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THE SOLUBILIZATION OF MICROSOMAL ANTIBODY ACTIVITY BY THE SPECIFIC INTERACTION BETWEEN THE CRYSTALLIZABLE FRACTION OF γ-GLOBULIN AND LYMPH-NODE MICROSOMES

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In a previous study,[1] it was shown that when 2,4-dinitrophenyl bovine γ-globulin is injected into guinea pigs there appears in the microsomal fraction of regional lymph nodes an antibody activity which is specific for the 2,4-dinitrophenyl group. The present work is concerned with the questions: (1) is the antibody activity of lymph-node microsomes[2] due to conventional serum antibody linked to microsomes, and, if so, (2) what kind of linkages are responsible? In order to examine these questions it was necessary to determine the conditions required to separate antibody activity from microsomes and to compare the solubilized material with serum antibody of the same specificity.

The recent work of Porter[3] describing three fractions of rabbit γ-globulin and antibodies, obtained by papain digestion and chromatography, has led to a significant advance in understanding the structure of antibodies and has provided methods which may prove applicable to studies of antibody synthesis. In the present report, evidence is presented that microsomal antibody activity is the result of a specific association between lymph-node microsomes and that segment of γ-globulin which corresponds to Porter's fraction III.

Materials and Methods.—The materials used and the procedures followed for immunization of guinea pigs and preparation of lymph-node microsomes have been described.[1] In principle, the method used for measuring microsomal anti-DNP[4] antibody activity involves incubation of the microsomal fraction with an indicator antigen (I^{131}-DNP-BSA), washing the particles by suspension and sedimentation (105,000 × *g*), and, finally, elution of the radioactive indicator antigen from the microsomal pellet with a homologous hapten (ε-DNP lysine; see Table 1). This assay, which was described previously in detail,[1] has been simplified in the present work by eliminating one of the two washing procedures (step 3, Fig. 1 of ref. 1) and by eliminating bovine serum albumin from the medium used for washing and eluting the microsomes (steps 2 and 4 of Fig. 1, ref. 1). The basic medium used for homogenization of lymph nodes and for isolation and assay of microsomes was medium A of Littlefield and Keller:[5] 0.35 *M* sucrose, 0.05 *M* Tris, pH 7.6, 0.004 *M* $MgCl_2$, and 0.025 *M* KCl. Microsomal protein was isolated by the method of Schneider,[6] dissolved in 5.2 *M* acetic acid, and measured by its absorbance at 280 mμ; its extinction coefficient, $E^{1\%}_{1cm}$, is 10, not 13.6 as previously given.[1,2] In experiments in which mitochondria and microsomes were compared, these 2 fractions were isolated from the same lymph-node homogenate (0.25 *M* sucrose) by the method used for rat liver.[7] The mitochondria were washed 3 times to reduce contamination by microsomes.

Fractions of rabbit γ-globulin (I, II, and III) were prepared and isolated as described by Porter.[3]

H^3-labeled antibody, specific for the 2,4-dinitrophenyl group, was prepared biosynthetically by incubating 4,5-H^3-DL-leucine with lymph-node cells that were isolated from guinea pigs immunized with DNP-BγG. The procedures for carrying out the incubation, for isolation of H^3-anti-DNP antibody and for preparing a particulate fraction from the incubated cells are given elsewhere.[8]

For some control experiments, lymph-node microsomes were isolated from guinea pigs immunized with an antigen lacking the DNP determinant. The latter antigen was prepared by coupling BγG with diazotized *p*-arsanilic acid; the conjugate (R-azo-BγG) was purified by alternately precipitating at pH 4 and dissolving at pH 8.[9] Guinea pigs were injected with 0.8 ml (distributed evenly among the 4 footpads) of complete Freund's adjuvant containing a total of 1.0 mg R-azo-BγG. Lymph nodes were removed 3–6 weeks later and microsomes were prepared as usual.

BγG (fraction II of bovine plasma) and RγG (fraction II of rabbit plasma) were obtained from Armour and Co., Chicago, Ill., and Pentex Corp., Kankakee, Ill., respectively. Twice recrystallized bovine hemoglobin and protamine sulfate were obtained from Sigma Chemical Co., St. Louis. Twice recrystallized lysozyme and crystalline papain were products of Worthington Biochemical Co., Freehold, N. J. 4,5-H^3-DL-leucine, specific activity 3,500 μcuries per μmole, was obtained from New England Nuclear Corp., Boston. I^{131} was counted in a well-type scintillation counter and H^3-labeled proteins were counted in a Packard liquid scintillation spectrometer.

