

THE CHEMISTRY OF THE ANTI-PERNICIOUS ANEMIA
SUBSTANCES OF LIVER

BY

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THE efficacy of whole liver in the remission of pernicious anemia was first demonstrated in 1926, an observation which has since then been fully confirmed. In spite of several attempts by various investigators, the isolation of the active principle in a state of chemical purity and integrity has so far been unsuccessful but there has been a notable advance in the purification and potency of liver extracts. In view of the several divergent points of view regarding the chemical nature of the active material, it appears desirable to describe and review the work of closely allied groups of investigators together, before passing to that of another. This method of preparation, it is hoped, will, in preference to the strictly historical one, give a clearer perspective of the contributions of each worker in this field.

We shall not discuss the clinical aspects of pernicious anemia nor deal with the now widely accepted theory of the roles of the "extrinsic" and the "intrinsic" factors which, from a chemical view-point, have not so far been identified.

The claim of Klein and Wilkinson⁴⁷ that the active thermostabile principle of liver can be synthesized *in vitro* by incubating beef muscle and hog stomach extracts, could not be confirmed by Castle.⁴

We will not concern ourselves with the antianemic substance reportedly present in normal urine^{88,70,15,77,53,43} nor attempt to decide whether the antianemic-principles found in the kidney,⁵⁹ lungs,³⁶ brain, salivary glands, saliva,⁷² pancreas and other organs, are the same chemically as that associated with liver.

We will only refer to "a new therapy of pernicious anemia" with a spinach extract, a report, which is not supported by experiment.⁶⁴ No attempt is made to review the interesting observations of Massa and Zolezzi,^{55,56,57} and of Mermod and Dock⁶⁰ on the use of congo red.

We shall deal only with the substance or substances in liver which exert a beneficial influence in cases of pernicious anemia.

EARLY THERAPY AND ASSAY

Before Minot and Murphy, there had been scattered suggestions in literature that pernicious anemia is a deficiency disease.^{18,47} Whipple *et al.*, during the treatment of severe secondary anemia in dogs induced by repeated hemorrhage, observed the favourable influence exerted by the feeding of beef liver; they suggested that food factors be given serious consideration in the clinical management of pernicious anemia.^{82,83} In 1926, Minot and Murphy reported the prompt and distinct improvement in a large number of pernicious anemia patients on a diet in which liver was an important constituent. Within two to eight days of such treatment there occurred an increase in the reticulocytes of the circulating blood, reaching a maximum on the third to tenth day and subsequently returning to the lower original level. With continued liver therapy, this reticulocyte response was followed by a rise in hemoglobin and total red cell count, with a return to an approximately normal blood picture in about two months. This blood response, chiefly the reticulocyte rise and return of red cells to normal number, has formed the basis of the clinical assay of potent preparations. Inasmuch as the chief difficulty in the work on the purification of active materials has been the relative scarcity of pernicious anemia patients, many worthy attempts have been made to develop an animal assay; the guinea pig, dog, cat, pigeon, swine, monkey and rabbit have all been tried without definite success in any. Creskoff and Fitz-Hugh have covered this subject admirably in their review on the standardization and assay of liver extract.¹⁰ The clinical assay still is the only reliable way of following the fractionation procedures.

FRACTIONATION OF LIVER BY COHN, MINOT
AND ASSOCIATES

In 1927, Cohn, Minot and their collaborators attacked the problem of the isolation of the active material from beef liver.^{5,6,7,8,9} Table I summarizes their steps of fractionation. A major step in the purification procedure was the treatment of a concentrated

TABLE 1
Raw Minced Liver
brought to pH 9.0

INACTIVE FRACTIONS**ACTIVE FRACTIONS**

Insoluble Residue (A)	Water Soluble Extractives brought to pH 5.0
Protein Precipitate (B) (acid-precipitable proteins)	Water Soluble Extractives heated to 70° C.
Heat Coagulable Proteins (C)	Water Soluble Extractives (D) extracted with ether
Ether Soluble Extractives (EE) [Removes 2 pct. of solids (D)]	Non-ether Soluble Extractives (E) extracted with strong alcohol
Alcohol Soluble Extractives (F) [Removes about 30 pct. of solids (D)]	Alcohol Precipitable Extractives (G) dialyzed
Dialyzed Extractives (H)	Dialysate (I) treated with silicic acid gel (pH 5)
Extractives Adsorbed by Silicic Gel (J)	Filtrate (K) extracted with <i>n</i> -butyl alcohol
Residues of <i>n</i> -butyl Alcohol Extraction (M)	Extractives (L) precipitated with basic lead acetate
Lead Precipitable Extractives (N)	Filtrate (O) precipitated with phosphotungstic acid
Filtrate from Phosphotungstate (Q)	Precipitate (P) phosphotungstates treated with 90 pct. acetone
Acetone-soluble Phosphotungstates (S)	Acetone-insoluble phosphotungstates (R)
Starting with precipitate (P) regenerated and treated with 95 pct. alcohol	
Insoluble Residue (peptones, proteoses, polypeptides)	Extract concentrated Intravenous Extract "I.E."
"I.E." dissolved in 90 pct. alcohol, added equal volume of ether	
Filtrate (contains tryptophane, tyrosine)	Precipitate. Treated with 1 volume H ₂ O: 9 vol. alcohol: 4 vol. ether
Precipitate (large number of substances giving diazo test)	Filtrate. Treated with 1 vol. H ₂ O: 12 vol. alcohol: 6 vol. ether
Filtrate (extracts phosphorus- containing substances)	Precipitate (Z). Treated with 11 vols. alc.: 6 vol. ether: 1 vol. H ₂ O.
Precipitate (this is the fraction that has hitherto been precipitated by HgSO ₄ in acid solution). Treated with picric acid.	
Precipitate (extremely active). (Effective in 140 mg. dose).	

aqueous extract of liver (D) with an amount of absolute alcohol to result in a final alcohol concentration of 95 per cent.—the active precipitate is Cohn's Fraction G—which proved effective when fed daily in amounts of 9–14 grams (equivalent to about 200–400 grams of whole liver). A later modification was the treatment of the aqueous extract with alcohol to 70 per cent., the active principle remaining in solution with the precipitation of a good deal of inactive solids. They were able to eliminate proteins, fats and carbohydrates from the raw beef liver without a noteworthy loss in activity. The purer fractions contained less phosphorus and a greater percentage of nitrogen leading to the inference that "the active principle is either a nitrogenous base or a polypeptide".⁶ On purer fractions the biuret test was negative and hydrolysis produced no increase in amino nitrogen. In highly purified solutions, heavy metals appeared to destroy activity. They believed that the active principle is free from proteins, carbohydrates, lipoids, tryptophane, tyrosine, arginine, cystine, creatinine, iron, sulfur and phosphorus.⁸ Cohn's group concluded in 1930^{8,9} that the active principle was a nitrogenous base, the nitrogen in which exists as in a secondary or tertiary amine. The low nitrogen content (10.8 per cent.) appeared to exclude purine or pyrimidine bases, but not ring compounds of the pyrrole or pyridine types. It seemed unlikely that it was a pyrrole because it gave no pine-splinter test characteristic of certain pyrroles. Their most active preparation (picric acid precipitate) proved active when given parenterally in a 140 mg. dose.

