TABLE I.

Effect of Time and Temperature on Inhibition of Rat Brain Oxygen Consumption by Ethyl Alcohol. See text from experimental details. Oxygen consumption expressed as Q_{O_2} for time periods given.

Temp. °C	Time after addition of alcohol, min.	Conc. of ethyl alcohol—% by volume					
		0	1.90	3.80	5.70	7.60	9.50
37.7	30- 60	11.02	11.74	10.82	9.89	8.24	3.70
	60-120	11.02	11.74	9.68	7.82	3.61	1.39
	120-180	11.02	11.74	8.14	4.58	1.03	1.03
30.0	30- 60	5.25	6.59	5.30	5.46	4.74	4.02
	60-120	5.25	6.08	5.30	5.46	4.17	2.68
	120-180	5.25	5.87	5.30	5.46	3.45	1.91
20.0	30-180 (Constant)	1.96	2.27	1.96	1.85	1.55	1.55

termed the "inhibitor stable" respiration. It has been found to be of approximately equal magnitude in brain slices maintained for over 3 hours without added substrate[4] and in the presence of a number of other inhibitors.[3,5-7]

It may be seen from Table I that low concentrations of alcohol appeared to produce an increase in the rate of oxygen consumption at all 3 temperatures. The effective concentration for this augmentation was about 2%. A similar increase in brain QO_2 with alcohol concentrations of from 1.6 to 3.2% has been reported by Levy and Olszycka.[8] It is possible that this increased rate of oxygen consumption is due to alcohol metabolism by brain tissue.[9]

Summary. The inhibition of oxygen consumption of rat cerebral cortex slices by ethyl alcohol was studied at 37.7°C, 30°C and 20° C. The inhibition becomes progressively greater with time at 37.7°C; such progressive inhibition is less marked at 30°C and is absent at 20°C. The minimum effective inhibitory concentration increases with decrease in temperature. The percentage inhibition with a given concentration of alcohol decreases with decrease in temperature.

[4] Fuhrman, F. A., and Field, J., 2nd., unpublished results.

[5] Fuhrman, F. A., and Field, J., 2nd., *J. Cell. and Comp. Physiol.*, 1942, **19**, 351.

[6] Fuhrman, F. A., and Field, J., 2nd., *J. Pharm. Exp. Therap.*, 1943, **77**, 392.

[7] Fuhrman, F. A., *Abst. of Dissertations, Stanford University*, 1943, **19**, 3.

[8] Levy, J., and Olszycka, L., *Compt. rend. Soc. Biol.*, 1940, **133**, 370.

[9] Himwich, H. E., Nahum, L. H., Rakieten, N., Fazekas, J. F., DuBois, D., and Gildea, E. F., *J.A.M.A.*, 1933, **100**, 651.

16710

Hemagglutination by Botulinal Toxin.

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In the course of studies of the properties of type A botulinal toxin we have observed that the addition of toxin to red cell suspensions results in agglutination. Crystalline and amorphous preparations of toxin have proven to be equally capable of causing hemagglutination. Observations for agglutination have been positive with red cells from all animal species tested (chicken, guinea pig, rabbit, sheep, man.)

TABLE I.
Influence of Temperature on Hemagglutination Titer.

Dilution	0*	2	4	8	16	32	64	128	Control
Sheep cells:									
0°C	+	+	+	sl+	0	0	0	0	0
45°C	0	0	0	0	0	0	0	0	0
Chicken cells:									
0°C	+	+	+	+	sl+	0	0	0	0
45°C	+	+	sl+	sl+	0	0	sl+	0	0

Each tube contained a mixture of 1 ml of diluted toxin and 1 ml of red cells. Saline was used as diluent. There were 131 × 10^6 cells per tube in the case of the sheep cells and 38 × 10^6 cells per tube in the case of the chicken cells. These numbers of cells were chosen so as to give about 3.45 times as many sheep cells as chicken cells which is about the reported difference in surface area of these cells. Agglutination was read by "pattern" test.

* This tube contained about 25,000 mouse MLD, so that as little as 1-3,000 MLD are giving macroscopic evidences of agglutination in the endpoint tubes.