Results.—Characteristics of the microsomal antibody system: In a previous report,[1] the assay for microsomal antibody activity was described with particular reference to its specificity and sensitivity. For purposes of the present work, it was necessary to characterize some other parameters of this assay. The advantage of using the hapten, ε-DNP lysine, to elute the indicator antigen after it has been bound to microsomal antibody is brought out in Table 1. If the combined radioactivity of the final supernatant and pellet for any given tube, eluted with or without hapten, is taken as the total quantity of indicator antigen bound to the microsomes, then microsomes from DNP-BγG–injected animals obviously take up far more I^{131}DNP-BSA than do the lymph-node microsomes from control animals. However, measurement of low levels of antibody activity based on the total indicator antigen bound to microsomes would be hazardous and also laborious, as it would necessitate the routine isolation of microsomes from control animals to provide an analytical blank. The requirement for the latter control is eliminated when the elution technique is used; i.e., the value for control microsomes is zero (Table 1). Since most of the indicator antigen bound to microsomes of DNP-BγG–injected animals is specifically eluted by hapten, the assay is insensitive to microsomal contamination of the final supernatant.

The selection of a 30-minute incubation period for elution (with and without hapten) is based on an experiment in which elution for 15, 30, 45, and 60 min yielded, respectively, 61, 81, 77, and 81 mμg indicator antigen specifically eluted per mg microsome protein. Elution is performed at 37°, since at 4° the release of bound indicator antigen occurs very slowly. For example, after 90 min at 4°, less than 15 per cent of the total elutable indicator antigen is released. The temperature dependency of hapten displacement of antigen from microsomes is in accord with previous observations on hapten dissociation of antibody-antigen complexes.[10]

The dependence of the assay on the amount of microsomes was shown in an experiment wherein 1.4, 2.8 and 3.7 mg microsomes protein yielded, respectively, 67, 140, and 167 mμg indicator antigen specifically eluted.

TABLE 1

EFFECT OF ELUTION ON THE DISTRIBUTION OF INDICATOR ANTIGEN BETWEEN SUPERNATANT AND MICROSOMAL PELLET

Immunized with	Eluted with	Fraction*	Radioactivity, cpm — Total	Radioactivity, cpm — Specifically eluted†
Freund's adjuvant	Medium A	Supernatant	1,100	<150
		Pellet	7,850‡	
	ε-DNP lysine§	Supernatant	975	
		Pellet	7,490‡	
DNP-BγG in Freund's adjuvant	Medium A	Supernatant	6,260	41,400
		Pellet	54,700**	
	ε-DNP lysine§	Supernatant	47,700	
		Pellet	16,000**	

The experimental procedure is described in *Materials and Methods.*

* Refers to the last step in the microsomal antibody assay in which the supernatant and microsomes (pellet) are separated by centrifugation at 105,000 × *g*.

† Difference between supernatants obtained by elution with Medium A alone and by elution with ε-DNP lysine in Medium A. 41,400 cpm corresponds to 78 mμg I^{131}-DNP-BSA eluted/mg microsomal protein.

‡ The pellet contained 2.65 mg protein.

§ 10^{-3} *M* ε-DNP lysine in Medium A.

** The pellet contained 1.95 mg protein.

For routine assay it is desirable to use an amount of the I^{131}-indicator antigen which allows saturation of the microsomes but which is not in unnecessary excess. As shown in Figure 1, the binding capacity of a representative preparation of active microsomes is estimated, by extrapolation, to be 72 mμg indicator antigen. While the amount of indicator antigen normally added (about 15 μg) does not saturate the binding capacity of this preparation, the yield of specifically eluted antigen is relatively insensitive to small variations in the quantity of indicator antigen added in the range 7–20 μg. For example, the three highest levels of antigen eluted in Figure 1, i.e., 52, 56, and 59 mμg, were obtained when 7.6, 14.2, and 17.1 μg, respectively, of indicator antigen were added.

The microsomal antibody is stable at −20° and at 4°, with a loss of less than one-third of the initial activity over a 9-day period. Ordinarily, the microsomes were stored at 4° as a suspension in medium A. Although most of the data presented herein were obtained with preparations stored up to 7 days, repetition of some of the more critical experiments (e.g., Table 5) with fresh microsomes yielded the same results.

A comparison of the antibody activity associated with the microsomal and mitochondrial fractions of a lymph node homogenate is shown in Table 2. The distribu-

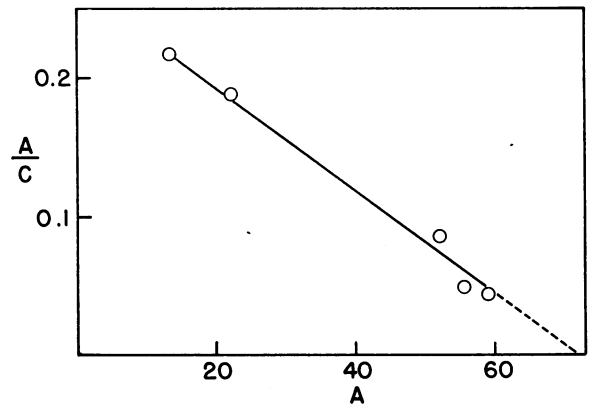

FIG. 1.—Titration of microsomal antibody activity with indicator antigen. To a constant amount of microsomes (0.85 mg protein in final pellet) varying quantities of indicator antigen were added and the microsomal antibody activity determined (see *Materials and Methods*). *A* refers to the indicator antigen specifically eluted (mμg) from the microsomes, and *C* refers to the concentration of unbound indicator antigen (mμg/ml). Total volume per tube: 12.5 ml.

tion of diphosphopyridine nucleotidase was determined in the same fractions, since this enzyme is known to be associated principally with the microsomal fraction in several rat tissues.[11] Both the enzyme and antibody activities (expressed as specific activities relative to protein) are about 4–5 times higher in microsomes (Table 2).

