that barley was improved prior to incorporation in the diet and improvement did not depend on microbially produced enzymes acting in the digestive tract of the chick.

Only 100 ml of 20-hr peptone washed culture significantly ($P<.01$) improved the nutritional value of sterile barley in Exp. 2 (Table II). Thus, it is highly probable that microorganisms grew in wet barley, perhaps producing enzymes, which could have acted on the barley substrate during drying. Exp. 3 (Table III) showed that 400 ml of nutrient broth culture gave a significant ($P<.05$) growth response, presumably a result of enzymes produced during incubation of the stock cultures.

The data demonstrate that microbial fermentation is effective in improvement of barley by water-treatment. This, however, does not eliminate the possibility that enzymes endogenous to barley may also play a role in improving the nutritional value of this grain by water-treatment. Both phenomena could conceivably take place simultaneously.

Summary. Water-treated barley taken from drying oven contained many more bacteria than untreated barley, even though drying temperature was 70°C. The nutritional value of sterilized barley inoculated with Gram-positive rod isolated from dust, or with *B. subtilis* (ATCC 9943) and dried at 70°C was highly significantly improved. Large inoculations of the organism isolated and grown in nutrient broth elicited a significant growth response. This response was not as great as that obtained by water-treatment or addition of fungal enzyme to the feed. Autoclaving did not lower nutritional value of water-treated barley. This fact indicates that the change(s) responsible for improvement was effected prior to feeding and was not due to presence of an enzyme(s) produced during treatment process that later acts on barley in the digestive tract of the chick.

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Antibody Response to Influenza Vaccines Combined with Hexadecylamine. (25459)

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Certain lipids, including cholesterol, palmitic acid, stearic acid, and longer aliphatic amines, such as hexadecylamine (HDA) have been shown by Youngner and Noll to possess an affinity for several myxoviruses(1,2,3). Such virus-lipid complexes increase antigenicity, in an as yet unknown manner, to a level greater than that of equal amount of virus alone. This reaction is also notable in that large amounts of viral antigen can be adsorbed to a given quantity of HDA(3). These observations have an immediate bearing on the problem of enhanced potency of influenza vaccines. In an earlier study we reported preliminary observations on cholesterol containing vaccines(2). The following report describes the responses of man and animals to formalin-inactivated influenza viruses mixed with hexadecylamine, since, in comparative adsorption experiments, this compound ex-

hibited high affinity for a wide range of influenza strains.

Materials and methods. Hexadecylamine, ($CH_2(CH_2)_{15}NH_2$) technical grade, was obtained from Eastman Kodak Co., Rochester, N. Y., as a waxy, white solid, insoluble in aqueous solutions. After thorough grinding with mortar and pestle and subsequent sifting through triple layers of surgical gauze, slurries were prepared by slowly adding influenza vaccines to finely ground lipid and further grinding the combination until a fine suspension resulted. Since HDA separates from suspension, it was necessary to agitate the preparation just before use to ensure homogeneity. Polyvalent influenza vaccines employed were 4-strain "civilian" (Pfizer lot #80431) and 6-strain "military" (Pfizer lot #88305) formulae containing 500 and 1000 CCA (chick cell agglutinating units)/ml, respectively.

Results. HDA in concentrations greater than 5 mg/ml was irritating when injected subcutaneously into guinea pigs. Therefore, to determine minimal amount of lipid that would enhance vaccine potency, varying amounts of HDA from 5 mg/ml to .1 mg/ml were combined with constant amount of 4-strain influenza vaccine and guinea pigs were injected with 1 ml of the mixture by intramuscular route. Animals were bled at 2, 4, and 8 weeks thereafter and sera assayed for hemagglutination-inhibition (HI) antibody titers using periodate and trypsin to destroy non-specific inhibitors. Levels were determined for all 4 strains in the vaccine, but only those for B/GL strain are illustrated as typical in Fig. 1. Vaccine mixed with .5 mg/ml or more HDA elicited higher geometric mean titers than found with control vaccine.

Toxicity of HDA was studied in mice, guinea pigs, monkeys, and subsequently in man. Although injection of 1 mg/ml in guinea pigs resulted in focal deposit of HDA which persisted for several weeks, lesser amounts were tolerated with no gross reaction. Histological examination of site of subcutaneous injection in guinea pigs and monkeys at intervals to 4 weeks revealed a transient inflammatory reaction of only slightly greater intensity than resulted from injection of influenza vaccine alone. After 21 days, the site of injection was difficult to find and fibrosis or abscess formation has never been observed in several hundred guinea pigs.

