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Pharmacokinetics of Heparin V: *In Vivo* and *In Vitro* Factors Affecting the Relationship Between Concentration and Anticoagulant Effect of Heparin in Rat Plasma

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Abstract □ There are appreciable interindividual variations in rats of baseline activated partial thromboplastin time (APTT) and of the anticoagulant effect of heparin added to plasma (as reflected by the slope of the regression line describing the essentially linear relationship between ln APTT and heparin concentration). Determination of baseline APTT and slope value on two occasions, 7 days apart, in the same rats revealed that (unlike in humans) these characteristics were subject also to considerable intraindividual variation. To explore the possible reasons for the observed variability, the effect of citrate concentration (acid citrate solution is used as a blood anticoagulant in the collection of plasma), calcium concentration (in the recalcifying solution used to initiate coagulation), and plasma incubation time (for activating the coagulation system) was determined. All three variables had pronounced effects on the anticoagulant response to heparin. Since rat erythrocytes are almost totally impermeable to citrate, hematocrit is a determinant of plasma citrate concentration when acid citrate solution is added in constant proportion to rat blood. Accordingly, inter- and intraindividual differences in baseline APTT and slope values were measured in another experiment in which the citrate solution to plasma (rather than blood) volume ratio was held constant and blood samples were obtained 30 days apart to permit the return of hematocrit values to normal. Intraindividual variation of the coagulation characteristics was appreciably decreased under these conditions. There are important differences between rats and humans with respect to the effect of citrate concentration and plasma incubation time on baseline APTT and on the anticoagulant action of heparin, as well as with respect to the relationship between these two characteristics.

Keyphrases □ Heparin—pharmacokinetics, concentration and anticoagulant effect, *in vivo* and *in vitro* factors □ Pharmacokinetics—heparin, concentration and anticoagulant effect, *in vivo* and *in vitro* factors □ Anticoagulants—heparin, effect of concentration, *in vivo* and *in vitro* factors, pharmacokinetics

Safe and effective anticoagulant therapy with heparin is complicated by the chemical and pharmacological heterogeneity of this natural product (1–3), by inter- and intraindividual differences in anticoagulant response (4–8) that necessitate individualization and frequent changes of the dosing rate of this drug, and by questions concerning the suitability of the various *in vitro* clotting tests used as intermediate therapeutic end points to serve as indices of therapeutic efficacy (prevention of thrombosis) and safety (absence of hemorrhagic episodes due to excessive anticoagulation) (9, 10). An individual's anticoagulant response to a given dose or dosing rate of heparin is subject to two sources of considerable variation, one pharmacokinetic and the other pharmacodynamic: the disposition (systemic clearance and biological half-life) of the drug and the relationship between heparin concentration in plasma (the site of anticoagulant action) and the magnitude of anticoagulant effect (6). Practical and ethical considerations impose limitations on exploration of these problems in humans and make it desirable to use animal models for certain pharmacokinetic and pharmacodynamic studies of heparin. The rat appears to be promising for this purpose. Like humans, rats exhibit dose-dependent elimination kinetics of heparin (11). The anticoagulant response to this drug as reflected by the activated partial thromboplastin time (APTT) is log-linearly related to the concentration of added heparin in plasma over a wide concentration range in both humans and rats (4, 11).

Studies in normal human adults (4) have shown a significant correlation between hematocrit and an index of the anticoagulant response of plasma to added heparin (the slope of the essentially linear relationship between ln APTT and the concentration of heparin added to plasma, to be referred to in this article as the slope or slope value). It has also been observed that there are pronounced interindividual differences in both baseline APTT (*i.e.*, APTT of plasma without added heparin) and slope value, but that intraindividual differences are relatively small in humans (4).

Contrary to these findings, it was found in the initial phase of the present study that baseline APTT and slope values in individual rats, while exhibiting similar interindividual differences as in humans, were poorly reproducible when measured again 7 days later. To explore the reasons for these intraindividual differences in rats, the relationship of hematocrit, citrate concentration (acid citrate solution is added to blood to prevent coagulation),

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and calcium concentration (calcium chloride is added to the citrated plasma to initiate the clotting process) to baseline APTT and slope value were determined. Based on the results of these experiments, the assessment of inter- and intraindividual differences in baseline APTT and slope value was repeated under conditions in which the volume ratio of citrate solution to *plasma* (rather than blood, as is the usual practice) was kept constant. Since citrate is almost totally excluded from erythrocytes (12), this prevents any variation of plasma citrate concentration due to differences in hematocrit. Another variable studied in this investigation was the effect of the incubation time of citrated plasma with APTT reagent (before recalcification) on baseline APTT and on slope. The results of these studies (*a*) serve to identify those variables which have to be carefully controlled in pharmacodynamic and pharmacokinetic (based on bioassay) experiments on rats, (*b*) provide an indication of the magnitude of inter- and intraindividual differences of baseline APTT and anticoagulant effect of heparin in rats, and (*c*) permit a comparison of differences between humans and rats with respect to inter- and intraindividual variations of baseline APTT and slope, and the relationship of these two indices to one another and to certain other physiological and methodological variables.

