lecithin, sphingomyelin, and lysolecithin, only lysolecithin moved completely from the point of application. In 2-dimensional runs with different solvent systems, lecithin and sphingomyelin left origin spots at both first and second separations (Fig. 1). The relatively pure lecithin (dipalmitoleyl-glycerylphosphorylcholine) and lysolecithin (β-monostearoyl-glyceryl-phosphorylcholine) were gifts of Dr. D. J. Hanahan. The faintness of the sphingomyelin spots indicated that only slight amounts were bound at the origin. This was found to be 2% in one analysis in which 1.20 μg sphingomyelin phosphorous were applied. However the intensity of the lecithin origin spots warranted special analysis of the effect of varying the amounts of phospholipid applied.

Table I gives percentage recovery of lecithin at the origin in runs in which varying amounts of lecithin were applied in a mixture containing equal amounts of pure lecithin, sphingomyelin, and lysolecithin phosphorus (P). At least 10%, and as much as 30% of the lecithin was left at the origin when 1.20 μg of lecithin P (29 μg lecithin) or less were applied to the paper. Approximately 7% was left at the origin when 2.40 μg lecithin P (58 μg lecithin) or more were applied.

A means of avoiding this loss at the origin is provided by the technic of "running start" chromatography. Since on regular Whatman #1 filter paper, with the solvents used, all lipid material travels with or close to the solvent front, papers have been prepared so the application of material is made on an unimpregnated area of the filter paper. The applied material travels 2.0-2.5 cm before reaching the silicic acid impregnated portion where separation of the individual phospholipids begins. No Rhodamine G staining material is left at the origin, and separations of individual compounds are favorably affected in shape, so delineation of the boundaries of the spots is more definite. Fig. 2 contrasts the "running start" method with the standard application to silicic acid impregnated paper. The component traveling with an R_f between lecithin and lysolecithin is sphingomyelin. It gives a positive choline test and has the same R_f as pure sphingomyelin prepared from egg yolk by the method of Rhodes and Lea(4). Our recoveries of lecithin have improved 7-10% by the use of "running start" chromatography with loadings up to 5 μg of lecithin P. Repeated applications of extracts may be made, as the variation in the area of loading is not critical. Our recoveries of lysolecithin and sphingomyelin have been essentially the same with or without the "running start" procedure. The technic may have application in other areas where fixation of compounds may occur.

Summary. A technic is described for avoiding loss of lecithin at point of origin during quantitative separation of phospholipids on silicic acid impregnated paper.

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Effect of Drugs on Cx-Protein Responses in the Rabbit.* (26160)

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Availability of appropriate blocking agents commonly provides a valuable tool in studies of sites of action of drugs and sequences of biochemical and physiological reactions. Studies directed toward an elucidation of the role of C-reactive protein, which appears during inflammatory episodes, would be aided if

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blocking agents for formation of C-reactive protein were available. Cortisone and salicylates(1,2) have been reported to be ineffective or only partially effective in this regard while extensive blockade of the reticuloendothelial system with colloidal thorium dioxide (Thorotrast) has been reported to depress the Cx-reactive protein (CxRP) response initially provoked by intravenous injection of Thorotrast in the rabbit(3). In this study a number of substances were screened for their ability to prevent the appearance of CxRP in rabbits. One of the active compounds, fluorometholone, was examined for its ability to block production of CxRP triggered by various stimuli, and some data were obtained with regard to the relationship of CxRP production to antibody formation.

