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Research Article

DETERMINATION OF HUMAN PLATELET SURVIVAL UTILIZING C^{14}–LABELED SEROTONIN *

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All of the methods available for estimation of platelet survival have certain disadvantages. Isolation and considerable manipulation of platelets *in vitro* are necessary with the Cr^{51} technique (1, 2). The P^{32} (3), diisopropylfluorophosphate^{32} (DFP^{32}) (4), or S^{35} (5) methods are all *in vivo* labeling procedures. Studies with P^{32} or S^{35} require special subjects for study (patients with polycythemia vera or leukemia). Finally, both autologous and homologous platelet survival cannot be studied with facility.

The use of serotonin [5-hydroxytryptamine (5-HTA)] as a platelet label appeared to have certain advantages. It is preferentially concentrated by blood platelets *in vivo* and *in vitro* (6). Isolation of platelets is not required to achieve an *in vitro* label, and *in vivo* labeling of platelets can be accomplished without labeling their precursors. We have used radioactive labeled (C^{14}) serotonin to study platelet survival.

MATERIALS AND METHODS

C^{14}-labeled 5-hydroxytryptamine.[1] C^{14}-labeled 5-HTA [5-hydroxy-3-indolyl-(ethyl-2-amino-1-C^{14})] creatinine sulfate monohydrate complex (specific activity 6.87 mc per mmole) was made up in sterile, pyrogen-free, isotonic saline solution in concentration of 2.5 μc per ml. Each milliliter of solution contained approximately 64 μg of 5-HTA. After filtration through a cellulose bacteriological[2] filter and bacterial culture, the solution was stored in a refrigerator at 4° C in a multi-dose vial. Paper chromatography of the material as originally received confirmed that greater than 95 per cent of C^{14} activity was associated with 5-HTA. Deterioration of the substance was noted on prolonged storage. After 3 months only 50 per cent of the C^{14} activity was still associated with 5-HTA.

We were initially concerned with the pharmacological and radiation hazards of C^{14}-5-HTA. On the basis of *in vitro* studies, it was determined that a usable label for *in vivo* studies could be achieved with 1.0 to 2.0 μc of C^{14}-5-HTA. 5-HTA has profound physiological effects when given intravenously in amounts of 2 mg or more (7). In these studies, however, the amount given is much smaller (25 to 50 μg) and in greatest part is bound to platelets and unavailable for immediate pharmacological action. Local venospasm has not been observed in any of the experimental subjects. The radiation hazard is negligible. The maximal permissible body burden of C^{14} is 400 μc (8). In these studies no more than 2 μc was used. Since it is probable that the end-products of 5-HTA catabolism are excreted practically *in toto,* it seems unlikely that C^{14} injected as 5-HTA would enter other metabolic pathways to any appreciable extent (9).

Preparation of C^{14}-5-HTA-labeled platelets. Blood, 250 ml, was drawn directly (via a 16 gauge needle) into a plastic bag. Initially, 30 ml of 0.1 M sodium citrate was prepared and used as the anticoagulant, because Born and Gillson had indicated (10) that uptake of 5-HTA by platelets was maximal under these circumstances. Subsequently, commercially prepared plastic bags (Cutter) containing acid citrate dextrose as the anticoagulant have been used with results similar to those obtained with 0.1 M sodium citrate. After withdrawal of the blood, 1 to 2 μc of C^{14}-5-HTA solution was injected into the bag and the mixture was incubated at 37° C for 30 to 45 minutes in a water bath with occasional gentle swirling. At the end of the incubation period the contents of the bag were rapidly infused into the experimental subject, retaining approximately 15 ml in siliconized conical volumetric centrifuge tubes for sampling of platelets and plasma for radioactivity.

Determination of amount of C^{14} activity in the infused platelets. A hematocrit determination and platelet counts using the method of Brecher and Cronkite (11) were done on the retained sample. The remainder of the sample was then immediately centrifuged and platelet-rich plasma obtained. The platelet-rich plasma was separated and platelet counts performed. A 2-ml aliquot of platelet-rich plasma was transferred to a sample container (20-ml liquid scintillation counting bottle). The remaining volume of platelet-rich plasma was measured and centrifuged to obtain a platelet plug. The plate-

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[1] Nuclear-Chicago Corporation.

