ministration of a combination of 2 strains of *Bacteroides*. The specific effect of *Clostridium difficile* on the cecum of the axenic animal has not yet been determined, other than that it will restore the cecum to normal size.

Summary. An anaerobic, gram-positive, spore-forming bacillus has been isolated from the cecal contents of conventional mice. This organism causes reduction in size of the enlarged cecum usually associated with axenic mice. It has been identified as *Clostridium difficile*. A combination of 2 strains of *Bacteroides* isolated from cecal contents of conventional mice was also effective in this respect.

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Curtain Electrophoretic Separation of Phenyl Acetate Hydrolysing Esterases of Human Serum. Demonstration of an EDTA-Dependent Enzyme.* (27549)

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Using cellulose column electrophoresis for separation of plasma from various species, Augustinsson(1-4) found A- and C-esterases in human plasma. The A-esterase hydrolyzes phenyl acetate at a high rate and is resistant to inhibitors such as organophosphorus compounds and physostigmine; however, it does not cleave aliphatic esters. This esterase was found to migrate as a separate fraction with an electrophoretic mobility similar to that of albumin in a veronal buffer. The C-esterase of human plasma was shown to be a typical butyrylcholinesterase with low substrate specificity and sensitive to various inhibitors, physostigmine being the most potent one.

In a large number of electrophoretic preparations of human serum we found high phenyl acetate hydrolyzing activity corresponding to the A-esterase as described by Augustinsson (1-4). Using continuous flow curtain electrophoresis this A-esterase was found to migrate slightly faster than the albumin fraction; furthermore since the prealbumin region is relatively low in protein content, high specific A-esterase activity was thus obtained. Studies of the resistance to heat and EDTA of this fraction revealed that more than one distinct A-esterase was present. Based upon these observations(5) Lundblad(6) separated serum on Sephadex and by using differential heat resistance as a criterion, he was able to confirm the existence of at least 2 A-esterases in human serum.

In the present study repeated continuous flow curtain electrophoresis of the A-esterase fraction yielded 2 distinct A-esterases of high specific activities.

Material and methods. Human serum was obtained from healthy blood donors. The blood was drawn directly into sterile bottles without additions. Each bottle was kept at 35°C for 1 hour to secure maximum clot formation. The clot was then loosened aseptically from the glass wall and the specimen left overnight in a refrigerator. The next day

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serum was drawn aseptically and centrifuged twice at 1000 × *g* for 30 min at 2-4°C. The sera were kept in a deep-freeze in 25 ml portions. Pooling was done after thawing. Approximately 40 different sera were pooled for each preparation.

One litre of pooled serum in 200 ml aliquots was dialyzed in cellophane bags (Visking, no jax) in a rocking compartment for 3 days at 5°C against 120 litres of streaming M/150 phosphate buffer pH 7.9, made up by 10-fold dilution of Sørensen's M/15 phosphate buffer. A small precipitate was formed corresponding to 4.6% of the total dry weight of untreated serum. After centrifugation at 1000 × *g* for 2 hours at 2°C the clear supernatant was subjected to continuous flow curtain electrophoresis.

Continuous flow curtain electrophoresis was performed with 2 Spinco Model CP apparatuses. Before each run the apparatus was disassembled and thoroughly washed with a bactericidal agent ("Colgon" 1:1000, Colgafabriken Aktiebolag, Stockholm) and rinsed repeatedly with distilled water. Curtains and wicks were always autoclaved before use.

Autoclaved M/150 phosphate buffer of pH 7.9 was used for electrophoresis. By using 10 litre portions of this buffer and exchanging 10 litres twice daily, a *"wash-out effect"* was obtained which prevented bacterial growth. In some experiments test tubes were placed under the 32 curtain drip-points and the fractions were tested immediately for esterase activity. In other experiments plastic tubing was attached to small funnels placed under the drip-points and in this way the fractions were transferred directly into pre-weighed glass containers, submerged in a −15°C ethanol bath, causing each drop to freeze immediately as it came off the curtain. After weighing the bottles at the end of the run, and volume obtained, the fractions were lyophilized.

Before placing the dialyzed serum in the 30 mm diameter sample container in the Spinco CP apparatus, filtration was performed through a G 5 M sintered glass sterile filter. The flow rate was kept at 1.5 ml per hour, the current at 35 mA and the voltage close to or above 600. Usually the apparatus was permitted to work continuously for 5-7 days.

