hepatitis patients were also inactivated by these treatments.

From these studies, the nature of hemagglutinins against *Macacus rhesus* erythrocytes found in the serum of hepatitis patients appears to be 19 S antibody. The macroglobulin nature of this agglutinin was further confirmed when it was detected in the first cut of serum passed through the column of Sephadex G-200(5).

Detailed studies are under way exploring the hypothesis that the *Macacus rhesus* hemagglutinins are actually autoimmune antibodies.

Summary. Agglutinin titers for *Macacus rhesus* erythrocytes were higher in sera of infectious hepatitis patients than those of normal persons. The agglutinin was found to be a macroglobulin antibody (19 S), as shown by ultracentrifugation, zone electrophoresis, gel-filtration and sensitivity to heating and mercaptoethanol.

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Effect of Condensed Phosphates on Vitamin D-Induced Aortic Calcification in Rats.* (31269)

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It has been shown(1) that inorganic pyrophosphate and longer chain condensed phosphates inhibit calcium phosphate precipitation *in vitro* at concentrations as low as 10^{-6} M. Subsequently pyrophosphate was found to be a normal constituent of urine(2) and plasma(3), and it was suggested that this substance might be of importance in preventing collagen and other nucleating substances from calcifying(3,4). In favor of this hypothesis was the finding that pyrophosphate inhibited mineralization of chick embryo femurs grown in tissue culture(5). Further work(6,7) showed that pyrophosphate or polyphosphates inhibit calcification of the aorta induced by massive doses of vitamin D *in vivo*. In the present study we have attempted to investigate the mechanism of this inhibition of aortic calcification in more detail.

Material and methods. The experimental design was based on that of Gillman *et al*(8) who obtained heavy calcification in the aortas of rats after dosage with a large amount of vitamin D_2. Forty-eight female Wistar rats, weighing from 200 to 250 g and fed stock rat chow (Altromin, Lage, Germany) throughout, were divided into two groups. One group received daily subcutaneous injections of Graham salt, a long chain polyphosphate (J. A. Benckiser, Ludwigshafen/Rhein, Germany) in saline at pH 7.4, given at a dose level of 10 mg P/kg body wt, over the 15 days of the experiment. The other group did not receive Graham salt. From the third to the seventh days inclusive, both groups were

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given 75,000 i.u./kg body wt of vit D_3 (Wander AG, Berne, Switzerland) per day by stomach tube. Two rats were taken for examination daily from each group, 24 hours after beginning vit D and on each succeeding day. Since 4 rats in the group receiving Graham salt died before the end of the experiment, only one could be taken on each of the last 4 days.

To study the earliest histological changes occurring during the development of the vitamin D-induced aortic calcification, a further 25 rats were treated as above and 6 were killed at 36 hours, 6 rats at 48 hours, 6 rats at 60 hours and 7 rats at 72 hours after the last dose of vit D. These animals received no Graham salt.

Before killing, blood was withdrawn from the heart under ether anesthesia. Plasma was analyzed for total calcium by EDTA titration with calcein as indicator. The thoracic aortas were removed and part of each was lyophilized, weighed, ashed with H_2SO_4, HCl and H_2O_2 and analyzed for calcium. The other part of each aorta was examined histologically with the following techniques: hematoxylin and eosin for general histology, periodic acid-Schiff (P.A.S.), and alcian blue with chlorantine fast red as a counter stain for mucopolysaccharides, Krutsay's(9) modification of the von Kossa method for calcification and Sudan black for lipids(10). The tissues for the P.A.S., alcian blue and Krutsay methods were all embedded in paraffin and could thus be taken from the same area of the aorta. Since the embedding for Sudan black staining cannot be done in paraffin, another part of the aorta had to be used; however, an attempt was made to choose these two areas as close as possible.

Results. Symptomatology. As in previous experiments(7) the clinical symptomatology induced by vit D was not influenced by the condensed phosphates. The animals looked sick, stopped eating and lost weight. The death rate was somewhat higher in the polyphosphate-treated group.

