ished hepatic and other glucose output in the observed hypoglycemia. The calculation of phosphorus changes using the 15 minute rather than the starting value as the reference point may not be valid. If it is valid, then acetohexamide would appear to exert most and perhaps all of its additional hypoglycemic effect through an increased secretion of insulin.

The lack of consistently greater hypoglycemic effect of one drug or the other in diabetes mellitus suggests that individual susceptibilities to these two agents vary. This may be of import in their clinical applications in specific patients with or without a poor response to tolbutamide or sulfonylureas, though the impression to date is that the therapeutic effectiveness of acetohexamide and tolbutamide is comparable, and that control of hyperglycemia with the former requires less drug and perhaps only a single daily dosage (3-9).

*Summary and conclusions.* 1. Intravenous injection of one gram of the sodium salt of acetohexamide or of tolbutamide produced the same degree of hypoglycemia in human adults without diabetes mellitus but the acetohexamide-induced hypoglycemia was more prolonged. 2. In patients with diabetes mellitus the blood sugar and inorganic phosphorus decreases following acetohexamide and tolbutamide were of the same order of magnitude, though in some patients one or the other drug had a greater effect.

1. Danowski, T. S., Leinberger, M., Mateer, F. M., *Ann. N. Y. Acad. Sci.,* 1959, v74, 979.

2. Anderson, R. C., Root, M. A., Welles, J. S., Lilly Research Laboratories, Feb. 22, 1961, Private Printing.

3. Maha, G. E., Kirtley, W. R., Root, M. A., Anderson, R. C., *Diabetes,* 1962, v11, 83.

4. Weller, C., Donesa, A., Linder, M., *Metabolism,* 1962, v11, 551.

5. Balodimos, M. C., Stimson, W. H., Tanner, C. C., Reid, J. A., Williams, R. H., *ibid.,* 1961, v10, 1063.

6. Owen, J. A., Jr., *ibid.,* 1962, v11, 475.

7. Dobson, H. L., *ibid.,* 1962, v11, 1282.

8. Radding, R. S., McHenry, J. I., Purdie, C. B., Robins, O., *ibid.,* 1963, v12, 311.

9. Boshell, B. R., Wilensky, A. S., Barrett, J. C., Almon, J. V., *Clin. Pharmacol. Ther.,* 1962, v3, 750.

Received September 3, 1963. P.S.E.B.M., 1964, v115.

## A Study of Rabbit Antibody Against Rous Sarcoma Virus.* (28976)

MARY ALEXANDER FINK

*Laboratory of Viral Oncology, National Cancer Institute, Bethesda, Md.*

The effect of antibodies against chicken tissues on the neutralization of Rous sarcoma virus (RSV) has most recently been reviewed by Rubin(1). Previous investigators used either antisera prepared against normal chicken tissues, or antisera prepared with much less potent RSV preparations than are available today.

The present work was undertaken to determine whether or not the more potent preparations of RSV available in this laboratory could engender a good antibody response in the rabbit; and to measure the reactivity of this antibody by complement-fixation and neutralization techniques before and after exhaustion of antibody reactive with Forssman antigen and with the antigens of normal chicken tissue.

*Materials and methods. Preparation of antigens.* The RSV used to immunize and as antigen in the complement-fixation tests was prepared from wingweb tumors in chickens. The microsomal fraction was obtained by differential centrifugation in a manner which yielded a preparation with an infectivity titre 100 times greater than previously standard preparations. The method was essentially the same as that previously used(2), except that the ultracentrifugation was carried out at 30,000 *g*. One ml of this preparation rep-

* National Institutes of Health, P.H.S., U. S. Dept. of H.E.W.

resented the virus recovered from one gram of tissue, together with host cell components sedimentable under these conditions.

A similar preparation from normal chicken liver, obtained by differential centrifugation at 18,000 *g*, was used to immunize rabbits, and as an antigen in the complement-fixation tests.

An acetone-extracted chicken liver powder (3) was used in the absorption studies.

A preparation of chicken breast tissue prepared similarly to the RSV was used as a complement fixing antigen; a lyophilized preparation of this was used in the absorption studies.

Three times washed sheep erythrocytes previously stored for less than one month in sterile Alsever's solution were used in the absorption studies.

*Immunization of rabbits.* Two 9-month-old male rabbits were injected intra-abdominally with 0.75 ml of a mixture of 1 part RSV and 2 parts of Freund's adjuvant. Three weeks later they were given a subcutaneous injection of 0.35 ml of a similar preparation. They were bled from the heart 2 weeks later, the serum collected, and a pool of equal amounts of each serum made. This pool was used for all studies.

A similar preparation from normal chicken liver was injected in a like manner into 2 other rabbits.