Protein estimations were made for each drip-point fraction by determining the extinction at 280 mμ (Beckman DU) and/or by ninhydrin determination according to the method by Moore and Stein(7).

Fluorescence spectrometry was used in some instances to characterize fractions of high specific activity (Aminco-Bowman, American Instrument Co., Inc.).

Phenyl acetate esterase (PAE) assay: 0.5 ml of sample plus 3.0 ml 0.04 molar phenyl acetate in M/15 phosphate buffer (pH 7.4) were incubated for 60 min at 25°C and then diluted with 15 ml of H_2O and immediately titrated against 0.01 N NaOH to pH 8.3. Duplicate samples were always used. An automatic titrator (TTT 1 c, Radiometer, Copenhagen) was employed. A-esterase released HAc from phenyl acetate by hydrolyzing at the ester linkage. Therefore, the amount of phenyl acetate being hydrolyzed was equivalent to the consumed NaOH.

Experiments and results. When serum was subjected to electrophoretic separation, a fairly constant pattern was seen as determined by spectrophotometry and assay of phenyl acetate esterase (PAE). Fig. 1 represents a typical result. A detailed analysis of all the active regions was not attempted. Only the area of highest PAE-activity corresponding to Augustinsson's A-esterase and situated in the prealbumin region was subjected to further study.

The A-esterase fractions from each one of 8 separate runs were lyophilized and subjected to repeated electrophoretic separation. In preparatory studies using curtain or paper strips, the conditions for the second electrophoresis were varied as regards pH, ionic strength, current flow, flow rate and position of sample, and flow rate of buffer in curtain and wicks. The path travelled in the curtain was increased by starting the sample from the utmost left. Using a current of 50 mA, less separation was seen than with 35 mA. However, at 40 mA in conjunction with a decreased flow rate of buffer and sample, separation was appreciably improved, even when pH was kept at 7.9, which seemed to be optimal for retainment of activity of the fractions. Possibly these measures, or a differential

