# THE NATURE OF THE MATERIAL IN LIVER EFFECTIVE IN PERNICIOUS ANEMIA. II.*

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## INTRODUCTION.

Advances in practice, resting upon observation and experience, often precede exact knowledge of the underlying reactions and mechanisms of natural phenomena. Such an advance was the demonstration by Minot and Murphy (12–14) that the feeding of large amounts of liver is followed by an increase of concentration of red cells in the blood in cases of pernicious anemia. This discovery raises the question of the nature of the substance, or substances, which are thus effective, as well as that of the character of the disturbed physiological processes which are modified by the addition of liver or kidney to the diet.

Accordingly the chemical dissection of the liver has been undertaken as a means of eliminating, one by one, those of its constituents not involved in producing the prompt acceleration of blood formation (4). In the absence of any hypothesis regarding the

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nature of the active principle the empirical procedure, now to be described, was resorted to. The decision was made always to divide the liver into the smallest possible number of fractions, to discover which one of these fractions was most active, and to continue by subdividing the active fraction.

The facts that were relied upon to indicate whether or not the active constituent, or constituents, of the liver had been extracted, were furnished by clinical observations. Particularly important is the observation that the feeding of adequate amounts of liver to patients with pernicious anemia in relapse is regularly followed by a prompt and marked rise in the concentration of red blood cells, for the most part distinguished by their reticulation (15). Although our preparations had to be fed to patients with pernicious anemia in order to determine whether or not they were effective, the results were rarely ambiguous, and proved to be of a semiquantitative nature. This was due, in part, to the experience previously gained by Minot and Murphy while treating pernicious anemia with whole liver. These earlier observations on the increasing concentration of red blood cells in blood, together with those made while feeding the extracts, lend themselves to quantitative formulation, since the reactions initiated by the active principle take place in a very large number of cells. Many processes not yet entirely understood are involved; they occur within the human body, and they are occasionally modified by the state of the patient and by complications of the disease. Thus a certain variability is introduced into the results. The body must be considered the environment in which the reactions with which we are concerned take place. These reactions result in the rapid and prompt formation of several hundred thousand red blood cells per c.mm. of blood, following the ingestion of a small amount of material. Although the mass of the material is small, it contains, of course, a large number of molecules of the active substance. Because of the large numbers of cells and molecules involved there is reason to believe that the results may be successfully subjected to statistical treatment.

*Erythrocyte and Reticulocyte Response.*

In recording the response of the patient's red blood corpuscles in quantitative terms the following algebraic symbols have been

employed. The concentration of red blood corpuscles, or erythrocytes, has been designated by the symbol $E$, expressed as millions per c.mm. $E_0$ represents the number of red blood corpuscles per c.mm. in the capillary blood of a patient before the administration of a fraction of liver. This figure was obtained from a series of observations. The difference, $(E_{10} - E_0)$, thus represents the net increase in the number of red blood corpuscles per c.mm. in the capillary blood during the first 10 days of feeding.

The percentage of erythrocytes that are reticulated corpuscles is designated $r$. The concentration of reticulocytes present in the blood on any day is given by the product $Er$. In so far as there has been no destruction of red blood cells, no change in the distribution of body fluids, no maturation of reticulocytes to adult red blood cells, and no entrance into the circulation of non-reticulated erythrocytes, it must follow that:

$$Er = (E - E_0) \tag{1}$$

In the records of the cases presented, and in their graphical representation, both the quantities $Er$ and $(E - E_0)$ are often given.

Within the first few days after feeding of the extract the quantity $(E - E_0)$ frequently became negative, indicating either an increase in volume of the fluid at the point of removal or a destruction of red blood cells. This period rarely exceeded the first 4 or 5 days following the feeding of a potent fraction. Thereafter both $(E - E_0)$ and $Er$ increased. The amount of the increase in total erythrocytes continued at a comparable rate, but the number of reticulocytes diminished. Apparently maturation of the reticulocytes that first appeared had begun in the blood, and most of the newly formed erythrocytes had had time to mature beyond the reticulated stage before their extrusion from the bone marrow. The largest number of reticulocytes appeared in the blood in from 5 to 10 days, depending in part upon the potency of the extract. Within a subsequent 5 to 10 day period the reticulocytes had generally decreased to a low level in the blood stream. It would appear to be a fair deduction from these observations that the reticulocytes in the blood did not lose the reticular material, upon which their vital staining depends, for from 5 to 10 days. The rate at which reticulocytes lose the material that identifies them may however be a

function of the concentration in the blood of the active principle effective in pernicious anemia.

The algebraic notation adopted in this paper had not been devised when these observations were begun. Accordingly the importance of daily measurements, not only of the percentage of reticulocytes, $r$, but also of the concentration of erythrocytes, $E$, was not understood. The percentage of reticulocytes is, however, as elsewhere demonstrated (11, 14), largely a function of the level of the red blood cell count. The number of reticulocytes is more nearly independent of the red blood cell concentration, revealing the potency of the extract, although this quantity still shows a definite variation with the red blood cell level, and a lesser variation between cases with the same erythrocyte concentration. The clinical experience derived from the feeding of liver extract is discussed in another paper (11).

The erythrocyte level has occasionally been estimated on days when it was not measured, by graphical interpolation, in order to permit the calculation of the number of reticulocytes, $Er$. The error in estimating the percentage of reticulocytes is usually not greater than 3 per cent, whereas the error in counting the number of red blood cells is frequently as great as 150,000 per c.mm. or more. The number of erythrocytes formed within the first few days generally ranges from 300,000 to 500,000 per c. mm., whereas the percentage of reticulocytes may increase from a fraction of 1 per cent to 50 per cent, or more. It follows that a slight error in the estimation of $E$ by interpolation will introduce no great error in the product $Er$.

Another method may be suggested for estimating the erythrocyte concentration, within the first few days, from the reticulocyte concentration. Provided the only source of the increase in erythrocytes is through the formation of reticulocytes, it follows from equation (1) that:

$$E = E_0 + Er \tag{2}$$

Transposing, we have the relation:

$$E = \frac{E_0}{(1 - r)} \tag{3}$$

and

$$Er = \frac{E_0\, r}{(1 - r)} \tag{4}$$

Combining equations (1) and (4) we obtain:

$$Er = \frac{E_0\, r}{(1 - r)} = E - E_0 \tag{5}$$

These conditions can only be expected to obtain provided there has been no significant destruction of red blood cells, nor change in their concentration as a result of redistribution of body fluids. In fact not only must these conditions obtain, but during the length of time that these three equations yield identical results there can have been no appreciable change of the reticulocytes to adult cells in the blood, nor delivery of adult forms from the bone marrow. In the majority of cases these quantities have not been identical, but their relations to each other are none the less significant.

Accurate information as to whether an extract was effective was usually revealed by the reticulocyte count within a week. Information of comparable accuracy could not have been gleaned from the erythrocyte count in less than from 2 to 3 weeks.

The change in the concentration of reticulocytes and of erythrocytes in the blood of patients with pernicious anemia that were fed various fractions of beef or pig liver may now be considered. These responses revealed something of the nature of the disturbed reactions in the blood in pernicious anemia, whereas the chemical fractionation yielded information regarding the specific substances which initiated the responses.

*Chemical Fractionation.*

*Insoluble Residue* (*A*).—The first fractionation of the liver was attempted in collaboration with Dr. John F. Fulton. It was decided as a beginning to isolate the characteristic copious casein-like liver protein. This protein is soluble in alkaline solution, but insoluble in the neighborhood of pH 5. Accordingly fresh minced beef liver was rendered alkaline by the addition of sodium hydroxide. Since Bradley (3) has shown that the liver enzymes are rela-

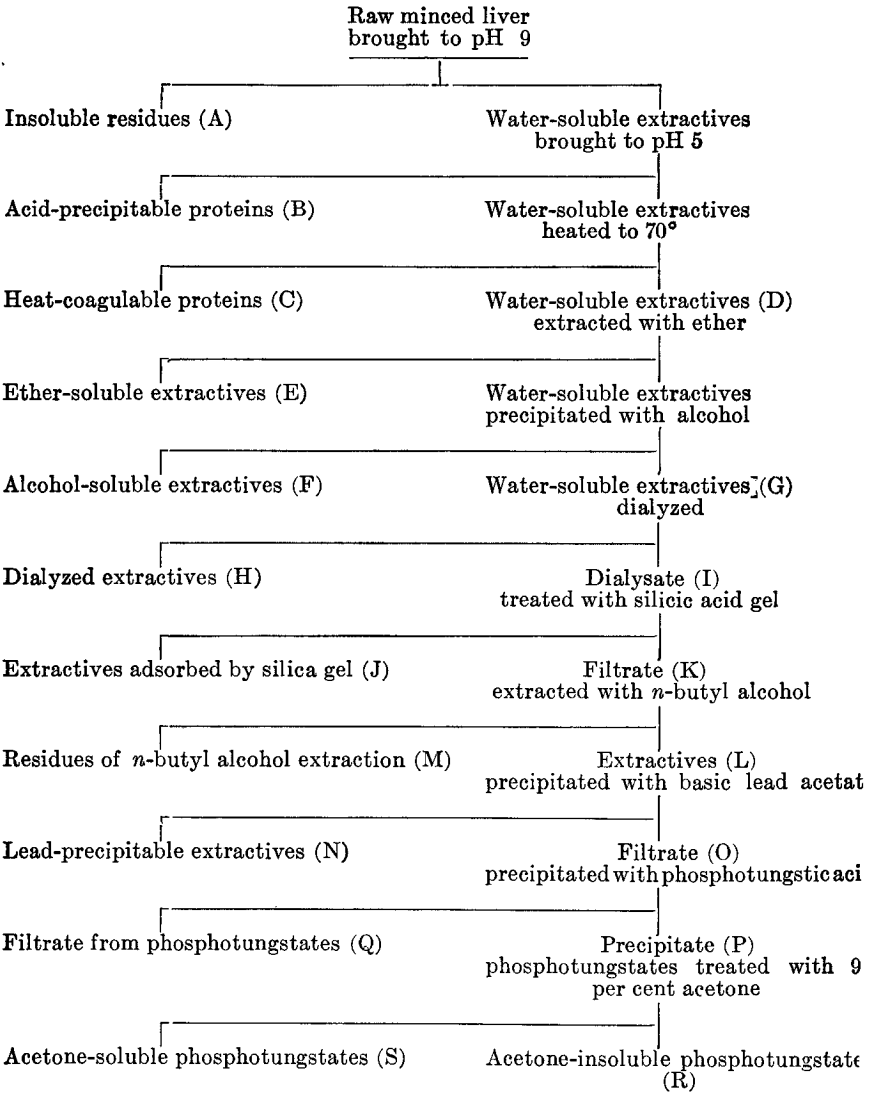
tively inactive at pH 9, enough alkali was added to bring the tissue to this reaction, as it seemed wise to effect a separation under such conditions that the liver would be as little altered as possible during the procedure. The soluble extractives were then separated from the insoluble residues. These fractions and those subsequently prepared have for convenience been designated by letters essentially in the order in which they were attempted. Their relation to each other is represented in the accompanying diagram. The procedures represented are not equally useful. Some are now rarely used, and all have not so far been employed in any one preparation.

