Retention of Preservative Levels of Formaldehyde in Desiccated Biological Products

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Concentrations ranging from 8 to 100% of the preservative level of formalin (37% formaldehyde solution) were retained by desiccated biologics, with most products retaining about 50% regardless of the amount originally present.

Most bacterins, vaccines, and antisera used as prophylactic or diagnostic reagents contain antimicrobial preservatives to kill organisms accidentally introduced during processing. Some of these products are subsequently desiccated to enhance their stability. Occasionally it is desirable to form multicomponent products by mixing these desiccated biologics with products containing live organisms that might be susceptible to preservatives. Sometimes it is desirable to evaluate these desiccated products with test systems sensitive to preservatives, such as those containing tissue culture cells, embryos, lymphocytes, etc. For these reasons, we felt that it was desirable to know how much preservative was retained in desiccated biologics.

Formaldehyde is a gas under normal conditions and even the dilute formaldehyde solutions used to preserve biologics give off the typical pungent odor. Because of this, it is likely to assume that the majority of formaldehyde would evolve along with the water during the lyophilization process. However, we have had several instances when we found significant amounts of formaldehyde in experimentally produced desiccated products. It is known (3) that the kinetics of formaldehyde distillation are very complex. During vacuum distillation under certain conditions, formaldehyde concentrates as a residue with eventual formation of a solid mixture of polymeric hydrates known as paraformaldehyde. Therefore, the following experiments were designed to evaluate the retention of formalin (37% formaldehyde solution) in desiccated biological products.

The samples used in this study were commercial biologics which are not normally lyophilized plus several samples made to contain ingredients often found in biologics. *Bacillus subtilis* bacterins containing several levels of formalin were made 5 days prior to desiccation to determine if recently added formalin acted dif-

ferently than formalin added to products many months previously. Several samples of phosphate-buffered saline (PBS) were evaluated to determine the effect of pH. Some of the samples were treated with a quantity of NaHSO_3 which was estimated on the basis of molar equivalence to exactly neutralize the free formalin. A brief description of each type of sample is presented in Table 1. These samples were divided, with part being retained as liquid and the remainder dispensed in four vials which were lyophilized for about 24 h. Two additional vials containing serum were used to monitor the lyophilization process. The moisture content of these serum samples was estimated by the vacuum-oven method to be <3%.

The fuchsin-sulfurous acid procedure (2) was used for the estimation of unbound formalin. Briefly, 1-ml aliquots of diluted sample were added to tubes containing 10 ml of 2.5% HCl, and then 2 ml of fuchsin-sulfurous acid reagent (0.05 g of basic fuchsin, 1 g of NaHSO_3 , 1 ml of concentrated HCl diluted to 1 liter) was added to each tube at 1-min intervals. After exactly 30 min of reaction time, the resulting color was determined colorimetrically at 570 nm and compared to standard solutions treated in a similar manner. Normally, an automated procedure (1) is used for routine testing, but the manual procedure is more versatile for testing samples containing a wide range of formalin levels. A few samples were analyzed using the chromotropic acid method to determine the "total" amount of formalin in samples containing NaHSO_3 . For this test 1 ml of diluted sample was added to 3 ml of chromotropic acid reagent (0.5 g of 4,5-dihydroxy-2,7-naphthylenedisulfonic acid dissolved in cold 80% sulfuric acid) and heated for 15 min in boiling water. After cooling, 4 ml of water was added to each tube, and the color was determined at 580 nm. Results obtained were compared to standard solutions treated in a similar manner. The chromo-