TABLE 2

COMPARISON OF ANTIBODY AND DIPHOSPHOPYRIDINE NUCLEOTIDASE ACTIVITIES ASSOCIATED WITH MICROSOMES AND MITOCHONDRIA

Experiment no.	Cell fraction	Antibody Activity* per mg of Protein	Antibody Activity* per mg of RNA	Diphosphopyridine Nucleotidase† units per mg of Protein	Diphosphopyridine Nucleotidase† units per mg of RNA
1	Mitochondria	11	390	64	1,280
	Microsomes	45	364	204	582
2	Mitochondria	10	353	50	1,008
	Microsomes	57	485	213	646

The experimental procedure for the preparation of cell fractions and the measurement of antibody activity is described in *Materials and Methods*.

* mμg antigen specifically eluted per mg protein or RNA of the cell fractions.

† Diphosphopyridine nucleotidase activity was measured in a 0.5 ml reaction volume containing 0.3 ml cell fraction in Medium A, 0.1 ml *M* Tris pH 7.6, and 0.1 ml diphosphopyridine nucleotide (6 μmoles/ml). The reaction was terminated at 15 min by the addition of 3.5 ml *M* KCN^{21}, and absorbance was determined at 325 mμ. A unit of enzyme is that amount causing the hydrolysis of 0.1 μmole of diphosphopyridine nucleotide under these conditions.

When the activities are expressed relative to RNA, antibody activity is equal in mitochondria and in microsomes, and enzyme activity is slightly higher in mitochondria than in microsomes. The mitochondria used in these experiments contained 4–5 per cent RNA, which is considerably higher than the 1–2 per cent level reported for liver mitochondria.[12] Since aggregated microsomes would tend to be recovered in the mitochondrial fraction during preparation of these cell components, the data are consistent with the view that the antibody activity is associated principally, if not exclusively, with the microsomes.

Effect of serum and some serum proteins on microsomal antibody activity: The conditions required to separate antibody activity from microsomes were most easily evaluated by measuring the loss of activity from the microsomes. In preliminary attempts to achieve the desired separation, soluble extracts of guinea pig lymph

nodes were tested on the assumption that enzymatic activity might be required. The effectiveness of several lymph-node extracts was inconstant, and the possibility that variable serum contamination of the extracts was responsible for the observed dissociation was tested with whole serum. Incubation of microsomes with normal guinea pig or rabbit serum resulted in a substantial loss of microsomal anti-DNP antibody activity (Table 3). Similar results were obtained by treating the micro-

TABLE 3

LOSS OF MICROSOMAL ANTIBODY ACTIVITY ON INCUBATION WITH NORMAL SERUM, γ-GLOBULINS, AND PURIFIED ANTIBODY

Experiment no.	Treated with	Pellet protein* (mg)	Antibody activity† (mμg/mg protein)
1	Control	0.45	46
	Normal serum‡	0.46	12
2	Control	1.56	64
	γ-globulins§	1.56	30
3	Control	0.49	44
	Antibody††	0.48	14
	Antibody**	0.50	19

Microsomes from lymph nodes of guinea pigs immunized with DNP-BγG were suspended in 5.0 ml. of medium A containing either normal serum, γ-globulins, or antibody. The controls were suspended in 5.0 ml of medium A alone. After incubation at 37° for 1 hr, the tubes were filled with medium A (12.5 ml final volume) and centrifuged for 15 min at 105,000 × g. After discarding the supernatant, the pellets were rinsed twice with medium A and then resuspended and assayed for microsomal antibody as described in *Materials and Methods*.

* The term pellet protein is used to emphasize the fact that microsomal protein, to which all values are normalized, refers to the residual pellet at the termination of the assay.

† mμg antigen specifically eluted per mg microsomal protein.

‡ 0.1 ml normal rabbit serum. Rabbit and guinea pig sera were equally effective. Microsomes treated with 1.0 ml whole guinea pig serum had exactly the same diphosphopyridine nucleotidase activity as untreated control microsomes.

§ 1.0 mg rabbit γ-globulin. Guinea pig γ-globulin was as effective as rabbit γ-globulin.

** 0.58 mg rabbit antiphenyl (*p*-azo-benzoylamino)-acetate antibody.[13]

†† 1.67 mg rabbit antiphenyl (*p*-azo-benzoylamino)-acetate antibody.[13]

somes with γ-globulin fractions of normal rabbit and guinea pig sera or with a highly purified rabbit antibody[13] whose specificity was unrelated to the DNP determinant (Table 3). The dependency of this effect on time and temperature of incubation is shown in Table 4. At 37°, the reaction is essentially complete in 15

TABLE 4

EFFECT OF TIME AND TEMPERATURE ON THE REDUCTION OF MICROSOMAL ANTIBODY ACTIVITY BY TREATMENT WITH A PURIFIED ANTIBODY

Treated with	Conditions	Pellet protein,* (mg)	Antibody activity† (mμg/mg protein)
Control	16 hr 4°	0.38	50
Antibody‡	16 hr 4°	0.38	39
Control	45 min 37°	0.35	45
Antibody‡	15 min 37°	0.40	24
"	30 min 37°	0.38	23
"	45 min 37°	0.37	22

The procedure used is the same as described in Table 3 except for the changes in time and temperature shown.

