A NATURAL ANTIBODY THAT REACTS IN VITRO WITH A SEDIMENTABLE CONSTITUENT OF NORMAL TISSUE CELLS*

I. Demonstration of the Phenomenon

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(Received for publication, July 6, 1942)

While making serological studies of transplanted rabbit cancers by the methods that disclosed a distinctive substance in the Brown-Pearce carcinoma (1), the blood serum of normal rabbits has been found to fix complement when mixed with fresh saline extracts of normal rabbit tissues, and control tests have shown that anticomplementary effects are not responsible for the reaction. The phenomenon appears to be due, as will now be shown, to a natural antibody that reacts *in vitro* with a sedimentable constituent of many normal tissue cells.

Methods and Materials

The natural antibody and the substance with which it reacts were studied by means of a standardized complement fixation test in which 2 units of complement were employed with 2 hours at room temperature for fixation. The procedure was in most respects the same as used in previous studies from this laboratory (1), but differed in the method whereby the test antigens were prepared. Fresh or frozen tissues instead of glycerolated ones were employed for this purpose, and the antigen-extracts were not heated at 56°C. as in the preceding work,—variations in method which proved significant, as will become evident further on.

The *test antigens* were made from normal tissues (liver, kidney, brain, etc.), which had been procured with aseptic precautions; these were used fresh or after preservation in the frozen state (−22°C.) for periods up to several months. They were ground with sand in a mortar, and the ground paste suspended in physiological saline (1:10 to 1:40 or more) and spun for 20 minutes at 4400 R.P.M. in the 51° angle-head International centrifuge. The unheated supernatant liquids, slightly to moderately opalescent but free from gross particles, proved notably effective in the complement fixation tests, especially when used the same day.

Normal sera were procured by bleeding rabbits either from an ear vein or from the heart, allowing the blood to clot in paraffined tubes and subsequently clearing in the

* Preliminary note in the *Proceedings of the Society for Experimental Biology and Medicine*, 1941, **47**, 128.

centrifuge. The specimens were stored in stoppered tubes in the refrigerator at about 4°C. Many of them were tested repeatedly during periods of several months without perceptible change in effectiveness and without becoming anticomplementary. All were heated at 56°C. for 30 minutes immediately prior to use, to inactivate complement.

Complement was got by bleeding 10 to 20 guinea pigs. As soon as the serum could be collected and pooled, it was distributed in small vials and dried from the frozen state in the Flosdorf-Mudd apparatus. Immediately before each experiment, the dried serum was dissolved in physiological saline and titrated with the actual hemolytic system to be employed. Two full units of complement were invariably used.

The *hemolytic system* consisted of rabbit serum containing immune sheep hemolysin in high titer, and thrice washed sheep erythrocytes. The hemolysin was titrated at infrequent intervals; it was diluted so that 0.2 cc. contained 4 hemolytic units. The 5 per cent washed sheep cells were sensitized with an equal volume of the diluted hemolysin, the mixture standing 10 minutes at 37°C. immediately before use in the test.

The serum, complement, and test antigens were mixed so that complement was present when the other two reagents came together. The mixtures, containing 0.2 cc. of each reagent, were allowed to stand 2 hours at room temperature, then 0.4 cc. of sensitized erythrocytes was added. Readings were made after 30 minutes at 37°C. and again after the tubes had stood overnight in the refrigerator. The final readings, which seldom differed significantly from the first ones, are recorded in the published tables in terms of fixation: ++++ = complete fixation (no hemolysis), +++ = about 75 per cent fixation, ++ = about 50 per cent fixation, + = about 25 per cent fixation, 0 = no fixation (complete hemolysis).

Control tests were made in every experiment for *anticomplementary effects*, using double volumes of each dilution of serum and of test antigen. In general the sera were practically never anticomplementary in dilutions of 1:4 or higher and usually not at all or only very slightly so at 1:2, the largest amount utilized. The test antigens occasionally manifested slight or moderate anticomplementary effects in dilutions of 1:10, but practically never in dilutions of 1:20 and never in our experience at 1:40. Neither the sera nor the antigens were anticomplementary in any of the experiments recorded in the present paper.

