preferable reaction for activity is found to be slightly alkaline, the fluid itself in which the ferments are elaborated is distinctly acid. It seems to be true, in addition, that the stability of both ferments is greatest in the presence of weak acid. The degree of acidity of the fluid was found to be nearly the same for the three animals investigated, but there are doubtless fluctuations here, as in the blood, which call for fuller investigation later.

Our thanks are due to Dr. Frederic Fenger for his courtesy in aiding us in securing the pancreases, to Mr. William Johnson for the determinations of the starch-converting power and to Professor A. I. Kendall for the use of the centrifuge of the bacteriological laboratory.

Conclusions.

We have explained the method of separating a minced organ like the pancreas into three fractions by the aid of a powerful centrifuge. In the case of the pancreas these fractions have different properties, especially with reference to the distribution and amount of the ferments present. The general composition of the three fractions for the organ of the hog, beef and sheep is given. It is likely that the method can be applied with advantage to the study of the fluids contained in other tissues, and especially to the enzymes present.

CHICAGO, ILL.

[HARRIMAN RESEARCH LABORATORY, ROOSEVELT HOSPITAL, NEW YORK CITY.]

THE NEPHELOMETRIC ESTIMATION OF PURINE BASES, INCLUDING URIC ACID, IN URINE AND BLOOD.[1]

BY SARA STOWELL GRAVES AND PHILIP ADOLPH KOBER.

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CONTENTS: I. Introduction, II. Method: *A*. Precipitant; 1. Reagent, 2. Protective Colloid, 3. Use of Precipitant, *B*. Separation of Uric Acid from Purine Bases, III. Directions, IV. Applications; *A*. Urine, *B*. Blood, V. Summary.

I. Introduction.

The estimation of purine bases with the exception of uric acid, is at present a long and often inaccurate process. Recently Folin[2] and collaborators have developed a colorimetric reagent for uric acid, which does not react with the other purines, but gives reactions with phenols and allied substances. To circumvent the difficulty they precipitated the uric acid with Salkowski's reagent and, after washing by decantation, make the colorimetric estimation. This method is sensitive to small amounts of uric acid but the present methods for determining the other purine bases, xanthine, hypoxanthine, adenine and guanine require such large

[1] Read before the Am. Soc. Biol. Chem., Dec., 1914, St. Louis, Mo.

[2] Folin and Macallum, *J. Biol. Chem.*, 13, 363 (1912); Folin and Denis, *Ibid.*, 14, 95 (1913).

amounts of material as to make them unsuitable for blood analysis and their estimation in urine consumes the better part of two days.

While developing technic for the determination of guanine and adenine in the study of nucleases along nephelometric lines,[1] it was found possible to estimate both uric acid and other purines in a few minutes. The requirements for such a method were (1) a nephelometric precipitant[2] for these substances and (2) a means of eliminating either uric acid or the other purines from reacting with this precipitant.

II. Method.

A. Precipitant.—The reagents used now, ammoniacal silver nitrate and magnesium mixture in Salkowski's method, and cupric sulfate with sodium hyposulfite in the Krüger-Schmidt method, have heretofore been considered specific. The difficulties of these methods were therefore not in the precipitation but in the subsequent isolation or analysis of the purine bases after precipitation. As colors are developed with the Krüger-Schmidt reagents, no attempt was made to apply them nephelometrically, attention being confined to Salkowski's reagent.

(1) **Reagent.**—Salkowski's reagent consists of equal volumes of magnesia mixture and ammoniacal silver nitrate (26.0 g. in 1 liter and sufficient NH_3 to prevent precipitation of AgO). Ammoniacal silver nitrate precipitates the purine bases as a white silver complex, but black reduced silver is formed with uric acid, unless chlorides are present.

After careful study of pure material, uric acid,[3] xanthine,[4] hypoxanthine,[5] guanine,[6] and adenine,[7] separately or in mixtures, it was found necessary to modify the reagent as follows:

(*a*) The concentration of the silver nitrate was reduced.

(*b*) Ammonium chloride alone was found more suitable than magnesium mixture.

(*c*) The amount of ammonium hydroxide had to be redetermined.

In 100 cc. of reagent, silver nitrate was varied from 10 to 50 cc., magnesia mixture from 0.5 to 50 cc. (0.5 cc. being the smallest amount which would prevent the reduction of silver upon adding uric acid) and the ex-

[1] Kober and Graves, "Nephelometry in the Study of Nucleases," THIS JOURNAL, 36, 1304 (1914).

[2] For the definition of "Nephelometric Precipitant" see THIS JOURNAL, 35, 1585 (1913).

[3] Kahlbaum's.

[4] Merck's.

[5] Prepared in this laboratory from beef extract.

[6] Prepared in this laboratory from yeast nucleic acid according to Walter Jones whose directions, given in his "Monograph on Nucleic Acids," were found complete in every respect, and very satisfactory results were obtained.

[7] Also from yeast nucleic acid according to Jones. We received from Dr. P. A. Levene, of Rockefeller Institute, sufficient adenine picrate to make our preliminary experiments, for which we wish to express our thanks.

cess of ammonium hydroxide was varied from 2 to 12 cc. It was found that the uric-acid-silver complex was somewhat soluble in a large excess of ammonia, but some excess was necessary to prevent the formation of any insoluble silver chloride and to allow for ammonia evaporation while making the readings. The difficulty with the magnesia mixture was the formation, upon standing with an excess of ammonium hydroxide, of a white precipitate, possibly magnesium hydroxide and silver either in combination or occluded, which interfered with the work unless the solution was allowed to stand over night and filtered. This trouble was obviated by using ammonium chloride, only. Ammonium chloride solution was varied from 1 to 8 cc. per 100 cc. The reagent finally adopted as suitable for the precipitation of purines for nephelometric estimation consists of:

Fifty cc. of ammoniacal silver nitrate solution[1] (26.0 g. in a liter, with sufficient ammonium hydroxide—26 to 27 cc. s. g. 0.90—to prevent AgO from settling out).

Eight cc. of ammonium chloride solution (containing 16.5 g. in 100 cc.).

