CHEMICAL FACTORS IN PERNICIOUS ANÆMIA

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I. Pernicious Anæmia: Ætiology

Minot and Strauss [1] have dealt recently and authoritatively with the physiological aspects of pernicious anæmia; the present review aims at discussing those aspects of the problem which lie more in the field of the chemist. Nevertheless, a brief survey of the whole field will be included, as this is essential if the chemical data are to be viewed in their proper perspective.

"Anæmia" is a popular rather than a scientific term. It covers a wide range of conditions which appear to be in many cases pathologically unrelated and united only by the single common symptom of a low blood hæmoglobin content. The hæmatological picture may vary widely; the immediate cause may be hæmorrhage, hæmolysis or inadequate hæmopoiesis, and this, in turn, may be only one aspect of some underlying disorder—chronic infection, dietary deficiency, endocrine dysfunction, or even industrial poisoning. However, the anæmia is often the most manifest feature of the syndrome, the most susceptible to quantitative study, and may also constitute the gravest immediate threat to life. There is therefore classified together as anæmia a group of conditions, unrelated either pathologically or ætiologically.

Pernicious anæmia was one of the first of these to be clearly defined when Addison, nearly a hundred years ago, described the disease which now bears his name. Its ultimate cause is still obscure, beyond the establishment of familial tendencies [2] and, though it proceeds if untreated to a fatal termination through a series of apparently spontaneous remissions and relapses, up to twenty years ago no effective treatment was known. Then Minot and Murphy [3] made the classical observation that the condition could be cured by massive feeding of liver—250-500 g. daily. Later, it was found that suitable extracts of liver were effective parenterally in small amounts.[7,8] These clinical observations provided the first clue to the cause of pernicious anæmia. Three years later, our knowledge was greatly extended by Castle's work.[4,5] It was known that pernicious anæmia was accompanied by failure of the gastric secretions, and Castle demonstrated that simultaneous administration of gastric juice from a normal subject together with certain protein foods (*e.g.* beef muscle), produced beneficial results comparable with those obtained with liver; though neither the protein nor the gastric juice were effective *per se*. He suggested

that in the normal stomach an " intrinsic factor," probably a proteolytic enzyme, reacted with an " extrinsic factor " in the dietary proteins to produce a hæmopoietic principle similar to, if not identical with, that present in liver. This substance was presumably absorbed in the intestine, stored in the liver and released from there for transport to the hæmopoietic bone-marrow tissues. This theory is supported by the fact that gastric failure *precedes* the development of the anæmia itself and gastric achylia is frequent in relatives of pernicious anæmia patients.[2]

The hæmopoietic " hormone " (usually referred to as the " liver factor ") is therefore regarded as being essential for normal formation of erythrocytes in the bone marrow. When it is deficient, the normal type of erythrocyte (the " normocyte ") is no longer produced, its place being taken by a pathological form, the " megalocyte," larger in size and irregular in shape. As these are produced only in much reduced numbers, the erythrocyte count falls from its normal figure of 5,000,000 per mm.^{3} to 1,000,000 or even less. Though the increased hæmoglobin content of the megalocyte provides partial compensation, the colour index does not rise above 1·5, so the drop in hæmoglobin content is hardly less disastrous than the fall in the erythrocyte count. Specific treatment, by administration of liver extracts or stomach preparations, results in immediate release from the bone marrow of large numbers of immature erythrocytes, known on account of their characteristic staining reactions as " reticulocytes." A few days after the commencement of treatment these may account for 30 per cent. or more of the total erythrocytes. As they mature to normocytes, this figure falls again to a normal value of 1 per cent. or less. This reticulocyte crisis is widely used for assessing the success of treatment—or the potency of the extract used. It is discussed in detail later.

