A DISTINCTIVE SUBSTANCE ASSOCIATED WITH THE BROWN-PEARCE RABBIT CARCINOMA

II. Properties of the Substance: Discussion

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A complement-fixing antigen is regularly present in saline extracts of the Brown-Pearce carcinoma, which reacts specifically with the sera of certain rabbits bearing the tumor, as the experiments of Paper I have shown. What are the properties of the active material? How does it compare, in general traits, with other tissue antigens (the Wassermann, Forssman, organ- and species-specific antigens, for example), and especially with the specific complement-fixing antigen present in extracts of the virus-induced papillomas of rabbits (1)? Is it pathogenic? These questions will now receive attention, and afterwards the significance of the findings will be discussed.

Effect of Alcohol on the Complement-Fixing Antigen

It was deemed of importance, as bearing upon the general nature of the complement-fixing antigen of the Brown-Pearce tumor, to learn whether it is effective after alcoholic extraction of the tumor tissue as well as in saline suspensions, for it will be recalled that the Wassermann and Forssman antigens, as also some other tissue haptens (2), are soluble in alcohol.

Table IX shows the results of an experiment in which an emulsion of the residue after alcoholic extraction of the Brown-Pearce tumor tissue was compared as to complement-fixing ability with a saline suspension of the same growth. The latter, it will be seen, was notably effective, fixing complement completely in all of several dilutions in mixture with the specific antisera, while the residue of the alcoholic extract failed to react, as did also the suspension of the alcohol-insoluble fraction of the material. Other tests using alcoholic extracts of dried Brown-Pearce tumors of other rabbits yielded identical results, and it was later determined that the complement-fixing antigen of the Brown-Pearce tumor is rapidly inactivated by alcohol.

The fact that the complement-fixing antigen is not soluble in alcohol

may be taken as an indication that the material is different in nature from many tissue antigens, notably the Wassermann and Forssman antigens and certain other tissue haptens. It is worth mentioning in this relation that the complement-fixing antigen of the Brown-Pearce tumor was not present in the many saline extracts of normal rabbit tissues that have been

TABLE IX

Complement Fixation Tests with Saline and Alcoholic Extracts of the Brown-Pearce Tumor

Extracts			Complement fixation tests				
			Brown-Pearce antisera		Normal sera		Controls (no serum)
Preparation	Centrifugation	Dilution	D.R. 71	D.R. 73	D.R. 1-07	D.R. 1-08	
Saline extract	(*a*) Supernatant (3500 R.P.M.—15 min.)	1:20	++++	++++	0	0	0
		1:40	++++	++++	0	0	0
		1:80	++++	++++	0	0	0
	(*b*) Sediment of (*a*) resuspended	1:20	++++	++++	0	0	0
		1:40	+++±	++++	0	0	0
		1:80	+	++++	0	0	0
Alcoholic extract	(*c*) Supernatant (3500 R.P.M.—15 min.)	1:20	0	0	0	0	0
		1:40	0	0	0	0	0
		1:80	0	0	0	0	0
	(*d*) Sediment of (*c*) resuspended in saline	1:20	0	0	0	0	0
		1:40	0	0	0	0	0
		1:80	0	0	0	0	0
Controls (no antigen)			0	0	0	0	0

2 units of complement in all tubes.

Sera diluted 1:4.

Preparation of antigens: Portions of the frozen muscle tumors of D.R. 4-72 had been extracted respectively in alcohol and saline and centrifugalized. The alcohol was allowed to evaporate off from specimen (*c*) at room temperature, the residue being resuspended in the original volume of saline.

tested (Paper I), differing in this respect also from the tissue antigens just mentioned.

Effect of pH on the Complement-Fixing Antigen

As bearing further on the nature of the serologically active substance present in extracts of the Brown-Pearce tumor, an experiment was made to determine the effect of pH upon it.

Experiment 10.—1:10 saline extracts were made as usual of the omental metastases of the Brown-Pearce tumor of D.R. 69, which had been preserved frozen, and of those

of D.R. 35, which had been kept in glycerol. Portions of each extract were adjusted to pH 2.5, 4.5, 8.5, 10.0, and 11.5, as determined with indicator dyes, by the addition of suitable amounts of HCl or NaOH. The treated extracts, along with control samples of each material (pH 6.6 and 6.0, respectively) were incubated at 37°C. for 1 hour, kept overnight in the refrigerator, and heated at 56°C. for 30 minutes. Immediately before the tests, the pH of all of the specimens was adjusted to about pH 6.8 with NaOH or HCl as required and the volume of each adjusted finally to a dilution of 1:20 by the addition of isotonic saline.

TABLE X

The Effect of pH on the Complement-Fixing Antigen of the Brown-Pearce Tumor

Antigens		Complement fixation tests						
Rabbit No.	pH	Sera of rabbits with Brown-Pearce tumors			Sera of normal rabbits			Controls (no serum)
		71	73	1-79	1-07	1-08	3-05	
69	2.5	0	0	0	0	0	0	0
	4.5	0	0	0	0	0	0	0
	6.6	++++	++++	+++±	0	0	0	0
	8.5	+++±	++++	0	0	0	0	0
	10.0	±	++++	0	0	0	0	0
	11.5	0	±	0	0	0	0	0
35	2.5	0	0	0	0	0	0	0
	4.5	0	0	0	0	0	0	0
	6.0	++++	++++	+++±	0	0	0	±
	8.5	+++±	++++	0	0	0	0	0
	10.0	0	0	0	0	0	0	0
	11.5	0	0	0	0	0	0	0
Controls (no antigen).....		0	0	0	0	0	0	

2 units of complement in all tubes.
Sera diluted 1:4.
Antigens, 1:20 saline extracts of Brown-Pearce tumors from 2 rabbits.

