Separation of Components of Guinea Pig Complement by Chromatography.* (24758)

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(Introduced by M. M. Mayer)
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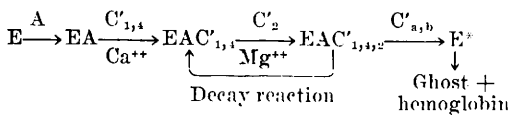
The hemolytic action of guinea pig complement (C′) on sheep erythrocytes (E) sensitized with rabbit antibody (A) to the Forssman antigen has been resolved into a series of successive reaction steps represented schematically as follows(1,2): In this scheme,



C'_1, C'_2 and C'_4 represent the first, second and fourth components of C′, as defined by the conventional component reagents(3), while C'_a and C'_b are provisional designations for 2 components of C′ whose existence has been reported recently(2). Both C'_a and C'_b are distinct from C'_1, C'_2 and C'_4 and either one or both of them in combination are identical with C'_3, as defined in the conventional manner. The conversion of $EAC'_{1,4,2}$ to E* by $C'_{a,b}$ has been found to proceed without requirement of a divalent cation such as Ca^{++} or Mg^{++}, and hence can take place in the presence of 0.01 *M* ethylenediamine tetraacetate (EDTA)(1,2). Further investigation of the C′ system, as well as studies in related fields, would be facilitated by availability of the C′ components in purified form. Pillemer *et al.* (4) have reported isolation of C'_1 and of a fraction possessing C'_2 and C'_4 activity from guinea pig and human sera by precipitation with ammonium sulfate. Jonsen *et al.*(5) have used precipitation with alcohol for separation of C'_3 from pig serum. These methods are cumbersome and the fractions so prepared have not found extensive application in studies of C′ action. Recently Peterson and Sober(6) introduced a technic for chromatographic separation of proteins with cellulose derivatives, such as diethylaminoethyl (DEAE)-cellulose. In particular it was shown that this method is useful for separation of serum proteins(7). In the present initial report the separation of C'_2, C'_4, C'_a and C'_b by chromatography of guinea pig serum, following in essence the simplified technic of Fahey *et al.*(8), is described. In a recent review by Sober(9) on the use of ion-exchange chromatography for protein fractionation, unpublished data of Becker are cited which also indicate successful separation of C′ components.

Methods. Chromatography was carried out by the method of Fahey *et al.*(8), except for omission of the pH gradient and other minor modifications cited below. Sixteen grams of N, N-Diethylaminoethyl cellulose (Eastman Organic Chemicals, No. 7392) were washed several times with 0.0375 *M* NaCl and adjusted to pH 7.4 with HCl, washed again as before, and poured into a pyrex glass chromatography tube (2.7 cm I.D.). After sedimentation of adsorbent under gravity for one to 2 hours, further packing was achieved by suction at 8-10 psi. Ten ml of pooled guinea pig serum were dialyzed overnight against 2 changes of 2 liters each of 0.0375 *M* NaCl. After removal of a white precipitate by centrifugation at 0°C for 20 minutes at 3000 g, the supernatant fluid was applied to the column in a cold room at 2-4°C. Approximately 60-70 ml of 0.0375 *M* NaCl were then passed through during 3 to 4 hours. Fractions were collected at 30 minute intervals during this stage and during the subsequent elution with a gradient of NaCl solution varying from 0.0375 *M* to 0.300 *M* (flow rate about 20 ml per hour). Collection was continued until about 1000 ml had been passed which gave a recovery of more than 97% of the material responsible for absorp-

