LXXXVIII. INVESTIGATIONS ON THE NATURE OF HAEMOPOIETIN, THE ANTI-ANAEMIC PRINCIPLE IN HOG'S STOMACH

III. THE INTERACTION OF HAEMOPOIETIN AND PEPSIN WITH MYOGLOBULIN AND CASEINOGEN

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KLEIN & WILKINSON [1933; 1934] described the isolation from hog's stomach of fractions which, when administered orally in relatively small doses, were effective in producing remissions in cases of pernicious anaemia. Further, when these fractions were incubated with beef, a relatively thermostable and therapeutically effective concentrate could be made from the products of digestion. Castle *et al.* [1929; 1930] had suggested that normal gastric juice contained an enzyme, the absence of which from the stomach was an important aetiological factor in pernicious anaemia. Klein & Wilkinson [1934] ascribed an enzymic nature to the substance (haemopoietin) obtained by them from stomach which they considered to be identical with Castle's hypothetical gastric juice enzyme.

The suggestion that the haemopoietic activity of normal gastric juice is due to the presence of an enzyme raises the question of its optimum pH ; a range of pH from 2.5 to 10 has been employed in digestion experiments involving gastric juice and its possible substrates. Lately Castle has exclusively used reactions around neutrality and has claimed [Castle *et al.* 1937] that no activity of mixtures incubated at $pH < 2.5$ has been demonstrated. This is in accordance with our own views and those of Meulengracht [1935] who believes that the enzyme is secreted by the Brunner's glands of the pylorus and duodenum. It thus appears probable that the site of action of haemopoietin is in the neutral or more alkaline regions of the gastro-intestinal tract rather than in the more acid regions of the stomach.

The nature of the substrate has been widely investigated. Castle [1929] first employed beef muscle on account of its accessibility and the fact that Cohn *et al.* [1928] had stated their belief that the liver principle, supposed to be the end product of the enzymic reaction, was a nitrogenous base or a polypeptide. Castle *et al.* [1930] obtained a satisfactory response to the incubation products of normal gastric juice and a crude preparation of the globulin from muscle similar to that described by Edsall [1930]. Using reprecipitated beef muscle globulin, they could produce no remission and thought that this was due to denaturation of the protein. Several other substances have been shown to contain the substrate necessary for the action of the enzyme and it is interesting that liver extract (that is the proteose fraction) has been said by Helmer *et al.* [1933], Reimann [1931] and others to contain this "extrinsic factor".

Griffiths [1934], by incubating beef myoglobulin with normal gastric juice from patients with pernicious anaemia and achlorhydric secondary anaemia at pH 6, claimed to have obtained proteolysis with the normal juice only and suggested that the proteolysis was due to interaction between the intrinsic and extrinsic factors. This has been criticized by Emerson & Helmer [1936] who showed that any change in non-protein nitrogen at pH 6 could have arisen from peptic hydrolysis. Lasch [1937] claimed to have obtained similar results, using a preparation of washed, dried beef as substrate, but this has not been confirmed by us. More recently still, Taylor *et al.* [1937], reported a progressive change in acid-soluble N when caseinogen was incubated with normal gastric juice at pH 7.4, under conditions in which the possible action of pepsin was excluded.

Castle *et al.* [1930] showed that the products of peptic digestion of beef failed to produce remissions in pernicious anaemia. While the peptic and haemopoietic activities of ordinary desiccated whole hog's stomach run parallel [Wilkinson, 1932], active fractions of hog's stomach have been prepared which have little peptic activity when tested by standard methods [Klein & Wilkinson, 1933]. Further, the oral administration of pepsin even in large quantities (up to 30 g. daily) with and without an acid medium has been repeatedly tried by one of us (J. F. W.) in several hundred patients with pernicious anaemia without ever producing any evidence of remission; intramuscular injection of crystalline pepsinogen was also shown to be inactive by Flood & West [1936]. Moreover, the removal of pepsin and rennin from gastric juice by means of various reagents such as caseinogen [Castle *et al.* 1931], calcium triphosphate and Lloyd's reagent [Helmer & Fouts, 1937] or by incubation at pH 10 [Flood & West, 1936] failed to remove the intrinsic factor. It is, therefore, quite certain that the intrinsic factor, haemopoietin, is not identical with pepsin.