Toxicity studies in man were begun by injecting HDA subcutaneously or intramuscularly at 48-hour intervals using following increasing dosages: .01, .02, .05, .10 and .50 mg. Thus, each of 20 volunteers received a total of .68 mg. The material was well tolerated with no local or systemic reaction.* In further human trials, none of 60 men injected with .5 mg/ml alone or mixed with influenza vaccine exhibited any local or generalized ill effects or manifested any increased sensitivity to egg protein when given a repeated dose of vaccine after a 90-day interval.

To determine adjuvant action with a wider variety of influenza strains, HDA was incorporated into a 6-strain vaccine in concentration of .5 mg/ml. Fig. 2 illustrates geometric mean titers in sera from groups of 20 guinea pigs vaccinated intramuscularly with this preparation and assayed for HI antibody at bi-weekly intervals to 8 weeks. With the PR 301 and PR8 antigens, peak titer was reached in 2 weeks contrasting with results obtained when measuring with the Asian and 2 B strains, where maximal titers were not obtained until after 4 weeks. The response to swine antigen was particularly disappointing, even though the vaccine contained 100 CCA units, a concentration shown to be satisfactory in eliciting antibody in the mouse potency assay for this biologic. Therefore, for reasons unknown at present, the swine component appeared to be a poor antigen in guinea pigs.

Influenza vaccine containing HDA not only elicited higher antibody titers than control vaccine but did so within a relatively short period of time. The elevated level did not persist, although titers in the test group were higher than controls for at least 8 weeks. Similar results have been obtained repeatedly in other experiments utilizing 4-strain vaccine. Typical data are summarized in Fig. 3 where guinea pigs were again the test species. Geometric mean titers found with 4 strains were cumulatively plotted so that a clearer com-

* We thank Dr. D. Iezzoni, Clinical Research Dept., Chas. Pfizer and Co., who conducted this study.