Sufficient ammonium hydroxide to redissolve any silver chloride formed, usually 9 cc. of s. g. 0.90.

An excess of 5 cc. of ammonium hydroxide of s. g. 0.90, and then sufficient water to make 100 cc. volume.

This reagent, if kept in well stoppered bottles, keeps indefinitely.

(2) **Protective Colloid.**—While the reagent was being worked out it was found that suspensions formed with dilute solutions of the purine bases either flocculated at once (uric acid and adenine), or in very few minutes (xanthine, hypoxanthine and guanine). It was therefore necessary to find a medium in which these precipitates remained in suspension long enough for making readings (7 to 30 minutes). In other words a protective colloid was sought which would hold the substances in suspension. After trying Witte's peptone, gelatin, soluble starch and casein, egg albumin in dilute solution was found to serve the purpose satisfactorily.

We know of no previous work in which protective colloids have proved an aid rather than a hindrance in quantitative analysis.

Albumin.[2]—From work with albumin, as a precipitant for nucleic acids, it was assumed that fresh egg white would have the strongest protective action, but the reverse proved true and after experiments with eggs of varying age, it was proven that albumin from eggs several months old (cold-storage eggs) was a stronger protective colloid than albumin from fresh eggs. That old eggs have a stronger protective action is probably

[1] It is best to filter the ammoniacal silver nitrate solution before using from traces of reduced silver or filter the reagent finally.

[2] Commercially dried egg albumins were dried in a few experiments but seemed unsatisfactory

due to the action of ferments. The incubation of fresh eggs improved their protective action, but the results were not as good as with eggs which had been some months in storage. For example, fresh egg albumin in the form of a 2.0% solution kept uric acid in suspension for 4–6 minutes, while fresh eggs that had been incubated at 40° for 4 days were efficient for about 18 minutes, and storage eggs prevented agglutination for more than 60 minutes. Eggs that had been in storage for a number of weeks were very much improved by three to four days' incubation. Longer incubation did no harm, except that it made the separation of the yolk from the white more difficult. Care must be taken to keep the yolk out of the albumin to be used, as much of it tends to produce cloudy solutions.

The isolation and purification of the active principle in egg white both for this purpose, and for use as a precipitant with nucleic acids would be a further advantage in the methods.

A weighed amount of egg white[1] was well shaken with 0.1 *N* acetic acid (0.5 cc. per gram of egg white[2]) and sufficient water was added to make a 2.0% albumin solution, the egg white being calculated as containing 10% of albumin. The solution was then thoroughly mixed and filtered until clear.

The protective action of this albumin solution varied with particular purines and mixtures of purines. Table I give the strength of albumin most suitable in each case and the time before flocculation takes place.

TABLE I.

Substance.	Albumin. %.	Time before flocculation. Min.	Mixtures of equal parts of	Albumin. %.	Time before flocculation. Min.
Uric acid	2.0	30+	Xanthine, Hypoxanthine, Guanine, Adenine	0.0	20+
Xanthine	0.5	15+			
Hypoxanthine	0.05	10			
Guanine	0.05	20+			
Adenine	2.0	8	Xanthine, Hypoxanthine	0 3	30+
			Adenine, Guanine	0.07	30+

Ten cc. 0.01% solution of the purine bases or mixture were used in each instance, with the exception of adenine sulfate, which in that strength gave too strong a precipitate to be held in suspension by 2.0% albumin. Adenine sulfate standard solutions were therefore only 0.005%. To 10 cc. of the solution were always added 10 cc. of the correct albumin solution and 10 cc. of the reagent. It is to be noted that mixtures as a rule re-

[1] For a long series of estimation, it is best to take the whites from many incubated eggs, thoroughly mix, and keep in an ice box as stock solution. In this way the albumin seems to keep indefinitely.

[2] From 5 to 10% excess of acid sometimes gives a clearer solution, but for the purpose of this method, a slight cloud in the albumin is of no consequence as long as it is completely soluble in a little ammonia.

quire much less albumin than the purine bases singly. An ideal protective colloid would be one which would be active with equal efficiency with all the substances. In practical work this inequality does not cause much difficulty, as one uses the smallest amount of colloid necessary for the desired protective action. Under these conditions we have convinced ourselves that precipitation with solutions as weak as 0.0025% is very nearly quantitative. Since the standard is treated in the same way, any deficiency in this direction is eliminated.

Use of Precipitant.—To determine the completeness of precipitation, of each purine by the reagent, nephelometric readings were made, using solutions of different strength, varying from 10.0 to 5.0 mg. per 100 cc. The standard in each case was a 0.01% solution[1] (10 mg. per 100 cc.) of the substance examined, except adenine, which was one-half that strength. The usual care was taken to precipitate the standard and "unknown" under the same conditions. In every instance 10 cc. of the purine solution with 10 cc. of albumin of suitable strength were precipitated with 10 cc. of reagent—prepared according to the preceding directions.

The readings shown in Table II and corresponding curves were obtained.

TABLE II.

	Concentrations in mg. per 100 cc.						
	10.0.	9.0.	8.0.	7.0.	6.0.	5.00.	
Substance.	Readings in millimeters.						Remarks.
Uric acid 1	13.0	14.3	15.7	17.4	19.7	23.1	
Uric acid 2	13.0	14.2	15.9	17.7	[illegible]	23.2	Curve No. 1
Xanthine	12.0	12.8	14.2	16.0	18.0	22.0	
Hypoxanthine	12.1	12.7	14.5	16.0	18.1	[illegible]	
Guanine hydrochloride	12.1	12.7	14.3	16.3	18.9	21.8	
Mixtures of equal parts of							
Xanthine and hypoxanthine 1	12.0	12.6	13.9	15.6	18.1	21.1	
Xanthine and hypoxanthine 2	12.1	12.9	13.8	15.0	16.9	20.1	Curve No. 3
Adenine and guanine 1	12.0	12.8	14.3	16.1	19.0	22.9	
Adenine and guanine 2	12.1	12.8	14.1	16.0	18.9	22.8	Curve No. 4
Adenine, guanine, xanthine and hypoxanthine 1	13.0	13.9	15.3	16.9	18.9	20.7	
Adenine, guanine, xanthine and hypoxanthine 2	13.0	14.1	15.6	17.2	19.3	21.7	Curve No. 5
	5.00.	4.50.	4.00.	3.50.	3.00.	2.50.	
Adenine sulfate	12.1	13.0	14.6	16.4	19.2	23.1	Curve No. 2

