I. RENAL THRESHOLDS FOR HEMOGLOBIN IN DOGS

Depression of Threshold Due to Frequent Hemoglobin Injections and Recovery during Rest Periods

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For a number of years in this laboratory there has been an interest in the *conservation of hemoglobin* in experimental anemia. It would seem probable that the body would utilize every agency to conserve and reconstruct hemoglobin or hemoglobin building material under the conditions of a long continued anemia due to bleeding. It is very easy to demonstrate experimentally in dogs (8) that during prolonged anemia there will be very efficient conservation of hemoglobin introduced into the blood stream whether the blood hemoglobin comes from the dog, sheep or goose. In fact the introduced hemoglobin, to the extent of 90 to 100 per cent will be conserved (7) and rebuilt into finished red cells which must be removed to maintain the fixed anemia level in the injected dog. It is obvious that the sheep and goose hemoglobin must be broken down to intermediates of unknown nature before the new dog hemoglobin and red blood cells are rebuilt. This observation reveals a very beautiful conservation of useful materials within the body.

The *renal threshold* obviously is related to this conservation of hemoglobin set free in the blood stream and a more complete understanding of the renal threshold for hemoglobin would be of especial interest to workers in this field. It was shown recently from this laboratory (3) that certain hemoglobins have widely different threshold values. In the normal dog the threshold for dog blood hemoglobin was determined as about 215 mg. hemoglobin per kilo body weight while the renal threshold for dog muscle hemoglobin was only about

11 to 15 mg. per kilo. Sheep and goose blood hemoglobin fell about midway between these extremes or 115 mg. per kilo.

Moreover the observation (5) that kidney feeding is almost as potent as liver feeding to produce new hemoglobin in experimental anemia due to bleeding, gives an added interest to a study of the kidney elimination and possible conservation of products coming from hemoglobin. Obviously materials are stored within the kidney which can be used to build up much new hemoglobin during long anemia periods in dogs. It is possible that some of these "building stones" are inherent in the individual cell protein of the kidney and have no relation to hemoglobin conservation but it is also possible if not probable that other "building stones" held in the kidney are related to hemoglobin breakdown and salvage within the body.

No more detailed exposition need be given to explain our continued interest in a study of renal thresholds for hemoglobin within the blood stream. It was decided to determine accurately the renal threshold for dog blood hemoglobin in normal dogs over long periods of time and from this base line to study variations which might be caused by agents injurious to the renal structures.

Our first surprise came when an attempt was made to establish a fixed base line for the hemoglobin threshold by daily injections of dog hemoglobin close to threshold values—sometimes a little above and alternately a little below. It became obvious that under these conditions with daily injection of hemoglobin the renal threshold was depressed. In other words the kidney filter seemed to become a less efficient barrier and let some hemoglobin through at a lower threshold value (Table 1). This renal threshold after subsiding to a certain level then became relatively constant if the frequent hemoglobin injections continued, but rose toward the initial higher level if the hemoglobin injections were discontinued for weeks or months (Charts B, C, D). This was all very confusing at first but experimental data given below will help toward a clearer understanding of these phenomena.

It will be observed that the threshold values for dog hemoglobin given by Manwell and Whipple (3) are somewhat higher than the initial values tabulated below. These differences are due to two factors, both related to the method used in these experiments. Manwell

and later Taylor used as their threshold a hemoglobin color in the urine recognizable by the eye although the color was always checked by the spectroscope. Urines which in gross were clear were recorded as negative and not examined by the spectroscope. In the experiments given below the urine was examined for hemoglobin bands as a routine whether it was grossly tinted or not and all specimens giving the characteristic bands were recorded as positive, although frequently grossly not colored. In other words the experiments below are based on a threshold of hemoglobin appearing in the urine in traces giving a micro spectroscopic but not necessarily a gross color test. This is probably more accurate and dependable.

The second factor is related to the *fading* of the hemoglobin color in the urine. Manwell and Taylor used a 3 hour collection period following the intravenous injection of hemoglobin, the dog being given water by stomach tube and placed in a metabolism cage. The experiments below are based on a $1\frac{1}{4}$ hour collection period, the urine being collected in a beaker or dish rather than in a cage. Small amounts of hemoglobin in the urine will fade or become invisible to the standard micro spectrometric test during an hour at room temperature or within the bladder.

These two differences in technique explain the differences in hemoglobin threshold values published by Manwell and Whipple (3) and tabulated below. We believe these later values to be more accurate and to approximate more nearly to the absolute renal threshold values.