[2] Millipore type HA filter, Millipore Corporation.

let-poor plasma was decanted from the plug and 2 ml transferred to a sample container. The silicon-coated conical tubes containing the residual platelet plug were inverted and the excess plasma allowed to drain thoroughly. One ml of distilled H_2O was added to the platelet plug and the plug dispersed using an ultrasonic vibrator.[3] This last step yielded a homogeneous suspension which was decanted into a third sample bottle. One ml of distilled H_2O was used as a washout of the tube and added to the sample container to bring the total volume of the disintegrated platelet suspension to 2 ml. Approximately 0.6 to 1.0×10^9 platelets were suspended in 2 ml of platelet-rich plasma and 1.0 to 1.5×10^9 platelets made up the platelet plug obtained from normal subjects. The platelet-poor plasma rarely had more than 0.003×10^9 platelets in the 2-ml aliquot (corresponding to fewer than 1,500 platelets per mm^3 of plasma).

The sample bottles into which the 2-ml volumes of platelet-rich plasma and the dispersed platelet plug were decanted had been previously prepared by the addition of 18 ml of a liquid scintillation mixture of dioxane, anisole and dimethyloxyethane (6:1:1 by volume) containing 7 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP [2,2-*p*-phenylenebis (5-phenyloxazole)]. In order to maintain the intact platelets in suspension, this mixture had been modified by addition of Thixcin[4] in concentrations of 2.5 g per 100 ml to form a gel. Counting efficiency in a liquid scintillation counter[5] with this system was approximately 30 per cent for aqueous colorless solutions. Efficiency was lower (approximately 20 per cent) for the samples containing colored solution (i.e., plasma). Samples were counted long enough to achieve a counting error of 3 per cent or less (usually 10 to 30 minutes). The use of liquid scintillation counting methods for *in vivo* studies was dictated by the relatively low C^{14} activity in platelets after dilution in the blood volume of the study subjects had occurred. The major advantage was in the number of platelets which could be conveniently placed in a liquid counting system as opposed to solid sample beta counting systems. Recovery of radioactivity could be enhanced by extraction of C^{14}-labeled serotonin from platelets or by use of a more efficient liquid counting system. The method used, however, allows rapid preparation of samples and is adequate in terms of efficiency.

C^{14}-5-HTA in red cells was not counted directly. *In vitro* experiments confirmed previous findings that red blood cells concentrate and retain 5-HTA poorly (6). It is unlikely that the small number of red cells in the samples contributes any of the radioactivity observed. Further confirmation of this point came from the use of reference standards for the total amount of radioactivity infused. Virtually all of the injected activity could be accounted for by totaling platelet and plasma activity.

Sampling of the recipient. Fifteen ml of whole blood was drawn with silicon-coated equipment and processed in the same manner as the aliquot of infused bag blood. The recipient was sampled until the radioactivity per 10^9 platelets was less than 20 per cent of the amount present 24 hours after infusion. In addition, 24-hour urines were collected from some subjects and total C^{14} activity in the urine determined.

Experimental subjects. Eleven normal males who had never received blood transfusions were infused with C^{14}-labeled platelets. Two experiments in normals were cross-transfusion studies. The remaining experiments were infusions of the subject's own labeled platelets. An additional normal subject received C^{14}-labeled 5-HTA infused in saline rather than in whole blood to determine the efficiency of *in vivo* labeling. Finally, four patients with thrombocytopenia were studied on five occasions. Three of these patients received homologous platelet infusions. The fourth patient was reinfused with his own platelets that were labeled *in vitro*, as in the normal subjects.

RESULTS

Yield of infused platelets. In nine experiments in normal subjects, it was possible to calculate the expected level of radioactivity in the circulating platelets following infusion of C^{14}-5-HTA-labeled platelets. Two experiments lacked data essential for this calculation. The radioactivity in circulating platelets was determined for each sampling period and the per cent yield was calculated according to the formula:

$$\text{per cent yield} = \frac{(\text{cpm, platelets observed})\ (\text{platelets/ml})\ (\text{blood vol, ml})}{(\text{cpm, platelets infused})\ (\text{platelets/ml})\ (\text{ml infused})} \times 100$$

Per cent yield for 2, 6 and 24 hours, summarized in Table I, included seven autologous and two homologous platelet infusions. The lowest yield, 39 per cent (Subject SH), differs from the re-

TABLE I

Per cent of calculated expected yield observed at 2, 6, and 24 hours in nine normal subjects

Subject	Time		
	2 hrs	6 hrs	24 hrs
	%	%	%
SH	39	49	36
KY	80		62
HB	75	80	75
LF	73		65
BD	54	58	51
NF	47		47
PF	51		59
EG*	59		61
JP*	56		57
Mean	59	67	57

* Homologous platelet infusions.