EXPERIMENTAL

Adult, male Sprague–Dawley rats[1] were used in this investigation. They had free access to food[2] and water at all times. The APTT was determined as described previously (11): Samples of frozen citrated plasma were thawed at 37° and the tubes were then transferred to an ice-water bath. Within 1 hr, 0.25 ml of the plasma was transferred to a 0.75-ml polypropylene tube containing 10 μl of normal saline solution. One-tenth milliliter of this plasma was mixed with 0.1 ml of APTT reagent[3], and this mixture was incubated at 37° for exactly 15 min (except when incubation time was studied as a variable). One-tenth milliliter of calcium chloride solution (0.025 *M* except when calcium concentration was studied as a variable) was then added to initiate clotting. The incubation and subsequent determination of APTT were done with a coagulation timer[4]. All samples were prepared and APTT was measured in duplicate. The duplicate values, which usually varied by <4%, were averaged.

To determine the effect of added heparin on APTT, the 10 μl of normal saline solution added to a plasma sample for determination of baseline APTT was replaced by 10 μl of sodium heparin (bovine lung origin)[5] in normal saline solution. The heparin concentration in this solution was varied such as to yield plasma heparin concentrations of 0.05–1.0 U/ml, usually in nine increments. The slope of the relationship between ln APTT and heparin concentration was determined by least-squares linear regression analysis of APTT values obtained from plasma samples in the 0.1 to 1.0-U/ml heparin concentration range. The statistical significance of differences between slope values of plasma obtained on different days from the same animal was determined by analysis of covariance (13).

To determine the effect of plasma citrate concentration on baseline APTT and on the anticoagulant activity of heparin, 10-ml blood samples were collected from each of seven rats, weighing 470–567 g. The blood was drawn from the abdominal aorta, during ether anesthesia, into a plastic syringe containing 0.6 ml of 0.1 *M* acid citrate solution (14). The blood samples were pooled in a plastic beaker immersed in an ice-water bath and kept well mixed by magnetic stirring. Aliquots (9.6 ml) of the pooled, citrated blood were transferred by polypropylene pipet to screw-capped polycarbonate centrifuge tubes containing 0.45 ml of either isotonic saline solution or acid citrate solution of six different (66–330 m*M*) concentrations. Since citrate is almost totally excluded from rat erythrocytes (12), the plasma citrate concentration was calculated from the known whole blood citrate concentration and the hematocrit. Plasma was obtained by centrifuging the blood samples at 14,000×*g* for 5 min in a temperature-controlled centrifuge at 15°, and baseline APTT and slope value were determined as described in the preceding paragraphs.

The effect of calcium chloride concentration in the recalcifying solution used to initiate plasma clotting was determined at a constant citrate concentration (*i.e.*, changing calcium–citrate concentration ratio) and at a constant calcium–citrate concentration ratio[6]. Pooled citrated plasma was obtained from eight rats, weighing 442–530 g, using the aforementioned procedure except that the final citrate concentration in half of the plasma samples was kept constant. The calcium chloride concentration in the recalcifying solution ranged from 12.5 to 46.6 m*M*, in six increments. Baseline APTT and slope values were determined.

To determine the effect of incubation time on baseline APTT and slope, 10-ml blood samples were obtained from each of 15 rats weighing 344–382 g. A 30-μl sample of blood was first drawn from the tail artery into a heparinized microhematocrit tube; the tube was centrifuged (4) and the hematocrit determined. A sufficient volume of acid citrate solution was then drawn into a plastic syringe such that on collection of 10 ml of blood from an animal, the volume ratio of citrate solution to plasma was 1:6. Individual plasma samples from five of the rats were divided into three equal volumes, and plasma samples from the other 10 rats were divided into two equal volumes. One of the plasma aliquots from each of the initial five rats was used to determine the time required for maximum activation of the intrinsic coagulation pathway. This was done by incubating the citrated plasma–APTT reagent mixture at 37° in covered tubes for 1–20 min before recalcification. Except for the variation of incubation time, the APTT measurements were performed by the standard procedure. Based on the results of the five-animal study, baseline APTT and slope value determinations on plasma from the other 10 rats were made after 3 and 15 min of incubation. (It should be noted that these experiments were not done with pooled plasma, but with individual plasma samples from a total of 15 animals.)