EARLY WORK BY WEST AND ASSOCIATES

West and Nichols in 1928⁷⁸ reported a product which showed a positive biuret test, a weakly positive Hopkins-Cole reaction and a positive test for arginine. West and Howe in 1930^{79,80} prepared a product which proved active on intravenous injection of 680 mgm.; their best fraction appeared to be rich in a nitrogenous body with acid properties which on hydrolysis set 50 per cent. of its nitrogen free as amino-nitrogen. A crystalline quinine salt which at first seemed active clinically turned out later to be inactive.⁸¹

LATER WORK BY DAKIN, WEST AND ASSOCIATES

In 1935 Dakin and West¹² introduced the precipitation of the active material by

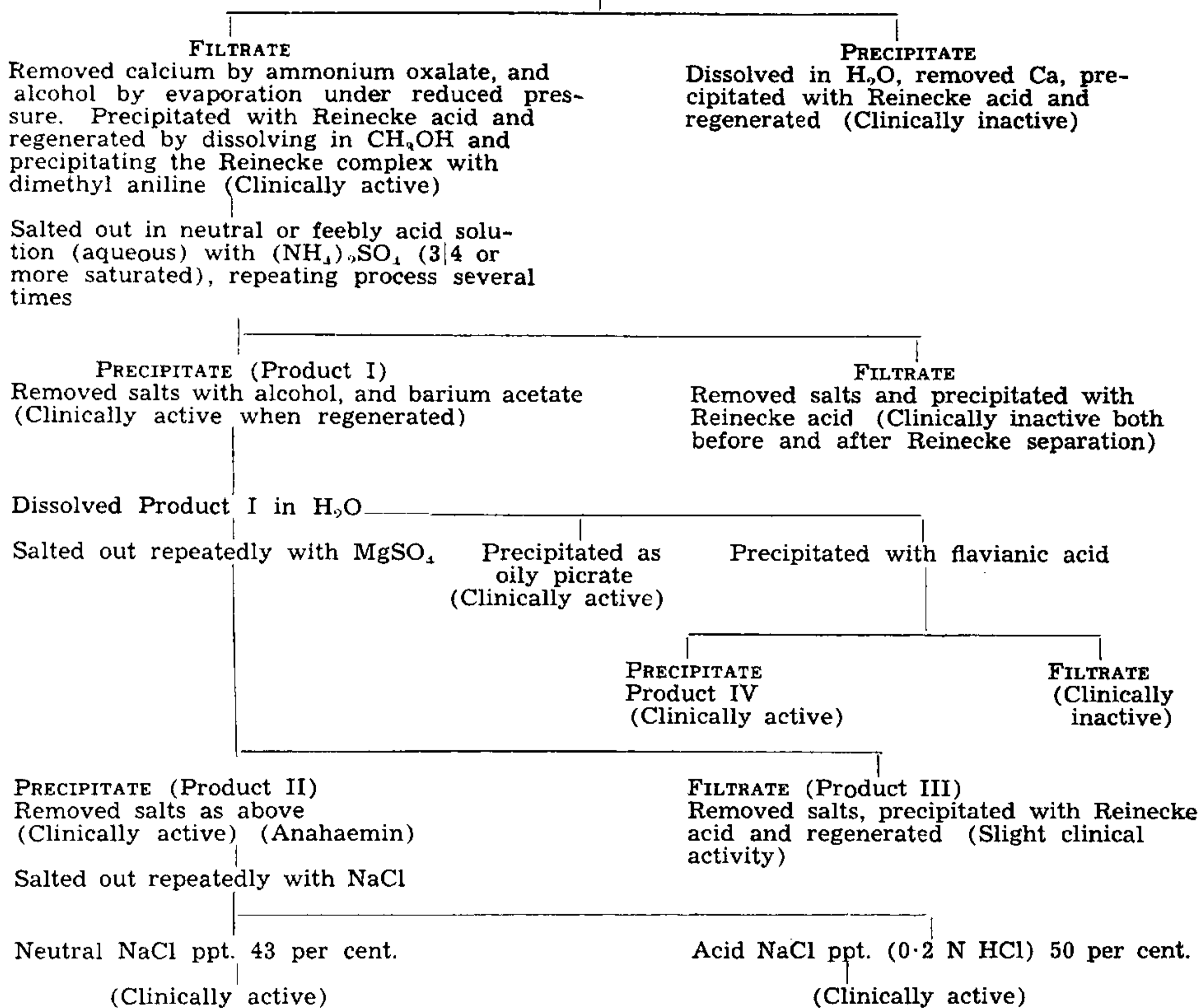
Reinecke salt in acid solution, with subsequent removal from a weak alcoholic solution of the Reinecke acid as the sparingly soluble salt of a tertiary base (e.g., dimethyl aniline) or by extraction with amyl alcohol. Table II summarizes the steps in their fractionation. 80 mg. of their Ana-haemin gave a maximal clinical response. Hydrolysis of this active preparation yielded a group of amino acids. Complete absence of pyrimidine and purine bases, pentoses and desoxyglucose was reported. Later work by Dakin, Ungley and West in 1936¹³ convinced them that the amino-hexose (glucosamine), which their previous preparation contained, was not an integral part of the active principle; they obtained further purification by introducing the precipitation of the active material by uranium (Table III). Their purest preparation at this time yielded on hydrolysis: arginine, glycine, leucine, aspartic acid, hydroxyproline and perhaps proline. There was also indication of a dicarboxylic acid easily soluble in water and giving a very soluble copper salt precipitable by alcohol—possibly hydroxyglutamic acid. From dialysis experiments through membranes of known pore diameter, they tentatively assigned a molecular weight between 2,000 and 5,000. The conclusion reached by Dakin, Ungley and West, as stated in 1936, was "that the hematopoietic substance in liver is, or is associated with, a peptide, possessing many but by no means all of the properties of an albumose". Recently Dakin and West¹⁴ reported a few experiments on the precipitation of their material with albumose precipitants, including nucleic acid bile, taurocholic and other bile acids, and the barium carbonate reaction of Seigfried; the first three reagents yielded precipitates containing much active material.

WORK OF LALAND, KLEM, STRANDELL AND ASSOCIATES

Strandell and associates in 1935 and 1936^{66,67} have reported the clinical assays of materials isolated by Laland and Klem.⁵² Their procedure of fractionation—as summarized in Table IV employs phenol for the elution of the active material after adsorption on charcoal.

Dakin and West in 1935 found they could salt out the active substance by three-fourths to complete saturation with ammonium sulfate. Laland and Klem also found that

TABLE II
Commercial Liver Extract
treated with calcium acetate in 75% alcohol



such treatment of their fractions yielded active material, although the antianemic principle was not precipitated quantitatively even by full saturation.

