CIRCULAR PAPER CHROMATOGRAPHY

Part V :—The separation, identification and quantitative determination of urea with application to human urine.

BY MISS TARA RAO AND K. V. GIRI

(*Department of Bio-chemistry, Indian Institute of Science, Bangalore 3.*)

Summary

A method for the separation, identification and quantitative determination of urea by means of circular paper chromatography is described. Samples to be tested are chromatographed, the position of urea on the chromatogram is indicated by spraying one half of the paper with phenol-hypochlorite reagent. The urea band on the unsprayed half of the chromatogram is cut out, eluted with water and the urea is determined colorimetrically with α-isonitrosopropiophenone reagent.

The method has been applied to the determination of urea in urine. It is obvious that the method in principle can also be used for the analysis of other constituents in urine.

Urea is one of the important nitrogenous constituents excreted in urine as an end product of protein metabolism, and is of considerable importance in the study of urine composition. Urea is usually determined by converting it into ammonia with the enzyme urease and estimating the ammonia nitrogen by usual methods. Another method is to convert urea by urease to ammonium carbonate and the carbon dioxide formed from the latter is determined manometrically. There are certain inherent difficulties for accurate determination of urea by these methods as the impurities in the enzyme preparations used and the presence of other constituents present in biological fluids such as pyruvate which also liberate Co_2 by the action of Cocarboxylase present in enzyme preparations interfere with the method of estimation and as such these methods have to be applied with care and attention to these details. A less accurate method which is used in renal function tests is the one based on the conversion of urea to nitrogen by sodium hypobromite and estimating the nitrogen manometrically. Archibald (1945) has described a simple colorimetric method for the determination of urea based on the red colour formed when urea is heated in acid with α-isonitrosopropiophenone. The application of this method to urine is limited to cases in which approximate values only are required.

Further, the presence of proteins and allantoin if present in high concentration interfere with the colorimetric determination of urea. This method is not recommended by the author for exact determination of the nitrogen distribution among urinary constituents.

Paper chromatographic method possesses certain advanages over the usual methods of analysis for the quantitative determination of biological constituents, as they can be separated from other interfering substances. Chromatographic methods are, therefore, preferred to other chemical methods, as the errors due to the interference of other substances on the quantitative determination of the substance under test are considerably minimised. In view of the successful application of the circular paper chromatographic technique to the separation of amino acids (Giri *et al.*, 1952[a]), organic acids (Giri *et al.*, 1953), carbohydrates (Krishnamurthy and Venkatasubramaniam, 1952), inorganic substances (Airan, 1952), and the quantitative analysis of amino acids (Giri *et al.*, 1952[b]) in this laboratory, investigations on the application of this technique, to the separation and quantitative determination of constituents occurring in urine and blood were undertaken. A brief description of this technique as applied to the study of the amino acid patterns of blood has been reported (Giri *et al.*, 1952[c]). The present report outlines a simple method for the separation, identification and quantitative determination of urea in urine by circular paper chromatographic technique.

Experimental

Reagents: 1. *n-Butanol-acetic acid-water (40 : 10 : 50)* (developing solvent mixture). The preparation of the solvent mixture has been described in Part I of the series (Giri and Rao, 1952).

2. *Colour developing reagents for urea:* (phenol-hypochlorite).

(*a*) 5 per cent phenol in absolute alcohol.

(*b*) Sodium hypochlorite solution—prepared by passing chlorine gas into a 10-15% solution of sodium hydroxide for 2 hours till the solution turned pale green and stored in the refrigerator.

3. *Reagents for quantitative colorimetric determination of urea:*

(*a*) *Sulphuric-phosphoric acid-water mixture.*—Prepared by mix-

ing 1 volume of concentrated sulphuric acid, 3 volumes of syrupy phosphoric acid and 1 volume of water.

(*b*) α-*isonitrosopropiophenone* ($C_6H_5.CO.CNOH.CH_3$).—3 gms. of the reagent was dissolved in 100 c.c. of alcohol.

