the review by Lamanna(6) on adhesion phenomenon, he pointed out the necessity for adding unheated normal serum to bring about adhesion reaction. Ross(7) discussing enhancement by normal serum of the cytotoxic action of antiserum on certain cancer cells, referred to normal serum factor as complement.

If this normal chicken serum factor is a component of complement, indications are that it is C′ 1 or associated with C′ 1. It is heat labile and found in water insoluble fraction of serum. Perhaps turkey antibody-antigen complexes cannot activate guinea pig C′ 1(8). However, it is difficult to understand why this labile factor must be added before the heated antibody and antigen combine. If chicken C′ 1, is substituting for guinea pig C′ 1, one would not anticipate the order of addition to be of such importance.

The normal factor also had some characteristics of properdin(9). It was adsorbed on kaolin, was destroyed at 56°C, tolerated 45°C for 2 hours and was found in water insoluble fraction of serum. Pillemer *et al.*(10) state that properdin was not destroyed at zero or −20°C. In contrast, this factor was inactivated after a few days at zero to 4°C and was greatly reduced in titer after several weeks at −20°C. Nor did this substance appear to form a complex with inulin(11). Neither whole serum nor serum diluted 1/10 lost activity after treatment with inulin in the range of 25 to 200 mg/ml of serum.

The N-ch factor appeared to be specific for systems in which turkey and chicken antiserum were used. It did not alter the titer of heated human, bovine, sheep, or in fact, pigeon antiserum nor did it always prevent zoning in all chicken sera.

Studies in progress will attempt to identify this normal component and to investigate a similar relationship in other serum species.

Summary. A factor in normal chicken serum when added to heated or unheated immune turkey serum-antigen complexes caused fixation of guinea pig complement. The factor was labile at 56°, unstable at room or refrigerator temperatures but remained active for several weeks at −20°C. It was found in the water insoluble fraction of serum and could be removed from diluted serum on kaolin.

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Antibody Response to Homografts. I. Technic of Lymphoagglutination and Detection of Lymphoagglutinins upon Spleen Injection.* (25220)

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There is considerable evidence that serum antibody responses of various types occur following transplantation of homologous tissues

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TABLE I. Lymphoagglutinating Activity of Sera from Chickens Injected with Pooled Homologous Splenic Cells.

Immunization			No. of positive agglutinations/No. tested	Lymphoagglutination titer (reciprocal) with lymphocytes from chicken No.: (4-fold dil. of serum)		
Total No. of spleens inj.	Time between inj. (days)	Time between last inj. and bleeding (days)		337	323	305
8	6 (Freunds adjv. added)	6	14/14			
30	43 (Freunds adjv. added)	16	6/8			
14	13-11-13-13 (Freunds adjv. added)	14	13/13	256	16	4
22	0	8, 12	11/11	64	256	64
22	0	8	3/3	16	4	"
22	0	8	2/3	64	0	16
20	8	19	9/10	"	64	64
8	11	14	9/9	"	"	"
8	11	14	13/13	16	4	4
62	8-9-8-8	8	9/9	1024	64	64
1	0	8-15	3/3*			
	Various		11/11			
	Total		103/107			
	Single skin graft	12-16	5/6*			
Controls:	Autografted with skin		0/9	0,0,0	0,0,0	0,0,0
			2/21	2	2	
	Untreated chickens		3/52			
Total controls			5/82			

* Lymphocytes for test from spleen or skin donor.

(1-3). However, there is little evidence that these humoral antibodies are causally connected with rejection of homografts(4-7). To reexamine this problem, we found that chickens injected with homologous splenic cells or given skin homografts regularly produce serum antibodies which will agglutinate the donor's lymphocytes. In demonstrating antibodies against homografted cells, the lymphoagglutinin test described here is thought to offer 2 advantages over hemagglutination and leucoagglutination tests used previously(6,8, 9). First, there is substantial evidence that red cells lack some transplantation antigens (10). Second, the difficulty with non-specific agglutination found in leucoagglutination technics is avoided by using lymphocytes. It has been found that lymphocytes do not agglutinate non-specifically even after repeated centrifugation (350 g, 10 min) and resuspension.

