THE RENAL LESION ASSOCIATED WITH HEMOGLOBINEMIA

I. Its Production and Functional Evolution in the Rat*

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The precise mechanisms involved in the association of hemoglobinemia with acute renal failure remain unknown. The difficulties in studying this problem in patients are manifold, consequently the bulk of the investigation in this area has necessarily involved experimentally produced lesions in the laboratory animal. Largely as a result of such studies a variety of differing and conflicting hypotheses concerning the pathogenesis of this renal lesion have arisen. Perhaps the most favored of these are the suggestions that (*a*) tubular blockage by precipitated hemoglobin is the primary cause of excretory failure in the affected kidney, and (*b*) some undefined factor or factors produce a primary reduction in glomerular filtration rate, presumably as a consequence of renal ischemia. Carefully performed studies may be cited in support of either the first (1, 2) or second (3, 4) of these hypotheses. In addition, other explanations for the renal failure have been considered, particularly the role of abnormal back diffusion of tubular fluid through damaged tubular epithelial structures and the effect of renal edema in reducing renal blood flow and filtration rate. The establishment of an experimental model of a clinical renal disease in the laboratory animal affords the opportunity of making accurate functional measurements of the evolution of the lesion and of correlating structural alterations at each stage of the disorder with the corresponding functional data. This does not appear to have been accomplished with acute renal failure associated with hemoglobinemia. This lack of serial functional and morphological studies of a standard lesion may in part account for the conflicting views regarding the pathogenesis of this disorder. Consequently the present study was designed, with the ultimate objective of defining the mechanisms underlying the renal functional failure associated with this lesion.

The present report describes a relatively simple method for the production of acute renal failure in the rat by the intravenous injection of hemoglobin. The lesion is reversible and relatively uniform in its functional and structural manifestations. Certain of the factors which influence its production and degree of severity have been examined. The functional evolution of the lesion, from its

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onset to virtual recovery, has been characterized in detail by the use of inulin clearance measurements. This report forms the basis for an interpretation of morphological changes observed at various stages of the lesion (5), which in turn permits the formulation of a working hypothesis to guide further study of this problem.

Methods and Procedure

All experiments were performed on female Wistar rats, of the Rochester strain, weighing 200 to 250 g. Animals were fed Purina chow (Purina Mills, St. Louis, Missouri) and allowed water ad libitum, except as noted.

Rat hemoglobin solution was prepared immediately before the time of injection. Previously harvested rat erythrocytes, washed with isotonic saline, were lysed in two volumes of distilled water. Following lysis, a volume of 1.5 M NaCl solution equal to one tenth of the distilled water was added in order to enhance the solubility of the hemoglobin. The preparation was centrifuged twice at room temperature, yielding a clear supernatant. A final hemoglobin concentration of 4 to 6 g per 100 ml resulted. Analysis of multiple samples just prior to injection revealed no detectable methemoglobin in the solution. A standard dosage of 40 mg hemoglobin per 100 g body weight was used in all of the present experiments. Injection was made into the jugular vein, through a small skin incision, in rats anesthetized either with ether or intraperitoneally administered sodium pentobarbital. Following injection, rats were placed in individual metabolism cages, water was withheld for 1 to 2 hr, and thereafter supplied ad libitum. Food was withheld during the initial 24 hr after injection, during which period water intake and urine output were measured. Animals carried beyond this time period were then allowed free access to food until the time of final study and sacrifice. Urine collected during the initial 24 hr period was analyzed for hemoglobin; none was detectable in urine passed subsequent to this period. Rats deprived of water prior to hemoglobin injection were permitted free access to food during the period of dehydration.