By a series of steps, the exact details of which have not yet been reported, Laland and Klem have obtained an active fraction "BBaBFu.s.E", 0.00035 grams of which correspond to 100 grams of liver. This material is a bright reddish-yellow, non-crystalline acidic substance, easily soluble in water, partly soluble in alcohol and insoluble in ether. They reported absorption bands in two regions of the ultraviolet range at 2500–2650Å and 3450–3500Å. The ninhydrin reaction was negative; the orcin test, positive. After hydrolysis, amino nitrogen

as well as acidic and basic amino acids have been detected.

53 mg. of Heptomin II (corresponding to about 200 grams of liver) showed antianemic activity when injected intraglutarially.⁶⁶ Their purest preparation "BBaBFu.s.E" when administered parenterally gave a satisfactory hematopoietic response in a dose of 0.7 mg. (corresponding to 200 grams of liver).⁶⁷ The Scandinavian workers regard their product as a biuret negative peptide.

APPLICATION OF LALAND AND KLEM PROCEDURES TO DAKIN AND WEST MATERIAL

Ungley in 1936 further purified an active Dakin and West fraction by the methods of

TABLE III

Liver Extract Power

1 kg. dissolved in warm H_2O (2.5 L); added $(NH_4)_2SO_4$ (1.4 kg.);
in refrigerator overnight

ACTIVE	INACTIVE
PRECIPITATE	FILTRATE
Washed with saturated $(NH_4)_2SO_4$ soln. and dried; stirred in H_2O (500 c.c.); filtered	
FILTRATE	RESIDUE
Reprecipitated by saturation with $(NH_4)_2SO_4$	
PRECIPITATE	FILTRATE
Suspended in H_2O (200 c.c.); added alcohol gradually (500 c.c.); in refrigerator	
FILTRATE	PRECIPITATE
Concentrated under diminished pressure (less than $50^\circ C.$); added basic lead acetate (200 gm. in saturated aqueous soln.) and NH_4OH until solution was alkaline to litmus; filtered immediately	
FILTRATE	PRECIPITATE
Acidified to Congo red with H_2SO_4 ; filtered to remove $PbSO_4$; precipitated active material with Reinecke acid (about 25 gm.); regenerated the Reineckate; added uranium acetate (150 c.c. saturated solution)	
CURDY PRECIPITATE	FILTRATE
Washed well with cold H_2O ; dissolved in H_2SO_4 (0.5 N); added $(NH_4)_3PO_4$ (2 gm.) and neutralized with NH_4OH to remove uranium as its insoluble phosphate; solution again subjected to precipitation with basic lead acetate (15 gm.), freshly prepared lead hydroxide (5 gm.), and NH_4OH added so long as a precipitate was formed.	
FILTRATE	PRECIPITATE
(Contained no glucosamine) Peptide recovered by acidifying with H_2SO_4 ; filtered off the $PbSO_4$; precipitated with Reinecke acid (3 gm.); regenerated the Reineckate; concentrated the decomposed Reineckate and precipitated with absolute alcohol	
SOLID PEPTIDE	
(4.02 gm.) (Glucosamine-free)	
On decomposition with H_2SO_4 , contained all of the glucosamine present in the product of the previous step.	

TABLE IV
FRESH HASHED LIVER
Extracted with H₂O with addition of acetone
to 50 pct. by volume (tissue H₂O inclusive)

ACTIVE	INACTIVE
<p>EXTRACT I strongly concentrated</p> <p>CONCENTRATE (II) filtered</p> <p>FILTRATE (III) freed from protein—different methods used: precipitation by metal sols or gentle heat coagulation with the addition of acid</p> <p>PROTEIN-FREE FILTRATE (IV) (PERNAMI I) free from albumin but not from salts. 3-4 gm. dry matter from original 100 gm. liver shaken with phenol—this extract, (IV) gives off the active substance quantitatively to phenol along with other substances. Add ether and H₂O to the phenol solution—the antianemic substance can be quantitatively shaken into the water layer</p> <p>FRACTION VI (Clinically very potent (HEPTOMIN —I—dark)</p> <p>0.27 gm. dry matter from 100 gm. liver Treat with active “coal” (charcoal)</p>	RESIDUE
<p>COAL ADSORBATE extracted by phenol and regenerated by shaking with ether and H₂O</p> <p>FRACTION VIII (Fully active) 0.028 gm. dry matter from 100 gm. liver combined the phenol extraction with ex- traction with H₂O containing phenol</p>	FILTRATE
<p>FRACTION B 0.001 gm. dry matter from 100 gm. liver additional adsorption on active charcoal and combined extraction with phenol water</p> <p>FRACTION BB 0.001 gm. dry matter from 100 gm. liver evaporated to dryness, dissolved in glacial acetic acid and precipitated with excess ether</p> <p>FRACTION BBa (nearly colorless) 0.001 gm. dry substance from 100 gm. liver</p>	<p>FRACTION A 0.019 gm. dry substance from 100 gm. liver (inactive)</p>

Laland and Klem.⁷⁵ Further purification was achieved by employing phenol and methyl alcohol for fractionation. 50 and 75 mg. of this purified preparation gave satisfactory results. By these methods Ungley purified Dakin and West's Anahaemin at least two and one half times.

In 1936 Wilkinson⁸⁶ applied Reinecke acid precipitation as employed by Dakin and West to the preparation of active liver extract^{84, 85, 86, 47}; he was able to elicit maxi-

mal response with total doses of 18 to 36 mg. equivalent to 661 to 1332 grams of fresh liver.

WORK OF SUBBA ROW, JACOBSON AND ASSOCIATES—THE MULTIPLE FACTOR HYPOTHESIS

The investigators whose work we have so far considered have worked on the general hypothesis that the active portion of liver is a single chemical entity. The fact that

the reports of the chemical properties of their active preparations differed might lead to some doubt that a single substance is involved. The possibility exists that the activity of material effective in the treatment of pernicious anemia rests upon a certain type of compound or linkage common to more than one substance as is believed to be the case of some of the vitamins. Then again, one cannot neglect the consideration that successful therapy may depend upon the interaction of several factors.

In 1935 Fiske, Subba Row and Jacobson presented results which suggested that therapy in pernicious anemia could be achieved most successfully by a combination of two or more substances.^{22,68} This led to the development of a multiple factor hypothesis. A report by Jacobson and Subba Row in 1937⁴¹ indicated that there is (a) a primary, active hematopoietic factor in liver, and (b) at least three accessory factors, in themselves inactive, but whose presence materially augments the activity of the primary substance. They observed that continued purification of active liver extract, in the absence of significant losses and of destructive procedures, led to a partial or complete loss of therapeutic activity, but that combining these purified preparations of reduced potency with certain inactive fractions, resulted in the restoration of activity.

The four factors believed to be concerned are:

- A. The primary factor of unknown chemical nature active in amounts of 0.2 to 0.4 mg. per day.
- B. Three accessory factors: Inactive, singularly or in combination
 - (1) Fraction A—*l*-tyrosine⁶⁸
 - (2) Fraction C—probably a complex purine⁶⁸
 - (3) Fraction F—probably a peptide.⁷⁰

Fraction C (11 mg. from 100 grams of fresh liver) consisted of light yellow crystals showing positive xanthine, murexide and diazo tests and containing 33.1 per cent. nitrogen. The tentative conclusion was drawn that the substance is a complex purine resembling members of the pterine series of Wieland and Schöpf. Later work by Subba Row indicates that fraction C is composed, for the most part, of xanthine, accompanied by several other difficultly separable substances.

