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Observations on the Effects of Cytochalasin B and Cytochalasin D on ADP- and Chymotrypsin-Treated Platelets (41776)

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Abstract. Cytochalasin B has been reported to inhibit fibrinogen binding and aggregation of rabbit platelets in response to ADP. The present study was designed to ascertain whether cytochalasins B and D inhibit aggregation by interfering with the exposure of fibrinogen receptors or more directly by inhibiting binding to available receptors. Aspirin-treated, washed, human platelets stimulated with ADP or chymotrypsin were used for these studies. Neither cytochalasin B nor D significantly inhibited the binding of fibrinogen to chymotrypsin-treated platelets when these agents were added to platelet suspensions before (16 ± 8% (mean ± SD) inhibition, $N = 8$), or after (15 ± 10% inhibition, $N = 13$) chymotrypsin treatment, i.e., before or after fibrinogen receptor exposure. This apparent lack of cytoskeletal involvement was consistent with the observation that chymotrypsin-treated platelets were unable to retract reptilase-induced fibrin clots, an activity that was restored by adding ADP. In contrast, incubating platelets with either cytochalasin B or D for 30 min before or after stimulation with ADP decreased fibrinogen binding by 42 ± 16% ($N = 13$) and 27 ± 11% ($N = 8$), respectively, compared to DMSO-treated controls. Platelets stimulated with ADP and incubated with DMSO for 30 min, however, became refractory and aggregated poorly in response to a second dose of ADP. In comparison, platelets stimulated with ADP, but incubated with cytochalasin B or D, aggregated more extensively when stimulated by a second dose of ADP despite diminished fibrinogen binding. The data suggest (1) microfilament polymerization is important not only for the exposure of fibrinogen receptors by ADP, but also for preserving the ability of exposed receptors to bind fibrinogen, (2) exposure of fibrinogen receptors by chymotrypsin is not accompanied by significant cytoskeletal activation, and (3) cytochalasins may impart partial protective effects against the development of ADP-induced refractoriness.

The morphology and function of human blood platelets depend in part on the nature and location of their cytoskeletons, particularly the microtubules and microfilaments (1–3). As visualized by electron microscopy, platelet activation is accompanied by the depolymerization of microtubules and rapid polymerization of actin filaments in the pseudopods of platelets that have undergone a change in shape from discs to spheres with spiny protrusions. Under the appropriate conditions, these actin filaments can combine with myosin to provide the contractile mechanism necessary to retract fibrin clots (4, 5). In addition, there is evidence to suggest that the polymerization of actin filaments may be crucial for platelet aggregation and the release reaction (6, 7).

The importance of actin filaments and actomyosin cables in platelet function has been investigated largely using cytochalasins B, D, and E (CB, CD, and CE, respectively). These mold metabolites inhibit the extent of microfilament polymerization (8–10). Since it has been suggested that cell cytoskeletons may have important functions not only in motility, phagocytosis, and secretion (11, 12), but also in stabilizing plasma membrane receptors (13), the present study was designed to determine whether cytochalasins inhibit the aggregation/agglutination of human blood platelets stimulated with ADP or treated with chymotrypsin by interfering with exposure of fibrinogen receptors, or more directly by inhibiting the binding of fibrinogen to available receptors. As the binding of fibrinogen to specific platelet receptors is thought to be essential for platelet aggregation (14–16), results from these studies were correlated with cytochalasin-induced effects on platelet aggregation. Both CB and CD were used because of the well-known inhibitory effects of CB also on hexose transport (9).

Materials and Methods. *Platelet preparation.* Blood was obtained from apparently healthy volunteers in accordance with the

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principles of the Declaration of Helsinki. It was collected into 0.1 vol of 3.2% sodium citrate and 0.05 vol (1 m*M*) acetylsalicylic acid. Platelet-rich plasma was prepared by centrifugation (280*g*, 12 min). The platelets were separated from plasma by centrifugation (1000*g*, 20 min) at pH 6.5 and were washed once in Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered modified Tyrode's solution at pH 7.5 (HBMT) (16).

Cytochalasin preparation. CB and CD were obtained from Sigma Chemical Co., St. Louis, Missouri. The following lots were used: CD—Lot 101F-40001, 111F-4005, and 128C-0413; CB—Lot 61F-4017, and 79C-0040. Stock solutions of CB and CD (10 mg/ml) were prepared fresh daily in DMSO (dimethylsulfoxide, Aldrich Chemical Co., Milwaukee, Wisc.).