Inter- and intraindividual differences of baseline APTT and slope values were determined in two experiments. All rats had a silicone rubber cannula implanted in the right jugular vein under ether anesthesia 3 days before the start of the experiment (15). Their body weight and hematocrit were determined on each study day. Thirteen animals were used in the first experiment. On day 1, 4 ml of blood was collected through the cannula in a plastic syringe containing 0.44 ml of 0.1 *M* acid citrate solution using the technique previously described (11). On day 7, 9 ml of blood was obtained from the abdominal aorta, under ether anesthesia, in a plastic syringe containing 1 ml of the acid citrate solution. The second experiment, with 12 rats, consisted of blood withdrawals on days 1 and 30. In this experiment, hematocrit was determined first from a microsample of blood taken from the tail artery, and the volume of acid citrate solution was individualized to yield a constant acid citrate solution–plasma volume ratio of 1:6. In all cases, the blood and citrate anticoagulant solution were mixed gently and platelet-poor plasma was separated by centrifugation at 14,000×*g* for 5 min at 15° in polycarbonate tubes. These plasma samples were transferred to stoppered polypropylene tubes, frozen in a dry ice–methanol bath, and stored at −80° until assayed.

RESULTS

The baseline APTT in a group of 13 rats ranged from 11.1 to 27.1 sec, averaging 19.5 sec. Identical average APTT and a similar range of individual values were found in the same animals 7 days later, but there was no significant correlation between the day 1 and day 7 baseline APTT values in individual rats (Table I). An essentially linear correlation between ln APTT and the concentration of heparin added to plasma was consistently found over a heparin concentration range from 0.1 to 1.0 U/ml. However, the APTT at a heparin concentration <0.1 U/ml and the baseline APTT were usually above the linear regression line (Fig. 1). Slope values ranged from 1.14 to 2.31 ml/U on day 1 and from 1.36 to 2.49 ml/U on day 7; 7 of the 13 animals had significantly different slope values on these 2 days and there was no apparent correlation between day 1 and day 7 slope values for individual rats (Table I). On day 1, baseline APTT showed a positive correlation with hematocrit; on days 1 and 7, the slope correlated negatively with hematocrit (Table II).

[1] Blue Spruce Farms, Altamont, N.Y.
[2] Formula R-1000; Charles River, Syracuse, N.Y.
[3] Automated APTT; General Diagnostics, Morris Plains, N.J.
[4] Fibrometer; Baltimore Biological Laboratories, Cockeysville, Md.
[5] Lot No. 955FW; The Upjohn Co., Kalamazoo, Mich.

[6] The constant calcium–citrate concentration ratio was chosen to be similar to the ratio obtained when 1 part of 0.1 *M* acid citrate solution is used to anticoagulate 6 parts of plasma and 0.025 *M* calcium chloride solution is used to recalcify the plasma.

Table I—Baseline APTT, Slope, Hematocrit, and Body Weight of Rats Determined Twice, 7 Days Apart When the Citrate Solution–Blood Volume Ratio was Constant

Rat	Baseline APTT, sec		Slope, ml/U		Hematocrit, %		Body Weight, g	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
1	26.8	17.7	1.14	1.80[a]	49	—[b]	464	—[b]
2	15.8	25.3	1.51	1.95[a]	47	—[b]	324	—[b]
3	18.3	19.0	1.67	1.86[a]	46	41	424	395
4	11.1	17.8	1.95	1.85	41	40	547	539
5	13.6	15.3	1.86	1.82	40	41	350	361
6	27.1	16.3	1.64	2.48[a]	50	36	312	381
7	20.0	22.5	1.61	1.68	51	44	548	552
8	16.5	18.3	2.26	2.49[a]	40	37	423	467
9	23.8	23.8	1.59	1.36	50	40	444	456
10	20.8	23.8	1.81	1.67	47	41	410	422
11	22.3	19.3	1.44	1.55	49	43	392	414
12	20.6	18.1	2.31	2.02[a]	44	40	388	402
13	16.2	16.8	1.97	2.32[a]	43	40	408	432
Mean	19.5	19.5	1.75	1.91	46	40	418	438
SD	4.8	3.2	0.32	0.34	4	2	72	61
Significance of difference between means	N.S.[c]		N.S.		$p < 0.01$		N.S.	
Correlation coefficient	0.04		0.44		0.29		0.94	
Significance	N.S.		N.S.		N.S.		$p < 0.001$	

[a] Significantly different ($p < 0.05$) from slope value on day 1. [b] Missing data. [c] N.S. = not significant.