In 1936, Subba Row and associates⁶⁹ reported the preparation of an active product by the elution with 65 per cent. ethyl alcohol after adsorption on charcoal—a method which they had already used in the isolation of fraction C⁶⁸ and one which is quite similar to that developed by Kyer at about the same time.⁵¹ A summary of the method of purification employed by Subba Row *et al.* is given in Table V.

Fraction I, a white microcrystalline material, had a negative biuret, Mellon's and Sagakuchi's tests; its absorption spectrum showed an inflection between 2480 and 2560 Å (note similarity to absorption spectrum of material of Laland and Klem). Fraction I proved effective clinically in total amounts of 4–8 mg. administered over a 10-day period.

In 1938, these investigators could report that by further purification the yield of total solids in the primary factor had been reduced to 1.2 mg. from 100 grams of liver, however with diminished potency.⁷¹ There was evidence that the diminished potency was due to the absence of additional accessory factors. Two such materials were isolated from the mother fraction of the primary factor and identified as tryptophane and guanosine. All five accessory factors together, but without primary factor, were inert in a pernicious anemia case. Yet administration of the five accessory factors along with the primary factor (in dosage of 0.12–0.26 mg. per day) proved active; of eight cases four responded maximally.^{71,42}

Further purification of the primary factor by readsorption on charcoal, elution and then precipitation by a mixture of alcohol and ether yielded an amorphous material which when tested on one patient showed good activity in dosage of 0.06 mg. per day. Chemical properties of the primary factor suggested that it is a complex pyridine derivative; in support of this view, synthetic nicotinic acid administered parenterally in dosage of 1 to 2 mg. per day to two patients along with three accessory factors (A, C and F) effected moderate hematopoietic response and clinical improvement.

OTHER EVIDENCE FOR MORE THAN ONE FACTOR

Eisler, Hammarsten and Theorell,¹⁷ using cataphoresis, obtained evidence of two active principles in liver preparations—one of which leads to reticulocytosis and the other,

TABLE Va

150 c.c. Commercial Liver Extract (equivalent to Cohn's G)

(3 c.c. from 100 gm. fresh liver)

Dissolved in 1 liter H_2O

Brought to pH 8 with NaOH, acidified to pH 6 with HCl

Added 50 grams ~~merite~~ and stirred 1 hour

Filtered

Charcoal + Adsorbate

Washed repeatedly with H_2O till washings colorless.

Suspended in 1 liter 65 per cent. ethyl alcohol, brought to the boiling point, stirred 5 minutes and filtered hot

Elution repeated

Combined Eluates

concentrated under diminished pressure at 40° C. to 150 c.c.

Fraction B

concentrated further under reduced pressure

White Granular Material
crystallized

Fraction C

Filtrate—Fraction D

TABLE Vb—I

Fraction D

(150 c.c. from 5 kg. liver)

(10-12 mg. total N per 100 gm. liver)

Acidified to pH 2 with HCl

Added 16 gm. fuller's earth

Stirred mechanically for 30 min. at room temp.

The ppt. was filtered and washed once with
50 c.c. H_2O

Filtrate + Washings

Added 10 volumes 95% ethyl alc. and 10 vols.
ether

Mixture left in cold room 24-36 hours

Filtered

Precipitate (Fraction H)

(20 mg. from 100 mg. of liver—12-13% N)

Dissolved in 50 c.c. H_2O Brought to pH 3 with 10 N H_2SO_4 Caused a crystalline ppt. to settle, mainly of
 $CaSO_4$

Filtrate

Added to 40 c.c. H_2O containing 1 gm. Reinecke
salt at 40°In cold room for 24 hours—brought down a
crystalline precipitate

Filtered

Precipitate

Washed once with 50 c.c. ice cold H_2O Suspended in 300 c.c. 0.03 N H_2SO_4 at 30-35°The Reinecke acid was removed by repeated
extraction with a 500 c.c. mixture of equal
vols. of amyl alcohol and ether

Concentrated in vacuo to a volume of 25 c.c.

Concentrate

Added 10 vols. acetone and 10 vols. ether

In cold room 48 hours

Precipitate (Fraction I)

(Yield of 100 mg.—i.e., 2 mg. from 100 gm.
fresh liver)

TABLE Vb—II

Fraction D (1 liter)

Added 7 liters 95% ethyl alcohol

At room temp. 5 hours

Filtered

Filtrate

Added 3 liters ethyl alc. and 10 liters ether
In cold room 48 hours

Precipitate (Fraction E)

Dissolved in 500 c.c. H_2O Added 200 c.c. 5% solution of rhodanilic acid
in methyl alcohol

In cold room 48 hours

Filtered

Crystalline Precipitate

Freed of rhodanilic acid by pyridine with sub-
sequent removal of pyridine by ether

Regenerated Rhodanilate Solution

Precipitated by Reinecke salt

Reineckate regenerated (as in other method)

Fraction I

(Yield about the same as in other method)

when administered with the first, to erythropoiesis as well. Hofer in 1934³⁶ separated a "reticulocyte response" factor from a general "curative" factor.

ON THE IMPORTANCE OF TRYPTOPHANE AND HISTIDINE

From the peptide character of the active material of Dakin and West, and of fraction F of Subba Row and associates as well as their evidence for the importance of tyrosine and tryptophane, and from the observations of Laland and Klem that amino acids are among the products of hydrolysis, it would appear that amino acids are of importance in the problem of pernicious anemia therapy. The use of tryptophane in the treatment of anemia (experimental) is first credited to Hirazawa.³⁴ In the early 1930's, Fontes and Thivolle presented evidence in favour of the hematopoietic action of both tryptophane and histidine.²⁴⁻³² They regarded the former as the precursor of the tetrapyrrole group of hæmatin and the latter as the amino acid essential for the formation of globin in the hemoglobin molecule. They first showed the hematogenic action of tryptophane and histidine by their observation that subcutaneous injection into normal rabbits and dogs resulted in "hyperhemoglobinemia" and "hypererythrocytosis"; this action seemed to depend upon the presence of indol and imidazol nuclei and not to be a function of amino acids in general. Alcock in 1933¹ could not produce anemia in experimental animals by tryptophane deficiency alone and was thus led to doubt that the pyrrole of hæmatin is derived from tryptophane. In 1936 Hamada³³ confirmed the results of Fontes and Thivolle by producing an anemia in rats on a tryptophane-poor diet. Levi has reported that the injection of tryptophane in rabbits with experimentally produced anemia caused a restoration of the red cells and hæmoglobin to nearly normal values.⁵⁴

Fontes and Thivolle reported results from the administration of histidine and tryptophane to pernicious anemia patients. They believed that the digestive disturbances in pernicious anemia may interfere with protein breakdown. Their treatment of six cases²⁹ with parenteral administration of 200-400 mg. histidine and 100-200 mg. tryptophane for a month was followed by a rapid remission of long duration in one case, a slow remission in another, a rapid remission fol-

lowed by relapse in the third, and complete inactivity in the other three cases—not an impressive record in a disease which may run a course of remissions and relapses as pernicious anemia does.