It will be seen from Table X that extracts of the Brown-Pearce tumor that had stood overnight at pH 2.5 and 4.5 had no capacity to fix complement when mixed with the specific antisera; whereas the control portions, which had remained at pH 6.6 and 6.0, respectively, retained their effectiveness. The aliquots made up to pH 8.5 had also remained effective, though in somewhat lesser degree. Of the two materials kept at pH 10.0, one had little capacity to fix complement, the other none. The materials kept at pH 11.5 had no capacity to fix complement in the tests.

It is obvious from the results of this experiment that the complement-fixing antigen derived from the Brown-Pearce tumor is inactivated by

standing in acid or alkaline solution. So too is the antigen derived from the virus papilloma (1). It may be mentioned in passing that the reaction of both materials to treatment with acid and alkali is quite similar to that of many proteins upon such treatment and remarkably like those obtained with purified preparations of tobacco mosaic and other plant virus proteins (3).

Effect of Heat on the Active Substance

As is well known, serologically active substances differ greatly in their reactions to heat, some withstanding much, others little. An experiment was made to note the effect of heat on the complement-fixing antigen of

TABLE XI

The Effect of Heat on the Complement-Fixing Antigen of the Brown-Pearce Tumor

Antigens		Complement fixation tests						
		Immune sera			Normal sera			
Rabbit No.	Temperature (30 min.)	71	73	1-79	1-07	1-08	3-05	Controls (no serum)
	°C.							
69	56	++++	++++	+++	0	0	0	0
	66	0	0	0	0	0	0	0
	76	0	0	0	0	0	0	0
35	56	++++	++++	++++	0	0	0	±
	66	0	0	0	0	0	0	±
	76	0	0	0	0	0	0	±
Controls (no antigen)		0	0	0	0	0	0	

2 units of complement in all tubes.
Sera diluted 1:4.
Antigens, 1:20 saline extracts of Brown-Pearce tumors from 2 rabbits.

the Brown-Pearce tumor. The results are set down in Table XI. Extracts of the Brown-Pearce tumors of 2 rabbits retained their capacity to fix complement in mixture with the specific antisera after they had been heated at 56°C. for 30 minutes, but lost it when heated for the same period at 66° and 76°C.

It would appear from the results of this experiment that the complement-fixing antigen of the Brown-Pearce tumor is inactivated by almost precisely the same amount of heating required to inactivate the complement-fixing antigen of the rabbit papilloma (1). In view of this fact an experiment was undertaken to compare directly the effect of heat on the antigens derived from growths of the two sorts.

Experiment 12.—1:20 saline extracts were made of the Brown-Pearce tumors of D.R. 7-33 and of the virus papillomas of cottontail rabbit 1-30. These were centrifuged 20 minutes at 4400 R.P.M., the slightly opalescent supernatant liquids removed and 4 cc.

TABLE XII

Effect of Heat on the Antigens Derived from the Brown-Pearce Tumor and the Rabbit Papilloma

Heating (30 min.)	Dilution	Brown-Pearce tumor antigen (D.R. 7-33)	Papilloma antigen (W.R. 1-30)
°C.			
Unheated	1:20	++++	++++
	1:40	++++	++++
	1:80	++++	++++
	1:160	++++	++
	1:320	+±	0
56	1:20	++++	++++
	1:40	++++	++++
	1:80	++++	++++
	1:160	+++±	++
	1:320	±	0
60	1:20	++++	++++
	1:40	++++	++++
	1:80	+++±	++++
	1:160	0	++
	1:320	0	0
65	1:20	0	0
	1:40	0	0
	1:80	0	0
	1:160	0	0
	1:320	0	0
70	1:20	0	0
	1:40	0	0
	1:80	0	0
	1:160	0	0
	1:320	0	0

2 units of complement in all tubes.
Brown-Pearce antiserum D.R. 5-04, 1:16 mixed with Brown-Pearce antigen.
Papilloma antiserum D.R. F 4, 1:32 mixed with papilloma antigen.
Antigens, 1:20 saline extracts heated as such then diluted as indicated.

portions sealed in ampoules and submerged for 30 minutes in water baths at temperatures of 56°, 60°, 65°, and 70°C. The heating caused no visible change in the gross appearance of the extracts, which were then tested along with unheated portions in various dilutions for capacity to fix complement. The Brown-Pearce tumor materials

were mixed with the serum of D.R. 5-04, a rabbit in which Brown-Pearce tumors had retrogressed, and the papilloma extracts were tested in mixture with the serum of D.R. 4, a rabbit carrying virus-induced papillomas.

