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RELEASE OF CREATINE PHOSPHOKINASE FROM MUSCLE—I

EFFECT OF POLYMYXIN B, COMPOUND 48/80, AND SEROTONIN*

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Abstract—Polymyxin B and compound 48/80 produce marked increases in the plasma levels of type III (skeletal muscle) creatine phosphokinase (CPK) in the Sprague–Dawley rat. Two other mast cell disrupters, dextran and ovomucoid, which also produce anaphylactoid shock, as well as the mast cell disrupters *d*-tubocurarine, diphenhydramine and tripellenamine, do not affect plasma CPK (PCPK) levels. The mast cell constituents histamine and heparin do not increase PCPK levels, although significant increases were noted following high doses of exogenous serotonin (5-HT). The effect of 5-HT on PCPK levels was inhibited by methysergide but that of polymyxin B was not. The neuromuscular blockade produced by polymyxin B was considered to have little if any role in the increase in PCPK levels, since neither succinylcholine or *d*-tubocurarine increases PCPK levels. Preventing the hypothermia secondary to polymyxin B or 5-HT did not block the increase in PCPK levels following treatment with these agents. Incubation of isolated extensor digitorum longus muscle *in vitro* in the presence of polymyxin B and compound 48/80 increases the rate of efflux of CPK from the muscle. It is postulated that polymyxin B and compound 48/80 have a toxic effect on muscle, one manifestation of which could be increased efflux of CPK from muscle.

CREATINE phosphokinase (CPK) is found mainly in skeletal and cardiac muscle, and brain.[1] Distinct isoenzymic patterns characterize these tissues.[2] Increased plasma activity of type III (skeletal muscle) CPK is one of the most sensitive indices of damage to skeletal muscle.[3] As part of a study of factors which affect the efflux of CPK and other enzymes from skeletal muscle into plasma, the effect of biogenic amines, including histamine, was of interest. O'Steen[4] has reported that administration of histamine to mice over a 21-day period produced evidence of muscle damage. Wells[5] has found that a 3–18 mg/kg, i.m., dose of histamine in the rabbit produced large increases in plasma lactic dehydrogenase (LDH) activity. Raab and Donhoffer[6,7] reported that histamine and the mast cell disrupter, compound 48/80, produced large increases in the activities of alkaline phosphatase and aspartate amino-transferase in rat urine, possible due to renal tubular damage.

Preliminary studies with administration of histamine or histidine to rats produced no changes in plasma CPK (PCPK) activity, but compound 48/80 and polymyxin B, another mast cell disrupter, produced large increases in PCPK activity. The studies reported in this paper were to determine if the increase in PCPK activity after polymyxin B was due to one or more of the following effects of the drug: (1) the release of

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histamine, heparin or serotonin (5-HT) from mast cells or other tissues; (2) "anaphylactoid shock"; (3) blockade of the neuromuscular junction; or (4) hypothermia.

MATERIALS AND METHODS

Studies in vivo

Male Sprague–Dawley rats, purchased from Sprague–Dawley, Inc., Madison, Wis., weighing 125–150 g, were used throughout these experiments, except for two experiments in which female rats of the same strain were employed. They were maintained in a temperature-controlled room at 22° and given Purina rat pellets and water *ad lib.*

Heparinized plasma was obtained at the end of an experiment by anesthetizing the rat with pentobarbital, 200 mg/kg, i.p., and removing blood from the inferior vena cava. (The CPK level of plasma obtained from rats following a blow on the head is not significantly different from that of the anesthetized rats, indicating that pentobarbital has no effect on CPK levels.) Plasma was obtained by centrifugation and frozen for analysis within 24 hr. CPK activity was determined spectrophotometrically at 30° by the method of Rosalki.[8] Frozen specimens retain full activity for at least two weeks.[8] Student's *t*-test was used for analysis of the results. The coefficient of variation of a series of duplicate determinations of plasma samples was 3·0 per cent. Samples with activity greater than 250 mμ/ml were assayed by using smaller aliquots of plasma so that the reaction rate was always linear during the period of study. As a control for specificity of the CPK assay, the apparent CPK activity of some plasma samples was also determined with the complete assay mixture except for the absence of the substrate, creatine phosphate (CrP). Aliquots of some plasma samples were heated to 56° for 10 min or dialyzed against 10 mM tris-buffer, pH 7·4, to determine the influence of these treatments on the apparent CPK activity of plasma. The isoenzymes of CPK plasma were determined by agar gel electrophoresis using the method of Van der Veen and Willebrands.[9]