Fontes and Thivolle have consistently maintained that the activity of raw liver and of the various liver extracts depends entirely on their contact of tryptophane and histidine in the free state. However, Cohn and associates obtained active preparations from which all tryptophane had been removed. Tryptophane and histidine were not found among the amino acids set free on hydrolysis of the material of Dakin and West. Aleksandrowicz and Gabryelski² found no tryptophane in a commercial preparation of proved activity (Pernaemon).

Negative results were obtained by Cuthbertson, Fleming and Stevenson,¹¹ who gave daily injections of 100 mg. tryptophane and 200 mg. histidine to two pernicious anemia patients. Dominici and Penati¹⁶ were also unable to confirm the favourable results of the French investigators. Tochowicz⁷³ agreed with Fontes and Thivolle that some of the trouble in pernicious anemia lies in faulty protein metabolism; he concluded that although tryptophane may play a role in pernicious anemia, histidine is of no importance in either the pathogenesis or the treatment of the disease.

Here again there are conflicting reports. Tentatively, we may conclude that tryptophane may play a role in the treatment of pernicious anemia, yet by no means a major one. The evidence for the importance of histidine in the "antianemic factor" is even less convincing.

WORK OF MAZZA AND PENATI

Mazza and Penati⁵⁸ have isolated active materials, containing, they believe, a nucleotide, a polypeptide and a pterine. Their steps of fractionation are summarized in Table VI.

Their substance C which they believe is of pterine nature showed maximal absorption at 2535 Å and 2490 Å.

Substance D contained 3 per cent. ash (traces of iron and copper); its microanalysis showed 50.1% C, 7.6% H and 12.4% N. It also contained phosphorus (about 3 %) and sulphur (trace). Ninhydrin and Mellon's tests were weakly positive; there were strongly positive tests for histidine and pentose; and a negative test

TABLE VI
Minced pig liver
Liver juice
Pressed at 500 atmospheres pressure
Treated with 96% ethyl alcohol to give a 70% alcohol content
Dilute H_2SO_4 to pH 5
Boiled for $\frac{1}{2}$ hour

Precipitate (proteins)	Filtrate
	Concentrated in vacuo to a syrup Extracted with trichlorethylene
Extract (lipoids)	Syrupy residue
	Taken up in 80% alcohol
Precipitate	Filtrate
	Neutralized Treated with saturated soln. of CaCl_2
Precipitate	Filtrate
	Calcium removed by ammonium oxalate
Precipitate	Filtrate
	Solvent removed in vacuo Treated with 5 vols. 98% alcohol Precipitate dried in vacuo
	Substance A (500 mg. from 500 gm. fresh liver) (active in total dose of 3 grams) Put in aqueous solution Treated with soln. of basic lead acetate and $\text{Ba}(\text{OH})_2$
	Filtrate
	Pb and Ba removed by H_2SO_4 Filtrate to pH 5 Charcoal adsorption Charcoal and Adsorbate Elution with warm (70°) 50% ethyl alcohol at pH 5 (acetic acid)
	Eluate
	Alcohol removed in vacuo Charcoal adsorption and elution repeated Resulting eluate evaporated to dryness
	Substance B (100 mg. from 500 grams liver) (active in total dose of 0.5-1.0 grams) (sky blue fluorescence)
4% Solution Substance B in H_2O	4% Solution Substance B in H_2O
Precipitated with H_2SO_4 and phosphotungstic acid	to pH with HCl Adsorbed on fuller's earth Eluted with 5% pyridine at 70°
Precipitate	Pyridine Extract
Decomposed by $\text{Ba}(\text{OH})_2$, the Ba being removed by H_2SO_4 solution concentrated to small volume Treated with 10 vols. absolute alc. and 10 vols. acetone	Evaporated to dryness Residue dissolved in 1 N NaOH Acidified with HCl
Precipitate	Filtrate
Dissolved in H_2O Saturated with $(\text{NH}_4)_2\text{SO}_4$	Adsorbed on fuller's earth and eluted with 5% pyridine Eluate concentrated in vacuo to small volume
Precipitate	Added NH_4OH , NH_4Cl and AgNO_3
Dissolved in 75% alcohol and reprecipitated with absolute alcohol and acetone Precipitate dried	Precipitate
Substance D (5 mg. from 100 grams liver)	Put in solution with 1N HCl Solution adsorbed with fuller's earth which is then eluted with 10% pyridine Pyridine eluate evaporated in vacuo to dryness
	Substance C (1 mg. from 5 kg. liver)

for tryptophane. It showed maximal absorption at 2600 Å and 2650 Å. Of the total nitrogen, 5.8% was amino nitrogen; after acid hydrolysis for 1½ hours amino nitrogen accounted for 68.2% of the total nitrogen. They believe that fraction D consists of a combination of an adenine nucleotide and a polypeptide containing proline, oxyproline, histidine, arginine, hydroxyglutamic acid and a monoamino-monocarboxylic amino acid.

Unfortunately, Mazza and Penati have as yet not reported adequate clinical tests for their material. In the single case which they do report for their purest preparations (C & D), substance D given alone (375 mg. over a period of 8 days) showed no activity. Then in the same case both D (total additional dose of 325 mg.) and C (total dose of 13 mg.) given over a two-month period showed only moderate activity, raising the red count from 2.0 million to 3.2 million. From this one case considering the relatively large amount of material used, their substances are most likely not very pure and thus the chemical properties they have described may be those of contaminating compounds rather than of the antianemic factor.

MISCELLANEOUS CONTRIBUTIONS

Buchanan in 1929³ reported that the active principle of liver extracts has apparently every chemical resemblance and physiological action of oxidized glutathione. Although Fleming²³ later declared that glutathione, mostly in oxidized form, enters into the composition of the antianemic factor, Koser in 1936⁵⁰ denied the importance of glutathione in this respect.

In 1933 Felix and Fruhwein²¹ fractionated active liver extract, following the activity by determining the reticulocyte response after injection in anemia (not necessarily pernicious anemia) patients or normal persons, and by methemoglobin formation *in vitro*. After repeated purification with mercuric sulfate, their preparation gave a negative test for tryptophane, and a negative biuret test. It is unfortunate that their preparations were not adequately assayed on pernicious anemia patients.

