Summary. The urinary excretion of trypsin inhibitor produced by equal amounts of glucocorticoid hormone administered to man at 2 different periods of the day was studied. The hormonally induced increase in excretion was much larger during daytime than during the night. It is concluded that the response, in terms of urinary trypsin inhibitor excretion, to a given amount of glucocorticoid, (*i.e.*, the sensitivity to the hormone) is much lower during the night than during the day.

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IgM and IgG Anti-Hapten Antibody: Hemolytic, Hemagglutinating And Precipitating Activity.* (30531)

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Antibody activity is found in several classes of globulin including those antibodies against simple haptenic groups. The presence of antibody activity against haptenic groups in rabbit macroglobulin (IgM‖) as well as in 7S γ-globulin (IgG‖) has been reported by Bauer(1) and by Onoue *et al*(2). In our previous study(3), rabbit IgM antibody against the *p*-azobenzenearsonate (R*p*) group was prepared in high purity and the number of hapten-binding sites of IgM antibody molecule was determined. It was shown that 6 hapten binding sites are present on the native IgM antibody molecule based on a molecular weight of 1,000,000. Little change was observed in the number of sites and constant for binding with the hapten after reduction and alkylation of native IgM antibody.

Using highly purified anti-R*p* antibody preparations of known binding properties, we compared, in the present study, the hemolytic and agglutinating activities of IgM and IgG antibody with erythrocytes to which R*p*-groups were attached(4). In agreement with previous reports by others on antibodies against erythrocytes or bacterial cells(5), the IgM antibody was shown to have a considerably higher activity than the IgG antibody in both hemolysis and hemagglutination. Reduction and alkylation of the IgM antibody resulted in loss of these activities as well as loss of precipitating activity with a polyvalent hapten-protein conjugate.

Materials and methods. Antisera. Antisera against the *p*-azobenzenearsonate (R*p*) group

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‖ In analogy to the nomenclature for human immunoglobulins recommended by WHO, we are using the symbols IgM and IgG for rabbit γ_1- or β_2-macroglobulin and 7S γ-globulin. Another antibody component previously called B_1-globulin antibody(2) was designated as IgA antibody because of its similarity to human IgA antibody in physical and biologic properties based on our more recent data.

were obtained from rabbits immunized intravenously either with a solution of a conjugate of R*p* with bovine γ-globulin (R*p*-BGG) or with plastic particles coated with R*p*-BGG. The methods of immunization have been described(3).

*Purified anti-R*p *antibodies.* The method of purification, purity and hapten binding properties of the IgM antibody have been described(3). In brief, the antibodies were purified by use of an immunoadbsorbent prepared by coupling *p*-azobenzenearsonate groups to an insoluble polymer of rabbit serum albumin (R*p*-poly RSA)(6). Antibodies adsorbed on the adsorbent were eluted with a benzenearsonate solution. The hapten was subsequently removed by dialysis. IgM and IgG antibodies were separated by gel filtration on Sephadex G-200 or BioGel P-300. IgM antibody was further purified by chromatography on diethylaminoethylcellulose. Three specifically purified antibody preparations with known binding constants, No. 3523, 3524, 3527 (Table I), were used without fractionation.

Reduced-alkylated IgM antibody was prepared by reduction with 0.1 M mercaptoethanol (for 40 minutes at room temperature, then for 6 hours at 4°C) and by subsequent alkylation with iodoacetamide. Procedures and properties of the product were described previously(3).

Hemolytic activity was measured with hapten-coupled erythrocytes(4) according to the method of Ingraham(7) with some modification. Alligator erythrocytes were used instead of sheep erythrocytes since Cohen *et al*(8,9) reported that the cells are remarkably durable under osmotic and mechanical stress. Although the cells were resistant to spontaneous hemolysis during and after coupling with haptenic groups, they were satisfactorily sensitive to immune hemolysis. R*p*-groups were coupled with erythrocytes by reacting one volume of washed, packed erythrocytes with 20 volumes of diazotized arsanilic acid (1.6×10^{-2} M) at pH 7.9 for 30 minutes in an ice bath. After washing, the R*p*-coupled erythrocytes were stable in Alsever's solution at least for 3 days at 4°C without any appreciable spontaneous hemolysis. The amount of antibody required for 50% hemolysis was determined according to the method of Taliaferro and Taliaferro(10) and Ingraham(7). In controls, the IgG fraction and whole macroglobulin fraction of normal rabbit serum were used in place of antibody solution.