Platelet incubation with cytochalasins. Washed platelets were incubated for 1, 5, and 30 min with CB or CD (10–100 μg/ml, final concentration) or DMSO either before stimulation with 10 μ*M* ADP to determine the effect of cytochalasins on the exposure of fibrinogen receptors, or 2 min after stimulation with ADP to assess the effect of cytochalasins on exposed but free fibrinogen receptors. Platelets stimulated with ADP for 2 min before incubation with CB or CD were designated refractory because they lost their ability to respond to a second dose of ADP. ADP-induced refractoriness has been extensively described (17, 18).

To determine the effects of cytochalasins on fibrinogen binding and platelet aggregation induced by mechanisms different from those involved in platelet stimulation with ADP or other traditional agonists (14, 19), a final suspension designated chymotrypsin-treated platelets was prepared. These platelets were incubated with 500 μg/ml chymotrypsin (α-chymotrypsin, 61 U/mg, Lot CDAG 35A634, Worthington Biochemical Co., Freehold N.J.) for 30 min at 22°C in the presence of 1 μ*M* PGE_1. The chymotrypsin was neutralized with a 10-fold molar excess of PMSF (phenylmethylsulfonyl-fluoride, Sigma). The platelets were centrifuged in the presence of 5 m*M* EDTA and resuspended in HBMT. These platelets were then incubated with CB, CD, or DMSO for 1, 5, and 30 min. In some experiments, platelets were first incubated with the cytochalasins or with DMSO, then with chymotrypsin.

Platelet aggregation. Platelet aggregability was examined using an aggregometer (ChronoLog Corp, Havertown, Pa.). Washed platelets were stimulated with 10 μ*M* ADP in the presence of fibrinogen (0.1–0.8 mg/ml, Kabi Diagnostics, Stockholm, Sweden). Chymotrypsin-treated platelets were agglutinated by adding fibrinogen alone.

Fibrinogen binding. The ability of washed platelets to bind fibrinogen in response to ADP (10 μ*M*) was assessed with ^{125}I-labeled fibrinogen (0.1–0.8 mg/ml, 1500 cpm/μg), purified and iodinated as described previously (16). Fibrinogen bound in the presence of 5 m*M* EDTA was taken as a measure of nonspecific binding (16). The ability of chymotrypsin-treated platelets to bind fibrinogen was determined by incubating them with ^{125}I-labeled fibrinogen alone or in the presence of EDTA.

[^{14}C]Serotonin release. In some experiments, platelets in plasma were prelabeled with [^{14}C]serotonin (5-hydroxy-(side chain-2-^{14}C)-tryptamine creatinine sulfate, Amersham Corp., Arlington Heights, Ill.), and prepared as described above. Imipramine (5 μ*M*) (Sigma Chemical Co., St. Louis, Mo.) was added to prevent the reuptake of released serotonin (20). Following stimulation, platelets were fixed with 1% formaldehyde, centrifuged (1000*g*, 5 min) and the supernatants examined for released radioactivity. The percentage of released serotonin was calculated as previously described (21).

Clot retraction. Suspensions of washed platelets or chymotrypsin-treated platelets incubated with Cytochalasin B, D, (final concentration 30 μg/ml), or DMSO were added to equal volumes of fresh plasma anticoagulated with 3.2% sodium citrate. Clotting was induced by the addition of 30 μl reptilase per ml (reptilase R from *Bothrops atrox,* Lot 28-029HJ, Abbott Laboratories, Chicago, Ill.; one vial was reconstituted with 350 μl of water). Parallel studies were performed in which ADP (10 μ*M*) and reptilase were added to platelet suspensions in rapid succession. The extent of clot retraction was assessed visually after 30, 60, 90, 120, and 180 min at 37°C.

Results. Results were obtained from studies performed at 22°C, except where otherwise

indicated. Since both CB and CD had identical effects on platelets, their results were averaged and expressed jointly.