In 1935, Erdős¹⁹ reported his work on the preparation of active material. He made an acid extract of finely hashed liver, the protein then being removed by iron. Inas-

much as his material gave a positive biuret and showed an increase in amino-nitrogen by about 300 per cent. after acid hydrolysis, he believed it to be a peptide. He isolated a water insoluble silver salt from the analysis of which he suggested the formula $C_{650}H_{720}O_{36}N_{30}S_2P_2Ag_3$ with a molecular weight of about 10,000. Unfortunately his method of assay was inadequate; he tested activity on the anemia produced in dogs and rabbits by the administration of phenylhydrazine.²⁰

We have thus far discussed the results obtained by investigators who employed the orthodox chemical procedure of isolation and identification. In 1937, Jacobs^{37,38,39} reported conclusions regarding the chemical character of the active liver material which he reached by employing less orthodox means. From a study of the properties exhibited by potent liver extracts, he tried to deduce the chemical nature of the anti-anemic principle. In his first paper³⁷ he suggested further investigation of the possible role of glucosamine and the common amino acids (especially leucine and tyrosine). By mixing raw potato scrapings (source of tyrosinase) and tyrosine he believed he could form the 5, 6-quinone of dihydroindole-2-carboxylic acid, the so-called "red substance" of Raper⁴⁵; feeding such a mixture to a pernicious anemia patient resulted in inconclusive results.³⁹ Later Jacobs concluded that the "red substance" of Raper was not concerned in the activity of liver extracts. In his most recent paper⁴⁰ he proposes to investigate choline for its antianemic effect inasmuch as he was able to isolate this compound from a commercial liver extract. However, we doubt that this substance will show any activity at all; we are of the opinion that the activity of the commercial liver extract was lost for the most part during his repeated decolorizations with animal charcoal.

Aleksandrowicz and Gabryelski² have proposed that heparin is one of the important therapeutic factors in liver preparations. Heparin administered intramuscularly to three pernicious anemia patients effected a moderate erythrocyte response in one. On the whole their results, both clinical and chemical, do not present convincing evidence for their thesis.

Jones, Phillips, Larsell and Nokes⁴⁴ reported that nuclear extractives from various

organ extractives—supposedly consisting chiefly of nucleoproteins and the sodium salts of nucleic acids—yielded satisfactory results in pernicious as well as other anemias when administered orally.

Karrer, Frei and Fritzsche in 1937⁴⁵ reported that liver preparations possessing full antianemic potency in a singly administered dose of 10–20 mg. contain amounts of phosphorus, pentose and adenine consistent with the presence of an adenine-nucleotide. However, a purer preparation active in doses of 8 to 10 mg.⁴⁶ contained no phosphorus or pentose. The ninhydrin test was positive, before and after hydrolysis, but the biuret was negative or at best only weakly positive. No polypeptides of the usual type were present. After hydrolysis, 2-amino acids were present only in small quantities, if at all. Koller⁴⁹ reported on the clinical use of Karrer's material.

In a short note in 1939, Tschesche and Wolf described the properties of an active material that resembled the preparation of Karrer et al. in that it showed a negative or only slightly positive biuret test, but a positive ninhydrine both before and after hydrolysis.⁷⁴ Their material, a white powder active in a dose of 40 mg., also had a negative Molisch and a negative Millon test; it was free of flavine, purine, pterine, reducing sugar and phosphoric acid esters. It analyzed: C, 50%; H, 7%; N, 14.5%; S, 0.6%. They have not as yet reported their clinical results in any detail.

COMPARISON OF ACTIVITIES OF PREPARATIONS

In the foregoing sections, the methods of preparation and, in some cases, the properties of various antianemic preparations have been described. There is as yet no agreement among the different investigators regarding what is the active material, or what are its properties. It may be of some interest, however, to compare the relative activities of some of the better preparations. How should such a comparison be made? The phenomenon of reticulocyte response is of little value in determining the comparative efficiency of extracts. It may or may not give an indication of the presence of potency, and it does not appear to indicate the degree of potency. Murphy suggests⁶³ that perhaps the most critical and important means of comparison is the determination of the amount of antianemic material that is necessary to maintain the erythrocyte count

of a pernicious anemia patient at a normal level. Inasmuch as there are few reports available concerning the long-time maintenance requirements of the extracts in which we are interested we cannot make our comparisons on this ideal basis.

In the absence of a more satisfactory basis of comparison, a few of the preparations may be compared in terms of the erythrocyte response elicited by definite quantities of different products (Chart 1, from J.A.M.A., 1941, 116, 367).