Table XII shows the results of the tests. It can be seen that each antigen retained its ability to fix complement in mixture with its specific antiserum upon heating at 56° and 60°C., but that both lost this capacity when heated at 65° and 70°C.

TABLE XIII

Complement Fixation Tests with Filtrates of Brown-Pearce Tumors

Antigens*		Complement fixation tests				
Rabbit No.	Preparation	Brown-Pearce antisera		Normal sera		Controls (no serum)
		34	4-69	1-07	1-08	
35	Unfiltered	++++	++++	±	±	±
	V filtrate	0	±	0	±	0
	W "	0	±	0	0	0
	S "	±	±	0	0	0
5-27	Unfiltered	+++±	+++	±	±	±
	V filtrate	+++	+++	±	±	0
	W "	±	±	0	0	0
	S "	±	±	0	±	0
Controls (no antigen).....		0	0	0	0	

2 units of complement in each tube.
Sera diluted 1:4.
* 1:20 saline extracts of Brown-Pearce tumors.
V, W, S = Berkefeld V and W, and Seitz, respectively.

Filtration of the Antigen

The specific complement-fixing antigen derived from the virus papilloma is retained by those filters which also hold back the virus (1), differing notably in this respect from the "soluble antigens" extractable from tissues infected with certain other viruses (4). Tests were undertaken to learn whether the same holds true for the antigen of the Brown-Pearce tumor.

In a first experiment, 10 cc. portions of 1:20 saline extracts of the Brown-Pearce carcinomas of 2 animals were passed through Berkefeld V and W candles and single Seitz EK disks, respectively, and the filtrates were tested, along with unfiltered samples, for capacity to fix complement in mixture with specific antisera. The results of the experiment are shown in Table XIII. The Berkefeld W and Seitz filtrates of both materials had practically

no capacity to react with the specific antiserum, nor had the Berkefeld V filtrate of one of the extracts, though the unfiltered materials were effective.

In a second experiment of the same sort an extract of the Brown-Pearce tumor was passed through collodion membranes of various pore diameters.

Experiment 14.—Equal parts of the frozen testicular Brown-Pearce tumors of 2 rabbits (D.R. 4-80 and 5-09) were ground together, made to 1:10 with saline, and left overnight in the refrigerator. The extract was spun at 4400 R.P.M. for 20 minutes and the supernatant fluid centrifuged a second time for 30 minutes. The final supernatant, which was free from gross particles though moderately opalescent, was mixed with an equal volume of sterile broth (pH 7.5). 8 cc. amounts of this mixture were run through collodion membranes of various pore diameters, after 10 cc. of plain broth had been allowed to filter slowly through each.[1] The filtration was done under nitrogen at a pressure of one atmosphere. 1 to 2 minutes was required for filtration through the membranes with large pore diameters, and as much as 2 hours in the case of some of those with smaller pores. The amount of the filtrates varied from 5.2 cc. to 7.3 cc.

Table XIV shows the results of tests with the filtrates that had been passed through the collodion membranes of various pore diameters and with an unfiltered sample of the tumor extract. The serologically active constituent of the extract passed readily through membranes with average pore diameters of 571, 397, and 383 mμ, but not at all in detectable amount through others with pore diameters of 348, 265, 193, 154, and 139 mμ.

In another experiment using extracts prepared from the Brown-Pearce tumors of 2 other rabbits, the complement-fixing antigen passed readily and in large amount through membranes with average pore diameters of 438 and 384 mμ, but not through others with pore sizes of 199, 150, 100, and 50 mμ.

An experiment was next done to compare directly the filtrability of the antigens derived from the Brown-Pearce tumor and the rabbit papilloma.

Experiment 15.—1:10 saline extracts of the Brown-Pearce tumors of 2 domestic rabbits and of the virus papillomas of 2 cottontails were made as usual and centrifuged at 4400 R.P.M. for 20 minutes. The supernatant liquids were spun at 4400 R.P.M. for 30 minutes and the final supernatants allowed to stand 31 days in the refrigerator to allow time for the dissociation of "soluble constituents" if such should be present (6). They were then mixed in equal parts with sterile broth (pH 7.5) and 10 cc. portions of the extracts were then filtered under nitrogen, as before, through various pairs of collodion membranes through which 10 cc. of plain broth had first been passed.

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[1] This filtration experiment and several others were done with membranes and apparatus generously made available by Dr. J. H. Bauer and Dr. T. P. Hughes, of the International Health Division of The Rockefeller Foundation. Dr. Kenneth C. Smithburn calculated the pore diameters of the various membranes at the time of the experiments, using the materials and methods described by Bauer and Hughes (5).