The agglutination can be followed either microscopically or macroscopically. Methods in current use for observation of virus hemagglutination[1-3] are applicable to studies with the toxin. A most interesting difference from the experience with viruses is that the toxin does not appear to be taken up by the clumped red cells under the conditions studied: chicken and sheep cells at 0°C and room temperature; guinea pig cells at 30°C. The suspending menstruum suffers no reduction in toxic titre by mouse titration when agglutinated cells are centrifuged off. The centrifuged red cells themselves show a low (less than 99% of the toxin) and irregular order of toxicity which in separate tests has been reduced or abolished by washing with saline. The toxicity can be as logically attributed to the cells being contaminated with slight quantities of toxin by reason of the residual fluid wetting the cells after centrifugation as to a real adsorption phenomenon.

The addition of formaldehyde to toxin solutions results in diminished toxicity and hemagglutination. Other conditions that reduce the toxic titre such as aging in a dilute solution of salt, also decrease the hemagglutination titre. A corollary observation is that the employment of a peptone-saline mixture as diluent in place of the use of saline alone often gives an increased hemagglutination titre. Such a protective colloid effect is not unexpected in view of the readiness with which purified toxin is biologically inactivated by surface denaturation. These observations make it highly unlikely that a non-toxic fraction can split off from the toxin molecule and cause hemagglutination independent of a toxic fraction which remains behind in solution.

The hemagglutination reaction is specifically prevented by type A antitoxin. Commercially prepared monovalent types A, B, and C horse antitoxins have been tested. The order of adding the reagents namely, toxin, antitoxin and red cells does not affect the inhibition by antitoxin. These observations provide conclusive proof that the hemagglutinating activity of the toxin preparations used was due to the toxin itself rather than to contaminating substances. Because ribonucleic acid is a major foreign material that accompanies the toxin fractions during several stages of purification, separate tests for hemagglutination by purified ribonucleic acid were performed. The ribonucleic acid was isolated from cultures of the same medium and strain of organisms as used for toxin production. Purified yeast nucleic acid was also tested. These nucleic acids did not agglutinate red cells.

The titre of a given mixture of toxin and red cells was found to vary with the temperature. But the influence of temperature was itself conditioned by the animal source of the red cells. Table I illustrates these findings. In attempting to compare red cells from different species, sheep and chicken cells were studied since among the common laboratory

[1] Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

[2] Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.

[3] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE II.
Relative Lack of Dependence of Hemagglutination Titer at a Given Temperature upon Prior Exposure at Another Temperature.

Dilution	0	2	4	8	16	32	64	128	256	512	Control
Temp. of exposure °C											
0	+	+	+	+	+	+	+	+	0	0	0
Room	+	+	sl+	sl+	0	0	0	0			0
39	0	0	0	0	0	0	0	0			0
45	0	0	0	0	0	0	0	0			0
Above tubes reshaken and placed at different temperatures:											
45°C set at 0°C	+	+	+	+	+	+	+	sl+			0
39°C set at room	+	+	sl+	0	0	0	0	0			0
Room set at 39°C	0	0	0	0	0	0	0	0			0
0°C set at 45°C	0	0	0	0	0	0	0	0	0	0	0

Sheep cells used. Agglutination read by "pattern" test.

TABLE III.
Effect of Washing Upon Agglutinated Red Cells.
Chicken cells: 38 × 10^6 per tube.

Dilution			0*	2	4	8	16	32	Control
Temperature of									
First agglutination, °C	Washing, °C	Agglutination after washing, °C	Agglutination results						
10	—	—	+	+	+	+	sl+	0	0
	45	10	+	+	+	+	hemolyzed	hemolyzed	0
45	—	—	+	+	sl+	sl+	0	0	0
	25	45	+	+	+	+	0	0	0
Sheep cells: 131 × 10^6 per tube.									
10	—	—	+	+	+	sl+	0	0	0
	45	10	0	0	0	0	0	0	0
45	—	—	0	0	0	0	0	0	0
	25	10	0	0	0	0	0	0	0
45	kept at 45°		0	0	0	0	0	0	
	then placed at 10°		+	+	sl+	0	0	0	0