The experiments described in this paper were designed further to elucidate the enzymic properties of fractions of hog's stomach. To this end we have examined chemically the interaction of these fractions with myoglobulin from beef and with caseinogen and compared the results with those of similar experiments using pepsin. Although pepsin is excluded from consideration as the intrinsic factor (haemopoietin), the properties of the crude fractions containing the latter were found to be so similar to those of pepsin as to merit careful comparison. Preliminary experiments showed that no significant change in amino-N takes place during incubation of myoglobulin with these fractions at various reactions (cf. Taylor *et al.* [1937]; Helmer & Emerson [1937]). We therefore decided to use the N soluble in trichloroacetic acid as a measure of the proteolysis, especially since the liver principle, the postulated end product of the reaction, is a nitrogenous substance soluble in that acid. A discrepancy between our results and the reported value of the Michaelis constant for pepsin [Haldane, 1930] led us further to study the influence of substrate concentration on the kinetics of action of pepsin on caseinogen.

EXPERIMENTAL

The enzyme preparations used in this work were:

(1) *Pepsin* (Parke Davis 1:10,000)—contained lactose and was assayed at 1:15,000 by the U.S.P. method. We are indebted to Dr K. Bullock for the assay and gift of this specimen.

(2) *Fraction P5* (Klein & Wilkinson)—this was a fresh preparation made from hog's stomach by the method of Klein & Wilkinson [1933].

(3) *Fraction P5 (ii)* [Klein & Wilkinson, 1933]. Several different preparations of P5 (ii) were made for the work; careful attention to detail, the precipitation

of P5 (i) at pH 3.8 measured by the hydrogen electrode and immediate dehydration of the alcohol precipitate at pH 6 with successively stronger solutions of alcohol gave a preparation of twice the enzymic activity of that described by Klein and Wilkinson. The yield of this material was increased by the means quoted to 30 g. from 100 g. of P5. It was found that substantially the same product was obtained if only 2 vol. of 92.5 % (by vol.) alcohol were added to the solution at pH 6 after the isoelectric precipitation of P5 (i) and filtration.

(4) *Fraction P5 (i)* (Klein & Wilkinson). In order to obtain a sample of P5 (i) uncontaminated by P5, the solution of P5 in *N*/15 acid was filtered before the addition of alkali to pH 3.8. The enzymic activity of this preparation was rather smaller than that of Klein & Wilkinson.

The substrates

(1) *Myoglobulin*. Edsall [1930] has described the preparation of the globulin of beef muscle in solution in a form as nearly as possible unchanged from that in which it occurs in the living tissue. For our work it was originally decided to follow this method as closely as possible (method A), especially as Castle *et al.* [1930] ascribed certain unfavourable results to denaturation of the protein. Later, however, a modified method was adopted (method B), by means of which a considerably larger yield of globulin was obtained. While the globulin fraction obtained by this method would not entirely correspond in physico-chemical properties with that obtained in method A, for our purposes it could be considered the same protein. It has, moreover, the merit of close similarity to the "beef muscle globulin" of Castle *et al.* [1930].