The residues (A) were subsequently fed, together with the acid-precipitable proteins (B), to a patient with pernicious anemia, without measurably influencing blood formation. The fraction designated (A) was thus shown not to contain any significant amount of the active principle. Besides connective tissue this residue contains such insoluble proteins, fats, and carbohydrates as are present in liver.

*Characteristic Liver Protein (B).*—The characteristic soluble liver protein represents the most abundant constituent of the filtrate. The liver protein that dissolved with the soluble extractives at pH 9 was next precipitated in the neighborhood of its isoelectric point. This was at first accomplished by adjusting the reaction nearly to pH 5 by hydrochloric acid, and at a later date by sulfuric acid. The copious flocculent precipitate that separates under these conditions may be removed by sedimentation, centrifugation, or filtration from the other soluble extractives. All three methods have been employed at one time or another. The protein precipitate was always repeatedly washed. In one preparation it was further purified by redissolving with alkali and reprecipitating with acid.

This protein, fraction (B), was fed to Patient 1 for 13 days, with negative results. A few days later when he was given 200 gm. of raw liver pulp daily he responded in the customary manner with a prompt increase in the concentration of reticulocytes and of erythrocytes.

Since the active principle was in neither fraction, (A) or (B), it was later decided to eliminate the preliminary alkaline extraction. In subsequent preparations the minced liver was immediately




brought to the apparent isoelectric point of the liver protein by means of acid, and the water-soluble substances repeatedly extracted from the copious precipitate consisting of certain proteins, carbohydrates, and lipoids of the liver. Approximately

40 cc. of normal acid sufficed to bring 1 kilo of minced liver to the desired reaction. The resulting precipitate was repeatedly washed by sedimentation and centrifugation. This process was carried out in the cold with the result that hydrolysis of carbohydrates and proteins was inhibited. In one case the precipitate, (A + B), after five successive extractions with 3 volumes of water, was fed to a patient. The benefit resulting to the patient and the increase of reticulocytes were so slight as to indicate that practically complete extraction of the active principle from the liver was possible. In practice it has not always been expedient to extract so completely.

Although the results with the liver protein (B) and with the combined precipitates (A + B) were negative, they indicated that large portions of the constituents of liver are inert, and that the active principle was soluble in water.

*Heat-Coagulable Proteins* (*C*).—The extractives soluble at slightly acid reactions still contain protein which coagulates upon being heated to from 60–70°. These proteins appear to be derived in large part from the blood contained in the liver, rather than from the tissue itself, and represent the albumins and globulins of the blood, together with any similar constituents of the liver. Accordingly their study was postponed until after that of the essentially protein-free filtrate obtained from the coagulum.

*Water-Soluble Extractives* (*D*).—The non-protein extractives dissolved in the filtrate from (C) had reached so large a volume that concentration was resorted to. The wash waters of the second preparation were therefore concentrated on a water bath. A part of the material was rendered insoluble during this process, but as much as dissolved was fed to a patient. The record of this case is in part presented in Table I. Feeding of this extract was promptly followed by increase of the reticulocyte concentration, though to a smaller extent than when a maximal amount of potent material is given. A second and much larger reticulocyte response followed the daily feeding of liver pulp, clearly indicating that the extract had been weak.

This first response, though incomplete, led to the belief that the active principle had been extracted, but had been largely destroyed during the prolonged concentration at high temperature. Accordingly a vacuum still was constructed and employed in concentrat-

ing the water-soluble extractives of Preparations IV to XVII. The temperature was maintained at 60°, since it had been shown that this temperature did not rapidly destroy the active principle. The resulting concentrate (D) has been prepared as a solution, as a viscous syrup containing but 25 per cent of water, and as a dry powder. It was fed to Patients 3, 4, and 5, with the results recorded in Table II.

TABLE I.

*Patient 2.*

| Days upon fraction. | Designation of fraction. | Amount of fraction given. | Reticulocytes. (*r*) | Concentration of erythrocytes. (*E*) | Concentration of reticulocytes. (*Er*) |
|---|---|---|---|---|---|
| | | *gm.* | *per cent* | *millions per c. mm.* | *millions per c. mm.* |
| 0 | | | 2.1 | 1.24 | 0.026 |
| 1 | II D | 9.45 | 3.0 | 1.41 | 0.042 |
| 2 | | 9.45 | 1.9 | 1.28 | 0.024 |
| 3 | | 9.45 | 1.0 | 1.10 | 0.011 |
| 4 | | 9.45 | 1.0 | 1.29 | 0.013 |
| 5 | | 9.45 | 0.3 | 1.40 | 0.004 |
| 6 | | 9.45 | 1.5 | 1.14 | 0.017 |
| 7 | | 9.45 | 2.9 | 1.37 | 0.040 |
| 8 | | 9.45 | 5.6 | 1.28 | 0.072 |
| 9 | | 9.45 | 10.1 | 1.38 | 0.139 |
| 10 | | 9.45 | 16.0 | | 0.221 |
| 11 | | 18.90 | 7.2 | 1.38 | 0.099 |
| 12 | | 18.90 | 3.1 | 1.47 | 0.046 |
| 13 | | 18.90 | 6.6 | | 0.091 |
| 14 | | 18.90 | 2.1 | 1.23 | 0.026 |
| 15 | | 18.90 | 2.8 | 1.32 | 0.037 |
| 16 | | * | | | |

* Patient given 200 gm. of raw liver pulp.

Each of these three patients received each day the extract from approximately 400 gm. of liver. The laboratory had not yet been adequately equipped for the preparation of extract, and accordingly but an uncertain amount of a weak extract was fed to the first of the three patients (Patient 3) who received extract (D), from the time when Preparation III D was finished until Preparation IV D was ready. This irregularity in the administration of the extract is reflected in the reticulocyte response, and graphically represented in Fig. 1. After taking a potent extract for 4 days,

TABLE II.

| Days upon fraction. | Designation of fraction. | Amount of fraction given. | Reticulocytes. (r) | Concentration of erythrocytes. (E) | Concentration of reticulocytes measured. (Er) | Concentration of reticulocytes estimated. $\left(\frac{Eor}{1-r}\right)$ | Change in concentration of erythrocytes. ($E - E_0$) |
|---|---|---|---|---|---|---|---|
| | | | **Patient 3.** | | | | |
| | | *gm.* | *per cent* | *millions per c. mm.* | *millions per c. mm.* | *millions per c. mm.* | *millions per c. mm.* |
| 0 | | | 2.5 | 1.83 | 0.046 | 0.047 | |
| 1 | III D | 8.10 | 1.1 | | | 0.020 | |
| 2 | | 8.10 | 1.6 | 1.88 | 0.030 | 0.030 | 0.05 |
| 3 | | 8.10 | 2.0 | | | 0.037 | |
| 4 | | 8.10 | 0.8 | (2.33)* | 0.019 | 0.015 | |
| 5 | IV D | † | 1.0 | | 0.023 | 0.018 | |
| 6 | | † | 1.5 | | 0.035 | 0.028 | |
| 7 | | † | 4.2 | | 0.103 | 0.080 | |
| 8 | | 15.72 | 5.6 | (2.47) | 0.138 | 0.109 | |
| 9 | | 15.72 | 4.9 | | 0.121 | 0.094 | |
| 10 | | 15.72 | 5.6 | | 0.134 | 0.109 | |
| 11 | | 15.72 | 9.0 | | 0.207 | 0.181 | |
| 12 | | 15.72 | 12.4 | 2.20 | 0.273 | 0.259 | 0.37 |
| 13 | | 15.72 | 11.6 | | 0.267 | | |
| 14 | | 15.72 | 7.8 | | 0.187 | | |
| 15 | | 15.72 | 6.5 | | 0.163 | | |
| 16 | V D | 16.54 | 6.7 | | 0.174 | | |
| 18 | | 16.54 | 1.3 | 2.88 | 0.037 | | 1.05 |
| 20 | | 16.54 | 2.1 | 2.95 | 0.062 | | 1.12 |
| 22 | | 16.54 | 0.5 | | 0.015 | | |
| 23 | VI D | 11.86 | | | | | |
| 24 | | 11.86 | | 2.98 | | | 1.15 |
| 25 | | 11.86 | | | | | |
| 26 | | 11.86 | 0.1 | 3.06 | 0.003 | | 1.23 |
| 30 | VII D | 19.42 | 1.0 | 3.40 | 0.034 | | 1.57 |
| 33 | | 19.42 | | 3.50 | | | 1.67 |
| 38 | VIII D | 14.06 | | 3.89 | | | 2.06 |
| 40 | | 14.06 | | 3.80 | | | 1.97 |
| | | | **Patient 4.** | | | | |
| 0 | | | 0.7 | 2.75 | 0.019 | 0.019 | |
| 1 | III D | 10.8 | 0.3 | 2.68 | 0.008 | 0.008 | −0.07 |
| 2 | | 10.8 | 0.7 | | | 0.019 | |
| 3 | | 13.5 | 0.8 | (2.93) | 0.023 | 0.022 | |
| 4 | IV D | ‡ | 2.2 | | | 0.062 | |
| 5 | | ‡ | 1.3 | 2.74 | 0.036 | 0.036 | −0.01 |
| 6 | | ‡ | 2.9 | 2.88 | 0.084 | 0.082 | 0.13 |
| 7 | | ‡ | 5.4 | | 0.156 | 0.156 | |
| 8 | | 15.72 | 7.6 | 2.87 | 0.218 | 0.226 | 0.12 |
| 9 | | 15.72 | 8.3 | | 0.238 | 0.249 | |

