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Report

Aluminum Compounds Used as Adjuvants in Vaccines

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The structure of nine commercially manufactured aluminum-containing adjuvants was investigated by X-ray diffraction, infrared spectroscopy, transmission electron micrography, and energy dispersive spectrometry. Seven samples which were labeled as aluminum hydroxide were identified as boehmite, a crystalline aluminum oxyhydroxide [AlO(OH)]. However, the degree of crystallinity varied between the samples. Two samples which were labeled as aluminum phosphate were found to be amorphous aluminum hydroxyphosphate. Buffer anions and sulfate anions substitute for hydroxyls in the amorphous aluminum hydroxide formed by the *in situ* alum precipitation method. Finally, the aluminum-containing adjuvant in diphtheria and tetanus toxoid, U.S.P., produced by three manufacturers was characterized.

KEY WORDS: adjuvants; vaccine; aluminum hydroxide; boehmite; aluminum hydroxyphosphate.

INTRODUCTION

The adjuvant action of aluminum compounds was first noted in 1926 when an alum-precipitated diphtheria vaccine was found to have greater antigenic properties than the standard diphtheria vaccine (1). Although aluminum compounds have a long history of use as adjuvants and are recognized as safe for this use by the Food and Drug Administration (2), the problem of inconsistent antibody production has been frequently cited (2–4).

An early study by X-ray diffraction and electron microscopy (5) of the structure of aluminum hydroxide gels used in preparation of vaccines showed that Schmidt's modification of Willstater's procedure for preparing C-gamma gel produced a high-surface area boehmite containing fibrils which was superior in terms of surface area to the bayerite and gibbsite aggregates of Willstater's C-gamma gel.

Vaccines containing aluminum compounds as adjuvants are prepared by two principal methods (2). A commercially prepared adjuvant, usually labeled aluminum hydroxide or aluminum phosphate, may be mixed with the antigen. Such products are termed aluminum hydroxide or aluminum phosphate adsorbed vaccines. The aluminum-containing adjuvant may also be prepared by *in situ* precipitation. A solution of alum, $KAl(SO_4)_2 \cdot 12H_2O$, is mixed with the antigen solution to form a precipitate which has been described historically as protein aluminate. These vaccines are termed alum precipitated vaccines.

This study was undertaken to examine the aluminum compounds which are used in commercial vaccines in the belief that the first step in producing a consistent adjuvant effect is to characterize the structure and properties of the aluminum compounds currently used as adjuvants.

MATERIALS AND METHODS

Nine commercially prepared adjuvants were studied. Adjuvants A–G were labeled as aluminum hydroxide and were obtained from E. M. Sergeant Co., Clifton, NJ, and Armour Pharmaceutical Co., Kankakee, IL. Adjuvants H and I were labeled as aluminum phosphate and were obtained from E. M. Sergeant Co., Clifton, NJ.

Standard buffer solutions (6) were used to prepare acetate, carbonate, citrate, phosphate, and Tris buffers.

Alum-precipitated adjuvants were prepared by dissolving potassium aluminum sulfate (analytical reagent, Mallinckrodt) in the appropriate buffer solution to obtain 0.02 *M*. The alum solution (100 ml) was titrated at room temperature with 0.1 *N* NH_4OH at 3.5 ml/min to pH 6.5. The

Table I. Alum-Precipitated Adjuvants Prepared in Phosphate Buffer at Various Molar Ratios of Phosphate to Aluminum

Phosphate buffer[a] (ml)	0.02 *M* $KAl(SO_4)_2$ (ml)	PO_4:Al
50	100	0.25
100	100	0.50
150	100	0.75
200	100	1.00

[a] Composed of 40.5 ml of 0.2 *M* Na_2HPO_4 and 9.5 ml of 0.2 *M* NaH_2PO_4 made up to 100 ml with double-distilled water.

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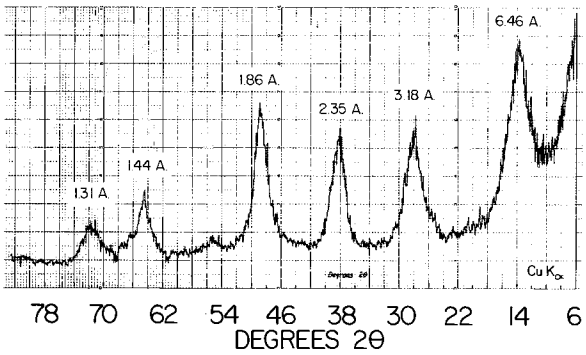

Fig. 1. X-ray diffractogram of adjuvant D.