These readings represent the average of readings (usually only 2) on two to three solutions of each concentration. Although all of the curves are below the hypothetical and therefore nearly quantitative, it will be observed that most of the purine curves tend to approach the hypothetical as the ratio of the solutions becomes smaller, while with uric acid and the

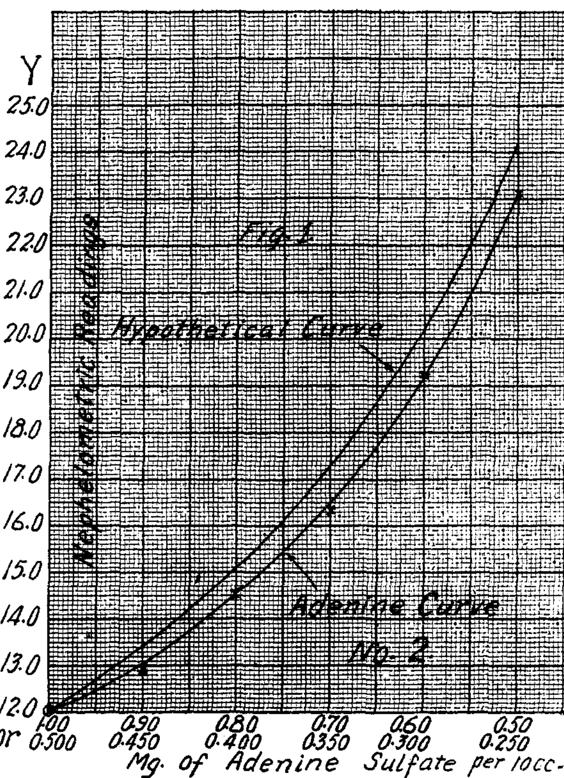
[1] In this paper throughout, the weights of substances refer with adenine to the sulfate, and guanine to the hydrochloride; the others to the free bases.

mixture of four purine bases this is not the case. This is best shown by calculating the nephelometric "constant"[1] at different points of the curve.

TABLE III.—NEPHELOMETRIC CONSTANTS.

Substance..........	Adenine	Guanine	Xanthine	Hypoxanthine	Purine mixt.	Uric acid
k at ratio of soln. 0.80	0.14	0.22	0.25	0.22	0.16	0.09
k at ratio of soln. 0.50	0.04	0.10	0.09	0.13	0.16	0.11

These facts are undoubtedly due to either the influence of the protective colloid or the solubility of the silver complexes, showing that uric acid precipitation follows the nephelometric curve accurately while the single purine bases show in the weaker solutions a distinct solubility or shift of equilibrium, due to the colloid. This was also demonstrated by adding a large excess of albumin. Thus, the reaction with the purine bases was very much weakened, whereas with uric acid, the reaction was unaffected.

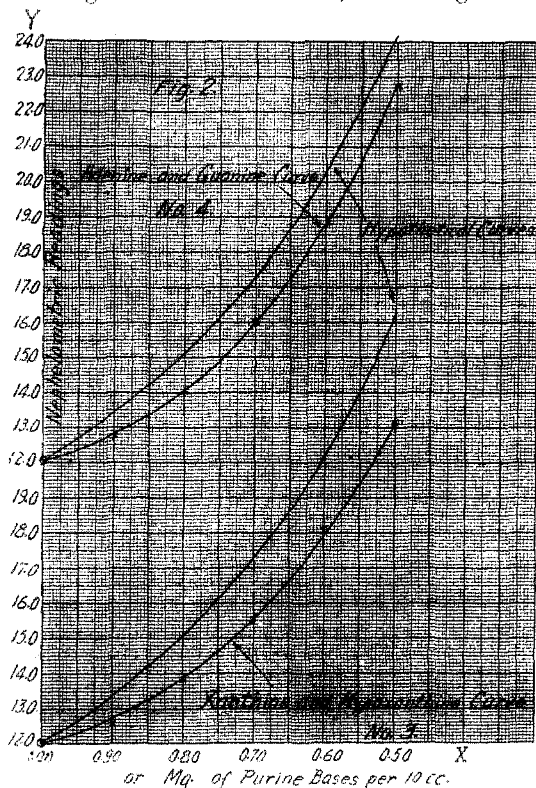


B. Separation of Uric Acid from Purine Bases. — As is well known, on adding Salkowski's reagent one obtains uric acid and the other purine bases as silver complexes, and although there are slight differences in solubility and the speed of reaction between uric acid and the other purine complexes, yet the differences are not enough to serve as a basis for their quantitative separation. The alternative was the elimination of either of these substances from reacting with the reagent. After trying several schemes, the oxidation of uric acid with a suspension of manganese dioxide, seemed very promising. This reagent is used in the Krüger-Schmidt method for a similar purpose. *On trying it as recommended by the authors in an acid solution.*

[1] Kober, *J. Biol. Chem.*, **13**, 491 (1913); also Kober and Egerer, THIS JOURNAL, 37, 2373 (1915).

it was found that, even without boiling, not only uric acid was oxidized but some of the other purines as well. On reducing the acidity, the difference between the action on uric acid and other purine bases became sharper, and quantitative.

Oxidatiyn Reagents.—The details as to the modified reagents and the separation are as follows: 25 g. of potassium permanganate are dissolved in 500 cc. of water, which, on bringing to boiling, are treated with 95% alcohol until all of the permanganate color has disappeared. After filtering and washing with distilled water, the manganese dioxide is suspended in 500 cc. of water. The reagent should give as a fine suspension indefinitely.