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tion of light at a wave length of 280 mμ. Preparation and standardization of sheep erythrocyte suspensions, hemolytic antibody and C′ have been described(10). The diluent for all reagents was veronal-NaCl buffer containing 0.1% gelatin, 0.025% sodium bicarbonate, 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++}, unless stated otherwise. $EAC'_{1,4,2}$ was prepared by the method described earlier(11) which involves interaction of EA with undiluted C′ in presence of Ca^{++} and Mg^{++} at 0°C for a brief period, usually about one-half hour, as determined by preliminary experiments for each lot of guinea pig C′. In some experiments the formaldehyde procedure(12) was employed as follows: To 1.0 ml of guinea pig serum is added .15 ml of a dilution of formalin (36.3% formaldehyde) chosen to be just sufficient to destroy lytic activity. Usually treatment with a 1/72 dilution of formalin for 40 minutes at 37°C ± 0.5° is sufficient, but this should be checked for each lot of C′. One ml of this inactivated C′ is added to 5.0 ml of EA containing 10^9 cells per ml, and the mixture is subjected to continuous, gentle agitation at 30.0°C ± 0.025° for 45 minutes. The suspension is diluted with about 2 volumes of ice-cold veronal buffer containing 0.01 *M* EDTA, centrifuged, washed and resuspended in ice-cold EDTA-buffer to a concentration of 1.5 x 10^8 cells per ml. Since no E* formation occurs on treatment of EA with formaldehyde inactivated C′, it is not necessary to hold the cells for 4 hours at 0°C prior to use. $EAC'_{1,4}$ was made from $EAC'_{1,4,2}$ by incubation at 37°C for 90 minutes during which time reactivity of the cells with $C'_{a,b}$ (C′ in 0.01 *M* EDTA) is lost, but lysability by C'_y (complement lacking C'_1 and C'_4) is retained(13). For assay of fractions for C'_2 activity, a 2-step procedure was employed. In the first step, $EAC'_{1,4}$ suspended in veronal buffer containing Ca^{++} and Mg^{++} were exposed to the chromatographic fractions which had been adjusted to isotonicity after collection. In the second step, the $EAC'_{1,4'2}$ so formed were treated with $C'_{a,b}$ to form E* which were allowed to lyse. Whole C′ in the presence of 0.01 *M* EDTA served as a source of $C'_{a,b}$. It is known that the amount of $C'_{a,b}$ required to achieve a given degree of lysis of $EAC'_{1,4,2}$ depends inversely on quantity of C'_2 used for generation of $EAC'_{1,4,2}$ from $EAC'_{1,4}$. Thus, it is possible to perform assays for C'_2 activity by determining the dilution of $C'_{a,b}$ required to yield 50% lysis in the second step. Specifically, 2 ml portions of each fraction to be tested were mixed with 1.0 ml of $EAC'_{1,4}$ containing 10^9 cells. The mixtures were shaken continuously for 20 minutes at room temperature (24°C) (First Step). After centrifuging and washing, the cells were suspended in veronal buffer containing 0.01 *M* EDTA to yield a concentration of 1.5 x 10^8 cells per ml, and 0.5 ml of this suspension was added at 0°C to 2.0 ml portions of whole C′ diluted in 2-fold steps in veronal buffer containing 0.01 *M* EDTA. The tubes were incubated at 37°C for 90 minutes with occasional mixing (Second Step). To each tube 5.0 ml of 0.15 *M* NaCl were then added and the mixtures centrifuged free of cells. Oxyhemoglobin concentration of the supernatant fluids was determined in the Beckman Model DU spectrophotometer at a wavelength of 410.5 mμ. Relative potency of each fraction with respect to C'_2 was expressed as the reciprocal of the final dilution of C′ necessary to achieve 50% lysis in the second step. For C'_a and C'_b assays each chromatographic fraction was tested on $EAC'_{1,4,2}$ as substrate in the presence of either partially purified C'_b or C_a obtained from guinea pig serum by acid precipitation and methanol precipitation, respectively, as previously described(2). Specifically, to assay for C'_a, 1.0 ml portions of appropriate dilutions of each fraction were mixed with 1.0 ml of C'_b (acid precipitable factor), followed by 0.5 ml of $EAC'_{1,4,2}$ containing 1.5 x 10^8 cells per ml. Conversely, assays for C'_b were performed with C'_a (methanol precipitable factor) as reagent and $EAC'_{1,4,2}$ as substrate. The mixtures were incubated at 37°C for 90 minutes, centrifuged and degree of hemolysis was measured as before. Assays for C'_4 activity were made with R_4 as described previously (3).

