glutamic acid(15). In idiopathic hyperlipemics, Furman(16) has shown that lipoproteins with the characteristics of alpha$_1$-lipoproteins can be dissociated by sonic vibration from the low-density beta-lipoprotein fraction separated at D 1.006.

Since there is evidence to suggest that chylomicron cholesterol plays little or no role in atherogenesis(17), further studies are in progress to determine if the presence of clinical atherosclerosis correlates better with "soluble" than with total serum cholesterol.

Summary. The effects of centrifugation at 20,000 × g for 2 hours on lipid distribution of various human sera have been studied. In general, when neutral fat was below 176 mg %, very little, if any, cholesterol or phospholipids rose to the surface. When neutral fat content was markedly elevated, as much as 90% of both cholesterol and neutral fat and 50% of phospholipids underwent flotation. However, subjects with similar degrees of marked hyperlipemia appeared to separate into 2 major groups represented by moderate and marked degrees of flotation of neutral fat, the latter group being composed primarily of subjects with familial hyperlipemia. An inverse correlation was found between whole serum neutral fat content and percentage of total cholesterol remaining in the subnatant serum. Variations from these general patterns and possible clinical interpretations are being studied.

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On the Hemolytic Activity of Mouse Complement.* (26586)

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As judged by the usual hemolytic tests, mouse serum appears to lack complement (C′). By use of a reaction system containing a very low concentration of sensitized cells, McGhee(1) demonstrated one unit of hemolytic C′ activity per ml in 7 of 12 mouse sera, and 2 units per ml in the serum of a single animal, but even with his sensitive test system, 4 out of 12 sera had no hemolytic activity. According to Ritz(2), the first component, C′1, is present since EA treated with mouse serum can be hemolyzed by subsequent incubation with guinea pig endpiece. Brown

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(3) claimed that C'3 and C'4 are also present, and that only C'2 is missing. Rice and Crowson(4) did not find C'2, C'3 or C'4 in tests with R2, R3, or R4 made from guinea pig serum; Ritz's demonstration of C'1 was confirmed. The finding by Marcus *et al.*(5) that mouse serum lacks bactericidal activity against certain Gram-negative bacteria was confirmed by Muschel and Muto(6), who showed, in addition, by bactericidal as well as hemolytic tests that C'2 and C'3 appear to be absent. Thus, there is agreement as to presence of C'1 and absence of C'2, but with regard to C'3 and C'4 results are contradictory.

The inadequacy of R1, R2, R3 and R4 as reagents for detection and measurement of the C' components has been pointed out(7) and, furthermore, attention has been drawn to the presence of inhibitory factors in fresh blood serum which may mask the cytotoxic activity of the C' system unless their interference is circumvented by appropriate design of the test system. Through detailed studies of the reaction mechanism of guinea pig as well as human C', improved analytical methods for detection and measurement of the C' components have been developed by use of the intermediates as substrates(7). We have initiated a reexamination of mouse C' by these new technical procedures, because mice are used widely in studies of the immunologic aspects of tissue transplantation, tumor rejection and resistance to microbial infection. The early results of this reinvestigation, presented herewith, indicate that all of the C' factors are present in mouse serum.

Materials and methods. Sheep erythrocytes (E), rabbit antibody (A) to boiled sheep erythrocyte stromata, and the diluent, veronal-buffered saline containing 0.00015 *M* Ca^{++} and 0.0005 *M* Mg^{++}, were prepared as described in(7). Technics for preparation of the intermediate EAC'1,4 and for separation of g.p. C'2 in a state of functional purity are given in(7,8). EAC'4(9) was made by treatment of 1.0 ml of EAC'1,4 (10^9 E/ml) with 0.11 ml of 0.1 *M* EDTA (ethylenediaminetetraacetate, pH 7.7) at 37°C for about 5 minutes. The cells were washed twice with cold diluent followed by suspension in sufficient diluent to make a cell concentration of 1.5×10^8 per ml. They may be stored at 0°C for about one week without loss of $SA_2C'4$ sites, but it is necessary to wash the cells and adjust their concentration just prior to use for detection and measurement of C'1. The theoretical principles and technical procedures for titration of C' components on molecular basis by use of the intermediates as substrates are described in(7,8,10). Reaction temperatures were maintained by use of water baths controlled within $\pm$ 0.02°C.