Effects of cytochalasins on fibrinogen binding and aggregation of washed human platelets. Incubating platelets for 30 min with either CB or CD (30 μg/ml) resulted in a significant decrease in their ADP-induced aggregability as shown in Fig. 1A. No further inhibition was achieved with increasing cytochalasin concentrations up to 100 μg/ml. CB- and CD-treated platelets were also unable to retract fibrin clots produced by adding 1 U/ml human thrombin (courtesy Dr. John Fenton, N.Y. State Department of Health, Albany, N.Y.) to a mixture of washed platelets in plasma (data not shown).

The inhibition of ADP-induced fibrinogen binding by CB or CD was time dependent (Table I). When platelets were preincubated with the cytochalasins for only 1 min, a 17% decrease in fibrinogen binding was observed. After 5 min, binding was decreased by 26% compared to DMSO-treated controls, and after 30 min, it was inhibited 46%, and did not decrease further with longer incubation.

Table II summarizes the extent of inhibition of ADP-induced fibrinogen binding and platelet aggregation obtained in 13 separate experiments. The amount of fibrinogen bound to platelets incubated with either CB or CD for 30 min before stimulation with ADP (17,000 ± 4909 (mean ± SD) molecules per platelet) was 55% less than that bound to DMSO-treated platelets (39,312 ± 7670), correlating with a 35% decrease in platelet aggregability. Similar results were obtained when platelets were incubated with the cytochalasins for 30 min, 2 min after platelet stimulation with ADP.

TABLE I. TIME DEPENDENCE OF CYTOCHALASIN-INDUCED INHIBITION OF ADP- AND CHYMOTRYPSIN-INDUCED FIBRINOGEN BINDING

Preincubation time (Min)	ADP-induced fibrinogen binding[a] (% Inhibition)[c]	Chymotrypsin-induced fibrinogen binding[b] (% Inhibition)[c]
1	17 ± 5	10 ± 29
5	26 ± 11	15 ± 38
30	46 ± 4	13 ± 12

[a] Platelets were incubated with CB or CD (30 μg/ml) for the designated times before stimulation with 10 μM ADP and fibrinogen.

[b] Platelets were incubated with CB or CD (30 μg/ml) for the designated times after chymotrypsin treatment.

[c] Compared to DMSO-treated control platelets; $N = 4$; values are means ± SD.

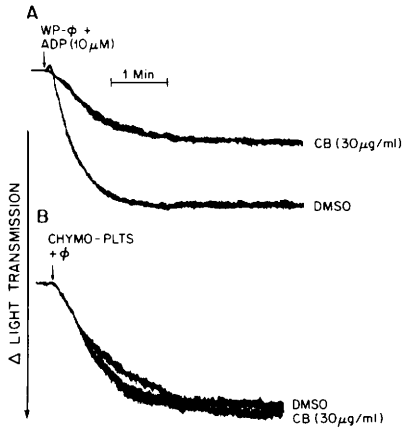

FIG. 1. Results from a typical experiment comparing the effect of CB (30 μg/ml) and DMSO on 10 μM ADP-induced aggregation (A) and chymotrypsin-induced agglutination (B) of washed platelets (WP). Platelets were aggregated/agglutinated in the presence of 0.2 mg/ml fibrinogen (Φ), with the aggregometer set so that a baseline was established at 10% light transmission using a buffer blank. CHYMO-PLTS refers to chymotrypsin-treated platelets.

Figure 2A depicts a Scatchard plot (22) of ADP-induced fibrinogen binding to cytochalasin- and DMSO-treated platelets. It illustrates a decrease in the average number of fibrinogen binding sites available on cytochalasin-treated platelets, and no change in receptor affinities. In three separate experiments, the affinity constants for high-affinity binding were 0.10 ± 0.08 and 0.11 ± 0.07 μM for cytochalasin- and DMSO-treated platelets, respectively, and 1.32 ± 0.8 and 1.07 ± 0.8 μM for low-affinity binding.

Effect of cytochalasins on platelets refractory to ADP. The effects of incubating platelets for 30 min with CB, CD, or DMSO, 2 min after stimulation with ADP are illustrated in Fig. 3 and Table III. Compared to platelets incubated with DMSO before stimulation with ADP, platelets incubated after ADP treatment became refractory (17, 18), and responded poorly to a second dose of ADP. They bound

TABLE II. EFFECT OF CYTOCHALASINS ON FIBRINOGEN BINDING AND AGGREGATION/AGGLUTINATION OF ADP- AND CHYMOTRYPSIN-TREATED PLATELETS

