chloride being the predominant urinary electrolytes.

Discussion. The data demonstrate that furosamide, given intravenously, is capable of markedly increasing the excretion of sodium, chloride, and water. A much smaller, but definite increase in potassium accompanies these effects. The diuresis is produced without a change in glomerular filtration rate as estimated by endogenous creatinine clearance, or the effective renal plasma flow (PAH clearance). Although very low creatinine clearances may not accurately measure filtration rate, the lack of change in clearance following furosemide argues for a corresponding lack of change in glomerular filtration rate.

One site of action of furosemide is clearly the proximal convoluted tubule as shown by some of the urine flow/creatinine clearance ratios presented. Additional sites are not excluded by the data although it is possible to examine the question clinically by observations of free water clearance and tubular reabsorption of free water during various states of hydration(4). Suki has presented data from dog studies from which he concludes that furosemide inhibits sodium reabsorption in the proximal convoluted tubule and the ascending limb of Henle's loop(5).

The eventual place of furosemide in clinical medicine will depend on accumulated experience with its oral usage in various edema producing diseases particularly in comparison with other diuretics. The present data demonstrate that it is a non-toxic, potent, diuretic under the conditions described above. It is evident that the very large diureses that may be produced by furosemide can lead to dehydration and on occasion to hypokalemia. Care is required with its use, just as with any powerful drug.

The evidence thus far is sufficient to warrant intensive clinical trials with furosemide, including observation in patients with substantially reduced kidney function.

Summary. A new diuretic compound, furosemide, has been studied in patients with renal disease. Doses of 40 mg intravenously were capable of producing large diureses of sodium, chloride, and water without adverse effects. Preliminary results with oral administration suggest that the drug may prove to be a useful therapeutic agent.

1. Wesson, L. B., Jr., Anslow, W. P., Jr., Am. J. Physiol., 1952, v170, 225.

2. Bonsnes, R. W., Taussky, H. H., J. Biol. Chem., 1945, v158, 581.

3. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., Graber, M., J. Clin. Invest., 1945, v24, 388.

4. Goldberg, M., McCurdy, D. K., Foltz, E. L., Bluemle, L. W., Jr., ibid., 1964, v43, 201.

5. Suki, W., Rector, F. C., Jr., Seldin, D. W., Clin. Research, 1964, v12, 260.

Received September 2, 1964. P.S.E.B.M., 1965, v118.

Hexose Content of Guinea Pig γ_1 and γ_2 Immunoglobulins.* (29836)

HERBERT F. OETTGEN, RUBEN A. BINAGHI AND BARUJ BENACERRAF†

Division of Immune Reactions, Sloan-Kettering Institute for Cancer Research, and Department of Pathology, New York University School of Medicine, New York City

A considerable number of investigations in recent years have been concerned with the problem of the heterogeneity of the immunoglobulins. Three main types of γ-globulins have been recognized, and have been designated γ_2, γ_{1A} (or β_{2A}) and γ_{1M} (or β_{2M}). They have been characterized in various ways, namely by their antigenic determinants, electrophoretic mobility, sedimentation constant, carbohydrate content and molecular structure(1,2).

* Supported by Grants AI-04983 and E-2094 from U. S. Public Health Service, and the Health Research Council of the City of New York, Contract I-138.

† Career Scientist of Health Research Council of City of New York.

Most of these studies have been performed with human or mouse globulins, since normal as well as pathological globulins (myeloma globulins) are available in both species. When immunoglobulins in these and other animal species were compared, some confusion and uncertainty frequently arose as to their nomenclature and equivalence.

In recent studies with purified guinea pig (3,4,5) and mouse 7S(6) antibodies, it was found that antibody populations with different physicochemical and antigenic properties also differ in some biological properties, such as complement fixation and participation in local and systemic anaphylaxis. The recognition of this fact introduces a new criterion for identification of immunoglobulins, so far characterized only by their physicochemical and antigenic properties.