Incidence and Characteristics of the Naturally Occurring Serum Principle

The results of many experiments have made plain the fact that the blood serum of nearly all normal adult rabbits will regularly fix complement in greater or less degree in mixture with unheated saline extracts of fresh or frozen normal rabbit tissues, whereas the sera of very young rabbits have always failed to do so. Table I gives the results of an experiment that can be considered typical. The sera of 10 normal adult rabbits all fixed complement in mixture with a freshly prepared 1:20 saline extract of normal rabbit liver, some in dilutions as high as 1:32; while specimens from 13 rabbits up to 24 days old of the same hybrid sort, from 7 litters, all failed to react with the test antigen. The positive reactions could not have been due to a summation of

anticomplementary effects; for the sera were not anticomplementary when tested concurrently, even in control tubes containing 0.4 cc. of the 1:2 dilutions; nor was the antigen anticomplementary in control tests containing two

TABLE I

Tests for the Serum Principle in the Blood of Adult and Very Young Rabbits

Source of serum	Rabbit No.	Age	Complement fixation tests					
			Serum dilution					
			1:2	1:4	1:8	1:16	1:32	1:64
		days						
Normal gray-brown domestic rabbits weighing 2 kg. or more	15-84		++++	++++	++++	++++	+++±	0
	15-87		+++±	++++	++++	+++±	+	0
	15-88		++++	++++	++++	++++	++++	±
	15-90		++++	++++	++++	++++	+	0
	15-98		++++	++++	++++	++++	0	0
	15-99		++++	++++	+++±	±	0	0
	16-00		++++	++++	++++	+++	0	0
	16-01		++++	++++	+++±	+±	0	0
	16-02		+++±	++++	++++	++	0	0
	16-03		++++	++++	++++	+++±	0	0
Normal gray-brown domestic rabbits less than 4 wks. old	1A*	24	0	0	0	0	0	0
	2B	18	0	0	0	0	0	0
	3C	19	0	0	0	0	0	0
	4D	20	0	0	0	0	0	0
	5D	"	0	0	0	0	0	0
	6D	"	0	0	0	0	0	0
	7E	"	0	0	0	0	0	0
	8E	"	0	0	0	0	0	0
	9F	16	0	0	0	0	0	0
	10F	"	0	0	0	0	0	0
	11F	"	0	0	0	0	0	0
	12G	"	0	0	0	0	0	0
	13G	"	0	0	0	0	0	0

Antigen, 1:20 saline extract of fresh normal rabbit liver (D.R. 5-73).

2 units of complement in all tubes, as also in all of the tables that follow.

++++ = complete fixation; 0 = no fixation.

None of the sera was anticomplementary when tested concurrently in double volume, nor was the antigen. The same holds true in all of the tables to follow.

* A, B, C, etc. = various litters.

and four times the amount used in the recorded experiment. This holds true also for the experiments that follow.

Like the generality of antibodies, the naturally occurring serum principle can be precipitated with ammonium sulfate (Table II). Yet it is inactivated when sera containing it are heated at 65°C. for 30 minutes (Table III)—a

procedure that has no noteworthy effect upon the generality of antibodies in rabbit blood, as is well known (2).

TABLE II

Precipitation of the Serum Principle with Ammonium Sulfate

Source of serum	Fraction	Serum dilution				
		1:4	1:8	1:16	1:32	1:64
Normal rabbit 16-23	Whole serum	++++	++++	++++	++++	+++
	Globulin fraction*	++++	++++	++++	++++	+++
Normal rabbit 16-28	Whole serum	++++	++++	++++	++++	±
	Globulin fraction*	++++	++++	++++	++++	±

* Precipitated with half saturated ammonium sulfate and dialyzed against 0.9 per cent NaCl.

Antigen, 1:40 saline extract of frozen normal rabbit liver (D.R. 3-72).