* Tube contains 25,000 mouse MLD of crystalline toxin per ml.
Results within each horizontal column are those obtained with a single set of tubes. A total volume of 2 ml was present per tube. Diluent and wash fluid was saline. Washing was done twice by centrifugation of the cells from 5 ml of fresh saline at the indicated temperature.

animals they represent cell types with extensive morphological differences in both structure and size. It was thought to be better to use concentrations of these cells with equivalent surface areas rather than equal numbers since the effects of the toxin are assumed to primarily involve surface phenomena or structures. The sheep cells were more sensitive than the chicken cells to the temperature variable. But with both types of cells the effect of temperature was found to be relatively independent of the previous temperature exposures of red cell-toxin mixtures. Data for 0°C about the same endpoint is recorded sheep cells given in Table II show that at whether the test is conducted initially at 0°C or the cell-toxin mixture is initially held at 45°C and then placed at 0°C. Exposures at the other 3 temperatures listed in Table II show the same characteristic.

An interesting difference between agglutinated sheep and chicken cells was in their behavior when washed free of the suspending toxic fluid (Table III). Once chicken cells have been agglutinated they tend to remain agglutinated irrespective of washing or the temperature of washing. With agglutinated sheep cells the effect of washing was more complex. Washing tended to reduce the persistence of agglutination but was conditioned by the temperature of washing. In one experiment a set of tubes of sheep cells which

TABLE IV.
Inability of Antitoxin to Redisperse Agglutinated Chicken Red Cells.

Dilution of toxin	0*	2	4	8	16	32	64	Control
Treatment								
Set 1: Toxin + 0.2 ml antitoxin for 30 min. at 25°C before adding red cells. Readings overnight at 10°C	+	0	0	0	0	0	0	0
Set 2: Toxin + 0.2 ml saline for 30 min. at 25°C before adding red cells. Reading overnight at 10°C	+	+	+	+	+	+	0	0
Set 2: Reshaken, 0.2 ml antitoxin added. Kept at 25°C 2 hr, then at 10°C overnight for agglutination reading.	+	+	+	+	sl+	0	0	0

Horse antitoxin used was commercially prepared. According to the manufacturer's statement of potency about 3 units of antitoxin would be present in the 0.2 ml added to each tube of the experimental set. Red cells per tube were 40×10^6 Agglutination was read by "pattern" on bottom of tube.
* 100,000 mouse MLD of toxin were present in the first tubes.

had been agglutinated at 0°C when washed at 45°C showed a complete loss of agglutination. A duplicate set washed at 5°C showed persistence of agglutination in the tubes originally containing the greatest amounts of toxin. There was only a one or two tube reduction in endpoint with the 2-fold dilution schedule employed.

Agglutinated sheep cells washed free of toxin at 45°C so that they were no longer agglutinable at 0°C remained capable of being reagglutinated by the addition of fresh toxin.

As already stated antitoxin was found to inhibit hemagglutination irrespective of the order in which the reagents were mixed initially. But it was of some interest to learn whether or not the addition of antitoxin could disperse cells which had been once agglutinated. Accordingly experiments to test this point were performed. The antitoxin proved relatively incapable of dispersing agglutinated chicken red cells. Typical data are given in Table IV. In still other experiments the agglutinated chicken cells remained agglutinated even when washed free of toxin with saline, and then resuspended in a quantity of antitoxin sufficient to inhibit agglutination if the reagents had been added together at the beginning of the trial. Experiments along similar lines with sheep cells were performed but are not recorded since results were irregular and inconclusive.