*Absorption of serums.* After inactivation at 56°C for 30 minutes in a water bath, the serum was cooled and diluted 1:5 with physiological saline containing 0.001 g of magnesium sulfate per liter. For every ml of original serum, 1 ml of packed sheep erythrocytes (SRBC) was added and allowed to react at room temperature for 2½ hours. This amount of SRBC demonstrably removed all SRBC agglutinins. A portion of this serum was stored, and another portion serially absorbed as follows.

For every 1 ml of original serum, 500 mg of acetone-extracted chicken liver powder was added, and the mixture was allowed to stand at room temperature for 3 hours before centrifugation.

A portion of the serum which had been absorbed with SRBC and chicken liver was then absorbed for one hour at room temperature and overnight at 0-4°C with 250 mg of lyophilized chicken breast microsomal fraction for every 1 ml of original serum. The final centrifugation was in a Spinco centrifuge for one hour at 30,000 *g*.

*Complement-fixation reactions.* Standard complement-fixation reactions were carried out on various dilutions of the absorbed and unabsorbed serums. The following conditions were maintained throughout: 1 ml system consisting of 0.2 ml antiserum dilution; 0.2 ml 1:80 antigen dilution; 0.2 ml guinea pig complement containing 2 hemolytic units; 0.2 ml 3% SRBC; and 0.2 ml hemolysin containing 4 hemolytic units. All dilutions were made with 0.9% NaCl containing 0.0001 gram of magnesium sulfate per liter. Incubation of the first 3 components was carried out overnight at 0-4°C. After addition of the hemolytic system the tests were incubated for 30 minutes at 37°C in a water bath and read for hemolysis. Fixation was not considered as positive unless at least a 2+ reading was made. These tests were run at least in duplicate; and in addition to the standard controls, anticomplementary control tubes of each dilution of each absorbed serum were always included.

*RSV-neutralization reactions.* The neutralization index (NI) of the anti-RSV serum before and after the various absorptions was determined with a pock-counting method in chick embryos previously adapted for routine use in determination of antibody levels in large numbers of serums(4). An equal volume of one or more dilutions of inactivated serum was mixed with serial 10-fold dilutions of standard RSV and incubated at room temperature for 30 minutes. Afterwards, 0.1 ml amounts of each mixture were inoculated onto the chorioallantoic membrane of six to nine 9-day-old embryonated hen's eggs. Controls included a known positive serum plus RSV; a known negative serum plus RSV; and diluent and appropriate dilutions of RSV. The NI was determined by subtraction of the log of the average number of pock-forming units per ml in eggs inoculated with virus-antiserum mixtures from that produced with the virus-diluent alone. The variation

TABLE I. Neutralization and Complement-Fixation Titres of Anti-RSV Serum After Absorption with Various Materials.

| Serum | RSV N.I.* (log) 1:100 dil | C.F. | Chicken liver C.F. | Chicken breast C.F. |
|---|---|---|---|---|
| Unabsorbed | 3.02 | 1:1280 | 1:40 | 1:640 |
| SRBC absorbed | 3.40 | 1:320 | 1:40 | 1:80 |
| Chicken liver absorbed | 2.87 | 1:160 | 0† | 1:80 |
| SRBC-chicken liver absorbed | 2.42 | 1:80 | 0† | 1:40 |
| SRBC-chicken liver—chicken breast absorbed | 2.42 | 1:80 | 0† | 0† |

* A difference of >0.5 log units is considered significant (see text).
† Also negative by precipitin test when tested by double diffusion in agar.

in titre of duplicate or separate assays of the same preparation of virus or of virus antibody mixtures done at weekly intervals for one year did not exceed 0.5 log units. In the present study, therefore, a difference of >0.5 log units was considered significant when comparisons were made between 2 or more neutralization indices.

In the experiments testing the effect of complement on the neutralization titre, 10 units of freshly titrated guinea-pig complement were added to the serum-virus mixture immediately prior to incubation.

*Results. Effect of absorption on complement fixation and neutralizing titre of immune serum.* Absorption with SRBC sufficient to remove all Forssman antibody significantly reduced the complement fixation titre to RSV and chicken breast without reducing the neutralizing antibody concentration.

Absorption with chicken liver alone did not significantly affect the neutralization titre, but lessened the complement fixation titre to RSV and chicken breast, and removed all reactivity with chicken liver. After absorption with SRBC and chicken liver, or SRBC, chicken liver and chicken breast, all of the complement-fixing antibody reacting with these normal chicken tissues was removed; but fixation with RSV persisted at a titre of 1:80 as well as the capacity to neutralize 2.42 logs of virus.