It does not seem necessary to review the literature as this was done in the first paper of this series (3).

Methods

Mongrel dogs were used in all experiments. These were of both sexes and ranged from 9 to 24 kilos in weight. They were kept in separate cages and fed on a diet of hospital scraps. Several control examinations of the urine of each dog were made to be sure that no detectable kidney damage was present before starting the series of experiments and checks were run at various intervals throughout their course. The clinical condition of the animals was normal at all times except where note is made to the contrary.

The dog blood hemoglobin used in practically all experiments was obtained from a colony of anemic dogs which were kept at a fairly low hemoglobin level by constant bleeding. It was prepared in a manner quite similar to that described

by Manwell and Whipple (3), the cells from the citrated blood being separated and washed twice with normal saline in a high speed centrifuge. They were then laked with approximately twice their volume of distilled water by moderately severe shaking for at least 15 minutes. Following this the sediment was thrown down by centrifugalization at high speed for at least 30 minutes, and the clear supernatant fluid was decanted through several layers of cotton gauze. For the most part these solutions of hemoglobin were not made isotonic before injection, but in the few cases in which this was done, a sufficient amount of 10 per cent NaCl solution was added and the resulting precipitate removed as thoroughly as possible by prolonged centrifugalization. Smith (6) has shown in blood volume studies that considerable amounts of distilled water can be introduced intravenously without causing hemolysis and our solutions were far below this amount and were given slowly. The isotonic hemoglobin solutions obtained were in any event never as clear as the distilled water solutions of the hemoglobin pigment. Carbon dioxide was never used to facilitate the separation of the stroma.

All hemoglobin solutions were immediately standardized by the acid hematin method of Robscheit-Robbins (4). 1 cc. was diluted to 100 cc. with 1/10 N HCl. This solution was allowed to remain at least 1½ to 2 hours at room temperature and then compared with a standard solution of acid hematin. To avoid the error of incomplete color development, the acid hematin solution was always left in the ice box overnight and a second comparison with the standard made.

For the injections a needle was carefully introduced into an external jugular vein and the injection mass (warmed to body temperature) allowed to flow in slowly from a standard 50 cc. burette. This method of injection was preferred to the use of syringes because it afforded greater uniformity of rate. Fresh hemoglobin was used in all experiments—usually 3 to 4 hours after preparation of solution. Immediately following this hemoglobin injection each dog (with a few exceptions) was given 250 to 500 cc. of warm tap water by stomach tube, the amount being roughly proportional to the size of the dog. This was to minimize any kidney damage produced by the hemoglobin (Barratt and York (2)) and to insure an early collection of urine. The dogs were fed once daily at about 4 p.m. and the injections were given in the forenoon or early afternoon, always before such feedings.

In securing urine specimens the following procedure was a routine. In most cases if the dog was removed from the metabolism cage 1¼ hours after injection, a fresh, uncontaminated sample of urine could be obtained in a clean beaker or dish. In the few cases when dogs refused cooperation the sample was obtained in the clean metabolism cage in which they were always placed following injection. It was found that the majority of dogs would pass urine about 1 to 1¼ hours after the injection and this was adopted as the optimum time for obtaining the urine. Variations of 15 minutes on either side of this time were accepted, but experiments with a greater variation in collection time were usually rejected, particularly if the urine showed no hemoglobin. Catheterization was never done because of the danger of producing slight traumatic hemorrhage and cystitis.

When necessary the urine was filtered, but in any event it was tested for hemoglobin immediately using the ordinary Duboscq colorimeter with a Leitz micro spectrometer replacing the eye-piece. The presence of the definite absorption bands characteristic of oxyhemoglobin was accepted as the sole criterion for hemoglobinuria.

Fading of Hemoglobin in Urine

The urine of these dogs after collection of the usual 1¼ hour sample was always neutral to litmus or faintly alkaline, never strongly acid. If the urine gave positive bands for oxyhemoglobin in the spectroscope it was recorded as positive. It was noted that many positive specimens put aside for 2 to 3 hours at room temperature would subsequently give negative readings. The hemoglobin had "faded" and no longer gave the characteristic bands in the micro spectrometer.