Blood chemistry. Hypercalcemia developed in both groups 3 days after starting vit D, the highest calcium values reaching a level of 16 mg per 100 ml after about 6 days. Twelve days after starting vit D, the blood calcium had returned to a normal value. In general, the hypercalcemia was slightly less in the animals receiving Graham salt.

Aorta histology. The first pathological sign was the appearance of edema and nuclear disorientation which began to appear in the aortas of the animals receiving vit D only, 3 days after beginning vit D treatment. Calcification began on the elastic fibers 6 to 7 days after the beginning of vit D, and became more intense with time. The calcified parts of the aortas usually showed a positive staining with Sudan black, P.A.S., alcian blue and silver nitrate. Positive staining with all these 4 stains seemed to appear at the same time (Fig. 1). It could not be determined, even when analyzed every 12 hours, which of the various stains appeared first. The spatial correlation was very good between silver nitrate and P.A.S. and alcian blue, less good with Sudan black. This could be explained by the fact that different parts of the aorta had to be taken for staining calcium phosphate and lipids, but that, on the other hand, it was possible to stain adjacent sections with $AgNO_3$, P.A.S. and alcian blue.

Although the location of the various stains was broadly the same, there was, however, some difference in the specific tissues stained. Thus Sudan black stained the elastic fibers, and this as far as could be seen in exactly the same place that had begun to calcify. In contrast, the P.A.S. and alcian blue reactions were between the elastic fibers. The staining with P.A.S. seemed to spread from the edges of the elastic fibers, which already normally stain strongly, to the interelastic area, leaving out the elastic lamellae, which could be seen coursing unstained through the stained masses. The same was seen with alcian blue, except that normally the edges of the elastic fibers were not stained by this method.

The aortas of the rats which received Graham salt in addition to vit D showed the same edema and nuclear disorientation 4 days after vit D had been started. These were, however, the only changes observed. The calcification and staining reactions with Sudan black, P.A.S. and alcian blue described above

