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Measurement of Toxicity of Antiseptics by the Method of Unstained Cell Counts.*

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The method of unstained cell counts is a simple, quantitative, and direct means of determining the capacity of an agent to kill cells in suspension.[1] The method seemed, therefore, suitable for studying the toxicity of antiseptics. The present work consists of the measurement of the cytocidal activity of a few antiseptics.

Method. Suspensions of cells were prepared by mincing rabbit thymus and bone marrow with scissors and filtering through wire gauze as described in detail in a previous paper.[1] To 0.2 ml of the suspensions were added 3.8 ml of an eosin solution (1:1000 in Tyrode's fluid). A drop of the mixture was placed in a hemocytometer and the stained, unstained, and red blood cells were counted. The suspensions were then diluted with Tyrode's solution, when necessary, to give a standard count of approximately 200,000 unstained cells per cubic millimeter.

The following commercial preparations of antiseptics were used: zephiran chloride, aqueous and tincture, stainless, 1:1000; merthiolate, aqueous and tincture, 1:1000; metaphen, aqueous 1:500 and tincture 1:200; and phemerol, aqueous, 1:1000. The following stock solutions were prepared in distilled water: mercuric chloride 1:100, crystal violet 1:1000, phenol 1:20, and sodium sulfathiazole 1:50. All of these antiseptics were diluted with Tyrode's solution.

To 0.2 ml of the cell suspension was added an equal volume of diluted antiseptic so that the final concentrations of the reagents were 1:100, 1:200, 1:500, 1:1000, 1:2000, etc. The mixtures were shaken mechanically in a water bath at 37°C for 30 minutes. After incubation, 3.6 ml of the eosin solution were added and counts of the unstained, stained, and red blood cells were again made. From these results it was possible to estimate the concentration of antiseptic that would kill 90% of the original viable cells.

Observations. Various dilutions of the antiseptics tested were titrated against suspensions derived from rabbit thymus and bone marrow. The predominating nucleated cells in these two suspensions were the lymphocyte and the polymorphonuclear leukocyte respectively. The results are summarized in Table I which shows the concentrations of antiseptic that killed 90% of the unstained cells.

It is seen from this table that the reagents differed considerably in their cytocidal action. Sodium sulfathiazole was the least toxic; a dilution of 1:100 killed only 30 to 40% of the cells of the thymus and bone marrow. Mercuric chloride was the most toxic; a dilution of 1:30,000 sufficed to destroy the viability of 90% of the lymphocytes.

A second finding was that the tinctures of merthiolate and zephiran chloride were more toxic than the corresponding aqueous solutions. In the case of metaphen, the solution and the tincture were equal in toxicity.

All the antiseptics tested were found to be less toxic to the polymorphonuclear leukocyte than to the lymphocyte. This difference in toxicity was particularly marked with crystal violet; 90% of the cells of the suspension from the bone marrow were killed by a dilution of 1:2,400 of the dye, while the cells of the thymus were killed by a dilution of 1:16,000. The resistance of the polymorphonuclear leukocyte was also evidenced by its capacity to withstand the addition of the undiluted commercial antiseptics, merthiolate and phemerol in aqueous solution.

Discussion. Four *in vitro* methods have been developed for measuring the toxicity of

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1 Schrek, R., *Arch. Path.*, 1943, **35**, 857.

TABLE I.
Concentrations of Antiseptics That Killed 90% of the Nucleated Cells in Suspensions Derived from Rabbit Thymus and Bone Marrow.

	Suspension from rabbit thymus		Suspension from rabbit bone marrow	
	Aqu. sol. of antiseptic	Tincture	Aqu. sol. of antiseptic	Tincture
Sodium sulfathiazole	(1:100)*		(1:100)*	
Phenol	1:260		1:300	
Mercurochrome	1:800		1:120	
Metaphen	1:7,000	1:6,000	1:2,400	1:2,800
Merthiolate	1:8,000	1:16,000	(1:2,000)*	1:8,000
Phemerol	1:12,000		(1:2,000)*	
Crystal violet	1:16,000		1:2,400	
Zephiran chloride	1:18,000	1:28,000	1:3,000	1:8,000
Mercuric chloride	1:30,000		1:3,800	

* Less than 90% of the cells were killed by the maximal concentration tested.

antiseptics on various physiologic functions of the animal cell. The tissue culture method utilized by Lambert[2], German,[3] Buchsbaum,[4] Salle,[5] Osgood,[6] Herrell,[7] and their associates studied the effect of the antiseptic on the growth and the microscopic appearance of cells. Bronfenbrenner, Hershey, and Doubly[8] compared the effect of reagents on the respiration of animal and bacterial cells by means of the manometric method. Nye,[9] Welch,[10] and Hirsch[11] and their co-workers utilized the inhibition of phagocytosis by polymorphonuclear leukocytes as a means of measuring the toxicity of antiseptics. Recently Witlin[12] and Dunham[13] have determined the effect of reagents on the viability of chick embryos.

There are, then, methods for studying the toxic effect of antiseptics on 3 physiologic functions, namely, the growth and respiration of cells and the phagocytosis by leukocytes. The method of the unstained cell count provides a means of determining the effect of antiseptics on a fourth important property of living cells, *i.e.*, their resistance to staining with eosin. Inasmuch as this resistance is a function of the viability of the cell and is rapidly lost when the cell "dies," the method permits the measurement of the cytocidal action of antiseptics.