TABLE II—*Concluded.*

| Days upon fraction. | Designation of fraction. | Amount of fraction given. | Reticulocytes. ($r$) | Concentration of erythrocytes. ($E$) | Concentration of reticulocytes measured. ($Er$) | Concentration of reticulocytes estimated. $\left(\frac{E_0 r}{1-r}\right)$ | Change in concentration of erythrocytes. ($E - E_0$) |
|---|---|---|---|---|---|---|---|
| **Patient 4.—Continued.** | | | | | | | |
| | | *gm.* | *per cent* | *millions per c. mm.* | *millions per c. mm.* | *millions per c. mm.* | *millions per c. mm.* |
| 10 | | 12.58 | 4.5 | 2.99 | 0.135 | 0.130 | 0.24 |
| 11 | | 12.58 | 4.3 | | 0.131 | 0.124 | |
| 12 | | 12.58 | 2.3 | | 0.072 | 0.065 | |
| 13 | | § | 4.2 | | 0.137 | | |
| 14 | V D | 16.54 | 3.5 | 3.54 | 0.124 | | 0.79 |
| 16 | | 16.54 | 1.6 | 3.69 | 0.059 | | 0.94 |
| 18 | | 16.54 | 0.7 | 3.53 | 0.025 | | 0.78 |
| 21 | | § | 0.8 | (3.04) | 0.024 | | |
| 22 | VI D | 11.86 | | (3.20) | | | |
| 23 | | 11.86 | 0.8 | | | | |
| 24 | | 11.86 | | 3.84 | | | 1.09 |
| 26 | | 11.86 | 0.3 | (3.53) | 0.011 | | |
| 29 | VII D | 19.42 | 0.1 | 4.31 | 0.004 | | 1.56 |
| 31 | | 19.42 | 0.2 | 4.28 | 0.009 | | 1.53 |
| 36 | | ‖ | | 4.48 | | | 1.73 |
| **Patient 5.** | | | | | | | |
| 0 | | | 0.9 | 1.58 | 0.014 | 0.014 | |
| 1 | IV D | 15.72 | 1.7 | | 0.027 | 0.027 | |
| 2 | | 15.72 | 0.8 | 1.58 | 0.013 | 0.013 | |
| 3 | | 23.58 | 1.9 | | 0.030 | 0.031 | |
| 4 | | 23.58 | 1.5 | 1.59 | 0.024 | 0.024 | 0.01 |
| 5 | | 23.58 | 3.0 | | 0.048 | 0.049 | |
| 6 | | 23.58 | 5.0 | | 0.080 | 0.083 | |
| 7 | | 15.72 | 11.0 | | 0.169 | 0.195 | |
| 8 | V D | 24.81 | 13.8 | 1.54 | 0.213 | 0.253 | −0.04 |
| 9 | | 24.81 | 14.0 | | | 0.257 | |
| 10 | | 24.81 | 9.9 | | | | |
| 11 | | 24.81 | 9.5 | 2.48 | 0.236 | | 0.90 |
| 12 | | 16.54 | 8.9 | | 0.231 | | |
| 13 | | 8.27 | 3.9 | | 0.105 | | |
| 14 | | ¶ | 6.2 | | 0.174 | | |
| 15 | | | 4.2 | 2.93 | 0.123 | | 1.35 |

* Erythrocyte counts that were not used in the calculation of $(E-E_0)$ are printed in parentheses.

† An uncertain amount was given for 3 days of the first part of Preparation IV D.

‡ Patient given 200 cc. per day of first part of Preparation IV D.

§ None administered.

‖ Patient given 180 gm. of raw liver pulp.

¶ Patient given 240 gm. of raw liver pulp.

and at a time when the reticulocytes were increasing in the peripheral blood, this patient received a weak extract for 3 days. The reticulocytes continued to increase in number for approximately 4 more days, but the response then lapsed until 2 days after the administration of the new and more potent extract, Preparation IV D. Thereafter the number of reticulocytes further increased until the peak of their rise was reached on the 12th day after the extract was first given. At that time there were 273,000 reticulated red blood cells in each c.mm. of blood. After the 13th day their number progressively diminished, and after

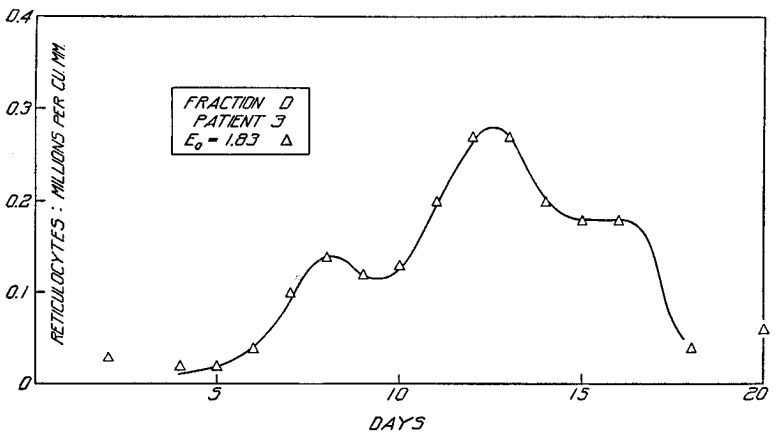



FIG. 1. Change in the reticulocyte concentration, *Er*, of Patient 3 upon varying amounts of fraction (D).

another period of about 8 days returned to normal. By this time newly formed erythrocytes, without reticulum, had begun to appear in the blood, which contained 1 million more red blood cells per c.mm. than at the beginning of feeding. The net increase in the total number of erythrocytes ($E - E_0$) of Patient 3, and of certain others, is graphically represented in Fig. 3. In Patient 3 after 40 days the red blood cell concentration had been increased by nearly 2 million per c.mm. That is to say, the number of corpuscles had more than doubled, and during this time the hemoglobin percentage had nearly doubled.

The next two patients (Nos. 4 and 5) who received the (D) type of extract responded slightly more rapidly than the first.

Although their red blood cell counts differed by approximately 1 million cells per c.mm. before and during the first days of feeding, the absolute numbers of reticulocytes at the peak of their rise were almost identical. Each had in his blood approximately 240,000 reticulocytes per c.mm. by the 9th day. Thereafter the number of these cells in the peripheral blood diminished, though more rapidly in one case than in the other. Patient 4 received for 36 days the successive extracts of the (D) type that were prepared. At the end of this time the number of red blood cells had increased 1.73 millions per c.mm. and had become almost normal (Fig. 3). The other patient (No. 5), with a much lower red blood cell count, was fed the fraction for only 13 days, and the red blood cell count

TABLE III.

*Solubility of Fraction (D) in Organic Solvents.*

| Solvents in order employed. | Per cent of fraction extracted from 2.098 gm. Preparation V D. | Solvents in order employed. | Per cent of fraction extracted from 1.872 gm. Preparation V D. |
|---|---|---|---|
| Ether. | 1.4 | Ether. | 1.0 |
| Acetone. | 6.2 | Acetone. | 7.9 |
| Ethyl alcohol. | 33.6 | Ethyl alcohol. | 34.4 |
| *n*-Butyl alcohol. | 30.9 | Glacial acetic acid. | 30.1 |
| Pyridine. | 0.8 | Pyridine. | 1.3 |
| Total extracted. | 72.9 | Total extracted. | 74.7 |

had increased by over 1 million cells per c.mm. The feeding of the fraction was then discontinued for lack of material, but the erythrocytes continued to rise to normal upon whole liver.

These results indicated that the active principle of the liver, effective in pernicious anemia, had been extracted and was contained in fraction (D). Of the solids in this crude extract approximately 20 per cent were inorganic substances, recoverable after ashing the material. From 5 to 8 per cent consisted of nitrogen, present neither as protein nor as ammonia.