This phenomenon was investigated further by observations on urine and dilute hemoglobin solutions set up in test tubes at room or incubator temperatures. A series of tubes containing 10 cc. normal dog urine was set up with increasing amounts of dog hemoglobin. After mixing the urine and hemoglobin, the solutions were read in the micro spectrometer and allowed to stand for some time, readings being made at intervals up to 5 to 6 hours at room temperature. It was found that in such mixtures of urine and dilute hemoglobin the fading amounted to approximately 1.5 mg. of hemoglobin per hour. At incubator temperature about twice as much fading would be recorded—that is the urine solution would lose about 3 mg. of hemoglobin each hour for 4 to 5 hours.

When the urine was boiled or when dilute solutions of phenol or formol were added to the mixture this fading was observed as usual. According to the observations of Baker and Dodds (1) the change from oxyhemoglobin to methemoglobin or acid hematin might be expected. We have made no effort to determine the end products but record the fading or disappearance of the oxyhemoglobin bands in urine mixtures.

We may record a typical experiment to illustrate the fading of hemoglobin within the bladder of a normal dog. A dog which had not been injected previously with hemoglobin was given intravenously a dose of 200 mg. dog hemoglobin per kilo body weight. Urine was retained in the bladder for 3 hours and when passed was negative for oxyhemoglobin bands. The same dose of dog hemoglobin was given the next day but the urine collected in a beaker at the end of 1¼ hours and this urine gave positive oxyhemoglobin bands. On the 3rd day the same dose of dog hemoglobin was given intravenously but the dog retained his urine for 5 hours. The sample was negative for oxyhemoglobin bands.

All this evidence makes it apparent that the urine in these experiments should be collected within 1¼ hours after injection and examined at once. Without doubt

there is a little fading even in this short time but by following a fixed routine this error can be held at a fairly constant level.

EXPERIMENTAL OBSERVATIONS

We use the term "renal threshold for hemoglobin" to indicate the smallest amount of hemoglobin which given intravenously will effect the appearance of recognizable hemoglobin in the urine. These values are given in terms of milligrams hemoglobin per kilo body

TABLE I

Depression of Renal Threshold for Hemoglobin Due to Frequent Injections of Dog Hemoglobin

Dog No.	Weight	Sex	Hemoglobin injections			Renal threshold for hemoglobin		
			Total	Urine Hb. +	Urine Hb. 0	Initial	Lowest	Decrease due to hemoglobin injection
	kg.					*mg. per kg.*	*mg. per kg.*	*per cent*
29-297	9.5	F	83	45	38	160	74	54
29-258	23.9	M	23	12	11	210	84	60
29-250	25.0	M	12	7	5	160	90	44
29-267	15.0	F	24	12	12	167	110	34
29-349	22.2	F	10	6	4	124	68	45
30-38	18.2	M	43	22	21	130	110	15
29-108	15.6	F	88	46	42	130	80	30
30-32	18.0	M	26	12	14	170	80	52
30-154	23.2	M	17	9	8	145*	60	59
Average						155	84	46

* A recovery threshold—see Chart D.

weight. To determine this renal threshold we usually inject alternately subthreshold and superthreshold amounts of hemoglobin, beginning with calculated threshold values. Injections are usually made daily at about the same hour and the 24 hour interval is ample to permit escape of all injected hemoglobin from the circulating plasma.

Table 1 is a summary of injections given to each dog. The *initial renal threshold* is recorded for each dog as well as the *lowest renal threshold* which is observed after a considerable number of daily hemoglobin injections. On the average the renal threshold is depressed

about 46 per cent as the result of continued daily hemoglobin injections. The initial renal threshold shows considerable differences in different dogs from a maximum of 210 to a minimum of 124 mg. hemoglobin per kilo. We can offer no explanation for such differences but may say that dogs vary greatly in their physical capacity for work and this difference in renal threshold may in like manner depend on inherent functional differences in these dogs.