Methods and materials. The hybrid albino male rabbits weighing 2 to 3 kilos were maintained on a diet of Purina rabbit chow and water, *ad libitum*. CxRP antiserum (CxRPA) was obtained from guinea pigs immunized with CxRP isolated from acute phase serum of adjuvant-treated rabbits(4). Samples of blood were obtained from the marginal ear vein of the experimental rabbits before and at various intervals after injection of test materials. Serum CxRP was assayed on dilutions of serum using a capillary precipitin test(5). CxRP levels are reported as the highest dilution giving a positive test with antiserum. To screen various substances for their ability to block the appearance of CxRP, the test substance was suspended in 1 ml of mineral oil and incorporated into 4 ml of ointment base (Aquaphor); and 2.5 ml of the drug-adjuvant mixture injected subcutaneously into pairs of rabbits. CxRP levels in serum were determined 24 hr after injection. Incomplete adjuvant alone elicited a good CxRP response 24 hr after injection and at the same time provided a suitable depot for the test substances. To test the ability of fluorometholone to block CxRP appearance after various stimuli, fluorometholone was injected subcutaneously in a 1% suspension of carboxymethylcellulose in physiological saline, a vehicle which did not usually provoke a CxRP response by itself. The particular stimulus to CxRP production was generally applied immediately after injection of the fluorometholone suspension. Whole body radiation, applied as one stimulus(6) in doses of 250 r, was administered from above and below by 2 X-ray machines operating simultaneously. The technical factors were: 250 kvp, 15 ma; FOD 100 cm; filters 0.21 mm Cu inherent, 0.5 mm Cu parabolic, and 1.0 mm Al; 14 to 17 r/min, measured in air. Seromucoid prepared from 24 hr post-irradiation (500 r, whole body) rabbit serum(7) was applied as another stimulus since it has been found to provoke a CxRP response in contrast to normal seromucoid(8). It was given in doses equivalent to 20 ml of post-irradiation serum. Finally fluorometholone, N-palmitoyl-2-aminoethanol and D,L-*erytho* sphingosine were examined for their ability to block the CxRP response to intravascular formation of antigen-antibody complexes(9). For this purpose rabbit serum from animals immunized with bovine serum albumin (BSA) was fractionated on DEAE cellulose to give the anti-BSA containing γ-globulin (preparation II(9)). A dose of 10 mg of this anti-BSA γ-globulin in 1 cc saline was injected intravenously followed immediately by a solution containing 10 mg of BSA. In experiments on the relationship of CxRP production and antibody production, 1 mg of tobacco mosaic virus (TMV) was injected intravenously into rabbits in which 1 mg/kg of fluorometholone was injected 72, 24 and 4 hr prior to and 4 and 7 hr after injection of TMV. Serum levels of anti-TMV were determined and the level expressed as the highest dilution of serum giving a detectable interfacial precipitation in a ring test with TMV. TMV, employed as an antigen, and as a trigger for CxRP appearance was strain U-2 or U-7 as indicated, which were kindly supplied by Dr. Irving Rappaport. Streptokinase streptodornase (Varidase)† was given intravenously in doses of 5000 units. Thorium dioxide as a colloidal 24-26% suspension (Thorotrast)‡ was injected intrave-

† Obtained from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

‡ Thorotrast was obtained from Testagar & Co., Inc., Detroit, Mich.

TABLE I. 24 Hour CxRP Response to Various Anti-inflammatory Compounds Incorporated in Adjuvant.

Compound*	Dose/rabbit, mg	24 hr CxRP response†
D,L-*erythro*-sphingosine	7.5	1:4, 1:16, 1:32
D,L-sphingine	7.5	1:4, 1:8, 1:16
Hydrocortisone	10.	1:2, 1:2, 1:4
N-palmitoyl-2-aminoethanol	7.5	neg., neg., 1:4
Methyl prednisolone	2.	neg., 1:1, 1:1
Fluorometholone	1.	neg., neg., 1:1

* Compounds were supplied by Dr. Paul O'Connell of The Upjohn Co.

† All rabbits were negative pre-injection. Responses are recorded for individual rabbits.

nously in doses of 2 cc/kg.

Results. The results of screening various substances for their ability to block a CxRP reaction 24 hours after stimulation with adjuvant are presented in Table I. Adjuvant alone in controls provided a good stimulant of reasonably consistent high serum levels of CxRP. In rabbits treated with adjuvant alone 12 of 15 gave CxRP titers of 1:8 or 1:16 24 hours after injection. The remaining 3 gave responses of 1:1, 1:2 and 1:4. The probability of recognizing appreciable blocking activity is therefore high in tests of a compound in a restricted number of animals in which adjuvant is used as a stimulus for CxRP. D, L-*erythro*-sphingosine and D, L-sphingine displayed no appreciable activity at the levels of drug employed. Hydrocortisone possibly showed some activity, while N - palmitoyl - 2 - aminoethanol and particularly methylprednisolone and fluorometholone showed appreciable activity at dose levels selected. No attempt was made to explore a range of doses for the inactive or weakly active substances since fluorometholone and methylprednisolone evidenced the type of activity we were seeking.

