Separation and Properties of Urinary Hemopoietine

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The presence in urine of rabbits rendered anemic by bleeding of a factor accelerating hemoglobin (Hb) recovery in rabbits after a standard bleeding has been described. More recently the presence of considerable erythropoiesis-stimulating activity has been found in the urine of patients with Cooley's anemia, and in cases of hypoplastic anemia. Urine from a child with aplastic anemia has been shown to have remarkable erythropoietic activity. Some properties and methods of separation of the active factor in human urine have been recently published. Work carried out in the author's laboratory has shown that the urine of rabbits made severely anemic by phenylhydrazine is a rich source of urinary hemopoietine of higher specific activity than that of urine of severely bled rabbits. Some data in methods of separation and properties of the factor in phenylhydrazinized rabbit's urine were also given. The purpose of this communication is to describe the methods used for separation of urinary hemopoietine from phenylhydrazinized rabbits, to relate some of the properties of the active material and to compare them with that of urinary hemopoietine obtained from patients with severe aplastic anemia.

Methods

Donor rabbits. The rabbits received five injections, one a day of 10 mg./Kg. of freshly prepared phenylhydrazine hydrochloride (Fisher certified reagent) solution, buffered to pH 7.4. Urine was collected on the two days following the last phenylhydrazine injection, using the technique previously described. Only rabbits showing severe anemia of less than 5 Gm. Hb/100 ml., were used as urine donors. Once the urine was collected, it was centrifuged to eliminate particulate material, and then stored in frozen state until used for separation of hemopoietine.

Urine from human patients. Freshly voided urine was collected into sterile flasks from five patients with severe anemia (less than 5 g Hb 100ml) of the aplastic type. The urine was kept in a frozen state until used for fractionation.

Assay procedure. The assay procedure used was the determination of the effects of two intraperitoneal injections, one a day, of the material to be tested, on distribution of Fe\textsuperscript{59} in grey a x c strain rats (Instituto de Biologia "Juan Noe", Universidad de Chile), starved for a total of 72 hours before administration of tracer doses of Fe\textsuperscript{59}(0.2 μc. per rat). The injections of extracts were made on the second and third day of fasting (table 1 shows a comparison between the 1 and 2 dose schedules). Groups of rats were exsanguinated at 3\textsuperscript{a} and 24\textsuperscript{a} hours after tracer injection and the fraction of injected Fe\textsuperscript{59} present in

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plasma and washed red cells was determined using a well type scintillation counter. From the fraction of injected Fe\textsuperscript{59} remaining at 3 hours in plasma (Fe\textsubscript{359}), plasma iron turnover rate (K, per cent/hr.) was calculated, 

\[ K = \frac{1}{3} \ln \frac{1}{\text{Fe}_{359}} \]

In some experiments extracts were also assayed for their acute (2 injections) effect on iron distribution in normal rats. In the results the dose of material is given as mg. protein. When the amount of protein injected was not indicated, the material tested per rat was that obtained from 30 ml. of urine.

**Methods of separation.** All operations were carried out in the cold (\(-0^\circ\text{C.}\)).

**Crude alcohol extract.** Urine is brought to pH 4.5 by slowly adding 0.5 M acetic acid. Next four volumes of cold 95 per cent ethanol are slowly added with constant stirring. The mixture is left at rest in the cold overnight, and then the precipitate is separated by centrifugation. The supernatant is discarded. The precipitate is extracted with ether, then dried in vacuo at 25°C. The clear yellow brown supernatant is referred to as the "crude ethanol extract" (Cr.E.Ext. R.U.).

**Alcohol fraction 0–50 per cent and 50–75 per cent.** The crude alcohol extract is dialyzed against distilled water, then against 0.1 M acetic acid. To this solution 1 volume cold 95 per cent ethanol is added, the precipitate is treated as above, and is referred to the 0–50 per cent fraction. To the supernatant is then added, 3 or 4 volumes cold 95 per cent ethanol and the precipitate obtained treated as in the above method is called the 50–75 per cent or 50–80 per cent alcohol fraction. In some studies ethanol precipitation was also carried in solutions dialyzed against acetate buffers at pH 4 and 7.