Method A. The cheek and jaw muscle of the ox, obtained within 30 min. of slaughter, is freed from fat and minced, ice being added to keep the tissue cold. The weighed and minced tissue (800–1000 g.) is stirred for 1 hr. with ice-cold water to remove blood, etc., fat being skimmed from the surface of the water. The tissue is removed on a sieve and squeezed to remove excess water and immediately placed in roughly four times its weight of a salt solution of ionic strength 1.3–1.4 and pH 7.8 containing 1.2 *M* KCl, 0.05 *M* K_2HPO_4 and 0.01 *M* KH_2PO_4 of 0–5°. After stirring mechanically for 2 hr., the thick suspension is put through a wire sieve to remove connective tissue, allowing adequate time for drainage, the solution being retained tenaciously by the gelatinous tissue. The liquid is filtered on a Büchner funnel through a pad consisting of one Whatman No. 41 filter paper, a 2 cm. layer of paper pulp and a layer of calais sand of approximately the same thickness. The clear filtrate (about 4 l. including some washings) is diluted to 48 l. with water and after 1 hr. the finely suspended protein is removed by a Sharples super-centrifuge. To facilitate separation of the protein from the bowl of the centrifuge, the latter is lined with a sheet of celluloid, which can be removed easily at the end of the operation and from which the protein can be washed by means of buffer solution. The yield of protein is small, amounting to 2–3 g.

Method B. The use of cheek and jaw muscle was recommended by Edsall on the grounds that the protein could be extracted in greater yield and in a less altered condition than if ordinary muscular tissue be used. Assuming the protein we extracted to be myoglobulin, the contrary has been our experience. In place of tissue taken from the freshly killed animal, we have used the cheapest frozen beef with markedly improved yield of protein.

Approximately 1 kg. of minced frozen steak is washed with water at pH 5 for 1 hr. and extracted as in method A at pH 7–8 for 1 hr. After this extraction

the pH is slowly raised to 9 and the mixture stirred for a further hour. The gelatinous tissue is now removed and the fluid portion filtered as before. The filtrate is diluted with water to 20 vol. and brought to pH 5. Careful adjustment to pH 6 results in a copious precipitate of protein, which flocculates and settles quickly. This is washed by decantation and much of the suspending fluid can be removed by simple filtration by gravity, taking care, however, that the precipitate does not become dry. The protein is soluble in alkaline and acetate buffers (the latter on warming to 40°). From 700 g. of meat there were obtained in one experiment 2.4 l. of 0.7 % protein, i.e. 16.8 g.

A series of suspensions of the finely precipitated protein of strength 0.56 mg. N per ml. (the whole of the N being present as protein) were made in *M*/12 Walpole's acetate buffers pH 1.5–6 and in *M*/15 phosphate buffers up to pH 9.

(2) *Caseinogen*. This was made from skimmed grade A milk by isoelectric precipitation according to Van Slyke and Baker [1918]. The caseinogen was collected by sedimentation, washed with distilled water twice daily for 4 days, redissolved in *N*/100 NaOH and reprecipitated. The second precipitate was washed as before and filtered off on cheese cloth, the excess fluid being removed on a cheese press. The resultant cake was shaken into a large volume of alcohol and left for several days, the alcohol being removed on a cheese press and replaced by ether and finally benzene and the protein was then dried in a vacuum desiccator. The caseinogen was easily soluble in dilute alkali and by rapidly taking to pH 2 with vigorous mechanical stirring and warming to 40°, solutions at that pH could be obtained.

For the investigation of the influence of pH on the activity of the enzyme preparations, a series of solutions in *M*/12 acetate buffers was made, containing 0.38 mg. N per ml., by diluting a solution prepared as above with *M*/5 acetate buffer and water.

The pH of these standard solutions of caseinogen and also of those of myoglobulin was determined by both the quinhydrone and hydrogen electrodes, with excellent agreement.

The pH activity curves of pepsin and hog's stomach fractions.

The determination of activity

Numerous preliminary experiments were carried out to find suitable conditions for the comparison of activities at different pH and the following method was finally adopted.

