smaller amounts of activity in the microsomes and mitochondria. There was no evidence for a separation of hydrolytic and esterifying activity and hence, no evidence for 2 separate enzymes catalyzing the 2 different processes of esterification and hydrolysis.

The occurrence of a major part of the esterase activity in the soluble fraction and nuclei-cell debris also suggests that esterification occurs in the mucosal cells rather than in the brush border. It is likely that cholesterol passes through the brush border and enters the mucosal cells in the free form(1). Then, the cholesterol is esterified within the mucosal cells and rapidly passes into the lymph as a part of the chylomicron(1).

Summary. A modified procedure for brush border preparation has been developed which gives 90-100% yields of invertase activity in the brush border. It was determined that the esterifying and hydrolytic activities of cholesterol esterase are concentrated in the supernatant fraction of the mucosa as opposed to the brush border. Further fractionation of the mucosal cells into nuclei and cell debris, mitochondria, microsomes, and soluble fraction, and assay of these fractions for esterase activity demonstrated that 60-66% of the esterase activity of the whole homogenate was in the soluble fraction. The remaining activity was distributed among the other fractions. The data suggest that esterification of cholesterol occurs in the mucosal cell rather than in the brush border. Cholesterol levels in rat intestinal wall, intestinal mucosa, and mucosal subcellular fractions are also reported.

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Immunological Specificity of the Secondary Response with Dinitrophenylated Proteins.* (28589)

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Haptens may be coupled to different carrier proteins to produce complete antigens. If the antibodies are assayed with an antigen made from the hapten coupled to an unrelated carrier protein, only the anti-hapten specificity is tested. Taking advantage of this principle, the role of the carrier protein in the secondary response may be examined. Employing dinitrophenylated proteins, it was seen that a secondary response was produced only with the same hapten-carrier protein combination as used for the primary immunization.

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Materials and methods. 1. *Animals:* Random-bred albino rabbits weighing approximately 3-4 kg were used for immunization. Hartley-strain albino guinea pigs, 250 ± 50 g, were used for passive cutaneous anaphylaxis (PCA) reactions.

2. *Antigens:* 2,4-Dinitrophenylsulphonic acid (DNPS) (Eastman Kodak) was twice recrystallized from water and used for coupling to 3 different carrier proteins, *i.e.,* bovine gamma globulin (BGG) (Armour Pharmaceuticals, Lot #T 30203); crystalline bovine serum albumin (BSA) (Armour Pharmaceuticals, Lot #W 69312); crystallized egg albumin (EA) (Worthington Biochemical Corp., Lot #A 598).

3. Guinea pig complement lyophilized (Certified Blood Donor Service, Inc., Jamaica, N. Y.) was used for passive hemolysis.

4. Veronal buffer for complement fixation was prepared as described(1).

5. *Coupling:* A modification of the method of Eisen *et al*(2) was used. One g of anhydrous potassium carbonate was dissolved in 30 ml distilled water. One g of protein was then dissolved in the carbonate solution. Two g of DNPS were dissolved in 20 ml distilled water and then mixed to the protein solution and stirred at room temperature on magnetic stirrer for about 4 hours. The mixture was dialyzed against 4 liters of distilled water (pH adjusted with potassium carbonate to 7.8) for 5 days, changing the dialysis water every 12 hours.

The conjugated antigens were analyzed for protein content by the micro-Kjeldahl method and the number of DNP groups estimated from the optic density at 360 mμ using 17,400 as extinction coefficient.

Two of the preparations were used for immunization, *i.e.*, dinitrophenyl bovine gamma globulin (DNP-BGG) with 46 groups per mole, and dinitrophenyl-egg albumin (DNP-EA) with 18 groups per mole. Dinitrophenyl bovine serum albumin (DNP-BSA) with 37 groups per mole was used to titrate serum antibody levels.

6. *Immunization:* Two experiments with 15 rabbits each were performed. All rabbits were immunized with intravenous injections of the antigens in saline. Each animal received one daily injection of 3 mg of antigen for 3 consecutive days. Sixteen days after the first injection, the animals were bled and received one booster injection of 5 mg of the antigen to be tested.

In the first series, the animals received DNP-BGG as primary antigen, and as secondary stimulant 5 animals received DNP-BGG, 5 received DNP-EA, and 5 received BGG (the carrier protein alone).

In the second series, the animals received DNP-EA as primary antigen, and as secondary stimulant 5 animals received DNP-EA, 5 received DNP-BGG, and 5 received EA alone.

Six or 7 days after the booster, all animals were bled and the sera assayed for anti-DNP antibody content.