Failure of the hæmopoietic hormone may arise from causes other than the idiopathic deficiency of the gastric secretions. In rare cases, it has been observed that surgical removal of the secretory region, or its destruction by cancer, produces the same effect. In severe and prolonged dietary deficiency, the stomach may be producing the intrinsic factor but be unable to form the hæmopoietic hormone owing to lack of the dietary extrinsic factor. This is common in the tropics, where the condition is frequently precipitated by pregnancy. The precise relation of this so-called " tropical macrocytic anæmia " to pernicious anæmia is not yet clarified.[6] In pellagra and some intestinal disorders—*e.g.* sprue—interaction of the intrinsic and extrinsic factors is normal, but absorption in the intestine is impaired; or again, in some liver diseases, *e.g.* cirrhosis, the liver may fail to store the hormone. In these megalocytic anæmias injections of liver extracts is helpful, though treatment should aim primarily at relieving the condition causing the anæmia. In the rare condition of achrestic anæmia, formation and storage of the hormone appear to be normal, but utilisation by the hæmopoietic tissues themselves is deficient; the

condition therefore does not respond to treatment with liver preparations and ends fatally.[7, 8, 9] But this and other " refractory megaloblastic anæmias " are now claimed to respond well to proteolysed liver extracts administered orally. It is suggested that these preparations may contain a further factor which is necessary for hæmopoiesis in these conditions.[10] The ultimate cause of pernicious anæmia has yet to be discovered, and attempts to correlate it with other endocrine disorders have led to no conclusive results. Nor is its connection with the common complication of degeneration of the spinal cord understood. This results in loss of sensation in the extremities and weakness in the limbs; the patient may approach the doctor first complaining of " rheumatism " or " sciatica."

II. Assay of Antianæmic Substances

Viewed from the chemists' standpoint, the problem of pernicious anæmia is no more formidable than many of the vitamin or hormone isolations and syntheses of the past two decades. The comparative slowness of progress is due entirely to one factor—the absence of a satisfactory assay procedure. The only acceptable test involves clinical trial on human cases of pernicious anæmia, which are forthcoming neither in sufficient number to permit more than occasional tests, nor with the uniformity of age, state of nutrition or genetic constitution necessary for the application of the statistical methods so vital for accurate bio-assay. Pernicious anæmia is also an inherently unsuitable condition for such studies; it does not run a steady course, but proceeds by apparently spontaneous relapses and remissions. This necessitates an observation period in hospital of seven days prior to treatment, to ensure that a spontaneous remission, or one stimulated by the rest and diet of the hospital regimen, does not invalidate results.[18] This drastically reduces the number of cases available for the testing of preparations, since many patients are too critically ill on admission to hospital to allow of this delay in treatment. Usually a " satisfactory response " in three cases is taken as evidence that the preparation used is " clinically active " at the dosage given, and in America an official unit, based on this principle, has been adopted.[19] There are separate units for oral and parenteral preparations, the liver factor being about fifty times more active by the latter route. The stomach factor is, of course, suitable only for oral administration. " Satisfactory response " may be defined either in terms of the maximum percentage of reticulocytes attained following treatment, or from the rate of increase in the erythrocyte count. In both cases the response is a function of the original erythrocyte count, and the " satisfactory " values are tabulated for the two methods by *New and Non-official Remedies* [20] and by Dellavida and Dyke [21] respectively. The two criteria do not always give corresponding results. A good reticulocyte response is not always followed by adequate erythrocyte regeneration,

and in any case weak reticulocyte responses may be elicited by a wide variety of non-specific agents, possibly through some irritant action on the bone-marrow cells.

The most urgent problem and the most formidable is the provision of a satisfactory method for assay of the antianæmic factors. The method must satisfy four criteria: (1) Quantitative response to the antianæmic factor; (2) Specificity; (3) Freedom from interference from accompanying impurities; (4) Simplicity and reliability. None of the many methods proposed in the past satisfies these criteria, but a brief survey of these attempts is instructive. The methods may be classified into five groups: (1) Stimulation of blood formation in normal subjects; (2) Cure of experimental anæmias; (3) Biochemical methods; (4) Chemical methods; (5) Tissue culture methods. Some methods are claimed as applicable to both stomach and liver factors, some to only one factor. In most cases it is the liver factor which has been investigated.

(1) The stimulation of hæmopoiesis in normal subjects has been investigated in both man and laboratory animals.[22-29] The usual criteria of activity has been the elicitation of a reticulocyte response, but as non-specific substances ranging from glucose [23] to congo-red [25] appear to be capable of doing this, and as the reticulocyte count is also subject to spontaneous variations,[23, 25, 26] results obtained by this method must be accepted with the greatest caution. The accurate enumeration of reticulocytes is also a difficult technique and failure to realise this may lead to spurious results.[28] Minot and Strauss [1] and Piney [30] discuss in full the effect of antianæmic factors on the blood picture.