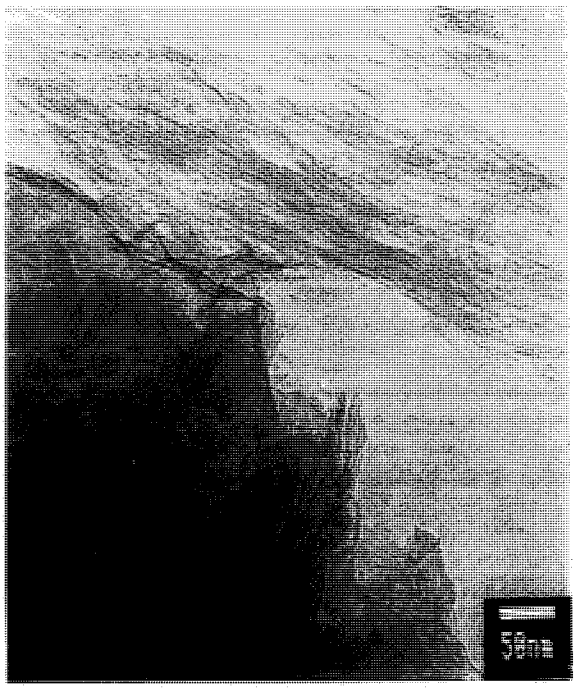

Fig. 3. Transmission electron micrograph of adjuvant D.

resulting precipitate was filtered under vacuum through Whatman qualitative filter paper and washed with 3 vol, each of 100 ml, of double-distilled water.

Aging studies of alum precipitated adjuvants prepared in the presence of phosphate buffer at molar ratios of phosphate to aluminum ranging from 0.25 to 1.0 (Table I) were conducted at 25 and 70°C.

Samples of diphtheria and tetanus toxoids, U.S.P., produced by three different manufacturers (Connaugh, Sclavo, and Wyeth) were obtained commercially.

The equivalent aluminum oxide content of the alum precipitated adjuvants was determined by ethylenediamine tetraacetate titration (7). The end point of the pH-stat titration (8) at pH 3.0 and 37°C was taken as the actual acid neutralizing capacity. The theoretical acid neutralizing capacity was calculated based upon the theoretical stoichiometry ($1Al{:}3H^+$) of the acid neutralization reaction.

Samples for X-ray diffraction were prepared as random powder mounts after gently grinding freeze-dried adjuvants in an agate mortar and pestle. The diffraction patterns were recorded (General Electric Model XRD-5 X-ray diffractometer) from 4 to 40° 2θ using CuK_α radiation. The breadth of the 020 reflection of each pattern was measured at half-maximum intensity after subtracting the background; the 2θ position of each 020 reflection was measured at the midpoint of the chord at half-maximum intensity. The 100 reflection of microcrystalline quartz (novaculite) was used as a standard for 2θ and as a measure of the instrumental broadening. The breadth (b) of the 100 reflection of quartz was subtracted from the measured breadth of the 020 reflection (B) of boehmite to give the pure diffraction breath (β), i.e., $\beta = B - b$ (9). The same samples were diluted in potassium bromide (2 mg/300 mg), compressed, and examined by infrared spectroscopy (Perkin Elmer Model 180 spectrophotometer).

