter through filter paper. 125cc. of the filtrate are collected and gently boiled for half an hour. Cool, and filter through Gooch filter. Wash the $BaSO_4$ precipitate, dry and ignite. The calculation is the same as for the total SO_4

THE ESTIMATION

of

CALCIUM, MAGNESIUM AND PHOSPHORIC ACID.

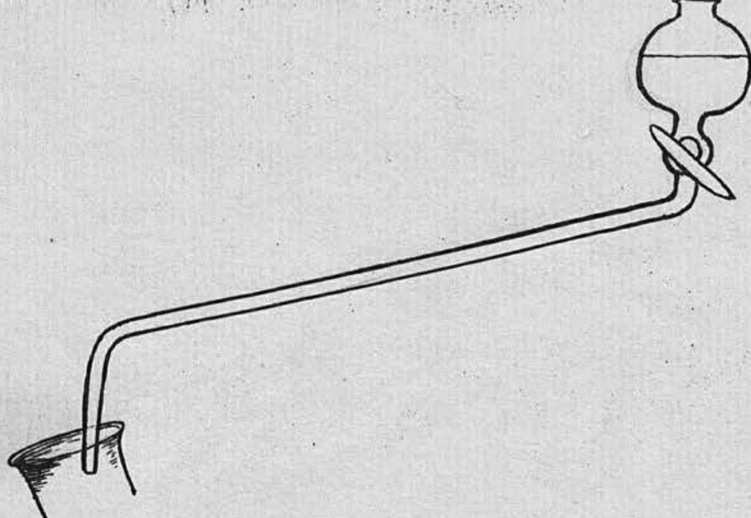
ESTIMATION of CALCIUM, MAGNESIUM and PHOSPHORIC ACID.

The estimation of calcium, magnesium and phosphorus has been grouped together as the first part of the process is the same, namely in that part of the process in which the organic substances of the urine are combusted, and the inorganic part left. The method used was that of Neumann. In this method the material is combusted in the presence of an acid mixture, without the urine being allowed to evaporate all its moisture. If this happens and the solid part of the urine becomes charred the time required for complete combustion would be greatly prolonged.

Therefore fresh acid is dropped in whilst the other is being combusted. This method is especially useful in urines containing albumin.

Details of Method;-

50-100cc. or even more of urine are taken and placed in a long-necked combustion flask (Kjeldahl) and 30cc. of pure nitric acid added, and the mixture heated. When the volume of the mixture has been reduced to about 30-50cc. ,the flame should be turned down somewhat, so that the mixture boils slowly. Whilst it is now boiling, a mixture of equal parts of nitric and sulphuric acid is allowed to drop into the flask slowly. For this purpose a fair sized separating funnel is used, with a long tube, bent twice so that the end of the tube dips into the neck of the combustion flask, whilst the reservoir end with



the stop-cock is some distance off, and the flow (of the acid) down the tube can be regulated without coming near to the acid fumes issuing from the flask.

At the commencement of the combustion, dense nitrous fumes issue from the flask, but as the combustion proceeds they diminish in amount. If the combustion is completed and one adds a few drops of the acid mixture or of nitric acid alone, then there is no dense evolution of brownish fumes and if no more acid is added, but the heating continued, the liquid remains colourless; if however the com- is not completed, then on the addition of acid mixture or of nitric acid, dense red nitrous fumes are evolved and on continuing to heat, the fluid becomes darkish and may even become black.

The points to be remembered about this process are; the slower one heats the quicker will the combustion proceed, and too much acid mixture must never be used. 10-15cc. of acid mixture should be sufficient, if more acid is required it is better to add nitric acid only, but not more of the acid mixture.

When the combustion is finished, the liquid is heated for another 5-10 minutes, this drives off the brown fumes from the decomposition of nitrosylsulphuric acid. After this if the sample is for the estimation of calcium, water is added to about three times the amount of acid mixture used in the combustion. If the sample is for the estimation of phosphoric acid, the amount of water added is about five times that of the acid mixture.

The second part of the process differs, according to whether calcium and magnesium or phosphoric acid is to be estimated.

