QUANTITATIVE ADSORPTION OF CRYSTAL VIOLET

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Received for publication December 10, 1952

The formula for crystal violet given in Conn's book (1940) implies that quantitative measurement of the dye adsorbed by bacterial cells should be possible by a suitable micro-Kjeldahl procedure.

At the present time there is no satisfactory method for estimating the degree of gram positivity although the need for such a procedure is recognized and is discussed by Bartholomew and Mittwer (1952). It is considered that the procedures reported here provide a useful tool for further quantitative studies on the gram reaction.

The present report shows that the amount of crystal violet combined with bacteria can be measured accurately by means of a simple micro-Kjeldahl procedure widely used in immunochemistry. The experiments reported were designed to determine the effect of the species and strain of the organism used, of the method of killing the cells, and of the time of contact with the dye, on the quantitative adsorption of crystal violet.

METHODS

All organisms used were cultivated at 30 C on a modified tryptose agar. Cells were harvested after 18 to 24 hours, killed, and washed with 0.85 per cent saline buffered at pH 7.1 with M/15 phosphate. Suspensions to be analyzed were killed either by heating to 99 C in a boiling water bath for 30 minutes or by the addition of 0.5 per cent formalin to the suspension and allowing to stand at 5 C for 48 or 72 hours. In the latter case, formalin in 0.025 per cent concentration was added to the buffered saline to avoid disintegration of the cells during washing.

The micro-Kjeldahl procedure used is described in detail by Kabat and Mayer (1948). The limit of error of this technique in the quantitative agglutinin method is about ±0.02 mg nitrogen.

The crystal violet used in all experiments was obtained from the National Aniline and Chemical Company, Inc., New York, N. Y., and labeled Certification no. NC 20, C.I. no. 681 with a total dye content of 89 per cent. A stock solution of the dye was prepared by weighing accurately 10 g of dye and dissolving it completely in 50 ml of ethyl alcohol. Fifty ml of M/15 phosphate buffer were added to yield a 10 per cent solution of crystal violet. The desired concentration of dye for use in the experiments was obtained by suitable dilution of the stock solution with buffer.

EXPERIMENTAL PROCEDURES AND RESULTS

Repeated micro-Kjeldahl analyses of dye solutions with various concentrations of crystal violet have yielded nitrogen values consistently within ±0.02 mg of the calculated value. Adsorption of the dye by bacterial cells may be measured quantitatively by micro-Kjeldahl analysis of thoroughly washed cellular suspension aliquots before and after treatment with the crystal violet solution; by analysis of a dye solution of known crystal violet content after the addition and subsequent centrifugation of a known quantity of cells; or by both procedures. In a typical experiment the cells are harvested, killed, and washed until 3 to 4 ml of the supernatant fluid yield no detectable nitrogen within the limit of error of the method (0.02 mg N). The suspension is filtered through cotton and adjusted to contain the desired concentration of bacterial nitrogen per ml. One ml of the bacterial suspension is added in triplicate to centrifuge tubes and centrifuged in the cold (5 C) at about 6,000 rpm (rcf 2,250) for 30 minutes. The supernatant fluid is discarded since it was found necessary to avoid the effect of saline on precipitation of the dye. The tubes are drained thoroughly, and either one or two ml of crystal violet solution are added to the packed cells. After thorough mixing, the tubes are centrifuged in the cold, the supernatants collected in Kjeldahl flasks, and the cells washed twice with buffer. The supernatant washings are added to the original supernatant fluid for micro-Kjeldahl analysis. The washed cells are transferred quantitatively to Kjeldahl flasks and similarly analyzed. Control suspensions to which buffer is added instead of stain are always set

up; analysis of control supernatants should give values within the limit of error of the micro-Kjeldahl method.