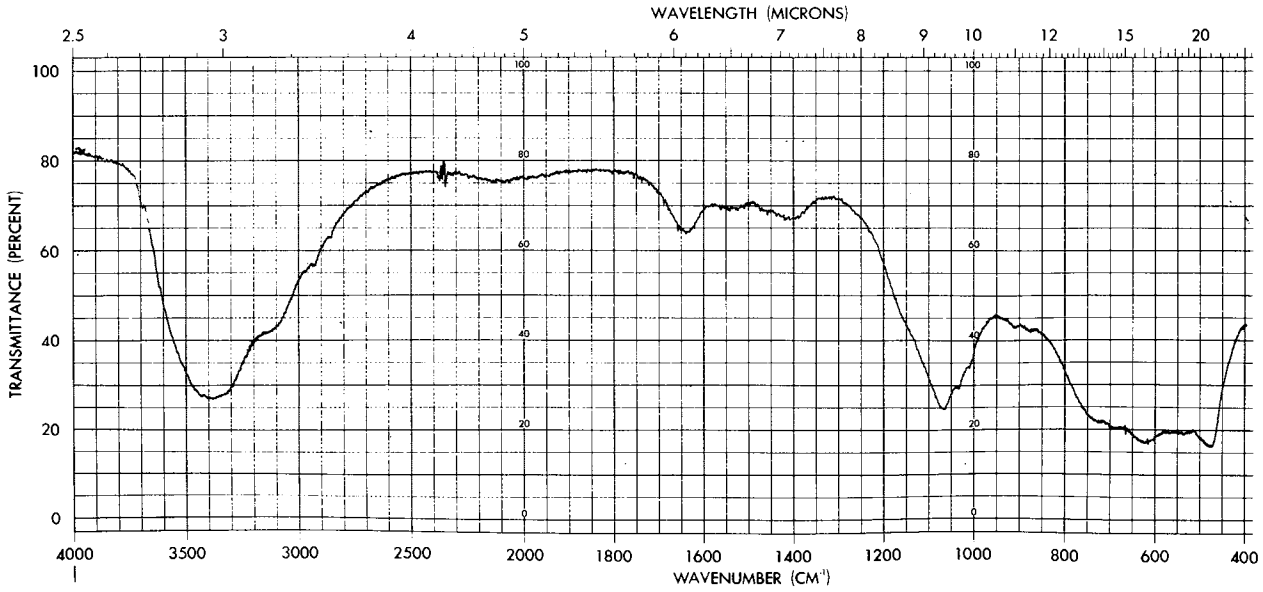

Fig. 2. Infrared spectrum of adjuvant D.

Table II. Line Broadening of the 020 Reflection of Boehmite-Containing Adjuvants

Identification	Adjuvant	Diffraction band width at half-height (° 2Θ)[a]
—	—	5.45[b]
A	Alhydrogel 85[d]	3.39
B	Rehydrogel[d]	3.39
C	Alhydrogel 85[d]	3.19
D	Rehsorptar[e]	2.99
E	Alhydrogel[d]	2.79
F	Alhydrogel[d]	2.59
G	Rehsorptar[e]	2.59
—	—	0.10[c]

[a] Corrected for instrumental broadening.
[b] Smallest crystallite size reported (10).
[c] Largest crystallite size reported (10).
[d] E. M. Sergeant Co., Clifton, NJ.
[e] Armour Pharmaceutical Co., Kankakee, IL.

Samples for transmission electron micrography (JEOL 2000 FX) were prepared by dipping a copper grid in a 0.01% aqueous suspension of the freeze-dried adjuvant which was dispersed by ultrasonic treatment. The grids were air-dried. After being examined by transmission electron micrography, energy-dispersive spectrometry (Link Analytical) was used to obtain elemental analysis.

RESULTS AND DISCUSSION

Commercial Adjuvants. The structure and properties of nine commercially available aluminum-containing adjuvants were studied. Adjuvants A to G were labeled as aluminum hydroxide. All of these adjuvants exhibited a similar X-ray diffraction pattern, having diffraction bands at 6.46, 3.18, 2.35, 1.86, 1.44, and 1.31 Å. These diffraction bands are in excellent agreement with those characteristic of boehmite (10,11), an aluminum oxyhydroxide [AlO(OH)]. Figure 1 is the X-ray diffractogram of adjuvant D. The first reflection at 6.46 Å is slightly displaced from the 6.11-Å spacing for well-crystallized boehmite. This displacement has been attributed to the effect of small crystallite size on apparent spacing (10,12).

The infrared spectra of adjuvants A to G also identify them as boehmite (11). Figure 2 shows the infrared spectrum of adjuvant D, which is typical of adjuvants A to G. The absorption band at 1070 cm^{-1}, in the O–H deformation region, is indicative of boehmite (11). The strong shoulder at 3100 cm^{-1} is also unique for boehmite and indicates the existence of structural hydroxyl environments which are characteristic of boehmite (11).