1)- Calcium and Magnesium:-

The liquid which has been got as a result of combusting the urine with the acid mixture, then diluted with distilled water and boiled for a few minutes, is taken, and to it is added concentrated ammonia in excess and a saturated solution of ammonium oxalate. The whole is left in a water-bath of boiling water for about one hour. During this time the calcium is precipitated as calcium oxalate. At the end of that time the supernatant liquid is decanted through a filter, avoiding as much as possible that any of the oxalate gets on to the filter. The residue in the flask is now washed with warm water, allowed to settle and the liquid filtered. This washing is repeated several times until a sample of the filtrate, acidified with nitrite-free nitric acid and warmed, does not colourize one drop of the pot. permanganate solution used for

titration. When this end is attained any calcium oxalate that may have got on to the filter, is dissolved and washed into the flask with hot, dilute nitric acid (nitrite-free), and so the residue in the flask is also dissolved. The filter is then washed with some hot distilled water to make sure that all the acid had been washed into the flask.

The contents of the flask are now heated, and when the solution just begins to bubble, it is taken and titrated with a known strength of pot. permanganate (preferably $\frac{N}{20}$), until the solution gets the first permanent rosy colour. When the pot. permanganate is first added, the solution gets a red colour which remains for some time, then suddenly disappears . After this the colour, on further addition of pot. permanganate, disappears more rapidly, until a sufficient amount has been added.

From the number of cc. $\frac{N}{20}$ pot. permanganate used, one can readily estimate the amount of calcium.

$$1 \text{cc. of } \frac{N}{20} KMnO_4 = \quad \text{gm.Ca.}$$

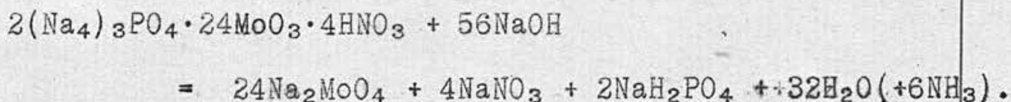
To estimate the magnesium the first and second filtrate, got from the first and second decantation of the solution from which the calcium as the oxalate has been removed, is used. These filtrates are put together and measured, and then treated with 10% ammonia solution (the amount of ammonia solution used being one third of the combined volume of the filtrates).

The solution is allowed to stand and the magnesium is precipitated as the ammonium magnesium phosphate. The

supernatant liquid is then filtered off, and the residue washed with dilute ammonia water. Any of the salt that may have got on to the filter is washed into a beaker and along with the remainder of the ammonio-magnesium phosphate dissolved with some acetic acid. If any uric acid acid be present, the solution should be filtered before it is titrated. The titration is carried out with a standard uranium nitrate solution, just as the ordinary phosphorus estimation which I shall describe later (pp. 42). The amount of phosphoric acid estimated is multiplied by 0·3426 and gives the amount of magnesium, or if multiplied by 1·5676 gives the amount of magnesium pyro-phosphate.

2)- Phosphoric acid (Neumann).-

In this method the phosphoric acid is precipitated as ammonium-phospho-molybdate, the reaction being according to the formula.-



$$2(Na_4)_3PO_4\cdot24MoO_3\cdot4HNO_3 + 56NaOH$$
$$= 24Na_2MoO_4 + 4NaNO_3 + 2NaH_2PO_4 + 32H_2O(+6NH_3).$$

The urine is combusted as described on pp. 37. The combusted solution is diluted with water, 5 times to the amount of acid mixture used. Now add 50cc. of ammonium nitrate (a 50% solution), and the mixture heated till bubbles begin tp appear (about 70-80°), then add 40cc. ammonium molybdate (19% solution, dissolved in the cold and filtered)*, shake the solution thoroughly for a minute or so (to get a good granular precipitate), allow the presipitate to settle for about 15 minutes, then decant the liquid and

wash the residue several times with cold water (about 150cc. each time) shaking it and the water thoroughly and allowing the residue to settle. Before filtering always pour some cold water through the filter, to close the pores, and there-fore the chance of any of the fine precipitate passing through is diminished. The washing is continued until the filtrate does not react acid to the litmus paper. The whole process of washing and filtering must be a continuous one, for otherwise if the precipitate is allowed to stand in touch with water, it dissloves to a slight extent.

When the washing is completed, the filter with any of the precipitate on it, is put into the flask containing the bulk of the precipitate, 150cc. of water are added, the whole thoroughly shaken up, and the precipitate dissloved by adding $\frac{N}{2}$-NaOH from a burette, shaking the whole time, until the solution becomes colourless, then add 5cc. of $\frac{N}{2}$-NaOH in excess. The liquid is now boiled for about 15 minutes (until the vapours give off no more ammonia, as tested with litmus paper). The flask and contents are cooled in a stream of cold water, and a few drops of phenolphthalein added, and the excess of $\frac{N}{2}$-NaOH titrated with $\frac{N}{2}$-H_2SO_4. The number of cc.-$\frac{N}{2}$-NaOH originally added minus the number of cc.-$\frac{N}{2}$-H_2SO_4 added to titrate the excess, when multiplied by 1·268, gives the number of milligrams of phosphoric acid(P_2O_5).