Hemagglutinating activity was determined in 2 systems, (a) alligator erythrocytes coated with R*p*-rabbit serum albumin (R*p*-RSA) and (b) sheep erythrocytes coupled directly with diazotized *p*-arsanilic acid.

(a) *Alligator erythrocytes* were coated with R*p*-RSA after treating the cells with tannic acid (0.005%) according to the method of Stavitsky(11). For coating, 1 volume of 2.5% tanned erythrocyte suspension was incubated with 5 volumes of R*p*-RSA¶ (0.04 mg/ml in phosphate buffer, pH 6.4), for 10 minutes at room temperature. The washed cells were used as a 1.5% suspension for the hemagglutination test.

(b) *Sheep erythrocytes* were coupled with R*p*-groups according to the procedure described for alligator erythrocytes, except that a more dilute diazonium reagent was used and the pH was lower during the coupling. One volume of the washed, packed erythrocytes was reacted with 10 volumes of diazotized *p*-arsanilic acid (1×10^{-3} M) at pH 7.6 ± 0.1 for 30 minutes in an ice bath. The cells were then washed and suspended to give a final concentration of 2.5% with Ingraham's #2 buffer (pH 7.3-7.4**). The same buffer solution was used as a diluent for dilution of antibody in hemagglutination test.

(c) *Hemagglutination test.* Each dilution of antibody solution (0.5 ml) was mixed with 0.05 ml of cell suspension. Agglutination of

¶ Number of R*p* group coupled on tyrosyl and histidyl residues of RSA molecule was determined as 7.8 by spectrophotometric method(12).

** Ingraham's #2 buffer was made as follows. To a 1500 ml solution containing 10.2 g sodium 5,5-diethyl barbiturate and 85 g NaCl, 10 ml of a solution containing 3.8 ml of 10 N/HCl (to adjust the pH to 7.3-7.4), 0.023 g $CaCl_2$ $2H_2O$ and 1.0 g $MgCl_2$ $6H_2O$ were added and made up to total 2,000 ml with water. For use, a mixture of 200 ml of the above solution, 10 ml of 20% bovine serum albumin (BSA) and 10 g dextrose was diluted freshly up to 1,000 ml with water.

alligator cells was judged after 3 hours at room temperature. Agglutination of sheep cells was judged after 3 hours at room temperature and after subsequent standing overnight in a cold room. A 2+ pattern of agglutination as defined by Stavitsky(11) was taken as an end point. In controls, the diluent and the IgG and whole macroglobulin fractions of normal rabbit serum were used in place of the antibody solution. Tanned alligator cells or mock-treated sheep cells (without R*p*) were also used in place of coated or R*p*-coupled cells to examine nonspecific agglutination.

Determination of amount of IgM antibody in IgG antibody preparations. Two-fold dilutions of each IgG antibody preparation were made with a standard normal rabbit serum. Radioimmunoelectrophoresis was carried out using the same amount of each dilution of antibody preparation and diffusing reagent. A mixture of sheep antiserum against the whole macroglobulin fraction of normal rabbit serum and I^{125}-labeled insulin coupled with *p*-azobenzenearsonate (I^{125}-R*p*-insulin, 2 μg/ml sheep antiserum) was used as a diffusing reagent. As a reference, a purified IgM antibody preparation (Prep. A) was diluted with the same normal rabbit serum and each dilution was submitted to radioimmunoelectrophoresis in the same way. All the slides were exposed simultaneously to the same sheet of X-ray film to obtain radioautographs. The amount of IgM antibody in an IgG preparation was estimated by comparing the limiting concentration of each preparation which gave an IgM arc on the radioautograph with the limiting concentration of purified IgM antibody Prep. A.