100 ml. of buffered protein solution (or suspension) were mixed with 10 ml. of the enzyme solution of suitable strength in a 500 ml. bolt-head flask; 15 ml. were removed immediately and run into 10 ml. of 20 % trichloroacetic acid and well mixed. The flasks containing the enzyme-substrate mixtures were placed in a thermostat maintained at $37.5 \pm 0.05^\circ$. The contents of the flasks were stirred mechanically. Samples (15 ml.) were removed from the flasks at suitable intervals, depending on the concentration of protein and enzyme, and the proteins precipitated as in the initial sample. Next day, the protein precipitate was filtered off on a small Whatman No. 42 filter paper, the first runnings being returned to the filter. No cloudy filtrates were obtained [cf. Taylor *et al.* 1937]. The non-protein N in the filtrate was determined by the micro-Kjeldahl method. Duplicate determinations carried through from the beginning usually agreed to one drop of *N*/100 alkali. The following blanks were carried out at the same time: (1) incubation of the protein with the addition of water in place of the enzyme solution, (2) incubation of the protein with a solution of the enzyme which had been heated at 100° for 10 min., (3) incubation of a solution of enzyme

similar to (2) with water and (4) incubation of a solution of the enzyme with water. It was found that no significant changes in the non-protein N occurred in any of these solutions during 7 hr.

Calculation of activity. The increase in non-protein N over the initial value was found for each sample and plotted against the time at which the sample was taken. Limiting the amount of hydrolysis to a small fraction of the protein present, by a suitable choice of conditions, the velocity curves approximate to straight lines (see Fig. 6). Haldane [1930] and Bodansky [1937] have stressed the importance of using the time taken to produce a given change rather than the change produced in a given time, as a measure of the activity of the enzyme. Where the velocity curves are not straight lines, we have therefore measured the time taken to produce the hydrolysis of one-third of the protein present in the system. The reciprocal of this time gives, in arbitrary units, the activity of the sample of enzyme used. Under conditions where the reaction velocity curves did not approach straight lines, Figs. 1 A and B show the relationship of the activity

Fig. 1 A.

Fig. 1 B.

Fig. 1. Comparison of activity- pH curves for the action of pepsin on caseinogen. A, activity calculated from reciprocal of half-time period. B, Calculated from initial velocity. Broken line: curve after Northrop [1922-23].

of pepsin acting on caseinogen to the pH of the medium. Fig. 1 A was obtained by plotting the reciprocal of the time taken to hydrolyse half of the protein present against the pH , while Fig. 1 B represents the variation of the initial velocity with pH . The curves are plotted on arbitrary scales chosen so that the activities at optimum pH are approximately the same in the two curves. It is seen that the curves are very similar, the optimum pH being 1.6-1.7 and the relative activity is slightly more in the alkaline regions, when calculated by the initial velocity method. For comparison, the broken line in Fig. 1 A shows the results obtained by Northrop [1922-23] for the same system, using a method whereby the unchanged caseinogen was removed from solution, dried and weighed.

Having established the conditions under which straight line reaction velocity curves are produced, we used the initial velocity method. In the absence of this condition, we always obtained the full time course of the reaction and compared the times for a given change.

Reproducibility of pH -activity curves

To test the reproducibility of the curves obtained by these methods and to compare the activities of two samples of P5 (ii) prepared under similar conditions at widely separated intervals, we have plotted the curve shown in Fig. 2. The activities of a solution of P5 (ii) of concentration of 0.01 % acting on solutions

of caseinogen containing 0.35 mg. N per ml. at the pH shown in the graph were determined simultaneously for each sample. The points for the two samples were obtained at an interval of 14 days. The points relating to sample I (triangles) and sample II (circles) are distributed at random about the smooth curve joining the mean at each pH. It is concluded that the method is adequate as far as reproducibility is concerned.

Fig. 2. Activity-pH curve of two samples of P5 (ii) acting on caseinogen. Ordinate mg. N produced in 30 min.

Caseinogen as the substrate

The curves in Fig. 3 represent the pH-activity relationships of pepsin and various fractions of hog's stomach using caseinogen 0.35 mg. N per ml. as substrate. The curves relate to:

- I. Pepsin 0.027 mg. per ml. (triangles)
- II. P5 0.10 mg. per ml. (circles)
- III. P5 (i) 0.73 mg. per ml. (diamonds)
- IV. P5 (ii) 0.10 mg. per ml. (squares)

Fig. 3. Comparison of activities of pepsin and hog's stomach fractions acting on caseinogen. Ordinates mg. N produced in 30 min. For explanation see text.

