INORGANIC ELEMENTS OF SPINACH IN THE TREATMENT OF NUTRITIONAL ANEMIA.*

BY HELEN S. MITCHELL AND LILA MILLER.

(From the Nutrition Research Laboratory, Battle Creek Sanitarium and College, Battle Creek.)

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Within the last 18 months there have developed two theories concerning the inorganic factors which influence hematopoietic processes. (1) In rats on a basal diet of milk and iron, supplementary copper alone will stimulate a maximum hemoglobin production. (2) On such a diet a group of elements including copper produces a maximum hemoglobin response while copper alone is slightly less effective. By comparing the effect of liver extract, H₂S fraction of the extract, and copper sulfate solutions on hemoglobin of rats, Hart and coworkers (1) have concluded that in the milk-iron diet inorganic copper is the only deficiency. However, Titus and coworkers (2, 3) have found that the rapidity of hemoglobin regeneration is greatly increased by the addition of copper-free manganese chloride to the milk-iron-copper diet and that there is a storage in the body of both copper and manganese which is later effective in the utilization of iron. Recently Myers and Beard (4) have reported that nickel, cobalt, and germanium as well as copper are effective in shortening the hemoglobin regeneration period.

In attempting to determine whether elements other than iron and copper influence hematopoietic function, it occurred to us that a clue to the proper balance of inorganic constituents might be found in the combination of minerals naturally present in a food generally conceded to be a good blood builder. If a material of natural source which supplies 0.5 mg. of iron and small quantities of other salts would bring about the regeneration of hemo-

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globoin in a shorter time than would equivalent quantities of iron and of copper in the form of pure salts, it would appear that other factors must be involved in this metabolic process.

For this purpose a concentrated water extract of dried spinach\(^1\) was used and the results of this work are presented in this paper.

**EXPERIMENTAL.**

In order to make the proper dilutions for feeding the extract in quantities which would supply 0.5 mg. of iron, the iron content was determined. At the same time quantitative determinations of the copper and manganese were made, it being realized that at least one and possibly both were significant. The ratio of the iron, copper, and manganese was found to be 39:1.1:1, respectively.

Young rats in which a condition of anemia had been induced by limiting the mother's diet to milk and wheat germ for several months, as has been described in previous publications from this laboratory (5, 6), were used when about 4 weeks of age. At that time, supplements were added to the whole milk diet unless the hemoglobin content of the blood was not sufficiently low. In that case, the mineral supplement was withheld for several weeks or until the hemoglobin readings were 6 gm. per 100 cc. of blood or below. Negative controls from each litter were maintained on milk alone or milk and pure iron salts.

Weekly weight and hemoglobin records were kept. Growth was always retarded on the milk ration but approached the normal in all animals receiving adequate mineral supplement. Variations in the weight curves were quite irrelevant to the type or amount of minerals added. For hemoglobin determinations, the regular acid hematin method with a Bausch and Lomb hemoglobinometer was followed. However, to insure maximum color development, the acid hematin samples were heated for 7 minutes by being placed in a water bath at 55–60\(^\circ\) and then were cooled to room temperature.

The results of some earlier work with spinach extract had shown that animals receiving this extract in doses supplying 0.8 mg. of iron recovered very rapidly. In order to ascertain whether the rapid recovery had been due to the excessive quantity of iron administered or to presence of other elements, investigation of

\(^1\) Prepared by the Battle Creek Food Company.
smaller dosages was made. A litter of rats was divided into two
groups. As daily supplement (6 days per week) those rats in
Group I were fed a quantity of spinach extract that furnished
0.5 mg. of iron and those in Group II were given but half that
dosage. Since 0.25 mg. of iron is below the quantity that has
been considered an optimum in most studies, it was expected that
the recovery of the animals in Group II would be slower than for
those of Group I. But as may be seen in composite Table I, it
required but 1 week longer for the hemoglobin of the animals in

| TABLE I. | Average Hemoglobin Levels at Time Supplement Was Added and at Intervals
|          | Thereafter, of Animals Receiving 0.5 and 0.25 Mg. of Fe from
|          | Spinach Extract, Ash, and HCl Solution of Ash.
|          | Figures represent gm. of hemoglobin per 100 cc. of blood.