[3] Narda "Sonblaster."

[4] Baker Castor Oil Company.

[5] Packard Tri-Carb.

TABLE II

Efficiency of in vivo labeling compared with in vitro labeling

Subject	Cpm/10^9 platelets at 24 hrs	Infused C^{14} activity in platelets at 24 hrs	C^{14} activity excreted at 24 hrs
		% total	%
RH (*In vivo* label)	61	7	53
EG (*In vitro* label)	385	61	8
JP (*In vitro* label)	630	57	6

mainder. This study was done with 75 ml of blood drawn into a silicon-coated glass bottle and incubated with C^{14}-5-HTA as usual. Unfortunately, it was necessary to reinject the blood with multiple syringes. The remainder of the experiments all utilized greater volumes of blood drawn into plastic bags and reinfused directly into the recipient. The lowest yield of the last eight experiments was 47 per cent. The mean yield for all nine experiments was 59 per cent at 2 hours and 57 per cent at 1 day. The results in two subjects (EG and JP) who received homologous platelets were similar to those of the autologous platelet infusions. The rise to peak activity at approximately 24 hours, seen in other studies (1–3) and interpreted as indicating temporary platelet sequestration *in vivo,* was not observed in these experiments. A small rise occurred in many between 2 and 6 hours. However, the 2-hour value usually was similar to or higher than the 24-hour value.

Calculation of per cent yield is subject to error since C^{14}-5-HTA free in plasma is present in the infusion, and *in vivo* labeling of platelets occurs. The magnitude of this error was assessed by infusing a normal subject with C^{14}-5-HTA in 250 ml of 0.85 per cent sodium chloride solution. Experiments done in Subjects RH, EG and JP are summarized in Table II. The blood of EG and JP was typed, cross-matched and cross-transfused after their platelets were labeled in plastic bags, as previously described. Less than 10 per cent of the infused C^{14} activity was free in plasma; the remainder was in platelets. RH received a similar amount of C^{14}-5-HTA in a volume of saline comparable to the whole blood infusions received by EG and JP. Less than 10 per cent of the infused

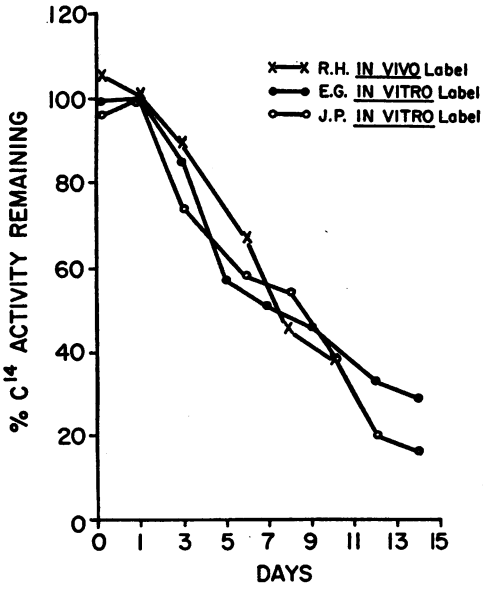

FIG. 1. DISAPPEARANCE OF C^{14}-LABELED 5-HTA FROM PLATELETS. EG and JP received *in vitro* labeled homologous platelets. RH was infused with 5-HTA in saline, an *in vivo* platelet label. 100 per cent = counts per minute per 10^9 platelets at Day 1.

