CONJUGATED FORMS OF MYELOKENTRIC AND LYMPHOKENTRIC ACIDS*

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The names in the title of this article are proposed for the two factors found in the urine of patients with leukemia, (1, 2) and subsequently in beef liver (3); these factors have the property of stimulating the proliferation of blood cells. The names are formed from a Greek root previously used by Coffin and van Dyke (4) for hormones of the pituitary.

Myelokentric acid, which occurs in the urine of patients with myeloid leukemia, has been shown to be a keto acid, and lymphokentric acid, which occurs in the urine of patients with lymphoid leukemia, has been shown to be a hydroxy acid, obtainable from myelokentric acid by reduction; lymphokentric acid can be oxidized to myelokentric acid. These acids are soluble in fat solvents, but not in water. Biological effects similar to those produced by myelokentric and lymphokentric acid have been obtained with water-soluble fractions prepared from acidified urine by adsorption on kaolin (5, 6) and benzoic acid (7, 8).

The present work was undertaken to determine whether myelokentric acid occurred in urine as the prosthetic group of a water-soluble conjugate, or whether the water-soluble substance and the fat-soluble myelokentric acid were totally different substances producing the same biological effects. For this purpose, we have used the crude adsorbate of Heinle et al. (7, 8), containing the water-soluble active material from the urine of patients with myeloid leukemia. Benzoic acid was used as the adsorbent in this procedure. The product was thoroughly extracted with acetone; this should remove any free myelokentric acid. To be sure of this, we have also extracted the adsorbate with ether. The material was then hydrolyzed by heating with acid or alkali, and the hydrolysis product was extracted with ether. If myelokentric acid has been released from a conjugate by hydrolysis, the acid fraction of the final ether extract should show biological activity.

In addition, we have purified the water-soluble material by a method which effects a 20-fold concentration, and then hydrolyzed the purified substance.

* We are grateful for the technical assistance of Nancy E. Herb.
Technique of Animal Testing—The aqueous solutions were given in equal
doses daily to male guinea pigs weighing 550 ± 80 gm. The animals were
killed 1 to 3 days after the last dose; sections were taken and examined as
described previously (2).

Normal butyl succinate¹ was found to be an excellent solvent for the oily
fractions from the ether extracts. The material was given in this solvent,
0.5 cc. being used for each animal. To make sure that the use of this
solvent does not change the biological effects observed in the animals,
various active and inactive fractions from our previous work (1, 3) have
been tested again with this solvent; the results of the new tests have agreed
with the old. The life of the animals was 3 to 4 weeks in the experiments
with oily solutions.

The histological changes observed in the animals described in this paper
have been the same as those described previously (2).

Preparation of Solution of Conjugated Material—The method of Katzman
and Doisy (9) was used as modified by Gurin, Bachman, and Wilson (10),
except that the procedure was conducted at room temperature and the
urine was not filtered after acidification. This method involves treatment
of acidified urine with the adsorbent, benzoic acid, and removal of the
benzoic acid from the adsorbate by washing with acetone. The dry
product, corresponding to Product A of Gurin et al. (10), was stored in the
refrigerator. From 200 liters of urine from patients with myeloid leukemia,
55 gm. of this product were obtained. This material, A, was injected into
guinea pigs in the form of an aqueous solution prepared by suspending
the dry product in water, adjusting to pH 7.0² with sodium hydroxide
solution, and centrifuging. The supernatant solution is designated
Product A-1 in Table I.

Alternatively, Product A was extracted three times with 5 times its
weight of absolute ether at room temperature. The extract was tested on
guinea pigs; it was negative, except in high doses (25 liters). The residue
from this was given to guinea pigs in the form of a solution brought to
pH 7 with 0.5 M phosphate buffer of pH 7.9 after purification by the borate+
extraction and acetone precipitation method of Hirschmann, Heinle, and
Wearn (8). This is designated Product A-3 in Table I.

