The Distribution of Diphtheria Antitoxin in Pepsin-digested Horse Antiserum

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It has been shown by many workers, both by salting-out methods and by electrophoresis, that antibodies to a single antigen are not confined to any particular fraction of the serum proteins, and that the immunological properties of the antibody may vary from fraction to fraction. Barr, Glenny & Pope (1931) showed that addition of euglobulin to diphtheria antitoxic serum, before salting out with ammonium sulphate, caused antitoxin to be precipitated by a lower concentration of ammonium sulphate than is necessary for antiserum alone; in the presence of added albumin more ammonium sulphate than normal is required to precipitate antitoxin, that is, there is an apparent redistribution of antitoxin when the proportions of the serum proteins are altered. Barr & Glenny (1931) showed that serum protein fractions obtained by successive precipitation with increasing amounts of ammonium sulphate carried antitoxin fractions which had decreasing *in vivo/in vitro* ratios. Van der Scheer & Wyckoff (1940*a, b*) and Van der Scheer, Wyckoff & Clarke (1940, 1941*b*) reported that, during the immunization of horses with bacterial toxins, a new serum component, *T*, with an electrophoretic mobility between those of γ - and β -globulins, made its appearance and that part of the antitoxin was associated with it. Immunization with polysaccharide or vaccine antigens did not evoke production of a *T* component but increased the γ -globulin fraction.

By electrophoretic analysis Kekwick & Record (1941) found that during immunization of horses with diphtheria toxin the γ -globulin fraction increased slightly, but in the early stages only, and that the β -globulin rose slowly at first and then more rapidly and to a greater extent in the later stages of immunization. Antitoxic activity was associated with both fractions but their immunological properties were different. The β -antitoxin had a long flocculation time (147 min. at the 7.5 Lf/ml. level), a low *in vivo/in vitro* ratio (0.9) and gave floccules containing only half the amount of antibody nitrogen found in floccules from the γ -antitoxic fraction, which had a short flocculation time (7 min. at the 7.5 Lf/ml. level) and a high *in vivo/in vitro* ratio (2.0). The more soluble fraction (pseudoglobulin) obtained by salt precipitation contained a higher pro-

portion of β -globulin than the less-soluble fraction (euglobulin) which contained more γ -globulin. The β -globulin of some normal horses contains two components, β_1 and β_2 , whilst all the immunized horses examined had both β_1 and β_2 ; the increase in globulin content was associated with the slower, β_2 -globulin, which corresponds to the *T* component of Van der Scheer *et al.* Kekwick, Knight, Macfarlane & Record (1941) showed that the peptic digestion of antitoxic sera by Pope's (1939) method, with a yield of about 80% of the antitoxin, gave a product having the short flocculation time of γ -globulin, but the low *in vivo/in vitro* ratio of γ -globulin, which they regarded as the source of the digested antitoxin. They found that rapidly flocculating raw sera with a high γ -globulin content gave poor yields of antitoxin after digestion, and that, from a serum rich in γ -globulin, only 15–20% of the antitoxin could be recovered. They suggested that peptic digestion eliminated most of the antitoxin associated with the γ -globulin and that the product was mainly a modified β -globulin. This opinion was based on the low *in vivo/in vitro* ratios and the low yields from sera from horses in the early stages of immunization in which most of the activity was associated with γ -globulin; the digestion products were not examined electrophoretically.

By electrophoresis Van der Scheer & Wyckoff (1940*c*) followed the digestion of horse antidiphtheria and antitetanus sera with pepsin at pH 4 and 37°, and found that the *T* peak, characteristic of antitoxin, disappeared within 10–30 min. and was replaced by one with a mobility corresponding to that of γ -globulin. The albumin was also rapidly destroyed. This was confirmed by Van der Scheer, Wyckoff & Clarke (1941*c*), who showed in addition that prolonged digestion reduced the amount of material constituting the newly formed γ peak. There was a 69% loss of coagulable protein and a 20% loss of antitoxic activity. When antiserum was heated at 37° at pH 4 in the absence of pepsin the γ -globulin peak, originally present, disappeared and was replaced by *T* or *T*+*T*₁ (*T*₁ having a mobility between those of γ and *T*). The globulins constituting this fraction were readily digested by pepsin. The *T*₁ component was regarded as being

due to aggregation of the γ -globulin; it could be precipitated on neutralization to pH 7 but redissolved at pH 7.6. Heating normal or antitoxic plasma at 53–65° caused the appearance of a new peak, *C*, with a mobility about the same as that of β -globulin (Van der Scheer, Wyckoff & Clarke, 1941*a*). The *C* peak of antitoxic plasma was formed at the expense of the γ and *T* components and was only slowly digested by pepsin.