Rectal temperature was determined by means of a Yellow-Springs Instrument Co. telethermometer with a small animal probe inserted at least 4 cm into the rectum.

Polymyxin B Sulfate and para-chlorophenylalanine (PCPA) were purchased from Pfizer Laboratories, New York, N.Y. Succinylcholine was purchased from Burroughs Wellcome & Co., Tuckahoe, N.Y., who also provided compound 48/80 as a gift. Dextran, molecular weight 40,000 ± 3000, and sodium heparinate were purchased from Nutritional Biochemicals Co., Cleveland, Ohio. Ovomucoid, 5-hydroxytryptamine creatinine sulfate (serotonin, 5-HT) and L-histidine were purchased from Sigma Chemical Co., St. Louis, Mo. Methysergide was a gift of Sandoz Pharmaceuticals, Hanover, N.J. Tripellenamine and reserpine were purchased from Ciba Pharmaceutical Co., Summit, N.J. Diphenhydramine was purchased from Parke, Davis & Co., Detroit, Mich. Pentobarbital was purchased from Abbott Laboratories, Chicago, Ill., who also provided pargyline hydrochloride as a gift. *d*-Tubocurarine chloride was purchased from E. R. Squibb & Co., New York, N.Y. All doses were calculated in terms of dry weight.

All drugs were dissolved in isotonic saline for administration, except for PCPA, approximately 300 mg/kg/day, which was mixed with ground chow and given to rats in separate cages for 3 days. This dose lowered brain serotonin levels to 10 per cent of normal (data not presented). In the PCPA experiments, controls received ground chow without PCPA.

Studies in vitro. The effect of polymyxin B, compound 48/80 and dextran on the efflux of CPK from the extensor digitorum longus muscle *in vitro* was determined. The method used has been described elsewhere.[10]

RESULTS

Effect of polymyxin B on serum CPK activity. The mean increase in PCPK activity 90 min after polymyxin B, 5 mg/kg, i.p., was more than 9-fold (Table 1). The levels at 90 min were the peak levels. PCPK activity was not significantly different from normal 3 hr after polymyxin B treatment. Ten of seventy rats given this dose of polymyxin B had no increase in PCPK activity, while in another 10 of the 70, the increase in PCPK activity ranged from 20 to 45 times the mean normal PCPK activity. In subsequent analyses, the trimmed mean (from the middle 50 values) of the PCPK levels following polymyxin B administration was utilized. Those animals whose PCPK rose markedly after polymyxin B treatment had the general appearance of being in anaphylactoid shock. The animals whose CPK activity did not rise after polymyxin B moved around vigorously, breathed normally, and had no fall in rectal temperature.

TABLE 1. EFFECT OF DRUGS ON PCPK ACTIVITY*

Drug	Dose (mg/kg)	Time of sacrifice (hr)	PCPK activity (mU/ml)	P†
Saline (36)‡		1·5	61 ± 13§	
Polymyxin B (50)	5	1·5	570 ± 143‖	< 0·001
Compound 48/80 (28)	3	2	320 ± 170‖	< 0·001
Serotonin (12)	20	2	371 ± 139§	< 0·001
Methysergide + (6)¶ Serotonin	5 20	2	149 ± 27§**	< 0·001

* Male Sprague–Dawley rats were injected with polymyxin B, compound 48/80 or saline, i.p. Methysergide and serotonin were administered s.c. At the stated time, blood samples were obtained from the inferior vena cava.