This reagent was prepared from propiophenone and butylnitrite by the method of Hartung and Munch (1929). This was stored in the refrigerator.

4. *Standard Urea solution.*—400 mg. of urea (Merck) dissolved in 100 c.c. of distilled water and stored in the refrigerator.

Procedure.—The chromatography was carried out according to the method described by Giri and Rao (1952). Whatman No. I circular filter papers (18.5 cm. in diam.) were used. A circle of 2 cm. radius was drawn with a pencil at the centre of the filter paper and a known volume (20-120μ l) of test solution containing urea was spotted on the circumference of the circle, diametrically opposite to each other. For spotting larger amounts of the test solution, the spot of the solution added before, was dried before another aliquot was spotted, so that the area of the spot was confined to less than 1 cm. diameter circle. The spots were dried at room temperature and the chromatogram was developed after inserting the 'wick', with n-butanol-acetic acid-water as the solvent. The solvent front was allowed to travel to a distance of 8.5 cm. from the centre. After marking the solvent boundary with a pencil the paper was dried and cut into two semi-circles taking care to see that each half contained the urea band. One half of the filter paper was used to locate the position of urea band by spraying with the hypochlorite reagent as suggested by Berry (1950). The chromatogram was sprayed first with 5% phenol solution in alcohol and dried at about 80-90° for about 10 minutes. It was then sprayed with hypochlorite reagent. A bright bluish green band developed indicating the presence of urea, with R_f value of about (0.60—0.66) when pure urea solution was used. In the case of urine two bands appeared on the chromatogram, one having an R_f value of about (0.60—0.66), and the other below this band with an R_f value of about (0.30—0.37). The

top band with the higher R_f value was identified as the urea band and the lower one corresponded to the position of the ammonium ion. Synthetic mixtures of urea and ammonium chloride chromatographed on the same paper along with urine, showed two bands, the higher one was due to urea and the lower one which occupied the same position as the lower band of the urine chromatogram, was due to the presence of the ammonium ion of the salt. Fig. II illustrates the chromatogram of urine showing the position of urea and the other substance reacting with the reagent. The lower band is probably due to the presence of the ammonium salt in the urine.

Quantitative estimation of urea separated on the chromatogram.— The semi-circular chromatogram containing the bluish green band of urea was dried. The paper was then superimposed on its corresponding half of the unsprayed semi-circular paper and the position of the urea band was marked with a pencil (Fig. II). The paper enclosed in the area outlined was cut out leaving about 1 mm. more marginal space around the pencil mark, in order to make sure that the urea band on the paper was completely enclosed within the area cut out for elution of urea. The urea was eluted from the paper with 2 c.c. of water for half an hour. The urea present in the aqueous eluate was then determined by the method of Archibald (1945) based on the red colour formed when urea is heated in acid with α-isonitrosopropiophenone.

Aliquots of 1 c.c. of the eluates were added into test tubes containing 9 c.c. of sulphuric acid-phosphoric acid-water mixture and 0.5 c.c. of α-isonitrosopropiophenone reagent was added to each tube. The test tubes were then placed in boiling water bath for exactly one hour. The tubes were then taken out and cooled in an enclosed container excluding exposure to day light. The colour intensity of the solutions was determined in a Klett-Summerson Photo-electric Colorimeter with a green filter (540 $m\mu$).

Different concentrations of standard urea solutions were chromatographed and from the colorimetric readings obtained a calibration curve was drawn (Fig. I.)