Radioimmunoelectrophoresis. The procedures as applied to this antihapten system have been described(2).

Determination of antibody content. This was done by adsorption with a specific immunoadsorbent as described previously(3).

Results. Hemolytic and hemagglutinating activities of one IgM and 2 IgG antibody preparations as well as 3 unfractionated antibody preparations were determined. All antibody preparations had been specifically purified with immunoadsorbents. Binding constants of these preparations with *p*-iodobenzenearsonate and their antibody contents are listed in Table I. The binding constants for all the antibody preparations were of similar order. All the preparations contained more than 90% antibody. The amount of contaminating IgG antibody in IgM Prep. A was only 2.5%. No IgM antibody was detected in the 2 IgG antibody preparations by radioimmunoelectrophoresis. Small amounts of IgM antibody were found in unfractionated antibody preparations. The amounts estimated by radioimmunoelectrophoresis are shown in Table I. None of the preparations contained antibodies of other classes (9S and 7S IgA-antibody‖).

TABLE I. Purity and Binding Constant of Anti-R*p* Antibody Preparations.

Antibody preparation	Antibody content: Total	IgM§ (%)	IgG‖ (%)	Binding constant with *p*-iodobenzenearsonate, $\times 10^5$ l/mole
IgM: Prep. A	93*	90	2.5	4.5
IgG: Prep. a	92*	<.13	92	—
Prep. b	99†	<.19	99	3.1
Unfractionated:				
No. 3523	100†	1.60	98	1.3
No. 3524	90*	.17	90	2.7
No. 3527	99*	.76	98	1.7
Reduced-alkylated IgM	68‡		2.5	~3

* Antibody content was determined by adsorption with an immunoadsorbent, R*p*-poly RSA.

† Antibody content was determined by equilibrium dialysis with *p*-iodobenzenearsonate on the basis of 2 binding sites per molecule of IgG antibody with a molecular weight of 160,000.

‡ A portion of IgM Prep. A used for equilibrium dialysis was extensively dialyzed to remove hapten and then used for reduction and alkylation. Some inactivation of antibody occurred in these procedures. Antibody contents were 93% for the original preparation A, 83% after removal of hapten and 68% after reduction and alkylation, as determined by adsorption with R*p*-poly RSA.

§ Values for IgG and unfractionated antibody preparations are those determined by radioimmunoelectrophoresis. Value for the IgM antibody preparation is: Total Ab(%)—IgG Ab(%).

‖ Values for native and for reduced-alkylated IgM antibody preparations are those determined by radioimmunoelectrophoresis. Values for IgG and unfractionated antibody preparations are: Total Ab(%)—IgM Ab(%).

Hemolytic activity. The amounts of IgM and IgG antibody required for 50% hemolysis are given in Table II. The relative

TABLE II. Hemolytic and Hemagglutinating Activity of Anti-R*p* Antibodies.

Antibody preparation	Hemolysis*			Hemagglutination†			
				R*p*-BSA-all. cells‡		R*p*-sheep cells§	
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 1	Exp 2
	μg antibody			μg antibody			
IgM: Prep. A	.16	.14	—	.018	.009	.055	—
IgG: Prep. a	7	5	6	.58	.29	.46	1.15
Prep. b	9.5	4.5	9.5, 11	—	—	—	5.0
Unfractionated:							
No. 3523	9.5	4.5	8.5	—	—	—	1.25
No. 3524	—	—	13.5	—	—	—	1.15
No. 3527	>15 ‖	>10.5‖	39, 43	—	—	—	2.45
Reduced-alkylated IgM		>5¶		~5			—

* Degree of hemolysis was determined from supernatants of mixtures containing 0.5 ml antibody, 0.25 ml erythrocyte suspension and 0.25 ml 10-fold diluted guinea pig serum (complement). Concentration of erythrocyte was adjusted to about 2% so that hemolysate gave an optical density of 0.95 at 541 mμ. Values indicate amount of antibody required for 50% hemolysis.