The solution containing purine bases and uric acid should be neutralized and to 10 cc. of solution containing about 0.001 g. of the substance, 0.1 cc. of 6% ammonium hydroxide (one part of ammonia s. g. 0.90 and three parts water) and 1 cc. of suspension of manganese dioxide are added. After 3 to 4 minutes' standing with frequent shaking, the mixture is filtered through paper of good grade until clear, and the nephelometric estimation of the purine bases in the filtrate is made. *The advantage of the reagent is that it accomplishes the oxidation of uric acid, without introducing any appreciable change in the solution, or leaving any interfering reagents, owing to its insolubility.* If the solution to be tested does not contain any electrolyte, it is well to add 1 cc. of a 4% solution of sodium acetate, with every cubic centimeter of the reagent, to flocculate the manganese dioxide and thereby increase the efficiency and speed of filtering. Using the reagent as modified, the following results were obtained with the different purines and with uric acid.

Action of Oxidizing Reagent on the Different Purines.—Solutions of the

purines were shaken with the reagent ten minutes, a mixture of the purine bases for thirty minutes, while uric acid oxidation, as Table IV shows, was complete in two minutes. The first column of figures gives the readings of the nephelometer with the solutions after treating with reagent, whereas the second column gives the results without the oxidation mixture, but after making the corresponding dilutions, etc.

TABLE IV.

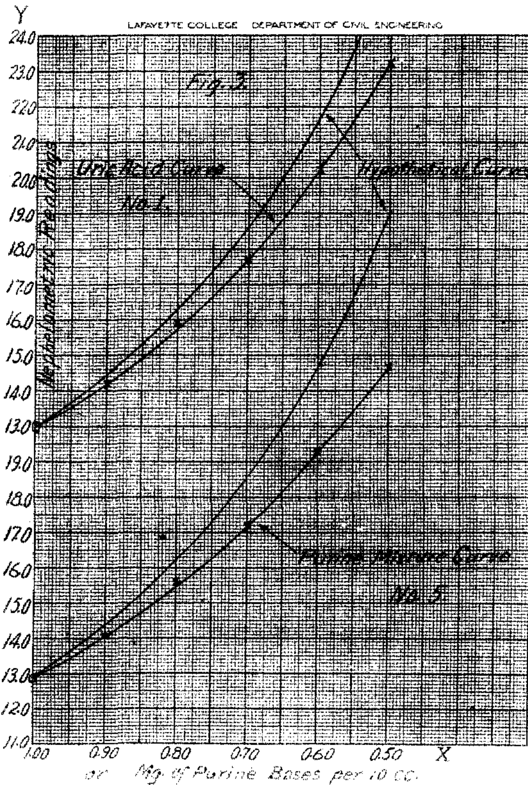
Substance. Soln. cont. 10 mg. per 100 cc.	Nephelometric readings. After shaking with manganese dioxide.	Nephelometric readings. Without shaking with manganese dioxide.	Substance. Soln. cont. 10 mg. per 100 cc.	Nephelometric readings. After shaking with manganese dioxide.	Nephelometric readings. Without shaking with manganese dioxide.
Xanthine	12.0	11.9	Mixtures of equal	12.3	11.9
	12.0	12.0	parts of xanthine	12.1	11.9
Av.,	12.0	12.0	hypoxanthine,	12.1	12.0
			adenine, and	12.1	11.9
Hypoxanthine	14.2	14.0	guanine	12.2	....
	14.0	13.9		12.2	....
	14.0	14.0			
Av.,	14.1	14.0	Av.,	12.2	11.9
			Uric acid.		
Adenine	12.0	12.4	0.5 minute	6.6	17.0
	12.4	12.3	1.0 minute	5.5	17.0
	12.2	12.0	2.0 minutes	3.1	17.0
Av.,	12.2	12.2	5.0 minutes	2.9	17.0
			5.0 minutes	3.1	17.0
Guanine	10.8	10.8	Reagents and water	3.2	17.0
	11.0	10.6			
	11.0	10.6			
Av.,	10.9	10.7			

The results show that the amount of purine bases oxidized was inappreciable, except with guanine, which showed a deficiency of about 2%. On treating the mixture of the purine bases for thirty minutes, the total loss was less than 2%, and it is therefore obvious that three minutes' shaking as prescribed will have no appreciable effect on the four purine bases, while the destruction of uric is quantitative.

As the amount of protective colloid necessary for uric acid is much greater than for the other purine bases it was expected that only a slight part of the other purine bases would be precipitated along with the uric acid, and that a control precipitation with the same amount of albumin would be necessary, after the uric acid had been eliminated. On experimentation it was found, however, that when uric acid was present, the other purine bases were thrown down with it quantitatively, notwithstanding the fact that the albumin was about ten times too strong for the purine bases alone.

Therefore, to determine uric acid and the other purine bases separately,

it is necessary (1) *to determine the total purine content, using enough albumin to prevent flocculation—that required for an equivalent amount of uric acid,* (2) *to estimate the purine bases separately, without uric acid, using the smallest amount of albumin that is necessary to give the desired protective action. The difference between* (1) *and* (2) *will give the uric acid content.*



At first sight this uncertainty concerning the amount of protective colloid may seem both troublesome and inaccurate, but in reality it is very simple. For definite applications, *i. e.*, for estimating these substances in urine, blood, and ferment work, definite amounts of albumin can be prescribed, for other and new work, albumin of two or three different strengths may be required, but the making of these solutions from a stock solution (2.0%) and their testing out, will only be a matter of from 15 to 20 minutes.

The correct amount of albumin can be determined very simply by adding three or four different strengths of albumin to the solution containing the uric acid and purine bases, and comparing these, after precipitation, with a standard in the nephelometer. The required strength will be known by the strongest cloud, *i. e.*, the lowest reading. An example will make this clear. When compared to a standard, 5 cc. of a purine mixture (10.0 mg. per 100 cc.) and 10 cc. of reagent gave, with 10 cc. 0.2% albumin, 26.4 mm.; 10 cc. 0.1% albumin, 18.6 mm.; 10 cc. 0.075% albumin, 19.8 mm.; 10 cc. 0.050% albumin, 21.5 mm. In this case 10 cc. of 0.1% of albumin was the necessary amount, while weaker albumin according to the readings allowed some flocculation.

To show that the precipitation is quantitative under these conditions the following results of Table V will suffice:

TABLE V.

