- Danforth, W. F. & Wilson, B. W. (1961). *J. gen. Microbiol.* **24**, 95.
- Dawes, E. A. & Holms, W. H. (1958). *Biochim. biophys. Acta*, **30**, 278.
- Dawes, E. A. & Ribbons, D. W. (1962). *Annu. Rev. Microbiol.* **16**, 241.
- Friedemann, T. E. (1957). In *Methods in Enzymology*, vol. 3, p. 414. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Ganguly, S. (1955). *Ann. Biochem.* **15**, 255.
- Gibbs, M. & Wood, W. A. (1952). *Bact. Proc.* p. 137.
- Gronlund, A. F. & Campbell, J. J. R. (1961). *J. Bact.* **81**, 721.
- Halvorsen, H. O. (1958). *Biochim. biophys. Acta*, **27**, 255.
- Kawamata, J. & Imanishi, M. (1961). *Biken's J.* **4**, 13.
- Lavenberg, B., Melnick, I. & Buchanan, J. M. (1957). *J. biol. Chem.* **225**, 163.
- Lowry, O. H., Rosebrough, J. N., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mandelstam, J. (1958). *Biochem. J.* **69**, 110.
- Midwinter, G. G. & Batt, R. D. (1960). *J. Bact.* **79**, 9.
- Norris, F. C., Campbell, J. J. R. & Ney, P. N. (1949). *Canad. J. Res.* **C27**, 157.
- Park, J. T. & Hancock, R. (1960). *J. gen. Microbiol.* **22**, 249.
- Sakami, W. (1955). *Handbook of Isotope Tracer Methods*, p. 158. Cleveland, Ohio: Western Reserve University.
- Salton, M. R. J. (1953). *Biochim. biophys. Acta*, **10**, 512.
- Stickland, L. H. (1956). *Biochem. J.* **64**, 498.
- Strange, R. E., Dark, F. A. & Ness, A. G. (1961). *J. gen. Microbiol.* **25**, 61.
- Stuy, J. H. (1958). *J. Bact.* **76**, 179.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, 3rd ed., p. 7. Minneapolis: Burgess Publishing Co.
- Urba, R. C. (1959). *Biochem. J.* **71**, 513.
- Warren, R. A. T., Ellis, A. F. & Campbell, J. J. R. (1960). *J. Bact.* **79**, 875.
- Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.

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The Action of Pepsin on Protein Fractions from Horse Antisera to Diphtheria Toxin

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The work on the action of enzymes on antitoxic horse sera described in the patents of Parfentjev (1936, 1938, 1939) and in the publications of Pope (1938, 1939*a*, *b*) stimulated a considerable amount of work on horse antitoxins and their peptic digestion in the years immediately following. From this it appeared that when horses are immunized with diphtheria toxin the γ -globulin increases slightly in the early stages and the β_2 -globulin (or T-globulin) to a greater extent in the later stages, and that antitoxic activity is associated with both (Kekwick & Record, 1941). The β -antitoxin has a long flocculation time and a low *in vivo/in vitro* ratio, and gives floccules containing only half the antibody nitrogen found in floccules from the γ -globulin antitoxin, which has a short flocculation time and a high *in vivo/in vitro* ratio. On peptic digestion the antitoxin gives a product with the short flocculation time of a γ -globulin, but with the low *in vivo/in vitro* ratio of β -globulin (Kekwick, Knight, Macfarlane & Record, 1941). Sera high in their proportion of γ -globulin give poor yields of antitoxin on digestion, and it was suggested that digested antitoxin comes from the β -globulin. On the other hand, on digestion, the β_2 -globulin peak disappears and is replaced by a γ -globulin peak (van der Scheer & Wyckoff, 1940; van der Scheer,

Wyckoff & Clarke, 1941; Amoureux & Yeu, 1951). The molecular weight of a pseudoglobulin fraction from horse diphtheria antitoxin (probably consisting largely of β_2 -globulin) was found by Petermann & Pappenheimer (1941) to be 184 000 and that of pepsin-digested antitoxin to be 98 000. They suggested that the antitoxin molecule is split in a plane normal to the long axis. Petermann (1942*a*) found papain to have a similar action.

Most of this work was done with unfractionated sera containing various proportions of β - and γ -globulins, as was true also of the dialysis-precipitated and ammonium sulphate-precipitated fractions used by Harms (1948) and Anderson (1955) in their studies on the pepsin-refining of antitoxins. Although there are suggestions that γ -globulin gives a poor yield on pepsin treatment, possibly because it is precipitated during the heat treatment (Harms, 1948), and that most of the refined antitoxin comes from the β -globulin (Kekwick *et al.* 1941), no studies, except those of Petermann & Pappenheimer (1941) and Petermann (1942*b*), have been published on pepsin treatment of separated globulins. Recently devised chromatographic methods allow of the separation of large amounts, at any rate of γ -globulins, in a fairly pure state, and the present work is a study of the pepsin-refining of

such materials. At the same time the opportunity was taken to reinvestigate the question whether it is pepsin or some impurity in the pepsin that is responsible for the refining activity (Pope & Stevens, 1951).