A simpler method but not quite so accurate is the one used clinically. It consists in titrating the urine with uranium nitrate solution, and from the number of cc. of uranium solution (which has been standardised) used, one

estimates the number of milligrams of phosphoric acid.

In carrying out this method cochineal tincture can be used as an indicator (it turns green when the reaction is completed), or the end-point can be tested by means of a solution of potassium ferrocyanide, a drop of the hot urine is added to a drop of the ferrocyanide solution, which has been placed on a white porcelain plate. When sufficient of the uranium solution has been added to the urine, then a drop of the hot mixture of urine and uranium solution will cause the ferrocyanide to turn brown. This latter method is more delicate than the cochineal tincture.

When doing the above 5cc. of the acetate solution (10gms. sod.acetate and 3 gms. glacial acetic acid) must also be added to the urine before heating and adding the uranium solution.

THE SUGAR ESTIMATION
IN THE BLOOD .

SUGAR ESTIMATION IN THE BLOOD

Rona and Michaelis describe a method in which 50cc. of serum or plasma are taken and diluted with water about 10-20 times. They then add 40 cc. "Ferri oxydati dialysati", drop by drop, shaking the while. Then filter, take the filtrate and evaporate to about 10-15cc. The sugar may be estimated by means of polarimeter.

Oppler andRona describe a slight modification of the above and one which gives more accurate results. This method was used by Mr Horne and myself to clear the blood prior to estimating the sugar.

In this method the blood is caught (about 50cc.) in a white porcelain basin, which had previously been rubbed with some (a pinch) sodium fluoride, to prevent coagulation of the blood. The blood should be weighed so as to approximately know the weight and how much iron solution is to be added. The blood is now poured into a flask and diluted about ten times with distilled water and iron solution added in small quantities at a time, shaking thoroughly (for every gram of blood one requires 3cc. of the iron solution). After having added all the iron solution and shaken the contents of the flask, allow it to stand for about ten minutes. Then add 1 gm. of finely powdered magnesium sulphate or Rochelle salt (it must be powdered in order that it may dissolve more easily) and again shake the flask with its contents forcibly. Then filter through a multi-folded filter paper. To the clear

filtrate add a few drops of concentrated acetic acid and evaporate (preferably in a vacuum distillation plant) to about 5cc.

The filtrate must be first tested to see whether there is any protein present. If there should be or if the filtrate is still markedly coloured, then a few more cc. of the iron solution must be added. Shake up and filter.

To estimate the amount of sugar present in the concentrated liquid, one can either use the polarimeter or estimate it by any of the copper reduction methods, such as Fehling, Pavy-Fehling, Bertrand etc. Of these the best is undoubtedly the Bertrand. This method I employed in the various sugar estimations of the blood. For the urines however I used the Ivar Bang method. ([illegible]).

In the method of Oppler and Rona, the blood is diluted down ten times, and then has to be evaporated to a bulk of about 5cc., and as this requires some time, a simpler method described by Moeckel and Frank (1910) was later used by Mr Horne and myself for treating the blood prior to estimating the sugar. The principle of the method is the same as that of Oppler and Rona, but smaller quantities are used and consequently the time required is considerably reduced, allowing f[illegible] an estimation to be completed in 2-3 hours. The results obtained are the amounts of sugar present in the plasma; the results are therefore a little higher than those obtained for the blood,

As this method was the one mostly employed, I shall explain it somewhat in detail.

In all those experiments the cats from which the blood was to be taken, were thoroughly anaesthetised (the anaesthetic used was chloroform); care had to be taken not to give too much chloroform, or otherwise the animal soon stopped breathing, the heart stopped or beat so feebly so that the blood stream would be very sluggish and so facilitate coagulation, and very little blood would be got. Having got the animal well under, an incision was made in the side of the neck, taking care not to cut any of the large veins, and the carotid artery exposed, and cleared from the surrounding tissues. A wooden spatula or the wooden handle of the scalpel was inserted beneath the artery to raise it from its surroundings. An incision was made into the artery with a pair of sharp scissors, the blood as it spurted out was collected in a glass vessel or porcelain basin, previously rubbed with some sodium fluoride the blood as it was collected was shaken (or stirred with a glass rod) about in the dish so as to come in contact with the sodium fluoride and to prevent coagulation taking place.