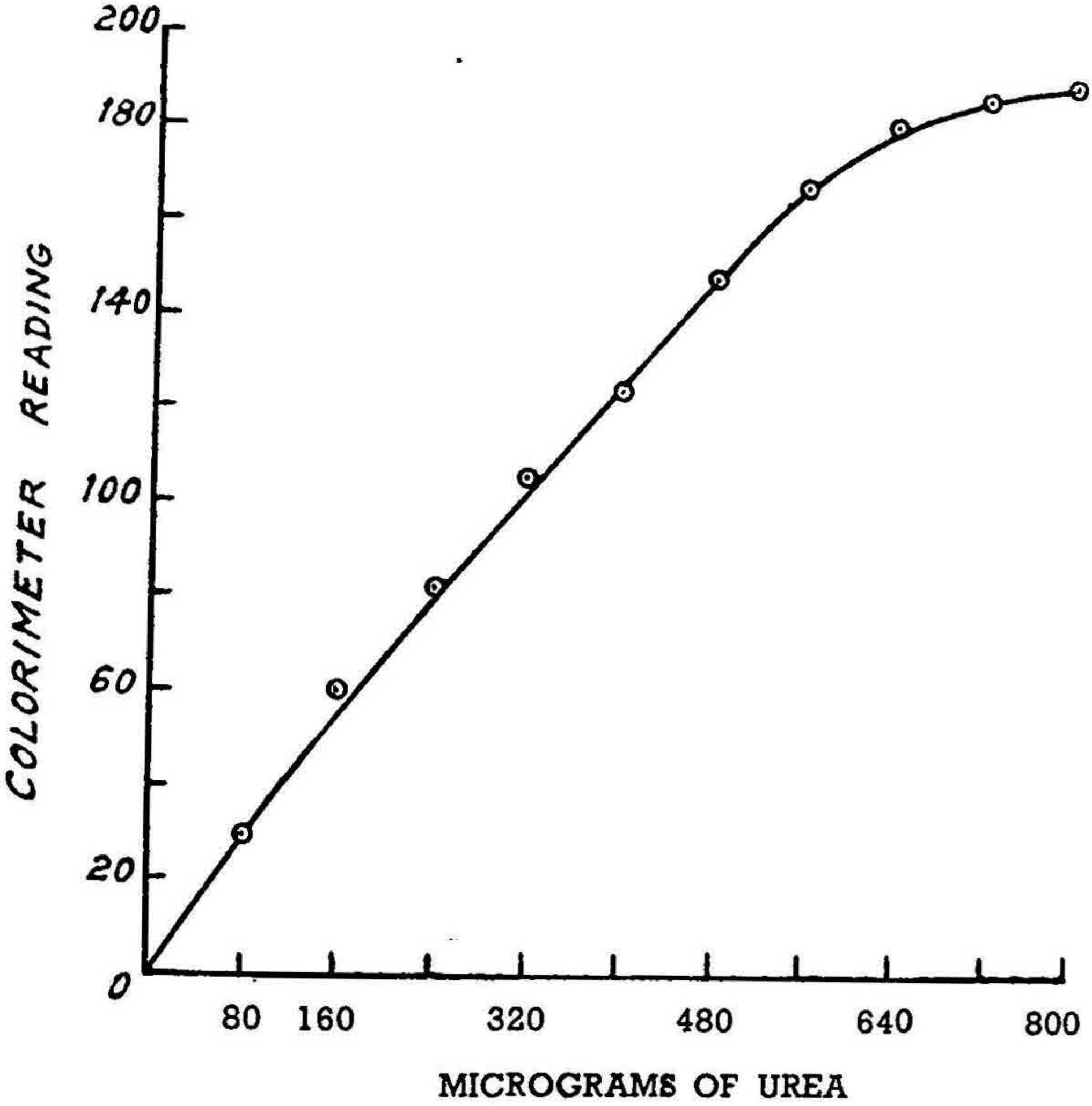

FIG. I. Urea standard curve showing the relationship between the colour intensity and the amount of urea.

The amount of urea present in the test sample was calculated from the standard curve. It is, however, desirable to run a mixed chromatogram on larger paper with the test sample and the standard urea solution and compare the colour intensities of the eluates, in order to obtain reliable values for the urea content.