**Kaolin preparation.** The procedure used was that developed by Bradbury et al.\textsuperscript{9} for the separation of urinary gonadotropins. A similar method has been applied by Winkert et al.\textsuperscript{5} for separation of E.S.F. from urine of patients with Cooley's anemia.

1) Adsorbtion: Urine is brought to pH 4.5 by adding slowly 0.5 M acetic acid with constant stirring. Then 100 ml. liter urine of a 20 per cent kaolin suspension (Kaolin American Standard, acid washed, Fisher Sc. Co.) in 0.1 M acetic acid acetate buffer pH 4.5 is added and the mixture is stirred for one hour, left at rest one hour, and then the precipitate is separated by centrifugation.

2) Elution: (a) 1 M. NH\textsubscript{4}OH elution.—The kaolin precipitate is extracted for 20 min. with 25 ml. of 1 M. NH\textsubscript{4}OH/20 Gm. kaolin. The precipitate is separated and the supernatant is brought to pH 4.5 by adding 1 M. acetic acid.

Elution: (b) At different pH.—In some experiments the kaolin precipitate was eluted serially with mixtures of NH\textsubscript{4}OH/NH\textsubscript{4}Cl at different pH. First it was treated with 1 M. NH\textsubscript{4}Cl, then serially with mixtures at pH 7, at pH 8, pH 9 and finally with 1 M. NH\textsubscript{4}OH.

3) Precipitation of elutectic Urinary Hemopoietine.—Two methods can be used, which give satisfactory results: precipitation with 4 volumes cold 95 per cent ethanol or precipitation with 5 volumes acetone at \(-4^\circ\text{C.}\). The latter procedure is more practical in that a dry acetone powder is obtained after washing the precipitate with more acetone and then ether, and drying in vacuo. After the alcohol precipitation technique, the precipitate is also extracted with ether and dried in vacuo. The material obtained by ethanol treatment is referred to as "kaolin extract". The material obtained by the acetone procedure is referred to as "kaolin acetone powder", and is the material which has been kept in powder form for periods of months at \(-25^\circ\text{C.}\), without losing activity.

4) Readsorption on kaolin.—In some experiments the urinary hemopoietine prepared by the kaolin technique was readsorbed on kaolin, and eluted serially with solutions of increasing pH.

**Adsorption on D.E.A.E. cellulose:** Urine is dialyzed for 24 hours against 25 volumes of 0.03 M. sodium acetate/acetic acid buffer pH 4.5. Next 1.5 Gm. D.E.A.E. (Eastman organic chemicals) per 100 ml. dialyzed urine is added, and the mixture is stirred for 20 min., left at rest for 1 hour, and the precipitate is separated by centrifuging. The separated D.E.A.
E. is eluted with 0.2 M Na₂HPO₄ in 0.5 M NaCl, with constant stirring for 20 min. The supernatant solution is then dialyzed against distilled water, and made isotonic with NaCl, before injection. The activity of this material is compared with that obtained from an aliquot of the dialyzed urine by precipitation with 4 volumes ethanol. The dialyzed solution can also be lyophilized to obtain a light brown powder.

### Chemical Studies

All studies were carried out using dialyzed extracts. Protein determinations were done using the Biuret method. Hexose and hexosamine content of the extracts were determined using the procedure described in Glick for determination in serumucoid; sialic acid was determined using the diphenylamine reagent, and the technique described by Burton for D.N.A. As no sialic acid standard was available, the absorbancy of solutions was compared to that of standard solutions of desoxyribose at 596 μ. The results are expressed as μg. desoxyribose equivalent per mg. protein. Ultraviolet absorption spectra were measured using a Beckman Model D.U. spectrophotometer.

Paper electrophoresis was done in Veronal buffer using the technic described in Block et al. Dialyzed solutions of extracts containing about 5 mg./ml. protein were used, and 0.06 ml. were applied to the paper. Tiselius electrophoresis runs were made in phosphate buffer pH 7.4; ionic strength 0.02.