MATERIALS AND METHODS

Purification and estimation of pepsin

In the initial stages of this work the pepsin was purified by adsorption on calcium phosphate and elution from this with phosphate-citrate buffer. In a typical preparation, 2000 g. of precipitated calcium phosphate (Hopkin and Williams Ltd., Chadwell Heath, Essex; batch no. 62198) was mixed with 10 l. of water and allowed to sediment, and the fines were removed with the supernatant. This process was repeated until the supernatant was clear. Then 1500 g. of pepsin (1:3000; Armour Laboratories, Eastbourne, Sussex; batch no. 20401) dissolved in 10 l. of water was mixed with the washed calcium phosphate, the adsorbent allowed to precipitate and the supernatant decanted. The precipitate was then washed five times by suspension in 10 l. of water and sedimentation. The pepsin was eluted by the addition of 5 l. of phosphate-citrate buffer, pH 4.3 (14.86 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 6.145 g. of citric acid/l.), sedimentation and removal of the supernatant. This process was repeated a further seven times. The combined eluates were treated with 400 g. of ammonium sulphate/l. and allowed to sediment overnight, the supernatant was removed, and the precipitate was centrifuged down and dissolved in the phosphate-citrate buffer. The yield was 4.2% by weight and the total activity 67% of that of the starting material, as measured by the Anson (1938) method at pH 2. The pH-activity curves of the original and purified materials, with haemoglobin denatured in urea as substrate, were identical, with maxima at pH 1.8, 2.5–2.6 and 3.5–3.6. The average recovery on six batches was 65.3%, and the proteolytic activity varied from 75% to 100% of that of pure crystalline pepsin.

Because this method was so time-consuming the pepsin used in later experiments was that purified on a large scale for the refining of antitoxins for therapeutic use, by a method for which we are indebted to Mr J. K. Green of the Lederle Laboratories, Pearl River, N.Y., U.S.A.; the product from this was of slightly lower purity than the pepsin prepared by calcium phosphate adsorption but the yields were higher and the method was simpler. In this method 12.7 kg. of pepsin (C. Zimmermann Ltd., Perivale, Greenford, Middlesex; 3:3000 granular, 1:3000) was dissolved in 120 l. of water, ammonium sulphate was added to give a concentration of 200 g./l. and the pH was adjusted to 3.0 with conc. HCl. After being stirred for 2 hr. the mixture was allowed to stand overnight, the pepsin forming a sticky layer on the surface. The liquid underneath was withdrawn and discarded, and the precipitate was dissolved by adding 2 l. of water and very cautiously bringing the pH up to 5.0 with 10N-NaOH under continuous stirring. The solution (5–6 l.) was diluted to 25 l. and the precipitation repeated. After standing overnight the supernatant was decanted from the bulky precipitate, which was centrifuged down and dissolved by cautiously bringing the pH to 5.0. The yields were 80–100%, the weights 2–5% of the original material and the activities 55–60% of crystalline pepsin.

Crystalline pepsin was produced directly from pepsin purified by either of these methods by dialysing the purified pepsin against distilled water to remove salts, filtering off any precipitate, and ultrafiltering until the protein concentration was 15–20%. The concentrated solution was then crystallized either by the method of Herriott (1955) or by the ethanol method of Northrop (1946) and freeze-dried.

Separation of parapepsins I and II from the purified pepsin was carried out as described by Ryle & Porter (1959), except that the DEAE-cellulose was Whatman DE 50 and the concentration of NaCl in the limit buffer was increased to 0.3M because elution was incomplete at 0.2M.

Pepsin activity. Proteolytic activity of pepsin was measured by the method of Anson (1938), with crystalline horse haemoglobin as substrate. Comparisons of the activity of pepsin preparations used for refining antisera were also made at pH 3.2, the pH used in the refining process, when the haemoglobin was buffered with 0.1M-glycine-NaCl adjusted to pH 3.2 with HCl.

Production and fractionation of antisera

Sera from individual horses hyperimmunized with diphtheria toxoid and toxin for the routine production of diphtheria antitoxin were used.