Methods. Immunization. Pooled splenic cells from White Leghorn (WL) chickens were injected into New Hampshire (NH) chickens or vice versa. Both breeds of chickens were from a non-inbred commercial stock. The spleens after removal were decapsulated and cut with scissors, Hank's solution was then added, and cell aggregates were further broken down by aspirations with a syringe. Splenic cells were mixed with Freund's complete adjuvant in roughly equal proportions for subcutaneous injection into 5 animals. All other chickens were injected subcutaneously without the adjuvant. After repeated injections of splenic cells, the birds were bled for sera as indicated on Table I. For tests with single spleen, 3 WL chickens were splenectomized, the spleens were dissociated into separate cell suspensions, and each was injected subcutaneously into 3 different NH chickens. Immunizations with homologous skin were done by a single 3 x 5 cm orthotopic graft. Sera were stored at 4°C, and in a few cases frozen at −14°C. All sera were inactivated by heating at 56°C for 30 minutes. The technic of isolation of lymphocytes from blood of chickens has been reported(11). Briefly, it consists of first incubating heparinized blood for about 30 minutes at 37°C, when large numbers of

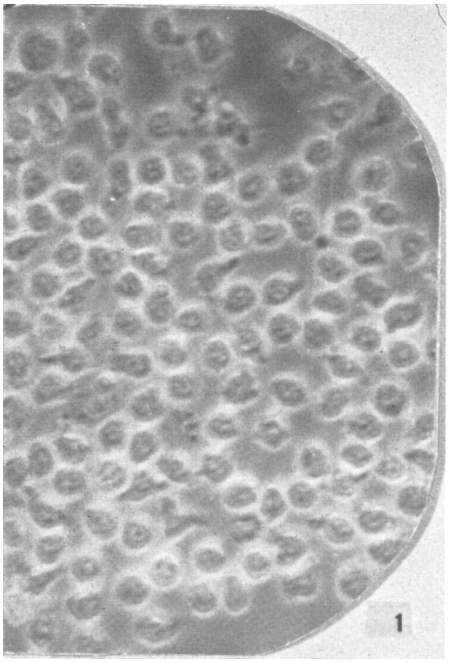

FIG. 1. Lymphocyte suspension. This is a suspension used for the lymphoagglutination test, prepared from blood of chickens by methods described in text. In this field, there are no red blood cells, no polymorphonuclear leucocytes, and 1 or 2 possible monocytes. All the other cells are lymphocytes (99%). (Phase contrast, 1000 ×.)

polymorphonuclear leucocytes and monocytes stick to wall of centrifuge tube. Blood is then centrifuged very lightly at speeds depending on height of blood in centrifuge tube so that red cells are packed to about ¾ of total volume (*e.g.* at 80 g for 10 min for 80 mm height of blood). The upper plasma layer containing lymphocytes is taken off carefully with pipette over thin, lightly packed, buffy coat. This step requires some experience in judging to what extent the pipette can be placed into the buffy coat without getting other white blood cells and red blood cells. The lymphocytes are concentrated by centrifuging at 350 g for 10 min and resuspended in Hank's solution. By these methods, suspensions of lymphocytes containing less than 1% contamination with other white blood cells have been routinely prepared. Red blood cell contamination has been less than 5% of lymphocytes. A photograph of an example of lymphocyte suspension is shown in Fig. 1. Total time required to prepare a suspension was 1-1½ hours. In all the tests, the lymphocytes were used within 3 hours after preparation. Although heparin seems to interfere with leucoagglutination technics(12,13), Bessis(14) found that heparin from certain sources does not cause non-specific agglutination. In our experience with heparin from Upjohn and Darwin laboratories, no non-specific clumps of lymphocytes were found at final concentrations of 10 u/cc to 50 u/cc. *Lymphoagglutination reaction* was carried out in: 1) chambers formed by microscope slide, a rubber gasket 1 mm thick, and coverglass; 2) agglutination slides with concavities 15 mm in dia. by 1.5 or 8 mm deep; or 3) test tubes—⅜″ dia. x 1¼″. To the test tubes and agglutination wells, 0.2 cc of serum dilution was added together with .02 cc of cell suspension (ca. 50,000/cu mm). All dilutions of sera were made with Hank's balanced salt solution. Upon incubation at 37°C for 2 hours, the cells were transferred onto a glass slide inside a wax ring .02-.04 mm in height and covered with coverglass. The wax ring served to prevent agglutinated clumps from being squeezed apart by pressure of coverslip. Agglutination was most easily detectable by using dark field illumination with 10 x objective (Fig. 2). All agglutination was then checked with phase contrast microscope employing a 43× objective, to determine whether clumps were lymphocyte clumps and not red blood cells or cells caught in microscopic pieces of clots or debris.