Fell, Stern & Coghill (1940) reported that antitoxic horse sera had increased β - or γ -globulin fractions and that pepsin-digested antitoxins consisted mainly of β - and γ -globulins. Modern & Ruff (1938*a, b*, 1940*a–d*, 1942) showed that almost all the albumin and 80 % of the euglobulin of normal and antitoxic horse sera, together with 75 % of the pseudoglobulin of normal sera and 66 % of that of antitoxic sera, were destroyed by peptic digestion at pH 3.8 and 45°. They considered it probable that most of the antitoxic activity lost on digestion was that associated with the less-resistant euglobulin. Harms (1948) digested euglobulin and pseudoglobulin fractions obtained by ammonium sulphate precipitation of horse diphtheria antitoxin with pepsin at pH 3.2 and found that a large proportion of the euglobulin fraction was precipitated by the subsequent heating in presence of ammonium sulphate, and considerably less from the treated pseudoglobulin fraction. He considered that this precipitated globulin was mainly γ -globulin and that the remaining antitoxin was largely β -globulin, although not all the β -globulin was antitoxin. The initial fractions were examined electrophoretically and shown to be very inhomogeneous, but the composition of the final digestion products was not investigated apart from nitrogen estimations. By electrophoresis, Amoureux & Yeu (1951) showed that diphtheria and tetanus antitoxic sera from hyperimmunized horses had high β -globulin and low γ -globulin contents, but that the enzyme-purified products consisted of almost pure γ -globulin. Wilson & Miles (1946) state that the enzyme digestion methods of purification of antitoxic sera favour 'the retention of the β - at the expense of the γ -antitoxin'.

Because of these discrepant opinions and because enzyme-purified antitoxins routinely produced at this Institute consist mainly of a component with the mobility of γ -globulin, it was thought desirable to follow the changes in the electrophoretic components of diphtheria-antitoxic horse serum during the various stages of preparation.

METHODS

A pool of plasma from eleven horses hyperimmunized with diphtheria toxin for the routine production of diphtheria antitoxin was used throughout. It had 850 antitoxin units/

ml., 15.34 mg. total N/ml., 0.31 mg. non-protein N/ml., 15.03 mg. protein N/ml. and 56.5 units/mg. protein N.

Fractionation of plasma

Ammonium sulphate. For comparative purposes the method of fractionation described by Harms (1948) was adopted. Plasma was diluted with an equal volume of water and a euglobulin fraction obtained by the addition of 22 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 ml. The precipitated globulin was separated by centrifuging and a pseudoglobulin fraction was precipitated by increasing the concentration of $(\text{NH}_4)_2\text{SO}_4$ in the supernatant fluid to 36 g./100 ml. After separation of this material, albumin was precipitated by bringing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 55 g./100 ml. All the precipitates were dialysed against 0.9 % (w/v) NaCl solution until free from $(\text{NH}_4)_2\text{SO}_4$.

Dialysis. To a sample of plasma were added 1.2 ml. of 56 % (w/v) CaCl_2 solution/100 ml. and the clot was removed by centrifuging. The serum was dialysed against three changes of 15 vol. of tap water and finally against two changes of 30 vol. of distilled water. The precipitated euglobulin was separated by centrifuging and taken up in 0.9 % (w/v) NaCl solution. The supernatant fluid, containing pseudoglobulin and albumin, was not further fractionated.