Application to urine.—The method was applied to the quantitative determination of urea in normal urine. Samples of morning urine were collected and 20-120μ l of the urine, without any treatment was spotted on the circumference of the circle drawn at the centre of the paper (Fig. II) and after development, urea was estimated as described above :—

Table I

The quantitative recovery and colorimetric estimation of urea in urine by Circular Paper Chromatography

No.	Urine + urea in μl	Urea added in μg.	Urea determined in μg. in urine spotted.	Urea recovered in μg.	Percentage recovery
		I			
1.	40 + 0	0	68	...	...
2.	40 + 20	80	...	80	100%
3.	40 + 40	160	...	160	100%
4.	40 + 60	240	...	252	105%
5.	40 + 80	320	...	304	95%
			Average recovery	...	100%
		II			
1.	40 + 0	0	160	...	...
2.	40 + 20	80	...	68	85%
3.	40 + 40	160	...	168	105%
4.	40 + 60	240	...	240	100%
5.	40 + 80	320	...	320	100%
			Average recovery	...	98%
		III			
1.	40 + 0	0	104	...	...
2.	40 + 20	80	...	68	85%
3.	40 + 40	160	...	160	100%
4.	40 + 60	240	...	240	100%
5.	40 + 80	320	...	312	98%
			Average recovery	...	96%
		IV			
1.	40 + 0	0	64	...	...
2.	40 + 20	80	...	88	110%
3.	40 + 40	160	...	146	91%
4.	40 + 60	240	...	248	103%
5.	40 + 80	320	...	312	98%
			Average recovery	...	100·5%