The observations discussed are preliminary to an effort to explain hemagglutination by the toxin, and to learn whether hemagglutination can be employed as a quantitative measure of toxin. They have been presented for the purpose of bringing the phenomenon into common knowledge. It is not possible at the moment to finally decide whether the agglutination is due to an enzymatic action by the toxin on the red cell, or to some purely physical effect of the toxin solutions that unsettles the balance of cohesive and repulsive forces normally operative at the red cell surface. Nor is it possible to conclude whether one type of mechanism is acting in the case of chicken cells and another for the sheep cells. But an additional explanation of the results exists which is probably not supported by the evidence at hand. Lamanna and Doak[4] have found that normal serum can be precipitated by botulinal toxin. If in the studies performed a contaminating layer of normal serum proteins was present at the cell's surface then the agglutination of the red cells might be more apparent than real. The reaction between toxin and the serum might well explain the results. Objections to the occurrence of this event are as follows: All the chicken and sheep red cells used were washed by centrifugation from saline or a buffer-saline mixture. The amount of residual serum proteins must be minimal. Guinea pig red cells when washed four times by centrifugation from mammalian Ringer's solution remained agglutinable by toxin. The addition of normal serum to a mixture of red cells and toxin did not affect the agglutination titre.

[4] Lamanna, C., and Doak, B. W., *J. Immunol.*, 1948, **59**, 231.

Yet the large excess of serum in these cases might logically be expected to compete for the toxin and thus reduce the hemagglutination titre. If normal serum proteins were in some way "bound" at the red cell's surface antitoxin might be similarly bound. The addition of toxin to a red cell-antitoxin mixture should then be expected to result in agglutination. Actually a mixture of sheep cells and horse antitoxin incubated for an hour before addition of toxin remains unagglutinated. Finally, the presence of serum at the cell surface should show some, if slight, toxin adsorption if such serum were actually responsible for agglutination. An experiment was performed in which 460 millions of sheep red cells were mixed with only 200 mouse MLD of toxin at 10°C. Putnam *et al.*[5] have determined that the number of molecules per LD_{50} is of the order of 2.1×10^7. An MLD would contain less than twice as many molecules.[6] Thus in the mixture used there was only of the order of 18 toxin molecules per cell. The adsorption of only a few molecules per red cell would have resulted in a large percentage adsorption of toxin. Yet when the red cells were centrifuged down and the supernate titrated no significant reduction of toxic titre was found.

Summary. Type A botulinal toxin causes agglutination of chicken, guinea pig, rabbit, sheep and human red cells. The agglutination is not accompanied by evidence of adsorption of the toxin by the red cells. Temperature, particularly in the case of sheep cells, influences the hemagglutination titre. When washed free of toxin, agglutinated chicken cells tend to remain agglutinated while agglutinated sheep cells are more readily dispersed.

[5] Putnam, F. W., Lamanna, C., and Sharp, D. G., *J. Biol. Chem.*, 1946, **165**, 735.

[6] Lamanna, C., McElroy, O. E., and Eklund, H. W., *Science*, 1946, **103**, 613.

16711

Regression and Reabsorption of Mammary Tumors by Extracts of Degenerating Amphibian Skin.

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The chemotherapy of cancer has received and still receives considerable attention. Most work has been directed towards the examination of substances which inhibit growth as contrasted with those which are curative in the sense of bringing about the complete regression of the tumor. The works of Roffo[1] and of Boyland[2] were of especial interest to the writer and led to the experiments here described. Roffo first found that the injection of fresh extracts of striated muscle into rats implanted with a transferable fibro-sarcoma 10-19 days previously were without inhibitory or regressional effects. The same negative findings were recorded when tumorous tissue was tested *in vitro*. However, striking regression and disappearance of tumorous tissue was obtained when applying hydrolysates of striated muscles both *in vivo* and *in vitro*. Boyland was able to demonstrate less striking results on grafted sarcomata and spontaneous carcinomata in mice following oral administrations of acid extracts of ox heart muscle. Pronounced inhibition of tumor growth was obtained but no cases of complete regression.

Reasoning from a general hypothesis that degenerating tissues might, in certain cases, contain a substance or substances detrimental to the growth or persistence of tumorous tissue, experiments were planned involving

[1] Roffo, A. H., *Biol. Inst. Med. Exp.* (Buenos Aires), 1938, **14**, 257.

[2] Boyland, Eric, *Biochem., J.*, 1941, **35**, 1283.