Fractionation of sera on diethylaminoethylcellulose. All processes were carried out at +4°. The method for the isolation of γ -globulin was modified from that described by Levy & Sober (1960). Horse serum (100 ml.) was dialysed for 3–4 days against two changes of 2 l. of 17.5 mM- NaH_2PO_4 , adjusted to pH 6.3 at 4° with NaOH. It was applied to a column (4 cm. diam. $\times$ 44 cm. long) of Whatman DE 50 DEAE-cellulose equilibrated with the same buffer. The same buffer was run through the column at 50 ml./hr. and collected in 25 ml. fractions. The protein content of the fractions was followed by their extinctions at 280 m μ . The peak containing γ -globulin emerged from the column at the hold-up volume of approx. 360 ml. After 800–1200 ml. the buffer was changed to 50 mM- NaH_2PO_4 containing NaCl (20 mM) to elute the β_2 -globulin, and this buffer was replaced after 1500 ml. had passed through the column by 0.1M- NaH_2PO_4 containing NaCl (0.5M), which eluted the remaining protein. The contents of all tubes containing one serum fraction were combined and concentrated by ultrafiltration in Visking tubing (8/32 in. inflated diam.) at a pressure of 70 cm. Hg, which this tubing will sustain without need of a support. The fractions were finally dialysed against isotonic boric acid-borate-NaCl buffer, pH 8.0, and stored at -10°.

Electrophoresis of fractions was carried out as a routine on cellulose acetate strips (Oxoid) in the tris-EDTA-boric acid buffer of Aronson & Grönwall (1957), and the purity of the fractions occasionally checked in the Tiselius apparatus at 0° in phosphate buffer, pH 8.0 and *I* 0.2, or sodium diethylbarbiturate buffer, pH 8.6 and *I* 0.1.

Pepsin treatment of serum fractions

Except where otherwise indicated, this followed the method for the routine refining of antisera (Harms, 1948), except that crystalline pepsin was used and the ratio of enzyme to protein was 1:400. The serum fraction was diluted to 2% (w/v) protein, brought to pH 3.2 and 30° and pepsin added. After 30 min. enzyme activity was stopped by adding (per 100 ml. of mixture) 13.5 g. of ammonium sulphate,

0.2 g. of sodium pyrophosphate and 0.1 ml. of toluene, the pH being adjusted if necessary to 4.3, and the mixture was heated for 1 hr. at 55°. The precipitated inactive protein was filtered off, the precipitate washed with one-third the original volume of 13.5% (w/v) ammonium sulphate at pH 4.3, and antitoxin in filtrate and washings precipitated by adding 185 g. of ammonium sulphate/l. and bringing the pH to 5.2. The precipitate was dissolved in water, dialysed against iso-osmotic borate-NaCl buffer, pH 8.0, concentrated by ultrafiltration and stored at -10°.

Nitrogen estimations

Nitrogen estimations were carried out by the micro-Kjeldahl method of Ma & Zuazaga (1942). The protein nitrogen of pepsin solutions was estimated from the difference between the total nitrogen and that in the supernatant after pipetting 2 ml. of enzyme solution into 8 ml. of boiling 6.25% (w/v) trichloroacetic acid and cooling immediately.

The protein nitrogen in serum fractions and digests was similarly estimated from the difference between total nitrogen and nitrogen in the supernatant after diluting the solution to 5–8 mg. of protein/ml. in acetate buffer, pH 4.8, and boiling for 15 min. (Siebenmann, 1937).

The nitrogen contents of pepsin, albumin, β -globulin and γ -globulin were taken as 14.6, 15.95, 15.24 and 16.03% respectively (Brand & Edsall, 1947).

Characterization of antitoxins

Titre. The flocculation titre was estimated by the classical Ramon technique at 50° with a standard toxin of 34 Lf/ml. and antitoxin dilutions at 10% intervals, the test being performed at least twice with different starting dilutions of antitoxin. The buffer was iso-osmotic borate-NaCl, pH 8.0. Phosphate buffers were found to give low results with fractions containing β -globulin. The time at which the first tube flocculated is called the Kf.

The L+ was estimated in 250 g. guinea pigs, deaths being recorded up to 6 days.

For both flocculation and L+ the working standard antitoxins were compared at intervals with the International Standards.

Purification. The degree of purification of pepsin-treated antitoxins is given as the 'R value' defined as the number of units of antitoxin/g. of protein in the purified material over the number of units of antitoxin/g. of protein in the original material.

Nomenclature. Cohen (1963) has suggested that the antibody-containing components of human serum usually known, on the basis of their immunoelectrophoretic behaviour, as β_2 A- and β_2 M-globulins should in future be called γ_1 A- and γ_1 M-globulins because one of the peptide chains of which they are composed appears to be structurally similar to one found in γ -globulin (7s γ). In the present paper the term β_2 -globulin has been retained because this component has been identified only electrophoretically as yet and it is not certain that the horse globulins are related to each other in the same way as the human globulins.

RESULTS

Purification of pepsin. Experiments on the precipitation of pepsin with ammonium sulphate, potassium dihydrogen phosphate or sodium chlor-

ide gave no evidence of separation into fractions with differing activity, either with crude or purified pepsin. Adsorption on ion-exchange resins, fuller's earth, alumina, kaolin, calcium phosphate etc. was also ineffective. Nor was it possible to isolate from the commercial swine pepsin used in these experiments the enzyme 'gastricsin' with optimum activity at pH 3.2, which Richmond, Tang, Wolf, Trucco & Caputto (1958) reported that they had isolated from human gastric juice. On the other hand, the parapepsins I and II of Ryle & Porter (1959) were readily isolated; the former was without action on serum proteins and the latter was present in purified pepsin at so low a concentration that it could not have been responsible for the splitting of antitoxin in the refining process.