Table II—Correlations of Data in Table I

Data Pair	Correlation Coefficient	Significance, p
Baseline APTT *versus* slope, day 1	−0.50	N.S.[a]
Baseline APTT *versus* slope, day 7	−0.66	<0.05
Baseline APTT *versus* hematocrit, day 1	0.79	<0.01
Baseline APTT *versus* hematocrit, day 7	0.42	N.S.
Slope *versus* hematocrit, day 1	−0.73	<0.01
Slope *versus* hematocrit, day 7	−0.75	<0.01

[a] N.S. = not significant.

The baseline APTT and slope values were found to be extensively affected by the concentration of citrate added as an anticoagulant to the blood to permit separation of plasma. The baseline APTT increased with increasing citrate concentration while the slope decreased (Table III). The opposite relationships were observed with respect to the concentration of calcium used to initiate the clotting process: baseline APTT decreased and slope increased with increasing calcium concentration (Table IV). When citrate and calcium concentrations were increased simultaneously, at a constant ratio, the citrate effect predominated with respect to the baseline APTT (which increased with increasing concentration), while the calcium effect predominated with respect to slope (which also increased with increasing concentration) (Table V).

Incubation time of citrated plasma with APTT reagent (before addition of calcium to initiate clotting), intended to activate the intrinsic coagulation pathway, had a pronounced effect on APTT. As reflected by the decreasing APTT, the activation process predominated during the first 3 min (Fig. 2). At longer incubation times, APTT gradually increased, suggesting degradation of one or more components of the coagulation system. Comparing the results obtained after 3 and 15 min of incubation, the change in slope values was relatively more pronounced than the change in baseline APTT (Table VI). However, the 3- and 15-min values for the same animals correlated strongly, and the ratio of the 15- to 3-min values varied little between animals (Table VI). The intercept of the ln APTT *versus* heparin concentration regression lines at zero heparin concentration determined in this experiment is lower, on the average, than baseline APTT (Fig. 1).

Based on the preceding results, the reproducibility of individual baseline APTT and slope values was reexamined in another experiment (Table VII). Compared with the first experiment (Table I), the citrate solution–plasma volume ratio rather than the citrate solution–blood volume ratio was held constant, and the time interval between repeated measurements was lengthened from 7 to 30 days. The mean baseline APTT values and standard deviations obtained in the two studies are similar. The slope values in the second experiment are slightly higher than those in the first experiment, but the coefficients of variation are similar.

Table III—Effect of Added Citrate on the Baseline APTT of Pooled Rat Plasma and on the Anticoagulant Response to Added Heparin[a]

Citrate Concentration in Plasma, m*M*	Baseline APTT[b], sec	Slope[c], ml/U
8.75	11.6	2.31
13.5	17.8	2.42
17.5	24.6	1.87
20.9	27.1	1.59
24.3	31.4	1.55
32.7	38.6	1.33

[a] Calcium concentration in recalcifying solution was constant at 25 m*M*. [b] Significantly correlated with citrate concentration ($r = 0.991$, $p < 0.001$). [c] Significantly correlated with citrate concentration ($r = -0.923$, $p < 0.01$).

Table IV—Effect of Added Calcium Concentration on the Baseline APTT of Pooled Rat Plasma and on the Anticoagulant Response to Added Heparin[a]

Calcium Chloride Concentration[b], m*M*	Baseline APTT[c], sec	Slope[d], ml/U
12.5	37.6	0.85
19.0	24.8	1.09
25.0	28.6	1.38
29.8	22.6	2.57
34.6	21.8	2.14
46.6	21.8	2.46

[a] Citrate concentration in plasma was constant at 18.75 m*M*. [b] Refers to concentration in the recalcifying solution added to plasma. [c] Not significantly correlated with calcium concentration ($r = -0.78$). [d] Significantly correlated with calcium concentration ($r = 0.864$, $p < 0.05$).

Based on the preceding results, the reproducibility of individual baseline APTT and slope values was reexamined in another experiment (Table VII). Compared with the first experiment (Table I), the citrate solution–plasma volume ratio rather than the citrate solution–blood volume ratio was held constant, and the time interval between repeated measurements was lengthened from 7 to 30 days. The mean baseline APTT values and standard deviations obtained in the two studies are similar. The slope values in the second experiment are slightly higher than those in the first experiment, but the coefficients of variation are similar.