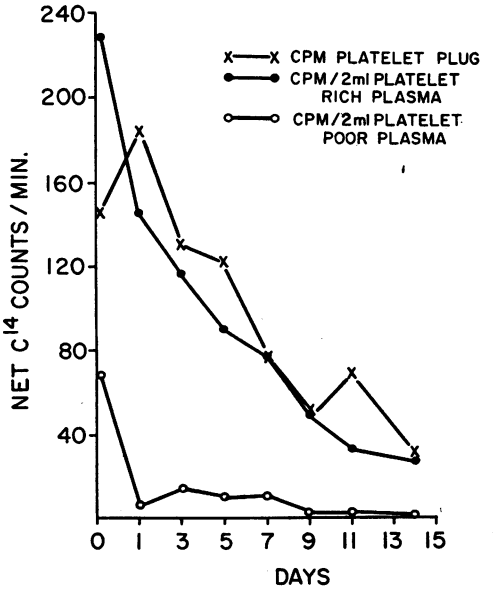

FIG. 2. COMPARISON OF C^{14} ACTIVITY IN PLATELET-RICH PLASMA, PLATELET-POOR PLASMA AND PLATELET PLUG. The total number of platelets in samples of platelet-rich plasma was approximately 0.7 to 0.8×10^9; in platelet-poor plasma 0.004×10^9; in the platelet plug 0.7 to 0.8×10^9 platelets.

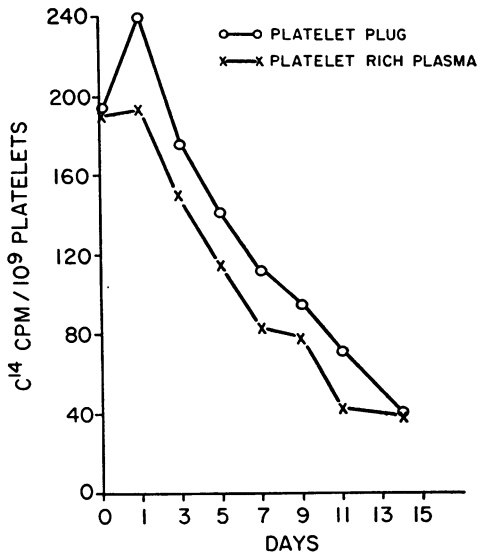

FIG. 3. PLATELET SPECIFIC ACTIVITY CALCULATED BY USING COUNTS PER MINUTE OBSERVED IN THE PLATELET PLUG AND IN PLATELET-RICH PLASMA. The lower specific activity seen in platelets suspended in platelet-rich plasma is due to the quenching effect of plasma as opposed to the aqueous platelet plug sample.

activity was present in urine (EG and JP) at the end of 1 day when C^{14}-5-HTA bound to platelets was infused. Fifty-three per cent of infused activity was in the 24-hour urine of the subject (RH) receiving C^{14}-5-HTA in saline. *In vivo* labeling of the platelets of RH was appreciable. This was, however, less than 20 per cent of the activity found when a comparable amount of 5-HTA labeled to platelets was infused. Since usually no more than 10 per cent of the injected activity is in plasma, free to label circulating platelets in the recipent, the magnitude of the error introduced by *in vivo* labeling is probably small. Overestimation of per cent yield by no more than 3 to 5 per cent results. The disappearance rates of the C^{14}-5-HTA from the circulating platelets of the three subjects were similar (Figure 1).

Platelet survival in normal subjects. Counts per minute per 2 ml of platelet-rich plasma, platelet-poor plasma, and counts per minute in the platelet plug are plotted against days for a typical experimental subject (Figure 2). Comparatively, the amount of activity in platelet-poor plasma was small. However, in calculating platelet specific activity, the platelet-rich plasma counts are corrected by subtracting platelet-poor plasma counts, because all of the C^{14} activity present in plasma on the first day cannot be accounted for by activity present in the few platelets which have remained in suspension in platelet-poor plasma. C^{14} platelet specific activity calculated by use of values obtained from the corrected platelet-rich plasma and platelet plug is plotted against days in Figure 3. Platelet specific activity based on the platelet plug is higher. This can readily be accounted for by the greater quenching effect of plasma in the scintillation solution as opposed to the aqueous platelet plug solution. In this case the quenching effect of the plasma resulted in reduction of observed counts to approximately 80 per cent of total counts in a colorless aqueous solution, as estimated by using internal standards in the scintillation solution. Correction of observed counts per minute per 10^9 platelets derived from platelet-rich plasma results in close agreement of the two values for platelet specific activity.