The morphology of adjuvants A to G is exemplified by the transmission electron photomicrograph of adjuvant D (Fig. 3). The fibrous morphology seen in Fig. 3 is characteristic of boehmite (13,14). Energy-dispersive spectrometry showed only the presence of aluminum in adjuvants A to G, a result which is consistent with boehmite.

Thus, adjuvants which have historically been termed aluminum hydroxide are actually a crystalline aluminum oxyhydroxide with the mineralogical name of boehmite.

Although adjuvants A to G are all boehmite, differences are seen in the width of the X-ray diffraction bands and in the width of the OH-stretching region (3100 to 3500 cm^{-1}) of the infrared spectrum. These differences are most easily quantified by the X-ray diffraction band width at half-height of the 020 reflection (6.46 Å). A sharp X-ray diffraction band (small width at half-height) characterizes highly ordered material, having a large crystallite size, while poorly ordered material, having a small crystallite size, has a broad X-ray diffraction band. The adjuvants are listed in Table II in order of increasing crystallite size. It is interesting to compare the crystallite dimensions for this group of adjuvants to the crystallite size seen in 32 synthetic boehmites (10). The line broadening in the samples studied by Tettenhorst and Hofmann ranged from 5.45 to 0.10° 2Θ. Thus, the boehmites which are used as adjuvants are in the middle of the possible range of crystallite size.

Adjuvants H and I were labeled as aluminum phosphate. These adjuvants did not exhibit any X-ray diffraction bands, indicating that they are amorphous. Transmission electron micrography of adjuvants H and I revealed a network of platy particles as shown in Fig. 4. The energy-dispersive spectrum of adjuvants H and I indicates the presence of both aluminum and phosphorous. Figure 5 is the infrared spectrum of adjuvant H, which is also typical of adjuvant I. The absorption band at 1100 cm^{-1} is characteristic of phosphate. In addition, the broad OH-stretching band around 3400 cm^{-1} suggests a highly disordered material. When the sample was heated to 200°C, a prominent OH-stretching frequency at 3164 cm^{-1} as well as a band at 3450 cm^{-1} was apparent. The band at 3164 cm^{-1} is evidence for the presence of structural hydroxyls. Thus, it is concluded that adjuvants H and I are amorphous aluminum hydroxyphosphate rather than aluminum phosphate as they have been historically identified.

Alum-Precipitated Adjuvants. When adjuvants are prepared by the alum precipitation method, the antigen is usually in a buffered solution when mixed with the alum solution. Since the influence of buffer anions on the precipitation

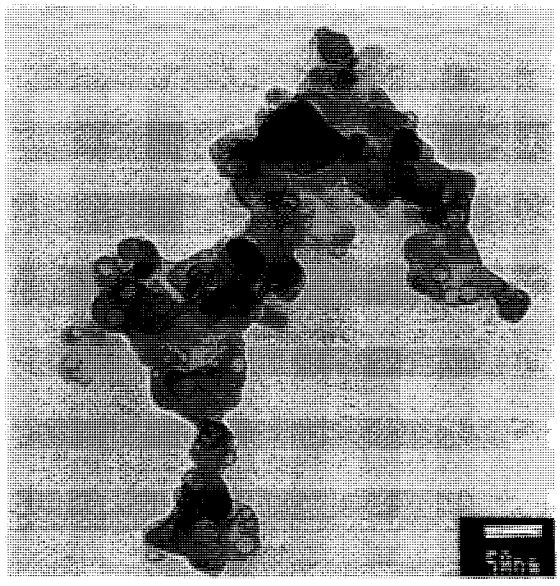

Fig. 4. Transmission electron micrograph of adjuvant H.