### RESULTS

1. **Assay of Fractions of Urine of the Phenylhydrazine Rabbits**

Table 1 shows some examples of the results of 24 hr. Fe⁵⁹ assays of fractions of urine of phenylhydrazine rabbits. The most active fraction obtained by the ethanol procedure is that which precipitates between 50-80 per cent ethanol concentration. This fraction has a specific activity (activity per mg.) twice as high as the crude extract, as can be deduced from figure 1. Other studies indicate that the material which precipitates with 1 volume ethanol at pH 4.5 has lower activity than the crude extract, while that precipitating with 1 volume ethanol at pH 4 is as active as the crude starting material.

The urinary extracts prepared by kaolin absorption (table 1) and elution with 1 M NH₄OH show specific activities in the order of three times the crude ethanol extract. When elution from kaolin is carried out with 0.1 M NaOH instead of 1 M NH₄OH the erythropoietic activity is lost. Readsoption of kaolin extracts on kaolin also results in considerable loss of activity. When elution from kaolin is carried out at different pH, the material of highest specific activity comes off at pH 7-8 (table 1). The yields of material obtained using the kaolin method are given in table 2.

Table 3 (a & b) illustrates the effects of extracts of urinary hemopoietine on Fe⁵⁹ distribution. Hemopoietine increases the rate of disappearance of plasma Fe⁵⁹ and the fraction of injected Fe⁵⁹ appearing in red cells of starved and normal recipient rats. The extracts prepared by D.E.A.E. treatment of urine show a similar specific activity to those prepared by the kaolin procedure. Figure 2 shows the linear correlation, \( r = 0.985 \), between log dose of

*The Tiselius electrophoresis was carried out by Mrs. G. Leyton, Immunochemistry Section, Instituto Bacteriologico de Chile, for whose collaboration we express our sincere appreciation.
Table 1.—Mean Value and Standard Error of 24 Hours Erythrocyte Fe\(^{59}\) Assay of Different Preparations of Rabbit Urinary Hemopoietine. The results included between two horizontal lines represent assays of material obtained from one urine pool.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Dose</th>
<th>% Fe(^{59}) Erythrocytes (24 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of 22 groups, 5 rats each</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (NaCl)</td>
<td></td>
<td>8.21 ± 3.0</td>
</tr>
<tr>
<td>Cr. Ethanol extr.</td>
<td>6 mg. 1 injection</td>
<td>22.8 ± 2.4</td>
</tr>
<tr>
<td>Cr. Ethanol extr.</td>
<td>6 mg. 2 injections</td>
<td>31.0 ± 2.0</td>
</tr>
<tr>
<td>Cr. Ethanol extr.</td>
<td>16 mg. &quot;</td>
<td>41.8 ± 2.8</td>
</tr>
<tr>
<td>0-50% Ethanol pH 4.5</td>
<td>16 mg. &quot;</td>
<td>19.7 ± 3.5</td>
</tr>
<tr>
<td>50-80% Ethanol pH 4.5</td>
<td>16 mg. &quot;</td>
<td>60.2 ± 5.2</td>
</tr>
<tr>
<td>Cr. Ethanol extr.</td>
<td>12 mg. &quot;</td>
<td>40.1 ± 4.54</td>
</tr>
<tr>
<td>Kaolin eluted NaOH 0.1M.</td>
<td>6 mg. &quot;</td>
<td>8.1 ± 1.03</td>
</tr>
<tr>
<td>Kaolin extract eluted NH(_4)OH 1 M.</td>
<td>6 mg. &quot;</td>
<td>48.0 ± 6.9</td>
</tr>
<tr>
<td>Kaolin extract eluted NH(_4)OH 1 M.</td>
<td>4 mg. &quot;</td>
<td>43.5 ± 7.65</td>
</tr>
<tr>
<td>Kaolin eluted NH(_4)Cl</td>
<td>7 mg. &quot;</td>
<td>18.1 ± 1.4</td>
</tr>
<tr>
<td>Kaolin eluted pH 7</td>
<td>7 mg. &quot;</td>
<td>49.3 ± 3.5</td>
</tr>
<tr>
<td>Kaolin eluted pH 8</td>
<td>7 mg. &quot;</td>
<td>41.5 ± 7.8</td>
</tr>
<tr>
<td>Kaolin eluted NH(_4)OH</td>
<td>7 mg. &quot;</td>
<td>39.7 ± 3.0</td>
</tr>
<tr>
<td>Kaolin acetone powder</td>
<td>6 mg. &quot;</td>
<td>60.9 ± 5.0</td>
</tr>
</tbody>
</table>

kaolin acetone powder and the increase in plasma iron turnover rate (\(\triangle K\) per cent/hr.).