TABLE XIV

Complement Fixation Tests with an Extract of the Brown-Pearce Tumor Filtered through Collodion Membranes of Various Pore Diameters

Filtration of antigen (A.P.D. of membrane)	Dilution of antigen	Brown-Pearce antiserum 71	Normal serum 5-00 A	Antigen controls (no serum)
Unfiltered	1:20	++++	0	0
	1:40	++++	0	0
	1:80	++++	0	0
	1:160	++++	0	0
	1:320	++++	0	0
	1:640	+++	0	0
571 mμ	1:20	++++	0	0
	1:40	++++	0	0
	1:80	++++	0	0
397 mμ	1:20	++++	0	0
	1:40	++++	0	0
	1:80	+++±	0	0
383 mμ	1:20	++++	0	0
	1:40	++++	0	0
	1:80	+++±	0	0
348 mμ	1:20	0	0	0
	1:40	0	0	0
	1:80	0	0	0
265 mμ	1:20	0	0	0
	1:40	0	0	0
	1:80	0	0	0
193 mμ	1:20	0	0	0
	1:40	0	0	0
	1:80	0	0	0
154 mμ	1:20	0	0	0
	1:40	0	0	0
	1:80	0	0	0
139 mμ	1:20	0	0	0
	1:40	0	0	0
	1:80	0	0	0
Serum controls (no antigen).....		0	0	

2 units of complement in all tubes.
Sera diluted 1:8.
Antigen, saline-broth extract of Brown-Pearce tumors from 2 rabbits.

TABLE XV

Comparative Filtrability of the Antigens Derived from the Brown-Pearce Tumor and the Rabbit Papilloma

Filtration (A.P.D. of membrane)	Dilution of antigen	Complement fixation tests	
		Brown-Pearce tumor antigen (D.R. 4-75-5-03)	Papilloma antigen (W.R. 56-1-56)
Not filtered	1:20	++++	++++
	1:40	++++	++++
	1:80	++++	++++
	1:160	+++	++++
	1:320	0	+++
	1:640	0	±
614 mμ	1:20	++++	++++
	1:40	++++	++++
	1:80	++++	++++
471 mμ	1:20	++++	++++
	1:40	++++	++++
	1:80	++++	++++
378 mμ	1:20	0	++++
	1:40	0	++++
	1:80	0	++++
331 mμ	1:20	0	++++
	1:40	0	++++
	1:80	0	+++±
293 mμ	1:20	0	++++
	1:40	0	+++±
	1:80	0	+
255 mμ	1:20	0	++++
	1:40	0	+++
	1:80	0	++
161 mμ	1:20	0	++++
	1:40	0	+++
	1:80	0	±
120 mμ	1:20	0	+±
	1:40	0	±
	1:80	0	0
96 mμ	1:20	0	±
	1:40	0	0
	1:80	0	0

3 units of complement in all tubes.

Brown-Pearce antiserum D.R. 71 (1:16) used in the tests with the Brown-Pearce tumor antigen; papilloma antiserum W.R. 8 (1:16) used in those with the papilloma antigen.

None of the materials was anticomplementary when tested concurrently in double amount.

Table XV shows the results of tests with the filtrates and with samples of the extracts that had not been filtered. Both of the antigens were highly active, the unfiltered specimen of each reacting with its specific antiserum in several dilutions. The Brown-Pearce tumor antigen passed readily through membranes with average pore diameters of 614 and 471 mμ, but not through any of those with smaller pores. The papilloma antigen passed readily through all of the membranes with average pore diameters of 161 mμ and greater, in small amount through the one with pore size of 120, and in very slight amount through the one with a pore diameter of 96 mμ,—results that agree well with those of Schlesinger and Andrewes on the filtrability of the papilloma virus (7).

The results of the filtration experiments leave no doubt that the complement-fixing antigen of the Brown-Pearce tumor is retained by filters and collodion membranes that are known to allow many viruses and presumably all of the "soluble antigens" to pass through. Hence it would appear that this antigen, like the one derived from the virus papilloma, has a comparatively large particle size, much larger it would seem than that of the papilloma virus and perhaps as great as that of some of the larger viruses.

Centrifugation of the Active Material

The following experiment was done to find whether the active material of the Brown-Pearce tumor can be thrown down in the high speed centrifuge as can many of the viruses.

Experiment 16.—A 1:20 saline extract of the Brown-Pearce tumors of D.R. 5-03 was made as usual, put into round bottom tubes with an internal diameter of 12 mm., and spun at 4400 R.P.M. for 30 minutes in the 51° angle head centrifuge. (Comparative tests had previously shown that this amount of centrifugation deposits relatively little of the active material, the supernatant liquid always being practically as effective as the whole extract in the complement fixation tests, and invariably much more so than the turbid sediment resuspended in the original volume of saline.) 14 cc. of the supernatant liquid was put into two lusteroid tubes with internal diameter of 12 mm. and overall length of 7.5 mm., and spun in the air-driven centrifuge at 30,000 R.P.M. for 130 minutes. This deposited a good sized button of transparent, jelly-like material, which was overlain by a clear buff-colored supernatant liquid having a thin, whitish, opaque pellicle of fat on top. The supernatant liquid and the fatty pellicle were carefully removed with a pipette and mixed together, forming an opalescent mixture almost identical in gross appearance with that of the first supernatant. The sedimented button was resuspended evenly in saline, which was added drop by drop at first with kneading and stirring by means of a flamed glass rod. After it had been made up to the original volume the resuspended sediment formed a moderately opalescent, pale bluish suspension, without visible particles. A part of this was saved as such for use in the test, and the remainder spun at 4400 R.P.M. for 30 minutes in round bottom tubes as

before. This threw down a considerable amount of rather opaque yellowish material, the supernatant liquid remaining quite opalescent. The latter was removed and spun again in a lusteroid tube at 30,000 R.P.M. for 100 minutes. A transparent button of