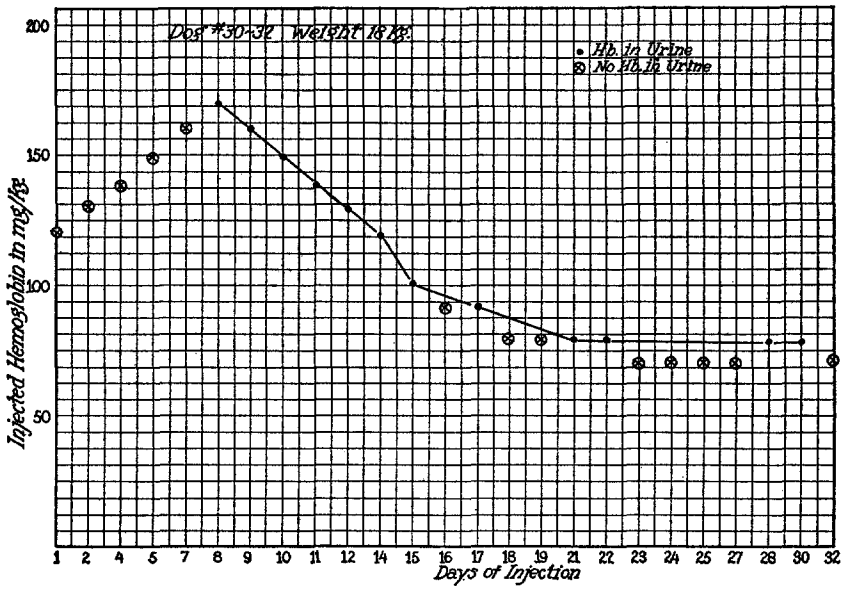

CHART A. Depression of renal threshold from 170 to 80 mg. hemoglobin per kilo.

The figures given for the *lowest renal thresholds* vary from a maximum of 110 to a minimum of 60 mg. hemoglobin per kilo but these values do not relate themselves to the values given for the initial thresholds. A dog having a high initial threshold may show a depressed threshold which is lower than the average (Dog 29-297, Table 1). A dog with a low initial threshold may show only a slight depression of that threshold and therefore a depression threshold that is above the average level (Dog 30-38, Table 1).

Many observations on hemoglobin injections in other dogs have

been made in the course of this study but it did not seem necessary to record these experiments some of which are incomplete in one respect or another. These unrecorded experiments are completely in harmony with those recorded in these papers and give added support to the published experiments. No differences were observed when isotonic solutions of hemoglobin were used. No differences were recorded when all water was withheld during an experiment and we

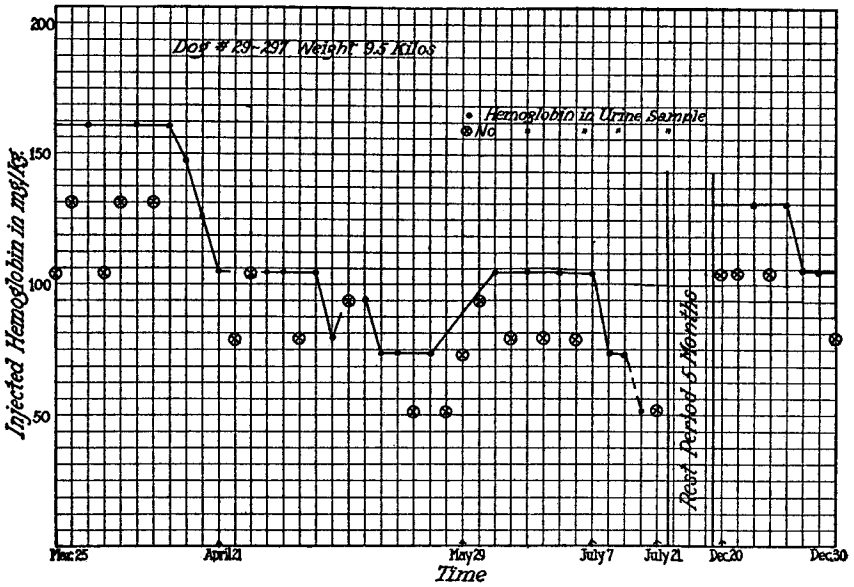

CHART B. Gradual depression of threshold and recovery during rest period.

have no evidence that moderate diuresis has any influence on the renal threshold for hemoglobin.

For sake of condensation in Charts A, B, C and D all the injections are not recorded. The black lines connecting the positive values are used to aid the observer in appreciating the approximate depression of the true renal threshold for dog hemoglobin in dogs.

Chart A shows a rapid drop in the renal threshold for hemoglobin between the 8th and 16th days of injection. If the initial injection had been higher, for example 180 to 190 mg. per kilo, it is possible that there would have been no escape of hemoglobin in the urine at

this higher level. The final level is quite satisfactorily established with a series of injections close to the threshold, some subthreshold and others superthreshold.