TABLE III

Incidence, Titer, and Heat Lability of the Serum Principle

Normal rabbit sera	Complement fixation tests									
	Sera heated at 56°C. for 30 min.					Sera heated at 65°C. for 30 min.				
	Serum dilution					Serum dilution				
	1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32
D.R. 5-65	++++	++++	++++	++++	++++	0	0	0	0	0
5-69	++++	++++	++++	++++	++++	0	0	0	0	0
5-70	++++	++++	++++	++++	++++	0	0	0	0	0
5-67	+++±	++++	++++	++++	++++	0	0	0	0	0
5-75	++++	++++	++++	++++	++++	0	0	0	0	0
5-71	+++	++++	++++	++++	+++±	0	0	0	0	0
5-74	++++	++++	++++	+++±	±	0	0	0	0	0
5-73	++++	+++	++±	0	0	0	0	0	0	0
5-68	++++	+++	++	0	0	0	0	0	0	0
5-66	+++	+++	+++	0	0	0	0	0	0	0
5-72	+++	++	+	0	0	0	0	0	0	0
5-76	++±	+±	0	0	0	0	0	0	0	0

Antigen, 1:40 saline extract of frozen normal rabbit liver (D.R. 3-72).

D.R. = domestic rabbit.

Since the fact has long been recognized that other naturally occurring serum principles, generally termed natural antibodies, are absent from the blood of newly born individuals and that they are more labile to heat than induced ones (3), it seemed possible that the serum principle now under study might also prove to be a natural antibody. Hence it was compared as to titer and to heat lability with two natural antibodies known to be present in the serum

of normal adult rabbits, *viz.*, natural sheep hemolysin and natural Wassermann reagin.

The sera of 6 adult gray-brown domestic rabbits were employed, which were known from the experiment of Table III to contain various amounts of the serum principle under study. Specimens were heated at 56°C. and 65°C. and tested in various dilutions for capacity to hemolyze 5 per cent washed sheep cells in the presence of 4 units of complement (natural sheep hemolysin), for capacity to fix complement in mixture with a 1 per cent cholesterolized Wassermann antigen[1] (natural Wassermann reagin), and for capacity to fix complement in mixture with a 1:20 saline extract of frozen normal rabbit liver (serum principle under study).

Table IV shows the results of the comparative tests. It will be seen that in general the titers of the three serum constituents varied together. The sera of rabbits 5-69, 5-75, and 5-65, for example, reacted notably well in all three tests, while the specimens of 5-72 and 5-76 did poorly. Serum 5-70 provides an exception in that it had little natural power to hemolyze sheep cells but much to react with the Wassermann and normal rabbit tissue antigens. (For other such exceptions see Table V further on.) Heating the sera at 65°C. rendered them completely ineffective, or nearly so, in all of the tests. It will be noted, however, that the serum specimen of rabbit 5-65 was exceptional in that it still retained a small proportion of its capacity to hemolyze sheep cells, and so too in slighter degree did those of rabbits 5-76 and 5-75.[2]

An experiment was next undertaken to find whether serum principles of the three types can be selectively absorbed.

Sera from 5 normal adult agouti rabbits were employed. 1.0 cc. volumes of each were mixed with 3.0 cc. of 50 per cent washed sheep cells, 3.0 cc. of 1 per cent cholesterolized Wassermann antigen, 3.0 cc. of 1:10 saline extract of normal rabbit liver, and 3.0 cc. of physiological saline, respectively. All of the mixtures were kept 2 hours at 37°C., then put overnight in the refrigerator. The ones containing sheep cells were next spun at low speed in the ordinary centrifuge and the others at 25,000 R.P.M. for 1 hour in the air-driven centrifuge—an amount of centrifugation that will not perceptibly throw down other rabbit antibodies, as has been repeatedly observed (4).

The absorbed and unabsorbed supernatant liquids were then tested as in the preceding experiment. Table V shows the results. Absorption with the sheep erythrocytes had removed completely the capacity of all of the sera to hemolyze sheep cells without affecting their ability to fix complement in mixture with the Wassermann

[1] Supplied by the Diagnostic Laboratories of the New York City Board of Health, through the generosity of Dr. G. I. Steffen.

[2] The exceptions are important as indicating the fact that 65°C. for 20 to 30 minutes does not always completely inactivate the natural antibodies of normal rabbit's blood. On several occasions sera with unusually high titers of the natural tissue antibody have retained slight activity when thus heated.