II. Assay of Alcoholic Extracts Obtained from Urine of Anemic Patients

Table 4 summarizes the results of assays of urinary hemopoietine from severely anemic patients. The first assay carried out with human urine showed a striking response by the rats; later assays confirmed these first findings. Figure 1 (quantitative bioassay) shows that the material from human sources has a much higher specific activity than that prepared from rabbit’s urine, and that transfusion notably decreases the specific activity of the material obtained from human urine. With reference to the activity of the 0-50 per cent and 50-80 per cent fractions it can be seen (table 4) that in 3 of 4 urines the specific activity was equally divided between these two fractions. In one case the 0-50 per cent fraction showed the highest specific activity.

III. Properties of Urinary Hemopoietine from Rabbit and Human Sources

The U.V. spectrums of both rabbit and human urinary hemopoietine preparations, as well as of extracts with no erythropoietic activity, show an absorption peak about 280 \(\mu\).

Figure 3 shows an example of paper electrophoretic patterns of a simultaneous run of (1) normal human serum; (2) kaolin acetone powder prepared from phenylhydrazine rabbits’ urine, by kaolin adsorption, elution with 1 M. NH\(_4\)OH, and acetone precipitation; (3) ethanol extract of urine from patient O. M.; (4) ethanol extract of a control inactive human urine. All three
C. HODGSON AND CO-WORKERS

Fig. 1.—Relation between increment of Fe\(^{59}\) (24 hr.) in erythrocytes of fasted rats (ordinate) and dose (mg.) of urinary hemopoietine (abscissa, log scale):

X Crude ethanol (0-80\%) extract of urine of phenylhydrazinized rabbits.
○ 50-80\% ethanol fraction obtained from the same urine.
■ 50-80\% ethanol fraction of urine of a patient with aplastic anemia.
▲ 50-80\% ethanol fraction of the urine of the same patient above, 3 days after transfusion had raised Hb from 4.2 to 7 Gm./100 ml.

Each point represents the average of at least 5 animals.

extracts show one component moving behind albumin while the kaolin preparation shows other components moving like \(\alpha_2\) and \(\beta\) globulins.

Figure 4 shows the pattern of Tiselius electrophoresis of an extract of rabbit urine prepared by kaolin adsorption and elution with 1 M. NH\(_4\)OH. Figure 5 shows the pattern of an extract obtained by kaolin adsorption and elution at pH 7. The latter is a more homogenous preparation.

The chemical studies carried out with both active and inactive extracts from rabbit and human urine showed that they all contained varying amounts of: (1) hexose ranging from .2 mg./mg. protein to 1 mg./mg. protein; (2) sialic acid ranging from .3 mg. to .05 mg./mg. protein; and (3) hexosamine

Table 2.—Yield of Material Obtained from 250 ml. Urine of Phenylhydrazine Treated Rabbits by Alcohol Precipitation, and that Obtained from 3,500 ml. by Kaolin Adsorption and then Serial Elution at pH 4.6, 7, 8 and with 1 M. NH\(_4\)OH. The results of the assays of these fractions are given in table 1

\[
\text{Yield} = \frac{\text{mg. protein in extract}}{\text{mg. protein in urine}} \times 100.
\]

<table>
<thead>
<tr>
<th>Extract</th>
<th>mg. Obtained</th>
<th>Calculated Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude ethanol</td>
<td>84</td>
<td>18%</td>
</tr>
<tr>
<td>Kaolin eluted pH 4.6</td>
<td>78</td>
<td>1.2%</td>
</tr>
<tr>
<td>Kaolin eluted pH 7</td>
<td>362</td>
<td>5.6%</td>
</tr>
<tr>
<td>Kaolin eluted pH 8</td>
<td>88</td>
<td>1.4%</td>
</tr>
<tr>
<td>Kaolin eluted 1 M. NH(_4)OH</td>
<td>72</td>
<td>1.1%</td>
</tr>
<tr>
<td>Kaolin (\Sigma)</td>
<td>600</td>
<td>9.3%</td>
</tr>
</tbody>
</table>
Table 3

a. Mean Value and Standard Error of Fe<sup>59</sup> in Plasma and Erythrocytes in Fasted Female Rats, 3 Hours after Tracer Injection, and Calculated Values of the Fraction of Plasma Iron Turned Over per Hour (K per cent/hr.)