TABLE XVI

Complement Fixation Tests with a Centrifugalized Extract of the Brown-Pearce Tumor

Extract of Brown-Pearce tumor*			Brown-Pearce antiserum 71	Normal rabbit serum 1-07	Controls (no serum)
Centrifugation	Material used	Dilution			
(*a*) 4400 R.P.M., 30 min.	Supernatant	1:20	++++	0	0
		1:40	++++	0	0
		1:80	++++	0	0
		1:160	++++	0	0
		1:320	+++±	0	0
(*b*) First high speed centrifugation (portion of supernatant of (*a*) centrifuged at 30,000 R.P.M. for 130 min.)	Sediment resuspended	1:20	++++	0	0
		1:40	++++	0	0
		1:80	++++	0	0
		1:160	++++	0	0
		1:320	++	0	0
	Supernatant	1:20	0	0	0
		1:40	0	0	0
		1:80	0	0	0
		1:160	0	0	0
		1:320	0	0	0
(*c*) Second high speed centrifugation (portion of the resuspended sediment of (*b*) centrifuged again at 30,000 R.P.M. for 100 min. after intermediate low speed centrifugation)	Sediment resuspended	1:20	++++	0	0
		1:40	++++	0	0
		1:80	++++	0	0
		1:160	++++	0	0
		1:320	+	0	0
	Supernatant	1:20	0	0	0
		1:40	0	0	0
		1:80	0	0	0
		1:160	0	0	0
		1:320	0	0	0
Controls (no antigen)			0	0	

2 units of complement in all tubes.
Sera diluted 1:4.
* Muscle tumors of D.R. 5-03.

jelly-like material was deposited as before, the supernatant liquid now being water-clear and colorless. This latter was removed with a pipette and the sedimented button was resuspended in the original volume of saline, a moderately opalescent suspension resulting. Sulfosalicylic acid tests showed that the supernatant liquid procured after

the first high speed centrifugation contained much protein, as manifested by the heavy precipitate forming upon addition of the reagent, and about as much as was present in the whole extract. Precipitates formed also upon addition of the sulfosalicylic acid reagent to the suspensions of the buttons that had been thrown down by the high speed centrifugations, but these were much less dense. Samples of the various materials were tested in several dilutions for capacity to fix complement in mixture with a specific antiserum.

As Table XVI shows, the supernatant liquid obtained after centrifugation of an extract of the Brown-Pearce tumor at 4400 R.P.M. for 30 minutes fixed complement specifically in all of several dilutions when mixed with a Brown-Pearce antiserum, and so too did the sedimented material resuspended in saline after centrifugation at 30,000 R.P.M. for 130 minutes. The supernatant liquid taken off after the high speed centrifugation had no capacity to fix complement. A suspension of the sedimented material after a second low and high speed centrifugation also fixed complement, its capacity to do so being practically as great as that of the suspension of the sediment after the first high speed run. The final supernatant liquid in turn manifested no such capacity.

An experiment was next done to compare directly the sedimentation at different speeds of the antigens derived from the Brown-Pearce tumor and the rabbit papilloma.

Experiment 17.—1:20 saline extracts of the Brown-Pearce tumors of 2 domestic rabbits and of the virus papillomas of 2 cottontails were made as usual, spun at 3000 R.P.M. for 10 minutes, and the supernatant liquids spun again at 4400 R.P.M. for 20 minutes. The supernatant liquids then removed for use were free from visible particles. Their gross appearance was characteristic, that of the Brown-Pearce tumor being buff-colored and moderately opalescent, while the extract of the papillomas was colorless and only slightly opalescent. 7 cc. samples of each extract were then spun for 60 minutes in lusteroid tubes in the air-driven centrifuge at 5000, 10,000, 15,000, and 20,000 R.P.M., respectively. The various supernatant liquids were removed, and the sedimented materials resuspended in the original volume of saline.

Table XVII shows the results of complement fixation tests with the various materials. The whole extracts were notably effective, each fixing complement in all of several dilutions in mixture with its specific antiserum. Centrifugation at 5000 R.P.M. for one hour threw down only moderate amounts of both antigens, the supernatant liquids in each instance being considerably more potent in the tests than the suspensions of the sedimented materials. Centrifugation at 10,000 R.P.M. for 60 minutes deposited more than half of the active materials from each extract, the suspensions of the sedimented materials being somewhat more potent in the tests than the

supernatant liquids. Spinning at 15,000 R.P.M. threw down greater amounts of the antigens, correspondingly less of each remaining in the supernatant liquids. After centrifugation at 20,000 R.P.M. for one hour practically all of the active material of each extract had been thrown down, the supernatant liquids being completely or almost completely inactive in the tests.

A number of other centrifugation experiments, in which extracts of the Brown-Pearce tumors of many animals were used, yielded precisely similar results. The findings as a whole make it evident that the serologically active material of the Brown-Pearce tumor can, like the antigen of the virus papilloma, be thrown down readily in the centrifuge. It is an interesting fact, however, that the Brown-Pearce tumor antigen comes down in the centrifuge under practically the same conditions as the antigen derived from the virus papilloma, and certainly no more readily than the latter, though it is retained by collodion membranes with pores much larger than those required to hold back the papilloma antigen. The fact will be commented upon in a later communication.