Renal function was evaluated by the measurement of inulin clearance. All clearance studies were performed under pentobarbital anesthesia. A priming injection and sustaining infusion of inulin, administered with a Harvard infusion pump, were given through a small polyethylene catheter in the jugular vein. Urine was collected from a polyethylene catheter sutured into the bladder through a small suprapubic incision. Bladder emptying was accomplished by manual pressure at the termination of each collection period. Clearance periods were started 35 to 40 min after beginning the inulin infusion. In most studies 3 clearance periods of 10 to 15 min duration were obtained. Urine was collected into weighed 2 ml centrifuge tubes, capped with parafilm to minimize evaporation. Each tube was reweighed immediately at the close of the collection period and the urine volume was computed by arbitrarily assuming a urinary specific gravity of 1.020. After weighing, 2 ml of water was pipetted into each specimen and further dilution for analysis was performed by pipetting directly from this volume. This procedure precluded micropipetting and essentially negated errors which might result from variations in the specific gravity of the urine. Blood was collected from the tail during the 1st and 3rd clearance periods. Since relatively stable plasma inulin levels were achieved, an interpolated value was used in calculating the clearance in period 2. This procedure, plus the use of 0.1 ml of plasma for the inulin determination, minimized blood loss during the study. In those occasional studies in which only two clearance periods were obtained, blood was collected at the midpoint of each period. Except as noted below, animals were sacrificed at the end of the clearance study and the kidneys taken for histological study.

In another set of experiments the above procedures have been somewhat modified, in order to measure serial inulin clearances in individual rats from one to 24 hr after hemoglobin in-

jection. Hemoglobin was injected in the usual manner, under ether anesthesia, and anesthesia subsequently maintained by intravenously injected pentobarbital. Blood was drawn from a jugular vein catheter, and urine collected from a catheter introduced into the bladder via the urethra. Following the completion of the initial clearance study, during the 4 hr after injection, the rat was returned to its cage, allowed to awaken, and restudied at 24 hr by the technique previously described, inulin being infused through one of the previously implanted jugular catheters which had been sealed and sutured in place. A similar technique was used in rats studied at 24 and 72 hr after hemoglobin injection. In the first determination, urine was collected from a urethral catheter and blood from a jugular catheter which was then left in place for use in the second study, at 72 hr.

Inulin was determined in urine and plasma filtrates with diphenylamine, following alkali digestion (6). Plasma blank determinations were not performed with each study, since analysis of numerous rat plasmas revealed negligible blank values at the 1:51 dilution of plasma used in the preparation of the filtrates. Hemoglobin was determined by conversion to cyanomethemoglobin and reading against standards in a colorimeter at 540 mμ. Urinary osmolality was determined by freezing point depression (7).

RESULTS

Inulin Clearance Values in Normal Rats.—The mean inulin clearance rates in 16 normal rats, studied during pentobarbital anesthesia, was 0.850 ml/min/100 g body weight. The standard error was ±0.036 and the range 0.666 to 1.118 ml/min/100 g. The value for each animal represented the mean of 3 consecutive clearance periods. Urine flow during these studies averaged 6.05 μl/min.

The Effects of Anesthesia and Dehydration on Production of the Renal Lesion.—The effect of the state of hydration on production of the renal lesion in 52 rats

TABLE I

The Influence of Prior Dehydration on the Renal Functional Response to Hemoglobin Injection

Comparisons of the hemoglobin-injected rats are made with similarly treated animals, injected with saline rather than hemoglobin. All animals were anesthetized with ether at the time of injection.

Dehydration	Hemoglobin injected		Saline injected		*P*
	No. of rats	Inulin clearance	No. of rats	Inulin clearance	
hr		*ml/min/100 g*		*ml/min/100 g*	
0	9	0.586 ± 0.044*			
24	14	0.315 ± 0.058	6	0.758 ± 0.054	<0.001
48	15	0.174 ± 0.038	7	0.692 ± 0.070	<0.001
72	14	0.119 ± 0.019	8	0.648 ± 0.050	<0.001

* SE.