TABLE IV

Titer and Heat Lability of Various Natural Antibodies in Normal Rabbit Serum

Normal rabbit sera		Tests for																	
		Natural sheep hemolysin*						Natural Wassermann reagin‡						Serum principle under study‡					
Heated (30 min.)	Rabbit No.	Serum dilution						Serum dilution						Serum dilution					
		1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:32	1:64
56°C.	5-69	++++	+++±	+++	++	+	0	++±	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++±
	5-70	+±	+±	+	±	0	0	+++±	+++±	+++±	++++	++++	++++	++++	++++	++++	++++	++++	+++
	5-72	+++	++	+	±	0	0	+±	++±	++	±	0	0	+±	+	±	0	0	0
	5-75	++++	++++	+++	++	+	0	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	0
	5-76	++++	+++	++	+	±	0	++±	+++	+++	++	±	0	++±	++	±	0	0	0
	5-65	++++	++++	++++	++++	+++±	+++	+±	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	+++±
65°C.	5-69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-70	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-75	±	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-76	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5-65	++±	++	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

See text for procedures of the three types of tests.

* Readings in terms of hemolysis.

‡ Readings in terms of fixation.

TABLE V

Selective Absorption of Various Natural Antibodies from Normal Rabbit Sera

Normal rabbit sera	Absorbed with	Tests for: Natural sheep hemolysin* — Serum dilution 1:4	1:8	1:16	1:32	1:64	Natural Wassermann reagin‡ — Serum dilution 1:4	1:8	1:16	1:32	1:64	Serum principle under study‡ — Serum dilution 1:4	1:8	1:16	1:32	1:64
16-00	Nil, saline control	++++	++++	+++	++	±	++++	++++	+++±	±	0	++±	+±	0	0	0
15-88	" " "	++++	++++	++++	++++	+++	++++	++++	++++	+±	0	++++	++++	++++	±	0
15-84	" " "	++++	+++±	+++±	+++	+	++++	++++	+++±	++	0	++++	++++	++++	+	0
5-75	" " "	+++	++	+	+	±	++++	++++	++++	++++	+++	++++	++++	++++	++++	+
5-65	" " "	++++	++++	++++	+++±	+++±	+	+	++±	+++	++	++++	++++	++++	++++	+++±
16-00	Sheep erythrocytes	0	0	0	0	0	++++	++++	++++	+	0	+++±	++±	0	0	0
15-88	" "	0	0	0	0	0	++++	++++	++++	++	0	++++	++++	++++	+	0
15-84	" "	0	0	0	0	0	++++	++++	++++	++±	0	++++	++++	+++±	±	0
5-75	" "	0	0	0	0	0	++++	++++	++++	++++	+++	++++	++++	++++	++++	+
5-65	" "	0	0	0	0	0	+++	+++	++++	++++	+±	++++	++++	++++	++++	+++±
16-00	Wassermann antigen	+++±	+++±	+++	++	±	0	0	0	0	0	+++	++	0	0	0
15-88	" "	++++	++++	++++	+++±	++	0	0	0	0	0	++++	++++	++++	+	0
15-84	" "	++++	+++±	+++±	+++±	+	0	0	0	0	0	++++	++++	+++±	±	0
5-75	" "	+++±	++	+±	±	±	0	0	0	0	0	++++	++++	++++	+++±	±
5-65	" "	++++	++++	++++	++++	+++±	0	0	0	0	0	++++	++++	++++	++++	+++
16-00	Rabbit liver extract	++++	++++	+++	+±	±	++++	++++	+++	0	0	0	0	0	0	0
15-88	" " "	++++	++++	++++	++++	+++	++++	++++	++++	+	0	0	0	0	0	0
15-84	" " "	++++	++++	+++	++	+±	++++	++++	++++	0	0	0	0	0	0	0
5-75	" " "	+++	++	+±	±	±	++++	++++	++++	++++	++±	++±	++	0	0	0
5-65	" " "	++++	++++	++++	++++	+++	++	+++	++±	±	0	++	+	0	0	0

* Readings in terms of hemolysis.