Results. A typical fractionation experiment is shown in Fig. 1. The contents of each tube collected during elution were anlyzed for protein concentration by measuring light absorption at a wave length of 280 mμ, and in addi-

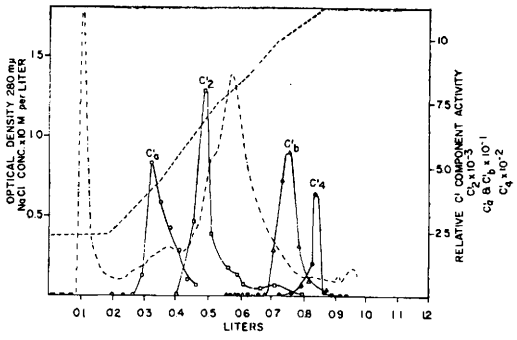

FIG. 1. Chromatography of dialyzed guinea pig serum on DEAE-cellulose. —•—•—, distribution of material responsible for absorption of light at 280 mμ. – – – –, sodium chloride concentration. ——, distribution of complement components.

tion, conductivity measurements were made in order to determine salt concentration. The approximate location of C′ components was known from preliminary experiments, and on this basis the contents of appropriately selected individual tubes were analyzed for their C′ component activities as indicated in the figure. It is evident that C'_a, C'_b, C'_2 and C'_4 can be separated effectively. The titers of the complement components given in the graph represent relative measurements of activity.

That C'_a and C'_b can be separated by chromatography confirms our earlier work in which it was shown that 2 factors so designated are required for transformation of $EAC'_{1,4,2}$ to E*. It is not known at present whether C'_a and C'_b, or both, correspond to C'_3 as defined in the classical sense with R_3 as reagent.

It is noteworthy that the C'_2, C'_a and C'_b fractions had good stability. C'_2 has been stored at 2-4°C for several weeks without appreciable loss of activity; C'_a and C'_b have been kept for several days at 2-4°C with good retention of activity. By contrast, C'_a and C'_b obtained by methanol and by acid precipitation lose their potency in a matter of hours.

The availability of these fractions in a state of functional purity and good stability makes possible new experimental approaches. For example, it has been found that conversion of $EAC'_{1,4}$ to $EAC'_{1,4,2}$ with purified C'_2 obtained by elution from the column, is a most convenient way of preparing $EAC'_{1,4,2}$ and yields a product which is far more reactive with $C'_{a,b}$ (C′ in the presence of 0.01 *M* EDTA) than preparations of $EAC'_{1,4,2}$ obtained by the methods used heretofore, *i.e.*, treatment of EA with C′ at 0°C in presence of Ca^{++} and Mg^{++} for about 30 minutes or by treatment of EA with formaldehyde inactivated C′. In these procedures the temperature of reaction or the concentration of formaldehyde must be controlled rigidly to minimize formation of E*, but these restraining measures are a source of inconvenience and they inevitably reduce the efficiency of formation of $EAC'_{1,4,2}$. These limitations are obviated by use of purified C'_2.

The ready separability of C'_a and C'_b makes possible new and more effective studies of conversion of $EAC'_{1,4,2}$ to E*, and of the relationship of these factors to C'_3, as defined by the reagent R_3. It may be postulated that an intermediate product exists between the $EAC'_{1,4,2}$ and E* stages and availability of C'_a and C'_b in a state free from the other components of C′ should facilitate the search for conditions appropriate for isolation of this postulated intermediate. This is desired, not only for clarification of the reaction mechanism, but also for improvement of analytical procedures of C′ component assay.

It is difficult to relate the activities of the fractions obtained by elution from the chromatographic column to the corresponding activities of complement components of the whole serum used as starting material. With respect to C'_2, difficulty arises from the fact that an assay for C'_2 based on conversion of $EAC'_{1,4}$ to $EAC'_{1,4,2}$ can only be performed in a valid fashion if the material to be assayed is free of $C'_{a,b}$, and if inhibitors or destructive agents are absent. It is known that serum contains inhibitors or destructive factors which can interfere with the hemolytic action of C′(14). In the present fractionation experiments, a factor which inactivates $EAC'_{1,4}$ has been detected and localized in the C'_b region. It is not known whether this destructive agent is related to the esterase studied by Ratnoff and Lepow(15).

