THE COLORIMETRIC DETERMINATION OF UREA IN THE BLOOD OF NORMAL AND UREMIC RATS*

BY HARLYN O. HALVORSON AND M. O. SCHULTZE

(From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul)

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In connection with other studies in this laboratory (1, 2), it became necessary to determine the concentration of urea in the blood of individual new-born rats. In view of the small quantities of blood available and the need for a considerable number of analyses, the colorimetric method developed by Archibald (3) appeared to be particularly suitable. This method is based upon the formation of a colored compound when strongly acid solutions of urea and α-isonitrosopropiophenone are heated together. As applied to human blood, this procedure was reported by Archibald to yield results which corresponded closely to those determined with a manometric urease method. Inasmuch as some other compounds, such as allantoin, citrulline, alloxan, and even proteins if present in sufficient quantities, give rise to a color similar to that produced by urea (3), the specificity of the Archibald method when applied to rat blood was not assured. When it was found that under certain conditions the urea concentration in the blood of very young rats, as measured by this method, rose to extremely high levels, further studies on the specificity of this method were clearly indicated. Such studies might also reveal extensive variations in the concentration of chromogens other than urea in the blood of rats. In this paper data are reported on (a) a comparison of the colorimetric method and a urease method for determination of urea in rat blood and (b) the extent of destruction by urease of substances which are chromogenic in the colorimetric method.

EXPERIMENTAL

Colorimetric Determination of Urea—Blood was collected from decapitated rats directly into dry 0.2 ml. Kahn serological pipettes graduated into 0.001 ml. divisions. The measured sample was then discharged into 2 ml. of distilled water and the pipette was rinsed with the water. The proteins were precipitated with zinc hydroxide according to Somogyi (4). The resulting suspension was then diluted so that the final volume was

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1 Halvorson, H. O., and Schultze, M. O., unpublished work.
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from 40 to 80 times that of the blood used and centrifuged. Urea was then determined in duplicate on 1 ml. aliquots of the deproteinized diluted blood as outlined by Archibald (3), with use of a Coleman junior spectrophotometer for measurement of optical densities at 540 m$\mu$. Standard solutions prepared from recrystallized urea and containing from 10 to 50 $\gamma$ of urea were run simultaneously. By close adherence to standardized procedure, the reproducibility of the method was very satisfactory. Thus, the mean optical density at 540 m$\mu$ of 62 external standards containing 10.7 $\gamma$ of urea was $0.155 \pm 0.0011$ (standard error of the mean); the mean optical density of fifteen internal standards of the same concentration was $0.156 \pm 0.002$. The optical density of the colored compound was directly proportional to the concentration of urea in the range of 10 to 50 $\gamma$ of urea.

Urease Method for Determination of Urea—The procedure used was that of Kinsey and Robison (5) in which the ammonia liberated by the action of urease (The Arlington Chemical Company, Yonkers, New York) diffuses into a drop of glycerol saturated with boric acid and is then titrated with standard acid and bromocresol green as an indicator. A micro burette was constructed from a 1 ml. syringe; it held a volume of 438.2 c.mm. and the plunger was moved by an attached micrometer screw graduated into 1000 divisions. The mean recovery of ammonia from six aliquots of a standard ammonium chloride solution containing 55 $\gamma$ of nitrogen per sample was $99.4 \pm 0.22$ per cent (standard error of the mean). When this procedure was tested with a standard solution of recrystallized urea, the mean recovery from seven aliquots containing 29.5 $\gamma$ of urea was $100.05 \pm 0.41$ per cent (standard error of the mean). For determination of urea in pooled blood specimens by this procedure the final dilution of blood to reagents plus water was 1:4.7 or 1:15.1 with normal and uremic blood, respectively. To 275 c.mm. of deproteinized, diluted blood 90 c.mm. of a freshly prepared 0.75 per cent solution of urease in 0.10 M phosphate buffer of pH 7.0 were added and the digestion was carried out at room temperature for 45 minutes in the closed system. After addition of 90 c.mm. of saturated potassium carbonate to raise the pH to $> 12.0$ the ammonia was allowed to diffuse into the boric acid for 2 hours at 37°C and then titrated with 0.02 \text{N} sulfuric acid.

Table I shows the precision obtained when the colorimetric and urease methods were applied to several aliquots of pooled samples of deproteinized normal and uremic blood.

Destruction by Urease of Chromogens Reacting with $\alpha$-Isomitrosopropionophenone—For these experiments a freshly prepared 0.75 per cent solution of urease in 0.1 M phosphate buffer, pH 7.0, was added to deproteinized blood with a final dilution of about 1:15. After 45 minutes at room
temperature the solutions were deproteinized again with zinc hydroxide and aliquots were treated with the reagents used for the determination of urea by the method of Archibald. The residual color was calculated in terms of urea, although it was produced by chromogens other than urea which react with α-isonitrosopropiophenone. This was ascertained by the observation that urea added to blood was destroyed completely by the quantity of urease used, even if urea were present in amounts exceeding 4 times that found in uremic blood. Blank determinations showed that the urease preparation contributed only traces of chromogenic material; these could not account for the color developed with α-isonitrosopropiophenone in many specimens which had been treated with urease.

### Table I

<table>
<thead>
<tr>
<th></th>
<th>No. of aliquots</th>
<th>Urea per 100 ml. whole blood</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Urease method</td>
<td>Colorimetric method</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>Normal rats</td>
<td>6</td>
<td>37.78 ± 0.18*</td>
</tr>
<tr>
<td>Uremic &quot;</td>
<td>7</td>
<td>190.0 ± 1.21</td>
</tr>
</tbody>
</table>

* Standard error of the mean.
† This specimen of pooled blood from 21 day-old rats is one of the two found which failed to produce a color with α-isonitrosopropiophenone after treatment with urease.
‡ This specimen gave a residual color after urease treatment equivalent to 16 mg. of urea per 100 ml. of blood.