Digestion. The raw plasma was treated by the routine method used to produce enzyme-purified antitoxins at these laboratories. The plasma, containing 0.3 % (w/v) of tricresol, was diluted with 2 vol. of tap water and the pH brought to 3.25. 1 in 10000 pepsin (Armour & Co., Chicago) (2.5 g./l. of plasma) was added, the mixture was allowed to stand for 2 hr. at room temperature (about 20°), then 14 g. $(\text{NH}_4)_2\text{SO}_4$ were added/100 ml., and the pH was adjusted to 4.30. After heating the solution for 2 hr. at 55°, a further 3.5 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 ml. were added and the precipitate formed during the heat treatment was removed by filtration. $(\text{NH}_4)_2\text{SO}_4$ was added to the filtrate to bring the concentration to 31 % (w/v). The precipitated antitoxic fraction was filtered off and dialysed against running tap water at about 4° for 8 days.

The plasma fractions were treated in essentially the same way, quantities and times being modified to agree with those described by Harms (1948) in order to make direct comparison with his results possible. The solution containing the fraction was diluted with water containing an appropriate amount of tricresol to give a final concentration of 2.5 % of protein and 0.12 % of tricresol, and the pH value adjusted to 3.25 with HCl. Armour's 1 in 10 000 pepsin (0.5 g./100 ml.) was added and the mixture kept at room temperature for 1 hr. $(\text{NH}_4)_2\text{SO}_4$ (13 g./100 ml.) was then added, and then NaOH solution to pH 4.25. The inactive material precipitated by heating the solution to 55° for 1 hr. was removed by centrifuging and $(\text{NH}_4)_2\text{SO}_4$ added to the supernatant fluid to give a concentration of 31 % (w/v). After standing at room temperature for 1.5 hr., the precipitated antitoxic protein was separated by centrifuging, taken up in 0.9 % (w/v) NaCl solution and dialysed until free from $(\text{NH}_4)_2\text{SO}_4$. The supernatant fluid was also dialysed, first against tap water and finally against 0.9 % (w/v) NaCl solution, until free from $(\text{NH}_4)_2\text{SO}_4$.

Samples were taken immediately after the peptic digestion, after the first $(\text{NH}_4)_2\text{SO}_4$ and heat treatment, after the second $(\text{NH}_4)_2\text{SO}_4$ precipitation and from the final supernatant fluid. Where necessary the samples were brought to pH 7 and dialysed to remove $(\text{NH}_4)_2\text{SO}_4$.

Table 1. *The results of fractionation of diphtheria antitoxic plasma*

The values are weights of the components/100 ml. of original plasma.

Fraction	Components (g./100 ml. plasma)						Antitoxin	
	Albumin	Globulins				Total	Units/ml.	% recovery
Original plasma	1.45	α -1.17	β -1.48	T -3.25	γ -2.05	9.40	850	100
Salt precipitated								
Euglobulin	0.23	0.43	0.83	2.66	1.53	5.68	550	77.7
Pseudoglobulin	0.89	0.45	0.33	0.52	0.27	2.46	250	21.2
Albumin	0.30	0.04	0	0	0	0.34	2	0.1
Total	1.42	0.92	1.16	3.18	1.80	8.48	—	99.0
Dialysis								
Water-insol. euglobulin	0	0	0.18	0.52	1.10	1.80	115	3.4
Water-sol. pseudoglobulin + albumin	1.01	0.64	0.87	2.14	1.25	5.91	660	78.9
Total	1.01	0.64	1.05	2.66	2.35	7.71	—	82.3

Total and non-protein N values and antitoxic potency were determined for the original plasma and fractions and for the samples taken at various stages of digestion by the usual methods.

Electrophoresis. The solutions for electrophoresis were diluted in phosphate buffer solution of pH 7.5 and ionic strength 0.1 to contain 1.5% (w/v) of protein and dialysed against a large volume of the same buffer solution overnight in the refrigerator. Electrophoresis was carried out in the Tiselius apparatus at 130 v and 10 ma at 1°. For evaluation, the photographed curves were enlarged by projection and traced on paper. The areas under each peak were measured with a planimeter and the proportion of each component was calculated.