Table V—Effect of Added Citrate and Calcium Concentrations[a] on the Baseline APTT of Pooled Rat Plasma and on the Anticoagulant Response to Added Heparin

Concentration, m*M*		Baseline APTT[b], sec	Slope[c], ml/U
Citrate in Plasma	Calcium Chloride in Recalcifying Solution		
8.74	12.5	9.7	1.47
13.5	19.0	15.8	1.83
17.5	25.0	19.0	1.92
20.9	29.8	25.6	1.92
24.3	34.6	27.0	2.59
32.7	46.6	39.0	2.75

[a] At a constant concentration ratio. [b] Significantly correlated with citrate and calcium concentrations ($r = 0.994$, $p < 0.001$). [c] Significantly correlated with citrate and calcium concentrations ($r = 0.943$, $p < 0.01$).

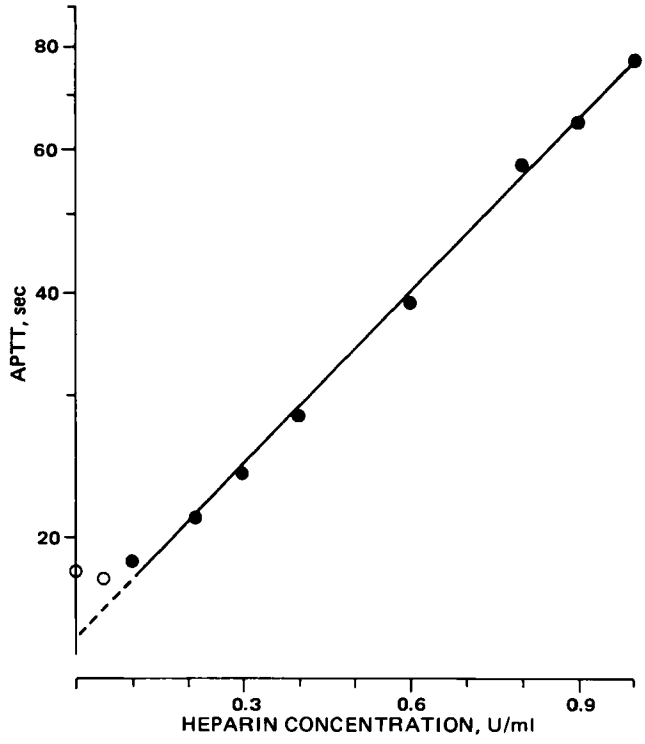

Figure 1—*Relationship between APTT and concentration of added heparin in plasma of a rat. The ordinate scale is logarithmic. The open circles at zero and very low heparin concentrations are usually above the regression line, which was calculated without these two data points. The incubation time was 15 min.*

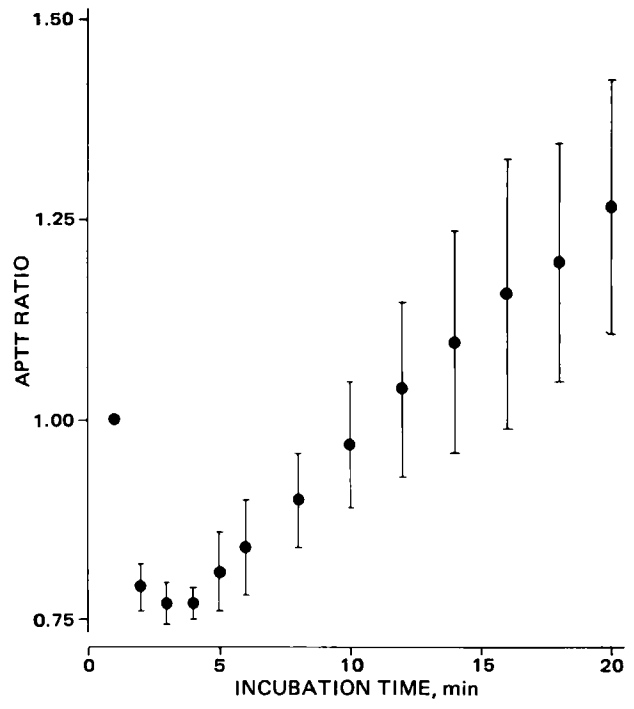

Figure 2—*Effect of incubation time on the baseline APTT of plasma from five rats. Plotted are the APTT ratios (APTT at a given time/APTT at 1 min), as mean ±SD, against incubation time. The APTT at 1 min ranged from 15.4 to 23.9 sec.*

The correlation coefficients for the baseline APTT and slope values, respectively, of the first and last day of each study were higher in the second study. Only the second study showed a statistically significant correlation between slope values obtained on different days from the same animals. During the 30-day interval of the second study, hematocrit values returned to normal, and body weight of the animals increased significantly (Table VII).