* See Table 3.

† mμg antigen specifically eluted per mg microsomal protein.

‡ 0.23 mg rabbit antiphenyl (*p*-azo benzoylamino)-acetate antibody.

min, while at 4°, the reaction is incomplete in 16 hr. In other experiments, after one hr incubation at 4° with the purified antibody of unrelated specificity there was no detectable loss of microsomal anti-DNP antibody activity.

Porter[3] has shown that papain splits rabbit γ-globulin and antibodies into three separable fractions which retain a high measure of structural integrity. Fractions I and II retain the ability to interact specifically with antigen (without precipitation), whereas fraction III is totally lacking in this activity. The availability of these

fractions made it possible to determine which segment of intact γ-globulin is responsible for reducing the level of microsomal anti-DNP antibody activity. The data of Table 5 (experiment 1) show that fraction III reproduces the behavior of

TABLE 5

EFFECT OF γ-GLOBULIN FRACTIONS ON MICROSOMAL ANTIBODY*

Experiment no.	Microsomes From	Microsomes Treated with	Pellet protein† (mg)	Antibody activity‡ (mμg/mg protein)
1	Guinea pig	Control	2.52	35
		I, II	2.42	36
		III	2.48	18
		I, II, III	2.57	20
2	Rabbit	Control	0.80	13
		I	0.75	10
		II	0.80	10
		III	0.75	3
3	Guinea pig	Control	1.73	30
		Hemoglobin	1.60	33
		Protamine	2.08	28, 34§
4	Guinea pig	Control	1.81	31
		Lysozyme	2.57	23, 32§

* Microsomes were prepared from lymph nodes of animals injected with DNP-BγG.
In Experiment 1, guinea pig lymph-node microsomes were suspended in 2.0 ml of Medium A which contained the indicated combinations of the fractions obtained on papain digestion of normal rabbit γ-globulin. The amounts of fractions I, II, and III were 1.0, 1.26, and 0.54 mg, respectively. The controls were suspended in 2.0 ml Medium A alone. All tubes were incubated for 1 hr at 37° and then processed as described in Table 3. The procedure followed in Experiment 2 was exactly the same as in Experiment 1, except for the use of rabbit lymph-node microsomes and the fact that the quantities of I, II, and III were 2.4, 2.9, and 0.6 mg, respectively.
Experiments 3 and 4 followed the same procedure as Experiment 1 but used 3.0 mg hemoglobin, 0.6 mg protamine, and 3.0 mg lysozyme.
† See Table 3.
‡ mμg antigen specifically eluted per mg microsomal protein.
§ The second value is corrected to the protein value of the control. Some of the added protamine and lysozyme apparently adheres to the microsomes yielding a higher pellet protein value than in the corresponding controls.

intact γ-globulin, whereas fractions I and II are ineffectual.

Following papain digestion of rabbit γ-globulin and dialysis of the reaction mixture, fraction III crystallizes as fragile plates in the shape of nonrectangular parallelograms.[3] Papain digestion of purified guinea pig antibody (anti-DNP) also resulted in the appearance of crystals which were similar to those obtained from rabbit γ-globulin except that they were rectangular plates. The solubility of fraction III of the guinea pig antibody was, however, so low (150 μg/ml 0.15 *M* NaCl—0.01 *M* phosphate pH 7.2) that this fraction could not be examined for its effect on guinea pig microsomal anti-DNP antibody activity. In order to examine the effects of papain-produced fractions of γ-globulins on microsomes of the homologous species, it was necessary, therefore, to use lymph-node microsomes from rabbits immunized with DNP-BγG. The results, shown in Table 5 (experiment 2), corroborate the observations made with guinea pig microsomes and fraction III of rabbit γ-globulin.

Specificity of γ-globulins in reducing microsomal antibody activity: Rabbit and guinea pig sera are about equally capable of reducing the antibody activity of guinea pig lymph-node microsomes. The data of Table 3 show that the effectiveness of these sera may be ascribed to their γ-globulins. Calf serum is, however, much less effective. Bovine serum albumin was entirely ineffectual, and while BγG had some slight capability, it was far less effective than RγG. With a single preparation of microsomes, the quantities of RγG and of BγG required to reduce antibody activity by 50 per cent were, respectively, 1 mg and more than 100 mg. Perhaps the strongest evidence for the specific character of the γ-globulin effect in

reducing the level of microsomal antibody is provided by the different capabilities of the three fractions obtained from rabbit γ-globulin by papain digestion (see above and Table 5).