Chromatography of antitoxic sera. On dialysis of antitoxic horse sera against 17.5 mM-sodium phosphate, pH 6.3, protein was precipitated which amounted to less than 1% of the total protein and contained less than 1% of the antitoxic activity. At first this was removed, but in later experiments it was put on the column with the rest of the protein.

A typical elution pattern from the chromatography of 100 ml. of horse antiserum on DEAE-cellulose is shown in Fig. 1, which also indicates the fractions into which the eluate was divided. The pattern of elution of fractions A, B and C was very consistent in different runs with a single serum and from one serum to another, but the results with

Fig. 1. Chromatography of 100 ml. of horse serum on DEAE-cellulose. Serum was dialysed against 17.5 mM-sodium phosphate, pH 6.3, at 4°, and run on a column (4 cm. $\times$ 44 cm.) equilibrated with the same buffer. The buffer was changed to 50 mM- NaH_2PO_4 containing NaCl (20 mM) at the first vertical arrow, and to 0.1 M- NaH_2PO_4 containing NaCl (0.5 M) at the second arrow. The volumes at which the effluent was divided into fractions A, B, C, D and E are indicated. Effluent was collected at 50 ml./hr. in 25 ml. fractions.

Table 1. *Recovery of protein and antitoxic activity in serum fractions after chromatography*

Experimental details are given in the Materials and Methods section. Fractions *A–E* correspond to those given in Fig. 1. The weight of protein was taken to be protein nitrogen multiplied by 6.25.

Fraction	Total protein recovered		Antitoxic activity recovered		
	(mg.)	(% of protein put on column)	(Total Lf)	(% of activity put on column)	(Lf/g. of protein)
<i>A</i>	1 212	14.7	600	1.6	495
<i>B</i>	529	6.4	5 150	13.5	9 750
<i>C</i>	2 018	24.5	28 700	75.2	14 200
<i>D</i>	1 677	20.3	2 400	6.3	1 400
<i>E</i>	2 476	30.0	0	0	—
Total	7 912	96.0	36 850	96.5	4 650
Original serum ('Donald')	8 240	100	38 200	100	4 650

fractions *D* and *E* varied considerably with slight variations in the volumes of buffer applied, in the packing of the column etc. The balance sheet of one run (Table 1) shows that 96.0 % of the protein (by nitrogen estimation) put on the column was recovered and 96.5 % of the antitoxic activity (by flocculation). Recoveries in 40 fractionations have varied from 84.5 to 100 % of protein and from 86.4 to 98.7 % of antitoxic activity put on the column. Whereas fractions *A*, *B* and *C* together formed 45–55 % of the serum protein recovered from hyper-immune horse sera, they formed only 25–30 % of the protein recovered from normal horse sera chromatographed under the same conditions.

Electrophoresis of the fractions on cellulose acetate strips at pH 8.9 showed fractions *A* and *C* to be apparently homogeneous, with mobilities corresponding to those of the γ - and β_2 -globulins respectively of the original serum. Moving-boundary electrophoresis of fractions *A* and *C* from a single horse, in sodium diethylbarbiturate buffer, pH 8.6 and *I* 0.1, gave mobilities of -0.8×10^{-5} and -2.8×10^{-5} cm.² v⁻¹ sec.⁻¹ respectively, which may be compared with mobility ranges of -0.8×10^{-5} to -1.3×10^{-5} cm.² v⁻¹ sec.⁻¹ for γ -globulin and -2.5×10^{-5} to -2.8×10^{-5} cm.² v⁻¹ sec.⁻¹ for β_2 -globulin from pools of human plasma under the same conditions of electrophoresis (Armstrong, Budka & Morrison, 1947). Analysis of fractions from another serum in phosphate buffer, pH 8.0 and *I* 0.2 (Fig. 2), showed that fraction *A* was composed to the extent of 96.4 % of a component with the mobility of γ -globulin, and fraction *C* to the extent of 92.2 % of a component with the mobility of β_2 -globulin, the remainder being γ -globulin. Despite this electrophoretic homogeneity, however, it appeared that denaturation of the protein in fraction *A* was taking place in some chromatographic runs, as the optimum flocculation time (Kf) of toxin–antitoxin mixtures was very variable; normally 12–20 min. with different sera, it was increased to 120 min. in one case and

Fig. 2. Electrophoresis of fractions *A* (*a*) and *C* (*b*) (see Fig. 1). The protein concentration was 2 %. Electrophoresis was carried out in phosphate buffer, pH 8.0 and *I* 0.2, at 0°; the current was 15 ma. Photographs were taken after 120 min.