Soln. cont. in 100 cc. Purine bases. Mg.	Soln. cont. in 100 cc. Uric acid. Mg.	Amt. for anal. Cc.	Amt. water added. Cc.	10 cc. albumin used. %.	Reading w. 10 cc. reagent. Mm.	Reading of standard. 1 S. Mm.	Reading of standard. 1/2 S. Mm.	Ratio of sol.	Total purine bases found in 100 cc. Mg.	Purine bases (net) in 100 cc. Mg.	Uric acid found in 100 cc. Mg.
00.0	10.0	5.00	0.00	1.0	17.5	....	....	....	....	....	....
10.0	10.0	2.50	2.50	1.0	18.5	17.5	....	0.938	18.7	9.2	9.5
7.5	10.0	2.50	2.50	1.0	19.7	17.5	....	0.873	17.5	7.0	10.5
5.0	10.0	2.50	2.50	1.0	16.0	12.4	....	0.748	15.0	4.9	10.1
2.5	10.0	2.50	2.50	1.0	14.3	....	17.0	1.21	12.1	2.3	9.8
10.0	7.5	2.50	2.50	0.5	14.5	12.8	....	0.873	17.5	10.0	7.5
10.0	5.0	2.50	2.50	0.25	17.1	12.8	....	0.720	14.4	10.0	(4.4)
10.0	2.5	2.50	2.50	0.25	19.2	12.8	....	0.628	12.6	10.0	2.6
9.2	0.0	5.00	....	0.10	18.9	17.5	....	0.916	9.2	9.2	....
7.0	0.0	5.00	....	0.05	24.0	17.5	....	0.696	7.0	7.0	....
4.6	0.0	5.00	....	0.05	22.8	12.4	....	0.485	4.8	4.8	....
2.3	0.0	5.00	....	0.05	32.5	....	17.0	0.463	2.3	2.3	....
0.0	5.0	5.00	....	1.0	17.0	....	....	....	....	....	....

These solutions were not treated with manganese dioxide, in order not to introduce any new factors. The first solution given in the table was used as a standard, and the last solution in the table as one-half standard. The results are as accurate as one could expect, when it is considered that an average nephelometric constant of 0.10 was used throughout. Since the albumin varied from day to day and the value for k differs for purines, an appreciable error may have been introduced. As soon as a standard source of albumin is obtained, the percentage accuracy can undoubtedly be considerably increased.

The results of Table VI were obtained by eliminating the uric acid, using the oxidation mixture previously described. Twenty cc. of a mixture of purine bases and uric acid, 2 cc. of sodium acetate and ammonia solution, and 2 cc. of a suspension of manganese dioxide, after shaking 3 to 4 minutes, were filtered and a nephelometric estimation made on the filtrate, as well as on the solution before treatment with oxidation reagents.

TABLE VI.

Soln. cont. in 100 cc. Purine bases. Mg.	Soln. cont. in 100 cc. Uric acid. Mg.	Reading of soln. Mm. I.	Reading of soln. Mm. II.	Reading of standard 0.01% uric acid. Mm. I.	Reading of standard 0.01% uric acid. Mm. II.	Ratio of soln. to standard. I.	Ratio of soln. to standard. II.	Total purine bases found in 100 cc. Mg. I.	Total purine bases found in 100 cc. Mg. II.	Purine bases (net) in 100 cc. Mg. I.	Purine bases (net) in 100 cc. Mg. II.	Uric acid found in 100 cc. Mg. I.	Uric acid found in 100 cc. Mg. II.
A													
10.0	2.5	14.5	14.7	18.0	18.0	1.26	1.25	12.6	12.5	....	....	...	...
Filtrate		20.5	21.0	18.0	18.0	0.863	0.838	....	....	10.4	10.1	2.2	2.4
B													
10.0	10.0	18.0	19.4	18.0	19.0	1.00	0.952	20.0	19.1	....	....	...	...
Filtrate		21.0	23.8	18.0	19.0	0.843	0 774	....	....	10.1	9.3	9.9	9.8
C													
2.9	10.0	14.5		18.0		1.26		12.6		....		....	
Filtrate		23.0		18.0		0.55		....		3.0		9.6	

In Group *A* the estimation was made by taking for the total purines, 5.00 cc. of the solution, 10 cc. of 0.25% albumin solution, and 10 cc. of reagent; for the filtrates,[1] 5.00 cc. of the solution, 10 cc. of 0.15% albumin and 10 cc. of reagent. In Group *B* the estimation was made by taking for the total purines, 2.50 cc. of solution, 2.50 cc. of water, 10 cc. of 1.0% albumin, and 10 cc. reagent; for the filtrate, 5.00 cc. of solution, 10 cc. 0.15% albumin and 10 cc. of reagent. In Group *C* the estimation was made by taking for the total purines, 2.50 cc. of the solution, 2.50 cc. of water, 10 cc. of 1.0% albumin and 10 cc. of reagent; for the filtrate, 5.00 cc. of the solution, 10 cc. of 0.05% albumin and 10 cc. of reagent, using one-half standard.

III. Directions.

As a standard cloud, uric acid might well be adopted, owing to the purity of the commercial product; and for this purpose we give below the nephelometric value of the different purines (as free bases) in terms of uric acid.

0.100 g. uric acid = 0.031 g. adenine; 0.079 g. guanine; 0.106 g. hypoxanthine; 0.104 g. xanthine; 0.071 g. purine mixture; 0.067 g. adenine and guanine; 0.104 g. xanthine and hypoxanthine.

As it is a safe principle in nephelometry to use as a standard a known amount of the same substance to be determined, the directions for making solutions of each of the purines follow:

One-tenth gram of finely powdered *uric acid* is weighed carefully in a 50 cc. beaker, to which is added 25–30 cc. saturated lithium carbonate[2] (about 1.0%), stirring and powdering with the flat end of a stirring rod until dissolved. The solution is then transferred to a 100 cc. graduated flask with a little water and made up to the mark with 0.2% tricresol.[3]

Xanthine and *hypoxanthine* solutions are made in the same way, except the commercial xanthine which seems to be slightly impure and therefore must be standardized.