† Hemagglutination was determined with mixtures containing 0.5 ml antibody and 0.05 ml 1.5% alligator or 2.5% sheep erythrocyte suspension. Values indicate amount of antibody required to give 2+ pattern of hemagglutination.

‡ Alligator cells coated with R*p*-RSA were used in this test.

§ Sheep cells coupled with R*p* groups were used in this test.

‖ Hemolysis was 10% at 15 μg and 2.2% at 10.5 μg.

¶ No hemolysis was observed at this concentration.

hemolytic activity of IgM antibody observed is about 30 to 50 times higher on a weight basis than the 2 IgG antibody preparations a and b. These IgG preparations contained no detectable amount of IgM antibody by radioimmunoelectrophoresis (Table I) although a small amount of material sedimenting slightly faster than 7S was seen on ultracentrifugation. This material is probably an aggregate of IgG antibody because no IgA antibody was detected by radioimmunoelectrophoresis. Thus, the large difference observed here in hemolytic activity is attributed to difference in the characteristics of IgM and IgG antibodies. The 2 IgG antibodies and 2 of the 3 unfractionated antibody preparations showed similar hemolytic activity. However, one of the unfractionated antibody preparations, No. 3527, showed significantly lower activity than IgG antibody preparations a and b, or other unfractionated antibody preparations.†† Although it is possible that hemolytic activity varies to some extent among antibody preparations of a particular immunoglobulin class, the present results indicate that the hemolytic activity of IgM antibody is much greater than that of IgG antibodies under the conditions used.

Hemagglutinating activity. Table II gives results of hemagglutination. The activity of IgM antibody on a weight basis was about 30 times higher than IgG, Prep. a, as measured with alligator cells coated with R*p*-RSA. It was about 9 times higher when sheep erythrocytes coupled with R*p* groups were used. The activity of different IgG antibody preparations or unfractionated antibody preparations was also compared by the use of the sheep cells. They showed similar activity except IgG, Prep. b, which showed somewhat lower activity. The difference of the ratio of IgM to IgG (Prep. a) in the 2 systems, namely, one with alligator cells coated with R*p*-RSA and the other with sheep cells coupled with R*p*-groups, may be due to differences in physical properties of the erythrocytes used, or the difference in distribution of the haptenic groups over the surface of erythrocytes, or both.

Hemolytic and hemagglutinating activity of

†† All 3 unfractionated antibody preparations had been prepared by the use of immunoadsorbents at the same time and stored in a refrigerator for about 10 months in borate buffer at pH 8.0 before use. Antibody contents were determined at the time of this experiment.

reduced-alkylated IgM antibody. No hemolysis was observed with 5 μg of the reduced-alkylated product of the IgM antibody preparation (Table II). Therefore, the activity was much lower than 3% of the native antibody. The hemagglutinating activity of the reduced-alkylated IgM antibody preparation was 0.2 to 0.4% of that of the native antibody. We had previously shown that essentially all the hapten-binding sites of the native IgM antibody remain intact after reduction and alkylation(3). Whether the small amount of residual hemagglutinating activity is due to the activity of reduced 6S subunits of IgM antibody or due to a small amount (2.5%) of contaminating IgG antibody in this preparation is not clear.

Attempts to demonstrate inhibitory activity of reduced-alkylated IgM antibody in hemolysis or hemagglutination tests were not successful under the conditions described below.

In hemolysis, no change in the 50% end point was observed with a series of mixtures consisting of reduced-alkylated IgM antibody (1.7 μg) and of dilutions of native antibody (2.9 to 0.36 μg) as compared with a control series without reduced-alkylated antibody. The reduced-alkylated antibody did not inhibit hemolysis by IgG antibody either, when the reduced-alkylated antibody (1.7 μg) was added to IgG antibody, Prep. a (6 and 10 μg).