It is possible to express per cent survival as a

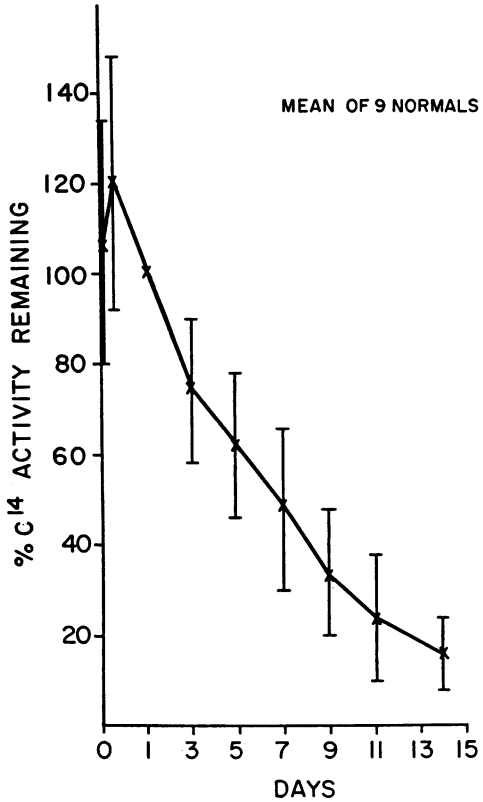

FIG. 4. MEAN ± 2 SD OF NINE AUTOLOGOUS C^{14}-LABELED PLATELET INFUSIONS. 100 per cent activity is taken arbitrarily as platelet specific activity at 24 hours.

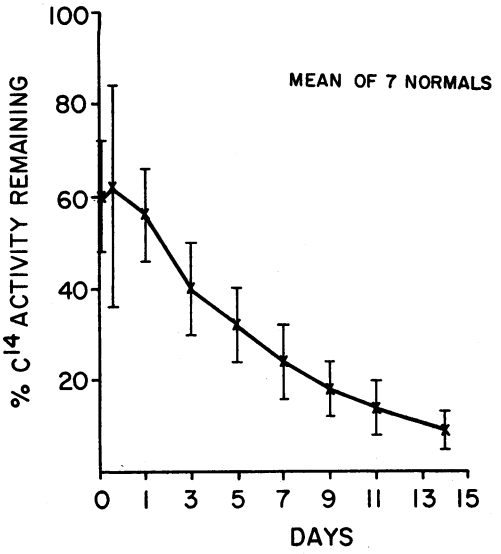

FIG. 5. MEAN ± 2 SD OF SEVEN AUTOLOGOUS C^{14}-LABELED PLATELET INFUSIONS. 100 per cent = calculated expected yield.

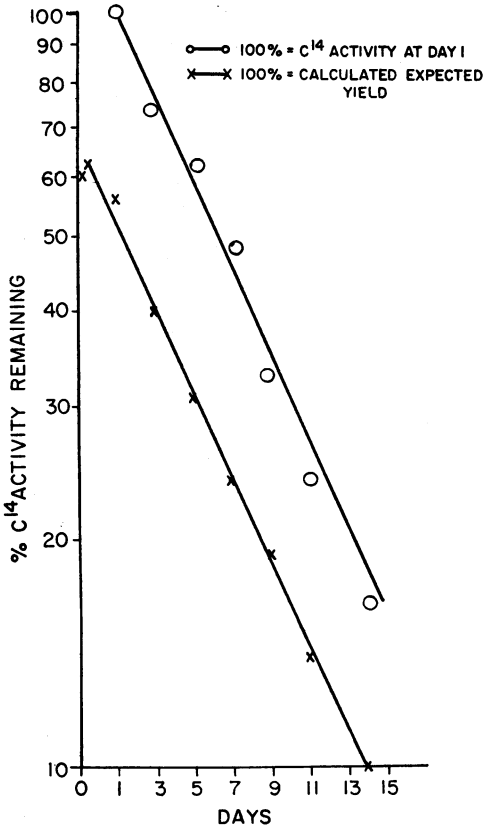

FIG. 6. VALUES FROM FIGURES 4 AND 5 PLOTTED ON SEMILOGARITHMIC COORDINATES. 100 per cent = value at Day 1; mean of 9 experiments (O——O). 100 per cent = calculated expected yield; mean of 7 experiments (×——×).