γ-globulins have relatively high isoelectric points. Of the three fractions obtained with papain, the fraction (III) which is capable of lowering the level of microsomal antibody is relatively enriched in respect to basic amino acids.[3] The question arises, therefore, whether other basic proteins are active. As shown in Table 5 (experiments 3 and 4), hemoglobin, lysozyme, and protamine had no effect.

The specificity of the γ-globulin effect is emphasized further by the observation that the diphosphopyridine nucleotidase activity of lymph-node microsomes is completely unaffected by incubation with guinea pig serum (see legend of Table 3).

The foregoing observations suggest that the antibody activity of lymph-node microsomes may be due to antibodies which are bound through noncovalent bonds. Accordingly, active microsomes were exposed to 8.0 *M* urea (pH 7.5) and to sucrose media at low pH. Both of these conditions markedly reduced the level of microsomal anti-DNP antibody (Table 6). It may be noted in this connection that

TABLE 6

EFFECT OF UREA AND pH ON MICROSOMAL ANTIBODY

Experiment no.	Treated with	Pellet protein* (mg)	Antibody activity† (mμg/mg protein)
1	Control	0.90	76
	pH 2.0	0.79	7
	pH 3.0	0.70	10
2	Control	2.43	18
	8 *M* urea	2.38	8

In Experiment 1, microsomes from lymph nodes of guinea pigs immunized with DNP-BγG were suspended in Medium A in which TRIS was replaced by HCl in a final concentration of either 0.01 *N* (pH~2.0) or 0.005 *N* (pH~3.0). The controls were suspended in 2.0 ml Medium A alone. All tubes were incubated for 10 min in an ice bath. The tubes were then filled with the same media used for incubation (12.5 ml final volume) and centrifuged and the pellet was then assayed for microsomal antibody as described in Table 3 and *Materials and Methods*.

In Experiment 2, microsomes from lymph nodes of guinea pigs immunized with DNP-BγG were suspended in 2.0 ml of Medium A (controls) or 2.0 ml of 8.0 *M* urea in 0.01 *M* TRIS pH 7.6. After incubation for 1 hr at room temperature, the contents of the tubes were dialyzed at 4° against three successive 2-liter volumes of Medium A over an 18-hr period. Following dialysis, the microsome suspensions were assayed for antibody activity as described in *Materials and Methods*.

* See Table 3.

† mμg antigen specifically eluted per mg microsomal protein.

rabbit antibodies retain their specific reactivities despite treatment with urea at a high concentration or brief exposure to pH 2.

Microsomal antibody activity was essentially unchanged by a variety of other conditions; e.g., by cysteine (0.01 *M*) and ethylenediaminetetracetate (0.01 *M*), ATP (0.001 *M*) or pyrophosphate (0.001 *M*).

Passive cutaneous anaphylaxis reaction of microsomal antibody: Passive cutaneous anaphylaxis offers a relatively simple and sensitive assay for detecting antibody. Ordinarily, this assay is performed by the intradermal injection of antibody followed several hours later by intravenous injection of the homologous antigen mixed with a dye. Specific interaction of antigen and antibody in the skin results in local increase in capillary permeability and extravasation of dye at the skin site.[14]

Skin sites that had been injected with a microsome suspension obtained from DNP-BγG–immunized animals reacted to intravenous injection of DNP-HSA, whereas sites that had been injected with 18 times more microsomes from control animals (immunized with benzene arsonate azo-protein) failed to react (Table 7). The positive skin reactions were completely inhibited, in other experiments, by

TABLE 7

PASSIVE CUTANEOUS ANAPHYLAXIS PRODUCED WITH A SUSPENSION OF MICROSOMES

Microsomes		Diameter (mm) of response for animal number					
From animals immunized with	Amount injected, mg protein	1	2	3	4	5	6
R-azo-BγG*	0.218	0	0	0	—	—	—
DNP-BγG	0.012	0	5	5	15	12	10

Guinea pigs (~250 gm) were injected intradermally on their ventral surface with 0.1 ml of microsome suspension in Medium A. Five hr later, the animals were injected intravenously with 0.5 ml containing 1 mg DNP-HSA and 2.5 mg of a blue dye (Evan's blue). Fifteen min later, the animals were sacrificed and diameters of extravasated blue dye at sites of intradermal injection were measured. For additional details of this assay, see text and reference 14. The microsomes injected had the usual protein/RNA weight ratio of ~3.0.[2,16]

* *p*-arsonic acid benzene azo-bovine γ-globulin.

intraperitoneal injection of hapten (35 μmoles ϵ-DNP lysine) 30 min prior to challenge with the antigen-dye mixture. Hapten inhibition of the skin test established its specificity for the DNP group and confirms the recent findings of Ovary and Karush in respect to the susceptibility of this skin response to hapten inhibition.[15]

Although in these experiments the skin sites were injected with microsome suspensions, it is not known whether anti-DNP antibody activity was still associated with microsomes at the time of challenge with antigen. Indeed, in view of the results of Table 3, it is likely that the antibody activity was separated from the microsomes in the skin sites by the γ-globulins of the test animals.

The smallest quantity of antibody detectable by passive cutaneous anaphylaxis has been reported to be about 0.01 μg.[14] If this sensitivity is assumed for the experiments in Table 7, the microsome preparation used had about 1 μg antibody per mg protein.