Collection of mouse serum. The jugular veins of adult white CFW mice were incised and the animals exsanguinated into a large Petri dish resting on crushed ice. Blood from about 45 mice was pooled. Clotting was rapid, and immediately after bleeding the last animal of the group, the blood was transferred to plastic tubes and centrifuged at 8.000 RCF for 30 minutes at 2-4°C. The serum was removed by pipette, centrifuged again for 15 minutes at 2-4°C at 1,000 RCF, distributed in test tubes, 0.5 ml per tube, and stored at −50°C.

Serum obtained in this fashion contained 2 to 3 times more C'1 than mouse serum from pooled blood allowed to clot at room temperature. The loss of C'1 on clotting at room temperature is probably due to fixation by isoantibody-isoantigen interaction. This difficulty could be avoided by pooling *after* careful separation of each individual serum from cells, but this is technically cumbersome and therefore we adopted the practice of immediate chilling and prompt centrifugation. Clotting appeared to be complete since the serum collected as outlined above showed no clot formation on incubation at 37°C.

Experimental and results. C'1. Becker (9) showed that EAC'4 does not react with C'2 to form EAC'1,4,2, unless treated with C'1. Thus, EAC'4 can be used for measurement of C'1. It should be noted that the EAC'4 cells used in the present experiments carry SA_2 as well as $SA_2C'4$ sites. Hence, on reaction with C'1, both $SA_2C'1$ and $SA_2C'1,4$ sites may be formed, but only the latter will react with C'2 to yield $SA_2C'1,4,2$. This re-

sults in waste of some C'1, the extent of loss depending on the ratio of SA_2 to $SA_2C'4$. Analyses for C'1 made with different preparations of EAC'4 will be comparable only if this ratio is kept constant.

Experiment MC020161. Ten ml portions of EAC'4 (1.5×10^8 E/ml) were mixed with equal volumes of whole mouse serum diluted 1/50,000, 1/100,000, 1/200,000, 1/400,000, and the mixtures were incubated at 37.0°C with gentle mechanical agitation. One ml samples, removed at suitable time intervals, were delivered into and mixed promptly with 5.0 ml ice-cold diluent to stop the reaction. The diluted samples were centrifuged immediately at 4°C, the clear supernatant fluids were removed as completely as possible by decantation and thorough drainage, and the cells were suspended in 1.0 ml of purified g.p. C'2 diluted to supply about 200 effective molecules of C'2 per cell. The reaction mixtures were incubated at 30.0°C for about 30 minutes, which suffices for complete conversion of $SA_2C'1,4$ to $SA_2C'1,4,2$, then 1.5 ml of a 1/37.5 dilution of g.p. C' in 0.005 *M* EDTA buffer (C'-EDTA) were added. After 90 minutes' incubation at 37.0°C for conversion of $SA_2C'1,4,2$ to S*, followed by lysis of all cells with at least one S*, 5.0 ml of 0.15 *M* NaCl were added with mixing, the tubes were centrifuged to sediment the cells and hemoglobin concentration of the supernatant fluids was measured photometrically at 412 mμ to determine degree of hemolysis. Results were corrected by subtraction of the appropriate controls which included: EAC'4 + diluent, EAC'4 + 1/50,000 mouse C', EAC'4 + 1/50,000 mouse C' + C'2, EAC'4 + 1/50,000 mouse C' + C'-EDTA. The control flask containing EAC'4 + diluent showed less than 1% lysis at the end of experiment, and none of the other controls exceeded this value. From the results shown in Fig. 1 it is evident that mouse serum is a potent source of C'1. The C'1 activity is quantitatively comparable to that of guinea pig serum which has been estimated to contain 9×10^{12} effective molecules of C'1 per ml(10). The data in Fig. 1 show the kinetics of reaction in terms of $y_{1,4} \rightarrow$ which denotes the fraction of cells in the state EAC'1,4 or further in the sequence of successive reactions which comprise the C' system. Thus, the symbol EAC'1,4 $\rightarrow$ refers to cells with at least one $SA_2C'1,4$ site, or at least one $SA_2C'1,4,2$, or at least one S*, or a combination of these. *C'2. (Experiment MC021361)* Six ml portions of EAC'1,4 (1.5×10^8 E/ml) were mixed with equal volumes of whole mouse serum diluted 1/1,000, 1/2,000, 1/4,000 and 1/8,000. Reaction mixtures were incubated at 30.0°C with continuous gentle agitation. At appropriate time intervals 1.0 ml samples were removed, mixed with 1.5 ml of g.p. C'-EDTA and incubated for 90 minutes at 37°C, followed by measurement of degree of lysis. Results were corrected by subtraction of appropriate controls which included: EAC'1,4 + diluent, EAC'1,4 + mouse C' 1/1,000, EAC'1,4 + C'-EDTA. None of the controls had more than 1% lysis. The corrected data are shown in Fig. 2 as a plot of $y_{1,4,2} \rightarrow$, the proportion of cells in the state EAC'1,4,2 $\rightarrow$, as a function of time of incubation at 30.0°C following addition of the mouse serum dilutions to EAC'1,4. For comparison, 2 similar experiments with guinea pig serum are included, one showing reaction of EAC'1,4 with a 1/2,000 dilution of purified C'2, the other depicting reaction of EAC'1,4 with a 1/12,000 dilution of whole g.p. C'.