DISCUSSION

The magnitude of interindividual variation of baseline APTT and slope values observed in this investigation is similar to that found previously in another investigation on rats (11)[7] and is similar also to the magnitude of interindividual variation of these characteristics in humans (4, 5). All of the cited studies were carried out, at various times, in this laboratory with reagents from the same sources and with the same coagulation timer and are therefore readily comparable. However, unlike the situation in humans (4), the reproducibility of baseline APTT and slope values determined twice, on different days, in the rats was poor (Table I). An examination of the results of the first crossover experiment in rats (Tables I and II) suggested that one reason for the relatively poor reproducibility of individual results may be related to the inter- and intraindividual variation of hematocrit values.

Baseline APTT and slope values were significantly correlated with hematocrit (Table II). Since the permeability of rat erythrocytes to citrate is almost negligible (13) and the standard method of blood collection for plasma APTT determinations involves the addition of acid citrate anticoagulant solution to *blood* in a constant volume ratio, increasing hematocrit values are associated with increasing citrate concentration in *plasma*. As citrate concentrations are increased (all else being constant by use of pooled blood), baseline APTT increases and slope values decrease (Table III). The positive correlation between hematocrit and baseline APTT and the negative correlation between slope and hematocrit, obtained by statistical analysis of individual results from 13 rats (Table II), are consistent with these observations. Thus, a modification of the experimental methodology whereby the citrate solution–blood volume ratio is individualized on the basis of the animal's hematocrit such as to assure a constant citrate solution–plasma volume ratio was indicated. Moreover, a 7-day interval between blood withdrawals was apparently not sufficient for the animals to regenerate the considerable volume of blood that was taken on the first day of the experiment, as reflected by the significantly lower hematocrit values on the seventh day (Table I). It is possible also that the levels of certain clotting factors, which are likely to have been lowered by the first blood withdrawal, may not have returned to normal within 7 days. It was therefore deemed advisable to increase the time interval between blood withdrawals considerably.

In the second experiment to determine inter- and intraindividual differences in baseline APTT and slope value, the citrate solution–plasma volume ratio was held constant, and the interval between the two blood withdrawals was 30 days. The results of this experiment (Table VII) yielded higher correlation coefficients for the individual pairs of baseline APTT and slope values, respectively, than in the first experiment. The hematocrit values had returned to normal within the 30-day period. However, while intraindividual variation was decreased relative to the first experiment, interindividual variation was not appreciably different. The correlation between slope values and hematocrit was no longer statistically significant (Table VIII). On the other hand, there remains a significant correlation between hematocrit and baseline APTT, but this correlation is now negative rather than positive. The reason for this is

Table VI—Effect of Incubation Time on the Baseline APTT of Plasma from Rats and on the Anticoagulant Response to Added Heparin [a]

	Incubation Time, min		Ratio	Correlation Coefficient	
	3	15	15:3 min		
Baseline APTT, sec	18.6 ± 2.8	24.5 ± 3.0	1.32 ± 0.08	0.933	$p < 0.001$
Ordinate Intercept, sec	17.3 ± 2.4	20.5 ± 2.5[b]	1.19 ± 0.09	0.855	$p < 0.001$
Slope, ml/U	0.98 ± 0.13	1.74 ± 0.26	1.78 ± 0.13	0.872	$p < 0.001$

[a] Data and ratio values are expressed as mean ± *SD*, $n = 15$. [b] Significantly different from baseline APTT, $p < 0.001$.

[7] The slope values reported by Bjornsson and Levy (11) are based on heparin concentrations in plasma before it was diluted 10-fold for APTT determination. The slope values in the present study are based on heparin concentration in plasma that was used undiluted for APTT determination. For comparison with the results of this investigation, the slope values reported by Bjornsson and Levy (11) must be multiplied by 10.