Two distinct populations of 7S guinea pig antibody have been described (γ_2 and γ_1) whose physicochemical and biological properties have been well established. Although it is tempting to conclude that the faster migrating antibody (γ_1), possessing the anaphylactic activity, corresponds to the γ_{1A} type of other species, no definite proof of such identity is available. One of the characteristics of the γ_{1A} globulins in other species is their high carbohydrate content as compared to the γ_2 globulin. It seemed desirable, therefore, to determine the hexose content of guinea pig γ_1 and γ_2 antibodies. This is the subject of the present report.

Materials and methods. Adult, Hartley strain guinea pigs of both sexes were used throughout. The antigen used was highly conjugated dinitrophenyl bovine gamma globulin (DNB-BGG). Preparation of the antigen, immunization and purification of the antibody were performed as previously described (3). Some hapten remained in the final antibody preparations even after prolonged dialysis. Human gamma globulin was also analyzed (Fraction II—E. R. Squibb & Sons, N. Y.).

Separation of specifically purified guinea pig anti-DNP antibodies can be obtained by starch block electrophoresis(3). The fractions resulting from this procedure, however, were contaminated with considerable amounts of polysaccharides and, therefore, were not suited for our purpose. Preliminary experiments indicated that the same was true for fractions obtained by zone electrophoresis in a polyvinyl block (Geon, Goodrich Rubber Co.)(7). The carbohydrate introduced by any of these methods could not be removed by repeated precipitations of the globulins with 18% sodium sulfate (see below).

Resort was made, therefore, to ion exchange chromatography, employing DEAE cellulose. The samples were dialyzed against 0.01 M phosphate buffer, pH 8.0, and were applied to a column equilibrated with the same buffer. Under these conditions, most of the γ_2 fraction comes off while the γ_1 is retained and can be subsequently eluted by increasing the salt concentration. When applied to preparations of purified antibody, 2 well separated peaks can be obtained containing the γ_2 and γ_1 globulins respectively. In this experiment, 10 ml containing 127 mg of purified guinea pig anti-DNP antibody, isolated from pooled guinea pig antisera, were applied to a column of 2 cm diameter and 15 cm length (approximately 6 g DEAE cellulose). Following the first peak, a gradient elution was started from 0.01 to 0.15 M phosphate buffer at pH 8.0 The first peak consisted only of γ_2 globulin and the second peak almost entirely of γ_1 globulin with only a trace of γ_2 globulin as shown by immunoelectrophoresis (Fig. 1). An additional indication of the purity of the γ_2 fraction was given by its inability to mediate passive cutaneous anaphylaxis in guinea pigs. It was found that the amount of γ_1 in this fraction was less than 0.5%.

Prior to analysis for hexose, all protein preparations were salted out in 18% Na_2SO_4. As a rule, one volume of a solution containing 10 or more mg protein per ml was mixed with 9 volumes of 20% Na_2SO_4, and the mixture was kept for 1 to 3 hours at 37°C. After centrifugation, the precipitate was dissolved in one volume of distilled water, centrifuged, and the supernatant precipitated again by adding 9 volumes of 20% Na_2SO_4, incubated at 37°C and centrifuged. The precipitate was then dissolved in distilled water. It was found that most of the contaminating hexose was