Results. Sera from chickens immunized by injection of splenic tissue almost invariably react with lymphocytes from other chickens (Table I). Thus 103 of 107 different serum-lymphocyte combinations tested showed positive lymphoagglutination. Substantial antibody titers were observed in most chickens injected with pooled spleens; in one instance a titer of 1:1024 was found. Spleens of one breed of chickens were used to immunize chickens of another breed. Sera of immunized chickens were tested with chickens of the spleen-donor breed and the recipient breed. Lymphoagglutination was obtained in 75 of 76 cases when antisera were tested with lymphocytes of spleen donor breed. However,

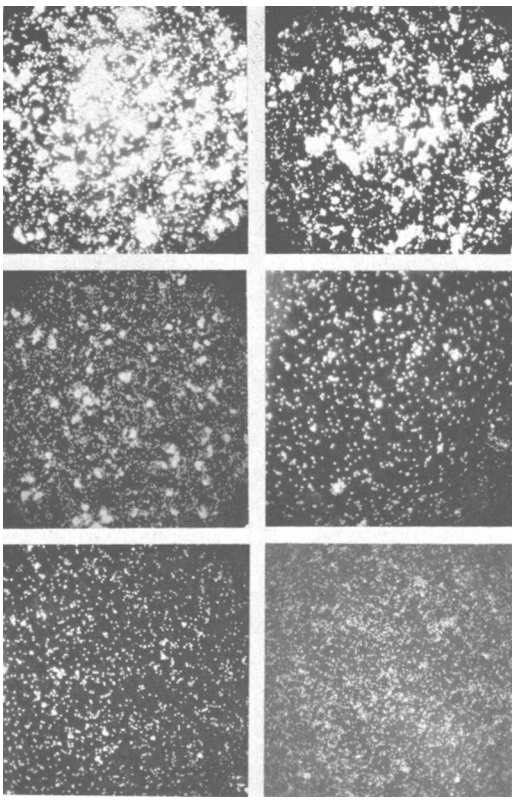

FIG. 2. Different degrees of lymphoagglutination (darkfield, 50 ×). Reading left to right: +4 agglutination, +3, +2, +1, ±, and —. In titration tests titer was taken as dilution of sera producing a +1 intensity of agglutination. About 98% of cells shown are lymphocytes. Occasional oval shaped cells about twice the size of lymphocytes are red blood cells.

even when antisera were tested with lymphocytes of recipient breed, lymphoagglutination was obtained in 28 of 31 cases. In other words, sera from NH chickens immunized with WL spleens reacted in most cases with lymphocytes from NH chickens. Although Freunds adjuvant was used in initial experiments to induce immunity, it was unnecessary for elicitation of lymphoagglutinins. In tests of 52 control sera from untreated birds, no reaction was obtained except in 3 cases. In these 3 instances it seemed that the clumping may have been caused by small clots formed around cells. It is also possible that natural agglutinins were responsible for the positive reaction. Sera from autografted chickens tested 2-3 weeks after grafting with lymphocytes from other chickens produced lymphoagglutination in 2 of 21 instances. In the 2 positive reactions, the titers of sera were 1 : 2.

Lymphoagglutinins were also demonstrated in chickens receiving a spleen or skin graft from one donor. Sera from 3 chickens injected with spleen, reacted in all 3 instances with the lymphocytes from the splenectomized donor. Sera of 5 of 6 chickens which were grafted with skin, also reacted with lymphocytes from the skin donor. Lymphoagglutinins produced after inoculation with splenic tissue appeared to be less specific than those detectable after rejection of skin grafts in that they reacted with 6 of 13 lymphocyte suspensions from chickens other than the spleen donor, while sera from skin-grafted animals reacted with lymphocytes from only 1 of 9 non-specific donors.

Discussion. In attempts to show that serum antibodies are at least in part responsible for rejection of homografts, probably the most direct evidence is that of passive transfer of immunity by serum antibodies. Many such attempts have been unsuccessful(15,16), although there are some recent reports of success(17,18). One of the difficulties in such tests is that large quantities of antibodies may be required to produce a gross visible effect on a graft. Using lymphocytes as target tissue, Woodruff and Forman were able to show that serum from rats either injected with lymph node cells or skin-grafted can cause a lowering of lymphocyte count in peripheral blood(19). Harris, Harris and Farber have recently shown that sera from rabbits immunized with pooled homologous leucocytes suppressed antibody forming capacity of passively transferred lymph node cells(20). It is likely, however, that dissociated cells would be more sensitive to small amounts of antibodies when tested *in vitro*. Hemagglutination tests(6,8) have the disadvantage that red cells are known to lack certain transplantation antigens since they do not evoke either immunity (10) or tolerance(21) to skin homografts. Recently, it has been clearly shown that hemagglutinating antibodies are not responsible for homograft breakdown by Mitchison and Dube(6) and Hildemann and Medawar(8). Leucoagglutinins appear in humans following