function of time in two ways: either as a per cent of activity per 10^9 platelets at some arbitrary time after infusion, or as a per cent of the calculated expected yield, as Aas and Gardner (1) and others (2) have done. The latter method has the advantage of compensating for changing platelet counts in the recipient. Figure 4 illustrates the mean, plus or minus 2 standard deviations, of nine experiments done in normals with autologous transfusions. Specific activity 24 hours after infusion has been arbitrarily taken as 100 per cent. Figure 5 represents seven of these nine experiments with per cent activity remaining plotted against time as a function of platelet yield. Figure 6 shows the data of Figures 4 and 5 plotted on semilogarithmic coordinates. An exponential disappearance of C^{14}-serotonin from the circulating

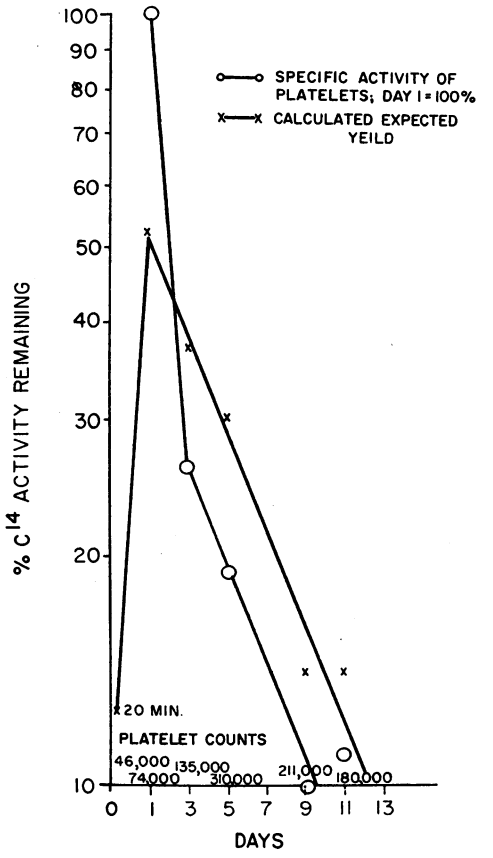

FIG. 7. PLATELET SURVIVAL IN DRUG-INDUCED (?) THROMBOCYTOPENIA IN RECOVERY PHASE. Survival (O——O) based on platelet specific activity appears markedly shortened initially, while the curve (×——×) based on calculated expected yield is basically normal. Platelet counts in peripheral blood at time of sampling are indicated.

TABLE III

Per cent of circulating platelet radioactivity, expected and observed platelet rise, and urinary excretion of C^{14} in four homologous platelet transfusions in thrombocytopenia

Subject	Yield at 24 hrs	Expected platelet yield/mm³	Observed platelet yield/mm³	Per cent of expected platelet rise observed	C^{14} excreted first 24 hrs
	%			%	%
EJ (DLE)	53	22,000	11,000	50	47
EC (Acute leukemia)	5	65,000	0	0	49
MD (ITP)					
1	4	7,650	0	0	80
2*	6	148,000	(?)2,000		92

* Infused with blood from patient with idiopathic thrombocythemia (platelet count of donor 1.5×10^6/mm³).

platelet mass was seen. The half-times for these curves were close to 5 days in both cases. They are similar to the *in vivo* and homologous labeled curves (Figure 1). One difference is in the time necessary for the curves to reach 20 per cent or less of initial activity. In the case of the per cent yield model, this occurred at approximately 8 days, while less than 20 per cent was not reached until 13 or 14 days after infusion, if all values are referred to the amount of activity present 24 hours after infusion.

The advantage of using per cent of calculated platelet yield is best illustrated (Figure 7) by a patient who developed acute thrombocytopenia, possibly drug-related (Librium [6]). A platelet survival study, utilizing the patient's own platelets, was initiated 2 days after steroid therapy was begun. Peripheral blood platelet count at that time was 46,000 per mm³. The curve based on per cent yield indicates a basically normal survival ($T_{\frac{1}{2}} = 5$ days), while that based on per cent residual activity per 10^9 platelets is initially suggestive of a markedly shortened survival. The impression of a relatively normal life span is further strengthened by the slope of the curve based on platelet specific activity after 3 days, when marked changes in platelet count were no longer occurring. This portion of the curve has a half-value of 5 days much like that in the normal situation.

[6] Roche; 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine 4-oxide hydrochloride.