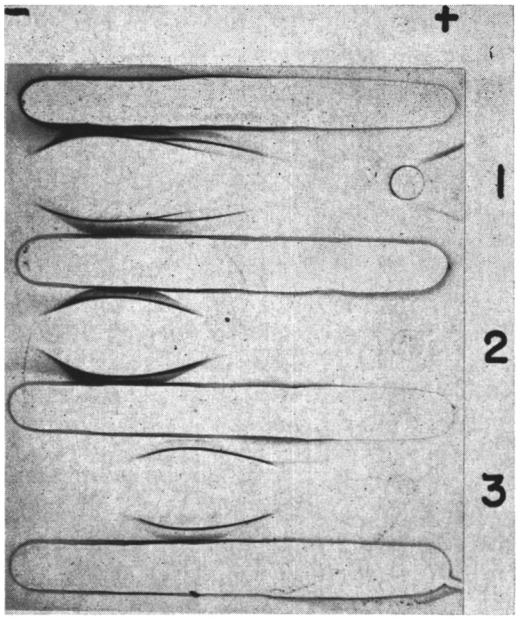

FIG. 1. Immunoelectrophoresis of purified guinea pig anti-DNP antibody (1) and its γ_2 (2) and γ_1 (3) fractions obtained by DEAE-cellulose chromatography, developed with rabbit anti-whole guinea pig serum.

removed by the first precipitation. Two precipitations were routinely performed with all preparations.

Digestion of antibody gamma globulin with pepsin was carried out according to the method described by Nisonoff *et al*(8). Pooled guinea pig anti-DNP-BGG serum containing 3-4 mg of anti-DNP antibody per ml was fractionated on a DEAE-cellulose column. The γ_2 fraction was concentrated by lyophilization, dissolved in a small volume of water and dialyzed against a buffer containing 0.07 M acetate and 0.05 M sodium chloride at pH 4.8. To 165 ml of this solution, containing 12.7 mg protein per ml, 2 mg of pepsin (2 × crystallized, Worthington) were added and the mixture incubated for 18 hours at 37°C. The pH was then adjusted to 8.0 with N KOH, and the digest was dialyzed against 0.16 ionic strength borate-saline buffer, pH 8.2. The material resulting from this digestion was precipitated twice in 18% Na_2SO_4. When the final product was tested with double diffusion in agar, it gave a single precipitin line of partial identity with undigested antibody when developed with rabbit anti-guinea pig serum (Fig. 2). It also gave a clear precipitin line with DNP-BSA, 250 gamma per ml, and with a rabbit anti-S serum (piece I and II of Porter) but gave only a trace reaction with a rabbit anti-F serum (piece III of Porter)(9).

The γ_2 fraction of purified guinea pig anti-DNP-BGG antibody, obtained from a DEAE cellulose column, was digested with papain as described by Porter. Fifty-four mg of antibody in 4 ml were dialyzed against 0.01 M phosphate buffer, pH 7.2, 0.002 M in ethylenedinitrilo-tetracetic acid disodium salt. Eight mg l-cysteine hydrochloride and 1 mg crystalline papain (L. Light & Co., England) were added, the mixture incubated at 37°C for 18 hours and then dialyzed against water. The digested mixture contained at this point both S and F fragments in solution. To obtain only the F fragment the following method was used: 23 mg DNP-fibrinogen were added to the digested mixture to specifically bind the S fragments which possess the combining sites. The soluble complexes so formed were then precipitated by adding streptomycin sulfate, 35 mg per ml, and adjusting the pH to 5.5. The DNP-fibrinogen preparation used(3) was highly conjugated and had been precipitated at pH 2.5 and redissolved in phosphate buffer pH 7.0 before use. After addition of streptomycin, the mixture was

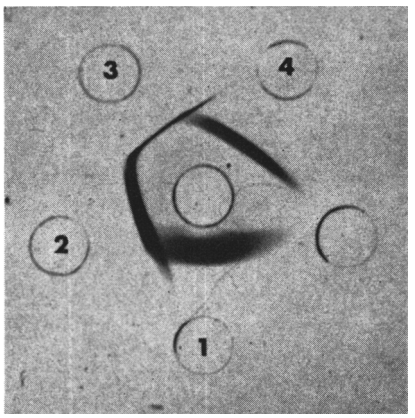

FIG. 2. Double diffusion in agar gel. Center hole: Rabbit anti-whole guinea pig serum. Peripheral holes: γ_2-fraction (DEAE column) of guinea pig anti-DNP-BGG serum. Undigested: Hole No. 2 (2.7 mg protein per ml) and hole No. 3 (0.7 mg protein per ml). Pepsin digest: Hole No. 1 (4 mg protein per ml) and hole No. 4 (1 mg protein per ml).

kept at 37°C for 20 minutes, at 4°C overnight and then centrifuged. The supernatant was dialyzed against 0.15 M sodium chloride with 0.01 M phosphate buffer at pH 8.0 and analyzed for hexose.