Considered together the results of the filtration and centrifugation experiments make clear the fact that the serologically active substance of the Brown-Pearce tumor, like that of the virus papilloma, appears to have a large particle size and weight, differing notably in this respect from the generality of "soluble antigens." Furthermore it would seem that the antigen of the Brown-Pearce tumor has a nearly uniform size and weight, since sharply defined and constant end-points were regularly got in the filtration and centrifugation experiments with active material procured from the Brown-Pearce tumors of several animals and prepared in various ways.

Pathogenicity Tests with Extracts of the Brown-Pearce Tumor

A number of tests were made to determine whether extracts of the Brown-Pearce tumor are pathogenic. The details of these will be given briefly.

In a first experiment the glycerolated Brown-Pearce tumors of 4 rabbits were ground together and suspended 1:20 in Tyrode. The suspension was then centrifuged and filtered through a Berkefeld V candle. 15 cc. of the filtrate was injected into a hind leg vein of 4 rabbits with ears that had been tarred twice weekly for 12 weeks. In addition, 0.2 cc. of the filtrate was injected into the skin at two situations on each flank, and 0.2 cc. was rubbed into a scarified area about 3 by 4 cm. on the skin of the abdomen. 0.5 cc. was injected into the testicles of 2 of the rabbits. None of the rabbits developed lesions at the sites of injection at any time and none had fever on the 4th day after injection. The tarred ears of the rabbits were examined at weekly intervals for 15

TABLE XVII

Sedimentation of the Antigens Derived from the Brown-Pearce Tumor and the Rabbit Papilloma Respectively

Centrifugation (60 min.)	Portion tested	Dilution	Brown-Pearce tumor antigen (D.R. 7-36-8-24)	Papilloma antigen (W.R. 1-68-1-70)
r.p.m.				
	Whole extract	1:20	++++	+++
		1:40	++++	+++±
		1:80	++++	++++
		1:160	++++	+++±
		1:320	++±	±
		1:640	0	0
5000	Supernatant	1:20	++++	+++
		1:40	++++	++++
		1:80	++++	++++
		1:160	+++±	+++±
		1:320	+	0
		1:640	0	0
	Resuspended sediment	1:20	++++	++++
		1:40	++++	+++±
		1:80	+++±	0
		1:160	0	0
		1:320	0	0
		1:640	0	0
10,000	Supernatant	1:20	++++	++++
		1:40	++++	++++
		1:80	+++±	+++
		1:160	+	±
		1:320	0	0
		1:640	0	0
	Resuspended sediment	1:20	++++	+++±
		1:40	++++	++++
		1:80	++++	++++
		1:160	+++	+
		1:320	0	0
		1:640	0	0
15,000	Supernatant	1:20	++++	++++
		1:40	+++	±
		1:80	±	0
		1:160	0	0
		1:320	0	0
		1:640	0	0
	Resuspended sediment	1:20	++++	+++
		1:40	++++	++++
		1:80	++++	++++
		1:160	+++±	+
		1:320	+	0
		1:640	0	0

2 units of complement in all tubes.
Brown-Pearce antiserum D.R. 5-04, 1:16 mixed with Brown-Pearce antigen.
Papilloma antiserum D.R. F 4, 1:32 mixed with papilloma antigen.
Antigens, 1:20 saline extracts, diluted as indicated after centrifugation.

TABLE XVII—*Concluded*

Centrifugation (60 min.)	Portion tested	Dilution	Brown-Pearce tumor antigen (D.R. 7-36-8-24)	Papilloma antigen (W.R. 1-68-1-70)
r.p.m.				
20,000	Supernatant	1:20	±	0
		1:40	0	0
		1:80	0	0
		1:160	0	0
		1:320	0	0
		1:640	0	0
	Resuspended sediment	1:20	++++	+++
		1:40	++++	++++
		1:80	++++	++++
		1:160	++++	+++±
		1:320	+	0
		1:640	0	0

weeks. None showed any changes except those referable to the tar, namely, generalized thickening and hyperkeratosis and a few tar papillomas of ordinary sort.

Three other experiments of the sort were done in which crude extracts of the Brown-Pearce tumor or concentrated suspensions of the active material deposited by centrifugation were injected into the testicles, scarified normal skin, and tarred skin of the ears and scrotum of 24 domestic and 5 wild cottontail rabbits. Many of the injected rabbits were kept for periods up to 6 months and examined periodically. None showed any changes referable to the injected materials.

No lesions resulted in any of the experiments from the injection into rabbits of extracts of the Brown-Pearce tumor. It can be concluded that under the circumstances described the extracts were not pathogenic.

DISCUSSION

The foregoing experiments have shown that a serologically active substance of highly specific character is regularly associated with a transplanted tumor of unknown cause (the Brown-Pearce rabbit carcinoma), and that the substance has certain properties which distinguish it from the generality of antigens studied heretofore and which appear to render it similar in type to the antigen of the virus papilloma (1). What implications have the findings? Can anything be said as to the origin and function of the active material? Does this play any special part in the activities of the tumor cells with which it is associated? Several possibilities suggest themselves immediately in relation to these questions.