[1] As the filtrates were diluted with 4 cc. of water, from the sodium acetate and ammonia and the suspension of manganese dioxide, the ratios of the filtrates were divided in each case by the factor 20/24 or 0.833. Owing to the presence of the salt solution (sodium acetate) it was necessary, as may be observed in the relatively strong filtrates, to increase the strength of albumin from 0.10% to 0.15%.

[2] S. R. Benedict, *J. Biol. Chem.*, 20, 619–27 (1915), uses a phosphate mixture, in connection with acetic acid, which permits the stock solution to be preserved for at least a month. This seems to be an advantage over previous methods of dissolving uric acid.

[3] S. S. Graves and P. A. Kober, This Journal, 36, 751 (1914). As a diluting liquid for organic substances liable to bacterial decomposition, we use an aqueous solution of tricresol (usually 0.2%). This is a much more powerful germicide than chloroform or toluene and causes no trouble in volumetric measurement. Both chloroform and toluene, as is well known, make measuring apparatus unclean and oily, so that they are unsuitable for accurate work.

For *adenine sulfate* and *guanine hydrochloride*, 25 to 30 cc. of hydrochloric acid (1 vol. of conc. made up to 100 vols. with water) are used instead of lithium carbonate. Warming the solution slightly will hasten solution, but if the substance is sufficiently powdered, this is not necessary.

Directions for making reagents have already been given and in the application of the method to blood and urine, specific details will be given.

General Precautions.—As egg albumin solutions have a tendency to form shreds, it is necessary to avoid their formation or to remove them when formed by careful filtration. The chief cause of their formation seems to be surface tension and therefore any increase of surface through the formation of air bubbles or shaking unnecessarily is to be avoided. In pipeting it is well to allow the solution to drain down the side of the containing vessel or better still to dip the draining pipet into the liquid. Mixing of the solution can be accomplished by gentle rotation. In case filtering is necessary, a long, wide-stem funnel is the most suitable to prevent air bubbles or the formation of drops.

IV. Applications.

A. Urine.—When the reagent is added to urine the complexes of the purines, including uric acid, are formed at once and agglutinate almost immediately (in 1–4 minutes) even when the urine solution is moderately dilute and contains the usual amount of protective colloid. This is doubtless due to the coagulative action of the inorganic salts in the urine. There are two ways to circumvent this difficulty: (1) to offset this action by further dilution and increased amount of albumin, or (2) to remove the interfering salts. This second possibility seems theoretically more desirable. Attempts to throw out calcium as oxalate and magnesium as a carbonate did not produce a solution satisfactory for nephelometric work, probably because other salts are present which cannot be directly precipitated. The final solution of the problem lay, as Folin and Dennis found in their work, in precipitating the purines with the uric acid as complexes and, after centrifuging for a minute or two, pouring off the supernatent liquid containing most of the interfering substances, dissolving the complexes and, after suitably diluting, reprecipitating them. This procedure gives complete control of the precipitating medium and is therefore conducive to accuracy.

Folin and Dennis redissolve uric acid from similar precipitation by means of hydrogen sulfide and hydrochloric acid, boiling off the excess of sulfide. The total elimination of silver at this point is not necessary in our method as our reagent contains silver. Therefore hydrochloric acid alone is sufficient to set free the purine bases which on heating[1] are all redissolved, but some suspended silver chloride remains even after filtering.

[1] Without boiling 98% or more of the purines redissolve, if the solution is not less than 35 cc. for 0.005 g. of purine bases.

In the estimation of total purine bases this silver chloride is of no consequence, but in the estimation of the purines excluding uric acid, the medium for oxidation must be alkaline[1]—in which case the silver chloride again forms complexes and in the absence of protective will be filtered off with the oxidation reagent, manganese dioxide. By using a solution of lithium carbonate saturated with hydrogen sulfide, to make the oxidation medium alkaline as given in the general directions, silver chloride is converted into silver sulfide and is completely filtered off with the manganese dioxide. Not only is the silver removed from the sphere of action by this operation, but the uric acid is completely oxidized, and after filtration it will be found that no trace of sulfide remains in the solution, the excess having been removed by the manganese dioxide or changed into some other insoluble substance, as, for example, sulfur.

The removal of alkaline sulfide by a suspension of manganese dioxide seems, as far as we are aware, not to have been used in practice before, and we can strongly recommend it for this purpose. Its advantages over the boiling technic are obvious.

Since the purine reagent is strongly ammoniacal, some inorganic bases of urine, as calcium and magnesium, are thrown down with the purine complexes and find their way into the final solution, but owing to the presence of protective colloid and their small concentration they do not, as some following experiments show, appreciably affect the estimation of the total purines. In the estimation of purine bases, the alkaline medium necessary for the oxidation of the uric acid also removes these inorganic bases, thus no extra precautions for this purpose are needed.

Technic.—Five cc. of urine are put into a 15 cc. graduated centrifuge tube, 5 cc. of reagent added and the solution centrifuged 1 to 3 minutes—the supernatant liquid is then poured off, 10 cc. of hydrochloric acid (1-100) are added to the residue and the tube placed into boiling water for 2 to 5 minutes. The tube is then cooled and the volume made up to 15 cc. The solution is then centrifuged, or allowed to settle, to remove the larger part of the silver chloride and the liquid drawn off with a pipet—Solution A.

I. Total Purines.—To 3 cc. of Solution A are added, in the order named, 7 cc. of water, 10 cc. of 2% albumin, 0.05 to 0.10 cc. of strong ammonia (s. g. 0.90) to dissolve any silver chloride and 10 cc. of reagent. The suspension is compared in the nephelometer with a standard suspension made by 5 cc. of 0.01% uric acid solution, 5 cc. of water, 10 cc. of 2% albumin and 10 cc. of reagent. This standard is of satisfactory strength for the comparison of most normal urines, but may be made stronger if the urine contains large quantities of purine bases.

II. Purine Bases (excluding Uric Acid).—To 10 cc. of Solution A are

[1] The acid or neutral medium permits oxidation of some of the purines.

added, in the order named, 2 cc. of 1% lithium carbonate solution saturated with H_2S, 2 cc. of lithium carbonate, and 2 cc. of suspended manganese dioxide (see general directions).