Alligator cells coated with R*p*-RSA were used for inhibition tests in hemagglutination. No shift of end point was observed in a series of mixtures consisting of reduced-alkylated IgM antibody (1.7 μg) and of dilutions of native antibody as compared with a control series without reduced-alkylated antibody. The end point was 0.009 μg for both series. Using the same amounts of reduced-alkylated IgM antibody, it was also not possible to demonstrate inhibitory activity in the hemagglutination test by first incubating erythrocytes with reduced-alkylated antibody and then mixing with native antibody. For comparison a univalent papain fragment (Fab) of purified anti-R*p* (IgG) antibody was examined under the same conditions. When 2.5 μg Fab was present, the end point shifted from 0.018 μg to 0.035 μg of antibody (one tube shift) indicating that 2.5 μg was the minimum amount for papain fragment Fab to show detectable inhibition in this system. On this basis the amount of reduced-alkylated IgM antibody added (1.7 μg) may not have been enough to demonstrate any inhibition. The test could not be carried out at a higher concentration because of a small residual hemagglutinating activity of the reduced-alkylated IgM preparation.

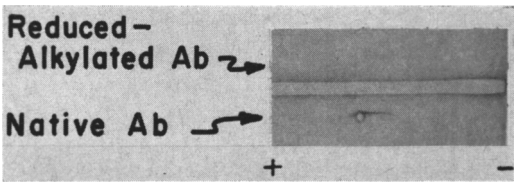

FIG. 1. Precipitating activity of native and reduced-alkylated IgM anti-R*p* antibody. Native and reduced-alkylated IgM antibody preparations (about 4 mg/ml) were electrophoresed. A solution of R*p*-RSA with a concentration of 50 μg/ml in RSA (1 mg/ml) solution was diffused from the center channel. Reduced-alkylated IgM did not show any arc at 100 μg/ml concentration of R*p*-RSA, either (not shown in Figure).

Precipitating activity of native and reduced-alkylated IgM antibody. As shown in Fig. 1, native IgM antibody formed a precipitate arc at a position typical for IgM antibody in immunoelectrophoresis on reacting with R*p*-RSA, while the reduced-alkylated antibody did not form any precipitate arc.

Discussion. A number of investigators have shown that high molecular weight antibodies are more efficient than 7S antibody in agglutination and hemolysis(5). They used antibodies against a complex antigen, such as erythrocytes, bacteria and protein antigens.

Our present work was carried out with antibodies against a known haptenic group, *p*-azobenzenearsonate (R*p*). Antibodies were purified with a specific immunoadsorbent and fractionated to isolate antibodies of the IgM and IgG classes. By use of such antibody preparations, the results clearly indicate that the IgM antibody is much more efficient than the IgG antibody in hemolysis as well as in hemagglutination of erythrocytes to which R*p*-groups are attached.

The amount of IgM antibody required for 50% hemolysis was approximately 1/30 that of IgG antibody. For positive agglutination

the ratio of the 2 antibodies was 1:30 or 1:10 depending upon the system used. On molecular basis, an IgM antibody molecule would be roughly equivalent to 180 to 60 IgG antibody molecules for these reactions (assuming a molecular weight of 1,000,000 for IgM and 160,000 for IgG). A difference by factor of about 750 was reported by Greenbury *et al* with anti-blood group A antibodies(13). Factors responsible for such large differences between IgM and IgG antibodies are beyond the scope of the present investigation. However, IgM antibody molecules being of a larger size and having a higher number of antigen-binding sites (6 sites per molecule (3)) might be expected to be more efficient than the smaller bivalent IgG molecules in forming antibody bridges between the greatly larger erythrocytes.

Although the binding constants for a simple hapten were found to be similar for IgM and IgG antibody preparations used, the l number of binding sites of the IgM antibodies may lead to multiple site attachment of an antibody with an erythrocyte and much greater apparent binding constant as pointed out by Greenbury *et al*(14,14a). For example 2 site attachments with two times the energy of a single site attachment would result in a square of the binding constant observed for a single site attachment.