Table 2. *Antitoxic properties of horse serum and of fractions derived from it*

Experimental details are given in the Materials and Methods section. The Lf and Kf₅₀ values for fractions *A* and *C* (see Fig. 1) are given as means $\pm$ S.D.

	Serum	Fraction <i>A</i>	Fraction <i>C</i>
Lf/g. of protein	8 300	480 $\pm$ 65	19 000 $\pm$ 600
Kf ₅₀ (min.)	27	21 $\pm$ 5	34 $\pm$ 5
L + /Lf	0.92	5.00	0.92

there was no flocculation with another. More consistent results were obtained when the salt concentration in fraction *A* was increased by putting 1 ml. of 17 % (w/v) sodium chloride in the first 50 tubes at the start of a chromatographic run.

A summary of some of the properties of fractions *A* and *C* from a number of fractionations of a single serum is given in Table 2. In accordance with expectation the flocculation time of the γ -globulin is less than that of the β -globulin and its L + /Lf ratio is much greater; the antitoxic activity/g. of protein is very much lower. Over a series of experiments

with four different sera fraction *A* possessed from less than 1% to 6.4% of the total antitoxic activity of the original sera and the activity/g. of protein was only 350–1200 Lf, compared with the 5000–8500 Lf/g. of protein of the original sera, which were themselves all of fairly low titre (350–700 Lf/ml.). The L+/Lf ratio ranged from 2.6 to 5.0 and flocculation time from 12 to 25 min.

The bulk of the antitoxic activity (61–86% of that of the original sera) was found in the β_2 -globulin of fraction *C*, which had 15 000–25 000 Lf/g. of protein, Kf 20–60 min. and L+/Lf ratio 0.92–1.11.

Of the other fractions, *B* contained a mixture of γ - and β -globulins and 2.5–21% of the total antitoxic activity, *D* some β -globulins with the remainder of the antitoxic activity and the bulk of the α_1 - and α_2 -globulins, and *E* the albumin and various globulins with a spread of mobility from γ - to α_1 -globulin and no measurable antitoxic activity.

Action of pepsin on serum fractions. Most of the experiments on peptic digestion of antitoxin fractions were carried out on fractions *A* and *C*, because they were the most homogeneous, contained the bulk of the antitoxin and permitted separate

studies of the effect of pepsin on β - and γ -globulins. Material from a number of fractionations of the same serum was stored at -10° until sufficient had accumulated for pepsin treatment. In early experiments the ratio of pepsin to serum protein was varied from 1:100 to 1:800, but as this seemed to have little effect on the results the ratio was later standardized at 1:400. Other conditions are given in the Materials and Methods section and are the same as those used in the routine production of refined antitoxins.

The average of results from four experiments on fractions from three different sera is shown in Table 3. Destruction of protein and loss of antitoxic activity are equal in fraction *A*, 40% of each being left, so that the purification as shown by the *R* value was negligible, and Lf/g. of protein remained the same as with undigested globulin. The L+/Lf ratio fell slightly (from 2.6 to 2.25 with another serum), but was still much higher than that of a β -globulin, and the flocculation time fell to about 10 min. The electrophoretic mobility on cellulose acetate remained unaltered. Some increase in the Lf/g. of protein took place on pepsin treatment of fraction *C*, but the average *R* value of 1.4 is much lower than that usually attained in the pepsin treatment of plasma, which is rarely below 2.5. There was, however, a striking change in Kf, which fell from 20–60 min. to 5–10 min., and the electrophoretic mobility changed to that of a γ -globulin, though it remained slightly greater than that of pepsin-treated γ -globulin.

The effect of pepsin treatment on the other fractions is illustrated in Table 4; fraction *B* shows results intermediate between those of fractions *A* and *C*, as was to be expected; two-thirds of the protein of fraction *D* was lost and nine-tenths of that of fraction *E*.

Finally, all the fractions from chromatography of a single serum were combined in the average proportions in which they were obtained from the column (Table 5) and were refined at the same time as the corresponding unfractionated serum. The results (Table 6) do not differ greatly from those expected from the refining of the individual fractions.

DISCUSSION

No one as yet has reported the isolation from pepsin preparations of an enzyme active at pH 3.2 but inactive at pH 1.0. There are, however, numerous reports of enzymes, other than pepsin, present even in crystalline pepsin preparations: Pope & Stevens (1951) separated from crude pepsin a fraction inactive at pH values above 3; and Richmond *et al.* (1958) isolated from human gastric juice an enzyme with optimum activity at pH 3.2. The present work is in agreement with that of Taylor

Table 3. *Recovery of antitoxic units and protein after treatment of fractions A and C with pepsin*

	Fraction A	Fraction C
Percentage of initial antitoxic units (Lf) recovered	40.1 (35.3–44.0)	63.5 (59.9–68.7)
Percentage of initial protein recovered	38.7 (32.6–42.0)	45.7 (41.9–50.4)
<i>R</i> value	1.04 (1.02–1.11)	1.39 (1.22–1.65)
L+/Lf ratio	4.00	0.99
Lf/g. of protein	615 (385–1000)	25 100 (19 200–29 800)

Table 4. *Recovery of antitoxic units and protein after treatment of fractions B, D and E with pepsin*

Experimental details are given in the Materials and Methods section. Fractions *B*, *D* and *E* correspond to those given in Fig. 1.