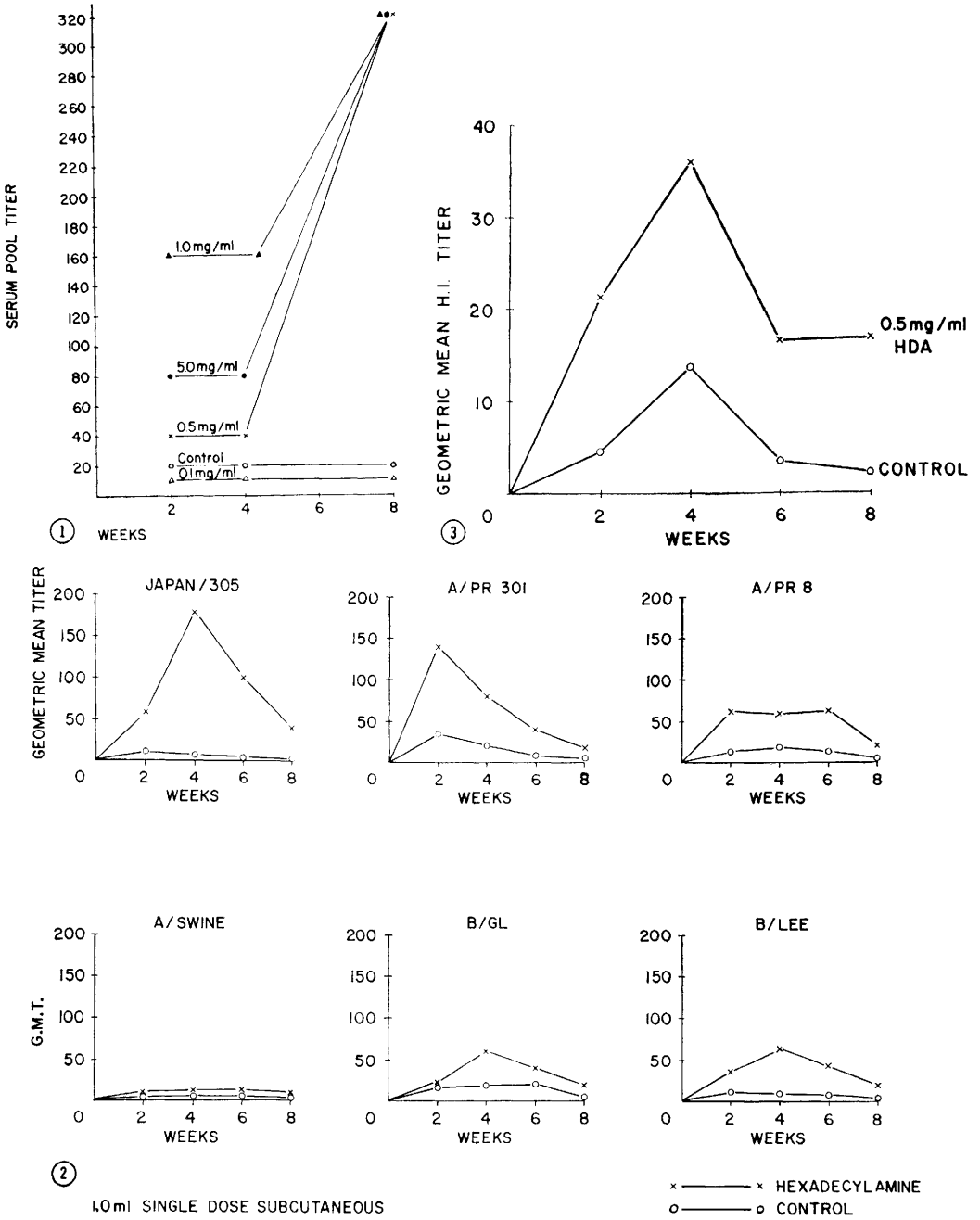


FIG. 1. Guinea pig responses to B/GL strain in combination with various amounts of hexadecylamine.

FIG. 2. Guinea pig antibody responses to 1000 CCA six strain influenza vaccine with or without hexadecylamine.

FIG. 3. Guinea pig combined antibody response to 500 CCA four strain influenza vaccines.

parison of the over-all response to a vaccine with and without added HDA could be obtained. The average titers of animals receiving HDA adjuvanted biologic remain 3- to 4-fold greater than control vaccine at each bleeding interval.

In experiments cited thus far, HDA was added directly to influenza vaccines, and the

TABLE I. Guinea Pig Antibody Titers Following Injection of Monovalent Influenza Vaccines Adsorbed to Hexadecylamine.

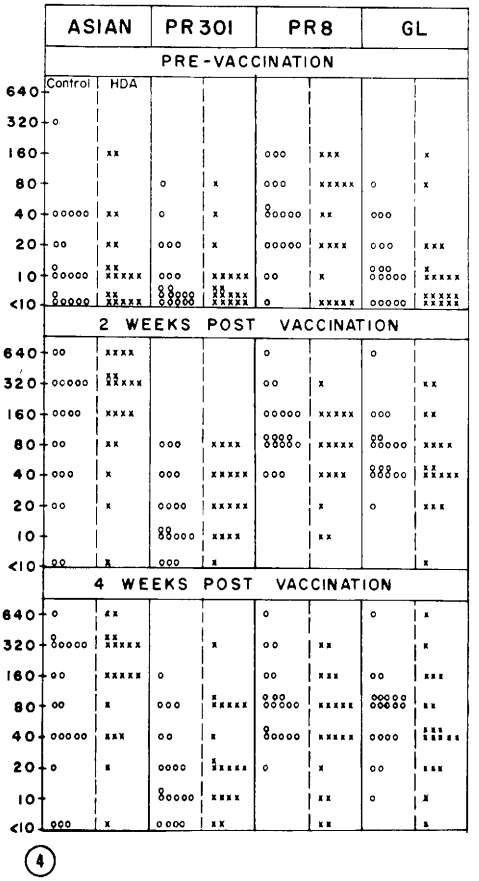
Type	HA, units/ml	Preparation	Conc. HDA/mg	Wk post-vaccination 2	4	7
Swine	639	HDA	.5	23*	93	59
	"	C†	.0	1	9	6
	148	HDA	.1	2	12	6
	"	C	.0	2	2	2
	30	HDA	.02	1	1	1
	"	C	.00	1	1	2
	6	HDA	.004	1	1	1
	"	C	.00	1	1	1
B/GL	494	HDA	.5	69	87	64
	"	C	.0	14	7	8
	99	HDA	.1	10	27	18
	"	C	.0	3	3	4
	20	HDA	.02	1	1	3
	"	C	.00	3	2	3
	4	HDA	.004	1	1	2
	"	C	.000	1	1	1
Asian	890	HDA	.5	24	160	50
	"	C	.0	16	29	21
	178	HDA	.1	5	95	11
	"	C	.0	2	5	2
	37	HDA	.02	1	6	2
	"	C	.00	3	8	9
	8	HDA	.004	1	4	1
	"	C	.000	1	7	2

* Reciprocal of geometric mean titer.
† C = Control.

actual amount of virus bound to the amine was not determined. To ascertain whether vaccine with only HDA-bound antigen might be superior, HDA was saturated with inactivated monovalent influenza concentrates and the hemagglutinating (HA) units/ml of adsorbed virus calculated. Vaccines were then diluted so that 1. ml contained .5, .1, .02, and .004 mg/ml HDA. Such dilution also reduced the number of hemagglutinating units by 5-fold increments. Comparable control vaccines without HDA were also prepared. Guinea pigs were injected intramuscularly with 1 ml of a given preparation, bled at intervals of 2, 4, and 7 weeks and sera assayed for HI antibody titer. Data for swine, B/GL, and Asian strains are presented in Table I. Addition of HDA to monovalent influenza vaccines in concentrations of .1 mg/ml or greater enhanced potency, while below that level adjuvant action was not obtained. Thus, it is clear that injection of 600 HA units firmly bound to HDA provided a pronounced adjuvant effect, even with the swine strain, a virus which had given poor results in previous experiments. It appears that when HDA-bound antigen is used as vaccine, a more striking enhancement occurs than with injection of mixtures of lipid-adsorbed and free virus antigens.