It should be noted that significant loss in activity occurs during dialysis of the serum prior to chromatography. With respect to

lytic activity on EA as substrate, the loss exceeds 95%; part of this is accountable as loss of $C'_{a,b}$ (60-90%), as detected with $EAC'_{1,4,2}$ as substrate. Currently, efforts are being made to reduce this loss by modification of the dialysis procedure.

Summary. Fractionation of guinea pig complement by chromatography on diethylaminoethyl cellulose has been explored. C'_2 and C'_4, as well as the components provisionally designated as C'_a and C'_b which convert $EAC'_{1,4,2}$ to E*, can be separated from one another in a single pass, and the fractions so obtained possess good stability.

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1. Mayer, M. M., *Progress in Allergy,* 1958, v5, 215.

2. Rapp, H. J., *Science,* 1958, v127, 234.

3. Kabat, E. A., Mayer, M. M., Experimental Immunochemistry, 1948, C. C. Thomas, Springfield, Ill.

4. Pillemer, L., Ecker, E. E., Oncley, J. L., Cohn, E. J., *J. Exp. Med.,* 1941, v74, 297.

5. Jonsen, J., Manski, W., Heidelberger, M., *J. Immunol.,* 1951, v67, 385.

6. Peterson, E. A., Sober, H. A., *J. Am. Chem. Soc.,* 1956, v78, 751.

7. Sober, H. A., Gutter, F. J., Wyckoff, M. M., Peterson, E. A., *ibid.,* 1956, v78, 756.

8. Fahey, J. L., McCoy, P. F., Goulian, M., *J. Clin. Invest.,* 1958, v37, 272.

9. Sober, H. A., Peterson, E. A., *Fed. Proc.,* 1958, v17, 1122.

10. Osler, A. G., Strauss, J. H., Mayer, M. M., *Am. J. Syph.,* 1952, v36, 140.

11. Levine, L., Mayer, M. M., Rapp, H. J., *J. Immunol.,* 1954, v73, 435.

12. Rapp, H. J., Sims, M. R., *Fed. Proc.,* 1958, v17, 531.

13. Levine, L., Mayer, M. M., *J. Immunol.,* 1954, v73, 426.

14. Leon, M. A., Plescia, O. J., Heidelberger, M., *ibid.,* 1956, v76, 28.

15. Ratnoff, O. D., Lepow, I. H., *J. Exp. Med.,* 1957, v106, 327.

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Drug-Induced Pulsus Alternans in Dogs.* (24759)

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Pulsus alternans has been recognized as a symptom of considerable clinical importance since it was first described by Traube(1), but the fundamental mechanisms underlying it are not yet clearly understood(2,3,4). Obscurity of mechanism has resulted from want of a reliable method for its experimental induction in laboratory animals. Poumailloux (5) emphasized that most authors before 1930 were satisfied with clinical description neither dependent on nor indicative of any causal relationship. With few exceptions later studies have been limited to observations on occasional patients in whom the condition was discovered at examination. The present paper presents a simple pharmacological method for inducing pulsus alternans in dogs.

Methods. Twenty-five mongrel dogs in apparent good health were used. Electrocardiograms were recorded from limb electrodes through Sanborn preamplifier on either Sanborn Polyviso recorder or on Heiland recording galvanometer. With direct cannulation of vascular structures and appropriate Statham transducers, pressure changes were converted into electrical changes for recording. Femoral arterial pressure was used to represent systemic arterial pressure to avoid interfering with carotid sinus mechanisms. Cannulation of the ventricles was accomplished by direct puncture with 16 gauge needle. Flexible connecting tubing of polyethylene permitted measurement for protracted periods without shifting of needle. Positive pressure artificial respiration was provided when spontaneous respiration was inadequate and in open chest

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