	Fraction B	Fraction D	Fraction E
Percentage of initial antitoxic units (Lf) recovered	56.2	0	—
Percentage of initial protein recovered	44.3	33.3	9.4
<i>R</i> value	1.27	—	—

Table 5. *Composition of mixture of fractions combined in the proportions in which they were isolated from serum*

Experimental details are given in the Materials and Methods section. Fractions A-E correspond to those given in Fig. 1.

Fraction	Protein		Antitoxic units			
	(mg.)	(% of total)	(Lf)	(% of total)	[Kf (min.)]	(Lf/g. of protein)
Original serum ('Crook')	2 000	100.0	13 000	100	38	6 500
A	336	16.8	383	2.8	12	1 140
B	178	8.9	2 740	20.0	38	15 400
C	488	24.4	10 500	76.7	57	21 500
D	388	19.4	77	0.5	—	—
E	610	30.5	0	0	—	—
Total	2 000	100.0	13 700	100	—	6 850

Table 6. *Recovery of protein and antitoxic units after refining the mixture given in Table 5 and the serum from which it was derived*

Experimental details are given in the Materials and Methods section.

	Protein		Antitoxic units				
	(mg.)	(% of total)	(Lf)	(Percentage recovery)	[Kf (min.)]	(Lf/g. of protein)	R value
Combined fractions A-E	576	28.8	7 750	57.5	9	13 500	1.97
Calculated from result of refining individual fractions	642	32.1	8 000	58.4	—	12 500	1.85
Original serum ('Crook')	515	25.8	8 600	66.2	8	16 700	2.57

(1962) in finding no evidence that the activity of pepsin at pH 3.2 is separable from that of pepsin itself.

The method of separation of γ -globulins applied on a small scale by Levy & Sober (1960) to sera of man, guinea pig, rabbit and horse has been shown in the present work to be applicable on a fairly large scale to horse sera and to produce under these conditions material over 95 % homogeneous on electrophoresis. The separation of β_2 -globulin with 50 mM-sodium dihydrogen phosphate containing sodium chloride (20 mM) was less satisfactory, but still gave over 90 % of a protein with a single mobility. Both these products eluted at their original elution volume on rechromatography and sera from different horses gave identical results. Rechromatography of the mixed β - and γ -globulins in fraction B led to an apparent decrease in the proportion of γ -globulin and increase in that of β -globulin, the latter coming off the column at the volume normally occupied by fraction C.

The low antitoxin yield and activity of the γ -globulin fraction are surprising, but Kekwick & Record (1941) also found low γ -globulin titres in horses immunized with diphtheria toxin, and it may be calculated from the results of Anderson (1955, Table 1) on the refinement of ammonium sulphate-precipitated serum fractions, on the assumption that all the antitoxin was in his T- (or β_2 -) and γ -

components, that the γ -globulin had a titre of 950 Lf/g. of protein and the T-globulin 20 000 Lf/g. of protein. On the other hand, Cinader & Weitz (1953) found up to 38 % of the tetanus antitoxin of similarly hyperimmunized horses in the γ -globulin fraction, and Kekwick (1941) as much as one-half. It thus appears that the response to the two toxins in horses is different.

The prime purpose of the present investigation was to discover the contribution of individual globulin fractions to the content of antitoxin and protein in the refined diphtheria antitoxin produced by pepsin treatment of pooled horse plasma at pH 3.2. The routine process in use in many parts of the world (Harms, 1948) yields a material possessing 50–70 % of the antitoxin and 14–20 % of the protein of the original plasma, giving an R value of 2.5–5.0. This material has the electrophoretic mobility, adsorption characteristics on DEAE-cellulose and flocculation time of a γ -globulin, but has the low L+/Lf ratio of a β -globulin. The present work shows that the γ -globulin of the original serum can play little part in this result, both because of its low initial antitoxic activity and because no purification takes place on pepsin treatment, but 60 % of its activity is lost. The conclusion of Kekwick *et al.* (1941) that most of the refined antitoxin comes from the β -globulin, is thus confirmed, but at the same time it appears that the antitoxic activity/g.

of protein of this fraction is increased only some 40 % and that the greater part of the purification comes from digestion and precipitation of protein fractions without antitoxic activity. The experiment on the refining of a mixture of the fractions (Table 6) showed that they exert no synergistic or inhibitory effect on each other, but the fact that both the yield and the purification are significantly lower than those of the original serum refined at the same time suggests that the fractionation causes some alteration in the constituent proteins.