<table>
<thead>
<tr>
<th>Extract</th>
<th>Dose</th>
<th>% Fe&lt;sup&gt;59&lt;/sup&gt; Plasma</th>
<th>K (%/hr.)</th>
<th>% Fe&lt;sup&gt;59&lt;/sup&gt; Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean value for 18 groups, 5 rats each</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (NaCl)</td>
<td></td>
<td>35 ± 6.6</td>
<td>35 ± 7.0</td>
<td>0.94 ± 0.48</td>
</tr>
<tr>
<td>D.E.A.E. extr.</td>
<td>3.7 mg.</td>
<td>3.54 ± 0.46</td>
<td>112 ± 3.4</td>
<td>18.6 ± 1.83</td>
</tr>
<tr>
<td>Kaolin Acetone powder</td>
<td>6 mg.</td>
<td>5.5 ± 0.33</td>
<td>98 ± 2.1</td>
<td>13.3 ± 0.90</td>
</tr>
</tbody>
</table>

b. Mean Value and Standard Error of Fe<sup>59</sup> in Plasma and Erythrocytes in Normal Female Rats 1 1/2 hr. after Tracer Injection and Calculated Value of K per cent/hr.

| Control                  |       | 38.1 ± 1.2               | 65 ± 2.23 | 4.5 ± 0.30                    |
| Kaolin Acetone powder    | 1 1/2 hr. | 18 mg. | 22.5 ± 1.13              | 100 ± 3.25 | 15.2 ± 1.49                  |

ranging from .03 mg. to .05 mg./mg. protein. A kaolin preparation, obtained from a 8 liter pool of phenylhydrazine rabbits urine, gave the following analysis: hexose .279 mg./mg. protein, sialic acid .115 mg./mg. protein, and hexosamine .011 mg./mg. protein. From the results of the chemical studies no clear correlation was apparent between gross chemical composition and erythropoietic activity.

**DISCUSSION**

It is apparent from the results that urine of rabbits made severely anemic by phenylhydrazine treatment, as well as urine of patients with severe anemia

![Fig. 2](image-url)
Table 4.—Mean Value and Standard Error of 24 hr. Fe\textsuperscript{15} in Erythrocytes of Fasted Rats Injected with Preparations of "Human Urinary Hemopoietine" from Cases M. C., A. L., T. R. and O. M. The extract from M. O. was tested for its effect on Fe\textsuperscript{15} at 3 hrs.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Dose</th>
<th>% Fe\textsuperscript{15} Erythrocytes (24 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. C.</td>
<td>0–80%</td>
<td>6 mg.</td>
</tr>
<tr>
<td></td>
<td>0–50%</td>
<td>1.4 mg.</td>
</tr>
<tr>
<td></td>
<td>50–75%</td>
<td>1.4 mg.</td>
</tr>
<tr>
<td>A. L.</td>
<td>0–80%</td>
<td>Equiv. 30 ml. urine</td>
</tr>
<tr>
<td></td>
<td>0–50%</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>50–75%</td>
<td>&quot;</td>
</tr>
<tr>
<td>T. R.</td>
<td>0–80%</td>
<td>Equiv. 30 ml. urine</td>
</tr>
<tr>
<td></td>
<td>0–50%</td>
<td>1.4 mg.</td>
</tr>
<tr>
<td></td>
<td>50–75%</td>
<td>1.4 mg.</td>
</tr>
<tr>
<td>O. M.</td>
<td>0–80%</td>
<td>3 mg.</td>
</tr>
<tr>
<td></td>
<td>0–50%</td>
<td>1.3 mg.</td>
</tr>
<tr>
<td></td>
<td>50–75%</td>
<td>3 mg.</td>
</tr>
<tr>
<td>Control</td>
<td>NaCl</td>
<td>8.2 ± 3.0</td>
</tr>
<tr>
<td>M. O.</td>
<td>0–80%</td>
<td>Equiv. 30 ml. urine</td>
</tr>
<tr>
<td>Control</td>
<td>NaCl</td>
<td>30.2 ± 1.10</td>
</tr>
</tbody>
</table>