	ADP-treated platelets		Chymotrypsin-treated platelets	
	Fibrinogen binding	Aggregation	Fibrinogen binding	Agglutination
Incubation	(% Inhibition)[a]	(% Inhibition)[a]	(% Inhibition)[a]	(% Inhibition)[a]
Cytochalasin before platelet stimulation	55 ± 16[b]	35 ± 15[b]	16 ± 8[c]	12 ± 4[c]
Cytochalasin after platelet stimulation	27 ± 11[c]	—	15 ± 10[b]	14 ± 10[b]

[a] Washed platelets were incubated with 30 μg/ml CB/CD for 30 min either before stimulation with ADP or chymotrypsin, or 2 min after ADP or chymotrypsin treatment. Their responses were compared to similarly incubated DMSO-treated platelets. Values are means ± SD.
[b] $N = 13$.
[c] $N = 8$.

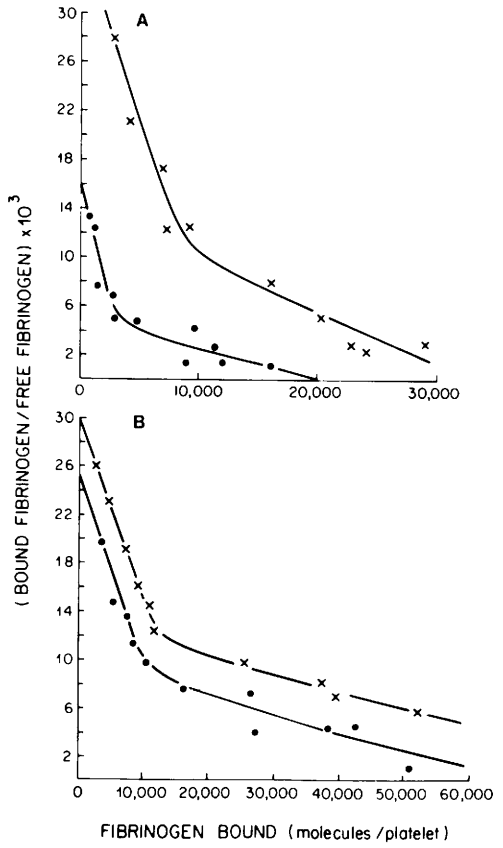

FIG. 2. Scatchard analysis of fibrinogen binding to ADP- (A) and chymotrypsin-treated platelets (B). (×) Platelets preincubated with CB; (●) platelets preincubated with DMSO.

42% less fibrinogen than nonrefractory control platelets (20,000 molecules of fibrinogen bound per platelet compared to 34,000 molecules) and aggregated poorly. In comparison, platelets incubated with CB or CD following stimulation with ADP, bound even less fibrinogen (27% less, or 15,250 molecules of fibrinogen bound per platelet compared to 20,890 molecules), but aggregated more extensively. These effects could be demonstrated at cytochalasin concentrations up to 100 μg/

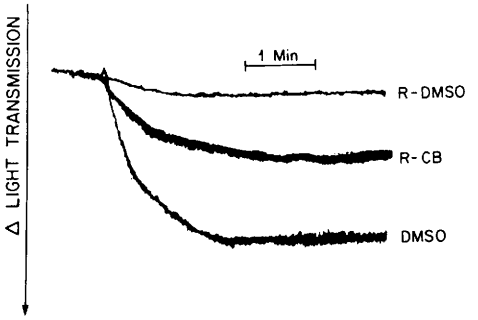

FIG. 3. Results from a typical experiment illustrating the effect of CB on the aggregability of refractory platelets (R-DMSO): platelets incubated with DMSO for 30 min after stimulation with ADP; (R-CB): platelets incubated for 30 min with CB (30 μg/ml) after stimulation with ADP; (DMSO): platelets incubated with DMSO for 30 min before stimulation with ADP. Aggregation was induced by adding 10 μ*M* ADP and 0.2 mg/ml fibrinogen to the above-mentioned platelet suspensions.