† Significance of the difference between the mean PCPK levels of the saline-treated rats and the drug-treated rats using an unpaired *t*-test.

‡ Number of rats in parentheses.

§ Mean ± S.D.

‖ Trimmed mean ± S.D. (see Results).

¶ Methysergide was administered 15 min prior to serotonin. Rats were sacrificed 2 hr after serotonin.

** Significantly different from mean PCPK levels of rats treated with serotonin, using an unpaired *t*-test, $P < 0{\cdot}005$.

The comparative effects of administering polymyxin B, i.m. or i.p. are given in Table 2. It may be seen that the i.m. route is more potent in achieving increased PCPK levels. All 20 rats given Polymyxin B i.m. had increased PCPK levels. However, this was not significantly different from the incidence of responders following i.p. injections (60/70, Chi-square = 1·96, $P > 0{\cdot}10$).

No CPK activity was detected in plasma from polymyxin B-treated rats using the standard reaction mixture with the substrate CrP omitted. PCPK activity of polymyxin B-treated rats was not affected by dialysis but was destroyed by heating to 56°

for 10 min. Only type III CPK was found in the plasma from six animals following polymyxin B treatment (Fig. 1).

Effect of other mast cell disrupting agents on plasma CPK activity. Compound 48/80, 3 mg/kg, i.p., produced a 5-fold increase in PCPK activity which peaked at 2 hr (Table 1). With compound 48/80, as with polymyxin B, approximately one-seventh (6/40) of the rats had no increase in PCPK activity, and the same number had much larger increases in PCPK activity than the majority (PCPK activity, 12–20 times greater than normal levels). The rats which had markedly increased PCPK levels following compound 48/80 appeared to be in shock. Dextran, 250 mg/kg, i.v. or i.m., and ovomucoid, 25 mg/kg, i.v. or i.m., produced no significant increase in PCPK activity at 15 min, 1 hr and 2 hr. In the doses employed, dextran and ovomucoid produced anaphylactoid shock.

TABLE 2. COMPARISON OF EFFECTS OF INTRAMUSCULAR AND INTRAPERITONEAL ADMINISTRATION OF POLYMYXIN B ON PCPK LEVELS*

Dose (mg/kg)	No. of rats	Route	PCPK activity (mU/ml)	P†	
2	20	i.m.	1369 ± 802	< 0·001	
5	50	i.p.	570 ± 143		
0·5	6	i.m.	164 ± 48	< 0·001	NS
0·5	6	i.p.	70 ± 20‡		
1·0	6	i.p.	173 ± 92		

* Male Sprague–Dawley rats were injected with polymyxin B, i.p. or i.m. Blood samples were obtained from the inferior vena cava 90 min after injection.

† Significance of the difference between the means tested by means of a two-tailed unpaired *t*-test with correction for unequal variances where appropriate.

‡ Not significantly different from the mean PCPK levels of untreated rats.

Role of histamine and heparin in the increase in PCPK activity after polymyxin B. Histamine, in doses of 5, 50, 125, 250, 500 and 1000 mg/kg, i.p. or s.c., 10 and 20 mg/kg, i.m., or 250 mg/kg, i.v., produced no significant increases in PCPK activity when plasma samples were obtained at intervals of 15 min, 1, 2, 4, 8, 12 and 24 hr following histamine administration. (Not all doses and routes were tried at each interval except for 250 mg/kg, i.p.) There was still no increase in PCPK activity in male or female rats after an i.p. injection of 100 mg/kg of aminoguanidine, an inhibitor *in vivo* of diamine oxidase,[11] 1 hr prior to s.c. administration of histamine, 250 mg/kg. Animals were sacrificed at 1, 4 and 8 hr after histamine. Aminoguanidine pretreatment also had no effect on the PCPK levels of female rats who were subsequently given polymyxin B. L-Histidine, 75 mg/kg, i.p., did not increase PCPK levels at 1 or 2 hr after drug administration.