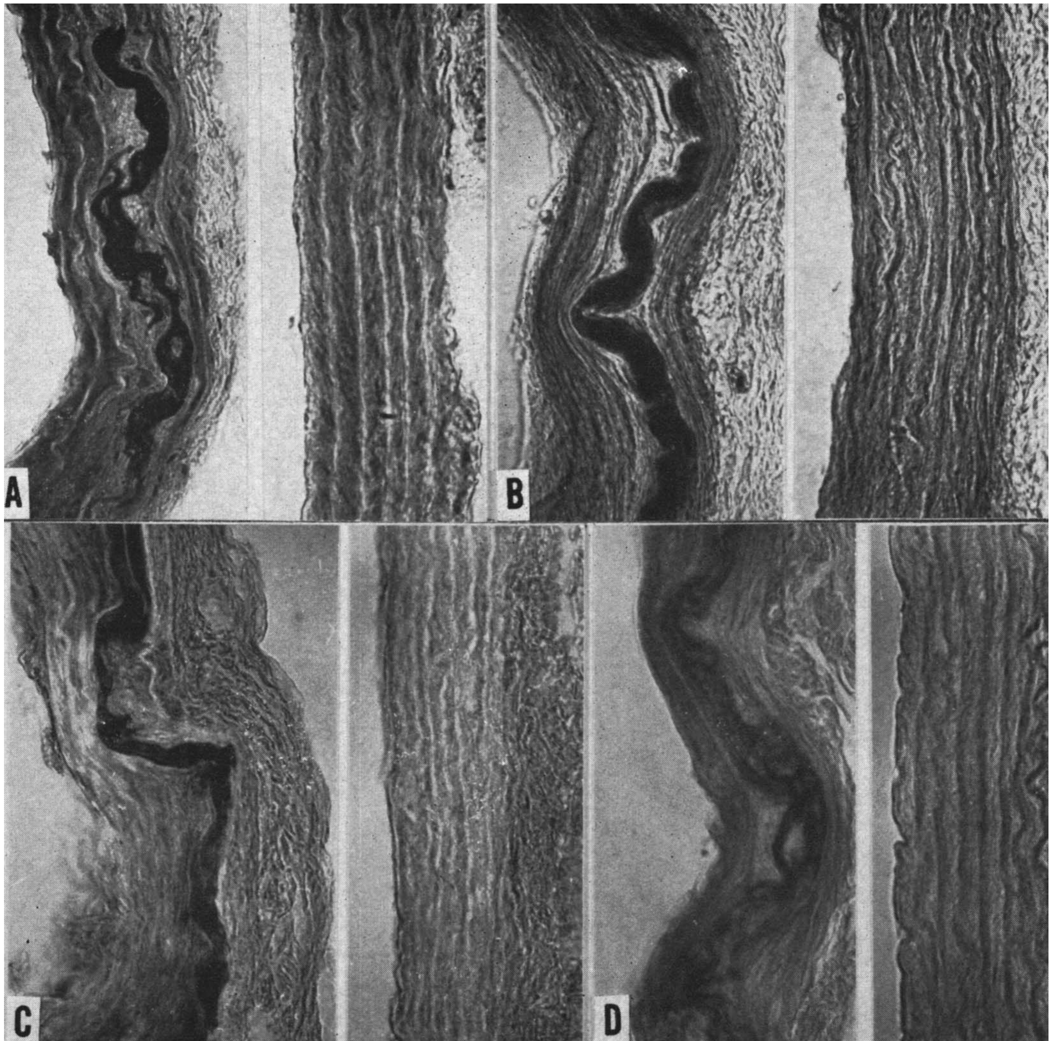

FIG. 1. Photomicrographs on left are of the aorta of a rat which was killed 7 days after beginning treatment with vit D. Figures on right are from a rat treated in the same way with vit D, but which also received Graham salt throughout experiment. Note complete inhibition of all the stains following treatment with Graham salt. A, von Kossa; B, Sudan black; C, Alcian blue; D, P.A.S. Magnification ×273.

were uniformly absent (Fig. 1).

Aorta chemistry. Five control rats receiving no vit D were found to have 1.2 to 3.1 γ calcium per mg of dry weight. The calcium levels in the aortas of the rats receiving vit D only remained at approximately the control level till the 5th day after starting vit D. At this time the values rose rapidly, and attained a maximum level of 48.0 γ/mg 2 days later. The calcium levels of the aortas of the rats given Graham salt were nearly all in the normal range. The Graham salt had thus almost completely prevented the aortic calcification caused by vit D.

With 2 exceptions, good correlation was found between the calcium content of the aortas determined chemically and the degree of silver uptake.

Discussion. These results clearly confirm our earlier work that ectopic calcification can be prevented *in vivo* by polyphosphates(6,7). In addition they show that the changes in the organic matrix associated with calcification are also suppressed. Since this finding

may cast some light on the sequences of calcification in general, it is worthwhile to consider in more detail the changes observed in the animals of both groups.

One explanation of the inhibiting action of condensed phosphates could be that they prevent vit D from acting. However, hypercalcemia occurred in all animals which received vit D, whether they had Graham salt or not. The few results of this work are supported by current investigations on a larger scale showing that administration of Graham salt does not suppress the vitamin D-induced increase of either ultrafiltrable or ionized calcium, or the increase of inorganic phosphate in the blood(7). It seems thus unlikely that the suppressive action of the condensed phosphates works through changing the Ca $\times$ P product in the blood. It is interesting to note that aortic calcification can apparently be obtained after treatment with vit D without any accompanying hypercalcemia(11).

A second explanation could be that condensed phosphates prevent the local action of vit D which renders the elastic fibers or other proteins calcifiable. The fact that vit D caused edema and cellular changes in the aortic well of all the experimental animals, whether they calcify or not, indicates that vit D did have some action on the aortas, even in the presence of Graham salt. On the other hand, the other changes of the matrix, *i.e.*, the appearance of a positive P.A.S., alcian blue and Sudan black stain, which are said to develop always at calcification sites(10,12,13), were prevented by administration of Graham salt. If these changes are the primary event in the calcification process, as is often thought(13,14), the lack of mineral deposition could be explained by a direct action on the calcifiable matrix. However, we see no explanation of how condensed phosphates could block the formation of such a matrix, the only known property of these compounds which could account for a decreased calcification being that of inhibiting crystal formation, probably due to adsorption of the compounds onto crystals or nuclei surfaces(1,15). It might thus be possible that the matrix change is actually a secondary event, following the calcification step, and not as usually suggested the primary one. Thus a treatment with condensed phosphates inhibiting crystal formation would necessarily prevent the matrix alterations. This might be the explanation for the prevention of the P.A.S. and alcian blue reaction. This explanation is supported by our findings that P.A.S. and alcian blue stainings did not occur on the tissues which calcified first, the elastic fibers, but between them. Furthermore workers who have induced renal calcification(16) with vit D suggest that the appearance of mucopolysaccharides is actually a consequence and not a cause of ectopic calcification.