The experimental results in table 1 represent the average of triplicate analyses and indicate that the amount of dye taken up by the cells is directly proportional to the quantity of bacterial N used (series 1 and 2 in table 1); that heat-killed *Micrococcus pyogenes* var. *aureus* has a significantly greater dye-binding capacity than formalin-killed cells (series 1 and 3) but this relationship does not exist in *Salmonella typhosa* (series 9 and 10); that the time in contact with the dye (incubation time) has no significant influence on the quantity of dye adsorbed over the range of 30 minutes to 18 hours (series 3 and 4); and that 3 strains of staphylococci and one strain of *Corynebacterium xerose*, the gram positive organisms, adsorbed 0.25 ± 0.02 mg crystal violet N per mg of bacterial N, one strain of *Corynebacterium diphtheriae*, a gram variable organism, adsorbed 0.20 ± 0.02 mg crystal violet N per mg of bacterial N, while one strain of *Salmonella typhosa* and one strain of *Escherichia coli*, the gram negative organisms, adsorbed 0.15 ± 0.02 mg crystal violet N per mg of bacterial N (series 3, 5, 6, 7, 8, and 10, 11).

TABLE 1

Micro-Kjeldahl determinations of crystal violet nitrogen adsorbed by bacteria

SERIES	SPECIES	STRAIN	METHOD OF KILLING	INCUBATION TIME	ANALYSES ON STAINED BACTERIA			ANALYSES ON SUPERNATANTS		
					Total N	Bacterial N	Crystal violet N adsorbed	Original N content	N after staining	Crystal violet N adsorbed
					mg	*mg*	*mg*	*mg*	*mg*	*mg*
1	*Micrococcus pyogenes* var. *aureus*	Oxford 202	formalin	30 min	0.79	0.69	0.10	0.23	0.12	0.11
2	*Micrococcus pyogenes* var. *aureus*	Oxford 202	formalin	30 min	0.41	0.37	0.04	0.11	0.07	0.04
3	*Micrococcus pyogenes* var. *aureus*	Oxford 202	heat	30 min	0.95	0.76	0.19	0.21	0.04	0.17
4	*Micrococcus pyogenes* var. *aureus*	Oxford 202	heat	18 hr	0.97	0.76	0.21	0.22	0.03	0.19
5	*Micrococcus pyogenes* var. *aureus*	903E	heat	60 min	0.77	0.62	0.15	0.34	0.17	0.17
6	*Micrococcus pyogenes* var. *aureus*	903D	heat	60 min	0.78	0.64	0.14	0.34	0.18	0.16
7	*Corynebacterium xerose*	513	heat	30 min	0.36	0.29	0.07	0.17	0.10	0.07
8	*Corynebacterium diphtheriae*	Park 8	heat	30 min	0.82	0.68	0.14	0.17	0.03	0.14
9	*Salmonella typhosa*	O-901	formalin	30 min	0.79	0.68	0.11	0.16	0.04	0.12
10	*Salmonella typhosa*	O-901	heat	30 min	0.85	0.73	0.12	0.21	0.10	0.11
11	*Escherichia coli*	ATCC 9980	heat	30 min	0.72	0.62	0.10	0.17	0.07	0.10

DISCUSSION

The development of the quantitative chemical method for the estimation of adsorbed stain makes it possible to measure the amount of stain combined with bacteria on a weight basis and to study and describe the reaction as a function of the cell and the stain. The results show that the type of fixative or killing agent may have a marked influence on the stain-binding capacity of some organisms. In addition to its inherent interest and its significance to technicians working with tissue sections, this finding indicates that heat fixation should be preferred to formalin fixation in investigations concerned with the gram reaction.

Six of the studied strains fell into two well demarcated groups based on their crystal violet-binding power. The seventh strain, a known gram variable culture (Bartholomew and Mittwer, 1952), assumed an intermediate position.

While suggestive, this grouping may or may not be related to the true gram differentiation. Regardless, extension of these data should prove useful for classification purposes.

SUMMARY

The uptake of crystal violet by bacterial cells can be measured quantitatively by micro-Kjeldahl analyses.

Heat-killed staphylococci adsorbed more crystal violet than formalin-killed cells. Heat- and formalin-killed *Salmonella typhosa* cells adsorbed the same quantity of crystal violet.

Four strains of gram positive organisms adsorbed 0.25 ± 0.02 mg crystal violet N per mg of bacterial N; two strains of gram negative organisms adsorbed 0.15 ± 0.02 mg crystal violet N per mg of bacterial N; one gram variable strain adsorbed 0.20 ± 0.02 mg crystal violet N per mg of bacterial N.

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