(2) Pernicious anæmia has never been observed in experimental animals and attempts to produce it artificially have failed. The obvious method of gastrectomising the animal, and thus depriving it of its source of "intrinsic factor," has been tried without success in dogs;[31, 32, 33] whilst in pigs, pernicious anæmia is said to develop after this treatment, but only after a lapse of two to three years.[34] Results based on attempts to induce anæmias by other methods must be treated with great caution since the anæmias so produced usually bear no pathological relation to true pernicious anæmia. It is true that Whipple's classical researches on blood-regeneration after hæmorrhage stimulated Minot and Murphy's first experiments with liver;[3] but the relation is probably accidental and Whipple's researches,[35-38] valuable though they are in other fields, contribute little to this problem beyond indicating the conditions for rapid blood regeneration once the primary treatment with liver or stomach preparations has been initiated. The tapeworm, *Bothriocepahalus*, produces a gastric failure and hæmatological condition similar to that of pernicious anæmia, but the "intrinsic factor" is still secreted and the parasite does not destroy the liver factor, at least *in vitro*.[39, 40] The use of this condition for assaying the anti-pernicious anæmia factors thus rests on a very

uncertain basis. Various poisons have been used in attempts to produce an anæmia responsive to the anti-pernicious anæmia factors, including phenylhydrazine,[41] hydroxylamine,[42] bile acids,[43] bacterial toxins,[44] saponin,[45, 46, 47] and lead.[48-53] Recently it has been suggested that splenectomy, whilst not rendering the animal anæmic, sensitises it so that injection of the liver factor elicits liberation of reticulocytes, and an assay method based on this claim has been described.[54] But these numerous attempts to employ artificial anæmias for the assay of anti-pernicious anæmia potency cannot be said to suggest anything more than that the liver factor may be capable of acting as a hæmopoietic stimulant under very varied conditions. They do not reveal under what conditions, if any, the response is sufficiently consistent, quantitative, and above all, specific to serve as the basis of an assay method.

(3) and (4) The chemical and biochemical methods have found but little favour. Schales' test—determination of the nitrogen in the material precipitated from liver extracts between 70 and 95 per cent. alcohol concentrations—is crude and unspecific.[55] A biochemical test for the liver factor, based on the reduction of methæmoglobin, has been discredited [56] and a like fate has overtaken Lasch's determination of the stomach (intrinsic) factor, based on the proteolytic activity of stomach preparations at neutral *p*H.[57]

(5) Tissue culture methods have the advantage of dealing directly with the hæmopoietic tissue in the bone marrow, and hence are more likely to be specific; on the other hand, they inevitably involve such formidable technical difficulties that only those with special experience of the technique of *in vitro* tissue culture can hope to apply them with success. Three claims have so far been put forward. One [58, 59] is based on the stimulation of the growth of bone-marrow cells by the liver factor. The method is complicated by the fact that, on increasing the concentration of the liver factor, the stimulation rises to a maximum at a critical value and then falls off. Another bone-marrow stimulation method was employed in working out the assay using spelectomised animals (see above [54]), but abandoned when the latter technique was perfected. The third method claims to overcome many of the difficulties involved in orthodox methods of *in vitro* culture by using bone-marrow cells suspended in a liquid culture fluid in special vials. It is claimed that bone marrow obtained by sternal puncture from a pernicious anæmia patient retains its pathological structure under these conditions, but returns to the normal type if liver factor is added to the medium.[60-63] None of these tissue culture methods have been confirmed or found their way into general use.

III. The Intrinsic (Stomach) Factor

The function of the stomach factor—also known as "Castle's Intrinsic Factor" or "Hæmopoietin"—has already been described.