A somewhat higher dose, 0.4-0.5 mg/kg, was chosen in an examination of the CxRP blocking effect of fluorometholone after various stimuli shown in Table II. Fluorometholone at this dose level was ineffective in blocking the response to Thorotrast and to 250 r of whole body irradiation. It was partially effective in blocking the response to injection of 5000 units of Varidase, and was most effective in blocking the response to injection of acute phase seromucoid, TMV, and to the intravascular formation of antigen-antibody complexes.

It was noted that the rate of appearance of anti-TMV was delayed in those rabbits which were challenged with TMV in presence of fluorometholone. Formation of new anti-BSA following immune elimination was also delayed in animals challenged with BSA after passive transfer of anti-BSA. These latter observations indicated that fluorometholone, like cortisone(10), possessed the ability to block or delay antibody formation.

As it has been reported that a rabbit's ability to form high titered antibody correlates with its ability to produce elevated serum levels of CxRP(11), it was of interest to note whether fluorometholone blocked both of these capabilities in parallel.

Rabbits given TMV intravenously responded in 24 hr with a marked CxRP response and had a maximum anti-TMV titer of 1:128 on the 7th day (Fig. 1). Rabbits treated with fluorometholone 72 or 24 hr before or 7 hr after TMV gave maximum anti-

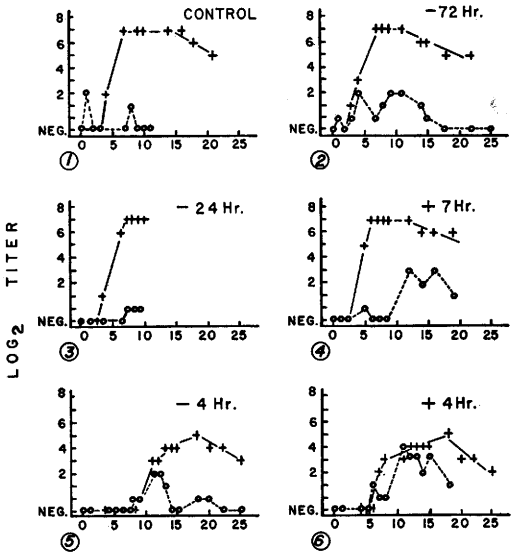

FIG. 1-6. CxRP (○−−○) and anti TMV (+—+) titers of rabbits following intrav. inj. of 1 mg of TMV at time 0 and a single intramusc. inj. of 1 mg/kg of fluorometholone at various times before or after TMV. (1) Control given no fluorometholone. (2) Fluorometholone given 72 hr before TMV. (3) Fluorometholone given 24 hr before TMV. (4) Fluorometholone given 7 hr after TMV. (5) Fluorometholone given 4 hr before TMV. (6) Fluorometholone given 4 hr after TMV.

TABLE II. Effect of Agents on CxRP Responses to Various Stimuli.

CxRP stimulus	Drug*	CxRP response 24 hr after stimulation†	
		Control (no drug)	Experimental
250 r whole body irradiation	Fluorometholone	1:8, 1:16, 1:16, 1:16	1:2, 1:4, 1:8, 1:8
Thorotrast	"	1:8, 1:32	1:8, 1:16
Varidase	"	1:2, 1:4, 1:8	neg., 1:1, 1:2, 1:4
Radiation seromucoid	"	1:4, 1:16, 1:32, 1:32	neg., 1:1
Tobacco mosaic virus (strain U-7)	"	1:8, 1:8, 1:16, 1:32	neg., 1:1
Passive intravascular antigen-antibody complex formation	"	1:4, 1:8	neg., neg.
Idem	N-palmitoyl-2-aminoethanol	1:4, 1:16	1:8, 1:8
"	D,L-*erythro*-sphingosine	1:4, 1:16	1:8, 1:8

* Fluorometholone was given subcut. at time of stimulation of CxRP response in a dose of 0.3-0.5 mg/kg. N-palmitoyl-2-aminoethanol was given the same way in a dose of 3 mg/kg 3 hr before and at time of stimulation and D,L-*erythro*-sphingosine was given in a dose of 10 mg/kg 6 hr before and at time of stimulation.

