PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITY OF BLOOD. I. ERYTHROCYTES [1]

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The presence in animal tissues of an enzyme which splits off purines from purine nucleosides has been recognized for many years (1). This enzyme, purine nucleoside phosphorylase (PNP), has been studied by Kalckar (2–4) and has been shown to be involved in the following reaction:

ribose-1-purine + phosphate ⇌ ribose-1-phosphate + purine

Purine nucleoside phosphorylase from rat liver also possesses a certain specificity with regard to the nitrogenous bases. Inosine and guanosine are the only ribosides which undergo phosphorolysis in the presence of the enzyme. Adenosine and xanthosine are inert in the system, as are pyrimidine ribosides. Hypoxanthine and guanine are the only nitrogenous bases which are incorporated into ribosides by the enzyme. Nucleoside phosphorylases fractionated by various means catalyze the phosphorolysis of purine desoxyribosides as well as of the ribosides. Cleavage of the purine base from the pentose does not occur in the absence of phosphate but takes place readily in the presence of either phosphate or arsenate.

Purine nucleoside phosphorylase activity has been demonstrated in a number of animal tissues and in micro-organisms (4–8) but the enzyme has been studied little in the red cell. Klein (1) demonstrated its presence in the bone marrow of the calf and ox. The presence of a phosphorylase in red cells is implied in the work of Dische (9) and Tsuboi and Hudson (10). Recently, Gabrio and Huennekens (11) partially purified a nucleoside phosphorylase from red cells.

It is the purpose of this paper (a) to demonstrate the presence of PNP activity in erythrocytes; (b) to indicate the comparative activity of PNP in the red blood cells of man, dog, rabbit, pig, and chicken; and (c) to report experimentally induced changes in the PNP activity of dog erythrocytes.

METHODS

Red cell enzyme preparations were made as follows. Blood obtained by venipuncture was anticoagulated with heparin and centrifuged for 30 minutes at 3,000 r.p.m. The plasma and buffy coat were drawn off. The cells were hemolyzed with distilled water. They were not washed prior to hemolysis since this procedure did not modify the activity of the preparations.

Xanthine oxidase was prepared from raw cream [4] by the method of Ball (12) as modified by Kalckar (13). The xanthine oxidase thus prepared was standardized against a 7.6 μg. per ml. xanthine solution. In our assays, that amount of xanthine oxidase was used which in 10 minutes produced complete oxidation of xanthine to uric acid. Rat liver PNP was prepared according to the procedure of Kalckar (2).

Purine nucleoside phosphorylase activity was determined by Kalckar's method (2). In this procedure, inosine is split to hypoxanthine and ribose-1-phosphate by PNP. In the presence of an excess of xanthine oxidase, the hypoxanthine is oxidized to uric acid. The latter is then measured spectrophotometrically. In order to be certain that the end product of the reaction was uric acid and not uric acid riboside, which is known to be present in erythrocytes (14), the presence of uric acid was demonstrated by noting the changes in optical density at 292 mμ after the addition of uricase. Uricase does not act on uric acid riboside (13). All of the uric acid formed by the red cell hemolysates was present as such.

The reaction mixture consisted of 3 ml. of 0.001 M inosine in 0.06 M phosphate buffer of pH 7.4, 0.1 ml. of xanthine oxidase and 0.05 ml. of red blood cell hemolysate. These substances were added in the order described. The mixture was incubated at 36° C. to 38° C. and shaken for exactly thirty minutes. The reaction was

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[4] The fresh cream was generously supplied by the Cloverleaf Dairy Company, Salt Lake City, Utah.

stopped by adding 1 ml. of 20 per cent perchloric acid. After centrifugation, the absorption at 292 mμ was determined. In each case a blank was measured simultaneously, and the difference between the optical density of the reaction mixture and the blank was recorded. The blank was prepared by adding the buffer, red cell hemolysate, perchloric acid, and then the xanthine oxidase in the order named.

For convenience in reporting purine nucleoside phosphorylase activity a "unit" of activity, in terms of the assay method used, is defined as follows: one unit of PNP activity is equal to 1,000 times that amount of the enzyme which will produce one μg. of uric acid in 30 minutes at a pH of 7.4 and a temperature of 37° C. from 3 ml. of 0.001 M inosine solution in 0.06 M phosphate buffer. A standard solution of uric acid in 5 per cent perchloric acid was prepared. Under these conditions and at a wave length 292 mμ, one μg. of uric acid gave an extinction of 0.056. Thus,

$$\frac{\text{PNP units}}{\text{ml. packed RBC}} = \frac{\text{optical density}}{0.056} \times \frac{4.15 \text{ (final vol.)}}{1{,}000} \times \frac{\text{dilution of hemolysate}}{\text{vol. of hemolysate (.05 ml.)}}$$

or,

$$\text{PNP units/ml. packed RBC} = 1.48 \times \text{optical density} \times \text{dilution of hemolysate.}$$

The volume of packed red blood cells and reticulocytes were determined by methods described previously (15).