Table VII—Baseline APTT, Slope, Hematocrit, and Body Weight of Rats Determined Twice, 30 Days Apart When the Citrate Solution–Plasma Volume Ratio was Constant at 1:6

Rat	Baseline APTT, sec		Slope, ml/U		Hematocrit, %		Body Weight, g	
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
14	10.6	12.9	3.12	3.12	46	47	394	477
15	24.3	20.8	1.83	2.32[a]	46	47	412	485
16	17.1	19.6	2.48	2.78	45	47	422	492
17	16.6	16.6	1.94	2.56[a]	49	50	397	458
18	22.3	15.6	2.49	2.87[a]	47	51	456	508
19	14.0	20.3	3.23	2.73	45	41	478	538
20	16.6	19.2	2.30	2.34	44	45	397	455
21	20.7	18.2	2.13	2.45	45	47	417	468
22	16.1	15.3	2.21	2.24	48	47	421	462
23	20.0	22.8	1.94	1.66	42	42	485	524
24	17.3	17.0	2.61	2.54	48	49	356	422
25	22.3	20.3	2.21	1.90	46	44	342	422
Mean	18.2	18.2	2.37	2.46	46	46	415	476
SD	3.9	2.8	0.44	0.41	2	3	43	36
Significance of difference between means	N.S.[b]		N.S.		N.S.		$p < 0.001$	
Correlation coefficient	0.50		0.68		0.73		0.95	
Significance	N.S.		$p < 0.05$		$p < 0.01$		$p < 0.001$	

[a] Significantly different ($p < 0.05$) from slope value on day 1. [b] N.S. = not significant.

unknown; it could reflect differences in dilution (by the addition of acid citrate solution to blood in variable proportions) of one or more endogenous substances that distribute readily between plasma and erythrocytes and whose concentration is relevant to the clotting of plasma.

One potential alternative to individualization of the citrate solution–blood volume ratio (to achieve a constant citrate solution–plasma volume ratio) is to adjust the concentration of calcium chloride in the recalcifying solution such as to achieve a constant molar ratio of citrate to calcium. Increasing the concentration of calcium while citrate concentration was held constant did in fact cause an increase in slope and may also have decreased baseline APTT, although the latter effect was not statistically significant (Table IV). However, maintaining a constant molar ratio of citrate to calcium did not abolish the citrate concentration dependency of baseline APTT and indicated a predominance of the calcium effect over the citrate effect on the slope (Table V).

It has been the practice in this laboratory to incubate the plasma–APTT reagent mixture 15 min, rather than the customary 3 or 5 min, for activation of the intrinsic coagulation pathway. This change was made to increase the sensitivity of the APTT to changes of heparin concentration. A detailed study of the relationship between APTT and incubation time revealed considerable complexity (Fig. 2), with the activation process predominating initially and degradation of component(s) of the clotting process predominating at later times (after ~3 min). Increased incubation time had a more pronounced effect on slope than on baseline APTT (Table VI) and contributed to a more pronounced difference between the actual and apparent baseline APTT, determined by back-extrapolation of the ln APTT–heparin concentration regression line to a heparin concentration of zero (Fig. 1, Table VI). Since there is a relatively constant relationship between baseline APTT and slope values, respectively, obtained after 3 and 15 min of incubation, the time variable should have little or no effect on the analysis and interpretation of the results of heparin studies such as the one described here. The 15-min procedure is more sensitive to heparin concentration (due to the increased slope value) and is more convenient.

The results of this investigation, assessed in conjunction with the results of corresponding clinical studies also performed in this laboratory (4, 5) provide interesting information concerning species differences between rats and humans (Table IX). These differences should be appreciated in the context of the two major similarities between these species: the pharmacokinetics of heparin are dose dependent and there is an essentially linear relationship between ln APTT and heparin concentration in both humans and rats (11). On the other hand, citrate concentration in plasma has a pronounced effect on baseline APTT and slope in rats, but not in humans. Both human and rat erythrocytes are essentially impermeable to citrate (12). In view of the pronounced citrate concentration effect in rats, the human experiments were repeated recently and the previously observed lack of a citrate concentration effect was reconfirmed (unpublished data).

Another striking difference between rats and humans is the effect of plasma incubation time on APTT. The APTT of human plasma decreased and eventually reached a constant value during incubation for up to 21 min (5), indicative of activation of the clotting system without noticeable degradation of clotting factors. On the other hand, the APTT of rat plasma first decreases (indicative of activation) and then increases,

Table VIII—Correlations of Data in Table VII

Data Pair	Correlation Coefficient	Significance, p
Baseline APTT *versus* slope, day 1	−0.70	<0.05
Baseline APTT *versus* slope, day 30	−0.67	<0.05
Baseline APTT *versus* hematocrit, day 1	−0.10	N.S.[a]
Baseline APTT *versus* hematocrit, day 30	−0.66	<0.05
Slope *versus* hematocrit, day 1	0.01	N.S.
Slope *versus* hematocrit, day 30	0.48	N.S.

[a] N.S. = not significant.