It has attracted much less attention than the liver factor, both from the chemist and the clinician, since its use does not extend to so wide a range of megalocytic anæmias; also, more frequent dosage is necessary. It may be administered only orally, and not by injection. A characteristically thermolabile enzyme, it is much less amenable to chemical investigation than the liver factor, which is probably a polypeptide. It is found in different parts of the stomach—the fundus and cardiac regions in man,[11] but the pylorus in the hog,[11, 12] whilst horse [13] and dog [14] stomachs are reported inactive. The commercial preparations comprise the appropriate part of the stomach dried under conditions which do not destroy the thermolabile enzyme. A more refined product is obtained by expressing the juice from the tissue under high pressures, precipitating the hæmopoietin along with the pepsin by addition of alcohol and removing the pepsin by isoelectric precipitation.[64] The stomach factor prepared thus shows the characteristic properties of a protein. It has been claimed to be a proteolytic enzyme, but the difficulty of completely eliminating pepsin and also trypsin, regurgitated from the intestine, has prevented conclusive demonstration of any specific proteolytic activity of the hæmopoietin itself. An assay method based on this supposed proteolytic action has been discredited.[57] Since this work, newer concepts of proteinase specificity have been developed [71, 72] and a study of the action of hæmopoietin on synthetic peptides suggested that the enzyme is a prolinase or prolidase, hydrolising specifically prolyl peptides, with a *p*H optimum at 6·0.[70] But another worker, employing hæmopoietin from intestinal mucosa, regards the enzyme as an aminopolypeptidase.[73] Our precise knowledge both of the nature and the mode of action of the intrinsic factor is thus very limited. As in every phase of the pernicious anæmia problem, lack of a satisfactory method of assaying antianæmic potency is the stumbling block to future progress. Nevertheless, the intrinsic factor has attracted less attention than it deserves.

IV. The Extrinsic Factor

Equally limited is our knowledge of the extrinsic factor [4, 5] with which the intrinsic factor reacts in the normal stomach to produce the hæmopoietic hormone found in the liver. Castle [74] recently reported its presence in a wide range of protein foods—milk, eggs, liver, yeast, rice-polishings and wheat germ, in addition to beef muscle—and he reports that it is extracted by dilute acetic acid and resists autoclaving. Sixty-five per cent. alcohol extracts it from casein. This and similar observations have suggested to some workers that the extrinsic factor may be not a protein as first considered, but a member of the vitamin B complex. None of the known B vitamins, alone or in combination, can replace the extrinsic factor however.[74] Also, if the extrinsic factor is not a protein derivative it is difficult to imagine how it reacts with the intrinsic factor which, as far as the evidence goes, is probably

a proteolytic enzyme, to fabricate the liver factor, which in turn is considered to be a polypeptide. Wills [6] considers that an unidentified factor of the B_2 group is involved in the so-called " tropical anæmia," a megalocytic anæmia due to dietary deficiency. But though it is frequently assumed that this condition is analogous to pernicious anæmia save that the extrinsic and not the intrinsic factor is deficient, Wills herself considers that the two conditions are not identical and makes no claim that her results are applicable to pernicious anæmia.

V. The Liver Factor

The attempts to isolate the anti-pernicious anæmia factor of liver account for the bulk of the chemical work, starting right from the time of Minot's discovery of liver treatment for the disease.[76] But even now it is not certain whether the modern highly active extracts are representative of the active principle, or consist mainly of inert material containing only traces of a still more intensely active substance. Much of the recent work has been done in commercial laboratories and methods of preparation and isolation are concealed. Cohn [77] early prepared the product known as " Fraction G " by heating the aqueous extract of minced liver to 80° C. at pH 4-5 to denature and coagulate the proteins, and fractionally precipitating the filtrate with alcohol; the active fraction was collected between 50-95 per cent. alcohol concentrations. This process was used in the manufacture of the well-known Eli Lilly " Liver Extract 343." Much of this classical work is now of only historical interest, since " Fraction G " contained only a small percentage of active material and the properties described are mostly those of the accompanying impurities. He established, however, that the factor was comparatively thermostable, though labile towards alkali, and precipitated by phosphotungstic acid but not by the majority of protein precipitants. He suggested that it might be a nitrogenous base or a polypeptide.

Shortly afterwards Gänsslen introduced a liver extract suitable for injection [78] under the trade name " Campolon." The mode of preparation was exceedingly simple—a press juice of liver was rendered free of proteins (which may cause " allergic " reactions) by heat-coagulation. As the liver factor is some fifty times more potent when administered parenterally than orally, this injectable extract enormously simplified treatment, an occasional injection taking the place of massive feeding with liver. At the same time it greatly stimulated research into the nature of the active factor, since crude extracts of the " Campolon " type sometimes produce severe reactions in the patient, and the preparation of the liver factor in a more purified form would reduce both the frequency of the injections and the risk of reactions. Felix and Frühwein [79] found the factor to be precipitable by heavy metals—Ag, Cu, Pb and Hg, the last proving the most satisfactory. But the most important papers were those of Dakin and his

co-workers.[80, 81] They employed salting-out with ammonium sulphate, sodium chloride and magnesium sulphate, and included a precipitation with Reinecke's salt. Their preparations, representing about 0·1 per cent. of the original liver (wet weight) initiated recovery in doses of the order of 50 mg. parenterally, as compared with the 500 g. of liver taken orally in Minot's original treatment ten years before. They describe their product as "a peptide, possessing some, but by no means all, the properties of an albumose." It was free of carbohydrate, yielded 15 per cent. N, two-thirds of which was liberated as free amino-N on acid hydrolysis. Nevertheless, the material failed to give a biuret reaction. Untrafiltration methods suggested a molecular weight in the range 2000-5000. This process was the basis of the first "refined" extract, "Anahæmin."