TABLE 1. Amount of formalin found in liquid and rehydrated samples^a

Sample description	Neutralized	% Formalin found in		% Original formalin retained in desiccated sample
		Liquid sample	Rehydrated sample	
Pullorum antigen, stained		1.06	0.47	44
Wart vaccine, high percentage of solids		0.16	0.10	63
Wart vaccine, low percentage of solids		0.22	0.08	36
<i>C. perfringens</i> bacterin, high percentage of solids		0.48 (0.47)	0.32 (0.46)	67
<i>C. perfringens</i> bacterin, low percentage of solids		0.48 (0.50)	0.02 (0.02)	8
<i>C. chauvoei</i> bacterin		0.25	0.15	60
<i>C. perfringens</i> bacterin, Al (OH) ₃ adjuvant		0.21	0.21	100
<i>C. chauvoei</i> bacterin, (Al(OH) ₃ adjuvant		0.15	0.10	67
Erysipelas bacterin, Al(OH) ₃ adjuvant		0.13	0.10	77
Erysipelas bacterin, Al(OH) ₃ adjuvant		0.09	0.09	100
Mixed bacterin		0.21	0.05	24
<i>B. subtilis</i> bacterin, no formalin		0	0	—
<i>B. subtilis</i> bacterin, ca. 0.2% formalin ^b		0.13	0.08	62
<i>B. subtilis</i> bacterin, ca. 0.2% formalin	NaHSO ₃	0 (0.16)	0 (0.14)	— (88)
<i>B. subtilis</i> bacterin, ca. 0.5% formalin		0.37	0.25	68
<i>B. subtilis</i> bacterin, ca. 0.5% formalin	NaHSO ₃	0.02 (0.38)	0.02 (0.38)	— (100)
<i>B. subtilis</i> bacterin, ca. 1% formalin		0.88	0.51	58
<i>B. subtilis</i> bacterin, ca. 1% formalin	NaHSO ₃	0.04 (0.80)	0.04 (0.86)	— (108)
H ₂ O + 2% glycerin, ca. 0.2% formalin		0.21	0.11	52
H ₂ O + 2% glycerin, ca. 0.5% formalin		0.52	0.25	48
H ₂ O + 2% glycerin, ca. 1% formalin		1.05	0.44	42
H ₂ O + 2% thioglycolate, ca. 0.2% formalin		0.19	0.04	21
H ₂ O + 2% thioglycolate, ca. 0.5% formalin		0.45	0.07	16
H ₂ O + 2% thioglycolate, ca. 1% formalin		0.95	0.27	28
H ₂ O, ca. 0.2% formalin		0.23 (0.24)	Trace (trace)	0
H ₂ O, ca. 0.2% formalin	NaHSO ₃	0 (0.25)	0 (0.20)	— (80)
H ₂ O, ca. 0.5% formalin		0.55 (0.55)	Trace (trace)	0
H ₂ O, ca. 0.5% formalin	NaHSO ₃	0 (0.61)	0 (0.50)	— (82)
H ₂ O, ca. 1% formalin		1.09 (0.93)	Trace (trace)	0
H ₂ O, ca. 1% formalin	NaHSO ₃	0 (0.97)	0 (0.90)	— (93)
PBS, pH 5.5, ca. 0.2% formalin		0.21 (0.21)	0.07 (0.09)	33
PBS, pH 5.5, ca. 0.5% formalin		0.52 (0.46)	0.16 (0.13)	31
PBS, pH 7.2, ca. 0.2% formalin		0.24 (0.25)	0.04 (0.05)	17
PBS, pH 8.5, ca. 0.2% formalin		0.22 (0.21)	0.01 (0.01)	5
PBS, pH 8.5, ca. 0.5% formalin		0.51 (0.51)	0.03 (0.03)	6

^a The percentages of formalin reported in the table represent the amount of free formalin as estimated by the fuchsin-sulfurous acid test, and the results in parentheses represent the total amount of formalin estimated by the chromotropic acid test.

^b Approximate quantity of formalin added to product.

tropic acid method was also used to verify the formalin content of two *Clostridium perfringens* bacterins which had very different retention characteristics.

The levels of formalin in the liquid and rehydrated samples are presented in Table 1. The rehydrated biological products retained between 8 and 100% of the original amount of formalin. There was a considerable variation among types of products. Most of the rehydrated products retained about one-half of the original formalin regardless of the original concentration. Generally, the products with a higher percentage of total solids and products containing Al(OH)₃ retained a higher percent-

age of formalin. There seemed to be little difference in percentage retained between the older commercial products and the recently made *B. subtilis* bacterins. Aqueous solutions of glycerin retained about 50% of the added formalin, thioglycolate solutions retained about 25%, and water retained practically none. The PBS solutions at pH 5.5 retained more of the formalin than the buffers at pH 7.2 or 8.5, although all the PBS solutions retained more than the water.

Some of the samples were assayed by the chromotropic acid method, which estimates the "total" amount of formalin in the sample even if it had been neutralized with NaHSO₃. The re-

sults obtained by the chromotropic acid method appear within the parentheses in Table 1. All the products in which the formalin had been neutralized with NaHSO_3 retained most of the added formalin. In some cases, the free formalin content of the desiccated samples that were neutralized was slightly higher than those of the liquid product. Possibly this indicates that the desiccation process interfered with the neutralization.

In summary, it can be seen that many biological products retain a large percentage of the formalin during desiccation which might influence certain uses of these products. Caution should be exercised when mixing these products with formalin-sensitive components (i.e., live virus vaccines, tissue culture test systems, etc.). Also, some deleterious effects caused by

excessive concentrations of formalin in the microenvironment of antigens could occur during certain phases of dehydration and rehydration. The exact molecular structure of the formalin in the desiccated samples is beyond the scope of this report, but those interested should refer to the discussion of formaldehyde polymers presented by Walker (3).

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