THE EFFECT OF CYSTEINE, HISTIDINE, AND METHIONINE ON THE PRODUCTION OF POLYCYTHEMIA BY COBALT*

BY JAMES M. ORTEN AND MARY C. BUCCIERO

(From the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit)

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The fact that cobalt administered daily in small amounts either orally or parenterally will produce a polycythemia is now well established. The polycythemia has been produced in a number of species of animals, including the rat, mouse, rabbit, dog, duck, and frog, and is characterized by an increase in the erythrocyte count and hemoglobin and hematocrit values without any significant alteration, either quantitatively or qualitatively, in the leucocytes (1). There is a distinct increase in the total blood volume due to an increase in the number of circulating erythrocytes, the plasma volume remaining essentially unaltered (2, 3).

The mechanism involved in the production of polycythemia by cobalt has received some attention. Previous work in this laboratory has indicated that there is some active stimulus to erythropoiesis, since a distinct reticulocytosis precedes the rise in the erythrocyte count (4). Barron and Barron (5) have suggested that cobalt may inhibit cellular respiration and thus produce a compensatory polycythemia for the purpose of increasing oxygen transport to the cells. In support of this hypothesis they have reported that the administration of ascorbic acid, allegedly involved in cell respiration, inhibits the production of polycythemia by cobalt in the rabbit. Other studies in this laboratory (6) add some indirect support to such a hypothesis by demonstrating that cobalt does not alter the oxygen-carrying capacity of hemoglobin nor does it form a “methemoglobin” in the rat. Thus if cobalt produces a compensatory polycythemia by interfering with the respiratory process, it must be the internal or cellular respiration which is affected rather than the external respiratory process.

The effect of several nitrogenous compounds on the action of administered cobalt in the animal organism has been investigated. Davis (7) reported that choline administered orally to dogs will completely inhibit the pro-

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duction of polycythemia by cobalt. This observation was not confirmed in this laboratory with the rat as the experimental animal (8). Griffith and his coworkers (9) have observed that cysteine, and to a lesser extent cystine, but not methionine, will greatly decrease the toxicity of orally administered cobalt in the rat as evidenced by growth response. However, they did not study the possible effects on hematopoiesis. These investigators attributed the toxic effect of cobalt to a "fixation of sulfhydryl compounds in tissues with resulting interference with oxidative mechanisms."

Another amino acid, histidine, has been investigated in connection with the toxicity of cobalt. Burk et al. (10) have observed that histidine decreases the toxicity of cobalt in certain bacteria and increases the growth and respiration of cobalt-treated microorganisms and cultures of various animal tissues. Burk et al. (11), as well as Michaelis (12), have found that cobalt forms a complex salt with histidine which combines irreversibly with oxygen. Thus, he also attributes the toxic effect of cobalt to an inhibition of cellular oxidation, perhaps by the formation of an oxygen-binding cobalt-histidine complex in the cell.

The purpose of the present investigation was to determine the possible effects of three of the previously mentioned substances, cysteine, methionine, and histidine, on the production of polycythemia by cobalt.

EXPERIMENTAL

Weanling, male, albino rats of the Connecticut Agricultural Experimental Station strain, weighing 40 to 50 gm., were used. They were housed in individual cages and were fed a synthetic basal ration having the following percentage composition: casein 20.0, sucrose 10.0, white corn dextrin 40.0, Crisco 25.7, Wesson's (13) salt mixture 4.0. Synthetic vitamin supplements were incorporated in the foregoing basal diet in the following amounts (in mg. per 100 gm. of diet): thiamine 1, riboflavin 2, pyridoxine 1, niacinamide 2, calcium pantothenate 4, inositol 200, p-aminobenzoic acid 60, folic acid 2, biotin 0.001, and 2-methyl-1,4-naphthoquinone 0.4. In addition, vitamins A, D, and E were supplied as haliver oil with viosterol fortified with α-tocopherol (100 mg. per 50 cc. of oil). 3 drops were administered individually to each rat twice weekly.