Purification of Conjugated Myelokentric Acid by Extraction with Alcohol—
Product A was mixed at room temperature with 10 times its weight of
50 per cent ethanol.³ The resulting sludge was brought to pH 0.0 by the

¹ The use of this solvent was suggested by the late Dr. W. Osler Abbott.
² The pH measurements were made throughout with a Beckman meter.
³ The term "per cent" is used in our papers in the sense of gm. per 100 gm. of
solution.
dropwise addition of a solution made by diluting 8.0 cc. of concentrated ammonium hydroxide to 100 cc. with 50 per cent ethanol. The mixture was allowed to stand for several hours at 10° in a refrigerator; it was then centrifuged. The extraction process was repeated four times in the same manner with the residue, with 50 per cent ethanol and adjusted to pH 6.0 only if the extract was more acid than this. The combined alcoholic extracts were precipitated by the addition of 2 volumes of commercial absolute ethanol. The mixture was allowed to stand overnight in the

### TABLE I

**Tests with Water-Soluble Products**

Two guinea pigs were used for each test, except as noted in the last column.

<table>
<thead>
<tr>
<th>Product</th>
<th>Dose of urine</th>
<th>Dose of material in aqueous solution</th>
<th>Duration</th>
<th>Result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugated myelokentric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-1</td>
<td>5</td>
<td>15</td>
<td>21</td>
<td>++ M.</td>
</tr>
<tr>
<td>A-3</td>
<td>0.5</td>
<td>1.7</td>
<td>9</td>
<td>+ M.</td>
</tr>
<tr>
<td>&quot;</td>
<td>1</td>
<td>3.6</td>
<td>9</td>
<td>++ &quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3</td>
<td>9.4</td>
<td>9</td>
<td>++ &quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>5</td>
<td>2.0</td>
<td>9</td>
<td>++ &quot;</td>
</tr>
<tr>
<td>B-1</td>
<td>4</td>
<td>10</td>
<td>21</td>
<td>+ to</td>
</tr>
<tr>
<td>B-2</td>
<td>3.5</td>
<td>7</td>
<td>25</td>
<td>++ M.</td>
</tr>
<tr>
<td>Conjugated lymphokentric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-1</td>
<td>2.3</td>
<td>7.5</td>
<td>16</td>
<td>+ to</td>
</tr>
<tr>
<td>B-1</td>
<td>2, 4</td>
<td>2.2</td>
<td>11, 21</td>
<td>++ &quot;</td>
</tr>
<tr>
<td>Material from Hodgkin's disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-1</td>
<td>2</td>
<td>12</td>
<td>25</td>
<td>+ L.</td>
</tr>
<tr>
<td>B-1</td>
<td>4</td>
<td>8</td>
<td>29</td>
<td>++ to</td>
</tr>
<tr>
<td>Material from monocytic leukemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-1</td>
<td>2.5</td>
<td>14</td>
<td>30</td>
<td>+++ &quot;</td>
</tr>
<tr>
<td>Mixture of material from myeloid and lymphoid leukemia</td>
<td>7 Myeloid</td>
<td>13</td>
<td>21</td>
<td>++ H.</td>
</tr>
</tbody>
</table>

* The significance of the + signs, and of the letters M., L., and H., is the same as has been described previously (1-3). M. designates infiltration in the organs of the animals of the myeloid type, L. indicates infiltration of the lymphoid type, and H. is used to designate a peculiar type of response differing from either, and having a superficial resemblance to lesions found in Hodgkin's disease.
refrigerator and was then centrifuged, giving a precipitate designated Product B-1.

Another product, B-2, was obtained by adjusting the alcoholic solution to pH 4.5 by the addition of a solution from 2 gm. of acetic acid made up to 100 cc. with 50 per cent ethanol, and then precipitating with 2 volumes of ethanol. From 30 liters of the urine of patients with myeloid leucemia, 360 mg. of precipitate were obtained.

A product, B-3, was made by substituting 1.25 volumes of acetone for the 2 volumes of ethanol used in making Product B-2. The extraction of Product A-3 with 50 per cent ethanol and precipitation at pH 4.3 with acetone was also carried out. This gave Product B-4.