‡ Readings in terms of fixation.

or the normal rabbit tissue substances. Likewise, the Wassermann substance absorbed the natural reagin completely without affecting the other two serum constituents. The extract of normal rabbit liver absorbed almost completely the capacity of the various sera to react with a similar extract, and it reduced their ability to fix complement in mixture with the Wassermann substance, but it had no effect upon the natural hemolysin.

In general the titer of the serum principle under study ran parallel to that of the two natural antibodies with which it was compared, though there were

TABLE VI

Reaction of the Natural Antibody with Saline Extracts of Normal Rabbit Tissues

Source of antigen		Antigen dilution					
Rabbit	Organ	1:20	1:40	1:80	1:160	1:320	1:640
5-69	Kidney	++++	++++	++++	++++	++++	+++
	Liver	++++	++++	++++	++++	+++±	+±
	Lung	++++	++++	++++	+++±	0	0
	Brain	++++	++++	++++	±	0	0
	Spleen	++++	++++	+++	0	0	0
	Heart	++++	++±	0	0	0	0
	Muscle	+±	0	0	0	0	0
5-76	Kidney	++++	++++	++++	++++	++++	++
	Liver	++++	++++	++++	++++	+++±	0
	Lung	++++	++++	+++±	0	0	0
	Brain	++++	++++	+++±	±	0	0
	Spleen	++++	++++	+±	0	0	0
	Heart	+++±	0	0	0	0	0
	Muscle	0	0	0	0	0	0

Antigens, saline extracts of frozen normal tissues as indicated.

Normal rabbit serum D.R. 5-65, 1:12,—known from previous tests to contain the natural tissue antibody (see Table III).

noteworthy exceptions (Tables IV and V). The results of absorption tests, however, proved that the new serum principle has affinities wholly distinct from those of the natural antibodies mentioned (Table V). Since its incidence and heat lability proved similar to those of the so called natural antibodies, and because it possesses a strict affinity for a sedimentable constituent of normal tissue cells (about which more further on), it has been termed the natural tissue antibody. More will be said about it in the discussion, after scrutiny of the tissue substance with which it reacts *in vitro*.

Characteristics of the Tissue Substance with Which the Natural Antibody Reacts

Normal rabbit livers provided the antigens for the preceding complement fixation tests. Will other rabbit organs serve as well? The experiment sum-

marized in Table VI yields an answer to the question. For saline extracts, made as already described from the kidney, liver, lung, brain, spleen, and heart tissues of two rabbits, all reacted with a serum known to contain the natural tissue antibody, their capacity to do so varying from much to little in the order given. Saline extracts of the voluntary muscles had comparatively

TABLE VII

Reaction of the Natural Antibody with Centrifugalized Extracts of Normal Rabbit Tissues

Source of antigen	Portion tested	Centrifugation (60 min.)	Antigen dilution 1:10	1:20	1:40	1:80	1:160	1:320	1:640
		R.P.M.							
Normal rabbit liver—frozen (D.R. 5-73)	Whole extract		++++	++++	++++	++++	++++	++++	++
	Supernatant	7,500	++++	++++	++++	++++	+++±	++	0
		15,000	++++	++++	++	0	0	0	0
		20,000	+	0	0	0	0	0	0
		25,000	0	0	0	0	0	0	0
		30,000	0	0	0	0	0	0	0
	Resuspended sediment	7,500	++++	++++	++++	++++	++++	+++	0
		15,000	++++	++++	++++	++++	++++	++++	++
		20,000	++++	++++	++++	++++	++++	++++	+±
		25,000	++++	++++	++++	++++	++++	++++	+±
		30,000	++++	++++	++++	++++	++++	++++	++
Normal rabbit kidney—fresh (D.R. 4-64)	Whole extract		++++	++++	++++	++++	++++	++++	+++
	Supernatant	7,500	++++	++++	++++	++++	+++±	0	0
		15,000	++++	++++	+++	±	0	0	0
		20,000	+++±	±	0	0	0	0	0
		25,000	±	0	0	0	0	0	0
		30,000	0	0	0	0	0	0	0
	Resuspended sediment	7,500	++++	++++	++++	++++	++++	+++	0
		15,000	++++	++++	++++	++++	++++	++++	0
		20,000	++++	++++	++++	++++	++++	++++	+±
		25,000	++++	++++	++++	++++	++++	++++	+±
		30,000	++++	++++	++++	++++	++++	++++	+±

Antigens extracted in dilute phosphate buffer (approximately 0.05 M, pH 7.3).