It is also possible that the matrix changes are primary but can develop to an extent visible in the microscope only if mineral deposition is taking place. If the latter is inhibited, for example with condensed phosphates, only a very small, histologically non-detectable, calcifiable matrix would be formed. This might be the case for the sudanophilic material. Indeed, as Irving has reported, sudanophilic material, almost certainly some form of lipid, and present in a masked form in tissues which normally calcify, always appears where calcification is in progress, both in tissues such as bone and teeth(10) and in pathological calcifications such as calciphylaxis(17). In our experiments it also developed where calcification started, *i.e.*, on the elastic fibers.

Which of these hypotheses is correct is not clear. Attempts to compare the first appearance of the various staining methods were unfortunately not successful, even in the earliest stages of calcification; all 4 stains, silver nitrate, Sudan black, alcian blue and P.A.S. seemed to appear simultaneously. However, the histochemical methods at present available are too crude to detect small differences.

Summary. Ectopic calcification of the aortas of rats was induced by large doses of vit D_3. The calcification was accompanied by sudanophilia of the elastic fibers, where the calcification started, and by the presence of mucopolysaccharides between the fibers, as indicated by P.A.S. and alcian blue stains. Concurrent subcutaneous administration of condensed phosphates prevented the calcification as assessed by chemical determination of calcium and by silver nitrate staining. The

development of the various matrix stains was inhibited as well. The most likely mechanism of this inhibition is thought to be the prevention of the formation of calcium phosphate crystals by the condensed phosphates. Possible mechanisms of the inhibition of the matrix changes are discussed.

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Inhibitory Effect of Cysteine on *Streptomyces griseus* Phage Reproduction.* (31270)

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The mechanism(s) whereby the amino acid cysteine inhibits the growth of bacteriophage deserves further clarification. The evidence presented by Spizizin *et al*(10) with the T2r+ phage of *Escherichia coli* B favored an interference mechanism in which cysteine was postulated as binding certain essential metal ions. Joklik(8) suggested a mechanism involving the release of mature phage. With the lambda phage of *E. coli* K-12, Gots and Hunt(6) hypothesized that cysteine interfered with the biosynthesis of threonine.

The present report on the cysteine effect with the *Streptomyces griseus* phage-host system(5) favors an interference mechanism centering on overall phage protein biosynthesis.

Materials and methods. S. griseus strain 3475 (Waksman) was used as the phage host for all experiments. Spores for inocula were harvested from 5-day-old slants grown on glycerol-asparagine agar and suspended in 0.25% peptone. The spores were filtered through gauze to remove mycelial debris and diluted to 20% light transmittance at 630 mμ. This value corresponded to approximately 5×10^8 spores/ml by plate count on glucose nutrient agar. Prior to each experiment, the spores were diluted in the following synthetic medium (CSM) to a cell count of 1×10^8/ml: glucose, 0.5%; $(NH_4)_2HPO_4$, 0.2%; K_2HPO_4, 0.1%; $CaCl_2$, 0.001 M; glutamic acid, alanine, and aspartic acid, 250 μg/ml; arginine and lysine, 100 μg/ml; valine, isoleucine, leucine and histidine, 50 μg/ml.

Phage strain 514-3 isolated by Gilmour and Buthala(4) and specific for *S. griseus* 3475 was maintained in 0.25% peptone at 2-3°C. A high titered phage stock was obtained by the soft agar layer method described by Adams(1). For experimental purposes,

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