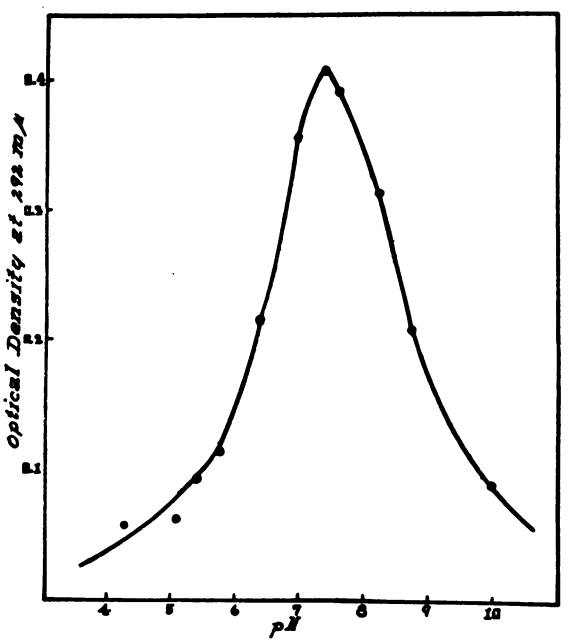

FIG. 1. THE EFFECT OF pH ON PNP ACTIVITY OF HUMAN RED BLOOD CELLS

Phosphate buffer and 0.05 ml. of a 1:100 hemolysate of red cells were used.

RESULTS

Characteristics of erythrocyte purine nucleoside phosphorylase

In the absence of red blood cell hemolysate xanthine oxidase did not split inosine. When xanthine oxidase was incubated with the hemolysate of either human or dog erythrocytes, there were only negligible changes in the optical density at 292 mμ. In none of the preparations used could any uricase activity be demonstrated by incubating the erythrocyte hemolysate with uric acid in glycine buffer of pH 9.4. The determination of uricase activity was necessary since the presence of uricase would nullify the results of these experiments. No xanthine oxidase activity could be demonstrated in the red cells of man or dog when the hemolysates were incubated with a solution of xanthine.

When glycylglycine buffer, pH 7.2 and 0.05 M, was used in the reaction, replacing the phosphate buffer, no splitting of inosine could be demonstrated. The addition of phosphate buffer resulted in an abrupt breakdown of inosine by the PNP of the red cell hemolysate. When arsenate buffer was used in the reaction in place of phosphate buffer, PNP activity was demonstrated (Table I).

TABLE I

PNP activity in phosphate buffer and in arsenate buffer *

Time *min.*	Phosphate buffer	Arsenate buffer
15	14	8
30	30	19
60	59	36
120	120	74
180	179	112

* The reaction mixture consisted of 3 ml. of 0.001 M inosine in 0.06 M phosphate buffer (pH 7.4) or in 0.05 M arsenate buffer (pH 7.4), 0.1 ml. of xanthine oxidase and 0.05 ml. of a 1:200 dilution of red cell hemolysate. The results are expressed in "units" of PNP activity.

The effect of varying pH on the PNP activity of red cells is shown in Figure 1. The optimum pH for this reaction is 7.4.

The effects of time and of different concentrations of inosine, phosphate, and xanthine oxidase on PNP activity of red cells are shown in Figure 2. Inosine was converted to uric acid in a linear fashion over a three-hour period. An inosine concentration of 0.001 M was used in these experiments and the activity at this concentration falls on the plateau part of the PNP activity-sub-