### RESULTS AND DISCUSSION

The results of these comparative studies summarized in Table II establish that (a) the colorimetric method as applied to rat blood gives results which are about 12.5 per cent higher than those obtained by the urease method, (b) rat blood usually contains one or more chromogens other than urea which react with α-isonitrosopropiophenone (the quantity of these non-urea chromogens increases approximately in proportion to the increase of urea in the blood of uremic rats), (c) the condition of “acute uremia of the newborn” described elsewhere (6) is a true uremia. Since the amount of urease used in these experiments was sufficient to decompose quantities of urea far in excess of those present in the blood filtrates obtained from uremic rats, the residual color developed after urease treatment must be due to non-urea chromogens present in rat blood. We have observed this residual color in all of the thirteen specimens of uremic rat
blood and in eight out of ten specimens of normal blood. The intensity of the residual color in the latter specimens was quite weak (optical density 0.02 to 0.04) and hence little significance should be ascribed to the magnitude of these values. In the uremic bloods, however, the mean residual color was equivalent to 14.8 mg. of urea per 100 ml. of blood.

Of the various compounds which have been shown by Archibald (3) to give a color with 2-isonitrosopropiophenone similar to that produced by urea, allantoin, citrulline, and traces of protein or polypeptides are those most likely to be present in the deproteinized filtrates from uremic blood. Allantoin has been reported to occur in the blood of rats older than those with which we have worked to the extent of 0.85 to 1.67 mg. per 100 ml. by Christman et al. (7) and 7.7 mg. per 100 ml. by Hawkins et al. (8). According to the data of Archibald (3), allantoin, under the conditions of the

| Table II |
|------------------|------------------|------------------|
| **Comparison of Colorimetric and Urease Methods of Analysis and Presence of Non-Urea Chromogens in Blood** |
| mg. | mg. |
| Normal rats, 10 specimens | Uremic rats, 13 specimens |
| (a) Colorimetric method | 37.4 ± 1.7* | 208.2 ± 11.2* |
| (b) Urease method | 33.2 ± 2.0 | 185.2 ± 10.0 |
| (c) Residual color after urease action, calculated as urea | 2.6 ± 1.1 | 14.8 ± 1.9 |
| 
| (a - b)/b × 100 | 12.7 | 12.4 |
| c/b × 100 | 7.8 | 8.0 |

* Standard error of the mean.

colorimetric method used here, gives an optical density of only about 7 per cent of that produced by an equal weight of urea. Even if it were assumed that the residual color observed in the samples obtained from uremic blood was due solely to allantoin, no valid estimate of its concentration in the blood could be made from the present data because it has been found that the urease preparation used reacts with allantoin. Thus when amounts of allantoin ranging from 0.24 to 1.7 mg. were treated with urease under the same conditions as were used for deproteinized blood and then deproteinized and treated with 2-isonitrosopropiophenone, the allantoin treated with urease yielded only from 49 to 56 per cent of the color obtained with untreated allantoin. The presence of allantoinase and other enzyme activity in commercial preparations of urease has been pointed out by others (9, 10). The fact that the sum of the urea equivalent of the
residual color and the urea determined by the urease method does not equal the values obtained colorimetrically (Table II) may reflect the action of the urease preparation on allantoin in the blood. The urease preparation which was used for these experiments, however, did apparently not contain allantoicase, since no ammonia was evolved from allantoin by the enzyme. Therefore, the presence of allantoin in the blood did not affect the urea determinations made with the urease method as reported here.

The interference by citrulline in the colorimetric determination of urea is even much less than that of allantoin (3). No data appear to be available on the concentration of citrulline in rat blood. In uremic human blood Archibald found a citrulline concentration of not more than 2 mg. per 100 ml. If this amount of citrulline were present in rat blood, it would not be sufficient to cause an appreciable residual color in the colorimetric analyses reported here. De Verdier and Ågren (11) have reported that filtrates prepared with zinc hydroxide from the blood of cattle contain small amounts of protein. Tests for protein in the blood filtrates prepared in this study were negative. The presence of appreciable quantities of polypeptides in the blood filtrates is made unlikely by the observation that the filtrate prepared with zinc hydroxide from a tryptic digest of soy bean protein, when treated with α-isonitrosopropiophenone, formed a color which had an entirely different absorption spectrum from that of the residual color observed with urease-treated filtrates from uremic blood. The latter, with α-isonitrosopropiophenone, yield absorption curves similar to those given by low concentrations of allantoin or urea with a maximum at 530 m. The absorption maxima of the colored compound obtained when urea or blood filtrates from uremic rats are treated with α-isonitrosopropiophenone are at 540 m. While the chemical nature of the non-urea chromogens present in uremic rat blood requires further study, the evidence obtained is not inconsistent with the view that allantoin is involved to a large extent.

SUMMARY

1. A comparative study of the determination of urea in the blood of normal and uremic rats by the colorimetric method of Archibald and the urease method of Kinsey and Robison has shown that the former yields, in general, results which are about 12 per cent higher than those obtained with the latter.

2. The chemical nature of the compounds responsible for the high values obtained in the colorimetric method has not been identified.

3. The "acute uremia of the newborn rat" has been characterized as a true uremia.
BIBLIOGRAPHY

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