Normal rabbit serum D.R. 5-70, 1:8, known to contain the natural tissue antibody (see Table III).

little or no such ability. The results have often been confirmed: saline extracts of the organs just mentioned, obtained from many normal rabbits, invariably fixed complement in mixture with normal rabbit sera known to contain the natural antibody, and their ability to do so was in general much as shown in Table VI. Numerous tests failed to disclose the effective substance in either the erythrocytes or the blood serum of normal rabbits, though it was found in noteworthy amounts in extracts of whole rabbit embryos. Experiments will be reported further on which show that it is also present in extracts of the tissues of alien species.

It seemed important to learn whether the material with which the natural antibody reacts can be thrown down readily in the high-speed centrifuge. For this is a property shared alike by many viruses and by certain constituents of normal and neoplastic tissues (5), including the distinctive substance of the Brown-Pearce tumor (1).

To decide about this, 1:10 extracts were made in dilute phosphate buffer (Sørensen's, approximately 0.005 M, pH 7.3) of the frozen normal liver of rabbit D.R. (domestic rabbit) 5-73 and of the fresh kidney of D.R. 4-64. The extracts were spun briefly to throw down the coarse tissue debris, and the densely opalescent supernatant liquids divided into 7 cc. lots. A 3 cc. portion of each material was saved as such (whole extract), and others were spun in the air-driven centrifuge for 1 hour at speeds

TABLE

Complement Fixation Tests with Sera of (a) Normal and (b) Syphilitic Ra

Source of serum	Rabbit No.	Alcoholic extracts								
		Normal rabbit liver						Normal		
		Antigen dilution						Antig		
		1:25	1:50	1:100	1:200	1:400	1:800	1:25	1:50	1:100
(a) Normal rabbits	13-94	0	0	0	0	0	0	0	0	0
	13-95	0	0	0	0	0	0	0	0	0
	13-92	0	0	0	0	0	0	0	0	0
	13-93	0	0	0	0	0	0	0	0	0
(b) Rabbits with experimental syphilis	3-25	++++	++++	+++±	+++±	±	0	++++	++++	+++
	3-17	++++	+++	+	0	0	0	++++	++++	++

Complement, 2 units in all tubes.
Sera diluted 1:4. Heated 56°C.
The antigens were made from the tissues of a single normal rabbit—D.R. 2-72.

of 7,500, 15,000, 20,000, and 30,000 R.P.M., respectively. The supernatant liquids were poured off and the sedimented materials resuspended in the original volume of dilute buffer. The materials were kept cold during the entire procedure, and the sedimented ones were resuspended carefully and uniformly, according to a procedure already described (1).

The results of tests with the various fractions are set down in Table VII. Both of the whole extracts proved notably effective in mixture with a 1:8 dilution of a serum that was known to contain the natural tissue antibody (D.R. 5-70). The supernatant fluids of the materials spun at 7,500 R.P.M. fixed complement in several dilutions in the tests, but they did so in lesser degree than did the corresponding resuspended sediments. The supernatant fluids of the materials spun twice as fast were much less effective and the resuspended sediments much more so. Practically all of the effective substance was thrown down in the materials spun at 20, 25, and 30 thousand R.P.M., comparatively little or none of it remaining in the supernatant liquids.

From Tables VI and VII it becomes clear that the substance with which the natural tissue antibody reacts is widely distributed in the tissues of normal

rabbits and that it can be thrown down readily in the high-speed centrifuge, comparatively little or none of the effective material remaining in the supernatent liquid when extracts containing it are spun at 25,000 R.P.M. (45,400 *g*) for 1 hour. The implications of the finding will be discussed further on.

Certain of the other properties of the reactive tissue constituent will now be described.