These results indicate, of course, that mouse serum contains C'2, though less than guinea pig serum. The precise quantity cannot be estimated because of several complications, notably the difference in decay behavior between $EAC'1^{g.p.}4^{g.p.}2^{g.p.}$ and $EAC'1^{g.p.}4^{g.p.}2^{m}$. Fig. 2 shows that the former decays in a typical fade-away fashion, *i.e.*, closely approximating first-order kinetics with respect to loss of $SA_2C'1,4,2$ sites. The latter, on the other hand, shows no decay at all at the 1/8,000 mouse C' dilution and only partial decay with the lower dilutions. These matters will be considered in the discussion. *C'4. (Experiment MC020261)* This component was demonstrated in terms of EAC'1, 4,2 $\rightarrow$ formation on treatment of EA with mouse C' as follows: Flask A was charged with 6.0 ml EA (1.5×10^8 cells/ml) and 6.0 ml of mouse C' 1/750; Flask B received 15.0

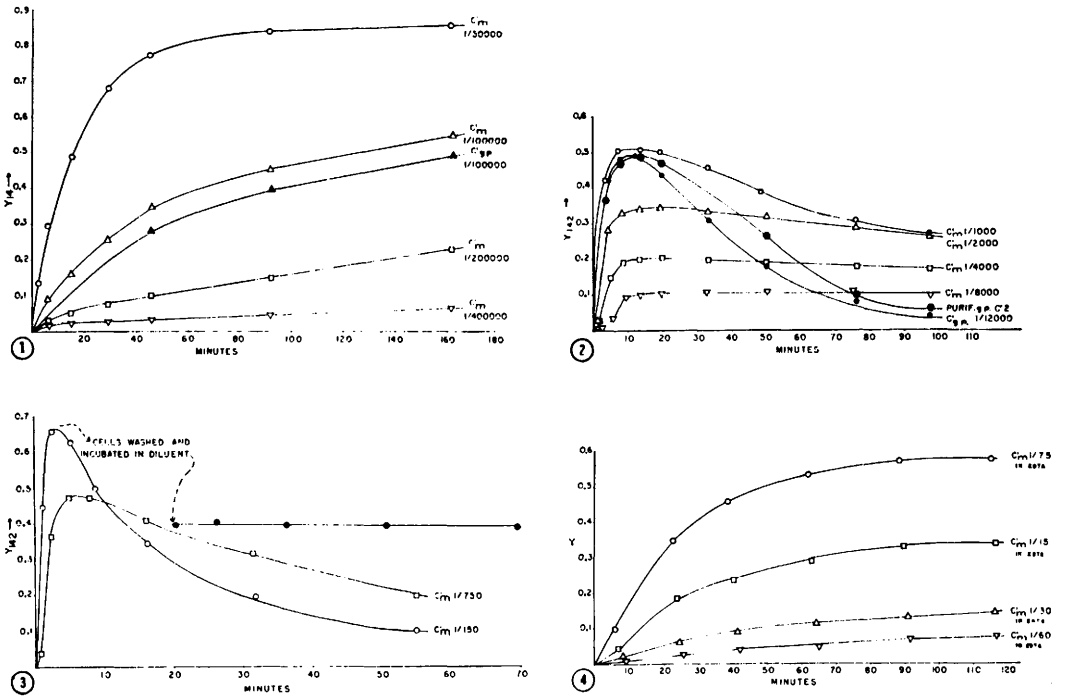

FIG. 1. Kinetics of reaction of g.p. EAC'4 with whole mouse or g.p. serum at 37.0°C.