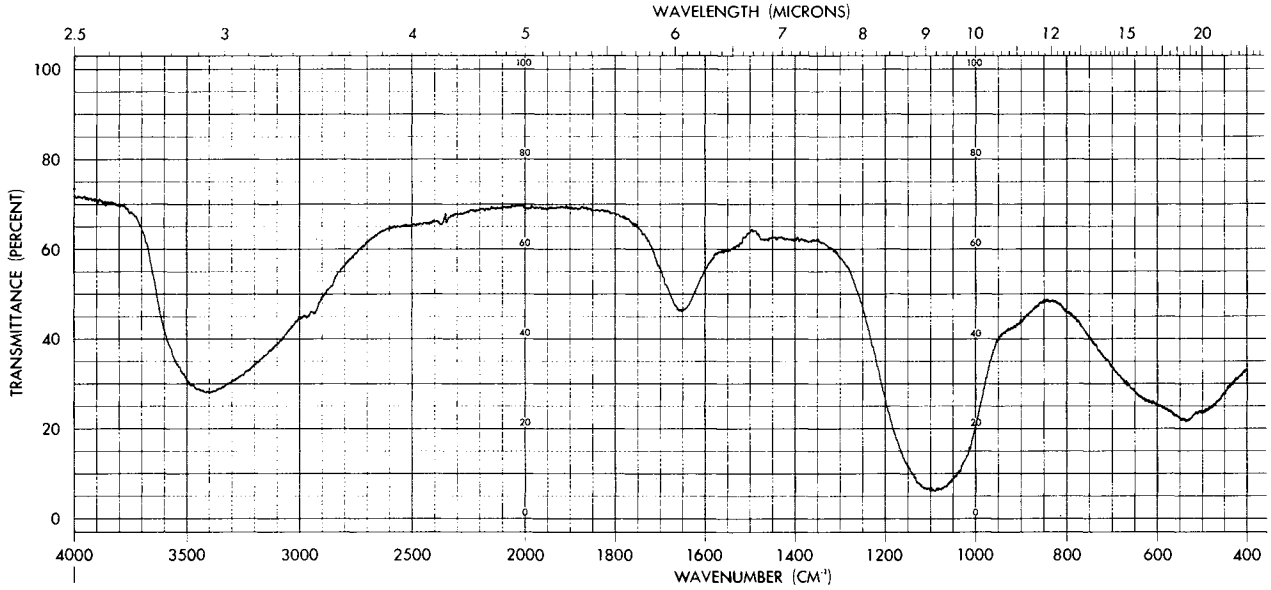

Fig. 5. Infrared spectrum of adjuvant H.

of aluminum hydroxide has been reported (15–17), the effect of buffer ions on the alum precipitation of adjuvants was examined. Acetate, carbonate, citrate, phosphate, and Tris buffers were studied. In each case the X-ray diffraction pattern of the alum-precipitated adjuvant prepared in the presence of a buffer was amorphous. Infrared spectra showed the presence of bands associated with sulfate, from the alum, as well as the buffer ion. Thus, the adjuvant produced by the alum precipitation method is an amorphous aluminum hydroxy (buffer ion) sulfate. The composition and properties of such a material would be variable and very dependent on precipitation conditions.

The effect of a phosphate buffer on the alum precipitation of adjuvants is reported as an example of the effect of buffer ions because of the wide use of phosphate buffers in vaccine production. The acid reactivity of the precipitates decreased as the concentration of the phosphate buffer increased (Fig. 6). The decreased acid reactivity is due to the specific adsorption of phosphate anion by aluminum hydroxide, displacing hydroxyl anion (18). The Al–OPO_3 bonds are more resistant to proton attack than the Al–OH bonds.

The acid reactivity of the alum-precipitated adjuvants which were precipitated in the presence of phosphate buffer decreased during aging. Figure 7 shows the change which occurred in the adjuvant precipitated at a molar ratio of 0.5 PO_4/Al. The decrease in acid reactivity reflects the increase in order which occurs during the aging of aluminum hydroxide (19).

The infrared spectra of adjuvants precipitated at molar ratios of phosphate to aluminum of 0.25, 0.50, and 1.0 are shown in Fig. 8. As the molar ratio of phosphate to aluminum increased from 0.25 to 1.0, the sulfate adsorption band was displaced from 620 cm^{-1} to lower wavenumbers, indicating a weakening of the coordination between sulfate anion and aluminum (20).

The precipitate formed at a 0.25 phosphate-to-aluminum molar ratio shows the presence of phosphate by the band at 1100 cm^{-1} and the two small bands at 1460 and 1385 cm^{-1}.

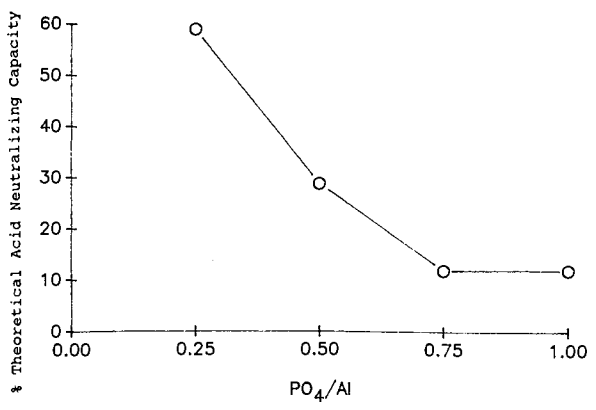

Fig. 6. Acid neutralizing capacity of alum-precipitated adjuvants which were precipitated in the presence of various concentrations of a phosphate buffer.