12-15cc. of blood are taken and placed in centrifuge-tubes and centrifugalised. Then the plasma is pipetted off; if a few red cells are also taken up it does not matter much. Of this 5cc. are measured exactly and placed in a measuring glass (of 100cc. capacity), now dilute with 50cc. of distilled water and add 10-14cc.

of liquor ferri oxyd. dialys. drop by drop and shake, then add a few crystals of magnesium sulphate (powdered) again shake thoroughly and acidify with one drop of concentrated acetic acid, make up to 100cc. with distilled water and mix. If any froth is present it can be removed by a drop of ether. Having shaken the mixture allow it to stand for one minute, filter through folded filter paper. Test for the absence of protein, take 50cc. of the filtrate and carry out the sugar estimation by the Bertrand method.

The Bertrand method for the estimation of sugar depends upon the fact that copper oxide which is formed when sugar is boiled with Fehling solution, becomes dissolved in a solution of ferrous sulphate in sulphuric acid and the iron salt formed is titrated against potassium permanganate.

The following solutions are required:-

Solution 1.

Pure crystalline copper sulphate	40 gm.
Distilled water to	1000 cc.

Solution 2.

Pure Rochelle salt	260 gm.
Sodium hydroxide (sticks)	150 gm.
Distilled water to	1000 cc.

Solution 3.

Ferrous sulphate (anhydrous)	50 gm.
Concentrated sulphuric acid	200 cc.
Distilled water to	1000 cc.

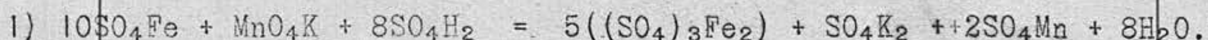
Solution /

Solution 4.

Potassium permanganate 5 gm.

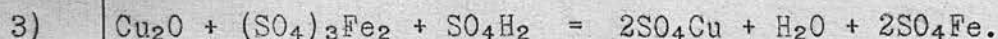
Distilled water to 1000 cc.

The strength of the permanganate solution is tested by carefully weighing out 0·250 gm. ammonium oxalate (pure). Put in into a beaker and dissolve in about 100cc. of distilled water, add 2cc. of conc. sulphuric acid and heat to about 60-80°. Add the potassium premanganate solution from a burette until a rose red colour is obtained. The reaction is worked out from the following formalae:-



1) $10SO_4Fe + MnO_4K + 8SO_4H_2 = 5((SO_4)_3Fe_2) + SO_4K_2 + 2SO_4Mn + 8H_2O.$

2) $5C_2H_2O_4 + 2MnO_4K + 3SO_4H_2 = 10CO_2 + 2SO_4Mn + SO_4K_2 + 8H_2O.$



3) $Cu_2O + (SO_4)_3Fe_2 + SO_4H_2 = 2SO_4Cu + H_2O + 2SO_4Fe.$

From this,

1 molecule of oxalic acid = 1 molecule of ammonium oxalate.
= 2 molecules of iron.

If the amount of ammonium oxalate (0·250gm.) be multiplied by $\frac{63 \cdot 6 \times 2}{142 \cdot 1}$ i.e. 0·8951 we get the amount of copper which equals the amount of pot. permanganate. 0·250 x 0·8951 = 0·223775 or in round figures 0·224.

Method of estimation:-

In an Erlenmeyer flask of about 250cc.,

20cc. /

20cc. of the sugar solution are poured, to this 20cc. of the copper solution (1) and 20cc. of the Rochelle salt solution are added, mix and boil gently for 3 minutes. Take away from the flame and allow the copper oxide to settle somewhat (about 15 minutes). The solution must still have a distinct blue colour. Carefully filter through a Gooch filter, partially filled with cleansed asbestos to prevent any of the copper oxide that may get on to the filter from being lost. When filtering as little as possible of the oxide must get on to the filter. Wash residue in flask with distilled water. If the water is allowed to flow down a glass rod on to the top of the copper oxide, so that very little of the latter is stirred up, then the supernatant liquid can soon be filtered, this washing and filtering off of the water must be repeated several times till all the copper sulphate solution is washed out.