It might be supposed, as a first possibility, that the serological findings

here recorded result from the presence of an extraneous parasitic agent of one sort or another, which had become associated with the Brown-Pearce tumor more or less by accident, flourishing in the transplanted growths and calling forth immune responses in the animal hosts though producing no perceptible change in the tumor cells. Extraneous viruses, pleuropneumonia-like microorganisms, and a variety of other parasites are capable of riding along in transplanted tumors in this way, as is well known (8). Virus III or the fibroma, papilloma, herpes, and vaccine viruses might conceivably have infected the Brown-Pearce tumor transplanted in our laboratory. But all of these were found to be unrelated antigenically to the active substance derived from the Brown-Pearce tumor. Furthermore, extracts of the growths containing the specific tumor antigen in large amounts gave rise to no lesions upon injection into rabbits; sections of the tumor showed no visible microorganisms nor any evidence of an extrinsic agent; and repeated cultures of the tumor were all negative, including those made under conditions favorable to the growth of pleuropneumonia-like microorganisms. Two further points may be mentioned which weigh against the possibility that microorganisms of the latter sort are responsible for the findings. The first is that visible flocculation or agglutinations, which might have been encountered had pleuropneumonia-like or larger microorganisms been involved, were never observed in mixtures of the specific antisera and extracts of the Brown-Pearce tumor. And secondly, centrifugation at 5000 R.P.M. for one hour, which should more or less completely sediment microorganisms as large as those mentioned, failed to throw down more than a small fraction of the active substance in extracts of the Brown-Pearce tumor. Nevertheless the presence of a non-pathogenic virus as a contaminant cannot be decisively ruled out as perhaps responsible for the findings.

A second possibility to be considered is that the complement-fixing antigen derived from the Brown-Pearce tumor may be a "normal" tissue component, comparable perhaps to one or another of the antigenic substances known to be present in normal tissues (blood group substances, Wassermann or Forssman antigens, organ- and species-specific tissue antigens, tissue globulins, for example). Some tumor cells undoubtedly contain "individual" tissue antigens (Loeb) that may call forth immune responses upon transplantation which lead to retrogression of the grafts; and some of these antigens are identical with those to be found in the normal tissues of the original tumor host and of animals genetically related to it, as Leo Loeb, Gorer, and others have pointed out (9). Such a material might be expected to elicit an immune response if tumor cells containing it were transplanted

to hosts lacking it, as the blood or tissues of an individual with one type of antigenic make-up may evoke an immune response when injected into another with a different type. But several of the facts already mentioned lead one to doubt that the antigen derived from the Brown-Pearce tumor is a tissue antigen of the sort mentioned. Firstly, the active substance has not been found, though extensively sought, in extracts of the normal or diseased tissues of rabbits. Secondly, its large particle size and weight, as manifested by the results of the filtration and centrifugation experiments, are properties that distinguish the Brown-Pearce tumor antigen from the normal and neoplastic tissue antigens studied heretofore, with the exception of the antigen of the virus papilloma (1). And thirdly, the fact that the antigen of the Brown-Pearce tumor is not soluble in alcohol indicates that it may differ in character from certain of the haptens present in normal tissues (blood group substances, the Wassermann and Forssman antigens, certain organ- and species-specific tissue haptens). On the whole, the facts make it appear unlikely that the serologically active material derived from the Brown-Pearce tumor is merely a component of normal rabbit tissues.

The further possibility must be considered that the results noted in the present study may have been due to "autoimmunization" as a result of the liberation of some constituent of normal rabbit tissue which had become altered as a result of the pathological activities of the tumor cells,—a phenomenon analogous perhaps to that underlying the Wassermann reaction (10), or similar to that of the precipitin reaction in yellow fever (11), or to the immunization against homologous tissues brought about by the injection of autolyzed brain (12). But the specific character and distinctive properties of the complement-fixing antigen of the Brown-Pearce tumor,—notably its absence from extracts of normal tissues, its large particle size and weight, and its inactivation by alcohol,—all render the analogy improbable. They do not suffice to rule it out, however, and it is conceivable that the Brown-Pearce tumor cells might somehow provide—either by the alteration of a normal tissue component or by the synthesis of a new one—a specific substance of large size and weight, which is sufficiently different from the normal constituents of rabbit tissue to account for the immune reactions noted in the present work.

Evidence has been adduced that transplantation leads to immunological differences in the leukemic cells of mice of inbred strain, since individuals rendered resistant to the long transplanted cells may develop spontaneous leukemia or may succumb to leukemic cells transplanted directly from spontaneous cases (13). The findings may merely indicate, however, that

a selection of tumor cells with special capabilities has taken place during the course of the repeated transfers; they provide no evidence that new antigenic potencies may be acquired by tumor cells as a result of long transplantation, and no instance of the sort was found in a search of the literature. Hence no reason exists to assume that the Brown-Pearce tumor antigen may have become manifest during the course of the transplantation of the tumor, but the possibility requires consideration.