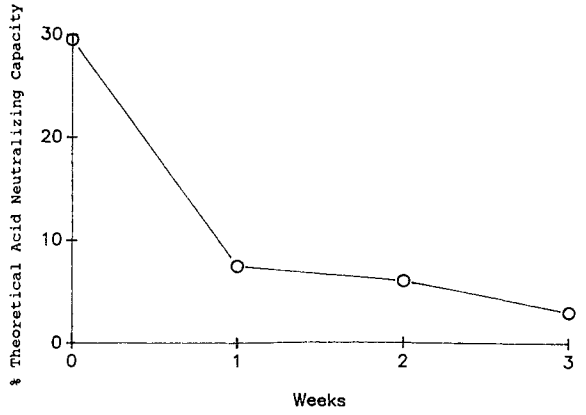

Fig. 7. Change in acid neutralizing capacity during aging at 25°C of alum-precipitated adjuvant which was precipitated in the presence of a phosphate buffer at a molar ratio of 0.5 PO_4/Al.

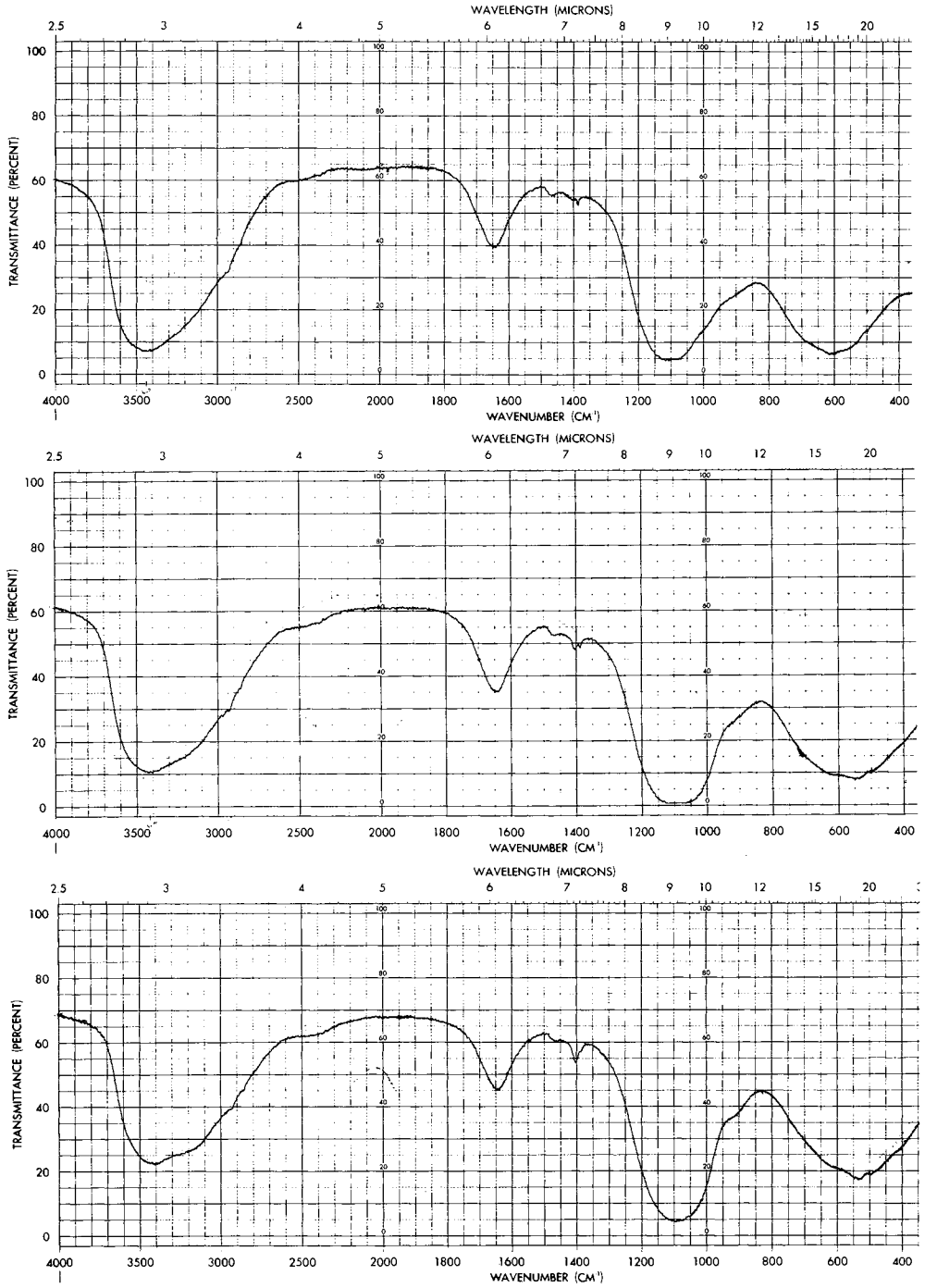

Fig. 8. Infrared spectra of alum-precipitated adjuvants which were precipitated in various concentrations of a phosphate buffer. Top, 0.25 phosphate:aluminum; middle, 0.5 phosphate:aluminum; bottom, 1 phosphate:aluminum.

At a ratio of 0.50, the small bands are replaced by slightly better defined bands at 1405 and 1390 cm^{-1}. When the phosphate to aluminum ratio was 1.0, a well-defined band at 1400 cm^{-1} becomes the dominant one. These changes suggest increasing phosphate inclusion in the structure. They could also be due to more ordered binding of phosphate to the gel.

All three infrared spectra in Fig. 8 show absorption at 1640 cm^{-1} in the O–H bending region. In the O–H stretching regions, the band at 3440 cm^{-1} moves to higher wavelengths with increasing phosphate, indicating stronger bonding of the lattice hydroxyls.

The X-ray diffraction pattern of an alum-precipitated adjuvant which was precipitated at a 1:1 molar ratio of phosphate to aluminum and aged at 70°C for 3 weeks failed to display any signs of crystallinity. Changes occurring during aging could not be detected by X-ray diffraction, possibly