To the residue in the Erlenmeyer flask 20cc. of the iron solution are added; when the copper oxide is dissolved the mixture has a green colour. The asbestos in the filter is taken with any copper oxide that may have got on to it and placed in the flask with the remainder of the oxide and the iron solution. The asbestos is shaken or stirred with a glass rod so as to make sure that any copper that may be in the meshes of the asbestos is dissolved up by the iron solution. /

TABLE V.

Sugar in mg.	Cu. in mg.	Sugar in mg.	Cu. in mg.
58	109·3	82	149·3
59	111·1	83	150·9
60	112·8	84	152·5
61	114·5	85	154·0
62	116·2	86	155·6
63	117·9	87	157·2
64	119·6	88	158·8
65	121·3	89	160·4
66	123·0	90	162·0
67	124·7	91	163·6
68	126·4	92	165·2
69	128·1	93	166·7
70	129·8	94	168·3
71	131·4	95	169·9
72	133·1	96	171·4
73	134·7	97	173·1
74	136·3	98	174·6
75	137·9	99	176·2
76	139·6	100	177·8
77	141·2		
78	142·8		
79	144·5		
80	146·1		
81	147·7		

TABLE IV

Sugar in mg.	Cu. in mg.	Sugar in mg.	Cu in mg.
10	20·4	34	66·5
11	22·4	35	68·3
12	24·3	36	70·1
13	26·3	37	72·0
14	28·3	38	73·8
15	30·2	39	75·7
16	32·2	40	77·5
17	34·2	41	79·3
18	36·2	42	81·1
19	38·1	43	82·9
20	40·1	44	84·7
21	42·1	45	86·6
22	43·9	46	88·2
23	45·8	47	90·0
24	47·7	48	91·8
25	49·6	49	93·6
26	51·5	50	95·4
27	53·4	51	97·1
28	55·3	52	98·9
29	57·2	53	100·6
30	59·1	54	102·3
31	60·9	55	104·1
32	62·8	56	105·8
33	64·6	57	107·6

solution. If all the copper does not dissolve then some more of the iron solution must be added. Then titrate with potassium permanganate till the first red colour pervades the whole solution.

From the number of cc. of permanganate used, and knowing how many cc. of it are equal to the 0·250gm. ammonium oxalate, one gets the amount of Cu in mgm. in the solution examined.

Example;- 5cc. Pot. permanganate are required for the titration.

Now it was previously found that 22·5cc. permanganate solution were required for the 0·250gm. ammonium oxalate, then,

$$\underset{KMnO_4}{22\cdot5 : 5\cdot5} \quad : \quad \underset{((NH_4)_2C_2O_4 - 0\cdot25 \times 0\cdot895)}{0\cdot224} \quad : \quad x(\text{mgm.Cu.})$$

From the amount of copper obtained, on comparing to the figures in the Tables IV-V, the amount(mgm.) of sugar is got.

THE ESTIMATION OF

GLYCOGEN CONTENT OF LIVER.

ESTIMATION OF GLYCOGEN IN THE LIVER

To estimate the glycogen in the liver, the glycogen must be extracted. This is not completely successful simply with water. Külz therefore introduced a method, in which the tissues are dissolved in caustic alkali separating the proteins and precipitating the glycogen with alcohol. Pflüger has modified this method and his method has been used in the present research. Pflüger and others ( ) have shown that glycogen boiled with caustic potash is not acted upon, even if continuously boiled with it for 24 hours or more.

To estimate the glycogen in the liver of rats, the whole liver is used; in the case of larger animals, such as cats, dogs etc., only an aliquot portion.

1. Liver of Rats:-

The liver is cut out and weighed, then it is placed in a flask (of about 100cc.) capacity, and some 60% caustic potash added (for each gram of liver tissue, 1cc. of the KOH solution), shaken and put on a water bath (boiling water) and left for 3 hours. The contents are now and then shaken to make sure that all the liver has been dissolved. When the flask has been on the water bath for about half an hour, a stopper (loose fitting) should be put int the flask to prevent a loss of water.

After the flask and contents have been on the

water-bath for 3 hours, it is taken off and allowed to cool, the contents are poured into a tall beaker and the flask washed out with 40cc. of distilled water. The glycogen is precipitated from this solution by means of 160cc. of rectified spirit. The glycogen is now allowed to settle and the supernatant liquid filtered within 24 hours. The settling of the precipitate can be accelerated (especially when a large quantity of glycogen is present) if after having added the rectified spirit, one stirs the solution vigorously with a glass rod for several minutes, the glycogen then separates out in large granules and quickly settles to the bottom of the beaker, and so allows the filtration to be commenced within half to one hour.