Table IX—Comparison of Healthy Humans and Rats with Respect to the Coagulation of Plasma and the Anticoagulant Effect of Heparin [a]

Characteristics	Humans	Rats[b]
Relationship between ln APTT and added heparin concentration	Linear	Linear
Effect of incubation time on APTT	Decreases asymptotically	First decreases, then increases
Effect of citrate concentration	No effect on baseline APTT or slope	Significant positive correlation with baseline APTT and significant negative correlation with slope
Slope–hematocrit correlation	Significant, negative	(*a*) Often significant, negative (*b*) no correlation
Slope–baseline APTT correlation	Significant, positive	(*b*) Significant, negative
Baseline APTT–hematocrit correlation	Not significant[c]	Variable
Citrate uptake by erythrocytes	Negligible	Negligible
Reproducibility of baseline APTT values on repeated testing at different times	Excellent	Very poor
Reproducibility of slope values on repeated testing at different times	Excellent	Poor

[a] Based on our previous studies (4, 5, 13) and on the results of the present study. [b] Different results were observed in some cases depending on whether plasma was obtained from animals with (*a*) citrate solution–blood volume ratio held constant or (*b*) citrate solution–plasma volume ratio held constant. [c] Significant negative correlation in patients (5, 7).

apparently due to the opposing effects of activation and subsequent degradation of coagulation factors. In a search for factors that may be useful for predicting an individual patient's response to heparin, baseline APTT has emerged as the variable which consistently correlates positively with slope value (4, 5, 8). Unlike humans, rats exhibit a negative correlation between baseline APTT and slope value. The reason for this species difference is not known.

In summary, coagulation studies in rats require considerably more attention to the control of the variables examined in this investigation than do similar studies in humans. The volume of blood required to determine baseline APTT and slope represents a significant fraction of a rat's total blood volume, but is negligible compared with the human blood volume. This is an additional complicating factor in studies on rats. The observed species variation may, however, be useful for exploring various aspects of the blood coagulation process. Parallel and coordinated studies on humans and rats, and perhaps also on other species, may facilitate the elucidation of important pharmacokinetic and pharmacodynamic aspects of the anticoagulant action of heparin.

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Distribution and Elimination of Poly(methyl methacrylate) Nanoparticles After Subcutaneous Administration to Rats

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Abstract □ Poly(methyl [1-^{14}C]methacrylate) nanoparticles were injected subcutaneously into rats. Almost all of the radioactivity stayed at the injection site. After an initial urinary and fecal excretion of ~1% of the administered dose per day, the rate of elimination dropped to a low level (~0.005%/day *via* the feces and ~0.0005%/day *via* the urine) within 70 days. After 200 days, the fecal elimination increased exponentially until a >100-fold increase was observed after 287 days in one rat. After this time, a tendency for an increase in fecal elimination was also observed in the other animals, and the radioactivity in all organs and tissue increased by ~100 times in all animals in comparison with the organ radioactivity determinations at earlier times.

Keyphrases □ Poly(methyl methacrylate)—^{14}C-labeled nanoparticles, distribution and elimination in rats, subcutaneous administration □ Distribution—^{14}C-labeled poly(methyl methacrylate) nanoparticles, subcutaneous administration in rats □ Elimination—^{14}C-labeled poly(methyl methacrylate) nanoparticles, subcutaneous administration in rats

Poly(methyl [1-^{14}C]methacrylate) nanoparticles were shown to be promising adjuvants for vaccines (1–4). In contrast to the rapidly biodegradable cyanoacrylates, they achieve a good adjuvant effect. The reproducibility of the adjuvant effect was much better than that of the presently widely used aluminum hydroxide (5). Moreover, in preliminary experiments (2), poly(methyl [1-^{14}C]methacrylate) nanoparticles seem to cause much milder tissue reactions than aluminum hydroxide.

However, the distribution and elimination of these nanoparticles after subcutaneous administration so far has not been studied. In a previous study (6) concerning the fate of poly(methyl [1-^{14}C]methacrylate) nanoparticles after intravenous administration, a strong affinity of the nanoparticles to the reticuloendothelial system, especially to the liver, was observed.

The elimination of radioactivity after implantation of poly(methyl [1-^{14}C]methacrylate) films was investigated in two studies (7, 8). In one study (7), an elevated elimination rate of radioactivity was observed in the urine between 2 and 8 weeks, decreasing to minimal values after this time. This elimination was probably caused by residual monomers or low-molecular weight components present in the polymer films. After 54 weeks, however, considerable radioactivity suddenly started to be eliminated (8), indicating a degradation of the polymer. This study investigates the elimination pattern and distribution

[1] Unpublished observation.

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