RESULTS

The results of fractionation are collected in Table 1, from which it can be seen that antitoxin is associated with both euglobulin and pseudoglobulin salt-precipitated fractions in the proportions 77.7 and 21.2% respectively. The euglobulin and pseudoglobulin obtained by dialysis not only have proportions of components very different from each other but also from the salt-precipitated fractions and a quite different distribution of the antitoxic activity, 3.4% being in the water-insoluble euglobulin and 78.9% in the water-soluble pseudoglobulin. It is interesting to note that the proportions of β -, T - and γ -globulins in salt-precipitated euglobulin are very close to those in the water-soluble pseudoglobulin fraction. The final products after digestion, heat treatment and ammonium sulphate precipitation all have approximately the same proportional composition whether derived from raw plasma or from any of the fractions, and contain about 70% of γ -globulin and 20% of β - and T -globulins (Table 2). The recovery of β - + T -globulins and of γ -globulin after digestion of raw plasma and of different fractions is shown in Table 3. It is obvious that the recovery of γ -

globulin is always greater, and usually very much greater, than that of β - + T -globulins.

The recovery of antitoxic activity (see Table 2) from raw plasma and from the salt-precipitated fractions is about 50–60%, but there is a wider difference in the recoveries from water-insoluble euglobulin (45.4%) and water-soluble pseudoglobulin (70.6%). The increase in purity, that is the ratio of antitoxin units/mg. of protein nitrogen before and after digestion, is fairly constant at 2.5 to 3 times.

DISCUSSION

During the heat denaturation stage of the process, the proportion of material having the mobility of γ -globulin increases and that of the other components diminishes; the loss is particularly associated with the β and T components. This becomes very obvious when the results are expressed as percentage recoveries of the amounts originally present in the starting material, as shown in Table 3. This finding is similar to those of Van der Scheer & Wyckoff (1940c), of Van der Scheer *et al.* (1941a) and of Amoureux & Yeu (1951) on digested plasma and heat-treated plasma, but is in conflict with the statements of Harms (1948) and of Wilson & Miles (1946).

After the fractionation of plasma by dialysis, the total absolute amount of γ -globulin present was greater than that in the original plasma, whilst the amount of the other globulin components was less (Table 1). Similar absolute increases in the amount of γ -globulin occurred during the digestion of raw plasma and of the water-soluble pseudoglobulin + albumin fraction and during the heat-denaturation stage of treatment of salt precipitated euglobulin (Table 2). Part of this increase during digestion is due to a component of the added pepsin, of which approximately two-thirds has the same mobility as

Table 2. *Results of peptic digestion of plasma and fractions*

Fraction	Components (g./100 ml.)						Protein nitrogen		Antitoxic activity		
	Globulins						mg./ml.	% of original	Units/ml.	% recovery	Units/mg. protein N
	Albumin	α -	β -	T ₁ -	γ -	Total					
Plasma	1.45	1.17	1.48	3.25	2.05	9.04	15.03	100	850	100	56.5
Immediately after digestion	0.57	1.32	1.26	1.80	3.21	8.16	4.35	28.9	230	81.5	53.0
After heat denaturation	0	0.15	0.36	—	1.05	1.56	0.83	5.5	190	67.5	230
Final product	0	0.13	0.37	—	1.24	1.74	33.40	—	5700	55.6	170
Discarded supernatant	—	—	—	—	—	—	—	—	1.5	—	—
Salt-precipitated euglobulin	0.19	0.36	0.73	2.22	1.28	4.78	7.63	100	550	100	72
Immediately after digestion	0	2.50	—	2.32	—	4.82	3.85	50.4	214	77.8	55.5
After heat denaturation	0	0	0	0.72	2.88	3.60	2.87	37.5	166	60.3	58
Final product	0	0	0	0.23	0.80	1.03	3.58	—	500	41.8	140
Discarded supernatant	0	0	0	0.02	0.04	0.06	0.04	—	0.2	0.1	5
Salt-precipitated pseudoglobulin	1.24	0.62	0.46	0.73	0.37	3.42	5.46	100	250	100	73
Immediately after digestion	0.56	0.38	0.19	0.20	0.65	1.98	3.57	65.3	155	82.8	43.4
After heat denaturation	0	0.11	0.12	0.15	0.49	0.87	1.57	28.6	136	72.4	86.5
Final product	0	0.02	0.05	0.07	0.35	0.49	4.31	—	620	59.5	144
Discarded supernatant	0	0	0.07	0.12	0.15	0.34	0.37	—	1.3	0.8	3.5
Albumin	0.36	0.08	0	0	0	0.44	0.71	100	2	100	3
Immediately after digestion	0.02	0.08	0.28	0	0.27	0.65	0.89	—	—	—	—
After heat denaturation	0	0.16	0.15	0	0.20	0.51	0.70	—	—	—	—
Final product	0	0.07	0	0	0.18	0.25	0.34	—	1	5.9	3
Discarded supernatant	—	—	—	—	—	—	0.08	—	—	—	—
Water-insoluble euglobulin	0	0	0.22	0.62	1.35	2.19	3.51	100	115	100	32.9
Immediately after digestion	0	0.56	0.58	0	0.89	2.03	3.25	93	110	87.0	30.7
After heat denaturation	0	0.12	0.14	0	0.36	0.62	0.57	16.2	56	48.7	98.5
Final product	0	0.11	0.10	0	0.43	0.64	1.52	—	136	45.4	89.5
Discarded supernatant	0	0	0	0	0	0	0	—	1.5	2.0	6.3
Water-soluble pseudoglobulin	1.06	0.67	0.92	2.24	1.31	6.20	9.89	100	660	100	66.7
Immediately after digestion	0.08	0.10	0.94	1.94	2.60	5.66	5.24	89.9	310	94.0	59.0
After heat denaturation	0	0.11	0.37	0.43	1.28	2.50	1.54	15.6	250	75.8	162
Final product	0	0.11	0.17	0.45	1.48	2.21	4.55	—	600	70.6	132
Discarded supernatant	0	0	0.06	0.15	0.21	0.42	0.18	1.82	0.5	0.3	2.8