Effect of Alcohol on the Tissue Substance.—Table VIII shows the results of an experiment in which the sera of 4 normal adult rabbits were tested in mixture with alcoholic and saline extracts of normal rabbit liver and heart. The sera of 2 rabbits with experimental syphilis were included for comparison. The alcoholic extracts were made by grinding the fresh tissues as usual and extracting overnight in 5 volumes

III

bbits in Mixture with Alcoholic and Saline Extracts of Normal Rabbit Tissues

rabbit heart			Saline extracts									
			Normal rabbit liver					Normal rabbit heart				
n dilution			Antigen dilution					Antigen dilution				
1:200	1:400	1:800	1:20	1:40	1:80	1:160	1:320	1:20	1:40	1:80	1:160	1:320
0	0	0	++++	++++	++++	+	0	++++	++++	±	0	0
0	0	0	++++	++++	±	0	0	++++	0	0	0	0
0	0	0	++++	++++	+	±	0	++++	±	0	0	0
0	0	0	++++	±	0	0	0	±	0	0	0	0
++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++
++++	±	0	++++	++++	+	0	0	++++	++++	±	0	0

of 95 per cent alcohol. The filtered alcoholic extracts (1:5) were then added slowly to 4 volumes of saline so as to form suspensions of maximum turbidity (1:25), which were used in the tests as such and after further dilution with saline, in comparison with saline extracts made as usual of the same tissues, which had been kept overnight at −22°C.

None of the 4 normal rabbit sera reacted with the alcoholic extracts, though both of the syphilitic rabbit sera did so (Table VIII). As was expected, all of the sera reacted with the saline extracts of the normal tissues. Manifestly, the tissue substance with which the natural antibody reacts is either destroyed by alcohol or does not come away into it.

Effect of Heat.—Table IX shows the results of one of several similar experiments. A 1:10 extract was made in dilute phosphate buffer (pH 7.3) of the frozen normal liver of rabbit 5–69, and likewise a 1:10 extract in 0.9 per cent sodium chloride solution of the frozen normal liver of rabbit 5–73. These were spun at 4400 R.P.M. for 20 minutes and the supernatant liquids, which were opalescent but free from gross particles, were removed. They will be referred to as whole extracts. A portion of each whole extract was spun at 25,000 R.P.M. for an hour and the sediments carefully resuspended

in buffer and saline respectively, to provide suspensions of the active material partially freed from extraneous materials. The two materials were then divided into several lots of 3.0 cc. One lot of each was kept unheated as controls and the rest were heated for 30 minutes in water baths at temperatures of 56, 60, 65, 70, and 75°C., respectively. Heavy precipitates formed in some of the heated materials (notably

TABLE IX

Effect of Heat on the Reactive Constituent of Normal Rabbit Tissues

Source of test antigen	Heating 30 min.	Gross appearance	Complement fixation tests* Antigen dilution 1:10	1:20	1:40	1:80	1:160	1:320	1:640
Whole liver extract in dilute buffer, pH 7.3 (D.R. 5-69)	Unheated	Opalescent	++++	++++	++++	++++	++++	++++	++++
	56°C.	Heavy precipitate	+	++	++	+	0	0	0
	60°	" "	0	0	0	0	0	0	0
	65°	" "	0	0	0	0	0	0	0
	70°	" "	0	0	0	0	0	0	0
	75°	" "	0	0	0	0	0	0	0
Whole liver extract in physiological saline (D.R. 5-73)	Unheated	Opalescent	++++	++++	++++	++++	++++	++++	++++
	56°C.	Heavy precipitate	+	++	++	++	++	±	0
	60°	" "	+	+	+	+	+	±	0
	65°	" "	0	0	0	0	0	0	0
	70°	" "	0	0	0	0	0	0	0
	75°	" "	0	0	0	0	0	0	0
Partially purified‡ liver extract in buffer (D.R. 5-69)	Unheated	Slightly opalescent	++++	++++	++++	++++	++++	++++	++++
	56°C.	" "	++++	++++	++++	++++	++	0	0
	60°	" "	++++	++++	++++	++++	+	0	0
	65°	Opalescent	++++	++++	+++±	++±	0	0	0
	70°	"	++	++++	+++±	+±	0	0	0
	75°	"	++++	++++	+++	+±	0	0	0
Partially purified‡ liver extract in physiological saline (D.R. 5-73)	Unheated	Slightly opalescent	++++	++++	++++	++++	++++	++±±	0
	56°C.	" "	++++	+++	++±	++±	++±	±	0
	60°	" "	++++	++±	++	++	+	0	0
	65°	Moderate precipitate	+±	±	±	0	0	0	0
	70°	Heavy "	+	±	±	0	0	0	0
	75°	" "	+	0	0	0	0	0	0

* Normal rabbit serum D.R. 5-69, 1:8, known to contain the natural tissue antibody in high titer.