Protein estimations and measurements of the optical density at 360 mμ were carried out after each step of the digestion procedure. The final preparation contained 12.5 mg protein (micro-Kjeldahl) in 9 ml, and its optical density at 360 mμ was 0.24 due to the yellow color either of DNP-lysine, still attached to the S fragments, or of DNP-fibrinogen. By correlating these values with those obtained at earlier steps of the procedure it was possible to estimate that S fragments could not constitute more than 30% of the protein finally analyzed for hexose.

The concentration of the different proteins used was estimated by measuring ultraviolet absorption at 280 mμ. The conversion factors were determined by analysis for nitrogen according to the micro-Kjeldahl method. The optical density per μg N/ml was 0.0083 for γ_2 globulin, 0.0094 for γ_1 globulin and 0.0096 for the pepsin digest.

Immunoelectrophoresis was done using a modification of Scheidegger's technique(11). Two per cent agar in barbital buffer pH 8.6, .075 M, was used. The precipitin lines were developed with rabbit anti-whole guinea pig serum.

Double diffusion in agar gel was performed according to the method of Ouchterlony(12).

Two methods were employed for the hexose analysis: the indol and the anthrone methods. As the range of hexose concentration available for analysis was close to the sensitivity limits of these methods, the utmost care had to be taken to avoid contamination by extraneous material such as, for instance, paper lint. All glassware used was carefully washed, kept for 20 minutes at 100°C in 75% sulfuric acid and rinsed with distilled water. This precaution proved essential. Analyses were performed at least in duplicate.

1. *Anthrone method.* Essentially the method described by Mokrasch was used(13). The reagent was prepared in an ice-water bath. Two hundred and fifty ml H_2SO_4 were added to 72.5 ml water, and 250 mg anthrone were dissolved in this mixture. For the assay, 0.5 ml samples were mixed with 3.0 ml of the anthrone reagent in an ice-water bath. The mixture was heated at 80°C for 25 minutes, cooled in ice water and the optical density read at 620 mμ. The measured optical density was corrected for the color of 2 blanks, one containing water as a sample and one containing the protein sample but only sulfuric acid and no anthrone.

2. *Indol method.* The method described by Dische(14) was employed. Five-tenths ml samples were mixed with 75% (in volume) sulfuric acid in an ice-water bath. Two-tenths ml of a 1% solution of indol in ethanol, freshly prepared, were added and thoroughly mixed. The mixture was heated for 10 minutes in a boiling water bath, cooled in ice water and the optical density read at 470 mμ. Corrections for blanks were made as indicated in the anthrone method.

Results. 1. *Comparison of anthrone and indol methods.* Most of the determinations of carbohydrate content of immunoglobulins have so far been carried out with the anthrone method. To express the results on a weight basis, standard curves have usually been prepared with a mixture of 45% galactose, 45% mannose, and 10% 6-deoxygalactose(7,15). This mixture represents approximately the composition of hexoses in human gamma globulin.

As the amount of material available in this study was limited, the indol method was preferred because of its higher sensitivity. The nature of the carbohydrates present in guinea pig γ-globulin being unknown, the results were expressed in terms of glucose equivalents.