Chart B shows a long series of experimental injections. Many given the animal are not included in this chart. There is an irregular slow depression of the renal threshold for hemoglobin. Injections were given several times every week with occasional short rest inter-

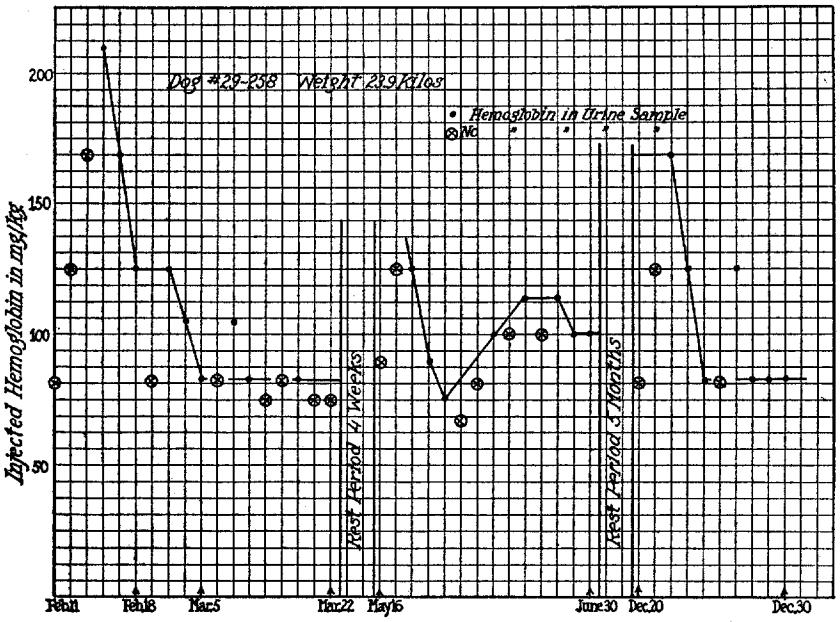

CHART C. Sharp depression of renal threshold and moderate recovery during rest periods.

vals throughout the 4 months preceding the rest period. There is a definite recovery toward the original threshold value during the 5 months rest period and then we observe a fall in threshold values as the hemoglobin injections are continued during December. During rest periods the dogs were given no injections (Charts B, C and D) but were kept in cages and fed a mixed diet of hospital scraps.

Chart C shows a marked depression in the renal threshold for hemoglobin within a month and a moderate degree of recovery during rest

periods but the recovery thresholds are never as high as the original threshold. The depression threshold or minimal levels are reasonably uniform at about 85 to 100 mg. hemoglobin per kilo.

Chart D brings out an interesting point. A considerable series of subthreshold injections (100 mg. hemoglobin per kilo) were given and eventually the renal threshold was depressed by this procedure to a level below 100 mg. per kilo. A rest period of 16 days showed a

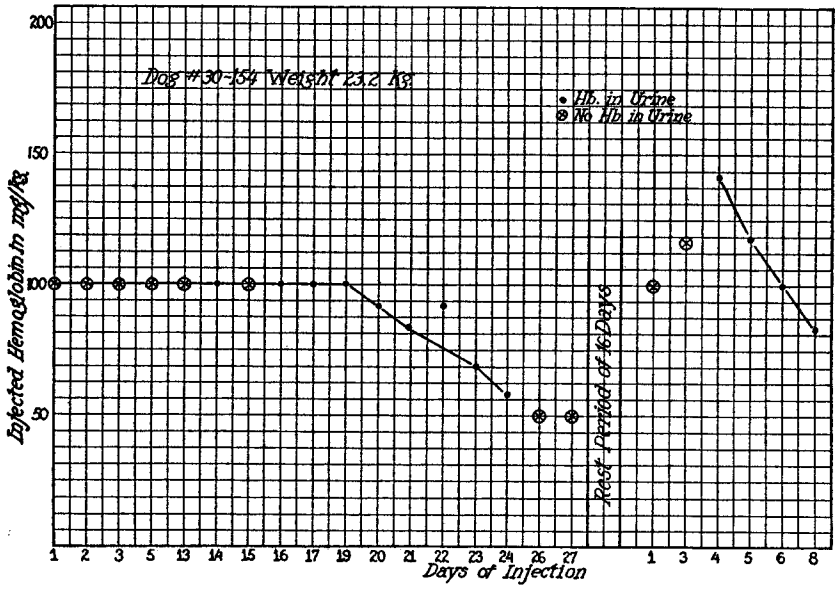

CHART D. Renal threshold depressed by subthreshold injections. Recovery threshold is above original injections level.

recovery threshold of 145 mg. per kilo which we may be sure is below the original threshold if we had determined it in the usual way.