The action of pepsin on the γ -globulin antibody is readily explicable in terms of recent work on the structure of γ -globulin. Nisonoff, Wissler, Lipman & Woernley (1960) have shown that pepsin, acting on rabbit γ -globulin antibody at pH 4-5, decreases the molecular weight from 160 000 to 100 000. This smaller molecule still precipitates specifically with its homologous antigen, but can be further split into two parts by reagents capable of reducing disulphide bonds. The fragments are unable to form precipitates with antigen because they are univalent, but they inhibit the precipitation of untreated antibody with homologous antigen. These fragments appear to be almost identical with the pieces I and II that Porter (1959) isolated after papain digestion of rabbit γ -globulin. Peptic digestion of human γ -globulin likewise produces a fragment with a molecular weight of 100 000 (Bridgman, 1946). Thus it appears probable that in the refining process pepsin splits off the non-antitoxic piece III of the γ -globulin (Porter, 1959) which has different solubility properties from the rest of the molecule, and that this is either precipitated during the period of heating at 55° or further digested by the pepsin. The antitoxin, composed of piece I or piece II, can then be precipitated with ammonium sulphate.

Little is as yet known about the structure of β -globulins, but it has been shown by Cohen (1963) that the fraction of human serum usually known as β_2 A-globulin can be dissociated by the reduction of some of its disulphide bonds and dialysis against acetic acid or propionic acid into two fragments, a large A chain and a smaller B chain; the application of the same process of γ -globulin (7S γ) results in similar fragments (Fleischman, Pain & Porter, 1962). The B chains of both these globulins appear to be of similar structure, but the A chains differ in electrophoretic behaviour and carry the antigenic determinants that are specific for each type of globulin. When γ -globulin is split enzymically part of the product comprises the pieces I and II, referred to above, which consist of the B chain combined with part of the A chain. If enzymic treatment of β_2 -globulin produces similar fragments their properties might be similar to those of the fragments from γ -globulin. This would explain the fact that

the protein recovered after pepsin treatment of β_2 -globulin in the present experiments had the mobility, flocculation time and elution volume from DEAE-cellulose of a γ -globulin. When Petermann & Pappenheimer (1941) treated with pepsin a fraction from horse antitoxic serum composed of purified pseudoglobulin, which must have contained a high proportion of β -globulin, they obtained a product with a molecular weight of 98 000, very similar to the molecular weight of 106 000 found for the product of pepsin treatment of rabbit γ -globulin (Nisonoff, Wissler & Lipman, 1960) and of 100 000 for that of pepsin treatment of human γ -globulin (Bridgman, 1946).

SUMMARY

1. Attempts to separate the activity of pepsin at pH 3.2 from its activity at pH 2.0 were not successful.

2. Chromatography of horse antitoxic sera on DEAE-cellulose with 17.5 mM-phosphate buffer, pH 6.3, followed by 50 mM-sodium dihydrogen phosphate containing sodium chloride (20 mM), separated the γ -globulin and β_2 -globulin, both possessing the antitoxic activity, from each other and from the remainder of the serum protein.

3. The antitoxic activity/g. of protein of the γ -globulin was very low and usually accounted for less than 5 % of the total activity. The L + /Lf ratio was high.

4. Most of the antitoxic activity was in the β_2 -globulin fraction with a much higher activity/g. of protein, but a lower L + /Lf ratio.

5. Treatment of the γ -globulin with pepsin at pH 3.2, under conditions used in the refining of antitoxin for therapeutic purposes, led to the loss of 60 % of its antitoxic activity without any increase in antitoxic units/g. of protein. Similar treatment of the β_2 -globulin led to the loss of 36 % of its antitoxic activity, but an increase of 40 % in antitoxic units/g. of protein.

6. Pepsin treatment of the remaining proteins from antitoxic sera showed that they were largely destroyed and that it is to this removal of inactive protein that the increase in activity/g. of protein achieved in the refining process for antitoxin is mainly due.

7. The properties of the pepsin-treated horse γ -globulins are compared with those of γ -globulin fractions obtained from other species by pepsin and papain treatment, and it is suggested that pepsin acts on β_2 -globulins to produce very similar fragments.