The rats were divided into five groups of ten animals each and were given supplements to the basal diet (per kilo of diet) as follows: (1) control, unsupplemented basal diet; (2) cobalt only (0.477 gm. of recrystallized CoSO₄·7H₂O); (3) cobalt plus cysteine (1.56 gm. or 4.68 gm. of L-cysteine hydrochloride); (4) cobalt plus histidine (6.25 gm. of L-histidine monohydrochloride); and (5) cobalt plus methionine 4.44 gm. of dl-methionine). The lower level of cysteine, 1.56 gm. per kilo of diet, is that found by Griffith and coworkers (9) to neutralize the toxic
effect of cobalt (equivalent to 0.477 gm. of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ per kilo of diet) in so far as response in body weight was concerned. The higher level of cysteine, 4.68 gm. per kilo of diet or 3 times the lower level, was used to determine whether a further effect might be obtained from the increased amount. The levels of methionine and of histidine are isomolar with the higher level of cysteine. All animals, with the exception of the controls, received cobalt as cobalt sulfate in an amount (0.477 gm. per kilo of diet) providing approximately 1.0 mg. of cobalt per rat per day, an amount found in earlier studies capable of producing a definite polycythemia.

In order to evaluate the possible effect of cysteine and histidine on the absorption of cobalt from the gastrointestinal tract, as will be discussed later, three additional groups of rats, of ten to fifteen animals each, in which cobalt with cysteine or histidine was given parenterally, were studied. One group (control) was injected with approximately 0.5 mg. of cobalt daily (1 cc. of an aqueous solution containing 250 mg. of recrystallized $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ per 100 cc.). The second group was given an equivalent amount of cobalt as the cobalt-cysteine complex (1 cc. of a solution containing 250 mg. of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ $+ 500$ mg. of l-cysteine hydrochloride $+ 240$ mg. of $\text{Na}_2\text{CO}_3$ per 100 cc.). The third group was given the same amount of cobalt as the cobalt-histidine complex (1 cc. of a solution containing 250 mg. of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ $+ 400$ mg. of l-histidine monohydrochloride $+ 175$ mg. of $\text{Na}_2\text{HCO}_3$ per 100 cc.). The solutions were injected subcutaneously in each case. The dosage of cobalt employed in these groups, 0.5 mg. of Co per day, was purposely reduced to approximately half that given orally to the preceding groups in order to compensate, partially at least, for the poor absorption of cobalt from the gastrointestinal tract (14). The amount of cysteine hydrochloride used in the preparation of the cobalt-cysteine complex is slightly in excess of the ratio 1:3, which Michaelis and Barron (15) have shown to be the combining ratio in the complex. Similarly, the amount of histidine monohydrochloride used in preparing the cobalt-histidine complex was slightly in excess of that required for a ratio of 1 part of cobalt to 2 of histidine (Michaelis (12)). In each case, the amount of $\text{Na}_2\text{HCO}_3$ added was slightly in excess of that needed to neutralize the hydrochloride of the amino acid preparation used. This also served to adjust the pH of the solutions to values at which the desired cobalt complexes form and at which irritation of the tissues by the injected solution was minimized.

Body weights and food consumption were determined weekly on the various groups of animals, and hemoglobin levels were determined bi-weekly. Hemoglobin was determined on blood obtained from a tail vein by an acid-hematin method with the Coleman spectrophotometer, previously calibrated by the oxygen capacity method. The animals were
followed for periods varying from 12 to 20 weeks, as stated in Tables I and II.

RESULTS AND DISCUSSION

The results obtained are summarized in Tables I to IV. Table I gives the average body weight for the five groups of rats given oral supplementa-

### Table I

Average Body Weights in Gm. of Control Rats and of Rats Fed Cobalt Alone or Supplemented with Cysteine, Histidine, or Methionine

<table>
<thead>
<tr>
<th>Group (10 rats each)</th>
<th>Initial weight</th>
<th>Wks. on experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44</td>
<td>124, 165, 258, 325, 376, 402, 463, 493, 517, 526, 542</td>
</tr>
<tr>
<td>Cobalt</td>
<td>45</td>
<td>77, 98, 135, 176, 205, 237, 269, 289, 309, 326, 347</td>
</tr>
<tr>
<td>&quot; + cysteine Low</td>
<td>42</td>
<td>70, 110, 166, 218, 256, 288, 333, 364, 380, 374, 395</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>67, 91, 163, 237, 289, 334, 370*</td>
</tr>
<tr>
<td>Cobalt + histidine</td>
<td>43</td>
<td>66, 98, 155, 199, 235, 280, 366, 332, 347, 366, 382</td>
</tr>
<tr>
<td>&quot; + methionine</td>
<td>42</td>
<td>92, 134, 218, 261, 293, 333, 359, 375, 394, 410, 425</td>
</tr>
</tbody>
</table>

* Group discontinued.