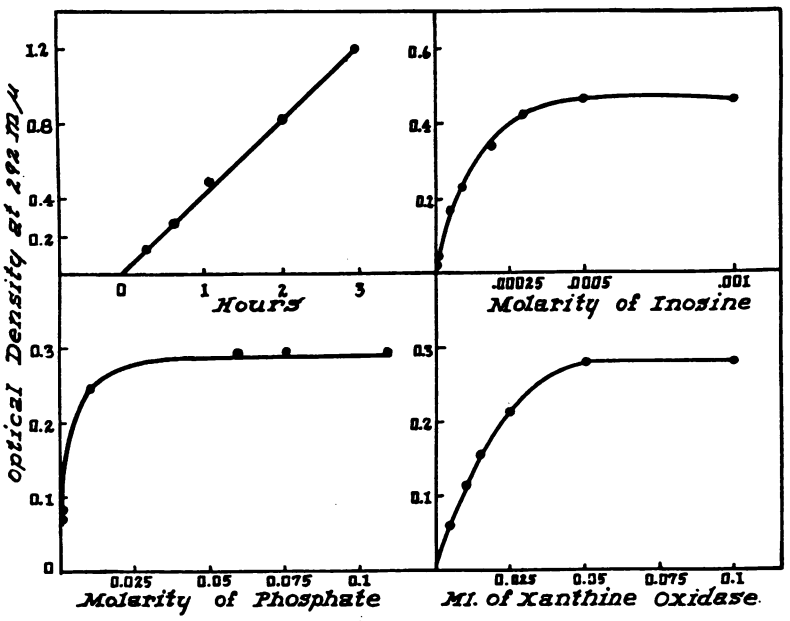

FIG. 2. THE EFFECTS OF TIME AND THE INFLUENCE OF DIFFERENT CONCENTRATIONS OF INOSINE, PHOSPHATE OR XANTHINE OXIDASE ON HUMAN RED CELL PNP ACTIVITY

Five hundredths ml. of a 1:100 hemolysate of red cells was used in all experiments.

strate molarity curve shown in Figure 2. A phosphate concentration of 0.06 M to 0.12 M was optimal. It is apparent from Figure 2 that, for every new preparation of xanthine oxidase, the optimal amount to be used should be determined.

Temperatures below 36° C. resulted in decreased PNP activity. For example, at 26° C., about one-fifth of the activity was present compared to that at 37° C.

When the rate of degradation of inosine by red blood cell PNP was compared with that of hypoxanthine desoxyriboside under identical conditions, it was found that inosine is split more readily than the desoxyriboside (Figure 3).

No phosphorolysis or hydrolysis of xanthosine or the ribosides of pyrimidines (uridine, cytidine) could be demonstrated with the hemolysates of red cells. The methods of Lampen and Wang (16) were used to follow the reactions with uridine and cytidine.

When hemolysates of red cells were incubated in 0.05 M glycylglycine buffer at pH 7.2 with adenosine, adenosine deaminase activity could be demonstrated. When the red cell hemolysate was incubated with xanthine oxidase, phosphate buffer, and adenosine as a substrate, some splitting of adenosine occurred. In a single experiment, the activity with adenosine as a substrate was about one-fifth to one-tenth of that which occurred when inosine was used as the substrate.

Partially purified PNP was prepared by lysing 10 ml. of packed red cells with 200 ml. of water. Solid ammonium sulfate was added to the hemolysate. The final concentration of ammonium sulfate was 1.6 M. The precipitate was discarded

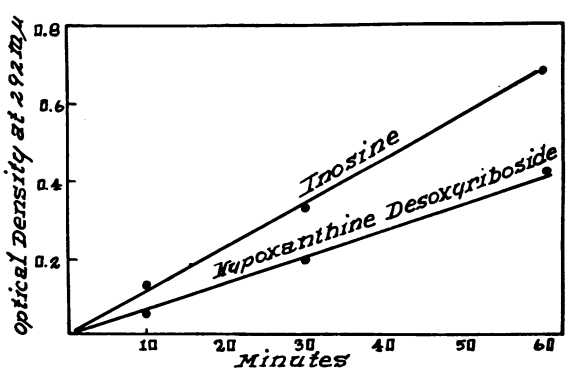

FIG. 3. THE CLEAVAGE OF INOSINE AND HYPOXANTHINE DESOXYRIBOSIDE BY RED CELL HEMOLYSATE

TABLE II

Synthesis of hypoxanthine riboside by partially purified red cell PNP

Experiment no.	Description	Hypoxanthine remaining μM	Hypoxanthine riboside μM
1	Complete system	0.23	0.12
2	Complete system minus ribose-1-phosphate	0.35	0
3	Complete system minus red cell PNP	0.35	0

Synthesis of hypoxanthine desoxyriboside by partially purified red cell PNP

Experiment no.	Description	Hypoxanthine remaining μM	Hypoxanthine desoxyriboside μM
4	Complete system	0.30	0.05
5	Complete system minus desoxyribose-1-phosphate	0.35	0
6	Complete system minus red cell PNP	0.35	0

and solid ammonium sulfate was added to the supernatant solution until the final concentration of ammonium sulfate was 2.4 M. The solution was then centrifuged and the non-dialyzed precipitate was used for assay. All fractionation steps were carried out at room temperature. The final precipitate was dissolved in water and diluted to the volume of the original hemolysate. The activity of the original hemolysate was then compared to the activity of the partially purified preparation. One ml. of this solution contained approximately 25 times the activity of one ml. of the original hemolysate.