The effects of dehydration in the hemoglobin-injected rats have been analyzed statistically, yielding the following *P* values for adjacent groups: 0 and 24 hr, $P = < 0.01$; 24 and 48 hr, $P = < 0.05$; 48 and 72 hr, $P = > 0.20$.

injected with a standard amount of hemoglobin (40 mg/100 g) during ether anesthesia is shown in Table I. The data reveal that incremental prior dehydration, up to 72 hr in duration, increased the severity of the functional defect. Although the difference between the 48 and 72 hr groups is not statistically significant, the lesion was more consistent in the latter group, as evidenced by the smaller standard error of the mean (Table I). Only a moderate reduction in inulin clearance is present in the normally hydrated animals injected with hemoglobin. The values in the dehydrated rats have been compared with corresponding groups of rats, handled in an identical fashion except that they were injected with 1.0 ml isotonic saline rather than hemoglobin. These saline-

TABLE II

Inulin Clearances 24 hr after Hemoglobin Injection in Rats Anesthetized with Sodium Pentobarbital

Dehydration	No. of rats	Inulin clearance	*P**
hr		*(ml/min/100 g)*	
0	6	0.664 ± 0.094‡	>0.40
24	7	0.465 ± 0.094	>0.10
48	7	0.493 ± 0.111	<0.01
72	7	0.408 ± 0.066	<0.001

* Comparison with comparably dehydrated rats (Table I) injected with hemoglobin during ether anesthesia.

‡ SE.

injected control rats showed a slight reduction in inulin clearance, apparently influenced by the extent of previous dehydration (Table I). In each group however, a marked and significant reduction in mean inulin clearance is seen in the hemoglobin-injected rats when compared with the comparably dehydrated control animals.

The injection of hemoglobin during pentobarbital anesthesia produces only a moderate reduction in mean inulin clearance rate (Table II). At each level of hydration lower clearances are observed in the ether anesthetized animals, however only in the 48 and 72 hr dehydrated groups does this difference assume statistical significance. There is less apparent effect of the degree of dehydration in these animals than in those injected under ether anesthesia. As shown in Fig. 1, a greater scatter of values is observed in the pentobarbital group, a fact which is also evident from the larger standard errors for these animals in Table II. It is evident from Fig. 1 that occasional animals injected under pentobarbital anesthesia manifested a reduction in inulin clearance comparable in degree with the ether anesthetized group. A similar observation was made in those rats dehydrated for 24 and 48 hr prior to injection.

The Functional Evolution of the Renal Lesion.—Serial studies in individual

rats, injected with hemoglobin during ether anesthesia, after 72 hr of dehydration, have yielded data relevant to the rate of development of and recovery from the renal functional defect.

Data from 9 rats on which clearance measurements were made during the initial 4 hr after injection and subsequently at 24 hr are shown in Table III. These reveal that the functional defect has a rapid onset. Inulin clearance was

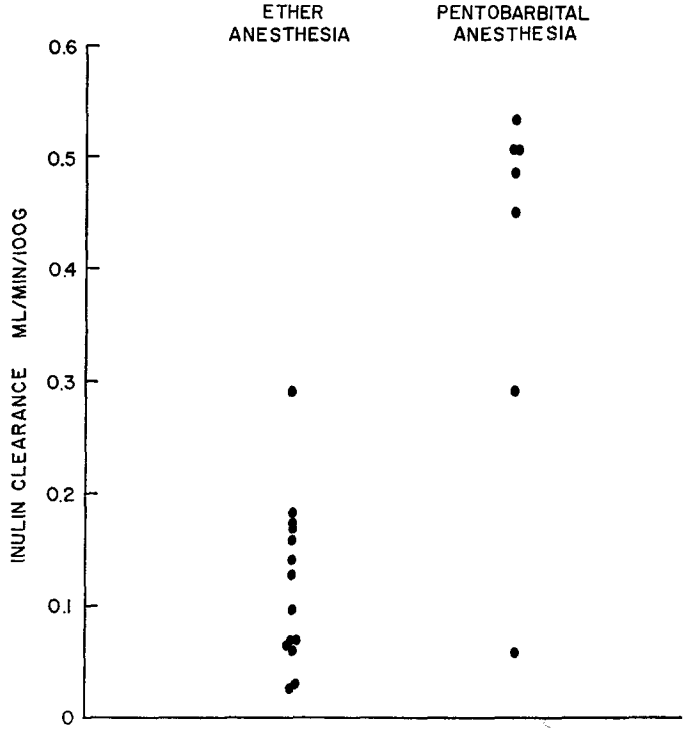

FIG. 1. The influence of the anesthetic agent used during hemoglobin injection on inulin clearance rate, measured 24 hr after hemoglobin administration. All animals had been dehydrated for 72 hr prior to injection.