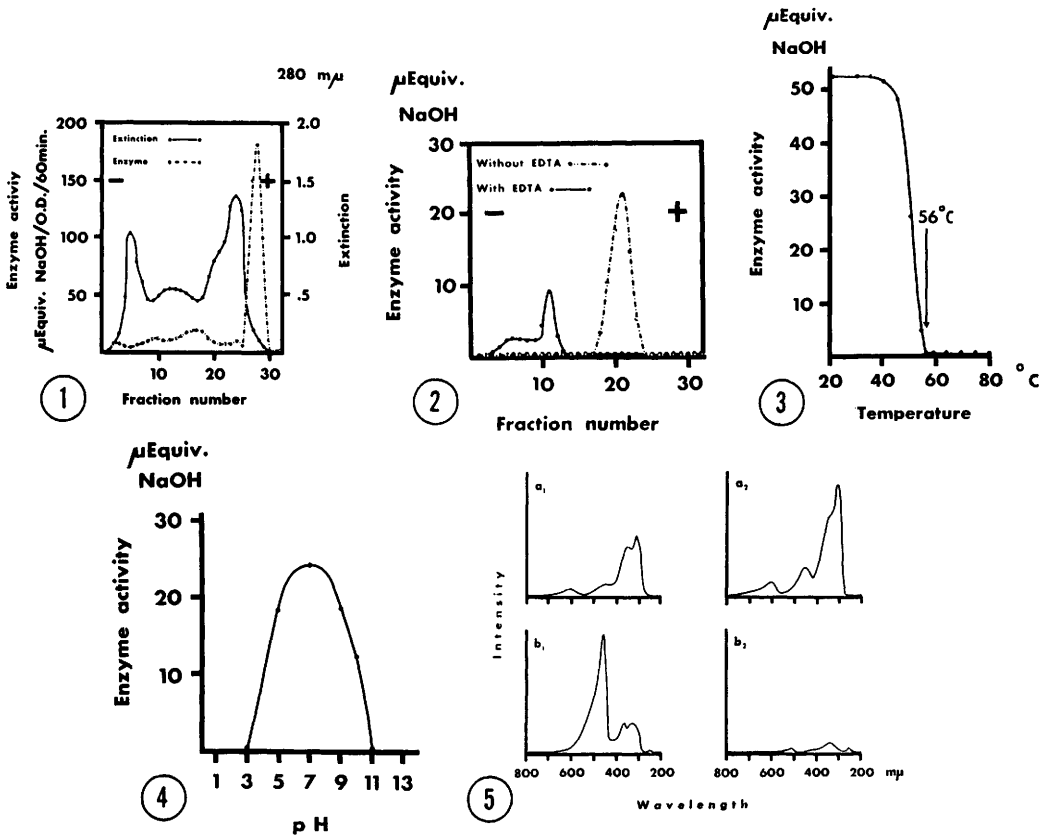

FIG. 1. Separation of human serum by curtain electrophoresis (M/150 phosphate pH 7.9, 35 mA) and assay of phenyl acetate (PAE) in each fraction from 32 drip-points. The high peak of esterase activity (broken line) in prealbumin region corresponds to Augustinsson's A-esterase.

FIG. 2. Separation by curtain electrophoresis (M/150 phosphate, pH 7.9, 40 mA) of the A-esterase containing prealbumin fraction obtained at separation of whole serum. Fractions 3-12 contain EDTA-dependent phenyl acetate esterase (PAE), while fractions 18-23 contain EDTA-sensitive enzyme.

FIG. 3. Heat-inactivation of EDTA-sensitive phenyl acetate esterase (PAE) activity. All activity disappeared after heating to 56°C for 30 min.

FIG. 4. Resistance of the enzyme to treatment with buffers of pH varying from 3-11 for 60 min at 35°C. After readjusting pH to 7.4 phenyl acetate esterase (PAE) activity was assayed.

FIG. 5. Fluorescence spectra for aromatic esterase fractions obtained by repeated curtain electrophoresis: a_1) EDTA-dependent enzyme. Activation at 200 mμ. a_2) Same preparation activated at 230 mμ. b_1) EDTA-sensitive enzyme. Activation at 200 mμ. b_2) Same preparation activated at 230 mμ. All spectra were obtained under identical conditions at pH 7.9 in M/150 phosphate buffer using an Aminco-Bowman Spectrophotofluorometer. (Light source: High pressure Xenon arc lamp; detector: RCA 1 P28; slit: 3; sensitivity: 50; uncorrected values.)

effect of lyophilization on the first heterogenous fraction, explain the pattern obtained at the second electrophoretic separation. It is also possible that the fast migrating fraction is highly influenced by an increase of the current flow, because 50 mA caused dissociation and reversed migration of this fraction. In paper strip electrophoresis similar phenomena were observed with decrease of pH.

The standardized second runs all gave similar results, a typical one being represented by Fig. 2. The PAE-activity was recovered in one peak, while another PAE-activity was brought forward by addition of 0.01 molar EDTA. Control titrations of all fractions in the absence of phenyl acetate revealed that

addition of EDTA in no instance caused a measurable increase of NaOH consumption. The relationships of these 2 PAE-activities to EDTA were inverse; one being dependent on EDTA for activity, while the other was active only in the absence of EDTA.

When tested in different chemical media EDTA-sensitive esterase activity varied. In the presence of Simms X-7 balanced salt solution,† activity was equal to that found in phosphate buffer. Dilution with NaCl and subsequent assay performed in TRIS buffer gave half the value of dilution with phosphate or Simms X-7. At low ionic strength, as produced by dialysis against distilled water for 3 days, irreversible denaturation and precipitation occurred.

Equilibrium dialysis against Simms X-7, M/150 phosphate or saline also showed that saline decreased the activity by 50%, while phosphate and/or Simms X-7 left the activity intact. All attempts to restore activity after dialysis against saline by addition of phosphate or Simms X-7 were unsuccessful.

The EDTA-inactivation of the EDTA-sensitive esterase was studied by addition of EDTA in the presence of various cations and anions, particularly those present in Simms X-7 balanced salt solution. Inactivation with EDTA was found to take place in the presence of 0.02 molar Na^+, K^+, Ca^{++}, Mg^{++}, Co^{++}, Cu^{++}, Pb^+, and Zn^{++}. However, 0.02 molar Mn^{++} prevented inactivation by 0.01 molar EDTA. Mn^{++} by itself did not influence the activity of the esterase.

These results indicate that the EDTA-sensitive PAE is influenced particularly by phosphate and manganese ions. The detailed nature of this interaction is not known.