These results indicate that when a preparation of RSV of chicken origin which contains both virus and components derived from the chicken, is injected into a rabbit, antibodies are formed against at least 4 antigenic components: The Forssman antigen, present in all normal chicken tissues; an antigen of chicken origin not associated with the Forssman antigen but removable by absorption with chicken liver; an antigen associated with breast tissue which also occurs in the RSV preparation; and an antigen strictly associated with RSV, antibodies against which are not removed by normal chicken tissue and which protect against an RSV infection.

*Effect of absorption on complement-fixing antibodies in anti-normal chicken serum.* The results presented in Table II show that the unabsorbed anti-normal chicken serum reacts equally well with either RSV or with chicken liver; and that absorption of the serum to remove Forssman antibody and antibody reactive with chicken liver, completely removes the complement-fixing antibodies reactive with RSV.

*Effect of complement on neutralization of RSV and anti-RSV serum and with anti-normal chicken serum.* The addition of complement (C′) to the 1:100 dilution of anti RSV serum which had been absorbed with SRBC chicken liver and chicken breast, increased the NI by 1.42 logs (2.42 to 3.85), indicating some enhancement of neutralization.

The unabsorbed anti-normal serum in a 1:10 dilution had no neutralizing effect (NI 0.25), with an equivocally significant increase when C′ was added (NI 0.77). The NI of complement alone was 0.3.

*Discussion and conclusions.* A large quantity of neutralizing antibody remained in rabbit anti-RSV serum after thorough absorption of the serum with sheep erythrocytes and

TABLE II. Complement-Fixing Antibodies in Rabbit Anti-Normal Chicken Serum.

| Serum | Complement-fixation titer RSV | Chicken liver |
|---|---|---|
| Unabsorbed | 1:160 | 1:160 |
| SRBC absorbed | 1:80 | 1:80 |
| SRBC-chicken liver absorbed | 0* | 0* |

* Also negative by precipitin test when tested by double diffusion in agar.

normal chicken tissues. The ability of the antibodies formed in response to RSV to fix complement with normal chicken tissues is reduced following absorption with sheep erythrocytes, and is completely removed following additional absorption with chicken liver and chicken breast. The complement fixation titre with RSV as an antigen, however, while considerably reduced by absorption, is not extinguished, and remains at a relatively high (1:80) titre.

Antibodies in a similar antiserum prepared against normal chicken liver fixed complement with RSV and with normal chicken tissues. These antibodies were completely removed by absorption. No neutralizing antibodies were demonstrable in this serum.

Dmochowski(5) prepared antisera in rabbits against Rous sarcoma tissue and normal chicken plasma and erythrocytes. He found a greater complement fixation capacity of the anti-RSV serum with RSV and Fujinami tumor extract than with normal chicken tissue extracts; and greater fixation of complement with normal tissue extracts when the anti-fowl plasma or anti-erythrocyte sera were used. Kabat and Furth(6) were unable to show that the neutralizing antibodies in an anti-RSV rabbit serum fixed complement, and attributed this to either the greater sensitivity of the neutralization test, or to the fact that the neutralizing antibody is incapable of fixing complement. The present findings would support the first explanation, especially since the 1:100 dilution of serum, while maintaining a high neutralizing capacity, would be negative in a complement fixation test. The fact that chicken anti-RSV neutralizing antibody does not fix complement very likely reflects the inability of chicken antibody to fix complement in any antigen-antibody system as measured by the usual complement-fixation procedure(7).

The finding that the anti-RSV serum after absorption with normal tissues neutralizes virus to a greater extent in the presence of complement is in keeping with the observation that complement interacts to enhance the protective capacity of many antibodies.

Absorption with chicken tissue may have had a slight effect on the neutralization titre of the antiserum for RSV; or the observed slight diminution in the neutralization index following absorption may reflect the non-specific physical removal of neutralizing antibody during the process of absorption. The persistence of the ability of the antiserum in a 1:100 dilution to neutralize 2.42 logs of the virus after absorption with massive amounts of normal chicken tissue suggests that if the RSV particle does contain an antigen derived from the host, antibodies against this host component are of minimal if any value as neutralizing antibodies, in comparison to neutralizing antibodies directed against distinct viral antigen or antigens. These findings are in agreement with those of previous workers including Rubin(1) who has shown that anti-chicken sera probably act against the cell in which the virus is growing, rather than neutralizing the virus itself; and Borsos(8), Kabat and Furth(6), Keogh(9) and Barrett(10) all of whom were unable to show any neutralization of Rous virus with antisera prepared against normal chicken tissues.

*Summary.* An antiserum against Rous sarcoma virus prepared in the rabbit was found to contain a large quantity of complement-fixing antibodies for RSV and for normal chicken tissue, as well as a high titre of neutralizing antibody against RSV. After absorption of the serum with sheep erythrocytes and normal chicken tissues, complement-fixing antibody reacting only with RSV remained, as well as a large quantity of the neutralizing antibody. These findings indicate that RSV antibody prepared in the rabbit is capable of fixing complement with RSV. They also support the thesis that the antibody neutralizing RSV is directed against viral and not against normal tissue antigens.