FIG. 2. Kinetics of reaction of g.p. EAC'1,4 with mouse or g.p. serum at 30.0°C. The purified g.p. C'2 furnished 1.35 effective molecules of C'2/cell.

FIG. 3. Kinetics of reaction of EA with whole mouse serum at 30.0°C. Solid circles represent a twice washed sample from a mixture of EA and C'm 1/150 which was incubated at 37.0°C and sampled into g.p. C'-EDTA.

FIG. 4. Kinetics of reaction of guinea pig EAC'1,4,2 with mouse C'-EDTA at 37.0°C.

ml of EA (1.5×10^8 cells/ml) and 15.0 ml of mouse C' 1/150 (a larger quantity of reaction mixture was used in Flask B in order to furnish sufficient material for a subsequent step in the experiment, as described below). Reaction mixtures were incubated at 30.0°C with gentle mechanical rocking. Samples of 1.0 ml were collected and mixed promptly with 1.5 ml of g.p. C'-EDTA, followed by incubation at 37.0°C for 90 minutes and photometric determination of degree of lysis in the usual manner. Controls, set up and sampled as in the other experiments, showed less than 1% lysyis at beginning and end of the experiment.

From preliminary experiments it was suspected that mouse serum contains a factor which destroys $SA_2C'1,4,2$ sites. To demonstrate this effect, 10.0 ml of reaction mixture were removed from Flask B at about 4 minutes, the cells were sedimented by centrifugation, washed twice with diluent at room temperature and suspended in 10.0 ml of diluent. This cell suspension was incubated at 37.0°C with agitation and 1.0 ml samples were mixed with 1.5 ml of g.p. C'-EDTA followed by incubation at 37.0°C for 90 minutes and photometric determination of extent of lysis.

The results, shown in Fig. 3, as a plot of $y_{1,4,2} \rightarrow$ *vs.* time, indicate that mouse serum contains C'4, as well as C'1 and C'2. It should be noted that the portion of cells removed from the reaction mixture at 4 minutes did not decay on incubation at 37°C. The interpretation of this effect will be taken up in the discussion. *C'3. (Experiment MC021561).* Initial attempts to demonstrate C'3 were made by incubating washed $EAC'1^m4^m2^m \rightarrow$ (*i.e.*, non-decaying) with a 1/37.5 dilution of mouse serum in 0.01 *M* EDTA-diluent, but these were unsuccessful. Appropriate tests after 9 minutes' incubation at 37.0°C revealed that the cells were not EAC'1,4, nor EAC'1,4,2 nor EAC'4, indicat-

ing destruction of the reactive sites by mouse C′-EDTA.

In view of this, subsequent experiments were performed with EAC′$1^{g.p.}4^{g.p.}2^{g.p.}$ as substrate. Four ml of EAC′$1^{g.p.}4^{g.p.}$ (10^9 cells/ml, t_{max} = 13 min) were treated with 4.0 ml purified g.p. C′2 (furnishing about 300 C′2 per cell) for 13 minutes at 30°C. EAC′1,4,2 prepared in this manner carry approximately 40 to 50 SA_2C′1,4,2 sites per cell. After washing in the cold, the cells were suspended in 0.01 *M* EDTA-diluent to a concentration of 1.5×10^8 cells/ml. Seven ml aliquots of this suspension were mixed with 10.5 ml portions of mouse serum diluted 1/7.5, 1/15, 1/30, 1/60 in 0.01 *M* EDTA-diluent, and incubated with gentle rocking at 37.0°C. Samples of 2.5 ml were pipetted into 5.0 ml portions of ice-cold 0.15 *M* NaCl, followed immediately by centrifugation in the cold and subsequent determination of degree of lysis. As a control, EAC′$1^{g.p.}4^{g.p.}$ were incubated with mouse C′-EDTA, diluted 1/7.5 and sampled in the same manner as the experiment proper. The extent of lysis in these control samples was exactly equal to degree of spontaneous cell lysis, which was less than 1%. The results plotted in Fig. 4, in terms of degree of lysis as a function of time of incubation, indicate presence of C′3 activity in mouse serum, though of much lower potency than that exhibited by g.p. serum. It should be noted that C′3 is defined as a group of several factors which lyse EAC′1,4,2; 3 distinct factors in guinea pig serum have been demonstrated by Rapp(11) and Taylor and Leon have made similar observations with human serum(12). It appears likely that mouse C′3 also comprises more than one factor, since a plot of the extent of lysis of EAC′1,4,2 at 120 minutes (*cf.* Fig. 4) as a function of concentration of mouse C′-EDTA yielded a sigmoidal curve.