Meanwhile Laland and Klem [82] had introduced a very different method. They observed that the liver factor was soluble in phenol; they adsorbed the material on charcoal and eluted with phenol. This, too, has become the basis of a commercial preparation. The claims that this material initiated recovery in doses of the order of a few milligrams [83] were not confirmed, and, in a comparative trial, it proved to be of the same order of activity as Dakin and West's material.[84, 85] Recently, a modification of this method, no details of which are disclosed, claims satisfactory responses to doses of the order of 5 mg.[86] or even less.[99] The use of phenol for eluting the adsorbed material involves difficulties, especially on a large scale, and some workers have tried dilute alcohol as an alternative.[87, 88, 89] Subbarow's product, representing only 2 mg. per 100 g. liver, initiated recovery on injection of 20 mg. Containing 13 per cent. N, four-fifths of which was liberated as free amino-N on acid hydrolysis, it yet failed to give a biuret reaction. Later, however, Subbarow reverted to phenol as eluent,[90] claiming to reduce his effective dose to 7 mg. But it must be emphasised again that the clinical method of assaying potency is inherently inaccurate, and the utmost caution should be exercised in comparing the active doses claimed by different workers in different laboratories.

Subbarow claimed that the liver factor is multiple in nature, the interaction of four factors being required for antianæmic action, viz. a "primary factor" of unknown nature, and three "secondary factors"—*l*-tyrosine, a peptide, and a complex purine. It is difficult to understand how so common a dietary constituent as *l*-tyrosine could play a specific part in the antianæmic complex, or how injection of minute amounts of this amino-acid should be necessary for antianæmic response. Another claim that the antianæmic principle may be separated into two parts, active together but not separately, is based on electrophoretic experiments,[91] but a more recent electrophoretic study indicates that the factor is homogenous under these conditions.[92]

Omission of all details of preparative methods renders Karrer's

papers of relatively little value.[93-96] He mentions absorption on charcoal and elution with phenol, and the removal of unspecified impurities by extraction with pyridine. He admits his material is not homogenous. It contained 14 per cent. N, two-thirds being liberated as amino-N on acid hydrolysis, but only 2 per cent. by tryptic digestion. The biuret reaction is described as "doubtful." The preparation was free of P, S, reducing sugars, flavines and pterines. An incomplete investigation of the amino-acids indicated that tyrosine was present but histidine, glycine, phenylalanine, proline and tryptophane absent. Molecular weight determinations by diffusion methods suggested three components with molecular weights of the order 3000, 6500 and 15,000 respectively, as compared with Dakin's figure of 2000-5000 and Mazza's [97] of 3000-4000. Tschesch and Wolf [98] prepared a product whose general properties followed those already described.

Our knowledge of the chemical factors involved in pernicious anæmia is thus extremely sketchy. Many authors, doubtless for commercial reasons, fail to describe their preparative methods adequately, and the preparations themselves are never homogenous and may indeed contain only a small proportion of the active substance itself. Were it not that the materials prepared by quite different methods share in common the general characteristics of a polypeptide, there would be strong grounds for regarding the properties described as appertaining not to the antianæmic factor but to the inert material masking it. The problem of isolation is probably one of separating a number of polypeptides closely resembling each other in physical and chemical properties. But even this is not certain; and a further complication is introduced by the suggestion that several factors may be jointly required for antianæmic action. In every direction, progress is blocked by the absence of an assay method other than that of human clinical trial—slow, difficult to perform, severely limited by the clinical material available, and giving results of barely more than qualitative significance. The solution of the chemical problem waits on the biological.