The present work was undertaken to determine the value of the method of unstained cell counts for measuring the toxicity of antiseptics. The findings are not sufficient to demonstrate the superiority of one antiseptic to that of another.

One observation made in this and other studies seems of particular interest. With all the reagents tested in this and other experiments the polymorphonuclear leukocyte proved much more resistant than the lymphocyte and testicular cells. Since the polymorphonuclear leukocyte is a specialized type of cell, the main function of which is prompt protection against deleterious agents, it is not surprising to find that this cell is much more resistant to a wide variety of toxic agents.

The greater susceptibility of the lymphocyte to antiseptics makes this cell particularly useful in measuring the toxic properties of antiseptics.

Summary. The method of unstained cell counts provides a simple quantitative means

2 Lambert, R. A., and Meyer, J. R., PROC. SOC. EXP. BIOL. AND MED., 1926, **23**, 429.

3 German, W. J., *Arch. Surg.*, 1929, **18**, 1920.

4 Buchsbaum, R., and Bloom, W., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 1060.

5 Salle, A. J., McOmie, W. A., Schechmeister, I. L., and Foord, D. C., *J. Bact.*, 1939, **37**, 639.

6 Osgood, E. A., *Arch. Int. Med.*, 1938, **62**, 181.

7 Herrell, W. E., and Heilman, D., *Am. J. M. Sc.*, 1943, **205**, 157.

8 Bronfenbrenner, J., Hershey, A. D., and Doubly, J., *J. Bact.*, 1939, **37**, 583.

9 Nye, R. N., *J. A. M. A.*, 1937, **108**, 280.

10 Welch, H., and Brewer, C. M., *J. Immunol.*, 1942, **43**, 25.

11 Hirsch, M. M., and Novak, M. V., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 376.

12 Witlin, B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 27.

13 Dunham, W. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 274.

of determining the toxicity of antiseptics to cells. The antiseptics tested differed markedly in their cytocidal activity, sodium sulfathiazole being the least and mercuric chloride the most toxic. Tinctures of merthiolate and zephiran were more toxic than the corresponding solutions. The polymorphonuclear leukocyte was more resistant to most of the reagents tested than the lymphocyte.

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Direct Stimulation of the Hypoglossal Nucleus by Acetylcholine in Extreme Dilutions.

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Acetylcholine (ACh.), when applied in minute amounts to the eserinized cerebral cortex, induces marked excitatory effects;[1] it was inferred that the ACh. stimulates or facilitates various cortical synapses. The experiments here reported indicate that ACh., on direct application in extreme dilutions, stimulates a motor nucleus, namely the hypoglossal nucleus; this nucleus is readily accessible, as the *trigonum hypoglossi,* immediately beneath the floor of the fourth ventricle.[2]

Methods. Decerebration under ether was by the Sherrington decerebrator. Both common carotids were tied or, in other experiments, both internal carotids were tied and also the branches of external carotids, but leaving lingual arteries intact; essentially similar results were obtained in both preparations. The skull was held in a special clamp, so that the entire tongue could be observed. The floor of fourth ventricle was exposed by removing median parts of cerebellum. Acetylcholine chloride (ACh.Cl) (Merck) was dissolved in buffered Ringer-Locke solution and was applied in small rectangles 2 mm x 6 mm of spot test paper to the XII nuclei of both sides.

Results. Preliminary localization of effects from the XII nucleus was by unipolar faradization with a fine silver wire, ending in a minute bulb; the diffuse electrode was a saline-soaked pad on animal's back. Secondary distances varied from 35 to 50 cm, using a large Palmer inductorium (Fig. 1).

The following effects of ACh. were obtained in a decerebrate cat (3100 g), having lingual arteries intact. Preliminary eserinization was by 0.25 mg eserine sulphate (Merck) intravenously. A rectangle of spot test paper soaked in ACh.Cl. 1:50 millions was applied to both XII nuclei: in 4 sec. a slight median furrowing appeared at base of tongue between circumvallate papillæ (Fig. 2); the furrowing, which coincided with inspiration, increased and was accompanied by simultaneous retraction of tongue base, elevation of soft palate and dilatation of remainder of mouth cavity. The ACh. increased respirations from 22 to 32/min.; these observations on respiratory stimulation agree with those of Worzniak and Gesell[3] and of Hansen, Worzniak and Gesell.[4]

Application of another rectangle of ACh.Cl. 1:10 millions intensified above effects within 5 sec.; in consequence of a further application of a rectangle of ACh.Cl. 1:10 millions and 2 successive rectangles of ACh.Cl. 1:1 million, with 0.25 mg eserine intravenously, the following symptoms developed (Fig. 2): a marked furrowing at base of tongue, coin-

[1] Miller, F. R., Stavraky, G. W., and Woonton, G. A., *J. Neurophysiol.*, 1940, **3**, 131.

[2] Winkler, C., and Potter, A., *An Anatomical Guide to Experimental Researches on the Cat's Brain,* W. Versluys, Amsterdam, 1914.

[3] Worzniak, J., and Gesell, R., *Am. J. Physiol.*, 1938, **123**, 222.

[4] Hansen, E. T., Worzniak, J. J., and Gesell, R., *Federation Proc.*, 1942, **1**, 36.