Heat- and pH-resistance were investigated. EDTA-sensitive PAE-activity disappeared after heating to 56°C for 30 min (Fig. 3). The EDTA-dependent PAE withstood 68°C for 30 min. After 60 min at 35°C in: a) M/30 molar HAc pH 3.0; b) M/30 acetate pH 5.0; c) M/30 phosphate pH 7.0; d) M/15 phosphate-NaOH pH 9.0; and e) M/15 phosphate-NaOH pH 11.0, EDTA-sensitive activity was assayed (Fig. 4) with results characteristic of proteins in general.

The above described PAE-activities were further distinguished by their UV fluorescence spectra (Fig. 5). The EDTA-sensitive enzyme, when activated at 200 mμ, emitted light with peak value at 465 mμ. The EDTA-dependent enzyme, when activated at 200 mμ, showed only weak fluorescence in this region. On the other hand, when the EDTA-sensitive enzyme was activated at 230 mμ, there was little fluorescence, while the EDTA-dependent enzyme activated at 230 mμ exhibited a characteristic fluorescence with peak values at 300 and 600 mμ. Although these fluorescence data cannot now be interpreted in detail, they have been useful for practical differentiation of the 2 enzyme fractions in the laboratory.

The specific activity of the PAE enzymes was calculated from the activities of whole serum with and without EDTA. One ml of serum in 60 minutes hydrolyzed 47.7 mg of phenyl acetate in the absence of EDTA and 6.3 mg in the presence of EDTA. For each millimol hydrolyzed phenyl acetate 223.2 and 1697.6 mg of serum protein (1 ml serum = 11.6 mg N × 6.73) were required, respectively. The corresponding amounts of purified enzymes were 1.8 and 2.0 mg, respectively, (calculated as equivalent to leucine), indicating purification of 120 and 850. The latter value is probably higher, because the protein contents, measured as γ leucine/ml, reached below the limit of sensitivity of the ninhydrin method.

Discussion. Relatively little is known about aromatic esterases in serum and knowledge of the natural substrate to this enzyme is still lacking. Much of the available information stems from Augustinsson's work on separation and classification of the plasma esterases in different species(1-4).

Further elucidation of the nature and properties of A-esterase in plasma will depend largely upon procedures permitting a high degree of purification of serum proteins. As such procedures improve, we are increasingly aware of the extreme complexity of plasma.

† Contains: NaCl 137.0; KCl 2.7, $CaCl_2 \cdot 6H_2O$ 1.0; $MgCl_2 \cdot 6H_2O$ 1.0; $NaH_2PO_4 \cdot 2H_2O$ 0.15; $Na_2HPO_4 \cdot 2H_2O$ 1.36; $NaHCO_3$ 6.0; Glucose 5.5 mM/l.

Therefore, it is advantageous when the conditions for separation of a particular serum constituent can be so changed that this constituent can be separated from the bulk of serum proteins and virtually free from both albumin and globulin. This was achieved in our study by using curtain electrophoresis in M/150 phosphate buffer at pH 7.9.

Curtain electrophoresis is free from the disadvantages of "tailing" and protein-protein interaction during the separation, because each electrophoretic component travels along its own path without being influenced by other components. This technic also permits an extremely mild, rapid and practically aseptic treatment of the individual fractions which can be frozen, lyophilized or tested as they appear at the different drip-points. Any one fraction can be re-run to give preparations of high specific activity. Here specific activities of 120 and 850, respectively, were obtained for A-esterases at the second run. These figures could probably be much improved by repetition at other ionic conditions. Therefore, separation of serum enzyme by continuous flow curtain electrophoresis seems to be a useful procedure.

The human A-esterase seems to consist of more than one enzymatic activity as indicated by this study, where it was possible to separate the A-esterase into 2 distinctly different enzymes, one being dependent on and the other being sensitive to EDTA.

Concerning the EDTA-dependent esterase one may argue that the activities obtained, being relatively weak, could perhaps have been due to acidification of the protein fractions themselves by removal of cations through interaction with EDTA. However, the amount of protein in each fraction was minute and in the actual fractions not exceeding 36 γ/ml, and control titrations of all fractions without substrate invariably resulted in no detectable increase of NaOH consumption. This, in combination with the fact that EDTA itself did not influence hydrolysis of phenyl acetate, shows that NaOH consumption after 60 minutes' incubation of the mixture of fraction and EDTA and substrate was due to enzymatic activity. Loss of activity after heat treatment before addition of EDTA also supports this concept.