Demonstration of soluble antibody released from microsomes: The assay for microsomal antibody can only be used to measure antibody activity associated with sedimentable particles. Other procedures were required to determine whether soluble antibody was released from the microsomes by those conditions which reduced the level of microsomal antibody activity (Tables 3–6). As shown in Table 8, microsomes exposed briefly to pH 2–3 yielded soluble antibody which was detect-

TABLE 8

PASSIVE CUTANEOUS ANAPHYLAXIS PRODUCED WITH ANTIBODY RELEASED FROM MICROSOMES

Microsomes treated with	Microsome supernatant dilution	Diameter (mm) of response for animal number 1	2
Control	1:3	0	3*
pH 2.5	1:3	10	15
Control	1:6	—	0
pH 2.5	1:6	—	5

Microsomes from guinea pigs immunized with DNP-BγG were suspended in 2.0 ml unbuffered Medium A (controls) or Medium A in which TRIS was replaced by HCl at a final concentration of 0.01 *N* (pH 2.5). After 10 min in an ice bath, both samples were adjusted to pH 5.2 with 0.4 *M* acetate buffer and centrifuged for 10 min at 9,000 × *g*. The supernatant was neutralized with NaOH to about pH 7.2 and diluted in Medium A. Dilutions of the supernatant were injected intradermally and passive cutaneous anaphylaxis was elicited and the response measured as described in Table 7.

* Faint color.

able by passive cutaneous anaphylaxis.[16]

An alternate and independent method for detecting the release of soluble antibody from microsomes involved the use of a particulate fraction derived from cells which had incorporated H^3-leucine of high specific activity into anti-DNP antibody. Using procedures described by Helmreich *et al.*,[8] isolated lymph-node cells from DNP-BγG–immunized guinea pigs were incubated with H^3-leucine and then

homogenized, and a particulate fraction was isolated by differential centrifugation (Table 9). After exposing the particulate fraction (mitochondria and microsomes) briefly to pH 2.5 at 4°, the particles were sedimented and the supernatants were assayed for H^3-labeled anti-DNP antibody by coprecipitation with carrier antigen and antibody.[8] The results, given in Table 9, demonstrate clearly the dissociation

TABLE 9

COPRECIPITATION OF ANTIBODY RELEASED FROM PARTICULATE FRACTION OF CELLS

Experiment no.	Particles treated with	Radioactivity in anti-DNP antibody,* cpm
1	Control	79
	pH 2.5	1,119
2	Control	109
	pH 2.5	3,480
3	Control	221
	pH 2.5	7,330

8–10 reaction mixtures consisting each of 2–3 × 10^8 guinea pig lymph-node cells in 2.0 ml of medium containing 88 μc H^3-DL-leucine were incubated at 37° for 1 hr under 95 per cent O_2–5 per cent CO_2. The cells were harvested by centrifugation (1,300 × g), lysed with a small volume of H_2O, and homogenized in Medium A. The homogenate was centrifuged at 2,500 × g for 10 min and the sediment discarded. The supernatant was divided in half and centrifuged in separate tubes at 105,000 × g for 1 hr. One of the pellets was suspended in unbuffered Medium A (control) and the other in Medium A in which TRIS was replaced by HCl at a final concentration of 0.01 N (pH 2.5). After 10 min in an ice bath, both samples were adjusted to pH 5.2 with 0.4 M acetate buffer and centrifuged for 10 min at 9,000 × g, and the supernatant was neutralized with NaOH to about pH 7.2.

In Experiments 1 and 2, 0.58 mg DNP-BγG and 3.5 mg anti-DNP antibody (in the form of a γ-globulin fraction) were added to the neutralized supernatant yielding a specific precipitate. In Experiment 3, the neutralized supernatant was subjected to further centrifugation for 4 hr at 93,000 × g before addition of the carrier antigen and antibody.

* Specifically eluted with ϵ-DNP lysine from the specific precipitate made with DNP-BγG and anti-DNP antibody.[8]

at low pH of soluble H^3-antibody from the particles.

Discussion.—The foregoing observations indicate that microsomal antibody activity is a function of microsome-antibody complexes; i.e., conventional antibody (γ-globulin) linked to microsome particles. The principal evidence in support of this interpretation is the fact that brief treatment of an active microsome preparation at low pH leads to a decrease in antibody activity of the particles with simultaneous appearance in the soluble phase of material which exhibits the behavior expected of conventional antibody, i.e., reactivity in passive cutaneous anaphylaxis and coprecipitation with homologous carrier antibody and antigen. This interpretation is further supported by the restricted nature of the proteins which are capable of reducing the level of microsomal antibody activity. Of the several proteins examined, from serum and other sources, only γ-globulins dissociated the complex. Rabbit and guinea pig γ-globulins were particularly effective, but bovine γ-globulin had hardly any effect. Moreover, of the fractions resulting from papain digestion of rabbit antibody or γ-globulin, only crystalline fraction III reduced the level of antibody activity on microsomes. These observations emphasize the specificity of the antibody-microsome interaction and imply that structural complementarity between microsomal sites and antibody (γ-globulin) is involved in this interaction.