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REFERENCES

- Amoureux, G. & Yeu, F. (1951). *Ann. Inst. Pasteur*, **80**, 165.
- Anderson, C. G. (1955). *Biochem. J.* **59**, 47.
- Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
- Armstrong, S. H., jun., Budka, M. J. E. & Morrison, K. C. (1947). *J. Amer. chem. Soc.* **69**, 416.
- Aronsson, T. & Grönwall, A. (1957). *Scand. J. clin. Lab. Invest.* **9**, 338.
- Brand, E. & Edsall, J. T. (1947). *Annu. Rev. Biochem.* **16**, 223.
- Bridgman, W. B. (1946). *J. Amer. chem. Soc.* **68**, 857.
- Cinader, B. & Weitz, B. (1953). *J. Hyg., Camb.*, **51**, 293.
- Cohen, S. (1963). *Nature, Lond.*, **197**, 253.
- Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys.* Suppl. **1**, 174.
- Harms, A. J. (1948). *Biochem. J.* **42**, 390.
- Herriott, R. M. (1955). In *Methods in Enzymology*, vol. 2, p. 3. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Kekwick, R. A. (1941). *Chem. & Ind.* **60**, 486.
- Kekwick, R. A., Knight, B. C. J. G., Macfarlane, M. G. & Record, B. R. (1941). *Lancet*, **i**, 571.
- Kekwick, R. A. & Record, B. R. (1941). *Brit. J. exp. Path.* **22**, 29.
- Levy, H. B. & Sober, H. A. (1960). *Proc. Soc. exp. Biol., N.Y.*, **103**, 250.
- Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.
- Nisonoff, A., Wissler, F. C. & Lipman, L. N. (1960). *Science*, **132**, 1770.
- Nisonoff, A., Wissler, F. C., Lipman, L. N. & Woernley, D. L. (1960). *Arch. Biochem. Biophys.* **89**, 230.
- Northrop, J. H. (1946). *J. gen. Physiol.* **30**, 177.
- Parfentjev, I. A. (1936). *U.S. Patent* 2065196.
- Parfentjev, I. A. (1938). *U.S. Patent* 2123198.
- Parfentjev, I. A. (1939). *U.S. Patent* 2175090.
- Petermann, M. L. (1942a). *J. biol. Chem.* **144**, 607.
- Petermann, M. L. (1942b). *J. phys. Chem.* **46**, 183.
- Petermann, M. L. & Pappenheimer, A. M., jun. (1941). *J. phys. Chem.* **45**, 1.
- Pope, C. G. (1938). *Brit. J. exp. Path.* **19**, 245.
- Pope, C. G. (1939a). *Brit. J. exp. Path.* **20**, 132.
- Pope, C. G. (1939b). *Brit. J. exp. Path.* **20**, 201.
- Pope, C. G. & Stevens, M. F. (1951). *Brit. J. exp. Path.* **32**, 314.
- Porter, R. R. (1959). *Biochem. J.* **73**, 119.
- Richmond, V., Tang, J., Wolf, S., Trucco, R. E. & Caputto, R. (1958). *Biochim. biophys. Acta*, **29**, 453.
- Ryle, A. P. & Porter, R. R. (1959). *Biochem. J.* **73**, 75.
- Siebenmann, C. (1937). *Biochem. J.* **31**, 205.
- Taylor, W. H. (1962). *Physiol. Rev.* **42**, 519.
- van der Scheer, J. E. & Wyckoff, R. W. G. (1940). *Proc. Soc. exp. Biol., N.Y.*, **45**, 634.
- van der Scheer, J. E., Wyckoff, R. W. G. & Clarke, F. H. (1941). *J. Immunol.* **41**, 349.

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Magnesium Deficiency in the Adult Rat

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Magnesium deficiency has been extensively studied in young rapidly-growing animals, where it is readily produced by specific dietary restriction. Although the symptoms of deficiency vary slightly between different species it always causes hyperexcitability leading eventually to convulsions (Kruse, Orent & McCollum, 1932; Orent, Kruse & McCollum, 1932; Blaxter, Rook & MacDonald, 1954). The magnesium concentration in the serum falls rapidly and about one-third of the bone magnesium may be lost, but most authors have observed little if any change in the magnesium concentration of soft tissues from either rats (Cunningham, 1936a; Watchorn & McCance, 1937; Tufts & Greenberg, 1938) or calves (Blaxter *et al.* 1954). Restitution of magnesium to the diet of magnesium-deficient weanling rats caused a preferential deposition of magnesium in bone (Duckworth, Godden & Warnock, 1940), indicating that the skeletal

magnesium acts as a reserve that can be mobilized during deficiency to meet the requirements of other tissues.

Very little information is available about the lability of bone magnesium in the adult animal, but Cunningham (1936b) observed normal concentrations in the skeletons of six dairy cows affected by naturally occurring magnesium deficiency. Contrary to the earlier reports, a significant fall in the magnesium concentration of skeletal muscle from young magnesium-deficient rats has recently been found (MacIntyre & Davidsson, 1958; Whang & Welt, 1963). These two considerations stimulated an investigation of the relation between the extracellular, soft-tissue and skeletal magnesium during prolonged deficiency in adult rats, with the object of defining the functional reserve of magnesium in the adult state. Disturbances in calcium and potassium metabolism have been reported during mag-