During the time when patients were being fed fraction (D), experiments intended to suggest a method of further purifying the extract were in progress. To this end the solubility of fraction

(D) was studied in a large number of organic solvents. The results of two such experiments[1] are recorded in Table III.

*Ether-Soluble Extractives (E).*—The extraction of the liver with water left the largest part of the lecithin and other lipoids undissolved in fraction (A). But from 1 to 3 per cent of the different preparations of fraction (D) was ether-soluble. None the less it seemed desirable to test one patient upon a fraction from which all the ether-soluble material had been extracted. The reticulocyte response of this patient although smaller than that obtained with the (D) type of extract, was, nevertheless, definite. The active principle effective in pernicious anemia appeared to be no more of a lipoidal than of a protein nature.

*Alcohol-Soluble Extractives (F).*—Approximately 7 per cent of the solids in fraction (D) were found to be soluble in acetone (Table III). Over 30 per cent dissolved in ethyl alcohol. Moreover the material dissolved by acetone was also soluble in ethyl alcohol. Accordingly fraction (D) was further purified by the elimination of the alcohol-soluble extractives (F).[2] This was first accomplished by pouring a concentrated aqueous solution of extract (D) into such an amount of absolute alcohol as to render the final alcohol concentration approximately 95 per cent by volume. The alcohol was maintained in violent agitation by a mechanical stirrer while the extract was slowly added. As a result the precipitate that appeared when the syrup was poured into the absolute alcohol was finely divided. After some time had elapsed the stirring was stopped and the precipitate removed by sedimentation and filtration. The precipitate was washed with a small volume of alcohol and the wash waters added to the filtrate (F) containing the alcohol-soluble extractives.

The concentrated alcoholic extract (F) has both a sharp taste and odor. It contains certain of the nitrogenous bases in the liver, but of those that are alcohol-soluble, such as choline and histamine, all are probably not extracted by this fractionation (2). Certain carbohydrates also dissolve and are removed in fraction (F).

[1] These experiments were conducted by Mrs. F. C. Sargent.

[2] The liver fraction fed to patients with pellagra by Voegtlin (20) was alcohol-soluble and was extracted directly from the dried liver by this solvent.

Fraction (F) was fed to one patient without producing beneficial results. At the same time the alcohol-precipitable material (G) was fed to a patient who responded characteristically with an increased concentration of reticulated red blood cells.

*Alcohol-Precipitable Extractives* (*G*).—The precipitate that appeared when fraction (D) was poured into sufficient alcohol to render the final concentration 95 per cent by volume, contained the active principle. In continuing the preparation this precipitate was generally not completely dissolved. Instead the type of fractionation employed by Osborne and Wakeman (17) in the concentration of water-soluble vitamin, was attempted. It was found that most, if not all, of the chemical substance effective in pernicious anemia was dissolved if sufficient water was added to the precipitate to render the concentration of alcohol 70 per cent. A large amount of inert material remained undissolved in this alcohol mixture, and thus a substantial concentration was achieved. The precipitate was repeatedly washed with 70 per cent alcohol to remove occluded material, and the washings were added to the solution containing the active principle.

Although fraction (G) was first successfully prepared in this manner, it was later found convenient to reverse the procedure, in order to obtain a dry powder as the final product. In the more recent preparations only enough alcohol was added to fraction (D) to render the alcoholic concentration 70 per cent. The resulting precipitate was repeatedly washed with 70 per cent alcohol and the combined filtrates concentrated *in vacuo*. This concentrate was then poured, in the manner previously described, into sufficient absolute alcohol to render the final concentration at least 95 per cent by volume. The resulting precipitate was collected upon a Buchner funnel, washed with 95 per cent alcohol, then with absolute alcohol, and finally with anhydrous ether. The precipitate, which proved to be very hygroscopic, was continuously covered with alcohol or ether until it had been transferred to a desiccator.

Fraction (G) appeared to be at least as effective as (D), or as whole liver, in the treatment of pernicious anemia (Table IV). The first patient (Patient 8) to whom fraction (G) was fed responded with a very prompt and marked increase of reticulocytes. This individual was given extract derived from ap-

TABLE IV.

| Days upon fraction. | Designation of fraction. | Amount of fraction given. | Reticulocytes. ($r$) | Concentration of erythrocytes. ($E$) | Concentration of reticulocytes measured. ($Er$) | Concentration of reticulocytes estimated. $\left(\frac{E_0 r}{1-r}\right)$ | Change in concentration of erythrocytes. ($E - E_0$) |
|---|---|---|---|---|---|---|---|
| | | | **Patient 8.** | | | | |
| | | *gm.* | *per cent* | *millions per c. mm.* | *millions per c. mm.* | *millions per c. mm.* | *millions per c. mm.* |
| 0 | | | 0.4 | 1.45 | 0.006 | 0.006 | |
| 1 | VII G | 9.79 | 0.2 | | | 0.003 | |
| 2 | | 9.79 | 0.4 | | | 0.006 | |
| 3 | | 9.79 | 2.1 | | | 0.031 | |
| 4 | | 9.79 | 1.6 | 1.44 | 0.023 | 0.024 | −0.01 |
| 5 | | 9.79 | 4.6 | | | 0.070 | |
| 6 | | 3.92 | 6.0 | | | 0.093 | |
| 7 | VIII $G_1$ | 5.72 | 11.7 | 1.65 | 0.193 | 0.192 | 0.20 |
| 8 | | 5.72 | 13.2 | | | 0.221 | |
| 9 | | 9.16 | 10.5 | | | 0.170 | |
| 10 | | 9.16 | 17.9 | 1.81 | 0.324 | 0.316 | 0.36 |
| 11 | | 9.16 | 15.9 | | | | |
| 12 | VIII $G_2$ | 9.06 | 16.4 | | | | |
| 13 | | 9.06 | 14.8 | 2.00 | 0.296 | | 0.55 |
| 14 | | 9.06 | 13.6 | | | | |
| 15 | | 9.06 | 11.7 | | | | |
| 16 | | 9.06 | 7.1 | | | | |
| 17 | | 9.06 | 4.5 | 2.19 | 0.099 | | 0.74 |
| 18 | | 9.06 | 2.8 | | | | |
| 20 | X G | 7.60 | 2.5 | 2.46 | 0.062 | | 1.01 |
| 24 | | 7.60 | 1.3 | 2.47 | 0.032 | | 1.02 |
| 27 | XI G | 13.90 | 0.4 | 2.74 | 0.011 | | 1.29 |
| 31 | | * | 0.2 | 2.83 | 0.006 | | 1.38 |
| | | | **Patient 10.** | | | | |
| 0 | | | 0.1 | 0.55 | 0.001 | 0.001 | |
| 1 | VIII $G_1$ | 9.16 | 0.8 | 0.62 | 0.005 | 0.004 | 0.07 |
| 2 | | 9.16 | 0.8 | | | 0.004 | |
| 3 | | 9.16 | 6.6 | 0.48 | 0.032 | 0.039 | −0.07 |
| 4 | VIII $G_2$ | 14.48 | 23.2 | | | 0.166 | |
| 5 | | 14.48 | 41.4 | 0.68 | 0.282 | 0.389 | 0.13 |
| 6 | | 14.48 | 30.0 | | | 0.236 | |
| 7 | | 14.48 | 35.8 | | | 0.307 | |
| 8 | | 14.48 | 14.3 | 1.50 | 0.215 | | 0.95 |
| 10 | | 7.24 | 9.6 | 1.61 | 0.155 | | 1.06 |
| 11 | X G | 7.60 | | | | | |
| 12 | | 7.60 | 4.6 | 1.80 | 0.083 | | 1.25 |
| 15 | | 7.60 | 1.0 | 2.21 | 0.022 | | 1.66 |
| 17 | | 7.60 | 3.0 | | | | |
| 18 | XI G | 13.90 | | 2.40 | | | 1.85 |
| 19 | | 13.90 | 1.8 | | | | |
| 22 | | * | 0.4 | 2.94 | 0.012 | | 2.39 |

* Patient given raw liver pulp.