depressed in the initial determination, 1 hr after hemoglobin injection. Only in rat 541 was the clearance at 24 hr depressed appreciably below the values observed at 1 or 2 hr. In 6 of the 9 rats the clearance rate was higher at 24 hr than during any of the initial periods, but only in rat 540 was this increment appreciable. The possibility exists that the increase in inulin clearance observed at 24 hr may be related to the improved state of hydration of the animals at that time. The markedly reduced inulin clearance observed during the initial hours after hemoglobin injection was not seen in animals which did not manifest a significant functional defect at 24 hr, as in 2 animals injected during pento-

barbital anesthesia. Thus the initial decrement in inulin clearance rate was not a nonspecific effect of the experimental procedure.

Similar studies have been done on individual rats at 24 and 72 hr after hemoglobin injection, and are presented in Table IV. The data reveal a marked and consistent rise in inulin clearance between these two time periods. Using 0.850 ml/min/100 g as a normal value, inulin clearance rate was reduced to 17% of normal at 24 hr in this group of animals. In the ensuing 48 hr, approximately 50% of the initial decrease in inulin clearance had been restored. Even in the presence of a depression of inulin clearance to 10% or less of normal at 24

TABLE III

Inulin Clearance Rates during the First 4 hr and at 24 hr after Hemoglobin Injection
Hemoglobin administered during ether anesthesia, after 72 hr of dehydration.

Rat No.	Inulin clearance Time after injection			
	1 hr	2 hr	4 hr	24 hr
	ml/min/100 g	*ml/min/100 g*	*ml/min/100 g*	*ml/min/100 g*
536	0.122	0.215	0.190	0.170
537	0.152	0.118	0.114	0.213
540	0.135	0.095	0.126	0.457
541	0.320	0.197	0.302	0.139
547	0.193	0.301	0.291	0.270
559	0.048	0.055	0.069	0.088
576*		0.021	0.026	0.082
598	0.183	0.169	0.246	0.298
604	0.080	0.139	0.114	0.189
Mean	0.154	0.161	0.182	0.228
SE	0.029	0.027	0.032	0.040

* Rat 576 was excluded from the calculation of the means and standard errors.

hr, as in rats 577 and 578, clearance rates always exceeded 30% of normal by 72 hr. These studies confirm earlier experiments in which clearances were performed only at 72 hr after hemoglobin injection in a comparable group of 13 rats. In that group, a mean clearance of 0.542 ml/min/100 g was found at 72 hr. One rat in the latter group, virtually anuric during the first 24 hr after hemoglobin injection, manifested the lowest clearance observed at 72 hr, 0.176 ml/min/100 g.

By 7 days after hemoglobin injection there has been an apparent further increase in inulin clearance (Table V). In an additional group of rats clearance studies were performed at 24 hr and 7 days after injection. Despite severe depression of function in some rats at 24 hr (rats 589 and 602 in particular) all animals in this group manifested clearance rates greater than 0.50 ml/min/100 g

at 7 days. In none of the animals was inulin clearance restored to normal however; moderate functional impairment persisted in all.

Additional Observations.—

Urinary excretion and plasma concentration of hemoglobin: Those animals manifesting the most severe depression of inulin clearance excreted minimal amounts of hemoglobin in the urine. This is illustrated in Fig. 2, in which a

TABLE IV

Inulin Clearance Rates in Individual Rats Studied at 24 and 72 hr after Hemoglobin Injection during Ether Anesthesia

All animals were dehydrated for 72 hr before injection.

Rat No.	Inulin clearance Time after hemoglobin injection	
	24 hr	72 hr
	ml/min/100 g	*ml/min/100 g*
564	0.229	0.661
567	0.183	0.618
572	0.167	0.404
574	0.183	0.672
577	0.017	0.374
578	0.085	0.276
579	0.139	0.507
Mean	0.143	0.502

TABLE V

Inulin Clearance Rates in Individual Rats at 24 hr and 7 Days after Hemoglobin Injection during Ether Anesthesia

All rats were dehydrated for 72 hr before injection.