When the partially purified PNP of red corpuscles was incubated with ribose-1-phosphate or desoxyribose-1-phosphate, synthesis of inosine or hypoxanthine desoxyriboside could be demonstrated (Table II). The incubation mixture contained 0.50 μM of ribose-1-phosphate or desoxyribose-1-phosphate and 0.35 μM of hypoxanthine in glycylglycine buffer (0.5 M, pH 7.4) and 0.1 ml. of partially purified red cell PNP. The mixture was incubated for thirty minutes. The reaction was stopped by heating in a boiling water bath for one minute. The hypoxanthine and the nucleosides in the supernatant were measured by differential spectrophotometry (13). The synthesis of inosine was of greater magnitude than that of its desoxyribose counterpart.

Purine nucleoside phosphorylase activity of erythrocytes of man and animals

A comparison of the PNP activity at various dilutions of erythrocyte hemolysate in man, chicken, and three different animals is shown in Figure 4. Of the species studied, porcine and human erythrocytes manifested the greatest activity. Rabbit erythrocytes showed intermediate activity. With chicken and dog erythrocytes there was minimal, but measurable, activity. The relatively low PNP activity of chicken red blood cells is interesting since the erythrocytes are nucleated in this species.

It is apparent that inhibition of PNP activity occurred in the lower dilutions of the cell hemolysates of the human subjects, the pig, and the rabbit. The nature of this inhibition is unknown. The activity of the chicken and dog erythrocytes was too low to permit any conclusions concerning inhibition.

The purine nucleoside phosphorylase activity of a 1:200 dilution of the erythrocyte hemolysate for six normal human subjects was found to range between 87 and 112 units per ml. of packed RBC with a mean value of 99 units. A value of 81 units per ml. packed red cells was observed in a patient with acquired hemolytic anemia and a reticulocyte count of 11.5 per cent. A 1:200 dilution of an erythrocyte hemolysate from a patient with paroxysmal nocturnal hemoglobinuria and a retic-

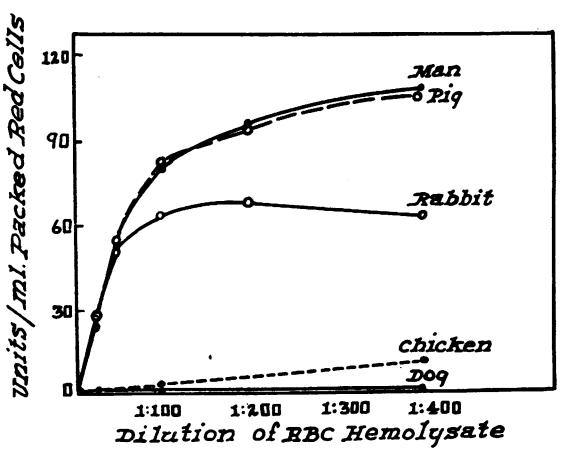

FIG. 4. VARIATION OF RED CELL PNP ACTIVITY OF VARIOUS SPECIES AT DIFFERENT DILUTIONS OF HEMOLYSATES

The data presented represent the averages of six humans (four male, two female), one pig, two rabbits, one chicken, and seven dogs.

ulocyte count of 23.1 per cent gave an activity of 115 units. Thus, it would seem that in human subjects there was no relation between the degree of reticulocytosis and the PNP activity.

No PNP activity could be demonstrated in the stroma of the red cells obtained by centrifugation at 8,000 r.p.m. (American Instrument Co. High Speed Angle Centrifuge) following lysis of the cells with water.

Experimentally induced changes in purine nucleoside phosphorylase activity of dog erythrocytes

When acute anemia was produced in dogs, either by phlebotomy or by the intravenous administration of phenylhydrazine, the PNP activity of the erythrocytes increased simultaneously with the increase in reticulocytes (Figures 5, 6, and 7). However, the PNP activity decreased to the preanemic levels more slowly than did the reticulocytes.