This solution, B, allowed to stand for 3 to 5 minutes with occasional shaking, is then filtered until clear.[1] Ten cc. of this solution, B, precipitated by 5 cc. of reagent, are then compared with the standard solution, as described above.

From the fact that the purine bases do not agglutinate in 8 to 10 min. without the addition of albumin, it is assumed that sufficient protective colloid is inherent in the urine to keep them in suspension—since only 5 cc. of 0.2% albumin supply the protection necessary for pure solutions of much greater strength.

III. **Uric Acid.**—The difference between the total purines and the purine bases is equivalent to the uric acid.

The two determinations are made from 5 cc. of urine, a quantity easily obtainable in normal urinary work. If desirable, however, a smaller quantity will suffice provided the reagents are used in the most concentrated form or the standard solutions made weaker.

Calculations of Results.—The readings of the standard and the unknown solutions are made according to the usual nephelometric directions[2] and are then substituted for s and y in the formula $x = \frac{s + sk + \sqrt{(s + sk)^2 - 4sky}}{2y}$ and the value of k being taken as 0.10. The equation is solved for x, the ratio of the solutions, by means of which the amount of substance in grams may readily be calculated.[3] The following figures were obtained and are examples of many similar experiments:

TABLE VII (GRAMS PER LITER).

Urine.	Total purines.	Purine bases.	Nephelometric method. Uric acid (by dif.).	Method of Folin and Dennis. Uric acid.
Normal I (human)	0.745	0.089	0.656	0.584
Normal I (human)	0.720	0.086	0.634	0.595
Normal I (human)	0.720	0.083	0.637	0.560
Normal I (human)	0.745	0.086	0.659	0.562
Normal I (human)	0.730	0.085	0.645	.....
Av.,	0.730	0.086	0.646	0.578

These results show that in our hands the Folin and Dennis colorimetric method gave from 8 to 10% lower values for uric acid than the nephelometric. We are not ready to decide which of these two methods is nearer

[1] Usually two filtrations are sufficient owing to the presence of salts in the urine but in pure solutions it is well to add some electrolyte, *e. g.*, 1 cc. of 4% solution of sodium acetate.

[2] Kober, *J. Biol. Chem.*, **13**, 491 (1913).

[3] The slide rule is a great time saver in these calculations.

the truth, but the following points have been observed: (1) that in pure solutions both methods seem equally accurate; (2) in practical urinary work, as indicated above, the nephelometric method may give results which are slightly too high, owing to the presence of some insoluble salts, but the error, as experiments below show, cannot be more than 2% and is probably very much smaller. The Folin and Dennis colorimetric method, however, in our hands and others, shows the possibility that it gives results which are too low. As Folin and Dennis have observed, their color fades on standing, but contrary to their statement, the color produced by the urine unknown, fades very much more rapidly than the color of the standard. In work done so far on urines with the colorimetric method it has been hard to get two readings which agree, the second reading always being higher than the first; thus, for example, a uric acid estimation in urine made according to their directions gave on the first reading 12.0 mm., on the second reading made as soon as possible after the first, 12.4; two minutes after the second reading, a third estimation gave 14.2 and the 4th reading made after the lapse of another five minutes, gave 17 mm.; thus in about ten minutes almost 50% of the color, as compared to the standard had faded. We are not sure that our technic was faultless but the estimations were made following the directions as closely as possible. If this fading,[1] which has been noted by others, follows the law of mass action, and it probably does, the fading during the first minute or two must be very appreciable and may account for the difference between the colorimetric and nephelometric methods.

Experiments to Show the Effect of Salts in Urine on the Total Purine Estimation.—I. Urine solutions, prepared according to the preceding directions, show none or a very slight cloud when treated with 2% albumin and reagent made without silver (other constituents being the same) in amounts prescribed for the estimation of total purines. It is to be remembered that the ammonia in the reagent is more than ample to dissolve any insoluble compound of silver with a urinary substance, such as phosphate, chloride, etc., except purine complexes.

II. Urine solutions, prepared according to the preceding directions, upon *oxidation in acid medium* which destroys uric acid and most of the purines after the addition of albumin and normal reagents, showed only a trace of cloud as compared with a standard containing only albumin and reagent.

III. To the filtrates from oxidized urine solutions (see II) were added known amounts of uric acid (0.0005 g.) which were recovered with a slight increase (less than 2%).

[1] S. R. Benedict, *J. Biol. Chem.*, 20, 619–27 (1915), obtains about 18% more color using KCN a a solvent for the silver complex. Benedict thinks this is due to the marked diminution in the rate of fading of the color. As the fading has not been entirely eliminated, one would expect the results to be still somewhat too low.

Readings found	15.9	15.7	15.8	16.2
Readings expected	16.2	16.2	16.2	16.2

These experiments show that in urinary medium, from which all of the uric acid and most of the purine bases had been oxidized and to which a known amount of uric acid had been added, we were able to obtain very nearly quantitative figures. Making no allowance for traces of unoxidized purines, the error is about 2%, but we are not certain that the acid medium permitted the oxidation of all of the purines, and if allowance is made for this factor, then the error in our nephelometric method is very much smaller.

B. Blood.—In the estimation of purine bases, as with most constituents of the blood, it is necessary to remove the bulk of the proteins. For the quantitative precipitation of the coagulable proteins 5 volumes of 3% sulfosalicylic acid[1] have proved very satisfactory—but, owing to the development of the yellow color[2] in the sulfosalicylic acid filtrate, when made alkaline, by the ammonia of the purine reagent, the direct estimation of the purines in the blood filtrates with this protein precipitant was found nephelometrically unsuitable.

Greenwald's[3] discovery of trichloroacetic acid as a reagent for the removal of blood proteins, is of great service for the estimation of purines in blood. It gives filtrates which are clear and free from proteins, and enables us to estimate the purine bases in them directly, after centrifuging off the calcium as oxalate.