Rat No.	Inulin clearance Time after hemoglobin injection	
	24 hr	7 days
	ml/min/100 g	*ml/min/100 g*
585	0.098	0.567
589	0.032	0.768
592	0.311	0.594
593	0.101	0.599
597	0.170	0.632
601	0.324	0.647
602	0.031	0.518
Mean	0.152	0.618

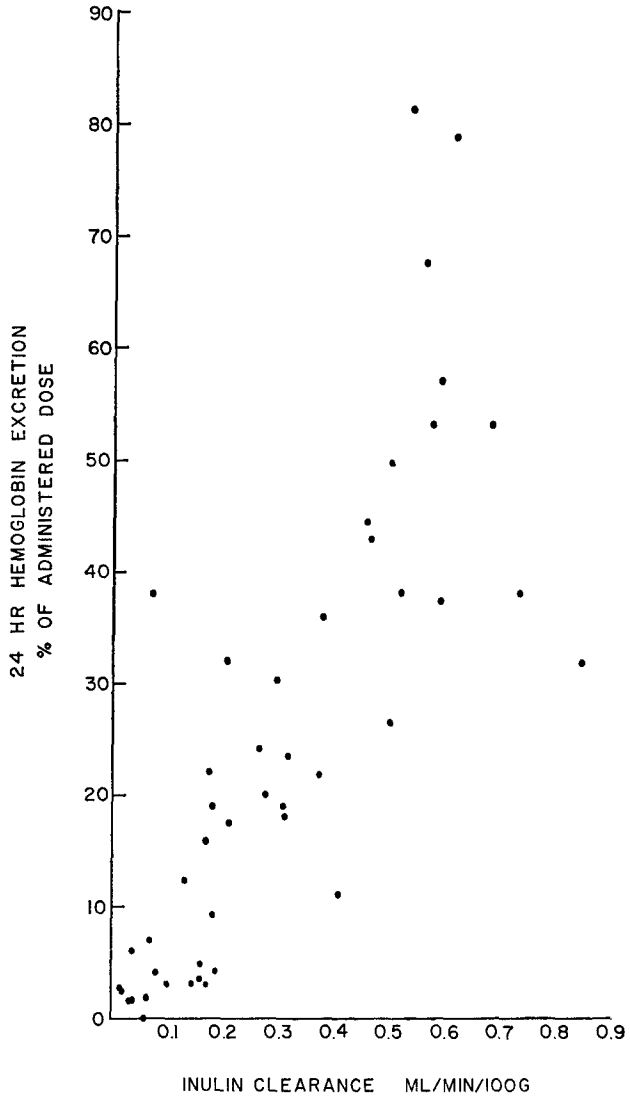

FIG. 2. The relationship of urinary excretion of hemoglobin during the 24 hr following its injection to the inulin clearance rate, measured 24 hr after hemoglobin administration.

close relationship is evident between inulin clearance at 24 hr after injection and the fraction of the hemoglobin excreted in the urine during that time period. Some animals showed no visible discoloration of the urine. A similar relationship was observed in more acute studies, performed 1 to 4 hr after hemoglobin injection.

Rats which did not develop a significant depression of function excreted hemoglobin in high concentrations, in the range of 10 to 15 g/100 ml, in the hour after injection. Thereafter the rate of excretion fell progressively, the urine containing immeasurably low concentrations by 5 hr after injection.

Plasma hemoglobin concentrations, in rats receiving 40 mg/100 g, ranged from 0.41 to 0.59 g/100 ml in the period 20 to 30 min after injection. By 6 hr after injection the concentration had fallen to less than 0.05 g/100 ml, and at 24 hr no hemoglobin was detectable in the plasma.

The relation of urine flow to renal function: Urine flow rate was not a generally reliable index of the renal function in these animals, except when oliguria was present. All animals with a 24 hr urine volume less than 5 ml or a flow rate less than 2.5 μl/min during clearance studies at 24 hr manifested an inulin clearance of less than 0.1 ml/min/100 g. However a number of rats with comparable

TABLE VI

The Effect of Dehydration on Urinary Osmolality in Normal Rats

Dehydration	No. of rats	Urinary osmolality
hr		*mOsm/kg H_2O*
24	9	2362 ± 68*
48	17	2524 ± 43
72	15	2637 ± 65

* SE.

