THE EFFECT OF OXIDATION OF FILTRATES OF A CHICKEN SARCOMA (CHICKEN TUMOR I—ROUS).

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(Received for publication, June 4, 1928.)

In the course of experiments with the Rous chicken sarcoma, results were obtained which suggested that infectivity of the filtrates was rather quickly destroyed by oxidation. Since the time of Rous' original work on the tumor it has been recognized that saline filtrates become inactive relatively soon at incubator temperatures, and the similar phenomenon in "primary cultures" of the tumor was ascribed by Gye (1) to oxidation in his first paper. Since that time, however, due in part to the work of Baker (2) on the effect of trypsin in Rous sarcoma filtrates, Gye has inclined to the belief that this "auto-inactivation" in saline at 37° was due to proteolytic ferments from the tissue acting on a non-living protein specific factor. He has shown (3) that it may be considerably delayed by HCN in small amounts, which he supposes to be due to a poisoning of the enzyme. Hydrocyanic acid in low dilutions, however, is stated by Oppenheimer (4) to have little deleterious influence on proteolytic enzymes, whereas from the work of Warburg and others it is known to inhibit certain oxidative phenomena almost specifically.

A preliminary experiment showed such striking results when free oxygen was excluded from candle filtrates of the tumor that it seemed worth while to study the phenomenon more carefully. There seemed to be some reason to hope that a partial explanation might be offered for the irregular and discordant results obtained by ourselves and other workers in attempts to repeat the experiments described by Gye (1) in support of his theory of tumor etiology. If, for example, it were possible to show that, like certain bacterial enzymes and toxins (5) an oxidative destruction could be reversed by reducing

agents, the relationship of the phenomenon to Gye's experiments would be evident.

EXPERIMENTAL.

Filtrates of healthy, rapidly growing Rous sarcomas were prepared by grinding the tissue with coarse carborundum in a mortar and suspending the ground mass in saline. The suspensions were centrifuged half an hour, filtered through a thin layer of paper pulp, and finally through a Mandler filter. Such filtrates were perfectly clear, and varied from yellow to pink in color depending on the amount of hemorrhage in the tumor.

Experiment I.

A filtrate prepared as described was divided into two parts. To one was added a solution of cysteine (pH 7.6) to give a final concentration of 1–2000, and after mixing it was covered with melted vaseline (6). A trace of methylene blue added to a similarly prepared test lot was decolorized in 3 or 4 minutes, and remained so for several days at 37°. Nothing was added to the second tube, nor was it sealed by vaseline. Both were incubated at 37°, and small portions removed at intervals for inoculation.

			Chick 1	Chick 2
1. Cysteine Mandler filtrate	$2\frac{1}{2}$ hrs. 37°		++++	+++
2. Plain " "			+++	++
3. Cysteine " "	$9\frac{1}{2}$ " 37°		+++	++
4. Plain " "			++	±
5. Cysteine " "	23 " 37°		+++	+
6. Plain " "			−	−

Experiment II.

The experiment was repeated and amplified by including a parallel test of HCN in a dilution of about 1–10,000.

Experiment II.

	1 hr.		2 hrs.		3 hrs.		5 hrs.		22 hrs.	
Chick....	3	4	5	6	7	8	9	10	11	12
1. Plain filtrate.....	++	+++	++	+	+	−	+	++	−	−
2. Filtrate + HCN.....	++	−	+	−	++	+++	++	++	−	−
3. " + cysteine	+++	+++	+++	++	+++	+++	+++	++++	++++	++++
4. " + HCN + cysteine	+++	+++	+++	+	+++	++++	+++	++++	+++	−

Experiment III.

This experiment was designed to compare the action of serum with that of anaerobic conditions induced by cysteine, and to test, at the same time, the effect of simple sealing with vaseline, and of an inert protein such as gelatin. Twelve tubes of 5.4 cc. of a Mandler filtrate were prepared, additions of rabbit serum, both fresh and heated to 56° for 20 minutes, saline, 3.0 per cent gelatin, and 0.05 per cent cysteine were made as indicated below. They were then incubated at 37° and portions removed at intervals for testing. Injections of 0.5 cc. were made in each case.

	8 hrs.				24 hrs.				70 hrs.				7 days			
Chick.....	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Tube																
1. Fresh rabbit serum 0.6 cc.....	+	+			−	−			†	†			−	−		
2. " " " " " sealed with vaseline.....	?	+			−	−							−	−		
3. Fresh rabbit serum 0.6 cc. + cysteine, sealed.....	+++	+++			+++	+++							−	−		
4. Heated rabbit serum 0.6 cc.....	?	±			−	−							−	−		
5. " " " " " sealed.....	+	+			−	−							−	−		
6. " " " " " + cysteine, sealed.....	++	+++			+++	+++							−	−		
7. Saline 0.6 cc.....			−	−			−	−			−	−			−	−
8. " " " sealed.....			−	−			−	−			−	−			−	−
9. " " " + cysteine, sealed.....			++++	+++			++	+++			++	++++			−	−
10. 3% gelatin 0.6 cc.....			−	−			−	−			−	−			−	−
11. " " " " sealed.....			−	−			−	−			−	−			−	−
12. " " " " + cysteine, sealed...			++++	+++			++	+++			+++	++++			−	−

† Both died in 9 days without tumors, and with no evident reason.

While there are some irregularities in the second experiment it is quite clear that (1) a few hours incubation at 37° will render Mandler filtrates of this tumor non-infectious, (2) that a concentration of 1–2000 of cysteine and protection from the air will defer this loss of infectivity for more than 24 hours and occasionally, at least, much longer, and (3) that cysteine is more effective than a dilution of 1–10,000 HCN, which, as a matter of fact, appears to have a slight deleterious action. Other experiments have shown that HCN in this concentration will regularly preserve infectivity of filtrates for some hours, but we have not seen it persist for as long as 24 hours in any experiment.