Our intention was to apply our reagent to the estimation of purines in different kinds of blood, normal and pathological, but two obstacles have prevented us. (1) For some time we lacked a suitable nephelometer[4]—one which held at least 100 mm. of liquid. (2) After getting such an instrument we now lack the opportunity to finish the work on blood. We are therefore compelled to content ourselves with an outline of our blood procedure and a few qualitative experiments.

As sheep blood contains no appreciable amount of free purine bases, it is well adapted for experimental work in developing this method. There-

[1] Kober, THIS JOURNAL, 35, 290, 1585 (1913).

[2] Probably a nitrate reaction.

[3] *J. Biol. Chem.*, trichloroacetic acid, while very efficient as a precipitant for strong solutions of protein, is, however, not so suitable for nephelometric estimation of proteins.

[4] As the amount of purine bases in some kinds of blood is extremely small, it was necessary to have about 100 mm. of liquid under observation. The instruments used by us heretofore allowed not more than 40 mm. of liquid. We were, therefore, compelled to design an instrument of larger capacity, and because of the difficulty of getting suitable optical glass, owing to the European war, the whole problem was delayed.

The new instrument, having many improvements over the Duboscq, such as a Lummer-Brodhun eyepiece, adjustable vernier, black glass plungers and other new devices, will be described in a separate paper, and can be obtained from Lenz and Naumann, 17 Madison Ave., New York City, for about 1/8 the original price, $36.00.

fore, the quantitative recovery of known amounts of purine bases added to the blood, will suffice to show the applicability of the method.

The following (Table VIII) qualitative preliminary experiments with the method were made with sheep blood, April 27, 1915:

TABLE VIII.

No.	Blood taken. Cc.	Purine bases added (mixture). Mg.	Trichloroacetic acid added for removal of protein. Cc. of 5%.	Filtrate taken for test. Cc.	Precipitate[1] found (Cloud).
(1)	20.0	0.25	100	80	+ + +
(2)	20.0	0.25	100	80	+ + +
(3)	20 0	0.25	100	80	+ + +
(4)	20.0	0.25	100	80	+ + +
(5)	20.0	....	100	80	....
(6)	20.0	....	100	80	....
(7)	H_2O	0.25	100 H_2O	80	+ + +

These experiments showed that the conditions for the direct nephelometric estimation of uric acid and other purine bases in blood, after the removal of the blood protein with Greenwald's reagent and of calcium with ammonium oxalate, to be very favorable.

V. Summary.

I. Salkowski's reagent for purine bases has been modified to meet nephelometric conditions, and it is shown that the reagent will precipitate xanthine, hypoxanthine, guanine, adenine and uric acid quantitatively in very dilute solutions (0.0002%).

II. The use of a protective colloid has been introduced—clear solutions of egg albumin—for the purpose of keeping the precipitates in suspension so that they may be estimated nephelometrically.

III. It has been shown that the suspension of manganese dioxide *in an alkaline, instead of in an acid medium*, as has been used heretofore, will oxidize uric acid completely in 1 to 3 minutes, and leave the other purines practically unattacked.

IV. It has been shown that manganese dioxide is an excellent reagent for the removal of alkaline sulfides from solution without the usual boiling technic, and without introducing any interfering reagents in the solution.

V. It has been shown that uric acid, and other purine bases in urine, may be quickly and fairly accurately estimated with the nephelometer.

VI. It has been shown that 5 volumes of 3% sulfosalicylic acid is an excellent reagent for removing all coagulable protein from blood. By

[1] In each case, to 80 cc. of clear filtrate were added 5 cc. of oxalic acid (saturated) solution and 5 cc. of strong ammonia (s. g. 0.90). After centrifuging off the precipitate of oxalates, and taking an aliquot portion (80 cc.), 5 cc. of a strong solution of ammoniacal silver nitrate were added (26.0 g. silver nitrate, 50 cc. of water, 66.0 cc. of ammonia (s. g. 0.90). This reagent contains no chlorides, as blood chlorides are sufficient to prevent the reduction of silver.

centrifuging 1 to 2 minutes after the precipitation with sulfosalicylic acid, the great bulk of protein can be removed, and if the supernatant liquid is shaken with a little talcum to cause agglutination of any remaining suspended protein, a perfectly clear filtrate can be obtained in 5 to 10 minutes without boiling.

VII. It has been shown that 5 volumes of Greenwald's reagent (5%) for the removal of blood protein is equally efficient, and that it has the advantage that it forms with ammonia no yellow color.

VIII. An outline of the technic for the estimation of purine bases in blood is given.

NEW BOOKS.

Surface Tension and Surface Energy and their Influence on Chemical Phenomena. By R. S. Willows and E. Hatschek. Pp. viii + 80; 17 illustrations. P. Blakiston's Son & Co., 1915. Price, $1.00 net.

This book, which is based upon a course of lectures delivered as a continuation of another set on colloidal chemistry, has for its object the consideration of the theory of those surface interactions which are evidenced experimentally by the phenomenon of adsorption. In the main, the treatment is excellent, and the book will be found an especially valuable résumé of the subject by those who are interested in the chemistry of colloids, whether from the biological or from the purely chemical side.

Unfortunately, by an oversight, it is made to appear on page 5 that the surface tension, itself of a liquid, is related to its critical temperature in the same way as Ramsay and Shields have found the more complicated function—surface tension times the two-thirds power of molecular weight over density—to be. This was proven long since to be incorrect, and in fact was the reason why the classical work of Ramsay and Shields was undertaken. J. L. R. Morgan.

The Electrical Nature of Matter and Radioactivity. By Harry C. Jones. Pp. viii + 212. Third Edition. Completely Revised. New York: D. Van Nostrand Company, 1915. Price, $2.00 net.

The viewpoint of this book is essentially that of about 1905, despite the two revisions which the work has undergone. The important work of Fajans on the electrochemical properties of the radioelements, and Soddy's rules for the effect of alpha and beta ray changes on the valence of the radioelements, have thrown much light upon the chemical nature of these elements, have made it possible to fit them into the periodic table and have given us a new notion in regard to certain elements—isotopes, as Soddy has called them. For example, thorium and ionium are isotopes, as are also radium B, radium D, radium G, and lead. These elements, of different atomic weight, are chemically and spectroscopically