7. *Antibody measurements:* Three methods were used to assay for antibody.

a. *Passive hemagglutination.* Tannic-acid treated sheep erythrocytes coated with DNP-BSA according to(3) were used. One ml packed, 3 times washed sheep erythrocytes were suspended in 40 ml of phosphate buffered saline, pH 7.2. This suspension was then added dropwise to 40 ml 1/40,000 dilution of tannic acid. After 10 minutes in 37°C water bath, the cells were centrifuged at 1500 rpm, washed once with buffered saline. 0.5 ml of the packed tanned erythrocytes was added to the DNP-BSA solution. This solution was prepared from a concentrated DNP-BSA preparation 10.5 mg/ml, 37 groups per mole. 0.25 ml of this preparation was added to 5 ml of pH 6.5, phosphate buffered saline. The mixture of erythrocytes — DNP-BSA — was gently shaken for 30 minutes at room temperature, then washed twice with pH 7.2 buffered saline and resuspended in 50 ml of pH 7.2 buffered saline to obtain a 1% suspension.

0.1 ml of these coated erythrocytes was used per tube, with 0.5 ml of antisera dilution. (The antisera dilutions were made in veronal buffered saline(1).)

Hand-made, round-bottomed homogeneous hemagglutination tubes (10 × 75 mm) (Scientific Glass Corp., Bloomfield, N. J.), were used. All antisera were inactivated at 56°C and absorbed twice with washed sheep erythrocytes (0.2 ml per ml of serum). They were all checked for absence of hemagglutination with tannic acid-treated, non-DNP-BSA-coated sheep erythrocytes.

The tubes were left overnight at the temperature of the laboratory and read the next morning. The results were marked from 0 to 4+ (according to the method described in (3)). Controls consisted of tannic acid-treated, non-coated sheep erythrocytes and DNP-BSA-coated erythrocytes mixed with normal rabbit serum dilutions.

b. *Passive hemolysis* was carried out according to(3). The same solutions which served for passive hemagglutination were also used for passive hemolysis. The cells were

TABLE I. Immunological Specificity of Secondary Response.

A. Primary Immunization with DNP-BGG.

Boosted with	Rabbit No.	Before			After		
		Agg	Lysis	PCA	Agg	Lysis	PCA
DNP-BGG	85	40*	0	100*	80*	10*	200*
	86	80	10	200	1280	160	1600
	87	40	±	100	160	40	200
	88	80	10	100	640	320	800
	89	80	10	50	640	160	400
DNP-EA	90	160	40	200	40	0	50
	91	80	0	100	0	0	0
	92	80	±	100	10	0	0
	93	80	10	10	10	0	0
	94	40	±	25	20	0	25
BGG	95	20	0	25	10	0	0
	96	80	40	100	160	20	25
	97	20	0	25	20	0	0
	98	320	80	200	160	40	100
	99	80	10	25	40	0	0

B. Primary Immunization with DNP-EA.

Boosted with	Rabbit No.	Before			After		
		Agg	Lysis	PCA	Agg	Lysis	PCA
DNP-EA	118	0	0	0	160	80	250
	119	0	0	0	5120	620	400
	120	0	0	0	620	80	250
	122	0	0	0	1280	80	400
EA	124	0	0	25	0	0	25
	125	0	0	25	0	0	25
	126	0	0	25	0	0	0
	127	320	0	25	40	0	25
DNP-BGG	128	20	0	25	20	0	0
	129	320	0	0	40	0	100
	130	0	0	0	0	0	0
	131	0	0	0	0	0	0
	132	0	0	0	0	0	0

Rabbits No. 121 and 123 died before second bleeding.
* Numbers refer to reciprocal of the highest serum titer showing antibody activity.

shaken and resuspended in each tube and 0.1 ml of a 1/15 dilution of complement (previously absorbed 2 times with packed sheep erythrocytes, 0.3 ml of erythrocytes/ml of complement) was added and the mixture incubated in a 37°C waterbath for 45 minutes. Hemolysis was estimated visually; the last dilution yielding close to 100% lysis was considered endpoint of the solution tested.

c. *Passive cutaneous anaphylaxis (PCA).* The sera were diluted by parallel dilutions and assayed as previously described(4). The latent period of sensitization was 4 to 5 hours and 500 γ of DNP-BSA per animal was used as challenging antigen. At least 4 animals were tested for each dilution and care was taken that the same dilutions before and after booster should be injected into the same animals on symmetrical sides, to decrease the effect of individual variations. As end point, the last dilution which gave a positive reaction was chosen. Sometimes, this last dilution did not give positive PCA reaction in every guinea pig, as occurs often with threshold doses in PCA reactions.

Results are presented in Table I. When DNP-BGG has been injected as primary antigen neither DNP-EA (the same hapten coupled to a different carrier protein) nor BGG (the homologous carrier protein alone) produced any increase of antibody titers against the DNP determinant. In a symmetrical situation, the rabbits initially immunized with DNP-EA showed an anamnestic

response only to DNP-EA and not to DNP-BGG or EA.

Discussion. The early literature on the specificity of the anamnestic response was reviewed by Dixon and Maurer(5), who studied quantitatively the anamnestic response to protein antigens. They observed that previous immunization with one antigen accelerates and magnifies the immune response to the first injection of another antigen only if the 2 antigens are related, and that subsequent immunization with one antigen may increase the amount of circulating antibody exclusively oriented to another antigen administered earlier only if the 2 antigens are related.