Both methods of analysis were compared, using glucose as well as the hexose mixture indicated above to prepare standard curves. The results of this comparison are shown in Fig. 3. It can be seen that the sensitivity of the indol method is approximately twice that of the anthrone method. Analyses of human gamma globulin were performed using both the indol and the anthrone method. Results were identical, within experimental error. The hexose content was found to be 0.78% with reference to the glucose standard or

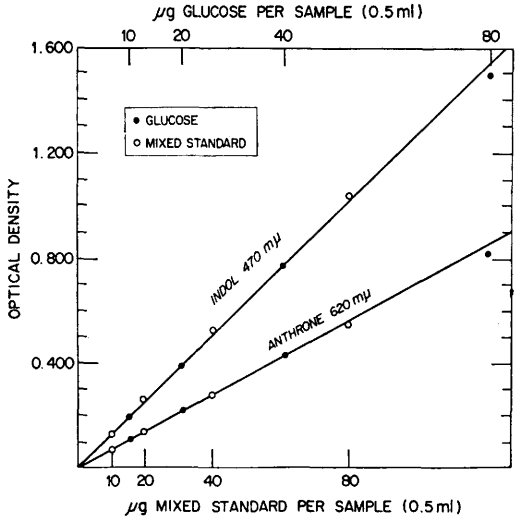

FIG. 3. Standard curves for glucose and mixed standard with indol and anthrone methods. Mixed standard: 45% galactose, 45% mannose, 10% 6-deoxygalactose.

1.19% with reference to the galactose-mannose-fucose mixture. This value is in good agreement with data reported in the literature(15).

2. *Hexose content of guinea pig* γ_2 *and* γ_1 *globulins.* Employing the indol method, the following guinea pig γ-globulin preparations were analyzed: a) non-specific γ_2 globulin; b) specifically purified anti-DNP antibody containing both γ_1 and γ_2 populations; c) and d) two specifically purified γ_1 anti-DNP antibody; e) and f) two specifically purified γ_2 anti-DNP antibody.

Samples a), c), and e) were obtained by DEAE-cellulose chromatography. Sample b) was not fractionated into its components and contained about 60% of γ_2 and 40% of γ_1. Sample d) was obtained from animals immunized without adjuvants which, as described previously(3), produced almost only γ_1 antibody. Sample f) was prepared from a pool of antisera from animals immunized with Freund's adjuvants that produced mostly γ_2 type antibody. Both samples d) and f) were specifically purified antibody preparations not subjected to DEAE chromatography fractionation. The nature of the antibody in these samples was verified by both electrophoresis and immunoelectrophoresis.

The results of the analyses are presented in Table I. They indicate that the hexose contents of guinea pig γ_1 and γ_2 globulin are identical, the average value being 0.74%, and correspond clearly to the hexose content of human gamma globulin.

3. *Hexose content of enzymatically obtained fragments of guinea pig gamma globulin.* The results of the hexose analysis of fragments obtained by enzymatic digestion of γ_2 guinea pig globulin are also shown in Table I. It can be seen that the fragment obtained by pepsin digestion contains very little hexose. The value obtained, 0.12%, is close to the sensitivity of the method, and represents probably a maximum estimate. On the other hand, the hexose content of the F fragment obtained by treatment with papain is more than twice that of the original molecule. The actual value might even be somewhat higher than that quoted in the Table, since the preparation analyzed contained a significant amount of contamination by S fragments.

Considering that the F fragment represents about ⅓ of the globulin molecule, it can be calculated that 90% or more of the total hexose in the molecule is contained in this fragment.

Discussion. Evidence has been presented that guinea pig γ_1 and γ_2 globulins have identical hexose contents. The average value obtained expressed in glucose equivalents was 0.74%. This is approximately the same as the values reported for γ_2 globulins of man, mouse, and rabbit. Analysis of different preparations of non-specific γ_2 as well as antibody γ_2 and γ_1 globulins obtained after fractionation with DEAE-cellulose, or specifically purified from sera of animals that produced mainly one type of antibody, have given identical results in all cases.