The supernatant liquid having been filtered off, (care being taken to get little of the glycogen on to the filter), the residue is now washed with 65% alcohol and again allowed to settle. This is repeated a second time and then the precipitate is washed with rectified spirit and lastly with absolute alcohol. Having filtered off the absolute alcohol the glycogen in the beaker and any that may have got on to the filter is dissolved in hot water and the solution made up to about 80-85cc. in a measuring flask (100cc.), 5cc, of concentrated hydrochloric acid added and the solution put in a water bath of boiling water (to hydrolyse the glycogen into dextrose) for 3 hours. The flask in then taken, allowed to cool, its contents neutralised with concentrated KOH solution, and made up to 100cc. with distilled water, shaken and

filtered. 20cc. of the filtrate are now used for the sugar estimation, the Bertrand method (as described on pp. 47) being used by me.

2. In the case of cats, dogs and larger animals, with large livers, the liver is taken, weighed and minced and a portion of this used. If very little glycogen is present then the whole liver can be used, (to each gram of liver substance 1cc. of the conc. KOH solution is used). The mixture is boiled for 3 hours, cooled, poured into a beaker and an equal volume of distilled water added. The glycogen is precipitated by adding twice the volume of rectified spirit to that of the glycogen solution. After this the method is identical with the foregoing.

To estimate the amount of sugar as glycogen, multiply the amount of sugar by 0·927 and the result will be the amount of glycogen.

RECENT METHODS INTRODUCED BY FOLIN ;
THEIR SUITABILITY FOR CLINICAL APPLICATION .

FOLIN'S NEW AND SIMPLE METHODS FOR THE ESTIMATION OF TOTAL NITROGEN, AMMONIA AND UREA

Folin has recently introduced a new technique for the estimation of the total nitrogen, urea and ammonia in the urine. The new method is comparatively simple and does not require any expensive apparatus beyond a colorimeter (of which there are a number of different kinds).

The apparatus can be made up of Jena test-tubes, connected together simply with glass and rubber tubing. The first part of the technique for the different substances varies, but in the latter part ~~in which~~ the ammonia that has been formed (in the case of free ammonia it is already preformed) is nesslerised or titrated. I shall describe the details for each substance separately.

1. Total Nitrogen - (Folin and Farmer)

5cc. of urine are measured into a 50cc. (graduated flask) if the specific gravity is above 1·018. The flask is then filled to the mark with distilled water, and the whole thoroughly mixed.

One cc. of the diluted urine is now pipetted into a large Jena test-tube 8" x 1" To this is added 1 cc. concentrated sulphuric acid, 1 gm. of pot. sulphate, one drop of a 5cc. copper sulphate solution, and a /

a small quartz pebble (to prevent bumping). This is boiled over a microburner for about 6 minutes (i.e. about two minutes after the mixture has become colourless). Allow to cool somewhat (but it must not be allowed to solidify), then add about 6cc. of water, a few drops at first, but more later, so as to make sure no solidification can occur (if more water has to be added to prevent a precipitation taking place, then the current of air passed through will have to be continued for a long time). To the acid solution an excess of sodium hydrate (3cc. of concentrated solution) is added, and the ammonia that is set free is driven over by means of a rapid air current (filter suction pump or force pump) into a flask containing about 20cc. of water and 2cc. of $\frac{N}{10}$ sulphuric acid. The principle here being much the same as in the case of the ammonia, only on a smaller scale. No calcium tube need be interpolated as there is not the same risk of moisture getting over.

The flask is now disconnected and the contents are diluted to about 60cc. and nesslerised against 1 mgm. of nitrogen as $(NH_4)_2SO_4$ solution. The combustion of the urine with sulphuric acid does not require to be carried out in a fume chamber. An old pipette (25 or 10cc. capacity) is taken and the one end invaginated with a small hole at the extremity.

This /

This is fixed to a water pump so that a constant current of air is drawn through. If it is now placed over the end of the Jena test-tube it will draw away all the fumes and keep the atmosphere of the laboratory free from the noxious acid fumes.