TABLE III. EFFECT OF CYTOCHALASINS ON WASHED PLATELETS REFRACTORY TO STIMULATION WITH ADP

Platelets	Fibrinogen binding (% Inhibition)*	Aggregation (% Inhibition)*	[^{14}C]Serotonin release (% Release)†
Refractory platelets			
CB/CD-treated	56 ± 9[a]	44 ± 10[c]	3.6 ± 2.6
DMSO-treated	42 ± 13[a,b]	72 ± 9[c]	1.3 ± 2.6
Control platelets			
DMSO-treated	100[b]	0	2.7 ± 3.3

* Compared to nonrefractory DMSO-treated control platelets; $N = 9$; values are means ± SD.
† Percentage of [^{14}C]serotonin taken up by platelets; $N = 9$; values are means ± SD.
[a-c] Letter pairs denote statistically significant differences using the paired student's t test.

ml, and in platelet-rich plasma, and were not accompanied by significant [^{14}C]serotonin release. At 37°C, similar effects were observed in only one of five experiments.

Effect of cytochalasins on chymotrypsin-treated platelets. Neither CB or CD significantly inhibited the agglutination or fibrinogen binding of chymotrypsin-treated platelets. Tables I and II illustrate that incubating these platelets for only one min with either of the cytochalasins reduced fibrinogen binding only 10 ± 29% (mean ± SD; $N = 4$) compared to DMSO treated controls ($p > 0.4$). No further inhibition of fibrinogen binding was achieved by incubating chymotrypsin-treated platelets for 30 min with CB or CD (16 ± 8% inhibition; 38,753 ± 4583 molecules per platelet, $N = 13$, compared to DMSO-treated controls, 45,800 ± 8459 molecules per platelet). Platelet agglutination in response to adding fibrinogen was also not significantly reduced (12 ± 4%, $p > 0.1$). Similar results were obtained when platelets were first incubated with the cytochalasins, then with chymotrypsin.

Scatchard analysis (22) of fibrinogen binding to chymotrypsin-treated platelets suggested that the slight cytochalasin-induced decrease in fibrinogen binding was more the result of a decrease in the average number of available fibrinogen receptors rather than changes in receptor affinity (Fig. 2B). High-affinity constants for cytochalasin and DMSO-treated platelets were 0.10 ± 0.05 μM and 0.14 ± 0.07 μM (mean ± SD, $N = 3$), and low-affinity binding constants were 1.95 ± 0.9 μM and 2.36 ± 0.87 μM, respectively.

Clot retraction. Figure 4 depicts the ability of ADP- and chymotrypsin-treated platelets to retract reptilase-induced fibrin clots. In three separate experiments, ADP-treated platelets retracted fibrin clots by approximately 80% after 3 hr, while chymotrypsin-treated platelets did so only minimally (5–10% retraction). If these platelets, however, were stimulated with 10 μM ADP immediately before adding reptilase to induce clotting, their ability to retract clots became similar to that of ADP-treated platelets. Incubating platelets with CB or CD inhibited clot retraction.

Discussion. The inhibition of fibrinogen binding to ADP-treated rabbit platelets by cytochalasin B was recently described (23). The present study expands these observations to

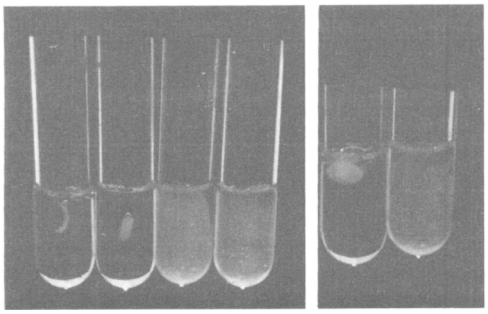

FIG. 4. Retraction of (A) thrombin- or (B–F) reptilase-induced fibrin clots after 3 hr at 37°C by washed or chymotrypsin-treated platelets. Washed platelets retracting a thrombin-induced clot (A). Retraction of reptilase-induced clots by (B) ADP-treated platelets, (C) ADP-treated platelets preincubated with 30 μg/ml CB, (D) unstimulated platelets, (E) chymotrypsin-treated platelets stimulated with 10 μM ADP, and (F) chymotrypsin-treated platelets.

suggest that in human platelets, the cytochalasin-induced inhibition of ADP-induced aggregation can be attributed to (i) the inhibition of fibrinogen receptor exposure, and (ii) the inhibition of fibrinogen binding to available receptors, or sequestration of exposed receptors. No change in the affinity of fibrinogen receptors for ligand was noted.