We are grateful to Dr. F. J. Rauscher, under whose supervision and in whose laboratory the neutralization tests were conducted; and to Mr. J. P. Kvedar for preparation of the RSV and chicken tissue antigens.

1. Rubin, H., *Virology,* 1956, v2, 545.

2. Bryan, W. R., Moloney, J. B., Calnan, D., *J. Nat. Cancer Inst.,* 1954, v15, 315.

3. Cherry, W. B., Goldman, M., Carski, T. R., Moody, M. D., Pub. Health Service Pub. No. 729, U. S. Govt. Printing Office, Washington, D. C., 1960.

4. Rauscher, F. J., Groupé, V., *J. Nat. Cancer Inst.*, 1960, v25, 141.
5. Dmochowski, L., *ibid.*, 1948, v9, 57.
6. Kabat, E. A., Furth, J., *J. Exp. Med.*, 1941, v74, 257.
7. Rice, C., *J. Immunology*, 1948, v60, 11.
8. Borsos, T., *J. Nat. Cancer Inst.*, 1958, v20, 1215.
9. Keogh, E. V., *Br. J. Exp. Path.*, 1938, v19, 1.
10. Barrett, M. K., *Cancer Research*, 1941, v1, 543.

Received September 3, 1963. P.S.E.B.M., 1964, v115.

## Histamine-Induced Increase in Fibrinolytic Activity.* (28977)

RUDOLF HOLEMANS AND ROBERT D. LANGDELL (Introduced by R. H. Wagner)
*Department of Pathology, University of North Carolina School of Medicine, Chapel Hill*

There appears to be a possible relationship between blood fibrinolytic activity and endogenous histamine release in a wide variety of conditions. In humans, following various forms of shock there is increased fibrinolytic activity(1) and depletion of cellular histamine(2). In dogs, endotoxin injection results in increased fibrinolytic activity(3) and plasma histamine(4). In mice increased tissue histidine decarboxylase activity follows stress, including endotoxin and epinephrine injection which tend to increase fibrinolytic activity(5).

To study the relationship between histamine and fibrinolysis we have determined fibrinolytic activity of blood in dogs following intravenous injection of histamine.

*Materials.* (1) Histamine phosphate USP (Burroughs Wellcome), dissolved in pyrogen-free water to a concentration of 0.5 mg/ml, or histamine phosphate USP (Abbott), 1 mg/ml in isotonic saline; (2) Bovine fibrinogen (Behringwerke, Marburg/Lahn, Germany); (3) Bovine Thrombin Topical (Parke-Davis), 50 NIH units/ml in buffer; (4) Bovine chloroform-activated plasmin (Parke-Davis), 10 Loomis units/ml in buffer; (5) Veronal-HCl-NaCl buffer, pH 7.35, according to Owren(6).

*Methods.* Histamine phosphate was given intravenously in a single dose (50-200 μg/kg) to 17 healthy mongrel dogs (14-23 kg) of either sex without anesthesia or sedation.

The euglobulin fraction of plasma was precipitated from a mixture of 1 ml plasma with 15 ml distilled water by adjusting the pH to 5.7 with 1% acetic acid solution. The euglobulin fraction was isolated by centrifugation and resuspended in one ml of buffer. All these steps were carried out at 4°C.

Fibrinolytic activity of the euglobulin fraction was determined on standard bovine fibrin plates, prepared according to a modification of the method of Astrup and Müllertz (7). Four and a half ml of bovine fibrinogen solution, 240 mg% in buffer, were added to 4.5 ml of 0.05 M $CaCl_2$ solution and the mixture clotted in a standard Petri dish with 0.1 ml thrombin solution. One drop (30 lambda) of euglobulin sample was placed on the fibrin layer. After incubation at 37°C for 20 hours the area of the lysed zone, expressed as the product of two perpendicular diameters, was measured. For each sample 3 separate determinations were made and the mean area determined.

Euglobulin lysis times were performed in 3 experiments. A volume of 0.9 ml euglobulin solution was clotted by addition of 0.1 ml thrombin solution and the time required for complete lysis at 37°C was recorded.

The antiplasmin activity of the plasma stored at 4°C for 24 hours was determined as follows. Two-tenths ml of plasma was mixed with 0.8 ml plasmin solution. After incubation of this mixture for 1 hour at 37°C, the residual fibrinolytic activity was determined on standard fibrin plates.

Glutamic-oxalacetic transaminase (GOT) in plasma was determined with the Sigma Chemical Co. reagents.

Protein content of the euglobulin fraction

* This investigation was supported by research grant from Nat. Inst. Health, P.H.S.