The ordinates are the relative activities calculated as mg. non-protein N per ml. produced in the solution in 30 min. (straight line velocity curves). The scale of the ordinates is equal for all the curves, but the zeros have been chosen so that the position on the graph at *pH* 3.5 is the same for all the preparations studied. The results will be discussed below.

Myoglobin as substrate

Fig. 4 shows the results using myoglobin as substrate. The substrate concentration was 0.56 mg. N per ml. and the curves relate to:

- I. Pepsin 0.045 mg. per ml. (triangles)
- II. P5 0.18 mg. per ml. (circles)
- III. P5 (ii) 0.18 mg. per ml. (squares)

Fig. 4. Comparison of activities of pepsin and hog's stomach fractions acting on myoglobin. Ordinate mg. N produced in 30 min.

The striking feature of the curves in both figures is their great similarity. While the optimum *pH* is approximately the same for all the preparations studied, there are minor differences between the ratios of the activity at the optimum *pH* to that at another *pH*. By the arrangement of the zeros to make the activity at *pH* 3.5 the same for all the preparations (Fig. 3), the heights of the optimum represent this ratio. This difference in the ratio is less marked with myoglobin as substrate. In both cases, the curve for P5 occupies a position intermediate between that of pepsin and P5 (ii). This is in accordance with the preparation of P5 (ii) which aims at the removal from P5 of the pepsin which it is known to contain. While the curves shown end at *pH* 3.5 in the case of caseinogen and at 4.3 for myoglobin, determinations were actually carried out up to *pH* 8.7. Prolonged digestion at the higher *pH* liberated amounts of non-protein N, which were negligibly small compared with the amounts liberated at more acid reactions and which were practically zero on the scale shown in Figs. 3 and 4. In the case of myoglobin, it is seen from Fig. 4 that the activity is already zero at *pH* 4.3. The results are similar to those of Northrop [1922-23] for the action of pepsin on caseinogen (dotted line in Fig. 1A), the activity of which becomes zero at *pH* 4 and remains so at higher hydrogen ion concentrations. Under our conditions, in no case was any activity observed at *pH* 7 comparable with that at *pH* 1.5.

The influence of substrate concentration

Substrate. It was found impossible to prepare solutions of myoglobulin of sufficient concentration for this work. The experiments were therefore confined to caseinogen as substrate. For this purpose, caseinogen was precipitated from alkaline solution as a fine suspension, allowed to settle and finally centrifuged at high speed. The caseinogen was then suspended in acetate buffer solutions of the optimum pH (1.55) in a series of descending concentrations from 6.2 mg. N per ml.

The velocity curves. Determinations of the velocity curves were carried out for several concentrations of the substrate using 0.18 mg. per ml. of P5 (ii) and 0.03 mg. per ml. of pepsin, exactly as described for the experiments on the pH-activity relationships. In the case of the stronger suspensions, since pipetting involved gross errors, we resorted to weighing the substrate suspension. Duplicate determinations of reaction velocity showed excellent agreement of an order

Fig. 5. Effect of concentration of caseinogen on initial velocity of the action of pepsin. Ordinate mg. N produced in 30 min.

Fig. 6. Effect of concentration of caseinogen on initial velocity of the action of P5 (ii). Ordinate mg. N produced in 30 min.

comparable with that of the more dilute suspensions. After precipitation of the protein with trichloroacetic acid, the mixtures were diluted with an appropriate solution of trichloroacetic acid in such a proportion that the amount of precipitate per unit volume was approximately the same for all solutions. This was intended to minimize errors due to unequal distribution of non-protein N between the solution and the protein precipitate. The non-protein N in the filtrate from the diluted deproteinized sample was then determined as usual.

The velocity curves for the two enzyme preparations studied are shown in Figs. 5 and 6 and the initial velocities corresponding to these curves are shown as points in Figs. 7 and 8, plotted against the logarithm of the concentration of substrate (calculated as protein N per ml.).