It was shown that IgM antibody lost both hemolytic and hemagglutinating activity upon reduction and alkylation although its hapten-binding activity was fully retained(3). The loss of these activities without loss of hapten-binding activity is consistent with the results reported by Chan and Deutsch(15) and by Jacot-Guillarmod and Isliker(16). In accord with the results of Josephson *et al*(17) and of Benedict *et al*(18) with anti-protein antibodies, IgM anti-hapten antibody formed a precipitate with a polyvalent hapten-protein conjugate and the precipitating activity was lost upon reduction. These results further support the view that reduced and alkylated 6S subunits of IgM antibody are univalent (3). Failure by Shulman *et al*(19) to demonstrate the precipitating activity of native IgM antibody may be due to low antibody concentration in their preparation.

Inhibition of the hemagglutinating activity of intact isoagglutinin by the reduced-alkylated product was shown by Jacot-Guillarmod and Isliker(16). No inhibitory activity could be demonstrated in both hemolytic and hemagglutinating systems in the present experiment. However, the amount of reduced-alkylated antibody used was apparently not enough to visualize the inhibition. 2.5 μg of the univalent papain fragment Fab of IgG antibody was required to cause a one tube shift of end point in hemagglutination, whereas a maximum of 1.7 μg of reduced-alkylated IgM antibody was used for the experiment.

Summary. Hemolytic and hemagglutinating activities of IgM and IgG antibody against the *p*-azobenzenearsonate (R*p*) group were compared using purified antibody preparations. IgM antibody showed a considerably higher activity in both hemolytic and hemagglutinating systems. The hemolytic and hemagglutinating activities of IgM antibody were both 180 to 60 times higher on a molar basis than those of IgG antibody when measured with erythrocytes to which R*p*-groups were attached. The reduced-alkylated product of IgM antibody showed little activity in both hemolytic and hemagglutinating systems, although the reduced-alkylated product had been shown previously to retain hapten-binding activity. IgM antibody also lost precipitating activity with a polyvalent hapten-protein conjugate upon reduction and alkylation. Loss of hemagglutinating and precipitating activities is probably due to univalency of 6S subunits produced by reduction.

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Plasma Enzyme Elevations with LDH Viruses from Different Tumors.* (30532)

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Many transplanted mouse tumors have been shown to be contaminated with a virus-like agent which produces 5-10-fold elevations in plasma LDH within 3-4 days of infection (1-8). The virus may be transmitted in the absence of tumor by injection of infected plasma alone and virus titer reaches 10^{10}-10^{12} infectious units per ml of plasma within 24 hours. Increase in plasma LDH enzyme does not begin until approximately 30 hours after infection, reaching a maximum in about 4-7 days, at which time virus titer has decreased to about 10^{6}-10^{7}(4). Infectious virus and elevation in plasma LDH enzyme persist throughout the otherwise normal lifespan of the mouse. There is no overt tissue damage and level of LDH in the tissues remains normal(6).

Following initial confirmation of the increase in LDH, it was shown that certain other plasma enzymes were also elevated(2,9, 10). A comparison of the results obtained by 4 groups of workers, each using a different tumor as a source of virus(10,9,2,7), shows that although there is qualitative agreement in the pattern of enzyme elevations, there is considerable quantitative disagreement for the different viruses. This is particularly evident for phosphohexose isomerase, where elevations ranging from 2.1-fold with the Moloney leukemia derived virus(2) to 6.7-fold with the Sarcoma 37 agent(9) have been reported.

Recent work has shown that plasma clearance of certain injected enzymes is impaired in infected mice(11,12,13,14) and blockage of plasma clearance mechanisms has therefore been proposed as a general mechanism for the action of LDH viruses. If such a common mechanism underlies the action of the virus, identical enzyme elevations should be produced by viruses from different tumors. If the viruses differ in their action, viruses from different tumors may produce different enzyme elevations. The present paper describes experiments in which LDH viruses were isolated from 8 different mouse tumors. Relative plasma enzyme elevations in the same strain of mice were measured at intervals following infection with each of the 8 viruses.

Materials and methods. Chemicals were from Sigma Chemical Co. or Nutritional Biochemicals, Inc. Mice used for measurement

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