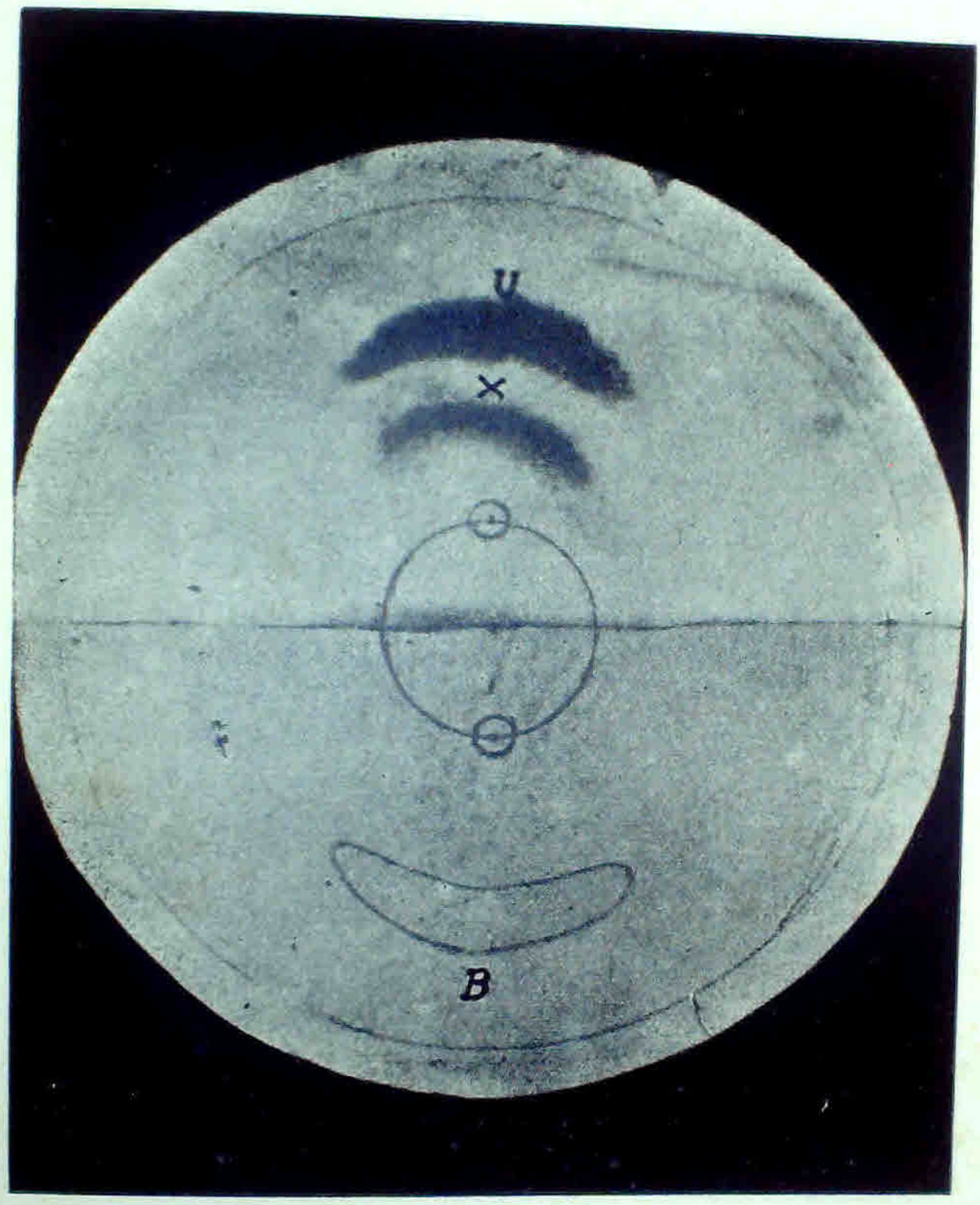

FIG. II. Circular Paper Chromatogram showing the separation, identification and cutting out technique of the urea band for the quantitative estimation of urea in urine.

U— Urea band coloured bluish green, on spraying the semi-circular chromatogram with phenol-hypochlorite reagent.

X— Band, identified as that of the ammonium salt, coloured bluish green on spraying with phenol-hypochlorite reagent.

B— Urea band (outlined with pencil) on the unsprayed half of the chromatogram.

In Table I are presented the results of the urea content of urine together with the values of recovery experiments on four different urine samples. The recoveries varied from 85—110 per cent.

A rough estimate of the amount of urea present in urine can be made by visual comparison of the colour intensity of the urea band with that of known standard urea samples run on the same paper.

The paper chromatographic method is simpler and gives reasonably accurate results in the quantitative determination of urea. This method which can apparently be applied to other compounds also present in urine and blood, is characterised by being more rapid than the usual methods of analysis.

It is hoped to investigate in more detail the accuracy of this method as compared with other existing methods and causes of any deviations in the values obtained by these methods. Further work is in progress on the application of this chromatographic method to the determination of urea in blood.

Our thanks are due to Dr. T. B. Subba Rao for having kindly supplied the urine samples.

References

1. Airan, J, W. ... *Science and Culture*, 1952, 18, 89.
2. Archibald, R. M. ... *J. Biol. Chem.*, 1945, 157, 507.
3. Berry, H. K. ... *Biochem. Inst. Studies*, IV, 1950, No. 5109, p. 88, (Univ. of Texas, Austin).
4. Giri, K. V. and N. A. N. Rao ... *Nature*, 1952[a], 618, 925, *J. Ind. Inst. of Science*, 34, 95.
5. Giri, K. V., Krishnamurthy, D.V. and Rao, P. L. N. *J. Ind. Inst. of Science*, 1953.
6. Giri, K. V., Radhakrishnan, A.N. and Vaidyanathan, C. S. *Anal. Chem.*, 1952[b], 24, 1677.
7. Giri, K.V., Krishnamurthy, K. and Venkatasubramaniam, T. A. *Lancet*, 1952[c], p. 562.
8. Hartung, W. H. and Munch, J. C. ... *J. Amer. Chem. Soc.*, 1929, 51, 2264.
9. Krishnamurthy, K. and Venkatasubramaniam, T. A. *Curr. Sci.* 1952, 21, 278.