‡ Spun down once in the high-speed centrifuge (25,000 R.P.M. for 1 hour) and resuspended in the original volume of fluid.

in the whole extracts—see the table) but these broke up readily and were resuspended before use in the complement fixation tests.

From Table IX it is seen that the whole extracts heated at 56°C. and 60°C. reacted very poorly in the tests and those heated at higher temperatures not at all. The results were different, however, with the partially purified materials. These were not visibly affected by 56°C. and 60°C., though the higher temperatures brought about an increase in opalescence of the ones suspended in dilute buffer and moderate and heavy precipitates in the materials suspended in physiological saline. The partially

purified materials heated at 56°C. were less than half as effective as the corresponding unheated ones, and the higher temperatures resulted in a progressive loss in activity. But this was not completely destroyed even at 75°C. It is noteworthy that the material suspended in the dilute phosphate buffer was more effective to begin with than that suspended in physiological saline.

Other experiments yielded similar results. In one, 37°C. and 45°C. for 30 minutes brought about no perceptible change in the activity of materials derived from normal rabbit liver and kidney that had been washed twice in the high-speed centrifuge and resuspended in dilute phosphate buffer. But 56°C. reduced their effectiveness by more than half, and the higher temperatures reduced it still more, though 75°C. failed to inactivate the materials completely. In still another test, twice washed liver material which had stood 2 weeks in the refrigerator in suspension in dilute buffer proved moderately effective when used unheated but was almost completely devoid of activity after heating for half an hour at 56°C. and at 65°C.

Effects of Various Extractives.—Incidental observations revealed that much of the active material comes away from ground tissues into physiological saline and even more into dilute phosphate buffer (Sørensen's, pH 7.3, approximately 0.005 M). Tyrode's solution, carefully adjusted to pH 7.3 with CO_2, proved poor as an extractive, less than half as much of the active substance being present in Tyrode-extracts as in 0.9 per cent saline-extracts of identical normal tissues.

Inactivation upon Standing.—Standing several days or even overnight in the refrigerator at about 4°C. had a markedly deleterious effect upon saline suspensions containing the active material from kidney or liver. These soon became more opalescent than when fresh, and after a day or two flocculations were visible in them, and the materials were found on test to have lost much or all of their ability to react with sera containing the natural antibody. The deleterious effects were noted also in buffer extracts, but were less marked in these.

Glycerolated and Frozen Tissues as a Source of Antigen.—A comparison of glycerolated and frozen tissues proved that the latter were superior as a source of the tissue substance. The liver, kidney, and testicle tissues from 2 normal rabbits, diced in pieces a few millimeters across and kept in 50 per cent glycerol-Locke's solution for 10 days, yielded less than half as much of the active material as did specimens of the same tissues kept frozen at −22°C.

From the observations just recorded it is plain that the constituent of normal tissues with which the natural antibody reacts is either inactivated by alcohol or does not come away into it; it is destroyed by mild heating (56–70°C. for 30 minutes); its effectiveness diminishes upon standing a few days in the refrigerator; and it is adversely affected by glycerol and by certain salts. Further consideration will be given to its properties in an associated paper.

SUMMARY

The foregoing experiments have shown that complement fixation takes place when the blood serum of normal adult rabbits is mixed with fresh saline ex-

tracts of normal rabbit tissues under controlled conditions. A natural antibody, which reacts *in vitro* with a sedimentable constituent of normal tissue cells, is responsible for the phenomenon.

Further observations on the theme are reported in an associated paper, and the findings as a whole are discussed.

References to both papers are given at the end of the second paper (page 576).