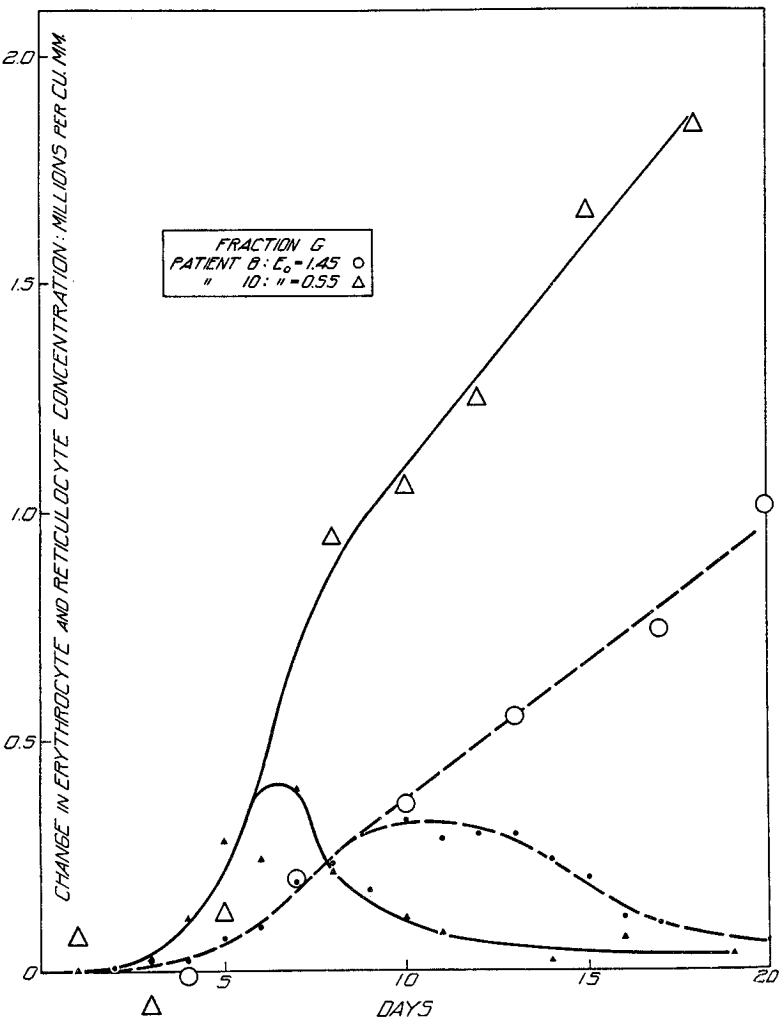



FIG. 2. Change in the reticulocyte concentration, $Er$, and in the erythrocyte concentration ($E - E_0$) of Patients 8 and 10 upon different amounts of fraction (G). The errors in determining reticulocyte concentration are far smaller and smaller solid symbols are therefore employed in designating this quantity, whereas large hollow symbols are employed in designating the erythrocyte concentration.

proximately the same amount of liver as the patients given fraction (D), and the rates of reticulocyte, and of erythrocyte, increase under the two treatments were almost identical. In this patient given fraction (G) the concentration of reticulocytes per c.mm. of blood, *Er*, was slightly greater at the peak of the rise than in the patients given fraction (D), but the red blood cell level before feeding ($E_0$) was lower (11, 15). The net increase in erythrocytes, ($E - E_0$), is compared in Fig. 3 with the results obtained with fraction (D). The concentration of red blood cells in the peripheral blood increased by 1 million per c. mm. in the first 20 days in this case.

The erythrocyte increase during the first 10 days appears to have been due entirely to the formation of reticulocytes (Fig. 2). These results thus appear to conform to the ideal conditions expressed by equations (1) and (5).

The second patient (Patient 10) to receive fraction (G) had only about 0.5 million red blood cells per c.mm. and was in a comatose condition when the material was first administered. Accordingly it was decided to feed larger amounts of the extract (G) than had been given to the first case. The more rapid reticulocyte response that resulted (Fig. 2), depended in part upon the larger amount fed, but in part, also, upon the lower erythrocyte level. Within 7 days 300,000 reticulocytes per c.mm. were in the blood, and by the 8th day the concentration of erythrocytes had more than doubled. By the 15th day the net increase in erythrocytes was 1.66 and by the 22nd day, 2.4 million per c. mm. Since then similar results have been regularly obtained when this amount of the fraction has been given (Fig. 3).

Although fraction (G) has been prepared free of iron, of proteins, and of lipoids, and although it was considered satisfactory from a therapeutic standpoint, it was still a relatively crude extract judged either from the chemical or from the physiological standpoint. The larger molecules had for the most part been removed, but the ash still represented nearly 20 per cent of the solids. Evidence, though inconclusive, suggested that the molecule of the active principle is of dialyzable size. After electrodialysis the fraction retained between parchment membranes, designated (H), proved inactive when fed to a patient. Another patient had an unexpectedly weak reticulocyte response when fed extract after

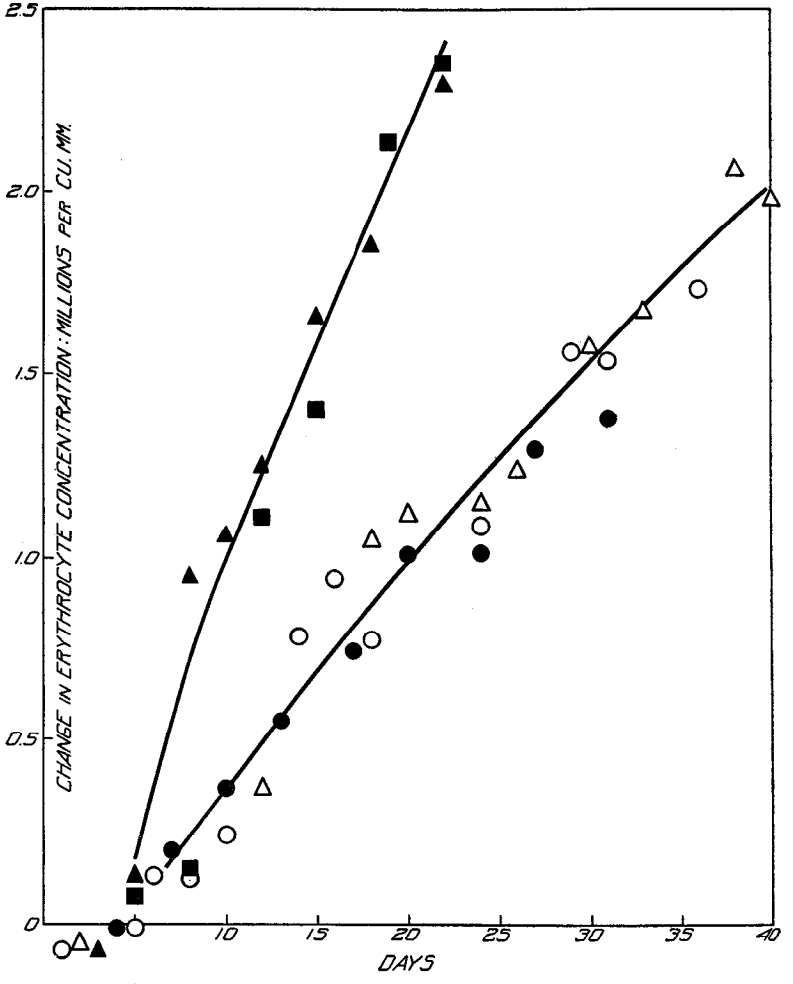



FIG. 3. Change in erythrocyte concentration ($E - E_0$) of patients given different amounts of extract.

△ Patient 3, $E_0$ = 1.83 fraction (D) derived from about 400 gm. of liver.
○ " 4, " = 2.75 " " " " " 400 " " "
● " 8, " = 1.45 " (G) " " " 400 " " "
▲ " 10, " = 0.55 " " " " " 600 " " "
■ " = 1.22 Liver Extract No. 343 derived from about 600 gm. of liver.

ultrafiltration through a collodion membrane at a low temperature (I). The observation that the substance is effective when given

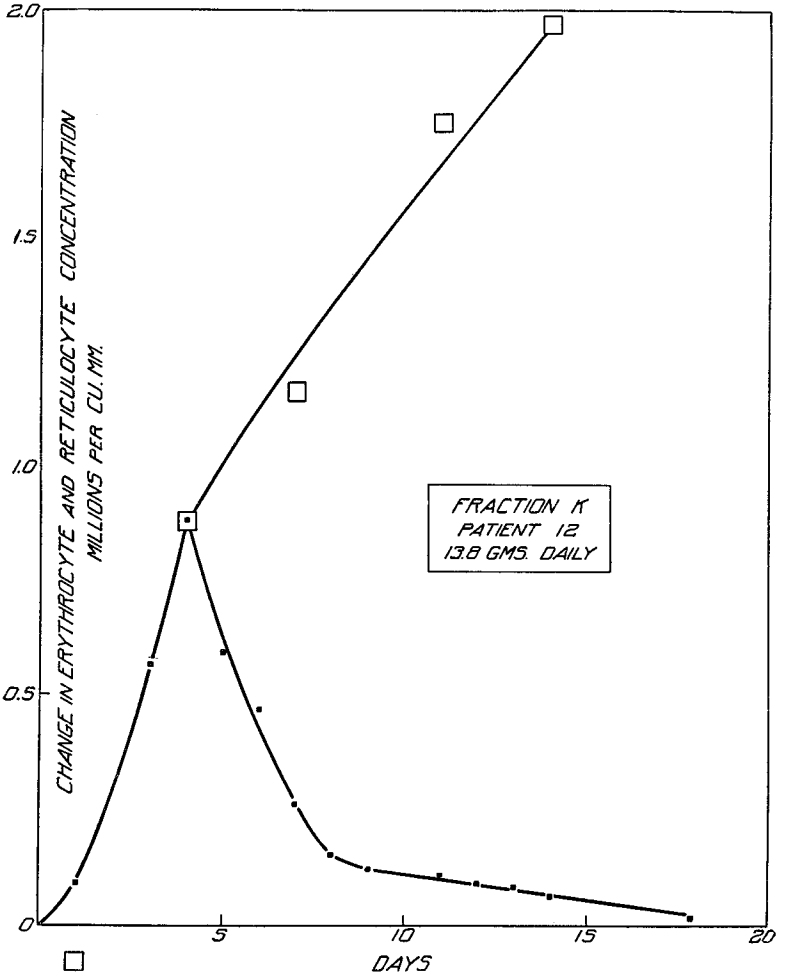



FIG. 4. Change in the reticulocyte concentration, $Er$, and in the erythrocyte concentration ($E - E_0$) of Patient 12, $E_0 = 1.26$.

by mouth, suggested that the dimensions of the molecule are not such as to interfere with its absorption.