The action of pepsin on caseinogen

The unbroken curve shown in Fig. 7 is plotted from the equation of Michaelis & Menten [1913], K_m having the value 0.316 mg. N per ml. which corresponds to a concentration of 0.20 % assuming caseinogen contains 15.62 % N. It is evident that in the range of concentration studied, there is excellent agreement between the experimental results and the theory of Michaelis. Haldane [1930] quotes a value of 4.5 % for K_m in the action of pepsin on ovalbumin, derived from certain results of Northrop [1919-20]. Allowing for the differences between suspensions of caseinogen and ovalbumin, it is unlikely that the value of K_m for the latter should be more than 20 times our value for the former. Inspection of Northrop's paper fails to reveal sufficient data on which the calculation of K_m could be based.

Fig. 7.

Fig. 7. Relationship of initial velocity and logarithm of substrate concentration (mg. caseinogen—N per ml.) for the action of pepsin.

Fig. 8.

Fig. 8. Relationship of initial velocity and logarithm of substrate concentration (mg. caseinogen—N per ml.) for the action of P5 (ii).

The action of P5 (ii) on caseinogen

The theory of Michaelis and Menten covers the data obtained for this system up to a concentration of about 1 mg. N per ml.; above this concentration the velocity is less than would be expected on the simple theory. Haldane [1930] has attempted to explain the inhibiting effect of high concentrations of substrate by combination of the compound ES with further substrate to give ES_2 , ES being the only compound which breaks down to form the end products of the reaction. The proportion of such unions to form ES_2 increases with substrate concentration and since they are unfruitful from the point of view of proteolysis, the velocity falls off. Consideration of the kinetics of this system leads to the equation

$$\frac{v}{ke} = \frac{1}{1 + \frac{K_1}{x} + \frac{x}{K_2}},$$

where the additional constants K_1 , K_2 , k and e are respectively the Michaelis constants of the reactions $E + S \rightleftharpoons ES$, $ES + S \rightleftharpoons ES_2$, the velocity constant of the slow reaction $ES \rightleftharpoons E + P$ (where P = products) and the total concentration of the enzyme in the system.

The unbroken curve in Fig. 8 has been fitted to the points by suitable choice of k' according to the equation

$$\frac{v}{k'} = \frac{1}{1 + \frac{0.398}{x} + \frac{x}{17.78}}.$$

The behaviour of the enzyme accords well with the theory of Haldane and the values of K_1 and K_2 are 0.398 and 17.78 mg. N per ml. respectively. The value of the constant for the reaction $E + S \rightleftharpoons ES$, viz. $K_1 = 0.398$ mg. N per ml. or 0.256 % caseinogen concentration, is 28 % higher than that for the action of pepsin on caseinogen.

DISCUSSION

In the above experiments, haemopoietin has been assumed to be a proteolytic enzyme and has been compared with pepsin but our attention lately has been increasingly drawn to the possibility that haemopoietin acts, not on protein, but on some other nitrogenous substance, possibly a product of other (peptic?) proteolytic action. If the assumption be true that it is a proteolytic enzyme, three possibilities present themselves: (a) that haemopoietin is identical with pepsin, (b) that it differs from pepsin but has some very similar properties, and (c) that the fractions used in the work contain so little of the active enzyme that its properties are masked by those of pepsin.

The first is easily disposed of since the non-identity of the two enzymes has been abundantly proved by us and others in a large series of clinical tests.

The expected optimum pH for the action of haemopoietin depends on our views as to its site of action; if the stomach is the usual site, then the optimum would reasonably be expected to be similar to that of pepsin; on the other hand the possibility that haemopoietin may be secreted in the pylorus and even in the Brunner's glands of the duodenum suggests that the enzyme might act at neutral or even slightly alkaline reactions. It must be admitted that our own experience, like that of Castle suggests an optimum action around the neutral point. Consequently, the question arises whether, in view of the absence of any proteolysis at pH > 4 comparable with that at pH 1.5, we are actually measuring the action of the haemopoietin known to be present in the preparations. The close similarity of the pH-activity curves, in spite of minor differences, and of the kinetics of reaction of preparations of haemopoietin and pepsin, supports the view that we are measuring the action of pepsin alone. While a careful attempt has been made in the preparation of P5 (ii) to remove all pepsin, it is possible that the salt produced by neutralization of the HCl used for solution of the P5 might aid in keeping some of the pepsin in solution [cf. Fenger & Andrew, 1927]. Contamination of the haemopoietin of P5 (ii) with pepsin is therefore to be considered when explaining the similarity of the properties of the two enzymes under discussion.