† All rabbits were negative pre-injection. Responses are recorded for individual rabbits.

TMV titers of 1:128 on the 6th or 7th day after TMV, as the controls, but *unaccompanied by a CxRP response* (Fig. 2, 3, 4). Rabbits given fluorometholone 4 hr before or 4 hr after TMV also showed no CxRP response but maximum anti-TMV titer reached was only 1:32 on the 18th day (Fig. 5, 6). The rabbits in which the CxRP response was blocked subsequently showed CxRP responses at a time when the effect of the fluorometholone had worn off.

Discussion. The sphingosines have previously been reported to have anti-inflammatory activity(12,13). It is of interest, therefore, that this was not reflected in the CxRP responses measured under our conditions. In a study by Ganley *et al.*(14) N-palmitoyl-2-aminoethanol exhibited no effect in the cotton pellet and capillary permeability (Evan's blue) tests, although anti-inflammatory activity in Coburn's guinea pig joint anaphylaxis test was noted. In contrast, we noted that N-palmitoyl-2-aminoethanol moderated the CxRP response stimulated by adjuvant but not by intravascular formation of antigen-antibody complexes. The doses used were somewhat higher than those in which they have shown activity in other tests.

Reduction of the acute phase response and attendant disappearance of C-reactive protein has been observed many times clinically in treatment of collagen diseases with ACTH, cortisone, salicylates or aminopyrine. In contrast, cortisone and salicylates have not shown appreciable activity in experiments with rabbits(1,2). The reduction of CxRP levels obtained on stimulation with adjuvant with incorporated hydrocortisone, methyl prednisolone or fluorometholone can probably be attributed to the greater anti-inflammatory activity associated with these drugs(16).

The inability of fluorometholone to block CxRP responses to X-irradiation and Thorotrast in distinction to the activity displayed against other stimuli could be attributed to some difference in site of action on alternate pathways or target organs which each lead to CxRP appearance. It could equally well be attributed to a large difference in the intensity of the inflammatory processes elicited by the various stimuli. Data are not available to support either contention although the latter is now susceptible to experimental test with the availability of a blocking agent. It will be desirable to ascertain this since determination of CxRP responses is relatively rapid and straight forward and could possibly supply a useful adjunct to other methods employed in screening of potential anti-inflammatory compounds. Hill(17) has previously made a similar suggestion.

Wood(11) has noted a useful correlation in that rabbits that produce high levels of CxRP in the serum on immunization with foreign proteins produce good levels of antibody and that rabbits that respond with a

poor CxRP response produce smaller amounts of antibody. A possible causal relationship was pointed out. Subsequently Good(15) noted that individuals with either congenital or acquired hypogammaglobulinemia, including the most severe cases, were capable of giving a number of normal acute phase responses, including a C-reactive protein response. He suggested that formation of C-reactive protein is not directly linked with formation and liberation of circulating antibody. In the same vein, we have noted that doses of X-irradiation sufficient to inhibit antibody synthesis do not alter the ability of the rabbit to synthesize CxRP(6). Since we have now found that the CxRP response to injected TMV can be prevented without modifying the course of anti-TMV production as measured in the circulating blood, it would appear that CxRP does not stand in a precursor relationship to circulating antibody or its production.

Two further points may be made from data presented in Fig. 2-6. First from the duration of action of fluorometholone on CxRP and antibody production, it is seen that fluorometholone more effectively blocks Cx RP than antibody formation and that it inhibits antibody production when given as late as 4 hours after antigen, behaving in this regard as does cortisone(10). The lack of CxRP response in one rabbit given the steroid 7 hours after TMV suggests that the process leading to appearance of CxRP can be blocked after many of the events which would otherwise trigger its appearance have already transpired.

Summary. Methyl prednisolone and fluorometholone prevented the appearance of CxRP in rabbit serum following subcutaneous injection of mineral oil-Aquaphor emulsion. Fluorometholone was unable to block CxRP responses to 250 r whole body irradiation or to intravenous injection of Thorotrast or Varidase. It did block CxRP responses to intravenous injection of post irradiation seromucoid or TMV and the intravascular formation of antigen-antibody complexes. Under appropriate time dose conditions fluorometholone inhibited both antibody production and CxRP appearance while under other time dose conditions CxRP appearance was prevented without alteration of antibody production.

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