Whilst this review was in course of preparation, claims appeared that synthetic crystalline folic acid, administered parenterally in doses of the order of 100-500 mg., initiated hæmatological and clinical remission in both pernicious anæmia [15, 16] and in Sprue.[17] Folic acid, a member of the vitamin B group, is found in liver. The molecule includes xanthopterin [100] and recent work claims to correlate antianæmic potency with pterine content in liver extracts.[101] It will be noted that the dosage used is considerably higher than that required to produce remission with the most active liver preparations. If, however, these claims are substantiated, our views on the chemistry of the liver factor may require drastic revision.

VI. Summary and Conclusions

Pernicious anæmia is a megalocytic anæmia. Idiopathic failure of the stomach to produce an "intrinsic factor," which normally interacts with a dietary "intrinsic factor," prevents formation of the "liver factor," a hormone which is stored in the liver and is essential for the production of normal erythrocytes. Similar conditions may result from other causes, preventing normal supply of this hormone to the erythropoietic bone-marrow tissue. The condition may be treated by oral or parenteral administration of concentrates of the hormone prepared from liver, or by oral administration of stomach preparations, which replace the deficient "intrinsic factor."

Progress in isolating and investigating the chemical factors involved has been seriously impeded by the lack of any adequate means of assaying antianæmic potency.

The intrinsic (gastric) factor is a typically thermolabile protein, possibly a proteolytic enzyme. The nature of the intrinsic (dietary) factor is unknown.

Various methods of fractionating the liver factor yield material which exhibits high clinical potency, but is probably grossly impure. The liver factor is relatively thermostable; the available evidence suggests a polypeptide structure.

References

[1] Minot, G. R., and Strauss, M. B. (1944), *Vit. Horm.*, **1**, 269. [2] Wilkinson, J. F., and Brocklebank, L. (1931), *Quart. Journ. Med.*, **24**, 319. [3] Minot, G. R., and Murphy, W. P. (1926), *Journ. Amer. Med. Assoc.*, **87**, 470. [4] Castle, W. B. (1929), *Amer. Journ. Med. Sci.*, **178**, 748. [5] Castle, W. B. (1937), *Cold Spring Harbor Symposia* **5**, 414. [6] Wills, L. (1945), *Biochem. Journ.*, **39**, xxxii. [7] Wilkinson, J. F., and Israels, M. C. G. (1935), *Brit. Med. Journ.*, **1**, 139, 194. [8] Wilkinson, J. F., Klein, L., and Ashford, C. A. (1938), *Quart. Journ. Med.*, **31**, 555. [9] Israels, M. C. G. and Wilkinson, J. F. (1940), *Quart. Journ. Med.*, **9**, 163. [10] Davis, L. J., and Davidson, L. S. P. (1944), *Quart. Journ. Med.*, **13**, 53. [11] Fox, H. J., and Castle, W. B. (1942), *Amer. Journ. Med. Sci.*, **203**, 18. [12] Wilkinson, J. F. (1940), *Brit. Med. Journ.*, **1**, 414. [13] Steiner, H., and Grunfeld, M. (1937), *Z. Kiln. Med.*, **131**, 444. [14] Singer, K. (1935), *Klin. Wochschr.*, **14**, 200. [15] Vilter, C. F., Spies, T. D., and Koch, M. B. (1945), *Southern Med. Journ.*, **38**, 707. [16] Vilter, C. F., Spies, T. D., and Koch, M. B. (1945), *Southern Med. Journ.*, **38**, 781. [17] Spies, T. D. (1946), *Southern Med. Journ.*, **39**, 30. [18] Wilkinson, J. F. (1932), *Brit. Med. Journ.*, **1**, 325. [19] *U.S. Pharmacopœia*, 12th ed. (1942), p. 553. [20] *New and Non-official Remedies* (1938), p. 321. [21] De la Vida, B. L., and Dyke, S. C. (1942), *Lancet*, **2**, 275. [22] Berglund, H., Watkins, C. H., and Johnson, R. (1928), *Proc. Soc. Expt. Biol. Med.*, **25**, 814. [23] Overbeek, G. A. (1936), *Arch. Intern. Pharmacodynamie*, **54**, 340. [24] Jacobson, B. M. (1935), *Journ. Clin. Investigation*, **14**, 665. [25] Lassen, H. C. A., Jacobsen, E., and Neilsen, A. K, (1936), *Acta. Path. Microbiol. Scand.*, **13**, 543. [26] Bachrach, W. H., and Fogelson. S. J. (1937), *Journ. Lab. Clin. Med.*, **22**, 395. [27] Vaughan, J. M., Muller, G. L., and Zetzel, L. (1930), *Brit. Journ. Exp. Path*, **11**, 456. [28] Gurd, M. R. (1935), *Quart. Journ. Pharm. Pharmacol.*, **8**, 39. [29] Shaw, G. E., and Pritchard, J. (1937), *Quart. Journ. Pharm. Pharmacol.*, **10**, 371. [30] Piney, A. (1939), *Recent Advances in Hæmatology*, 4th ed., London. [31] Petri, S., Ohlson, A. S., and Boggild, D. (1935), *Acta. Med. Scand.*, **87**, 14. [32] Fontes, G., Kumlin, J., and Thivolle, L. (1935), *C.R. Soc. Biol.*, **120**, 1219. [33] Vlasos, H., and Bagsdasarov, A. (1936), *Acta. Med. Scand.*, **88**, 295. [34] Bence, J. (1936), *Z. klin. Med.*, **130**, 275. [35] Whipple, G. H., and Robscheit-