This type of experiment has an important bearing upon our conception of the mechanism of the renal threshold for hemoglobin. Our first thought was that a supertheshold dose of hemoglobin might cause slight injury of the renal filter with increased permeability and lowering of threshold values. This experiment (Chart D) shows that a series of subthreshold injections may eventually decrease the renal threshold so that hemoglobin appears in the urine. The histological

findings (Paper IV) and iron analyses (Paper V) given below show that the *tubular renal epithelium* is responsible for this reaction.

Experiments with sheep hemoglobin have been performed but have not been carried out in sufficient detail to warrant publication at this time. It is probable however that sheep hemoglobin behaves like dog hemoglobin in experiments dealing with renal threshold values.

DISCUSSION

For the sake of clarity it may be worth while to outline our conception of the renal threshold for dog hemoglobin and its various fluctuations due to repeated intravenous injections of hemoglobin. This concept applies only to dog blood hemoglobin and its renal threshold in the normal dog kidney. We speak of the *initial renal threshold* by which term we mean the least amount of hemoglobin which early in the experiment will give a recognizable escape of hemoglobin into the urine—this figure averages in these experiments about 155 mg. hemoglobin per kilo body weight. As injections of hemoglobin are given daily we note a *depression of the renal threshold* which may show an average decrease of 46 per cent below the initial threshold. With rest periods there is a tendency for the threshold to return toward the initial level and we may term this the *recovery threshold.*

The lowest renal threshold or *depression threshold* reached only after many repeated hemoglobin injections is fairly constant and we believe this approximates the *glomerular threshold* by which term we mean the standard level of hemoglobin in the blood plasma which will cause escape of hemoglobin into the glomerular capsule. This glomerular threshold may be expressed in terms of milligrams of hemoglobin per kilo body weight and presumably is a little below the lowest or depression threshold which in our experiments (Table 1) averages about 84 mg. hemoglobin per kilo body weight.

This term *glomerular threshold* indicates our belief that in these experiments the hemoglobin escapes through the glomerular tuft whose cells act as a barrier and establish the absolute renal threshold for dog hemoglobin. The evidence for our belief is scattered through this group of papers but it seemed best to outline this thesis and the experimental data will fall into place as the argument proceeds.

From evidence above and in Paper IV it would appear that when

hemoglobin first escapes into the renal tubules it is rapidly taken up and in part at least deposited within the tubular epithelium. As this process continues with repeated daily injections the epithelium can take up less and less hemoglobin, therefore the renal threshold in bladder urine falls or approaches the glomerular threshold.

From evidence in Paper II we note that the lowest or *depression renal threshold* is not modified by moderate renal injury due to mercuric chloride poisoning which is known to injure almost specifically the tubular epithelium of the kidney. It seems difficult to explain this unchanged renal threshold for hemoglobin after bichloride injury of the kidney if it is assumed that the dog hemoglobin passes through the tubular epithelium into the lumen of the tubules.

From evidence in Paper IV it is seen that doses of hemoglobin definitely below the glomerular threshold although given over long periods of time never cause any deposit of iron containing pigment within the tubular epithelium of the kidney, although all the other organs are pigmented exactly as though a superthreshold series of hemoglobin injections had been given.

One can deplete the renal tubular epithelium of its pigment, iron and probably other stored intermediates related to hemoglobin by periods of simple anemia due to bleeding. This is further evidence—Papers IV and V—that renal epithelium has to do with conservation of hemoglobin factors under certain conditions.

SUMMARY

We use the term "renal threshold for hemoglobin" to indicate the smallest amount of hemoglobin which given intravenously will effect the appearance of recognizable hemoglobin in the urine.

The *initial* renal threshold level for dog hemoglobin is established by the methods employed at an average value of 155 mg. hemoglobin per kilo body weight with maximal values of 210 and minimal of 124.

Repeated daily injections of hemoglobin will depress this initial renal threshold level on the average 46 per cent with maximal values of 110 and minimal values of 60 mg. hemoglobin per kilo body weight. This minimal or *depression threshold* is relatively constant if the injections are continued.

Rest periods without injections cause a return of the renal threshold

for hemoglobin toward the initial threshold levels—a *recovery threshold* level.

Injections of hemoglobin below the initial threshold level but above the minimal or depression threshold will eventually reduce the renal threshold for hemoglobin to its depression threshold level.

We believe the *depression threshold* or minimal renal threshold level due to repeated hemoglobin injections is a little above the *glomerular threshold* which we assume is the base line threshold for hemoglobin. Our reasons for this belief in the glomerular threshold are given above and in the other papers of this series.

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