Fraction (G) was free of the blood sugar-reducing substances of

liver (4, 16), but it still accelerated the denervated heart. Although free from such anticoagulating effects as might be expected were heparin present, it appreciably lowered blood pressure when injected intravenously in a cat.[3] Indeed certain of the methods employed in this investigation have not been dissimilar to those described for the extraction and purification of blood pressure-reducing substances in liver (8, 10). The active principle effective in pernicious anemia is for the most part not precipitated, however, by the same reagent concentrations.

From the solubility of the material effective in pernicious anemia and that of known vitamins it became evident that the liver extract was free of those vitamins that are lipoid-soluble, but that it might still contain a water-soluble vitamin. The instability and the other properties of vitamin C appeared to exclude its consideration. With the possibility in mind that so called "vitamin B" might be responsible for the effectiveness of liver extract in the treatment of pernicious anemia, the method of concentration of vitamin B described by Levene and van der Hoeven (9) was attempted. The extract was treated with silicic acid gel at pH 5.0. The active principle was not adsorbed by the gel (J), but remained in the filtrate, designated (K), which when fed to Patient 12 produced the results graphically represented in Fig. 4. The gel however was inactive.

This kind of negative evidence concerning so called vitamin B was not considered satisfactory. Large amounts of different sources of the antineuritic and the pellagra-preventive factors were therefore fed to three patients with pernicious anemia. The first individual was given about 1000 gm. of yeast-cake in 8 days. The second received 407 gm. of a dried aqueous yeast extract in 8 days.[4] In these two patients there occurred in 15 days no

[3] We are indebted to Dr. Paul M. Harmon for testing the blood pressure-reducing effects of the various extracts. In the course of these observations acceleration of the denervated heart was observed.

[4] We are indebted to Dr. Joseph Goldberger for supplying us with this preparation—a commercial dried aqueous yeast extract. This material has been shown by Goldberger, Wheeler, Lillie, and Rogers (6) to be potent in but a few gm. a day as a preventive and curative agent in pellagra and experimental black tongue of dogs. Thus the pellagra-preventive factor appears not to be identical with the material which is effective in pernicious anemia.

change in their blood or general condition, but they later responded, one to liver, and one to liver extract. The third received 80 gm. of a vitamin extract derived from over 9 kilos of yeast, and 720 cc. of an extract derived from over 7 kilos of wheat embryo in 8 days. This patient showed some slight improvement, but not such as would be expected with even moderate amounts of liver extract. None the less the effects of different sources of the factors of so called vitamin B are being further investigated.

*Butyl Alcohol Extractives* (*L*).—Certain of the constituents of (G) can be extracted by means of such organic solvents as normal butyl alcohol, pyridine, and glacial acetic acid (Table III). The attempt was made at this point in the investigation to effect a separation by means of normal butyl alcohol. The type of Kutscher-Steudel apparatus described by Dakin (5) was employed under reduced pressure, and the extractives, (L), freed of the butyl alcohol by means of ether, were given to a single patient. A slight reticulocyte rise followed, but the experiment could not be continued at the time because sufficient extract was not available. The results suggested that the active principle had been extracted by means of butyl alcohol, though not, perhaps, without a certain amount of loss of potency. Because of the inadequate facilities available for the preparation of fractions, this process was abandoned in favor of those that were beginning to yield more striking results.

*Lead-Precipitable Extractives* (*N*).—Slightly more than half the constituents of fraction (G) were precipitable by lead. Sufficient alkali, either barium hydroxide or sodium hydroxide, depending upon whether or not it was desired to keep the salt content of the filtrate at a low level, was added to bring fraction (G) to approximately pH 8.0. Basic lead acetate was then added until precipitation was complete. The basic lead acetate yielded a copious precipitate which readily separated upon centrifugation or filtration. The precipitate, (N), was repeatedly washed with small quantities of water and the wash waters added to the centrifugate or filtrate (O). Basic lead acetate, a generally useful precipitant, was also employed by Best, Dale, Dudley, and Thorpe in their study of "The Nature of the Vaso-Dilator Constituents of Certain Tissue Extracts" (2).

The precipitate was slightly acidified, and the lead and barium

were removed as sulfate and sulfide.[5] This fraction (N) was then given to a patient with pernicious anemia, without, however, producing any significant reticulocyte response.

*Lead Filtrate* (*O*).—The filtrate from the lead precipitate was slightly acidified, generally with sulfuric acid, and the lead removed as sulfate and sulfide. The lead-free filtrate (O) was concentrated under reduced pressure to the desired volume, and

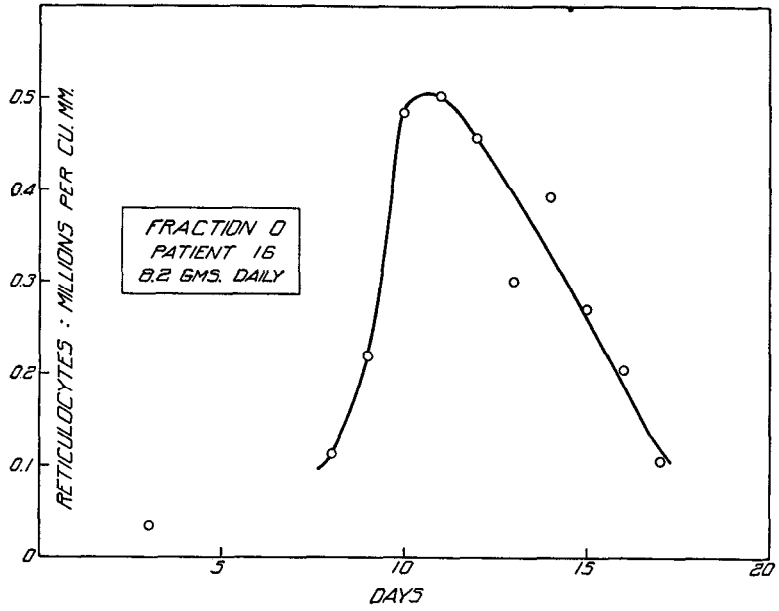



FIG. 5. Change in the reticulocyte concentration, *Er*, of Patient 16, $E_0$ = 1.73.

administered to Patient 16 with the results graphically represented in Fig. 5. In the belief that a part of the active material might have been lost in the copious precipitates involved in this purification, the extract derived from 1 kilo of liver a day was fed. Even so, but 8.2 gm. of solids were given each day, and within 11 days 0.5 million reticulated red blood cells per c.mm. of blood were present. The diminishing erythrocyte count of the patient prior

[5] We are indebted to Dr. Reid Hunt for testing these, and subsequent fractions, for freedom from poisonous substances.

to feeding had by this time been arrested and compensated. The reticulocyte response did not begin until the 8th day but its

TABLE V.

*Analyses of Fractions.*

| Designation of fraction. | Total solids. | Ash. | Total P. | Total N. | Amide and ammonia N. | Protein N. | Non-protein N. |
|---|---|---|---|---|---|---|---|
| | *per cent* | *per cent solids* | *per cent solids* | *per cent solids* | *per cent solids* | *per cent solids* | *per cent solids* |
| II D | 6.30 | 30.00 | | 5.80 | 1.62 | | 5.80 |
| III D | 5.40 | 29.30 | 3.20 | 11.57 | 2.53 | 1.40 | 10.17 |
| IV D | 15.72 | 19.62 | 2.10 | 5.56 | 0.66 | 0.34 | 5.22 |
| V D | 16.54 | 21.56 | 3.08 | 8.48 | 1.35 | 1.82 | 6.66 |
| VI D | 11.86 | 28.10 | 3.56 | 8.44 | 1.38 | 1.58 | 6.86 |
| VII D | 19.42 | 19.10 | 2.36 | 9.65 | 1.22 | 1.21 | 8.44 |
| VIII D | 14.06 | 16.74 | 2.17 | 7.98 | 1.27 | 1.62 | 6.36 |
| VII G | 19.59 | 20.50 | 2.97 | 8.96 | 1.14 | 0.90 | 8.06 |
| VIII G | 11.45 | 20.27 | 3.50 | 9.54 | 1.56 | 1.04 | 8.50 |
| IX G | 6.94 | 16.00 | 3.20 | 11.60 | 1.23 | 2.20 | 9.40 |
| X G | 38.00 | 20.79 | 1.70 | 8.05 | 1.13 | 0.66 | 7.39 |
| XI G | 55.60 | 20.78 | 2.27 | 9.51 | 0.97 | 0.87 | 8.64 |
| XII G | 37.62 | 22 33 | 1.64 | 8.78 | 1.09 | 0.47 | 8.31 |
| XVII G | Solid. | 15.51 | 3.17 | 10.05 | 1.15 | 0.00 | 10.05 |
| XIII O | 6.58 | 29.45 | 0.72 | 10.84 | 1.25 | 0.00 | 10.84 |
| XIV O | 22.03 | 27.06 | 1.00 | 8.51 | 1.31 | 0.00 | 8.51 |
| XV O | 39.86 | 39.05 | 1.81 | 8.40 | 1.00 | 0.00 | 8.40 |

magnitude at the peak was very great. After a fortnight the patient was given raw liver pulp owing to lack of a sufficient supply of extract.[6]

[6] At about this time Eli Lilly and Company of Indianapolis began to manufacture an extract for the Committee on Pernicious Anemia of the Harvard Medical School. As prepared at present this very satisfactory Liver Extract No. 343 is essentially the same as fraction (G), modified however to facilitate manufacture, in part in accordance with a suggestion for which we are indebted to Drs. Best and Scott of the Connaught Laboratories of the University of Toronto. The present extract, though more copious than (G) and richer in carbohydrates, is as satisfactory, therapeutically. Prepared from Liver Extract No. 343, fraction (O) is, however, not free of carbohydrates.