### Table II

Average Hemoglobin (Gm. Per Cent) Values for Control Rats and for Rats Fed Cobalt Alone or Supplemented with Cysteine, Histidine, or Methionine

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial</th>
<th>Wks. on experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.5</td>
<td>12.5, 13.2, 14.7, 15.8, 15.2, 15.7, 15.6, 15.6, 15.5</td>
</tr>
<tr>
<td>Cobalt</td>
<td>11.5</td>
<td>12.5, 15.5, 16.0, 17.3, 18.6, 19.0, 19.6, 19.6, 19.7, 20.4</td>
</tr>
<tr>
<td>&quot; + cysteine Low</td>
<td>10.6</td>
<td>14.4, 15.7, 16.5, 17.3, 17.6, 17.0, 17.7, 17.6, 17.3</td>
</tr>
<tr>
<td>High level</td>
<td>10.9</td>
<td>13.5, 14.3, 15.0, 16.3, 16.7, 17.1*</td>
</tr>
<tr>
<td>Cobalt + histidine</td>
<td>11.3</td>
<td>14.0, 16.0, 16.5, 16.8, 17.9, 18.2, 17.9, 18.2, 18.2</td>
</tr>
<tr>
<td>&quot; + methionine</td>
<td>10.5</td>
<td>12.9, 16.6, 17.2, 17.5, 19.2, 19.8, 19.5, 19.9, 19.9, 19.9</td>
</tr>
</tbody>
</table>

* Group discontinued.
control animals. The rats given the higher level of cysteine showed somewhat higher average body weight during the 12 weeks they were observed than did the animals receiving the lower dosage. Such a result was not unexpected.

The average data for the daily food consumption (not given in the tables) for the various groups, indicated that the cobalt-treated rats ate more food per 100 gm. of body weight than did the control animals. The average group values for the 20th week of the study were as follows (gm. of food intake per day per 100 gm. of body weight): controls 2.5, cobalt only 3.4, cobalt + cysteine 3.2, cobalt + histidine 3.6, cobalt + methionine 3.2. These data suggest that cobalt decreases the retention or utilization of some dietary constituent or constituents, since the animals ingested more food per unit (100 gm.) of body weight. An improvement in "food utilization" evidently occurred in the rats given either cysteine, methionine, or histidine as a supplement to cobalt. It is interesting that the above values for food intake correspond, inversely, with the terminal average body weights shown in Table I.

Table II gives the average biweekly hemoglobin values. The controls show the normal progressive increase with age, reaching a constant adult level of about 15.6 gm. per cent. The cobalt-fed rats, on the other hand, developed a typical polycythemia as evidenced by the final hemoglobin value of 20.4 gm. per cent. The addition of cysteine, particularly at the higher level, lessened the increase of the hemoglobin values above the controls, as did histidine to a lesser extent. Methionine, on the other hand, had no noticeable effect. This was rather surprising in view of the favorable effect of methionine on the growth and food utilization of the cobalt-fed animals. A statistical analysis of the data, Table III, shows that the effects of cysteine and, to a lesser extent, of histidine, are highly

<table>
<thead>
<tr>
<th>Group</th>
<th>Average hemoglobin</th>
<th>Standard deviation</th>
<th>Probable error of mean</th>
<th>Probable error of difference between means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.6</td>
<td>±0.17</td>
<td>±0.08</td>
<td></td>
</tr>
<tr>
<td>Cobalt</td>
<td>19.7</td>
<td>±1.23</td>
<td>±0.24</td>
<td></td>
</tr>
<tr>
<td>&quot; + cysteine, low level</td>
<td>17.6</td>
<td>±1.60</td>
<td>±0.32</td>
<td>±0.40</td>
</tr>
<tr>
<td>&quot; + histidine</td>
<td>18.2</td>
<td>±1.11</td>
<td>±0.23</td>
<td>±0.33</td>
</tr>
<tr>
<td>&quot; + methionine</td>
<td>19.9</td>
<td>±1.23</td>
<td>±0.25</td>
<td>±0.35</td>
</tr>
</tbody>
</table>