2. Urea. - (Folin)

The urine is diluted so that 1cc, contains about 0.75mgms, to 1·5mgms. of urea nitrogen. Dilutions of 1-20 are usually adequate. 1cc. of diluted urine is measured into a large Jena test-tube, into which the following substances have first been placed. 7gms. of dry pot. acetate (must not be in lumps), 1cc. of 50% acetic acid, a little powdered zinc (to prevent bumping) and a temperature indicator.*

The test-tube is closed by a rubber cork fitted with a long glass tube (to act as a condenser) and the whole suspended about a micro-burner and heated. When the acetate has melted and begins to boil, the flame is regulated so that the boiling is continued in a gentle even manner, for ten minutes, The test-tube and condenser (glass tube) are then taken away from the flame, and the contents diluted with 5cc. of water (the water being poured down the side of the long tube into the test-tube to ensure that any ammonia acetate which may have got to the sides /

sides is washed down). 2cc. of a saturated solution of sodium hydrate are added to the contents in the test-tube and the apparatus fixed up, as described above for the total nitrogen, and air passed through to remove the ammonia, which is caught in 2cc. $\frac{N}{10}$ acid and about 35cc. of distilled water. The time required for the air current to pass through the solution depends upon the strength of the air current; 10-30 minutes according to the velocity of the air current and volume of the fluid. The amount of ammonia thus set free is determined colorimetrically against 1mgm. of nitrogen. Details on pp. 58.

This method can be used also when sugar is present in the urine, which is diluted down 20-100 times according to the amount of sugar present (1cc. should contain not more than 0·1mg. urea nitrogen), and Folin found that 0·1 to 0·3mgm. of urea nitrogen can be estimated when as much as 2mgms. of dextrose are present.

Two outstanding facts which Folin established are (1.) that the less the quantity of urea used for decomposition, the less the time required for its conversion to ammonia; and (2.) that the less the bulk of ammonia-containing solution, the less the time required to drive the NH_3 off by the air current.

The methods bring about an increased saving of time and are suited to sideroom work, while Folin shows /

shows that the use of such small quantities is quite compatible with accuracy - on which point we also have satisfied ourselves.

3. Ammonia - (Folin and Macallum)

1-5cc. of urine are pipetted into a long test-tube - (The volume taken should give 0·75 to 1·5mgms. ammonia-nitrogen), 2cc. are usually quite sufficient. A few drops of 10% potassium carbonate and 15% pot. oxalate solutions are added, as well as a few drops of kerosene or toluol to prevent foaming. The air current is passed through for about 10 minutes or more and the ammonia collected in 20cc. of water 2cc. of $\frac{N}{10}$ acid. The ammonia collected is then nesslerised.

In the three foregoing methods, ammonia was collected in each case and then estimated either by nesslerising or by titrating. The latter method has not been found to be quite so accurate, and so nesslerising is preferred.

In each of the above cases the test-tube or flask with the ammonia is taken and diluted with about 60cc. of distilled water, and at the same time one takes 1mgm. of nitrogen (in the form of ammonia sulphate solution), diluting it to about the same volume. Both solutions should be nesslerised at the same time, and to do this 5cc. of Nessler's reagent ✗

reagent (which has been diluted immediately beforehand with about 25cc. of distilled water) are taken and added to each of the solutions. The volume of the solutions is made up to 100cc. in a graduated flask, with distilled water, mixed, and the relative intensity of the colours determined in a colorimeter.

* To calculate the result, divide the readings of the standard by the readings of the unknown and the answer will be in milligrammes of nitrogen in the urine used.

In Table VI I give some figures on the estimation of the total nitrogen and urea in several specimens of urine. The total nitrogen by the Kjeldahl method and the urea by Folin's original method are given for comparison with the figures (obtained by R.G.Bannerman, M.A. for me) by Folin's new method.

TABLE VI

Total Nitrogen		Urea-Nitrogen	
Kjeldahl	Folin's new meth.	Folin's old and new method	
3·4gms.	3·5gms.	3·3gms.	3·2gms.
	3·7		3·6
	3·7		
4·2gms.	4·1gms.	3·36gms.	3·8gms.
	4·2		3·9
	3·8		
	4·3		
3·0gms.	3·08gms.	2·64gms.	3·2gms.
	2·80		2·84
	2·84	2·92	2·88
	2·76		2·80
	2·64		

In general the only nitrogenous constituent of the urine estimated clinically, is urea. Are the results obtained by the method in use (Doremus) accurate and the conclusions drawn therefrom of any value ? I contend that they are of little value and that for the following reasons.