The preparation fed to this patient was free of carbohydrates, as judged by the Molisch test. All three classes of substances, proteins, fats, and carbohydrates, had thus been eliminated without loss of the active principle effective in pernicious anemia. Moreover many organic acids form lead salts and would have passed into fraction (N). There remained chiefly neutral or pre-

TABLE VI.

*Analyses of Fractions, Calculated upon Ash-Free Basis.*

| Designation of fraction. | Total solids. | Total P. | Total N. | Amide and ammonia N. | Protein N. | Non-protein N. |
|---|---|---|---|---|---|---|
| | *per cent* | *per cent solids* | *per cent solids* | *per cent solids* | *per cent solids* | *per cent solids* |
| II D | 4.41 | | 8.28 | 2.31 | 0.00 | 8.28 |
| III D | 3.82 | 4.52 | 16.36 | 3.59 | 1.98 | 14.38 |
| IV D | 12.64 | 2.61 | 6.92 | 0.83 | 0.43 | 6.49 |
| V D | 12.98 | 3.93 | 10.80 | 1.72 | 2.31 | 8.49 |
| VI D | 8.53 | 4.95 | 11.73 | 1.92 | 2.19 | 9.54 |
| VII D | 15.71 | 2.91 | 11.92 | 1.50 | 1.49 | 10.43 |
| VIII D | 11.70 | 2.61 | 9.59 | 1.52 | 1.95 | 7.64 |
| VII G | 15.58 | 3.73 | 11.26 | 1.43 | 1.13 | 10.13 |
| VIII G | 9.13 | 4.38 | 11.95 | 1.95 | 1.30 | 10.65 |
| IX G | 5.83 | 3.81 | 13.81 | 1.46 | 2.62 | 11.19 |
| X G | 30.10 | 2.14 | 10.15 | 1.42 | 0.83 | 9.32 |
| XI G | 44.05 | 2.86 | 12.00 | 1.23 | 1.09 | 10.91 |
| XII G | 28.84 | 2.14 | 11.45 | 1.42 | 0.61 | 10.84 |
| XVII G | Solid. | 3.75 | 11.89 | 1.35 | 0.00 | 11.89 |
| XIII O | 4.64 | 1.02 | 15.36 | 1.76 | 0.00 | 15.36 |
| XIV O | 16.07 | 1.37 | 11.66 | 1.79 | 0.00 | 11.66 |
| XV O | 24.30 | 2.97 | 13.78 | 1.64 | 0.00 | 13.78 |

dominantly basic molecules, and in large part, the inorganic constituents of the liver that had passed into fraction (G).

The analyses of the first fifteen preparations are recorded in Table V.[7] If it be assumed that the active principle is not inorganic in nature, these can be recalculated on an ash-free basis. This has been done and the results appear in Table VI. Whereas

[7] These analyses were made by Mr. H. F. Ulrichs and have previously been reported in part (4).

the purer fractions contained less phosphorus, they were richer in nitrogen. The nitrogen content of Preparation XIII O, calculated on an ash-free basis, was 15.36 per cent, of which 1.76 per cent was recovered as ammonia nitrogen. The inference that was tentatively drawn from these results was that the active principle is either a nitrogenous base or a polypeptide.

*Phosphotungstate Precipitate (P).* — Nitrogenous bases and certain polypetides are precipitated by phosphotungstic acid. The former substances are far more soluble than the latter in acetone-water mixtures (1). Sulfuric acid was accordingly added to fraction (O) until it contained 5 per cent by weight, and phosphotungstic acid until precipitation was complete. The precipitate was then repeatedly extracted with 90 per cent acetone, and the acetone-insoluble (R) and soluble (S) phosphotungstates separately regenerated. This was accomplished by adding to each preparation enough 20 per cent barium hydroxide to give a pH of from 8.5 to 9.0 and by filtering off the insoluble barium sulfate and phosphotungstate. Excess barium was then removed by sulfuric acid, or according to a later procedure by a mixture of sulfuric acid and sodium sulfate.

Both the acetone-soluble and insoluble phosphotungstates, (P = R + S), were given to Patient 18. Unfortunately this individual proved to have a very unusual case. She failed to respond by a distinct increased production of reticulocytes after being given this extract, and later did not promptly improve when given whole liver (15). She responded slowly to the feeding of whole liver, however, with an increasing erythrocyte count, which finally reached over 5 million per c.mm.

Another patient was subsequently given this fraction, but it was prepared from a manufactured extract during the period when manufactured extract was not yet satisfactory. None the less the patient responded promptly by an increased, though relatively small number of reticulocytes. This preparation had been further fractionated by precipitating the picrates before the phosphotungstates. After being separately regenerated these fractions were given to the same patient (No. 41). The response in this instance, together with the negative results previously obtained with the phosphotungstate filtrate (Q), suggested that the active principle was precipitable by phosphotungstic acid.

This was made more certain by a communication to us at the time.[8]

*Phosphotungstate Filtrate* (*Q*).—The filtrate (Q) (separated from the phosphotungstate precipitate fed to Patient 18) was regener-

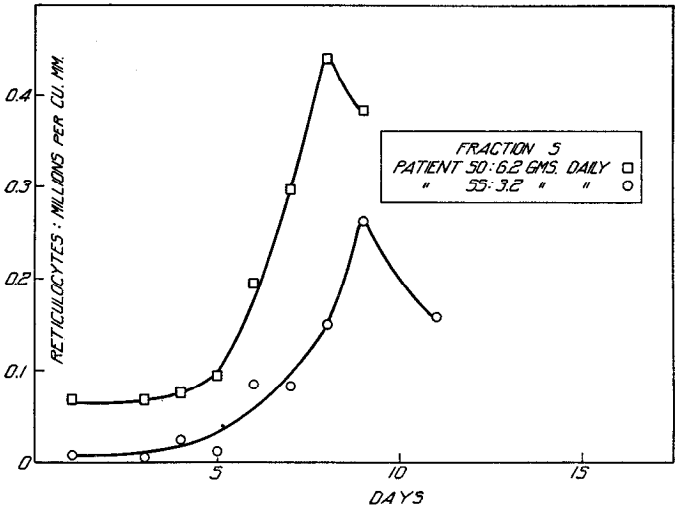



FIG. 6. Change in the reticulocyte concentration, $Er$, of Patient 50, $E_0$ = 1.86, and of Patient 55, $E_0$ = 1.22.

ated and given to another patient (No. 22). The results were entirely negative, suggesting that the active principle had been precipitated by phosphotungstic acid. This patient subsequently responded promptly to the feeding of potent liver extract.

*Acetone-Soluble Phosphotungstates* (*S*).—Of the substances that

[8] It is a pleasure to acknowledge the cooperation of Dr. Randolph West of the Presbyterian Hospital, New York. Following the announcement of Minot and Murphy, West attempted the fractionation of liver, and independently obtained an extract which contained the active principle (21). The Committee on Pernicious Anemia of the Harvard Medical School has provided him with the extract being manufactured for them by Eli Lilly and Company, so that he might the more easily continue his studies. At about the time that the reticulocytes of Patient 41 were responding weakly to the separately regenerated, but combined phosphotungstates, Dr. West informed us that a case of his had responded by the production of reticulocytes to the unfractionated phosphotungstates (P) made from a lead acetate filtrate (O) which he had prepared following our procedure.

are precipitated by phosphotungstic acid, the phosphotungstates of the nitrogenous bases are more soluble in acetone-water mixtures than are the phosphotungstates of the polypeptides (1). That part of the precipitated phosphotungstates which dissolved readily in 90 per cent acetone-water mixtures has been regenerated separately in our experiments. The solution containing the regenerated acetone-soluble phosphotungstates derived from 27 kilos of liver contained 55.9 gm. of solids. Of this Preparation XXXV S, 6.2 gm. were given daily to Patient 50. The reticulocyte response was so great as to indicate that this fraction was a concentrated source of the active principle (Fig. 6).

In studying Preparation XXXV S it was noted that a precipitate appeared in acid solution. Accordingly another preparation, No. XL S, was made and the acid-precipitable material removed from it before it was given to a patient. The extract from 120 kilos of liver was employed in this preparation and yielded 191.2 gm. of regenerated acetone-soluble phosphotungstates, from which the acid-precipitable material had been removed. The extract derived from 2 kilos of liver containing 3.2 gm. of solids was given to Patient 55 daily. The reticulocyte response clearly indicated that the acetone-soluble phosphotungstates contained the active principle.

*Acetone-Insoluble Phosphotungstates (R).*—After regenerating the acetone-insoluble phosphotungstates (R) it was noticed that a precipitate appeared at acid reactions. This precipitate appeared to reach its maximum in the neighborhood of pH 1.5 or 2, where it was readily flocculated. It was much more soluble at high than at low temperatures, and more soluble in salt solution than in distilled water. In Preparation XXXV, fraction (R) was concentrated and brought to pH 2 at a low temperature. The resulting precipitate was removed by centrifugation and filtration, washed with 0.1 N acetic acid, and redissolved by means of sodium hydroxide to a neutral reaction. The yield from 54 kilos of liver was 6.6 gm.

The acid-precipitable material appeared to be similar in fractions (R) and (S). The conditions affecting the separation and the regeneration of Preparation XL were therefore altered as we have seen so that the acid-precipitable material would pass largely into fraction (R). Hydrochloric acid was used as the precipitant

and as the wash water of the acid-precipitable material, which was dried by means of alcohol and ether. The yield of this acid-precipitable material from 120 kilos of liver was 59 gm. in this instance.