Statistical comparison of the difference between groups 24 and 48 hr, $P = <0.05$; 48 and 72 hr, $P = >0.10$.

clearances had urine flows similar to animals with minimal depression of function. Oliguria, as defined above, was invariably present only when the clearance was less than 0.03 ml/min/100 g. This was observed in 5 animals.

Oliguria was never present in clearance studies done 72 hr after injection, although this group included 9 animals which were oliguric during the initial 24 hr; i.e., they excreted less than 5 ml of urine during that period.

Studies on the effect of dehydration: It was postulated that increasing degrees of dehydration might influence the production of a renal lesion following hemoglobin injection by effecting an increase in urinary osmolality, which might in turn favor precipitation of hemoglobin within the lumen of the distal tubular system.

The effect of dehydration on urinary osmolality is summarized in Table VI. Some increase in osmolality is observed at each level of dehydration, however the 48 and 72 hr groups do not differ significantly. In 19 of these animals, urine was obtained just prior to hemoglobin injection and the osmolality related to the subsequent functional defect. No correlation was found within any group between urinary osmolality and the degree of depression of inulin clearance.

In order to evaluate further the effects of dehydration, inulin clearance was measured in a group of normal rats dehydrated for 72 hr. In 9 animals the mean clearance was 0.750 ml/min/100 g, a value only moderately lower and not significantly different from that in normally hydrated rats. However if the value in the dehydrated group is corrected for the approximately 15% body weight loss during the 72 hr dehydration period, the clearance becomes 0.638 ml/min/100 g, indicating a 25% reduction in filtration rate resulting from the dehydration process.

The influence of ether anesthesia: A systematic study of the renal functional effects of ether anesthesia has not been undertaken in the present study. However the effect of ether anesthesia on inulin clearance has been studied in 6 rats, 4 of which were dehydrated for 24 hr; the other 2 were normally hydrated. Each rat was virtually anuric for periods up to 60 min after induction and maintenance of anesthesia, precluding accurate clearance measurements during this period. Clearances were measured in 2 animals at 50 to 70 min, yielding extremely low values. The accuracy of such data in the face of extreme oliguria is questionable however.

DISCUSSION

The present study indicates that a reproducible and significant renal functional lesion can be produced in the dehydrated rat by the intravenous injection of hemoglobin. In accord with the observations of others (8, 9) hemoglobin injection in normally hydrated animals, not subjected to measures which produce renal ischemia, failed to produce significant renal failure. In the present study the combination of ether anesthesia and dehydration served to render the kidney susceptible to the deleterious effects of hemoglobinemia. The precise means by which these modalities exert this effect remains unexplained. Dehydration does not appear to operate solely via its effect on urinary osmolality, in view of the lack of correlation between osmolality prior to hemoglobin injection and the degree of renal function failure subsequently observed. A moderate reduction in filtration rate is observed in the hydropenic rat, but the data do not permit conclusions as to whether this is a critical determinant of the renal lesion. Deep ether anesthesia has previously been demonstrated to reduce glomerular filtration rate and para-aminohippuric acid (PAH) clearance in several species, including man (10), the dog (11), and the rabbit (12). This has generally been attributed to a renal vasoconstrictive response, mediated by an increase in endogenous epinephrine and norepinephrine release (13), and possibly influenced by a reduction in plasma volume during ether anesthesia (14). The effect of dehydration on the renal hemodynamic response to ether anesthesia has apparently not been studied, but the present data suggest that dehydration intensifies the renal ischemia occurring during ether inhalation. The technique employed in this study to effect renal susceptibility to hemoglo-

binemia has certain advantages, in that it is relatively simple and atraumatic, it produces a negligible effect on renal function in the absence of hemoglobinemia, and it leads to a reproducible lesion.