<table>
<thead>
<tr>
<th>At time of addition.</th>
<th>0.5 mg. Fe from spinach extract</th>
<th>0.25 mg. Fe from spinach extract</th>
<th>0.5 mg. Fe from ash of extract</th>
<th>0.25 mg. Fe from ash of extract</th>
<th>0.5 mg. Fe from HCl solution of ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 wk.</td>
<td>5.9 (7)*</td>
<td>6.0 (4)</td>
<td>4.2 (4)</td>
<td>4.2 (5)</td>
<td>4.4 (5)</td>
</tr>
<tr>
<td>2 wks.</td>
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<td>3 wks.</td>
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<td>4 wks.</td>
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<td>5 wks.</td>
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<tr>
<td>6 wks.</td>
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<td>7 wks.</td>
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<td>8 wks.</td>
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* The figures in parentheses indicate the number of animals used in
arriving at the average figure.

Group II to regenerate, as compared to the animals of Group I,
the average being 2.1 and 3 weeks, respectively.

As a means of comparison, 14 gm. of hemoglobin per 100 cc. have
been chosen as an indication of a fair degree of regeneration
although not as high as for our normal rats. The hemoglobin
levels reached in most of the animals were well above this figure.
For simplicity, we shall speak of it as having been regenerated
when the hemoglobin has increased to 14 gm. or more per 100 cc.
of blood.

The question arose of whether an organic factor might be
CHART I. Hemoglobin response of rats receiving a milk ration plus mineral supplement in the form of spinach extract or its ash. The broken line is used for the period on milk alone. 14 gm. of hemoglobin per 100 cc. of blood were chosen as indicative of a fair degree of blood regeneration although below the optimum.
involved; hence, the first step was the destruction of the organic matter by means of ashing the spinach extract and the subsequent feeding of this ash in graded quantities (0.5 mg. and 0.25 mg.). Since this ash was insoluble in water or milk, it was necessary that great care be exercised in feeding it. Complete consumption was secured by repeatedly mixing the ash with small amounts of milk until the dish had been licked clean before feeding larger quantities of milk.

The rats receiving 0.5 mg. of iron in the form of ash were much slower in responding than those receiving an equivalent quantity of the spinach extract, and in fact slower than those receiving but one-half dose of the extract, 6 and 9 weeks being required for regeneration, as compared with 2 and 3 weeks previously mentioned. It was also noted that the hemoglobin content of the animals receiving but 0.25 mg. of iron as the ash either did not reach or exceed the level of 14 gm. during the whole experimental period of 19 weeks, whereas on an equivalent quantity of the extract the hemoglobin attained levels of 18 or 19 gm. per 100 cc. of blood.

The much slower response of those rats receiving the ash supple-
Spinach Effect on Hb Building

ment might be taken as indicative of an organic factor involved in the spinach extract. Previous experiences, however, had suggested that the insoluble nature of the ash might alone be responsible for the longer regeneration period. Therefore, a solution was prepared by digesting the ash with dilute hydrochloric acid until completely dissolved which after further dilution could be fed in quantities supplying 0.5 mg. of iron.

The difference in the potency of the ash and the solution of the ash was very marked, the time for regeneration being 2 to 3 weeks longer for the ash than for the solution, whereas there was much less difference in the potency of the original spinach extract and the solution of the ash (Chart I). Thus from these preliminary data accumulated on twenty-six animals, we feel warranted in concluding that the potency of the hemoglobin-building material in spinach extract is increased by being in solution and that the active principle in the spinach extract must be of inorganic nature.

The quantities of copper and manganese carried by the extract
were 0.014 and 0.012 mg., respectively, corresponding to 0.5 mg. of Fe and 0.007 and 0.006 mg. corresponding to 0.25 mg. of Fe. In all the work on the supplementary value of copper and manganese salts, 0.05 mg. was the standard dose and the average hemoglobin levels of rats receiving these salts are shown in Table II. In a group of twenty-five animals receiving iron and copper the shortest regeneration period was 3 weeks, the longest 12 or more, and the average about 8 weeks. This recovery period averaged 4 times longer than that of the animals receiving iron from the spinach extract and $2\frac{1}{2}$ times longer than those receiving the HCl solution of the spinach extract ash. When copper and manganese together were fed with the iron to nine rats, the recovery period was somewhat shorter than with copper only (Chart II). The HCl solution of spinach extract ash, however, is still better than any combination of pure salts yet tried.