The dissociation of the microsome-antibody complex by urea or by brief exposure to pH 2.5 and the specific dissociation of these complexes by certain γ-globulins, including purified rabbit antibodies and crystalline fraction III, suggest that the linkages involved in establishing the complex are not covalent.

In view of the structural heterogeneity of the microsome fraction, the question arises whether antibodies found in this fraction are bound to the microsomes *per se*

(i.e., ribosomes) or to other components of the endoplasmic reticulum.[17] At present, it is not possible to answer this question, since a procedure which selectively separates the ribosomes from the endoplasmic reticulum without otherwise altering these components is not available. Although deoxycholate has been used to prepare ribosomes from mammalian tissues,[17] this reagent extracts considerable amounts of protein from the microsomal fraction,[18] and it also solubilizes or inactivates most of the microsomal antibody activity. In a representative experiment, when lymph-node microsomes[2] were treated with 0.25 per cent deoxycholate and then centrifuged at 12,000 $\times$ g for 15 min, an opaque white sediment was obtained (weight ratio protein/RNA = 6.3); when the supernatant was then recentrifuged at 100,000 $\times$ g for one hr, the transparent yellow pellet obtained was judged to be ribosomes on the basis of its having a protein/RNA weight ratio of 1.1. The low-speed RNA-poor sediment and the ribosomal pellet each had only 5 per cent of the antibody activity of the original microsomal fraction from which they had been derived. When the latter antibody activities are expressed as mμg indicator antigen specifically eluted per mg protein, or per mg RNA, or per mg protein + RNA, the residual antibody activity of the ribosomes was 12, 17, and 7, respectively, as compared to 69, 234, and 53, respectively, for the original microsomal fraction. Whether the 90 per cent of the microsomal antibody activity not accounted for after deoxycholate treatment was inactivated or solubilized was not determined. Although currently favored views might suggest that microsomal antibodies should be associated with ribosomes, we cannot exclude the possibility that these antibodies are, at least in part, associated with microsomal structures which in the cell constitute the endoplasmic reticulum.[19]

While there is no direct evidence that the antibodies associated with microsomes are synthesized on these particles, it may be noted that in cell-free lymph-node extracts, which incorporate isotopically labeled amino acids into protein, treatment of the microsomal fraction with a detergent yields soluble isotopically labeled protein that is immunologically indistinguishable from γ-globulin.[20]

The present findings and their interpretation furnish the following tentative explanation for the assay used for detecting microsomal antibody activity. We visualize that components of the microsomal fraction bind reversibly that portion of an antibody molecule (or γ-globulin) which corresponds to fraction III of a papain digest. The residual segments of the antibody molecule (fractions I and II) bear the two active sites which are then accessible for interaction with the indicator antigen. Since it is further visualized that the specific dissociation of the microsome-antibody complex by crystalline fraction III involves an exchange, it is expected that lymph-node microsomes from an unimmunized guinea pig should bind antibodies added to them *in vitro*. Binding of this type was described in a previous communication.[1]

Experiments concerned with identifying the particular microsomal constituent which specifically interacts with the fraction III moiety of rabbit and guinea pig antibodies are in progress.

Summary.—When lymph-node microsomes are treated under a variety of conditions, their antibody activity is diminished and antibodies may be identified in the soluble phase. The conditions which are effective in separating antibodies from microsomes indicate that the linkages involved are not covalent. It is inferred that

the microsome-antibody complex is reversible and due to a specific interaction between microsomal components and that segment of antibody (or γ-globulin) which corresponds to the crystallizable fraction obtained by papain digestion of rabbit γ-globulin.

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[1] Kern, M., E. Helmreich, and H. N. Eisen, these PROCEEDINGS, **45**, 862 (1959).

[2] The term microsome refers to that fraction of a lymph-node homogenate which is not sedimentable at 15,000 $\times$ g (15 min) but is sedimentable at 105,000 $\times$ g (1 hr). The weight ratio of protein/RNA in guinea pig lymph node microsomes is ordinarily about 3.2–3.5. At the termination of assay for microsomal antibody, this ratio is about 8.0–8.8. In ribosomes prepared by deoxycholate treatment of microsomes (see discussion), the protein has an $E^{1\%}_{1cm}$ value of 7.0 (280 mμ, 5.2 M acetic acid).

[3] Porter, R. R., *Biochem. J.*, **73**, 119 (1959).

[4] Abbreviations used: DNP is used generically to indicate 2,4-dinitrophenyl groups combined with proteins or amino acids; DNP-BγG is used for 2,4-dinitrophenyl bovine γ-globulin; DNP-BSA for 2,4-dinitrophenyl bovine serum albumin; DNP-HSA for 2,4-dinitrophenyl human serum albumin: ϵ-DNP lysine for ϵ-2,4-dinitrophenyl lysine; BγG for bovine γ-globulin; RγG for rabbit γ-globulin; Razo BγG for p-arsonic acid benzene azo-bovine γ-globulin; TRIS for tris (hydroxymethyl) aminomethane.