Discussion. The present experiments, though crude and lacking quantitative refinement, demonstrate unequivocally that mouse serum contains all of the recognized factors of the C′ system, despite the fact, observed by previous investigators, that over-all hemolytic activity of C′ is practically nil. It is not difficult to find reasons for the poor over-all C′ activity. First, and possibly most important, the C′2 and C′3 activities of mouse serum appear to be significantly lower than in g.p. serum. In addition, C′4 activity is lower, but it is not known whether this plays a significant role. Furthermore, mouse serum contains a factor which destroys SA_2C′1,4,2.

The inability of mouse C′-EDTA to lyse EAC′$1^m4^m2^m$, like the lack of over-all hemolytic activity of mouse C′, probably can be attributed to low multiplicity of SA_2C′1,4,2, low concentration of one or more of the C′3 components and destruction of SA_2C′1,4,2 by a factor in mouse serum. As a result, the probability of S* formation is low. On the other hand, mouse C′-EDTA was able to lyse EAC′$1^{g.p.}4^{g.p.}2^{g.p.}$, presumably because this substrate had a multiplicity of about 40 to 50 SA_2C′1,4,2.

These statements are tentative, subject to revision as more information becomes available on the concentration of each of the C′ factors in mouse serum, on optimal conditions with respect to temperature, concentration of reagents, time of incubation, ionic strength, concentrations of Ca^{++} and Mg^{++}, etc., applicable to each of the mouse C′ factors. Furthermore, there is no information on the efficiency of substitution of mouse C′ components for those of g.p. serum.

One of the most interesting and significant observations in these experiments is that EAC′$1^m4^m2^m \rightarrow$ does not decay on incubation at 37°C. As stated above, the arrow in this symbol indicates that the cells are in the state EAC′1,4,2 or beyond, *i.e.*, somewhere in the C′3 stage. The reason for introducing this new symbol derives from the definition of C′3, namely, a group of factors which convert EAC′1,4,2 to E*. It is obvious that the converse is not true, *i.e.*, cells which are converted to E* by treatment with the C′3 factors can be in the state EAC′1,4,2, or beyond. Thus, the new symbol EAC′1,4,2 $\rightarrow$, denotes cells which are lysable by C′-EDTA.

Müller-Eberhard(13) showed that a glycoprotein from fresh human serum, designated β_{1C}, stabilizes EAC′$1^{g.p.}4^{g.p.}2^{g.p.}$, and a likely interpretation of this observation is the assumption that β_{1C} is part of the group of C′3 factors and that its reaction with EAC′1,

4,2 produces a stable successor. The present observation that EAC'$1^{m}4^{m}2^{m}$, if washed to remove the destructive mouse serum factor, does not decay on incubation at 37°C, might be interpreted along the same lines, *i.e.*, that this intermediate is not EAC'1,4,2 but represents a state analogous to that formed on interaction of EAC'1,4,2 with Müller-Eberhard's β_{1C}. Admittedly, there are other possible interpretations, including the proposition that EAC'$1^{m}4^{m}2^{m}$ is inherently stable in contrast to EAC'$1^{g.p.}4^{g.p.}2^{g.p.}$, but on the basis of present information the hypothesis of stabilization by one of the mouse C'3 factors appears more reasonable.

Summary. By use of appropriate intermediates of the complement system, mouse serum has been shown to contain C'1, C'4, C'2 and the C'3 complex.

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