1.2 gm. of the acid-precipitable material in fraction (R) of Preparation XXXV, were given daily to Patient 53 for 5 days. There followed a slight reticulocyte response. On the 6th day no extract was available, but starting on the 7th day 2 gm. of the same fraction of Preparation XL, were given daily for 5 days. An additional reticulocyte response followed (Fig. 7). The patient thus received 16 gm. of fraction (R) in 11 days, or approximately 1.5 gm. per day. The experiment indicated that the amount fed per

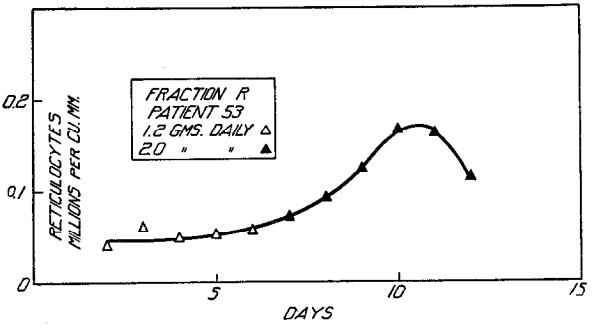



FIG. 7. Change in the reticulocyte concentration, $Er$, of Patient 53, $E_0 = 1.01$.

day was less than enough to produce the maximum reticulocyte response, but that this fraction contained the active principle.

Although fraction (O) had been high in nitrogen, the acid-precipitable material in fraction (R) contained but 7.11 per cent of nitrogen. Moreover it contained 1.10 per cent of phosphorus and 62 per cent ash. Since no acid-precipitable material could be observed in (O), it appeared probable that a small amount of phosphotungstic acid had not been removed as a barium salt at the alkalinity and in the volumes employed in regenerating these preparations. This hypothesis was rendered the more probable since the ratio of phosphorus to ash suggested the ratio of phosphorus to tungstic oxide in phosphotungstic acid. The conditions which prevented the complete precipitation of the phos-

photungstic acid as a barium salt are being investigated, as well as the precise conditions for the precipitation of the active principle by phosphotungstic acid.

Calculated on an ash-free basis, the nitrogen content of the acid precipitate in Preparation XL, fraction (R), was 18.7 per cent. This precipitate may thus be considered as consisting of phosphotungstates of nitrogenous substances. On this basis but 0.6 gm. constituted the average amount of nitrogenous substances given daily to Patient 53.

Of the regenerated phosphotungstates in Preparation XL, fraction (R), 94 per cent are precipitable by silver, and 92 per cent by mercuric acetate at an alkaline reaction. With a product as pure as fraction (R) it is hoped that the metal precipitations which have been repeatedly attempted upon less pure fractions may be more successfully employed. Earlier results are not reported in detail because of the losses in activity heretofore suffered in effecting these separations and regenerations. It may be recorded, however, that the regenerated silver precipitate (V) fed to a patient (No. 26) appeared inert, whereas the filtrate (W) produced a weak though definite reticulocyte response in Patient 23. On the other hand the mercury filtrate (Y) fed to Patients 34 and 51 was inactive, as was the material precipitated by mercuric sulfate from solution in sulfuric acid, whereas the material precipitated by mercuric acetate (X) from neutral solution has been shown to contain the active principle (Patient 65).

Like certain of the previous preparations, Preparation XL (R) was free of carbohydrates. A 1 per cent solution gave no Molisch test, no test with Tollens' reagent, and did not reduce Benedict's solution. It also failed to give the lead-blackening test characteristic of reduced sulfur. It gave a strong biuret,[9] a strong Millon's, but only a slight xanthoproteic reaction. It gave a strong reaction with diazobenzene sulfonate and with Folin's phenol reagent, but not with his uric acid reagent. It gave a faint Adamkiewicz reaction and a positive test with dimethylaminobenzaldehyde. The $\alpha$-naphthol-hypochlorite and the diacetyl tests were also positive. The absence of certain substances is thus demonstrated, but no final deductions will be drawn at this time regarding the

[9] Although Dr. West reported (21) that his extract gave no biuret test, he has informed us that his subsequent fractions have all given strong biuret reactions.

nature of the substances present in the purest fractions that have thus far been effective in pernicious anemia.

*Effect of the Active Principle upon Immature Red Blood Cells.*

Throughout this investigation the effort has been made to find some method of testing for the active principle effective in pernicious anemia that did not involve feeding patients fractions of liver. During the period when these fractions were being studied Dr. Sabin added certain of our extracts to chick embryo preparations and observed what seemed to be an accelerated division rate of certain cells.[10] She reports: "In a series of 266 early blastoderms mounted in Locke solution, to which had been added chicken bouillon as prepared by Lewis and Lewis, no second division of the cells of a given blood island was observed in intervals of from 3 to 6 hours" (19). "In three successive preparations in which extract [Preparation XL R] had been added to the medium a second cell division was seen. In two preparations the second division was of a blood island and the interval approximated three hours; in the third preparation the second division was of an endothelial cell, making the wall of the vessel, and the interval was approximately one hour. The preparations were fixed just as the second cell division was observed and they confirm the observation that the blood islands in the two preparations and the endothelium in the third were in a cycle of cell division." "In all of these chicks the heart action remained very vigorous throughout the experiment, so that there was no sign of any toxic substance involved." "This material is interesting inasmuch as the blood islands during the stages studied are made up entirely of the megaloblast." Megaloblasts and similar primitive cells crowd the bone marrow in pernicious anemia in relapse. Peabody concluded (18) from direct observations upon the bone marrow of pernicious anemia that liver feeding reduced the proportion of megaloblasts in this tissue. The appearance in the peripheral blood of reticulated red blood cells following the treatment of this disease by means of the active principle in liver has been interpreted as due to the maturation of these primitive cells (15).

[10] It is a pleasure to acknowledge the collaboration of Dr. Florence R. Sabin. We are indebted to her for the above report upon her experiments with Preparation LX, fraction (R).

These observations suggested that the active principle effective in pernicious anemia may have an effect in accelerating the development of primitive red blood cells. With this in mind small amounts of the purest fractions that had been prepared and were known to be effective in pernicious anemia were added to a suspension containing reticulated red blood cells *in vitro*. Histological observations were made by one of us (Minot) upon the staining of the reticulated cells by means of brilliant cresyl blue. Observations upon metabolism were made for us by Dr. Robert Emerson. The results that have been obtained thus far, though inconclusive, suggest that the purest fractions thus far prepared increase the metabolism and influence the staining of immature red blood cells either by reducing the dye or by modifying the reticular material.[11] Whether or not these phenomena depend upon impurities in even these preparations or upon the active principle effective in pernicious anemia must await further experimentation.

The purest fractions of the liver thus far prepared that contain the active principle effective in pernicious anemia have now been observed to accelerate one of the first stages in the formation of red blood corpuscles, namely the division rate of primitive cells, and may modify one of the last stages. These observations have so modified the course of this research as to render it desirable at this time to report so much of the attempt to isolate the active principle as has completely depended upon the feeding of fractions of liver to patients with pernicious anemia This part of the investigation suggests that the active principle is a nitrogenous base or polypeptide.

Further studies of the nature of the active principle will depend in large part upon observations of the action of this, or of a related substance, upon the modification of different types of cells. The

[11] Liver Extract No. 343 has been shown by Dr. West to reduce the dye under anaerobic conditions (personal communication). Our purest fractions exhibit but a very slight reducing action on brilliant cresyl blue however, or on methylene blue even in the presence of such substances as glucose, dihydroxyacetone, formaldehyde, or xanthine. The notion that we might be concerned with an oxidizing enzyme led to testing Preparation XL, fraction (R) by means of guiaconic acid, phenolphthalin, and pyrogallol in the presence of hydrogen peroxide. No peroxidase action was observed.

relation between the active principle effective in pernicious anemia and those substances extracted from liver by Heaton (7), that influence the growth of fibroblasts, must be considered. These considerations will involve studies of the metabolism and the kinetics of the maturation process. Thus we may pass from the study of a special problem of clinical medicine to one of general biology.

SUMMARY.

1. Studies are reported of the nature of the active principle effective in pernicious anemia. In these studies fractions of liver were fed to patients with this disease. Those fractions were considered to contain the active principle that produced an increased concentration of reticulocytes and erythrocytes in the peripheral blood.

2. The relations between the reticulocytes and the erythrocytes in the blood during the first days of feeding a potent extract have been mathematically formulated.

3. The active principle is soluble in water, insoluble in ether, and precipitable by alcohol.

4. Fractions have been prepared free of iron and of proteins, carbohydrates, and lipoids and have proved potent sources of the active principle. The active principle is found in the filtrate from basic lead acetate and is precipitable by phosphotungstic acid.

5. A fraction of the phosphotungstates contains the active principle. This consists of phosphotungstates of substances containing about 19 per cent of nitrogen. The active principle must therefore be considered either a nitrogenous base or a polypeptide of which but 0.6 gm. a day has sufficed to produce a pronounced response of reticulated red blood corpuscles in a patient with pernicious anemia.

6. The growth of immature red blood corpuscles has been stimulated by the purest fractions thus far prepared containing the active principle effective in pernicious anemia. The special methods and problems of clinical medicine are therefore being supplemented by those of general biology in the study of this constituent of liver.

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# THE NATURE OF THE MATERIAL IN LIVER EFFECTIVE IN PERNICIOUS ANEMIA. II

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