The PNP activity of dog plasma also increased considerably during the hemolytic phase following phenylhydrazine administration (Figure 5). In those animals which survived, the PNP activity of the plasma returned slowly to normal levels.

An acute hemolytic anemia was produced in three rabbits by the intravenous administration of phenylhydrazine. The mean reticulocyte levels increased from 3 to 37 per cent. The mean PNP activity (1:200 dilution of hemolysate) before the administration of phenylhydrazine was 51 units per ml. packed RBC and at the time of the reticulocytosis mentioned above was 52 units. Thus, there was no increase in PNP activity associated with the reticulocytosis in this species.

DISCUSSION

From the data presented, it appears that the purine nucleoside phosphorylase of red cells is similar to that which has been described in the tissues of animals and in micro-organisms (2–8, 17, 18). Whether or not it is identical with tissue PNP cannot be stated from the information available. Although the enzymatic action of PNP seems to be clear, the role of the enzyme in the metabolism of the erythrocyte is unknown.

The species differences in PNP activity of red cells are difficult to explain. They may be re-

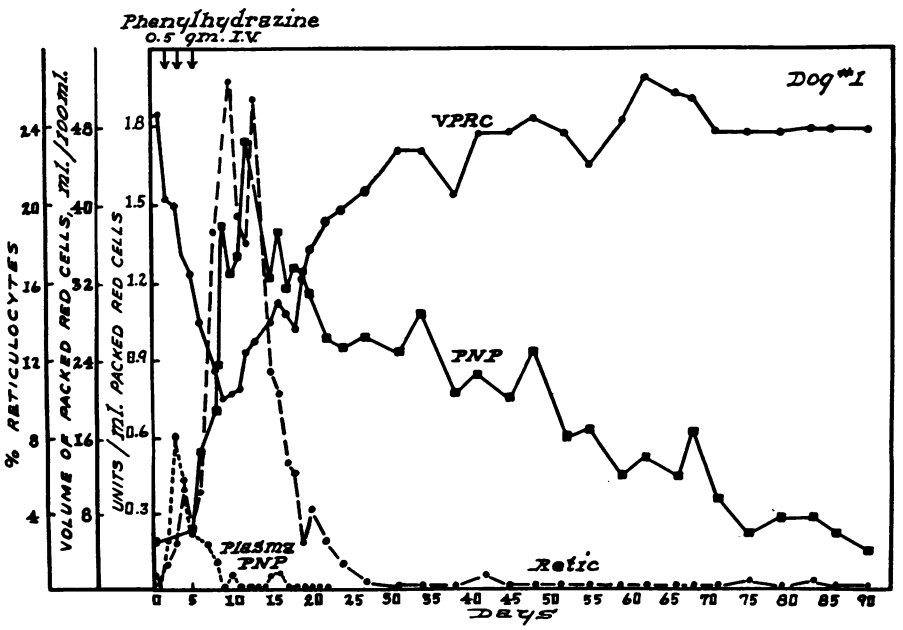

FIG. 5. CHANGES IN PNP ACTIVITY OF DOG RED CELLS DURING AND AFTER THE PRODUCTION OF ACUTE HEMOLYTIC ANEMIA BY THE ADMINISTRATION OF PHENYLHYDRAZINE

Note the high PNP activity in the plasma during the height of hemolysis.