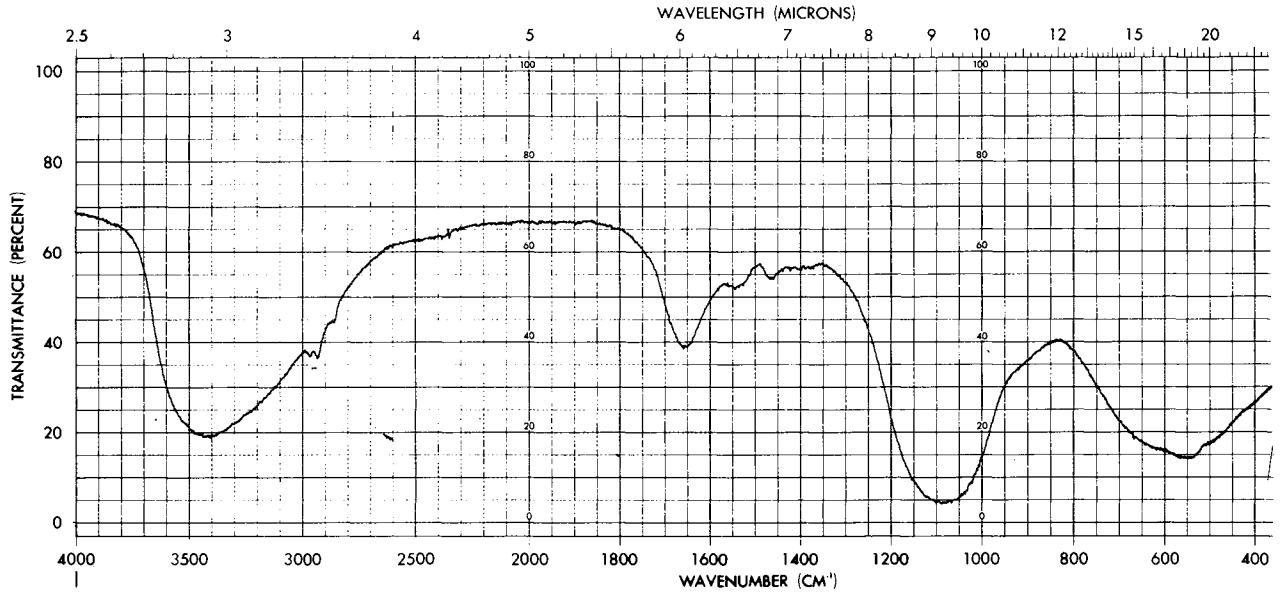

Fig. 9. Infrared spectrum of commercial diphtheria and tetanus toxoid, U.S.P., labeled as an alum-precipitated toxoid in an isotonic sodium chloride solution containing sodium phosphate to control the pH (vaccine A).

due to the fact that detection of order by this method depends on the regular successive stacking of the structural units (21).

Cheung *et al.* (22) studied the structure of aluminophosphates coprecipitated from solutions in which the phosphate-to-aluminum ratio was less than one by means of X-ray diffraction and high-resolution solid-state NMR spectroscopy using both ^{27}Al and ^{31}P nuclei. They concluded that the materials were not simple coprecipitated mixtures of Al_2O_3 and $AlPO_4$; no evidence for the presence of either species was detected. They appear to be amorphous structures in which the phosphate is randomly dispersed, and the aluminum exists in one octahedral and several different tetrahedral environments.

Transmission electron micrography of the alum-precipitated adjuvants which were precipitated in the presence of phosphate buffer reveals the presence of a continuous platy network. The morphology is similar to that of the aluminum phosphate adjuvant shown in Fig. 4. An energy-dispersive spectrum of these adjuvants shows the presence of both aluminum and phosphorus. These adjuvants are best described as amorphous aluminum hydroxyphosphate sulfate.

The alum precipitation experiments stress the importance of the possible effects of buffer ions on the structure of the aluminum adjuvant. Rigorous controls must be applied in order to manufacture adjuvants having consistent structures and physicochemical properties.

Commercial Vaccines. The aluminum-containing adjuvant in three commercial diphtheria and tetanus toxoids, U.S.P., identified as vaccines A (Connaugh), B (Sclavo), and C (Wyeth), was studied. Vaccine A was labeled as an alum precipitated toxoid in an isotonic sodium chloride solution containing sodium phosphate to control the pH. The X-ray diffractogram of vaccine A was amorphous. The infrared spectrum (Fig. 9) shows an absorption band at 550 cm^{-1}, possibly due to sulfate inclusion, and a large absorption band at 1100 cm^{-1}, indicating phosphate inclusion.