The hypobromite method as I have already indicated is affected by various factors, with the result that the estimations obtained by this method vary. I have tried this method myself and compared the results so got with those obtained by Folin's method. My idea was at first that even although the results were not the same as those obtained by the latter method, yet if they always were constantly proportional, the method would, by the employment of a factor for correction, give useful results. In Table V I present a series of results so obtained; when one examines them and compares the amount (which I have estimated as nitrogen) with the urea-nitrogen figures obtained by the Folin method, one is struck by the fact that it does not remain constantly higher or lower than the Folin estimations, but that it varies widely. This fact is also brought out if the percentage amount of the urea-nitrogen as estimated by the hypobromite method to the total nitrogen is observed. Here we see how much it varies; in the one case it was below 60% when the amount estimated by the Folin method gave a higher percentage (80); generally however it tends to give higher results than the Folin method , and even here there is no approximation to equality of the figures; on one occasion the result obtained even exceeded in amount that of the total nitrogen of the urine.

TABLE- VII

	Urine in ccs.	Total -N gms	Urea-N Folin method gms.	%	Urea+Ammonia-N Folin method gms.	%	Urea-N Doremus method gms.	%
	3324	7·72	6·03	78	6·51	84	6·16	79
	3290	7·46	5·38	72	5·87	78	5·79	77
	2500	9·72	7·08	73	7·91	81	7·69	79
	2600	8·22	6·26	76	6·62	80	5·40	54
	3182	8·73	6·57	75	7·03	80	6·62	71
2.	1365	7·83	6·07	77	6·49	83	7·44	89
	2415	10·82	7·87	78	8·49	106	11·50	86
3.	435	2·74	2·24	82	2·38	87	2·37	77
	460	2·49	1·92	77	2·07	83	1·93	98
	206	2·69	2·44	90	2·55	94	2·66	98
	435	4·28	3·62	84	3·77	88	4·33	93
4.	1275	8·57	7·45	87	7·74	90	8·02	88
	1600	7·21	6·01	83	6·23	86	6·36	93
5.	1080	5·62	4·75	84	5·01	89	5·33	90
6.	1306	7·38	5·83	79	6·25	85	6·69	93
7.	1600	14·83	11·71	79	12·40	83	13·79	91
	1075	8·91	7·74	87	8·06	90	8·11	

Recently whilst repeating some of the above results I found that the readings obtained in the hypobromite tube varied from hour to hour, after the bubbles of gas had disappeared, with the result that a reading obtained 3hours after the first reading exceeded ~~that~~ the latter by about 4gms.

From the foregoing, it will be seen that for practical reasons, the hypobromite method for the estimation of urea should be condemned. Still more definitely is this the case when we consider that a large personal factor comes into play in the clinical method of hypobromite estimation where the pipette is still used for inserting the urine into the tube. Whereas the results obtained by me were all got by means of the Hind modified ureometer, in which there is no loss of gas, if ordinary care be taken and in which the amount of urine passed in is accurately gauged. The apparent ease of the technique has been deceptive.

Apart from this practical consideration, the estimation of urea only has very little significance, if the dietetic factor is not considered; for as we have seen, the amount of urea may be reduced to very small amounts if the diet be protein poor, as I have shown in Tables XXXIV & XL of my researches. Also the absolute quantity is not quite so important as the relative amount, and in order to get the latter one has to estimate the total nitrogen. A substance which is of greater clinical moment is the ammonia, for it being one of the intermediate products of the protein metabolism and a precursor of urea may undergo great variations in conditions affecting the metabolism: and especially is this the case, where it has to

protect the organism, as in the various forms of acidosis. This substance is readily estimated by the titration method given on 32 (in about 1 minute), the figure so obtained is for ammonia + amino-acids, but it is a more constant one than the urea results got by the hypobromite method, and if ammonia estimations by the Folin method be simultaneously carried out, one gets an idea also of the amount of amino-acids.

In all metabolic disturbances however, one should have a fair idea of the amount of total nitrogen, as well as of the urea and ammonia. This is now within the reach of the clinician, by the methods recently introduced by Folin. The apparatus and working of the method are simple and with a little experience give accurate results as I have shown on pp. 59.

Before concluding I wish to point out, that if our knowledge of the various pathological disturbances of the metabolism is to advance at all, we will have to make a more thorough investigation of the relationship of the dietetic factor to the diseased organism apart from the use of more accurate analytical methods.

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