Platelet survival in thrombocytopenia. One of the studies in patients with thrombocytopenia has been referred to in the preceding section and was done with autologous platelets. The remaining three patients were studied a total of four times with homologous infusion of platelets. These included a 34 year old female with disseminated lupus erythematosus (DLE) and thrombocytopenia, a 54 year old male patient with acute leukemia in relapse and severe hemorrhagic manifestations who had previously received multiple transfusions, and two studies done in a 31 year old female patient with acute idiopathic thrombocytopenic purpura (ITP). Per cent yield at 24 hours, expected platelet rise, observed platelet rise, and per cent platelet rise, as well as C^{14} urinary excretion data are summarized for these three patients (Table III). The patient with idiopathic thrombocytopenic purpura had developed purpura 2 weeks prior to study with peripheral blood platelet counts of around 3,000 per mm³. She had received no blood transfusions at the time of these studies. The massive destruction of platelets previously observed (12) in idiopathic thrombocytopenic purpura was corroborated. The patient with leukemia had massive purpura at the time of study and had received multiple transfusions before the study was done. Rapid utilization of platelets due to his hemorrhagic diathesis, or destruction of platelets due to antibodies induced by prior transfusion of platelets, or both, could account for the result seen. The patient with lupus erythematosus had a defi-

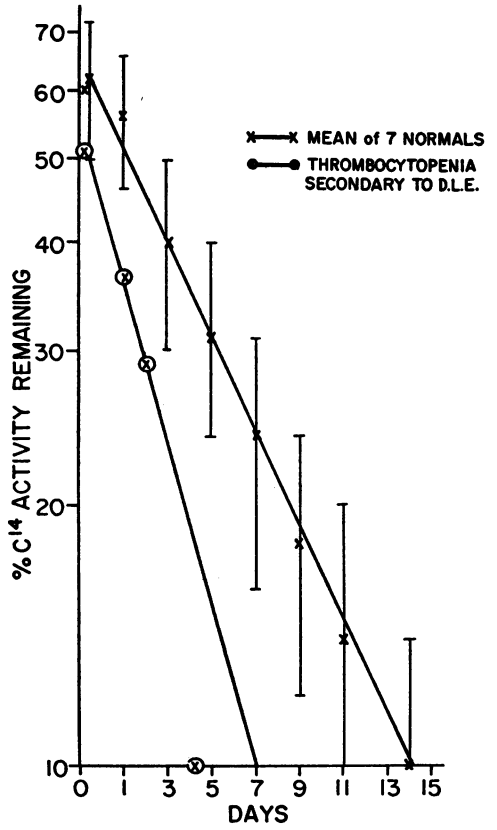

FIG. 8. PLATELET SURVIVAL IN A PATIENT WITH THROMBOCYTOPENIA (PLATELET COUNT 33,000 PER MM^3) SECONDARY TO DLE.

nitely shortened platelet survival with a half-life of less than 3 days. Platelet survival in this patient is plotted and contrasted with the seven autologous infusions done (Figure 8). The result may indicate simply more rapid utilization of platelets because of a decreased circulating platelet mass. Another possibility is a circulating platelet antibody due either to the basic disease or to three transfusions of whole blood received 4 months previously. The patient did not have a positive direct or indirect Coombs' test at the time of this study.

DISCUSSION

These studies were initiated because Udenfriend, Weissbach and Bogdanski noted the close correlation between the half-time of 5-HTA in platelets and platelet survival in rabbits and dogs (13, 14). The studies cited here in humans and similar studies by us in rats (15) extend these observations to two other species.

Still lacking is direct experimental proof that 5-HTA, once taken up and perhaps bound in platelets, is not lost *in vivo* until platelet lysis occurs. Born and Gillson (10) have shown that exchange of C^{14}-labeled for nonlabeled 5-HTA can occur between plasma and platelets *in vitro*. However, the conditions of their experiment would rarely, if ever, be found *in vivo,* as they properly pointed out. A study is cited by Zucker (16) on platelet 5-HTA disappearance rate in a patient with the carcinoid syndrome. Platelet 5-HTA disappeared in approximately 2 hours. The difference between that situation and our results is unexplained. It may relate to the basic disease process in the carcinoid syndrome.