The rate of inulin clearance has been utilized in the present study to evaluate the evolution of the functional lesion and its degree of severity. It is acknowledged that the clearance of inulin may not be an accurate reflection of glomerular filtration rate in the acutely diseased kidney, in view of the possible existence of abnormal back diffusion of filtered inulin through damaged tubular epithelial structures. Consequently it has been interpreted only as a measure of over-all renal excretory function, or effective filtration rate, and no conclusions have been drawn concerning the actual state of glomerular function. Functionally, the lesion produced in the present study has a rapid and abrupt onset. The inulin clearance rate is markedly reduced within 1 hr of the injection of hemoglobin. This is not a nonspecific response to ether anesthesia or dehydration, since animals which show little or no impairment of excretory function at 24 hr have essentially normal clearance rates during the initial hours after injection. The evolution of the functional lesion in the rat is similar to that observed in acute renal failure in man, with the exception that the rate of recovery is more rapid in this experimental model. The pattern of functional recovery observed in the present study is of interest, in that it resembles a biphasic process. Between 24 and 72 hr after hemoglobin injection there is a marked rise in the inulin clearance rate, so that approximately 50% of the initial reduction in clearance is restored. There is an apparent slow increment in inulin clearance rate between 72 hr and 7 days, but normal function was not restored in any animals at the latter time. These observations suggest that separate pathological processes are involved in the initial as opposed to the later and more persistent renal functional impairment.

It is of note in the present study that hemoglobinuria is least evident in those animals which manifest the most severe renal functional impairment. Indeed the urine from such animals may show no grossly visible hemoglobin pigment. This finding is readily explained by the rapid appearance of the functional lesion after hemoglobin injection. In the presence of a markedly reduced filtration rate, it is to be expected that the filtered load of hemoglobin and its ultimate urinary excretion rate will be accordingly reduced. This observation suggests the possibility that clinical acute renal failure associated with hemoglobinemia may occur despite the absence of discoloration of the urine. Reliance on the presence of gross hemoglobinuria to indicate the presence of a hemolytic state may consequently be unjustified, if an associated severe impairment of renal function has occurred. The data also indicate that under the proper predisposing conditions, renal failure may be induced by the administration of relatively small amounts of hemoglobin. Assuming a blood volume of 6.7 ml/100 g in the rat (15) and a hemoglobin concentration of 15.6 g/100 ml (16), the administered

amount of 40 mg/100 g is equivalent to 3.8% of the rat's circulating hemoglobin. In a 70 kg man, assuming a blood volume of 70 ml/kg and hemoglobin concentration of 15 g/100 ml, this is comparable to hemolysis of less than 100 ml of erythrocytes. Hemoglobinemia of this magnitude is relatively evanescent and will not be detected unless the plasma is examined within several hours of the initiating event. These considerations suggest that hemoglobinemia may be a more common contributory cause of acute renal failure in man than is generally recognized, although species differences in the solubility of hemoglobin and its degree of haptoglobin binding may preclude direct application of the present data to man.

SUMMARY

A method is presented for the production of a reproducible and reversible renal lesion in the rat by the intravenous injection of a relatively small amount of homologous hemoglobin (40 mg/100 g body weight). Production of the lesion is dependent on prior water deprivation and its severity is related to the degree of dehydration. Ether anesthesia, at the time of hemoglobin injection, predisposes to a severe and reproducible functional defect in the dehydrated rat. In contrast, injection of hemoglobin during pentobarbital anesthesia results in a significant lesion only sporadically. The functional evolution of the lesion has been characterized by inulin clearance measurements. Functional impairment occurs abruptly, within 1 hr after hemoglobin injection, and persists unchanged over the ensuing several hours. Some increase in inulin clearance rate is usually observed at 24 hr after injection, but severe functional impairment persists. Between 24 and 72 hr, a considerable increase in inulin clearance rate occurs, so that only moderate restriction of excretory function is present at the latter time. A further moderate increase in inulin clearance rate is apparent at 7 days after hemoglobin injection, but some reduction in function persisted in all rats studied at this time.

Hemoglobinuria is slight or inapparent in animals manifesting the most marked depression of excretory function, indicating that a severe renal lesion may exist in the absence of visible urinary pigment. Hemoglobinemia is evanescent at the dosage used in this study. These observations suggest that clinical acute renal failure secondary to hemoglobinemia may readily go unrecognized and that this may be a more frequent association than is now appreciated.

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