Analysis of the fragments obtained after digestion of guinea pig γ_2 globulin with papain have shown that most of the hexose is present in the fragment designated as "fast" (F), after papain digestion, corresponding to the piece III of Porter. On the other hand, the fragment obtained by digestion with pepsin, corresponding to the 5S fragment described by Nisonoff in the rabbit, contains very little if any hexose. These results are

TABLE I. Hexose Content of Guinea Pig γ-Globulins and Their Fragments.

Sample*	mg protein per sample (.5 ml)	Optical density at 470 mμ (Indol method) †		% hexose (glucose equivalent)
a) Nonspecific γ_2	.681	.10	.10	.74
b) Antibody $\gamma_1 + \gamma_2$	.557	.07	.09	.75
c) " γ_1	1.050	.12	.16	.75
d) " γ_1	1.300	.18	.20	.73
e) " γ_2	.900	.13	.15	.77
f) " γ_2	1.350	.22	.20	.77
g) Pepsin digest	1.300	.03	.04	<.12
h) F fragment	.706	.23	.26	1.77

* See text.

† Corrected for blanks.

in agreement with data reported for human and rabbit γ-globulins(16,9).

When guinea pig γ_1 globulins were first described(3,4,5), the problem arose of whether they corresponded to the γ_{1A} globulins of man and mouse which are characterized by carbohydrate contents higher than those of the γ_2 globulins of these species(2). The results of our experiments show that in the guinea pig both γ_1 and γ_2 globulins have identical hexose contents. Guinea pig γ_1 globulins cannot be considered, therefore, to be the counterpart in this species of human γ_{1A} globulins.

In a recent study of mouse antibodies(16), a situation has been found which is very similar to that in the guinea pig. In addition to a 19S component, two γ-globulins, designated γ_1 and γ_2, have been identified, whose relationship to one another, as far as electrophoretic mobility and biological properties are concerned, corresponds to that of guinea pig γ_1 and γ_2 globulins. Moreover, comparison of γ_1 and γ_2 with mouse γ_{1A} myeloma protein have indicated that all 3 are antigenically different. It was concluded that 4 types of γ-globulin are present in the mouse: γ_2, γ_1, γ_{1A}, and γ_{1M}.

These results suggest that γ_1-globulins may represent a separate family of immunoglobulins which mediate anaphylactic reactions within the species.

Summary. Various preparations of normal and antibody guinea pig γ_1 and γ_2 globulins have been analyzed for their hexose content, using the indol method. The value obtained, when expressed in glucose equivalents, was 0.74% for both types of γ-globulin. Analysis of the different fragments obtained by enzymatic splitting of the molecule of γ_2 globulin indicated that most of the hexose was contained in the fast (F) fragment obtained after digestion with papain (piece III of Porter). The fragment obtained after pepsin digestion contained very little if any hexose. The results indicate that guinea pig γ_1 globulin does not correspond to the γ_{1A} globulin of man and mouse. It is suggested that γ_1 globulin represents a distinct family of immunoglobulins, possibly also existent in other animal species, which mediates anaphylactic reactions within the species.