Fig. 3.—Paper electrophoretic patterns (scanned with registering densitometer) of simultaneous runs of: 1) Normal human serum. 2) Kaolin acetone powder from phenylhydrazinized rabbit’s urine. 3) Ethanol extract of urine of anemic patient O. M. 4) Extract of inactive control human urine.
Fig. 4.—Tiselius electrophoretic pattern of kaolin acetone powder from urine of phenylhydrazinized rabbits (eluted with 1 M. NH₄OH from kaolin).

Fig. 5.—Tiselius electrophoretic pattern of kaolin acetone powder from urine of phenylhydrazinized rabbits (eluted at pH 7 from kaolin).

of the aplastic type, contains material precipitable with alcohol and adsorbable on kaolin and D.E.A.E. cellulose, which when injected into normal or fasted rats produces striking increases in plasma iron turnover and uptake of Fe³⁺ in red cells. These changes can be taken as indication of increased erythropoiesis in the receptor animals and are a function of the dose of the material injected (Fig. 1 and 2). In general, the specific activity of the material obtained from rabbits’ urine is in the order of two or three times less than of the most active human preparations. The erythropoietic active material is nondialyzable and judged from its hexose and sialic acid content is of mucoprotein nature. On paper electrophoresis it has a component moving behind albumin. This is in agreement with the findings of Borsook for the material obtained from rabbit plasma. However, the material obtained by Winkert et al. by kaolin adsorption from cases of Cooley’s anemia differs markedly in electrophoretic mobility from the material obtained from urine of patients with aplastic
anemia. It must also be pointed out that inactive material can be obtained from human or rabbit urine with the same type of electrophoretic mobility as that of the active factor. The gross content of hexose and sialic acid of both active and inactive fractions is quite variable and no correlation is apparent between activity and hexose and sialic acid content.

The most active fraction from rabbits’ urine is that which precipitates between 50-75 per cent ethanol concentration, and that obtained by adsorption on kaolin and eluted with 1 M. NH₄OH, which has specific activity about three times higher than the crude alcohol fraction. If elution from kaolin is carried out at pH 7, material is obtained which is more homogenous (fig. 5) and has specific activity similar to that obtained by eluting at once with 1 M. NH₄OH; however, the total yield of material is smaller (table 2). The method of kaolin adsorption is practical for handling large volumes of urine: in a typical run 2.3 Gm. of an acetone powder were obtained from a pool of 8 liters of phenylhydrazinized rabbits’ urine, collected over a period of three days after cessation of phenylhydrazine treatment. Eighty per cent of this powder goes into solution on extraction with K₂HPO₄, 0.01 M. The minimum dose of this material giving detectable response was 0.75 mg. A total dose of 3 mg., given 1.1/2 mg. a day, produced a sixfold increase in Fe⁵⁹ uptake in red cells at 24 hours and a twofold increase in plasma iron turnover and uptake of iron by the marrow in fasted rats. The material obtained from urine of phenylhydrazine treated rabbits by D.E.A.E. adsorption shows similar specific activity as that of kaolin preparations. These activities per mg. material are comparable to those of the materials obtained from plasma of phenylhydrazine rabbits.¹⁴ The activity of material obtained from phenylhydrazine sheep plasma¹⁷ is stated to be on the average of 2 μg/mg. A unit of activity is that producing an effect on erythrocyte Fe⁵⁹ in fasted rats equal to that produced by 5 μg. cobalt. Judged from this criterion, the specific activity of rabbit urinary hemopoietine is of comparable activity. The fact that great numbers of rabbits can be given repeated series of phenylhydrazine injections with low mortality (10 per cent per course) and large volumes of urine can be collected would suggest that these animals might be a source of the abundant material which is required for the further purification and chemical characterization of urinary hemopoietine.