Transmission electron microscopy of vaccine A reveals a platy network similar to that of the aluminum phosphate adjuvant shown in Fig. 4. Energy-dispersive spectrometry confirms the presence of aluminum and phosphorous. Since the only identified source of phosphate in the vaccine was the sodium phosphate used to control the pH, it is hypothesized that the adjuvant in vaccine A is similar to the amorphous aluminum hydroxyphosphate sulfate produced when the alum precipitation was carried out in a phosphate buffer.

Vaccine B is labeled as a sterile suspension of toxoids adsorbed by aluminum hydroxide. The X-ray diffraction pattern of vaccine B shows the characteristic diffraction pattern of boehmite, i.e., 6.46, 3.18, 2.35, 1.86, 1.44, and 1.31 Å. The infrared spectrum is similar to Fig. 2. The band at 1070 cm^{-1} and the shoulder at 3100 cm^{-1} confirm the presence of boehmite. The transmission electron photomicrograph is similar to Fig. 3. Energy-dispersive spectrometry indicates only the presence of aluminum. Thus, the adjuvant in vaccine B is boehmite similar to adjuvants A–G.

Vaccine C is labeled as aluminum phosphate adsorbed. The X-ray diffraction pattern indicates an amorphous material. The infrared spectrum is similar to that of adjuvant H shown in Fig. 5, having a large band at 1100 cm^{-1} indicating phosphate and a broad OH-stretching band around 3400 cm^{-1}. The morphology is similar to that of the network of platy particles shown in Fig. 4. Energy-dispersive spectrometry indicates the presence of aluminum and phosphorous. The adjuvant in vaccine C is thus an amorphous aluminum hydroxyphosphate similar to adjuvants H and I.

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