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[8] Helmreich, E., M. Kern, and H. N. Eisen, *J. Biol. Chem.*, **236**, 464 (1961).

[9] We wish to thank Dr. C. W. Parker for preparing this reagent.

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[13] We are indebted to Dr. F. Karush of the University of Pennsylvania for his generous gift of purified antiphenyl (*p*-azo-benzoylamino)-acetate antibody.

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[15] Ovary, Z., and F. Karush, *J. Immunol.*, **84**, 409 (1960).

[16] In another experiment which followed the protocol of Table 8, the final microsomal pellets for the pH 2.5–treated sample and the control sample contained, respectively, 1.56 and 1.51 mg protein. This should be compared to a value of 1.5 mg protein for an equal aliquot of microsomes which was untreated (directly precipitated with trichloroacetic acid).

[17] Palade, G. E., in *Microsomal Particles and Protein Synthesis*, ed. R. B. Roberts (New York: Pergamon Press, 1958), p. 36.

[18] Peters has shown that deoxycholate extracts the chicken serum albumin that is bound to the microsomal fraction of chicken liver, *J. Biol. Chem.*, **229**, 659 (1957).

[19] The widespread view that ribosomes exercise a specificity-determining function in protein synthesis implies that ribosomal populations are heterogeneous and differentiated. If this is true, and if the antibody attached to microsomes is attached to ribosomes and is related to the synthetic process, it would be expected that only a small fraction of the lymph-node ribosomes are associated with antibody. This possibility may be tentatively evaluated from estimates of the amount of antibody found in lymph-node microsomes. From titration with I^{131} DNP-BSA (Fig. 1), this amount appears to be about 0.1 μg antibody/mg protein. This value is probably more valid than that estimated from passive cutaneous anaphylaxis (1 μg/mg protein), but actually both values are in fair agreement when normalized to RNA (1–3 μg antibody/mg RNA; see footnote 2 and

legend of Table 7). If it is assumed that the antibody is attached only to ribosomes and that all of the RNA of the microsomal fraction is in ribosomes, it may be estimated that there is about 1 antibody molecule per 100–200 ribosomal particles. Or, if about 10 per cent of the cells in the lymph nodes taken for homogenization contain microsomal anti-DNP antibody, it appears that in such cells only one out of about 10–20 ribosomes bears an antibody molecule. On steric grounds, it is unlikely that a single antibody molecule could simultaneously bind more than 1 or 2 ribosomes, but a single ribosome might carry many more than 1 antibody molecule. While these numerical speculations are consistent with the possibility of ribosomal differentiation, their acceptability is seriously limited by uncertainty as to what fraction of the total γ-globulin produced by an antibody-synthesizing cell is antibody of a given specificity.

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*ULTRAVIOLET LIGHT INDUCED LINKING OF DEOXYRIBONUCLEIC ACID STRANDS AND ITS REVERSAL BY PHOTOREACTIVATING ENZYME**

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The effects of ultraviolet light (UV) on compounds of biological interest have been the subject of numerous investigations. In a recent excellent and comprehensive review, Shugar[1] has described and evaluated the effects of UV on nucleic acids and its constituents. At the *chemical level* it would appear that of the deoxyribonucleic acid (DNA) bases, the pyrimidines are altered more readily than the purines.[2] Recent work[3] pertaining to the photochemistry of nucleic acids has opened a new dimension in the field by invoking thymine dimer formation when this pyrimidine is irradiated with UV either in the frozen state or when in its more natural habitat, DNA (in solution).[4–6] At the *physical chemical* level of UV induced DNA damage, many changes have been detected and described, but the most sensitive thus far would appear to be the lowering of the thermal denaturation temperature of native DNA.[7, 8] At the *biological level*, the inactivation of transforming factor DNA, while not as sensitive as the killing of bacteriophage, offers the most readily approachable means of studying the end result of the UV irradiation on DNA at the macromolecular level. The purpose of the present communication is to present some new data bearing on the effects of UV on DNA at the macromolecular level, using physical chemical, biological, and enzymatic tools, with the hope that it may be useful in explaining the effects that occur *in vivo*.

Materials and Methods.—Transformation: Transformation of *Diplococcus pneumoniae* (R-36A) was carried out according to the method of Fox and Hotchkiss.[9] *Bacillus subtilis* (168) transformation was carried out as described by Spizizen.[10]

Deoxyribonucleic acid: DNA was isolated from exponentially grown cells by the method of Marmur.[11] Hybrid N^{14}–N^{15} *Escherichia coli* B DNA was isolated from cells first grown for many generations in $N^{15}H_4Cl$ as the sole nitrogen source followed by one generation (doubling of the turbidity) in $N^{14}H_4Cl$.[12] P^{32}-labeled DNA was isolated from *B. subtilis* (WT $E_rA_rB_r$, resistant to the antibiotics erythromycin, amycetin, and bryamycin) grown on a medium containing the following ingredients: 1 mg $MgSO_4$, 0.1 mg $MnSO_4 \cdot 7H_2O$, 0.05 mg $FeSO_4 \cdot 7H_2O$, 500 mg NH_4Cl, 100 mg