The possibility exists that agreement between the half-time of disappearance of 5-HTA from circulating platelets and estimates of platelet survival *in vivo* is coincidental. We have been unable to devise a direct method for settling this point, other than radioautography of platelets. In humans, using the present amounts of C^{14}-labeled 5-HTA, it would take an estimated 40 years' exposure of the radioautographic material before a definite platelet label was detectable. We are now doing these studies in rats, since they have a much smaller platelet mass and a much heavier platelet label can be achieved. Another possibility is homologous infusion of labeled platelet-rich blood into an individual with thrombocytopenia due to marrow megakaryocyte aplasia and study of concomitant platelet and C^{14} disappearance rates.

Loss of platelet label from the circulation, studied by this method, is clearly exponential. Recently, much work has been done by several groups using various isotopic techniques to study the survival of platelets in the circulation in humans and animals. Disappearance of platelets from the circulation with the DFP^{32} (an *in vivo* label) method of Leeksma and Cohen (4) is reported as a linear phenomenon. Odell and Anderson (5), using S^{35} *in vivo* labeling of platelets in rats and humans, also have reported linear disappearance rates, but pointed out that the derived curves could as well be drawn as exponentials. Initially, Aas and Gardner (1) stated that the disappearance of platelets labeled *in vitro* with Cr^{51} was a linear phenomenon. In a more recent publication, however, Cohen, Gardner and Barnett

(17), using the Cr^{51} technique, have described the disappearance rate of platelets from the circulation as the sum of two exponentials multiplied by two constants rather than as a linear phenomenon. Adelson and co-workers (18) labeled platelets of polycythemic individuals *in vivo* with P^{32} and infused them into normal recipients. Exponential disappearance curves of platelet radioactivity were obtained. They have interpreted their results to indicate "utilization of platelets" in a constant process of *in vivo* clotting. They further presented evidence to show that platelet survival curves by their method can be altered from a clearly exponential function to a linear disappearance rate by anticoagulation with coumarin derivatives. Because of uncertainties with regard to re-utilization of C^{14}-5-HTA label, exchange of tracer between labeled and nonlabeled platelets or between labeled platelets and other body pools of serotonin, the question of whether platelets are utilized (random destruction) or age and die in the circulation cannot be settled from these studies.

The half-life of survival of platelets in the circulation has ranged, by these various methods, from 2 to 3 days (17, 18) to 5 days (2, 4, 5). Total life span has been estimated at from 8 to 14 days, the highest value being an upper limit reported by Pollycove, Dal Santo and Lawrence (19) using DFP^{32}. The results with C^{14}-5-HTA are in reasonable agreement with these estimates.

The yield of infused platelets of approximately 57 per cent observed at 24 hours is in accord with the concept of a relatively small reserve of noncirculating platelets (20). It has been estimated that the noncirculating platelet mass is only one or perhaps two times greater than the circulating platelet mass. Alternatively, the possibility exists that some of the infused platelets fail to circulate because of damage induced by manipulation. Platelet sequestration described by others is not a constant or a striking phenomenon in these studies. In normal subjects, the 24-hour period after injection appears to be more one of equilibration and redistribution than an active sequestration of platelets. Marked sequestration was noted in two abnormal subjects, one with drug-induced thrombocytopenia and one with lupus erythematosus. These differences again are unexplained.

The method described has several advantages over others. The most important is ease and simplicity of the labeling procedure and the minimal manipulation of platelets required. Either *in vitro* or *in vivo* labeling of platelets can be accomplished. Autologous or homologous studies may be done. The greatest disadvantage is the need for liquid scintillation counting techniques when studying as large an animal as man and the relatively expensive instrumentation required.

SUMMARY

1. The disappearance rate of C^{14}-5-HTA (5-hydroxytryptamine) from circulating platelets in normal subjects infused with both autologous and homologous C^{14}-5-HTA-labeled platelets has been studied. The half-time of C^{14}-5-HTA in circulating platelets was 5 to 6 days, with less than 20 per cent of the activity present at 8 to 13 days. These values correlate well with reported platelet survival in humans studied by other techniques.

2. If exchange of labeled for nonlabeled 5-HTA in platelets and re-utilization of label are minimal, the disappearance curves indicate an exponential loss of platelets from the circulation compatible with the concept of random disappearance of platelets from the circulation.

3. Calculation of the number of infused labeled platelets that circulate indicates that 50 to 55 per cent was in the peripheral blood at the end of the first day. This value is in reasonable agreement with other estimates of a relatively small total platelet pool.

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