The possibility now suggested itself that the extreme variability of infectiousness of candle filtrates of this tumor might be due in part to oxidative changes. Great annoyance is caused by these irregularities which may result in completely inactive filtrates over a considerable period of time, and more frequently in isolated cases. The following experiments were carried out.

Experiment IV.

Tissue from a rapidly growing tumor was minced finely and two lots of 4 cc. each ground in glass mortars with carborundum. One lot was then suspended in 200 cc. of a freshly prepared solution of cysteine hydrochloride 1–2000 in saline brought to pH 7.4 with NaOH. Both portions were centrifuged 15 minutes, the cysteine lot after covering with vaseline. Each lot was then filtered through a layer of paper pulp about 5 mm. thick, and then through Mandler candles. The portion containing cysteine was protected as far as possible from the air by vaseline during both filtrations. Although the paper pulp filtrate still contained enough unoxidized cysteine to decolorize methylene blue, the Mandler filtrate did not. That is to say, protection of the solution containing cysteine had been by no means thorough during the two filtrations. The reagent was gradually oxidized, and by the time the candle filtrate was obtained, it had been completely changed. Probably, however, it had spared to a considerable extent the oxidation of other components of the filtrate. The two Mandler filtrates were then tested.

		Chick 29	Chick 30
Plain	Mandler filtrate	+	+
Cysteine	" "	++++	++++

The filtrate in which oxidation had been partially prevented was strikingly more potent than that made in the usual way. Tumors

began to appear on the 8th day, grew rapidly, and killed in both cases on the 22nd day, while the control tumors appeared only after 14 and 20 days respectively, and were both small.

Experiment V.

The same type of experiment was repeated in such a way as to accentuate the differences due to oxidation. Tumor tissue was finely minced and 2 cc. transferred to each of two large test-tubes. A few glass beads were added to each tube and 50 cc. of saline. One tube was stoppered so as to include about 20 cc. air, while to the second, a fresh solution of cysteine, pH 7.4, was added to a final concentration of 1–2000, then completely filled with melted vaseline, and a stopper inserted in such a way that no air bubbles remained. After the vaseline had solidified, both tubes were shaken for ½ hour, fresh air being admitted to the first tube three times. The saline suspensions were then centrifuged, and filtered through Mandler filters without previous paper pulp filtration. The cysteine lot was protected throughout from the air by liquid paraffin, and a test lot of the Mandler filtrate decolorized methylene blue promptly, showing that unoxidized cysteine remained.

		cc.	Chick 31	Chick 32
Cysteine filtrate,		0.5	++++	+++
"	"	0.25	++++	+++
"	"	0.1	++++	+++
Plain	"	0.5	–	–
"	"	0.25	–	–
"	"	0.1	–	–

In this experiment one cannot be certain to what extent the mechanical effect of shaking may have played a part, since this was obviously greater in the tube containing air. The two experiments taken together indicate clearly that a loss in potency takes place during the preparation of filtrates of this tumor which is due to oxidation and may be largely prevented by adding cysteine and protecting from the air. It is therefore probable that variations in potency of filtrates made in the ordinary way are due, at least in part, to this factor.

Without considerable experience with this method it would be hazardous to assume that active filtrates may be obtained invariably by such means, and obviously in carrying out experiments of many kinds it is highly desirable to know that active filtrates are being used. Filtrates of this tumor which become inert within a few hours at 37°

do so only in the course of a few days in the ice box. If exclusion of oxygen will preserve infectivity for a considerable period at 37° it is reasonable to suppose that it would do so for a much longer time at a low temperature. If this proved to be the case, filtrates could be made and tested, then kept in the cold room preserved with cysteine and vaseline, and used 2 or 3 weeks later when the test inoculations had proved them to be infective. This has been tried with a few filtrates and it appears that tumors develop just as quickly after 3 weeks storage at 3–4° as they do in the case of fresh filtrates. How much longer they may be preserved has not yet been determined.

Reversibility of the Change.

Since Neill (5) has shown that many bacterial enzymes and toxins are readily inactivated by oxidation and in some cases, at least, reactivated by strong reducing substances, it is of importance to determine whether or not the same may be true of the Rous sarcoma agent. If such were the case it might go far toward explaining the results obtained by Gye, and also those of Murphy (7) and of Flu (8). The occasional ability of a "culture" of a tumor or of normal tissue to reactivate Gye's "specific factor" might be due simply to the presence of reducing substances from the tissue, and since this factor appears not to have been considered it is obvious that variations would be expected with such factors as area of surface exposed to air, depth of column, time of exposure, degree to which oxidative destruction of the agent had been carried, etc.

The writer has not been able, thus far, to secure clean-cut results. In experiments designed to show such reversibility indications that it may occur have appeared, but in such a way that other factors appear to be involved. We believe, however, that this possibility must be taken into consideration in carrying out more or less complicated experimental procedures with this virus.

CONCLUSIONS.

1. The rapid autoinactivation at 37° of candle filtrates of the Rous Foul Sarcoma I is due in large part to oxidation.

2. Such oxidation may be prevented by a dilution of 1–2000 cysteine hydrochloride brought to pH 7.4 with NaOH.

3. The variability of infectiousness of candle filtrates is due in part at least to oxidative changes which take place during their preparation; and more active extracts may be obtained by preventing these by means of cysteine.

4. For elaborate experiments it is possible to preserve filtrates in the ice box with cysteine and vaseline until test inoculations have established their infectivity.

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