A further possibility is that the serologically active substance may actually be responsible for the neoplastic activities of the Brown-Pearce tumor cells. Favoring this possibility is the fact that the antigen of the Brown-Pearce tumor is very similar in some of its general properties to the complement-fixing antigen derived from the virus-induced papillomas of rabbits, which latter in turn is closely associated or identical with the virus responsible for the activities of the papilloma cells. True, extracts of Brown-Pearce tumors containing the antigen in large amounts failed to induce any lesion upon injection into normal or tarred rabbits. But this negative result may merely mean that the extracted material lacked the capacity to induce neoplasia under the conditions of the tests; it does not preclude the possibility that the active substance may be responsible for the neoplastic activities of the tumor cells under the conditions of its natural association with them. The fact may be recalled in this connection that many papillomas and cancers induced experimentally with the Shope virus in domestic rabbits have failed to yield infectious virus on extraction, yet the virus is known to persist in the papillomas in "masked" form, increasing in amount as the growths enlarge (14), and its presence has been demonstrated by serological means in the two cancers that have been successfully transplanted.[2] When viewed as a whole, the evidence does not allow one to say whether the serologically active substance of the Brown-Pearce tumor plays any significant part in the activities of the tumor cells. A full understanding of its significance must await the results of further work.

Can it be supposed that substances of the sort associated with the Brown-

[2] The first cancer to be transplanted could not be carried beyond the second tumor generation (*J. Exp. Med.*, 1936, **64**, 63, 79). A second squamous cell cancer derived from a virus-induced papilloma has now been transplanted in collaboration with Dr. Peyton Rous, and is growing actively in our laboratory after 11 serial transfers during the past 22 months. The continued presence of the virus in the transplanted cancer has been demonstrated by the fact that the serum of every animal with progressively enlarging tumors tested in the 11 generations,—more than two score in all,—has developed specific antibodies capable of neutralizing the papilloma virus *in vitro* and fixing complement in mixture with it.

Pearce tumor are regular constituents of the generality of tumors, each growth having a specific component of its own? The serological studies of tumors made by others heretofore have disclosed no clear cut instances of the sort, though the controlled experiments of Andrewes and of Foulds (15) have recently shown that substances related antigenically to the chicken tumor agents are present in certain fowl tumors elicited with carcinogenic chemicals. Our tests revealed no evidence, or but very little, of the presence of any distinctive substance in extracts of the uterine cancers of rabbits (Paper I). Certain experiments of others with other tumors, however, are suggestive. Barratt has reported the fixation of complement in mixtures of an antigen made from a transplanted mouse cancer with sera of mice bearing the growth (16); and Ewing notes that the complement fixation test was positive in the majority of cases when the blood serum "of over 100 cases of carcinoma and 16 cases of sarcoma" were tested with antigens of the same or similar tumors (17). But many negative results have been obtained in similar experiments, as the literature attests, and often the findings have been irregular (18). Much of the irregularity of the recorded findings may be referable to the heterogeneous character of the materials used; for spontaneous tumors, even those which appear to fall into the same general class, need not all necessarily be of the same intrinsic character. It should be pointed out, furthermore, that the failure to demonstrate a specific, serologically active substance in extracts of a tumor does not necessarily imply that such a material is not associated with the growth. A negative result might mean no more, for example, than does the fact, now well recognized, that many viruses fail to react *per se* as complement-fixing antigens in mixture with their specific antisera. Furthermore many viruses are known which are extractable from tissues diseased by them in amounts too small or in forms unsuited to their disclosure by *in vitro* serological means. On the whole, the serological studies of tumors, though extensive, have not been controlled in such a way as to afford reliable tests for the presence of specific, serologically active substances similar to the one now shown to be associated with the Brown-Pearce carcinoma. Much further investigation of many other tumors will be required to decide whether the presence of a distinctive antigen in the Brown-Pearce tumor is unique or characteristic of tumors generally.

SUMMARY AND COMMENT

The serologically active substance of the Brown-Pearce tumor, a complement-fixing antigen, differs notably from certain other tissue antigens (the Wassermann, Forssman, and organ- and species-specific tissue haptens,

for example) in the fact that it is not effective after alcoholic extraction of the tissue containing it. Like many of the proteins and viruses it is inactivated upon heating to 65°C. for 30 minutes; and, like them as well, its activity is lost upon treatment with acid (to pH 4.5 or lower) or alkali (to pH 11.5 or higher).

Filtration and centrifugation experiments disclosed the fact that the antigen of the Brown-Pearce tumor passed readily through collodion membranes with average pore diameters of 383 mμ and more, but was retained completely by those with average pore diameters of 348 mμ and less. It was thrown down completely or almost completely upon centrifugation at 20,000 R.P.M. for one hour. The findings indicate that the Brown-Pearce tumor antigen has a large and nearly uniform particle size and weight,—as large as that of many of the viruses. They differentiate it sharply from the generality of "soluble antigens."

Upon direct comparison, the complement-fixing antigen of the Brown-Pearce tumor was found to be similar in a number of its general traits to the serologically active substance of the virus-induced papillomas of rabbits, which in turn is intimately associated or identical with the virus responsible for the papillomas. Extracts of the Brown-Pearce tumor containing the serologically active substance in quantity have given rise to no lesions, however, upon injection into normal or tarred rabbits.

The significance of the findings is discussed.

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