Table 3. *The recovery of β -+T- and γ -globulins in the final products of enzyme purification of plasma and different fractions, as % of the original amount present*

Fraction	Volume digested (ml.)	Volume of final product (ml.)	Globulin (g./100 ml.)					
			β - + T-		γ -		% recovery	
			Before digestion	Final product	Before digestion	Final product	β - + T-	γ -
Plasma	50 000	4150	4.73	4.49	2.05	14.80	7.9	60.0
Salt-pptd. euglobulin	100	46	2.95	0.50	1.28	1.74	7.8	62.6
Salt-pptd. pseudoglobulin	150	27	1.19	0.44	0.37	1.92	6.7	92.5
Water-insol. euglobulin	53	10	0.35	0.15	0.55	0.64	10.5	20.7
Water-sol. pseudoglobulin	75	39	3.16	0.80	1.31	1.90	13.1	75.6

serum γ -globulin; however, the maximum increase from this cause cannot be more than 0.3 g./100 ml. and therefore cannot account for the observed increases of 1.16, 1.60 and 1.29 g./100 ml. respectively. It is possible that similar 'manufacture' of γ -globulin also took place during treatment of the other fractions but was obscured by such factors as the degree of digestion and loss during heat denaturation.

It is becoming difficult to escape the conclusion that the substances commonly designated as α -, β - and γ -globulins are not the fixed entities that they are often considered to be. They seem, rather, to be the labile resultants of the physical conditions prevailing during their preparation or examination. The variability and heterogeneity of globin fractions shown by McFarlane's (1935) ultracentrifuge experiments are an early example of this interchangeability, and the altered patterns obtained by Perlmann & Kaufman (1949) on electrophoresis of serum proteins after treatment with dilute acetic acid form another. The effect of phenol or cresol, reported by Harms (1946), in reversing the order of precipitation of albumin and globulins by ammonium sulphate is, probably, in the same category. It is possible that the somewhat large proportion of albumin in the salt-precipitated euglobulin fraction used in the present work may be due to the presence of cresol in the plasma serving as starting material; although the composition of the fractions derived from the plasma may be dependent to some extent on the presence of this cresol, the conclusion that the final digestion products are of almost constant composition is not invalidated.

That the distribution of immunological properties of an antiserum is also subject to variation is shown by the salting-out experiments of Barr *et al.* (1931). Another manifestation of the same phenomenon is afforded by the fact that the antibodies in different batches of typhoid antiserum cannot be consistently separated in any given globulin fraction by salt precipitation or by adjustment of the ionic strength of the solutions (Anderson, unpublished results).

The terms euglobulin and pseudoglobulin are even less applicable as describing entities than are α -, β - and γ -globulins, as may be seen from the electrophoretic and antitoxic 'make-up' of the preparations shown in Table 1.