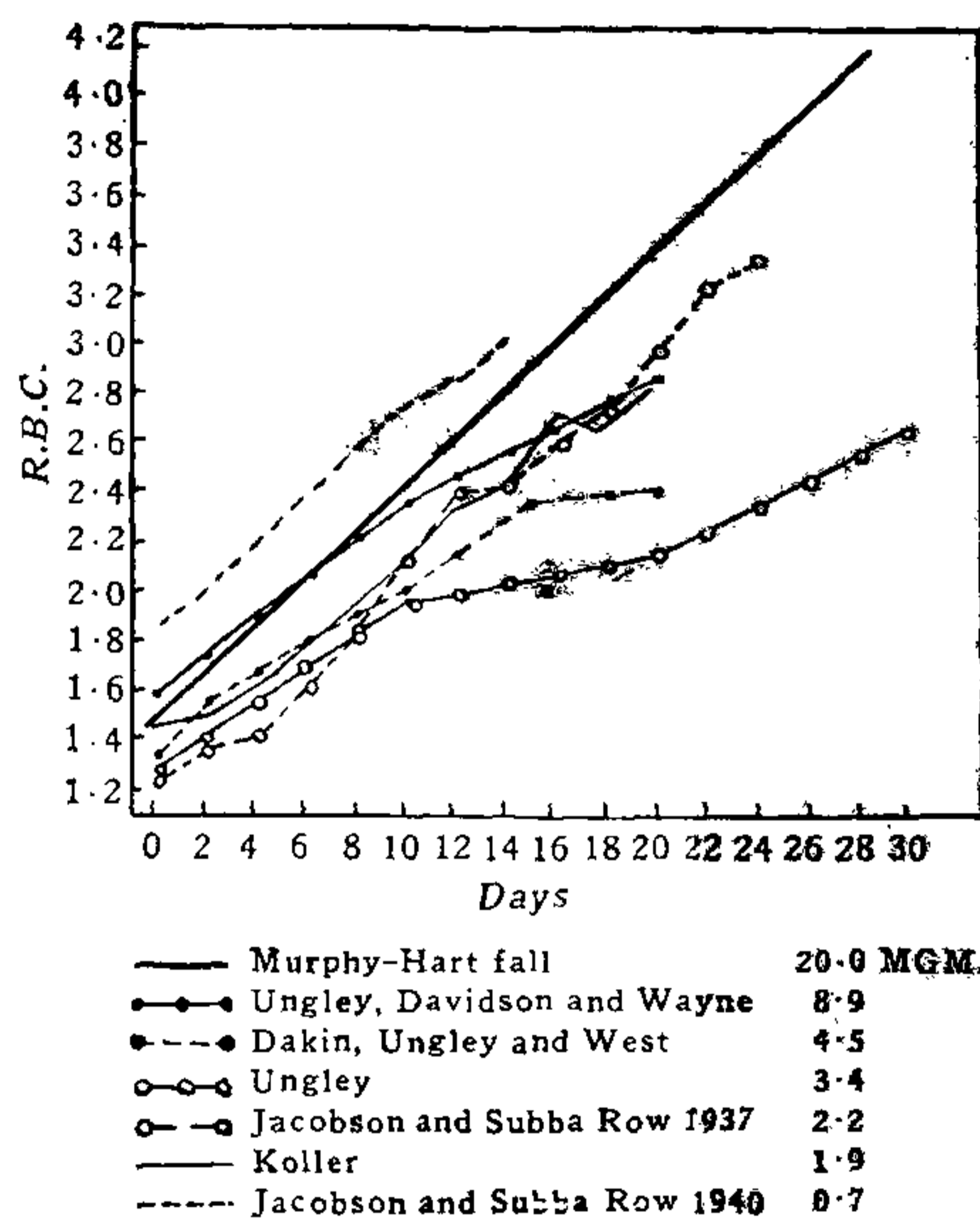


Chart 1.—Average erythrocyte regeneration curves following the administration of various purified liver extracts. The calculated average daily amount of material administered is recorded in the legend. The sources of these data are contained in the text. (The daily doses recorded for the curves of Jacobson and Subba Row refer only to the amount of primary factor administered; in addition, three accessory factors were administered in the 1937 curve, in a daily amount of 3.4 mg., and five accessory factors were administered in the 1940 curve in a daily amount of 6.2 mg.)

The data have been presented in the hope that further advances in the understanding of the chemical nature of the substances concerned will enable us to explain the discrepancies in the magnitudes of response achieved by different investigators. It is,

TABLE VII

Comparison of reported properties of certain liver extracts containing primary anti-pernicious anemia factors

	Cohn <i>et al.</i>	Dakin <i>et al.</i>	Laland <i>et al.</i>	Subba Row <i>et al.</i>	Karrer <i>et al.</i>
PHYSICAL PROPERTIES					
Color	Reddish Yellow	Colorless
Solubility	Sol in H ₂ O	Insol. in Absolute Alcohol	Insol. in ether Sol. in H ₂ O Partly soluble in alcohol	
	" " Acetic acid				
	" " Phenol				
	" " Formamide				
	" " Glycerol				
	" " 70% Alcohol				
	Slightly sol. in 95% Alcohol				
	Insol. in Abs. Alc.				
	" " Ether				
	" " Acetone				
Insoluble Salts with	Phosphotungstic Acid	Pptd. by High Conc. of Trichloroacetic acid but not by Low.	Phosphotungstic Acid Heavy Metals	
	Tannic Acid	Not pptd. by Rufanic Acid.			
	Sulfuric Acid	Not pptd. by Ferrocyanic Acid.			
	Picric Acid	Not pptd. by Metaphosphoric Acid.			
	Gold Chloride				
	Platinic Chloride				
	Silver Nitrate				
Precipitation by (NH ₄) ₂ SO ₄	Precipitated	Partially pptd.
Dialysis	Dialyzable	Dialyzable	Dialyzable	Dialyzable
Specific Rotation	(α) ¹⁶ D-112° to -133°		
Absorption of Light	250-265 m μ ; 345-350 m μ	248-256 m μ
CHEMICAL CONSTITUTION					
Composition in per cent.					
C	46.8-48.1	53.64	41.56	45.68
H	6.6-6.8	6.85	6.74	6.75
N	10.8	15.2-16.8	13.33	13.13	14.63
S	None		0.74	1.2	Present
Ash			2.05	(SO ₄ -10.2)	
Molecular Weight	2000-5000	
Presence of Pentose	None	Present	Present	None
Biuret Reaction	Negative	Negative	Negative	Negative	Negative
Amino Hydrogen Before Hydrolysis	None	0.5		5	
After Hydrolysis		10-10.4	Not Reported	75	Not Reported
Amino Acids Isolated	Not Reported	Arginine, Glycine, Leucine, Aspartic Acid, Hydroxyproline, Proline	Not Reported	Not Reported	Not Reported

unfortunately, apparently not possible at the present time to reconcile the various claims and facts regarding the material or materials which are present or capable of extraction from liver, and which are therapeutically active in pernicious anemia. Table VII summarizes the chemical properties of some of these preparations which

we have discussed. Although it is not yet possible to present the nature of the active material with chemical exactness, it is, nevertheless, proper to note with some satisfaction that since whole liver therapy was instituted, the amount of material needed by the patient per day had decreased from 400 grams to less than 10 mg. So much

progress makes it reasonable to expect the isolation of the active material in the near future.