Gilden(6), using protein antigens in fowls, also found that only related antigens produced secondary response. Moreover, he showed that only the cross-reacting antibodies were increased whereas the antibodies specific for the primary antigen were in lower amounts and the antibodies specific for the second antigen were in the range of antibodies obtained in primary immunizations.

In view of the possibility of similar antigenic structures being present in related protein antigens, more precise information should be obtained using well-defined haptens as antigenic determinants coupled to different carrier proteins used as immunogenic "backbone." Such an approach was used by Salvin and Smith(7), who studied delayed hypersensitivity and rate of antibody production in guinea pigs. They confirmed earlier findings(8) that delayed hypersensitivity is directed against an unknown portion of the carrier protein besides the hapten, whereas antibody specificity is primarily directed toward the hapten. They also showed that the rate of antibody production against a heterologous hapten (for example, para-aminobenzoic acid) was accelerated when the primary injection was made with another hapten (*i.e.*, para-amino arsonic acid) provided the carrier protein was the same (hen egg albumin). No increase in rate of antibody production was observed when the homologous hapten (para-amino benzoic acid) had been injected previously coupled to an unrelated carrier protein (bovine gamma globulin). They also found an accelerated response when the primary injection was the carrier protein alone (hen egg albumin).

However, Salvin and Smith used guinea pigs and only the method of PCA for antibody determinations. Since in guinea pigs two different families of antibodies can be produced of which only the electrophoretically faster moving component (γ_1) can sensitize the guinea pig for PCA(3,9,10), the use of only one method (in this instance, PCA) might be insufficient.

In the present study, amount of antibody produced by secondary injection rather than rate of antibody production was examined in rabbits. It is clearly seen that the antibody against the hapten (DNP) is increased only when the homologous carrier protein is used for secondary stimulus and that the carrier protein alone used as secondary stimulus does not produce a detectable anamnestic response in either system (EA or BGG).

The specificity of anamnestic response in hapten conjugated proteins must encompass both the hapten and carrier protein because neither of these two factors alone can produce an anamnestic response.

To obtain a secondary response against a well-defined hapten (in this case, DNP), it is necessary to use for secondary immunization the hapten coupled to the same carrier protein. The antibody is primarily directed against the hapten. The production of this anti-hapten antibody cannot be elicited unless the hapten is coupled to the primary carrier protein, in spite of the very high binding affinity which anti-DNP antibodies have been shown to possess against this hapten.

This situation is very similar to that seen in delayed hypersensitivity where it was also observed, with hapten-protein conjugates, that a high degree of carrier specificity characterizes these types of reactions.

The similar specific requirements of these 2 types of immunologic reactions may be due to the fact that in both cases they result from recognition of the antigen at the cellular level. The immunological mechanism which produces these responses is not well understood. These specific requirements may be explained either by the need for a stronger binding

energy in the case of immune reactions at the cellular level than is necessary for the reaction of antigen with free antibody, or by the fact that both delayed hypersensitivity or secondary response may require the same metabolism of the antigen by the macrophages(11) as the initial primary immunization.

Summary. The data presented clearly indicate that anamnestic response in both systems studied is related to the immunological backbone of the carrier protein as well as the hapten, and that the carrier protein alone injected secondarily does not cause production of antibodies directed against the hapten used for primary immunization.

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Lethal Toxicity and Dose-Related Azotemia Due to Amphotericin B in Dogs. (28590)

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Amphotericin B is a polyene antibiotic which is used in treatment of systemic fungal infections. Clinical use of this antibiotic is limited by its toxicity, particularly renal toxicity. Experiments were undertaken to determine if in dogs, renal function is impaired in proportion to the dose of amphotericin B administered. It was learned in experiments lasting from 4 to 235 days that time of death and rate of progression of azotemia were related to the dose administered.

Material and methods. Amphotericin B (solubilized with sodium desoxycholate and phosphate buffers, as Fungizone®, was reconstituted as a 0.5% suspension with sterile 5% dextrose in water, and was stored at 4°C until used, within 36 hours. Amphotericin B used in these experiments was obtained from lots 1H69482, 1K71002, 2D73768 and 8A80431. The drug was diluted to 1 to 2 mg/ml in 5% dextrose in water immediately prior to administration, and was given intravenously in a period of 3 to 10 seconds. Specimens for blood urea nitrogen determination were obtained immediately before and at 1- to 7-day intervals after administration of amphotericin B. The most frequent examinations were made when the dogs appeared ill or when the blood urea nitrogen (BUN) was known to be rising rapidly.

Healthy adult mongrel dogs of both sexes were used, after being held under observation for 12 to 140 days. They were kept in single cages and were offered daily 1 liter of water and 0.5 kg of NIH dry dog diet (NF 170, Type II, Class 2). Dogs were weighed at 7- to 14-day intervals, and daily weights were obtained during periods of acute illness.

The dogs were divided into 3 groups: Group 1 (Fig. 1, 4 dogs) received amphotericin B daily, in doses ranging from 0.25 to 2.0 mg/kg. Group 2 (Fig. 2, 5 dogs) received amphotericin B every 2 days, in doses ranging from 0.25 to 4.0 mg/kg. Group 3 (Fig. 3, 3 dogs)