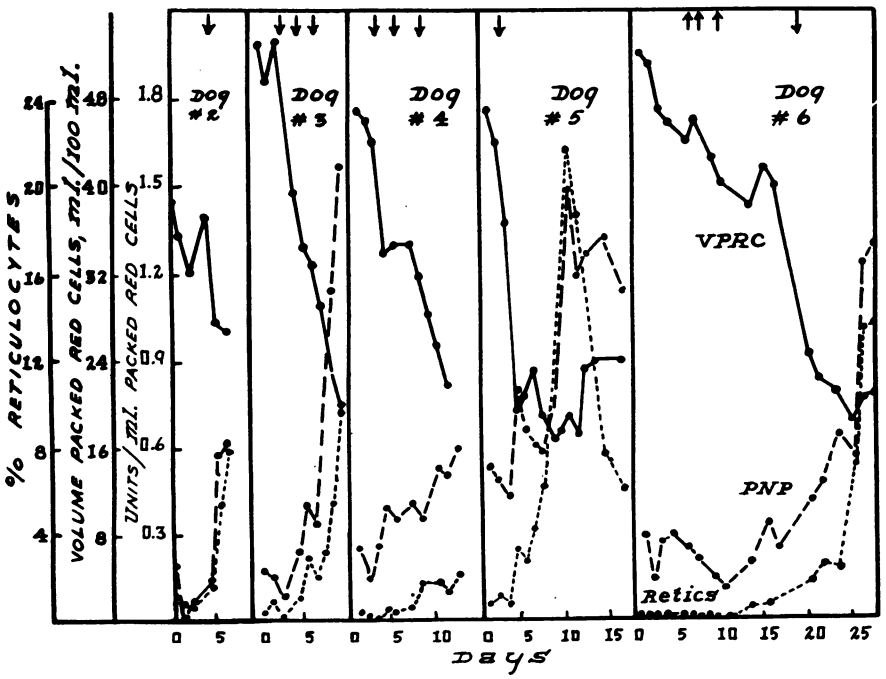

FIG. 6. CHANGES IN PNP ACTIVITY OF DOG RED CELLS DURING ACUTE ANEMIA

Note that there was only slight reticulocytosis and moderate elevation of PNP activity in Dog No. 4. The high initial activity in Dog No. 5 may have been due to the fact that the animal had been made acutely anemic ten weeks prior to the experiment. Dog No. 6 showed slight reticulocytosis after bleeding. The PNP activity rose only to a slight extent. When marked anemia and reticulocytosis were produced with phenylhydrazine, the PNP activity increased markedly.

The arrows pointing downward refer to the time of administration of phenylhydrazine. Those directed upwards refer to phlebotomy. The solid line represents the volume of packed red cells; the broken line, PNP activity; the dotted line reticulocytes.

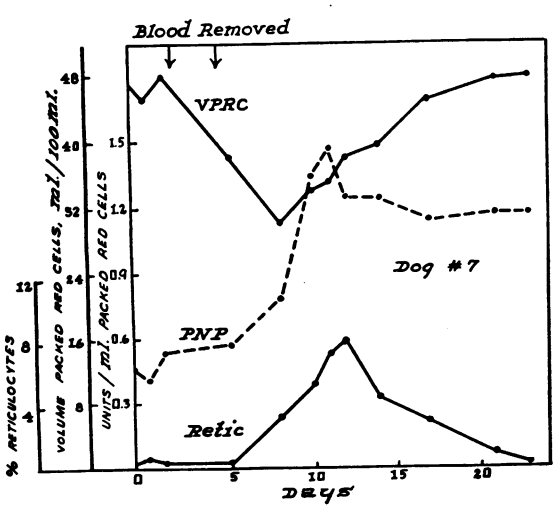

FIG. 7. THE EFFECTS OF PHLEBOTOMY ON DOG RED CELL PNP ACTIVITY

Note the persistence of high activity even after the percentage of reticulocytes returned to a normal level.

lated either to differences in PNP concentration or to the presence of various degrees of inhibition. From the limited data available, there does not seem to be an inverse relationship with the uric acid riboside content (19). However, this possibility deserves further study.

The activity of the enzyme in the blood of man and the rabbit was not related to the per cent of reticulocytes present. In the dog, as the percentage of reticulocytes increased following phenylhydrazine administration or phlebotomy, the PNP activity paralleled the reticulocyte count. The peak of PNP activity usually coincided with the reticulocyte peak. This suggests that red cells which are produced rapidly in the dog possess more PNP activity than do cells which are produced at the normal rate.

It has been demonstrated in the rabbit and the rat that cells which are rapidly produced are destroyed in a random fashion and survive in the

circulation for a shorter time than do cells which are produced at the normal rate (20, 21). If the same holds true for the dog, whose normal erythrocyte life span is about 100 days (22), the slow exponential type of decay curve of PNP activity over a period of about 70 days depicted in Figure 5 suggests that the increased PNP activity remained in the rapidly produced cells of the dog for the duration of their survival.

SUMMARY

1. Purine nucleoside phosphorylase activity, similar to that of other animal tissues, has been demonstrated in the erythrocytes of man, dog, rabbit, pig, and chicken.

2. The purine nucleoside phosphorylase activity was greatest in the erythrocytes of man and the pig. Dog and chicken erythrocytes showed much less activity. The activity of rabbit erythrocytes was intermediate between that of these two groups.

3. The purine nucleoside phosphorylase activity of dog erythrocytes was enhanced markedly following the production of acute anemia by the administration of phenylhydrazine or phlebotomy. Under similar circumstances no increase in PNP activity was noted in rabbit red cells. An increase in PNP activity was not observed in red cells from human blood in which the reticulocyte percentage was high.

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