- ¹ Alcock, R. S., *Biochem. J.*, 1933, **27**, 754.
- ² Aleksandrowicz, J., and Gabryelski, W., *Folia Haematol.*, 1937, **57**, 337.
- ³ Buchanan, J. A., *Amer. Med.*, 1929 **35**, (73).
- ⁴ Castle W. B., *Science*, 1933, **82**, 159.
- ⁵ Cohn, E. J., Minot, G. R., Fulton, J. F., Ulrichs, H. F., Sargent, F. C., Weare, J. H., and Murphy, W. P., *J. Biol. Chem.*, 1927, **74**, 69.
- ⁶ Cohn, E. J., Minot, G. R., Alles, G. A., and Salter, W. T., *Ibid.*, 1928, **77**, 325.
- ⁷ Cohn, E. J., McMeekin, T. L., and Minot, G. R., *Amer. J. Physiol.*, 1929, **90**, 316.
- ⁸ —, *J. Biol. Chem.*, 1930, **87**, 49.
- ⁹ Cohn, E. J., McMeekin, T. L., and Minot, G. R., *Trans. Amer. Physicians*, 1930, **45**, 343.
- ¹⁰ Creskoff, A. J., and Fitz-Hugh, T., *New International Clinics III*, Sept. 1938, 98.
- ¹¹ Cuthbertson, D. P., Fleming, J., and Stevenson, E. M. K., *Glasgow Med. J.*, 1931, **35**, 201.
- ¹² Dakin, H. D., and West, R., *J. Biol. Chem.*, 1935, **109**, 489.
- ¹³ Dakin, H. D., Ungley, C. C., and West, R., *J. Biol. Chem.*, 1936, **115**, 771.
- ¹⁴ Dakin, H. D., and West, R., *Proc. Soc. Exp. Biol. Med.*, 1939, **40**, 124.
- ¹⁵ Decastello, A., *Med. Klin.*, 1935, **31**, 377.
- ¹⁶ Dominici, G., and Penati, F., *Minerva Med.*, 1931, **2**, 213.
- ¹⁷ Eisler, B., Hammarsten, E., and Theorell, H., *Naturwiss.*, 1936, **24**, 142.
- ¹⁸ Eklers, C., *Lancet I*, 1925, 75.
- ¹⁹ Erdős, J., *Biochem. Zeit.*, 1935, **277**, 337.
- ²⁰ —, *Ibid.*, 1935, **277**, 342.
- ²¹ Felix, K., and Frühwein, H., *Zeit. Physiol. Chem.*, 1933, **216**, 173.
- ²² Fiske, C. H., Subba Row, Y., and Jacobson, B. M., *J. Clin. Invest.*, 1935, **14**, 509.
- ²³ Fleming, R., *Biochem. J.*, 1932, **26**, 461.
- ²⁴ Fontes, G., and Thivelle, L., *C. R. Acad. Sci., Paris*, 1930, **191**, 1088.
- ²⁵ —, *Ibid.*, 1930, **191**, 1395.
- ²⁶ —, *C. R. Soc. Biol.*, 1930, **105**, 965.
- ²⁷ —, *Ibid.*, 1930, **105**, 967.
- ²⁸ —, *Ibid.*, 1930, **105**, 969.
- ²⁹ —, *Le Sang*, 1930, **4**, 658.
- ³⁰ —, *C. R. Soc. Biol.*, 1931, **106**, 215.
- ³¹ —, *Ibid.*, 1931, **106**, 217.
- ³² —, *Ibid.*, 1931, **106**, 219.
- ³³ Hamada, T., *Zeit. Physiol. Chem.*, 1936, **243**, 258.
- ³⁴ Hirazawa, S., *Mitt. d. Med. Ges. Osaka*, 1921, **20**, 981.
- ³⁵ Hitzengerger, K., *Klin. Wschr.*, 1934, **13**, 1345.
- ³⁶ Hofer, W., and Hofer, I., *Ibid.*, 1934, **13**, 1601.
- ³⁷ Jacobs, H. R., *J. Lab. and Clin. Med.*, 1937, **22**, 371.
- ³⁸ —, *Ibid.*, 1937, **22**, 890.
- ³⁹ —, *Ibid.*, 1937, **22**, 892.
- ⁴⁰ —, *Ibid.*, 1938, **24**, 128.
- ⁴¹ Jacobson, B. M., and Subba Row, Y., *J. Clin. Invest.*, 1937, **16**, 573.
- ⁴² —, *J. A. M. A.*, 1941, **116**, 367.
- ⁴³ Jequierand, E., and Apsey, G. R. M., *Brit. Med. J.*, 1938, **2**, 934.
- ⁴⁴ Jones, N. W., Phillips, B. I., Larsell, O., and Nokes, H. T., *Ann. Int. Med.*, 1929, **2**, 603.
- ⁴⁵ Karrer, P., Frei, P., and Fritzsche, H., *Helv. Chim. Acta.*, 1937, **20**, 622.
- ⁴⁶ Karrer, P., Frei, P., and Ringier, B. H., *Ibid.*, 1938, **21**, 314.
- ⁴⁷ Klein, L., and Wilkinson, J. F., *Biochem. J.*, 1934, **28**, 1684.
- ⁴⁸ Kressler, K. K., Maurer, S., and Loughlin, R., *J. A. M. A.*, 1926, **87**, 476.
- ⁴⁹ Koller, F., *Helv. Medica Acta.*, 1938, **5**, 552.
- ⁵⁰ Koser, F. G., *Arch. exp. Path. Pharm.*, 1936, **180**, 183.
- ⁵¹ Kyer, J. L., *Proc. Soc. Exp. Biol. Med.*, 1935, **32**, 1102.
- ⁵² Laland, P., and Klem, A., *Acta Med. Scand.*, 1936, **88**, 620.
- ⁵³ Leiner, G., *Wiener Klin. Wschr.*, 1935, **48**, 559.
- ⁵⁴ Levi, A., *Arch. farmacol. sper.*, 1935, **60**, 437.
- ⁵⁵ Massa, M., and Zolezzi, G., *Giorn. d. clin. Med.*, 1933, **14**, 975.
- ⁵⁶ —, *Min. Med.*, 1934, **25**, 763.
- ⁵⁷ —, *Klin. Wschr.*, 1935, **14**, 235.
- ⁵⁸ Mazza, F. P., and Penati, F., *Archivo di Scienze Biologiche*, 1937, **23**, 443.
- ⁵⁹ McCann, W. S., *Proc. Soc. Exp. Biol. Med.*, 1928, **25**, 255.
- ⁶⁰ Mermod, C., and Dock, W., *Science*, 1935, **82**, 155.
- ⁶¹ Minot, G. R., and Murphy, W. P., *J. A. M. A.*, 1926, **87**, 470.
- ⁶² —, *Ibid.*, 1927, **89**, 759.
- ⁶³ Murphy, W. P., *Am. J. Med. Sci.*, 1936, **191**, 597.
- ⁶⁴ Okada, S., Shamoto, M., and Yamase, F., *Nagoya J. of Med. Science*, 1937, **11**, 147.
- ⁶⁵ Raper, H. S., *Biochem. J.*, 1927, **21**, 89.
- ⁶⁶ Strandell, B., *Act. med. Scand. suppl.*, 1935, **71**, 52.
- ⁶⁷ Strandell, B., Poulsson, S., and Schartum-Hansen, H., *Acta med. Scand.*, 1936 **88**, 624.
- ⁶⁸ Subba Row, Y., Jacobson, B. M., and Fiske, C. H., *New. Eng. J. Med.*, 1935, **212**, 663.
- ⁶⁹ —, *Ibid.*, 1936, **214**, 194.
- ⁷⁰ Subba Row, Y., Jacobson, B. M., *J. Biol. Chem.*, 1936, **114**, 111.
- ⁷¹ Subba Row, Y., Jacobson, B. M., and Hartfall, S. T., *J. Clin. Invest.*, 1938, **17**, 517.
- ⁷² Tempka, T., *Polsk. Gaz. lek.*, 1936, **15**, 865.
- ⁷³ Tochowicz, L., *Folia Haematol.*, 1936, **56**, 240.
- ⁷⁴ Tschesche, R., and Wolf, H. J., *Die Naturwissenschaften.*, 1939, **27**, 176.
- ⁷⁵ Ungley, C. C., *Lancet II*, 1936, 1513.
- ⁷⁶ Wakerlin, G. E., Bruner, H. D., and Kinsman, J. M., *J. Pharm. Exp. Ther.*, 1936, **58**, 1.
- ⁷⁷ Wakerlin, G. E., *Annals of Internal Med.*, 1937, **11**, 31.
- ⁷⁸ West, R., and Nichols, E. G., *J. A. M. A.*, 1938, **91**, 867.
- ⁷⁹ West, R., and Howe, M., *J. Clin. Invest.*, 1930, **9**, 1.
- ⁸⁰ —, *J. Biol. Chem.*, 1930, **88**, 427.
- ⁸¹ —, *Ibid.*, 1931, **94**, 11.
- ⁸² Whipple, G. H., and Robscheit-Robbins, F. S., *Am. J. Physiol.*, 1925, **72**, 395.
- ⁸³ —, 1925, **72**, 419.
- ⁸⁴ Wilkinson, J. F., *Brit. Med. J.*, 1932, **1**, 325.
- ⁸⁵ —, *Proc. Roy. Soc. Med.*, 1933, **26**, 1341.
- ⁸⁶ Wilkinson, J. F., and Klein, L., *Lancet II*, 1933, 629.
- ⁸⁷ Wilkinson, J. F., *Lancet I*, 1936, 354.
- ⁸⁸ Wilkinson, J. F., Klein, L., Ashford, C. A., Jones, T. S. G., Mainewaring, B. R. S., and Aylward, F. X., *Biochem. J.*, 1940, **34**, 698.