In contrast, neither CB or CD significantly inhibited the ability of chymotrypsin-treated platelets to bind fibrinogen and to agglutinate in response to it. Although the presence of PGE_1 during chymotrypsin treatment was used to prevent any effect of released ADP (20) on the platelet cytoskeleton, the possibility that the somewhat variable effects of the cytochalasins on chymotrypsin-treated platelets may have resulted from partial ADP-induced microfilament polymerization cannot be eliminated (19). Studies performed in the presence of apyrase, however, yielded similar results.

The apparent lack of cytoskeletal involvement in fibrinogen binding to chymotrypsin-treated platelets is consistent with the observation that these platelets were unable to retract reptilase-induced fibrin clots unless ADP was also present, presumably to activate actomyosin polymerization. Thus the ability of platelets to retract fibrin clots depends not only on their ability to bind fibrinogen but also on the interaction of fibrinogen receptors with activated platelet cytoskeletal components.

Platelet glycoprotein IIIa, which appears to be part of the platelet fibrinogen receptor (24), for example, has been shown to crossreact with an antibody to procine skeletal muscle α actinin (25), and has been found in association with cytoskeletal components of actin and myosin in thrombin stimulated platelets (26). It is thus thought to be a transmembrane protein with an external glycoprotein moiety involved in fibrinogen binding, and an internal protein moiety, similar to α actinin, capable of interacting with actin filaments in stimulated platelets.

Fox and Phillips (8) recently found the inhibition of actin polymerization by CB in intact thrombin-stimulated platelets to be extremely rapid (1 min). In this study, however, ADP-induced fibrinogen binding and platelet aggregation were inhibited maximally only after 30 min preincubation with cytochalasins, and fibrinogen binding and agglutination of chymotrypsin-treated platelets were not significantly altered, although slight inhibition was noted after 1 min preincubation. These findings further support the thesis (19) that the mechanism(s) whereby chymotrypsin exposes fibrinogen receptors is markedly different from that involved in ADP-induced exposure of the same sites.

In addition, the cytochalasins were found to partially protect platelets against development of ADP-induced refractoriness. It is interesting to note that while the aggregability of CB- or CD-treated refractory platelets was more extensive than that of their DMSO-treated counterparts, their fibrinogen binding was decreased. This not only provides additional evidence to suggest that fibrinogen binding while necessary, may not be sufficient for platelet aggregation (27), but also suggests that the interaction of platelet cytoskeletal components with fibrinogen receptors may contribute to the development of refractoriness. Chymotrypsin-treated platelets, for example with exposed available fibrinogen receptors can be agglutinated by adding fibrinogen several hours after the initial exposure of fibrinogen receptors, while ADP-treated platelets lose their ability to aggregate if not immediately stirred with fibrinogen.

Enhanced platelet aggregation following cytochalasin treatment of platelets has been reported by Haslam *et al.* (7). In their studies, CB was noted to enhance collagen induced aggregation only at low concentrations of CB and was accompanied by significant [^{14}C]serotonin release. The cytochalasin-induced enhancement of platelet aggregation of refractory platelets differs in that the potentiation could be demonstrated at low as well as high doses of CB or CD, and was not accompanied by significant [^{14}C]serotonin release. Similar effects could be demonstrated in platelet-rich plasma and suggest that the observations are not an artefact of the washed platelet preparation. These cytochalasin-induced effects may be temperature dependent, however, since the development of refractoriness was inhibited in only one of five separate experiments at 37°C. Although the mechanism whereby cytochalasins protect against the

development of refractoriness is obscure, the data do suggest that under certain conditions, cytochalasins can have protective effects.

In conclusion, data from the present study suggest that inhibition of platelet microfilament polymerization does not necessarily lead to inhibition of fibrinogen binding and aggregation, but can lead to enhanced aggregability of refractory platelets.

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    3. Langford MP, Weigent DA, Stanton GJ, Baron S. Virus plaque-reduction assay for interferon: Microplaque and regular macroplaque reduction assay. In: Pestka S, ed. Methods in Enzymology. New York: Academic Press, Vol Part A **78:** p000, 1981.

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calorie	cal	millimeter	mm
centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm^3	molal (concentration)	*m*
Curie	Ci	molar (concentration)	*M*

degree Celsius (centigrade)	°C	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
inch	in	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	μl	square centimeter	cm^2
micrometer	μm	square meter	m^2
milligram	mg	subcutaneous	sc
milliliter	ml	volt	V
		volume	vol

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