The material obtained from urine of cases of severe anemia of aplastic type is of a higher specific activity than that of rabbits’ urine, 1 mg. total dose producing uptake of Fe⁵⁹ in erythrocytes in the order of 25 per cent, while 3 mg. produce an increased uptake of Fe⁵⁹ in red cells at 24 hours from 8 to 70 per cent. Moreover (fig. 1), the slope of the response log dose curve, for extracts of human urine, is steeper than that for rabbit urinary hemopoietine. The finding of highly active material in urine of aplastic anemia, is in agreement with the results of Van Dyke¹⁸ and indicates that human urinary hemopoietine is one of the most potent preparations available to date. However, experience shows¹⁹,²⁰ that this type of hemopoietine preparation is obtained only from cases with very severe anemia, 3–5 Gm. Hb/100 ml., and that once anemia is corrected, even slightly to 7 Gm. Hb, the
SEPARATION AND PROPERTIES OF URINARY HEMOPOIETINE

Specific activity of urinary extracts drops very markedly (fig. 1). The depressing effects of transfusion on plasma erythropoietine levels had been previously noted by Medici et al.21 and Gurney et al.22 The fact that anemias of aplastic type have high urinary hemopoietine output would indicate that the humoral mechanism of control of erythropoiesis is functioning and that the mechanism of this disorder must be sought elsewhere. The fact that great amounts of erythropoietic stimulating factor appears in the urine in these conditions brings to mind the condition of animals with deficiencies of target organ (ovary, thyroid) which show large production of the respective trophic hormone (gonadotropin – TSH). In aplastic anemia the erythropoietine would be deficient, and large amounts of hemopoietine would be produced as a response to tissue hypoxia, due to the decrease of Hb, the product of erythroid marrow (target organ).

The results published in this communication accord with those of a recent publication by Lowy et al.21; these results indicate that at present the preparations of hemopoietine obtained either from plasma or urine are very crude, as judged from variability of composition and finding of inactive fraction with similar characteristics to the active. Most of the evidence available to date suggests that the material with erythropoietic activity obtained from either plasma16,17 or urine,19,5 is of mucoprotein nature, as judged from its hexose and sialic acid content, and the fact that activity is lost after exposure to acid24 or alkaline pH and when the material is submitted to the action of proteolytic enzymes25 and neuraminidase.26 It is possible that hemopoietine separates out, in the crude fractionation procedures used, with large quantities of other inactive mucoproteins. Thus, till fractionation procedures starting with gram quantities of active crude extracts are carried out, as has been done with urinary gonadotropins,27 it will be difficult to obtain more chemical information on the nature of this substance and a clearer picture of its physiologic role and its metabolism. Up to the present the only reproducible sources of hemopoietine are plasma and urine of animals made severely hypoxic, as a consequence of anemia or exposure to environments low in oxygen. The effects obtained with plasma from animals with moderate anemia of hemolytic type are very slight, even though their own erythropoietic system is responding maximally,28 and it is also difficult to pick up hemopoietine 48 hours after maintenance of rats in environments with low O2.29,30

In conclusion, it may be said that a great deal of information on hemopoietine has accumulated in the past years, but that a vast field of research lies open for the future on the physiological mechanism of action, biosynthesis, metabolic pathways, role in human anemias, etc. A necessary step towards this goal is the further purification and chemical characterization of hemopoietine; with this attained, the other research may be carried on a more solid basis.

SUMMARY

Methods are described for the separation of nondialyzable material with erythropoietic activity (urinary hemopoietine), from urine of rabbits made
anemic (<5 g Hb/100) by phenylhydrazine and of patients with aplastic anemia (<5 g Hb/100). The material obtained was assayed for its effects on Fe\textsuperscript{59} distribution 3 and 24 hours after tracer injection. The active extracts produced a marked increase in the rate of Fe\textsuperscript{59} clearance from plasma, and in the fraction of the injected Fe\textsuperscript{59} appearing in erythrocytes at 3 and 24 hours. Effect of the extracts was seen to be a linear function of log dose. The extracts prepared from urine of patients with aplastic anemia had a specific activity about 3 times higher than that obtained from urine of phenylhydrazine rabbits. The fractions of highest specific activity obtained from rabbit's urine were (a) that precipitating between 50-75 per cent ethanol at pH 4.5; (b) adsorbed by kaolin at pH 4.5 and eluted at pH 7-8; and (c) adsorbed by D.E.A.E. cellulose at pH 4.5 and eluted by 0.2 M Na\textsubscript{2}HPO\textsubscript{4} in 0.5 M NaCl. Only the ethanol procedure was used for human urine, and it was seen that specific activities of the 0-50 and 50-75 per cent fractions were similar. The fractions obtained from human and rabbit urine showed great variability in hexose, sialic acid and hexosamine contents, and no clear correlation was apparent between gross chemical composition and erythropoietic activity. Paper electrophoresis showed the presence of a single component moving behind albumin, in both active and inactive extracts of human urine.