**Table II**

*Hydrolysis of Ethanol Mother Liquor from Purification*

<table>
<thead>
<tr>
<th>Source of material</th>
<th>Material hydrolyzed; ethanol mother liquor from product</th>
<th>Dose of urine</th>
<th>Result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid leukemia</td>
<td>B-1, B-4</td>
<td>12, 15</td>
<td>Negative†</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>25</td>
<td>++ M.</td>
</tr>
<tr>
<td>Lymphoid &quot;</td>
<td>&quot;</td>
<td>12</td>
<td>+ L.</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>B-1, &quot;</td>
<td>6</td>
<td>++ H.</td>
</tr>
<tr>
<td>Monocytic leukemia</td>
<td>&quot;</td>
<td>2.5</td>
<td>++ &quot;</td>
</tr>
</tbody>
</table>

*See the foot-note to Table I.
†These animals are designated negative although they showed slight myeloid infiltrations in one organ (spleen). All the positive animals had infiltration in the liver, and at least two other organs.

Efficiency of Purification—The discarded fractions from the purification procedure could be tested only with difficulty.

The residue insoluble in 50 per cent alcohol at pH 6.0 was suspended in water and the mixture was adjusted to pH 7.0 with N sodium hydroxide solution; this was centrifuged. The supernatant solution gave a + myeloid response in guinea pigs in doses corresponding to 15 liters of urine and was negative in lower doses.

The alcoholic mother liquor from the precipitation of Products B-1 and B-2 and the acetone-alcohol mixture from Product B-3 could not be tested as conjugated myelokentric acid. These products were hydrolyzed on the steam bath after the addition of sufficient hydrochloric acid to make the
solution 2 N in acid. After heating for 3 hours, the mixture was cooled, diluted with water, and extracted with ether. Material from the ether solution was given to guinea pigs. The results are shown in Table II.

**Hydrolysis Procedure**

1. *Acid Hydrolysis*—The various products described above were suspended in water and made 2 N in hydrochloric acid. The resulting solution was heated on the steam bath for 3 hours. The mixture was cooled and extracted with ether. The ether was washed with distilled water until the washings were no longer acid to Congo red paper, dried over sodium sulfate, and distilled. The residue from the ether was given to guinea pigs.

2. *Fractionation of Hydrolysis Product*—The ethereal extract from Method 1 was separated into acidic and neutral material by extracting the ether with 5 per cent sodium carbonate solution. The alkaline layer was acidified and extracted with ether; this contained the acid fraction.

3. *Alkaline Hydrolysis*—The adsorbates and other Products A and B were heated in 100 cc. of 2 per cent potassium hydroxide for ½ hour on the steam bath. The solution was cooled, acidified to Congo red paper, and

### Table III

<table>
<thead>
<tr>
<th>Material hydrolyzed Product</th>
<th>Method No.</th>
<th>Dose of urine</th>
<th>Result*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conjugated myelokentric acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td>8</td>
<td>++ M.</td>
</tr>
<tr>
<td>A-3</td>
<td>2 Acid fraction</td>
<td>2, 5</td>
<td>+ “</td>
</tr>
<tr>
<td>“</td>
<td>2 Neutral fraction</td>
<td>15</td>
<td>++ “</td>
</tr>
<tr>
<td>B-1</td>
<td>1</td>
<td>8</td>
<td>+ M.</td>
</tr>
<tr>
<td>B-4</td>
<td>3</td>
<td>8</td>
<td>+ “</td>
</tr>
<tr>
<td>“</td>
<td>4</td>
<td>8</td>
<td>++ “, ++ M.</td>
</tr>
<tr>
<td><strong>Conjugated lymphokentric acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2 Acid fraction</td>
<td>4.5</td>
<td>+ L., ++ L.</td>
</tr>
<tr>
<td>“</td>
<td>2 Neutral fraction</td>
<td>4.5</td>
<td>Negative</td>
</tr>
<tr>
<td>“</td>
<td>1</td>
<td>10</td>
<td>++ L.</td>
</tr>
<tr>
<td>“</td>
<td>3</td>
<td>10</td>
<td>++ “, +++ L.</td>
</tr>
<tr>
<td><strong>Material from Hodgkin’s disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>2</td>
<td>++ H.</td>
</tr>
<tr>
<td>“</td>
<td>1</td>
<td>4</td>
<td>++ “</td>
</tr>
</tbody>
</table>

* See the foot note to Table I.
extracted with ether. The ethereal solution was washed, dried, and distilled.