It seems probable that the groupings on the protein molecules responsible for their immunological reactions and specificity are constant but that other parts of the molecule may be altered by the physical conditions in such a way that the antibodies appear to be differently distributed when the serum is fractionated or submitted to such processes as enzyme purification. The alteration in the 'carrier' part of the protein is probably also responsible for the observed changes in flocculation time, avidity and *in vivo/in vitro* ratios of the products of such manipulation.

SUMMARY

1. Electrophoretic examination shows that horse diphtheria-antitoxic plasma and euglobulin and pseudoglobulin fractions prepared from it by salt precipitation or by dialysis have quite different proportions of α -, β -, T- and γ -globulins and different distribution of antitoxin.

2. These changes of composition are at least partly due to conversion of one component into another and not only to selective precipitation or to loss.

3. Peptic digestion, followed by heat denaturation and ammonium sulphate precipitation, gives products from the original plasma and from all of the fractions with about 70 % of γ -globulin and 20-30 % of β -+T-globulins and containing 50-60 % of the original antitoxic activity. The recovery of γ -globulin is always greater than that of β -+T-globulins and is usually much greater.

4. It is suggested that fractionation and other physical conditions affect the apparent distribution of antitoxin and also alter the properties of the 'carrier' part of the antibody molecule in such a way as to modify such characteristics as flocculation time, avidity and *in vivo/in vitro* ratio without changing specificity and antitoxic properties.

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The Effects of Salicylate and Adrenocortical Preparations Added *in vitro* upon the Glycogen Content of Liver Slices

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The injection of salicylate has been observed to cause a marked depletion of the liver-glycogen level in normal rats (Lutwak-Mann, 1942; Smith, Meade & Bornstein, 1952) and to prevent the deposition of liver glycogen induced by the injection of cortisone in adrenalectomized rats (Smith, 1952). The present paper is concerned with the effects of salicylate and adrenocortical preparations added *in vitro* on the glycogen content of rat-liver slices incubated in buffered physiological salt solutions.

EXPERIMENTAL

Preparation of tissue. Male Wistar rats, weighing 150–300 g., maintained on a diet of cubes (Medical Research Council diet 41) were used. The rats were killed by stunning, the liver was removed and slices of thickness 0.3–0.4 mm. were prepared with a scalpel, always from the same lobe. The slices as cut were placed serially in a number of piles, equal to the number of samples needed, in a Petri dish over ice. Each sample, weighing 80–100 mg., consisted of about four slices. The samples were weighed at the beginning of the experiment on a torsion balance. Samples in duplicate were used throughout and the average difference between duplicates for the glycogen estimations was $\pm 6.3\%$.

Conditions of incubation. Two salt solutions were used: (a) phosphate-Ringer (Seckel, 1940): 8.0 g. NaCl, 1.0 g. NaHCO₃, 0.1 g. KCl and 0.1 g. CaCl₂, H₂O were dissolved in 1 l. of distilled water; 90 ml. of this solution were mixed with 7.0 ml. $m/15$ Na₂HPO₄ solution and 3.0 ml. $m/15$ NaH₂PO₄ solution before use; (b) solution containing K 110, Mg 20, Ca 10, HCO₃ 40 and Cl 100 m-atoms/l. (Hastings, Teng, Nesbitt & Sinex, 1952). The initial pH was 7.4 in all cases, an atmosphere of air or O₂ being used with (a) and 95% O₂–5% CO₂ (by vol.) with (b).

The samples, each with 2 ml. of medium, were placed in Warburg flasks which were attached to manometers and shaken continuously at 37°. At the end of the experiment the slices were transferred as rapidly as possible to 30% (w/v) KOH. The slices for initial values of glycogen were placed in 30% (w/v) KOH at the time the experimental samples went into the bath.

Materials. Salicylic acid (A.R.) was dissolved in the salt solutions to give final concentrations between 0.005M and 0.02M. 0.005M salicylate (69 mg./100 ml.) was chosen as a suitable initial concentration because plasma salicylate concentrations of 50–70 mg./100 ml. were found in rats at the time when maximum liver-glycogen depletion occurred following the subcutaneous injection of sodium salicylate.

The adrenocortical extract used was Eschatin, free from preservative (Parke, Davis and Co.), 1 ml. of which was