Since one of these 2 enzymes is inactive in absence of EDTA it is not likely that it played a role in Augustinsson's work. On the other hand, a situation can readily be visualized where the simultaneous presence of equal activities of these enzymes may give a misleading impression of resistance to EDTA. While one esterase is inactivated completely, the other, which normally is inactive, is activated to the same extent leaving a net result of unchanged activity. One cannot exclude the possibility that such a mechanism could play a role in maintaining A-esterase activity at a constant level during changing ionic conditions. In any case this should be considered in further exploration of plasma esterase.

Concerning heat-resistance it seems that the heat-labile EDTA-sensitive A-esterase may be similar to heat-labile serum esterase activity as described by Lundblad(6). Both activities reach zero at exactly 56°C for 30 min. It is not clear what relationship EDTA-dependent A-esterase bears to the heat-stable esterase. Even if the heat-stability is approximately the same, these enzymes cannot be identical unless passing of the heat-stable fraction through Sephadex effects changes of the same activating nature as obtained by addition of EDTA. *Per se* this is by no means impossible. Otherwise we will have to consider the possibility of 3 distinct A-esterases being involved.

The relative ease of preparation of fractions of reasonably high specific activity will facilitate further study, perhaps also dealing with the possible role of A-esterases for immunologic accessory reactions.

Summary. By repeated continuous flow curtain electrophoresis of human serum, 2 distinct phenyl acetate hydrolyzing esterases were purified 120 and 850 times. One esterase was heat-labile and inactivated by EDTA, while the other was heat-stable and required EDTA for activity. EDTA-inactivation could be prevented by manganese. The methods for purification and assay are described and the results discussed.

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Thrombocytosis-Promoting Activity of Normal Plasma.* (27550)

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Data from many laboratories have confirmed the existence of a humoral erythropoietic regulatory mechanism(1-3). Recent observations suggest that other aspects of hemopoiesis may be similarly influenced. The thermostable fractions of plasmas from patients with polycythemia vera(4) and from rabbits with acute phenylhydrazine-induced anemia(5) evoke thrombocytosis and leukocytosis in normal rats in addition to erythrocytosis and reticulocytosis. Thrombocytosis has also been reported in rats injected with plasma or serum from thrombocytopenic donors(6-8). These findings are in accord with the hypothesis that thrombopoiesis is subject to humoral homeostatic control; however, the concept of such a physiologic regulator demands that the responsible substance (or substances) also be demonstrable in normal blood. The experiments described herein bear directly on this problem.

In the majority of our studies on the humoral control of hemopoiesis, we have used, as measures of hemopoietic stimulation, changes in the peripheral erythroid values, reticulocyte, leukocyte, and thrombocyte counts, and marrow cytology in normal rats given multiple daily doses of test plasmas. For purposes of uniformity, individual doses usually have been calculated on the basis of 2 ml of the original plasma per 100 g of the recipients' body weights. These quantities of normal plasma are erythropoietically and leukopoietically inactive when assayed as just described, although erythropoietic activity has been detected in normal plasma with other technics(9) and is demonstrable in concentrates thereof(2,10). In contrast, however, such doses of normal human or rabbit plasma have consistently induced thrombocyte increases in normal rats(4,5). An example of the thrombocyte responses in recipients of normal human plasma extracts by gastric tube is shown in Table I. Two weeks after the treatment was stopped, normal baseline counts had been restored. In this report are described observations on the thrombopoietic effects of lesser amounts of normal plasma.

Materials and methods. Fresh normal human plasma was procured from the blood bank, pooled, adjusted to a pH of 5.5 by addition of 1N HCl, and boiled over a direct flame for 30 minutes. The material was filtered and the coagulum discarded. The filtrate was divided into 3 equal portions and reconstituted with distilled water so that 0.5 ml of the final extracts represented, respectively, 0.5, 1.0, or 2.0 ml of the unmodified plasma. Forty normal female Wistar strain rats were divided into 4 equal groups and given, over a period of 2 weeks (Saturdays and Sundays excepted), 10 doses of test materials by gastric tube. Three groups received normal plasma extracts in doses equivalent to either 0.5, 1.0, or 2 ml of the origi-

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