blood transfusions(Dausset,22; Payne,23). Following rejection of skin homografts between different strains of mice, leucoagglutinins were detected against peritoneal exudate cells of the skin donor strain(Amos *et al.*, 9). In our preliminary trials to repeat such experiments with chickens, considerable difficulty was experienced in obtaining sufficient cells from peritoneal washings and in spontaneous clumping of cells. Since blood lymphocytes could be resuspended without aggregation even after repeated centrifugations, the lymphoagglutination test was devised. With this technic, as in the work of Amos, *op. cit.*, antibodies were detected in sera of animals which had been homografted with skin. Also, serum antibodies which caused lymphoagglutination appear in 96% of animals either injected with cells from homologous pooled spleens or a single homologous spleen. Sera from chickens of one breed injected with pooled spleens of another breed reacted with lymphocytes from chickens of both donor and recipient breeds. This rather wide specificity of antisera would be expected from our previous work showing that noninbred White Leghorn and New Hampshire chickens are antigenically almost as different among chickens of one breed as they are from chickens of the other breed(24). The sera from animals injected with splenic cells from a single donor also reacted with lymphocytes from chickens other than the spleen donor, thus indicating chickens of breeds tested share a considerable number of antigens. Lymphoagglutinins were present in sera of only 6% of normal chickens and 10% of autografted chickens. Since 4 of the 5 positive tests in these controls were obtained in our early experiments, they may possibly be due to technical error. However, neither natural agglutinins nor technical error can explain agglutination reactions found with 96% of sera from homografted animals. Our results indicate that titers reach a maximum about 3 weeks after grafting(25,26). The occurrence of these antibodies do not, of course, necessarily implicate them as causal agents of the homograft reaction. If it is true that leucocytes and skin cells share transplantation antigens in common(10), it would seem that antibodies reacting upon leucocytes may also react upon skin cells. To what extent these antibodies react on grafted cells *in vivo* and what role this may play in the rejection of homografts is yet to be determined.

Summary. An agglutination method for detecting antibodies against blood lymphocytes is described. By this method non-specific clumping of polymorphonuclear leucocytes and/or monocytes usually encountered in leucoagglutination tests is avoided. The humoral response following homografting in chickens was reinvestigated using this lymphoagglutination reaction. In 82 combinations of normal sera or sera from autografted chickens, and lymphocytes, lymphoagglutination was found in only 5 instances. On the other hand, 103 cases of positive agglutination were obtained in tests with 107 combinations of immune sera and normal lymphocytes. These immune sera, including one with titer of 1:1024, were from animals previously injected with pooled homologous spleens. Sera from animals injected with splenic cells or grafted with skin from one donor, also contained lymphoagglutinins against lymphocytes of spleen or skin donor chicken. These lymphoagglutinins also reacted in some instances with lymphocytes of chickens other than spleen or skin donor.

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Comparative Threonine, Valine, Histidine, and Glucose Oxidation in Chicks.* (25221)

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There are very few reports of comparative studies on relative amounts of different essential amino acids which will be catabolized and oxidized, or synthesized into protein after ingestion by an animal. Mice injected intravenously with 0.7 to 2 mg of labeled amino acids(1) oxidized 28% of injected activity from glycine-1-C^{14}, 45% of that from L-histidine-2-imidazole-C^{14}, 55% of that from L-leucine-1-C^{14}, and 25% of that from L-lysine-1-C^{14} to expired CO_2 within 4 hours. Peak $C^{14}O_2$ production occurred within 30 minutes after injection. Fasted rats fed 25 mg of glycine-1-C^{14}(2) oxidized 50% of activity to CO_2 within 18 hours. Young turkey poults(3) had a maximum $C^{14}O_2$ production within 2 hours after subcutaneous administration of DL-lysine-2-C^{14} with total recovery of 20% of administered activity within 24 hours. Fasted phlorizinized rats given DL-lysine-6-C^{14} orally(4) oxidized 40% of activity to CO_2 in 8 hours. In a comparison of phenylalanine, tyrosine, and tryptophan metabolism(5), tracer doses of uniformly labeled amino acids administered orally to fasted rats showed a maximum $C^{14}O_2$ production during the first hour after administration. Within 8 hours, 45% of the C^{14} in tyrosine had appeared as CO_2, 20% of that from phenylalanine, and 25% of that from tryptophan. Tumorous mice injected intravenously with DL-tyrosine-3-C^{14}(6) oxidized 40% of the activity to CO_2 within 24 hours. Phenylalanine-deficient mice fed meals containing uniformly labeled L-phenylalanine(7) converted a smaller percentage of tracer doses to CO_2 than did normal mice when the meal contained a deficient level of phenylalanine, but a larger percentage when the meal contained a normal level of phenylalanine. This suggests that an animal tends to conserve an amino acid if it is deficient in its diet. This is also shown in the report that rats fed diets rich in methionine(8) oxidized 25% of methyl-C^{14} to CO_2 in 24 hours, but when diets contained a marginal level of methionine, only 6% of methyl-C^{14} appeared in CO_2 in the same time. Peak $C^{14}O_2$ production was reached 2 to 3 hours after oral administration of labeled amino acid in both cases. Since these reports involved various experimental animals, fed or fasted, with amino

* Part of data have been taken from thesis submitted to Graduate Division of Univ. of California in partial fulfillment of requirements for degree of Doctor of Philosophy.

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