A qualitative analysis of the extract has been made and the following elements were found to be present: copper, antimony, tin, iron, aluminum, zinc, manganese, strontium, sodium, potassium, calcium, magnesium, and phosphorus. The quantitative relations of these elements and their significance in hemoglobin building is being studied further. In order to determine whether these metals are normally present in spinach or whether the vessels and cans used in the course of preparing and storing the product are the source of some of the elements, the ash of local New Zealand spinach has been subjected to a similar analysis. Antimony, tin, strontium, and zinc were not present in this spinach.

**DISCUSSION.**

The rapid increase in the hemoglobin content of the blood of animals receiving but 0.25 mg. of iron from the extract indicates that the daily iron requirement may be less than 0.5 mg. in the presence of other metals such as copper, manganese, etc. Of course, in the early work on nutritional anemia, when the customary iron dosage was established as 0.5 mg. the rôle of copper and other elements was unknown. Small traces of these metals in the iron salts or the storage of these elements in the body may account for the results obtained by using that quantity of iron.

2 The assistance of Russell B. Cooper in checking these analytical data is hereby acknowledged.
There is a marked difference in the potency of equal quantities of the ash depending upon whether it is administered in the form of a solid or in the form of a solution, the latter showing distinctly better utilization. The very slow response of the animals on even half dosage of ash must, however, be attributed to a partial utilization of insoluble material rather than to any spontaneous recovery since such did not occur in the control group. It is theoretically probable that a portion of this ash may become dissolved during the process of digestion and thus become available.

When 0.05 mg. of copper was used as a supplement to the milk-iron diet, the recovery of the animals was slower than for either dosage of spinach extract or for the HCl solution of the ash. This quantity of copper is about 4 and 8 times as great respectively as that supplied by the two dosages of extract in its various forms. According to the data recently reported by Waddell, Steenbock, Elvehjem, and Hart (1) there is a shortening of the regeneration period with an increased copper dosage. If copper is the only deficiency in the basal milk-iron diet it is difficult to explain why the smaller dosages of copper in the extract should be more effective than the larger doses of copper as the sulfate.

Furthermore, the increased response on a combination of copper and manganese over and above that of copper alone is another point which may add weight to the theory that there is a group of elements that play a role in the regulation of hemoglobin building.

Robsheit-Robbins and coworkers (7) have reported that they have been unable to duplicate with artificial salt mixture the balance of inorganic salts in liver, kidney, and apricot ashes, which are effective in hemoglobin building. Furthermore, McHargue (8) has shown that iron, copper, manganese, and zinc as well as phosphorus, calcium, potassium, and sodium are contained in both cow’s blood and calf’s liver. All of these elements and additional ones are present in the spinach extract studied. It seems possible that at least some of the other elements present may have an influence as well as the iron and copper and that there may be an optimum balance of inorganic salts for hemoglobin regeneration. Further studies of this phase of the problem are now under way.
SUMMARY.

Spinach extract when used as a supplement to a milk diet for anemic animals in such quantities as to supply 0.5 mg. of iron, 0.014 mg. of copper, and 0.012 mg. of manganese or in half that quantity proved to be more potent than an iron-copper or iron-copper-manganese complex of pure salts in the building of hemoglobin.

Salts in solution are more effective than in the insoluble form as equal quantities of the spinach extract ash in these two states showed very marked differences in hematopoietic powers.

The analysis of the extract shows that Cu, Sb, Sn, Fe, Al, Zn, Mn, Sr, Ca, Mg, Na, K, and P are present.

Experimental data indicate that the daily iron requirement for a rat is less than 0.5 mg. if properly supplemented and that rather than copper alone there is a group of elements that is active in hemoglobin building.

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