Robbins, F. S. (1927), *Amer. Journ. Physiol.*, **79**, 260. [36] Robscheit-Robbins, F. S., and Whipple, G. H. (1929), *Journ. Exp. Med.*, **49**, 215. [37] Whipple, G. H. (1930), *Surg. Gynæcol. Obstet.*, **50**, 244. [38] Whipple, G. H. (1942), *Journ. Exp. Med.*, **76**, 283. [39] Homberg, C. F. (1941), *Acta. Med. Scand. Suppl.*, **123**, 255. [40] Bonsdorf, B. von (1939), *Acta. Med. Scand.*, **100**, 436. [41] Paschkess, K., and Taylor, G. (1934), *Klin. Wochschr.*, **13**, 1358. [42] Eisler, B., Hammarsten, E., and Theorell, H. (1937), *Scand. Arch. Physiol.*, **77**, 24. [43] Isaacs, R. (1940), *Proc. Soc. Exp. Biol. Med.*, **45**, 788. [44] Tschersche, R. (1938), *Angew. Chem.*, **51**, 349. [45] Rossi, A., and Contri-Marotti, R. (1939), *Atti. Acad. Ital. Rend. classe, sci. fis. mat.*, **1**, 295. [46] Schales, O., and Reimer, L. (1938), *Arch. Exp. Path. Pharmacol.*, **190**, 550. [47] Hindemith, H. L., and Muller, L. (1939), *Arch. Exp. Path. Pharmacol.*, **193**, 608. [48] Trizzino, E., and Polesse, I. (1940), *Sperimentale*, **94**, 509. [49] Maregiri, S. (1940), *Med. Lavaro*, **31**, 97. [50] Capsoni, M. T. (1939), *Biochem. Terap. Sper.*, **26**, 449. [51] Lourau, M., de Sacy, G. S., and Arthus, A. (1938), *C.R. Soc. Biol.*, **128**, 512. [52] Lourau, M., de Sacy, G. S., and Arthus, A. (1939), *C.R. Soc. Biol.*, **130**, 642. [53] Ferrari, G. (1940), *Arch. Sci. Med.*, **69**, 226. [54] Jacobsen, W., and Williams, S. M. (1945), *Journ. Path. Bact.*, **57**, 101. [55] Schales, O. (1936), *Berichte*, **69**, 359. [56] Deutsch, W., and Wilkinson, J. F. (1935), *Brit. Journ. Exp. Path.*, **16**, 33. [57] Jones, T. S. G., and Wilkinson, J. F. (1938), *Biochem. Journ.*, **32**, 1352. [58] Gaillard, P. J., Overbeek, G. A., and Yam, T. H. (1940), *Arch. Intern. Pharmacodynamie*, **54**, 340. [59] Pace, N., and Fisher, R. S. (1942), *Journ. Pharmacol.*, **74**, 256. [60] Osgood, E. E., and Brownlee, I. E. (1936), *Journ. Amer. Med. Assoc.*, **106**, 1888. [61] Osgood, E. E., and Muscovitz, A. N. (1936). *Journ. Amer. Med. Assoc.*, **107**, 123. [62] Osgood, E. E., and Brownlee, I. E. (1937). *Journ. Amer. Med. Assoc.*, **108**, 1793. [63] Osgood, E. E. (1939), *Symposium on Blood.* Wisconsin, p. 253. [64] Klein, L., and Wilkinson, J. F. (1933), *Biochem. Journ.*, **27**. 600. [65] Jones, T. S. G., Grieve, W. S. M., and Wilkinson, J. F. (1938), *Biochem. Journ.*, **32**, 665. [66] Griffiths, W. J. (1934), *Biochem. Journ.