1. Heremans, J., Les globulines seriques du systeme gamma. Bruxelles, Arscia, S. A., 1960.

2. Fahey, J. L., Advances in Immunology, 1962, v2 41.

3. Benacerraf, B., Ovary, Z., Bloch, K. J., Franklin, E. C., J. Exp. Med., 1963, v117, 937.

4. Ovary, Z., Benacerraf, B., Bloch, K. J., ibid., 1963, v117, 951.

5. Bloch, K. J., Kourilsky, F. M., Ovary, Z., Benacerraf, B., ibid., 1963, v117, 965.

6. Nussenzweig, R. S., Merryman, C., Benacerraf, B., ibid., 1964, v120, 315.

7. Muller-Eberhard, H. J., Kunkel, H. G., ibid., 1956, v104, 253.

8. Nisonoff, A., Wissler, F. C., Lipman, L. N., Woernley, D. L., Arch. Biochem. Biophys., 1960, v89, 230.

9. Porter R. R., Biochem. J., 1959, v73, 119.

10. Grabar, P., Williams, C. A., Biochem. Biophys. Acta, 1955, v17, 67.

11. Scheidegger, J. J., Arch. Allergy & Appl. Immunol., 1955, v7, 198.

12. Ouchterlony, O., Prog. Allergy (Basel, S. Karger), 1958, v5, 1.

13. Mokrasch, L. C., J. Biol. Chem., 1954, v208, 55.

14. Dische, Z., Popper, H., Biochem. Z., 1926, v175, 371.

15. Muller-Eberhard, H. J., Kunkel, H. G., Franklin, E. C., Proc. Soc. Exp. Biol. and Med., 1956, v93, 146.

16. Franklin, E. C., J. Clin. Invest., 1960, v39, 1933.

Received September 2, 1964, P.S.E.B.M., 1965, v118.

Renal and Adrenal Relationships in Compensatory Hyperplasia.* (29837)

R. J. Goss (Introduced by J. Walter Wilson)
Department of Biology, Brown University, Providence, R. I.

Growth in many organs can be stimulated either by partial ablation or by functional overload. In most cases, the two methods are known to be equivalent(1), but in physiologically complex organs such as the liver and kidney, it has never been discovered how the effects of partial ablation are translated into subsequent cellular proliferation in the residual tissues. Although functional overload has long been recognized as an obvious possibility, certain recent explanations(2) have favored the postulation of tissue-specific growth-regulating factors related more to maintenance of predetermined organ mass than to re-establishment of physiological proficiency. However, in view of vain attempts to demonstrate the existence of such factors in the case of the kidney, either in the serum or in renal tissue itself(3,4), the following experiments were designed to test the importance of strictly functional alterations on compensatory renal hyperplasia (CRH).

Methods. All experiments were carried out on male Sprague-Dawley rats weighing 100-120 g. Animals were kept in a temperature-controlled room at 76-78°F and illuminated from 0830 to 2230 daily. Operations and sacrifices were performed between 0900 and 1100. Nephrectomies and adrenalectomies were done under ether anesthesia *via* the dorso-lateral approach.

As previously described(4), results were assessed in terms of the mean per cent of renal cortical cells in mitosis ± S.E. Mitotic counts were made on the cortical areas of 7 μ kidney sections after fixation in Bouin's and staining with hematoxylin. Fifty randomly selected fields from 10 sections per kidney (5 fields per section) were scanned for mitotic figures at 430× magnification. The resulting counts divided by the total number of cells examined per kidney (approximately 21,400 cells) yielded a mitotic index expressed on a percentage basis. To reduce the effects of individual variations, rats were usually sacrificed in groups of 12, and their mitotic indices averaged. In experiments not involving nephrectomy, groups of 6 rats were used and their 12 kidneys were counted separately.

Role of salt and adrenals in compensatory renal hyperplasia. After subtotal nephrectomy, the incidence of cell division in the remaining kidney rises to a maximum of about 5 times normal during the second postoperative day(5-7). Williams(7) has shown that most of the mitotic figures at this time are in the cells of the proximal tubules. Recent investigations of the influence of the adrenal cortex on this process have yielded divergent results. Goss and Rankin(6) have claimed that bilateral adrenalectomy abolishes the CRH that normally takes place after unilateral nephrectomy. Williams(8), however, showed that nearly normal CRH occurred in adrenalectomized uninephrectomized rats. Subsequently, Astarabadi(9) observed compensatory increases in the relative weights of remaining kidneys during the 2 weeks following total adrenalectomy. Since the last 2 investigators maintained their rats on saline drinking water, while the first did not, it seemed plausible that this might account for the lack of agreement.

Accordingly, parallel series of rats were subjected to uninephrectomy and bilateral

* Supported by grant HD-00192 from Nat. Inst. of Child Health & Human Development.