4. Vigorous Acid Hydrolysis—In one experiment, Product B-4 from 20 liters of urine was taken up in 10 cc. of water. After the addition of 47 cc. of 2 N sulfuric acid, the mixture was autoclaved for 1 hour at 122° and 16 pounds pressure. After cooling, it was extracted with ether. The ethereal solution was washed thoroughly with water, dried, and distilled, giving 70 mg. of residue.

The products from the ether extracts of the various hydrolysis mixtures were not soluble in water. The doses shown in Tables II and III were 30 to 100 mg. The material was diluted with normal butyl succinate to 0.5 cc. for each animal. Each dose had a volume of 0.1 cc.

Results with Urine from Patients with Chronic Lymphoid Leucemia and Other Diseases—The method described for the purification and hydrolysis of the conjugated myelokentric acid was extended without change to material from the urine of patients with chronic lymphoid leucemia and Hodgkin's disease. The urine of one patient with monocytic leucemia was also used. The various preparations are shown in Tables I to III.

The residue from the extraction with 50 per cent ethanol was not active except in high doses, but the alcoholic solution from the precipitation showed activity (Table II). The hydrolysis of Product A from urine in lymphoid leucemia by Method 1 was incomplete. In one experiment, active material was recovered from the aqueous hydrolysis mixture after extraction with ether by adsorbing on benzoic acid (dose, 4.5 liters of urine). The conjugated material was thus not destroyed by heating with acid, but only partially hydrolyzed.

DISCUSSION

The purification method for the water-soluble materials, described above, is adapted from a procedure used by Gurin et al. (10) for the purification of gonadotrophic hormone. Extraction with 50 per cent ethanol effects an enormous purification, since nearly all the crude adsorbate remains in the residue. Thus, 200 liters of urine gave 55 gm. of Product A, and only 2 gm. of Product B-2.

After the hydrolysis of the water-soluble material with acid or alkali, activity was recovered in the acid fraction of the ether-soluble hydrolysis products, suggesting that myelokentric and lymphokentric acids were responsible for the biological effect of the water-soluble materials, in which they must exist as prosthetic groups. However, there was a diminution in activity after hydrolysis; this may be caused by the presence of additional water-soluble active substances, or merely by a poor yield in the hydrolysis of the conjugates. A poor yield in the hydrolysis of conjugated
products like pregnanediol glucuronide has been reported frequently (11). It is probable that the conjugated myelokentric acid is a more complex material, since it does not dialyze (8). However, the work reported here does not eliminate the possibility that other active water-soluble substances are present as well as conjugated myelokentric acid.

The production of lesions of the "Hodgkin's type" with mixtures of myelokentric and lymphokentric acids (1, 2) has been duplicated with the water-soluble materials. This confirms the results with the extracts of the urine of patients with lymphoid leucemia in indicating the existence of a conjugated lymphokentric acid. Our purification methods, like those of Heinle et al., were worked out with myelokentric acid and were then extended without change to lymphokentric acid. It is not surprising that lymphokentric acid has been found in rejected fractions, for example the alcoholic solution from the purified conjugate. Additional work is necessary with the object of finding a purification method adapted solely to the concentration of lymphokentric acid, free and conjugated.

SUMMARY

1. Myelokentric and lymphokentric acids, which cause the proliferation of cells of the leucopoietic system, occur in urine as the prosthetic groups of water-soluble conjugates.

2. The water-soluble conjugates have been partially purified and hydrolyzed.

BIBLIOGRAPHY

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