*, **28**, 671. [67] Emerson, C. P., and Helmer, O. M. (1936), *Amer. Journ. Digestive Dis. Nutrition*, **3**, 753. [68] Gessler, C. J., Dexter, S. O., Adams, M. A., and Taylor, F. H. L. (1940), *Journ. Clin. Investigations*, **19**, 233. [69] Taylor, F. H. L., and Castle, W. B. (1938), *Journ. Clin. Investigations*, **17**, 335. [70] Mazza, F. P., and Miglardi, C. (1941), *Schweiz. Med. Wochschr.*, **71**, 344. [71] Bergmann, M. (1942), *Advances in Enzymology*, **2**, 49. [72] Johnson, M. J., and Berger, J. (1942), *Advances in Enzymology*, **2**, 69. [73] Agren, G. (1944), *Nature*, **154**, 430. [74] Castle, W. B. (1944), *Science*, **100**, 81. [76] Minot, G. R., and Murphy, W. P. (1926), *Journ. Amer. Med. Assoc.*, **87**, 470. [77] Cohn, E. J., Minot, G. R., Alles, G. A., and Salter, W. T. (1928), *Journ. Biol. Chem.*, **77**, 325. [78] Gansslen, M. (1930), *Klin. Wochschr.*, **9**, 2099; *Brit. Pat.*, 372128. [79] Felix, K., and Frühwein, H. (1933), *Z. Physiol. Chem.*, **216**, 173. [80] Dakin, H. D., and West, R. (1935), *Journ. Biol. Chem.*, **109**, 489. [81] Dakin, H. D., Ungley, C., and West, R. (1936), *Journ. Biol. Chem.*, **115**, 771. [82] Laland, P., and Klem, A. (1936), *Acta. Med. Scand.*, **88**, 624; *U.S. Pat.*, 2,134,128. [83] Strandell, B., Paulson, L., and Schartum-Hansen, A. (1936), *Acta. Med. Scand.*, **88**, 624. [84] Wilkinson, J. F. (1936), *Lancet*, **1**, 354. [85] Ungley, C. (1936), *Lancet*, **2**, 1513. [86] Emery, W. B., and Hurran, W. J. (1945), *Brit. Med. Journ.*, **1**, 75. [87] Subbarrow, Y., Jacobson, B. M., and Prochownick, V. (1936), *Journ. Amer. Chem. Soc.*, **58**, 2234. [88] Subbarrow, Y., Jacobsen, B. M., and Fiske, C. H. (1936), *New England Med. Journ.*, **214**, 194. [89] Sladek, J., and Kyer, J. (1938), *Proc. Soc. Exp. Biol. Med.*, **39**, 227. [90] Jacobsen, B. M., and Subbarrow, Y. (1941), *Journ. Amer. Med. Assoc.*, **116**, 367. [91] Eisler, B., Hammarsten, E., and Theorell, H. (1936), *Naturwiss.*, **24**, 142. [92] West, R., and Moore, D. H. (1942), *Trans. Assoc. Amer. Physicians*, **57**, 259. [93] Karrer, P., Frei, P., and Fritzsch, H. (1937), *Helv. Chim. Acta.*, **20**, 622. [94] Karrer, P., Frei, P., Fritzsch, H. (1938), *Helv. Chim. Acta.*, **21**, 314. [95] Karrer, P. (1941), *Schweiz. Med. Wochschr.*, **71**, 343. [96] Karrer, P., and Keller, R. (1943), *Helv. Chim. Acta.*, **26**, 55. [97] Mazza, P. (1942), *Ricerca Sci.*, **13**, 84. [98] Tschesche, R., and Wolf, H. J. (1939), *Naturwiss.*, **27**, 176. [99] Emery, W. B., and Parker, L. F. J. (1946), *Biochem. Journ.*, **40**, iv. [100] Angier, R. B., *et al.* (1946), *Science*, **103**, 667. [101] Jacobson, W., and Simpson, D. M. (1946), *Biochem. Journ.*, **40** 1, 9.