* The values are those for the 18th week of the experiment.
† Comparison made with group given cobalt alone as the supplement.
significant, whereas that of methionine is not, as is also evident from a gross inspection of the data. Since cobalt forms insoluble complexes with cysteine and with histidine, it appeared possible that these two substances might prevent the production of polycythemia by cobalt by merely decreasing the absorption of cobalt from the gastrointestinal tract. Therefore, three additional groups of rats were studied. They received subcutaneously 0.5 mg. of cobalt, either as cobalt sulfate or as the cobalt-cysteine or cobalt-histidine complex each day for a period of 12 weeks. As was found in the group of rats given oral supplementation, the injection of cobalt sulfate decreased the rate of growth (data not included) as compared with that of the controls. Much less inhibition of growth was observed in the groups given the cobalt-cysteine or cobalt-histidine complex, particularly the former. The data on hemoglobin are recorded in Table IV. It is evident that injected cobalt sulfate produced a polycythemia, whereas the cobalt-cysteine complex in an equivalent amount of cobalt did not increase the hemoglobin level significantly above that of the controls. The average hemoglobin values of the group receiving the cobalt-histidine complex, on the other hand, differed little from that to which cobalt sulfate was given and a typical polycythemia resulted. This observation is in general agreement with that in the group given cobalt with histidine orally, although some inhibition of the production of polycythemia by cobalt was observed in the latter case. This may be due to the fact that the ratio of histidine to cobalt was much greater in the orally supplemented group than was possible in the injected group. These data therefore indicate that the inhibition of the polycythemia by cysteine because of a possible impairment of cobalt absorption from the gastrointestinal tract cannot be a determining factor, since cobalt bound as a cysteine complex and administered parenterally in an amount comparable to that given orally likewise does not produce a polycythemia.

Table IV

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Wks. on experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>13.3</td>
</tr>
<tr>
<td>CoSO₄</td>
<td>10</td>
<td>13.5</td>
</tr>
<tr>
<td>Cobalt-cysteine complex</td>
<td>10</td>
<td>14.3</td>
</tr>
<tr>
<td>Cobalt-histidine complex</td>
<td>15</td>
<td>13.2</td>
</tr>
</tbody>
</table>

* Daily injections omitted for 5 days during the 7th week.
† The cobalt-histidine complex solution injected during this interval did not contain added NaHCO₃, as described for the preparation of this solution in the text.
At least two explanations of the effect of cysteine and, to a lesser extent, histidine in preventing the production of polycythemia by cobalt appear possible. One is that these two substances, by forming complex compounds, may increase the excretion of cobalt and thus lessen its ability to produce polycythemia. However, such an explanation seems rather unlikely, because other substances such as choline and, under certain conditions, methionine also may form complexes with cobalt analogous to those of cysteine and histidine and thus presumably likewise increase cobalt excretion in the urine. Furthermore, under ordinary circumstances the excretion of parenterally administered cobalt is rapid and almost complete within 36 hours (14). Moreover, Stare and Elvehjem (16) have shown by analysis that at the height of the polycythemia there are present only 40 to 50 $\gamma$ of cobalt in the entire body of the rat.

Another explanation, the more likely in our opinion, is that the administered cysteine, and possibly also histidine, combines with cobalt to form insoluble or inert complexes in the organism, thus preventing its subsequent "blocking" of sulfhydryl and perhaps other groups active in cellular respiration, which, in turn, would prevent the development of a compensatory polycythemia. Such an interpretation is in accord with the observation (Burk, personal communication) that "the relative affinities of cobalt for the naturally occurring amino acids are, in decreasing order, cysteine, histidine, and then the others." Cobalt also has a relatively low affinity for choline. This would thus satisfactorily explain the failure of methionine and choline to prevent the production of polycythemia by cobalt. However, further work will be required to answer these questions in a positive manner.

**SUMMARY**

The oral administration of 1.0 mg. of cobalt as cobalt sulfate to rats fed a synthetic basal diet produces a sustained polycythemia, an inhibition of growth, and impairment in food utilization.

Supplementation of the cobalt-containing diet with cysteine inhibits the production of the polycythemia. Histidine has a similar effect but to a lesser extent. Methionine has no detectable effect when fed in equivalent amounts.

Parenterally administered cobalt sulfate (0.5 mg. of cobalt per day) likewise produces a marked polycythemia, whereas an equivalent amount of cobalt as the cobalt-cysteine complex does not. Histidine injected with cobalt as the cobalt-histidine complex has less effect in preventing the development of polycythemia.

It is proposed that cysteine inhibits the production of polycythemia by cobalt by the formation in vivo of an inert cobalt-cysteine complex. Hist-
dine may act in a similar manner but the cobalt-histidine complex is more active (less completely formed) than the cobalt-cysteine complex at the pH values existing in the animal organism.

The suggestion is made that cobalt may produce polycythemia by binding sulfhydryl or perhaps other groups active in cellular respiration, thus leading to a simulated cellular anoxia and, in turn, to a compensatory polycythemia.

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