Results of studies in human volunteers with a single one ml subcutaneous injection of commercial 4-strain influenza vaccine to which .5 mg/ml HDA was added are presented in Fig. 4. The distribution of antibody titers against the vaccine strains is shown for groups of 20 adult males with serum specimens obtained pre-vaccination, and at intervals of 2 and 4 weeks post-vaccination. The dots indicate subjects who received control vaccine and crosses are for those who were given vaccine with HDA. At 2 weeks, a slight enhancement of titer was observed with the Asian component, less with the PR301, none with PR8 and B/GL strains. A similar relationship held at 4-week bleeding interval. In light of the results obtained in animal trials, this pattern of human response was totally unexpected. The same vaccine preparation was, therefore, retested in guinea pigs and sera assayed at similar bleeding intervals. As in previous experiments with this animal host, a pronounced adjuvant effect was again observed at 2 weeks after vaccination, and this enhancement was further evident in the 4-week sera (Fig. 5). This difference between animal and human response appears to be related to prior experience of human subjects with influenzal antigens since many of the men studied were previously sensitized to each of the 4 vaccine strains.

Discussion. Combination of HDA with influenza vaccines does increase antigenic potency; however, our results illustrate a factor of significance in comparative studies of adjuvants in animals and man. When the test subject has had previous sensitization to a given antigen, such as is often the case in adults, the response may be only slightly increased. In contrast, enhancement will be observed with HDA-virus mixtures in a host undergoing primary immunization. The adjuvant effect appears to be more pronounced



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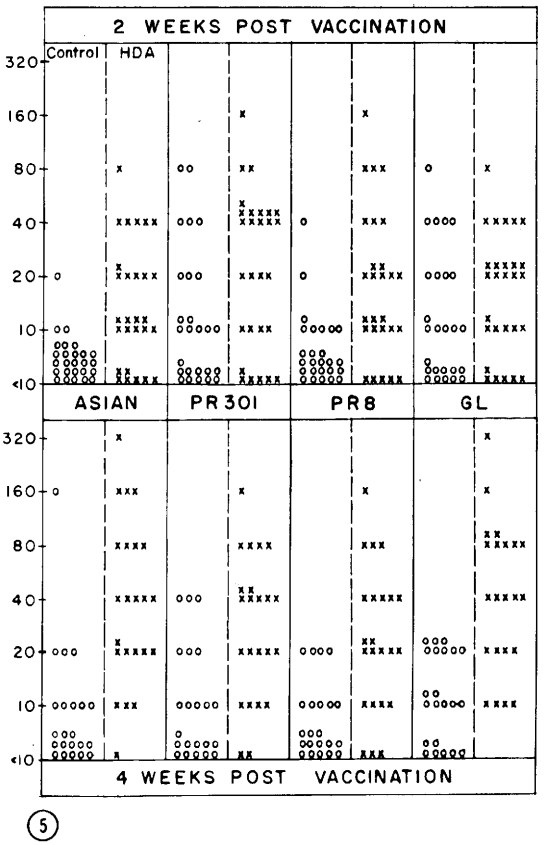

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FIG. 4. Antibody responses of adults to influenza vaccine with hexadecylamine.
FIG. 5. Antibody responses in guinea pigs to influenza vaccine with hexadecylamine.

when virus antigens are firmly bound to HDA. Studies are now in progress of responses in sensitized guinea pigs and men with virus-coated HDA vaccines.

Rapid antibody production can be an important factor in the control of influenza when new vaccines are being used to combat rapidly spreading new antigenic variants such as was the case in the pandemic of 1957. For this reason, the accelerated responses induced by myxovirus adsorbed to lipid is a valuable feature of the combination.

Summary. Hexadecylamine in levels up to .5 mg/ml is well tolerated by man and animals. A single injection into guinea pigs of influenza vaccines containing this material results in rapid production of antibody levels which exceed those elicited by control vaccines. Preliminary results in man suggest that adjuvants of this nature may be most efficacious in a population receiving primary influenza experience.

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