**SUMMARIO IN INTERLINGUA**

Methodos es describite pro le separation de non-dialysabile materiales con activitate erythropoietic (hemopoietina urinari) ab le urina de (1) conilios facite anemic (<5 g de hemoglobina per 100 ml) per medio de phenylhydrazina e de (2) patientes con anemia aplastic (<5 g de hemoglobina per 100 ml). Le materia obtenite esseva essayate pro su effectos super le distribution de Fe\textsuperscript{59} in rattos 3 e 24 horas post le injection del traciator. Le extractos active produceva un marcate acceleration del clearance de Fe\textsuperscript{59} ab le plasma e un marcate augmento in le fraction del injicite Fe\textsuperscript{59} apparente in le erythrocytos post 3 e post 24 horas. Esseva constatate que le effecto del extractos esseva un function linear del logarithmos del doses. Le extractos preparate ab le urina de patientes con anemia aplastic habeva un activitate specific circa 3 vices plus grande que illo obtenite ab le urina de conilios tractate con phenylhydrazina. Le fractiones del plus alte activitate specific obtenite ab le urina de conilios esseva (a) illo precipitare per inter 50 e 75 pro cento de ethanol a pH 4.5; (b) illo adsorbite per kaolin a pH 4.5 e eluite a pH 7 a 8; e (c) illo adsorbite per cellulosa D.E.A.E. a pH 4.5 e eluite per 0.2 M de Na\textsubscript{2}HPO\textsubscript{4} in 0.5 M de NaCl. Solmente le procedimento a ethanol esseva empletate pro le urina human. Il esseva trovate que le activitates specific del fractiones extrahite a 0-50 pro cento e a 50-75 pro cento esseva simile.

Le fractiones obtenite ab le urina de humanes e de conilios monstrava un grande variabilitate in lor contento de hexosa, acido sialic, e hexosamina. Nulle clar correlation esseva evidente inter le grossier composition chimic del extractos e lor activitate erythropoietic. Electrophorese a papiro monstrava le presentia, in extractos active e etiam inactive de urina human, de un mesme componente migrante post albumina.
SEPARATION AND PROPERTIES OF URINARY HEMOPOIETINE

REFERENCES


Urine from a patient with paroxysmal nocturnal hemoglobinuria was concentrated by ultrafiltration and injected into rats. True polycythemia with increase in hematocrit, hemoglobin and red cell mass was observed in normal rats after 14 daily injections. At the highest dose administered the degree of polycythemia was similar to that obtained in rats exposed to a simulated altitude of 20,000 feet for 14 days. When urinary erythropoietin was bioassayed in normal, hypophysectomized and fasted rats, a highly significant correlation between the 17 hour red cell utilization of radio iron and the dose of administered erythropoietin was observed. Furthermore, a high correlation was found between the 17 hour red cell utilization and the increase in total circulating hemoglobin in rats rendered polycythemic by means of erythropoietin. The usefulness of the 17 hour radioiron utilization test in estimating the rate of red cell production seems to be well established by now. However, its high degree of accuracy as an erythropoietic measure is indeed remarkable since it probably depends primarily on the early or late release of immature red cells from the marrow and only indirectly on the rate of red cell production (Lamerton, L. F., et al.: In The Kinetics of Cellular Proliferation. New York, Grune & Stratton, 1959, p. 301.).—A. J. E.


Studies by this group have demonstrated that a severe deficiency of acetylcholinesterase exists in the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH). In the current study an effort has been made to determine whether this enzyme deficiency influences potassium transport into and out of red cells. No defect in potassium transport could be demonstrated. —A. E.
Separation and Properties of Urinary Hemopoietine

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