The possibility that haemopoietin may not be a proteolytic enzyme, but may act on protein split-products has not been investigated with any thoroughness. Castle *et al.* [1937] have brought forward clinical evidence that the *in vivo* action of gastric juice on the products of peptic digestion of beef produces a remission in cases of pernicious anaemia. It is to be presumed that here the haemopoietin acts on some peptone-like substance, although we must not lose sight of the possibility that sufficient residual protein may remain as a substrate for the action of the haemopoietin.

SUMMARY

1. The effect of *pH* on the production of non-protein N in the interaction of beef muscle globulin and caseinogen with pepsin and preparations of hog's stomach has been analysed.

2. The effect of the substrate concentration on the hydrolysis of caseinogen by these preparations has been determined and shown to be in accord with the theory of Michaelis and Menten for the action of pepsin, and with Haldane's modification of the theory for an inhibited reaction in the case of the stomach fraction P5 (ii). The Michaelis constant for the former reaction is 0.316 mg. N per ml. or 0.20 % caseinogen while for the latter, the constants K_1 and K_2 have the values 0.398 mg. N per ml. and 17.78 mg. N per ml. respectively.

3. The bearing of these results on the nature of the enzyme haemopoietin is briefly discussed.

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REFERENCES

- Bodansky (1937). *Science*, **86**, 52.
 Castle (1929). *Amer. J. med. Sci.* **178**, 748.
 ——— & Townsend (1929). *Amer. J. med. Sci.* **178**, 764.
 ——— & Heath (1930). *Amer. J. med. Sci.* **180**, 305.
 ——— (1931). *J. clin. Invest.* **9**, 2.
 ——— Heath, Strauss & Heinle (1937). *Amer. J. med. Sci.* **194**, 618.
 Cohn, Minot, Alles & Salter (1928). *J. biol. Chem.* **77**, 325.
 Edsall (1930). *J. biol. Chem.* **89**, 289.
 Emerson & Helmer (1936). *Amer. J. Dig. Dis.* **3**, 753.
 Fenger & Andrew (1927). *J. biol. Chem.* **73**, 371.
 Flood & West (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 542.
 Gram (1936). *Ugeskr. Læg.* 655.
 Griffiths (1934). *Biochem. J.* **28**, 671.
 Haldane (1930). *Enzymes*. (Longmans, Green and Co.)
 Helmer & Emerson (1937). *Amer. J. Dig. Dis.* **3**, 906.
 ——— & Fouts (1937). *Amer. J. med. Sci.* **194**, 786.
 ——— & Zerfas (1933). *Proc. Soc. exp. Biol., N.Y.*, **30**, 775.
 Klein and Wilkinson (1933). *Biochem. J.* **27**, 600.
 ——— (1934). *Biochem. J.* **28**, 1684.
 Lasch (1937). *Klin. Wschr.* **23**, 810.
 Meulengracht (1935). *Proc. roy. Soc. Med.* **37**, 28.
 Michaelis & Menten (1913). *Biochem. Z.* **49**, 333.
 Northrop (1919–20). *J. gen. Physiol.* **2**, 595.
 ——— (1922–3). *J. gen. Physiol.* **5**, 269.
 Reimann (1931). *Med. Klinik.* **27**, 880.
 Taylor, Castle, Heinle & Adams (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 566.
 Wilkinson (1932). *Brit. med. J.* **i**, 325.
 Van Slyke & Baker (1918). *J. biol. Chem.* **35**, 147.