There was no significant difference in the increase in PCPK levels 90 min after polymyxin B, 5 mg/kg, i.p., in rats pretreated with saline, tripellanamine, 10 mg/kg, i.p., or diphenhydramine 25 mg/kg, i.p. (18 rats per group) 30 min prior to polymyxin B. Neither of these antihistamines increased PCPK levels at these doses, 30 or 90 min after drug administration.

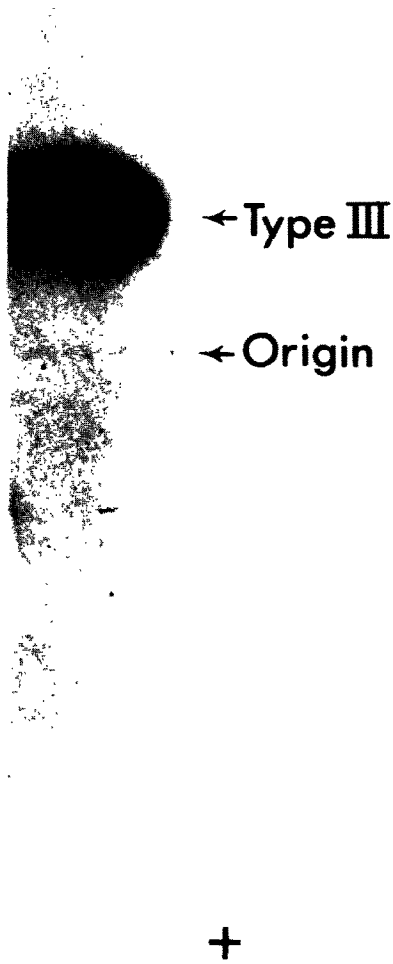

FIG. 1. Agar gel electrophoresis of rat plasma following polymyxin B demonstrating type III (skeletal muscle) CPK with the method of Van der Veen and Willebrands.[9]

Heparin, 4000 units/kg, i.p., also had no effect on PCPK activity 1 hr after administration of the heparin.

Role of 5-HT in the increase in PCPK levels after polymyxin B treatment. 5-HT, 20 mg/kg, s.c., increased PCPK activity 6-fold (Table 1). Although 5-HT release from mast cells is not likely to be the cause of the increase in PCPK activity after polymyxin B or compound 48/80 treatment, since polymyxin B is a relatively ineffective releaser of 5-HT from rat mast cells,[12] release of endogenous 5-HT from other tissue stores might be a factor. To determine if non-mast cell 5-HT might be involved in the increase in PCPK levels following polymyxin B, rats were treated with reserpine, 5 mg/kg, i.p., 24 hr prior to polymyxin B or with PCPA, approximately 300 mg/kg/day, p.o., for 3 days. Neither treatment itself raised PCPK levels, nor did they have any effect on the PCPK levels following polymyxin B treatment (data not presented). Methysergide, an antagonist of many of the peripheral effects of 5-HT,[13] given s.c. 5 mg/kg, 15 min prior to polymyxin B or 5-HT, did not inhibit the effects of polymyxin B, but it partially inhibited the increase in PCPK levels after 5-HT (Table 1).

Neuromuscular blockade and CPK release. Since neuromuscular blockade is a prominent effect of large doses of polymyxin B and compound 48/80,[14,15] the effects of the neuromuscular blocking agents *d*-tubocurarine and succinylcholine on PCPK activity were studied. *d*-Tubocurarine, 0·1 mg/kg or succinylcholine, 1·5 mg/kg, s.c., did not significantly increase the CPK activity of plasma samples obtained 15 min, 1, 2, 4 and 8 hr after drug administration despite marked signs of neuromuscular blockade such as weakness and labored breathing.

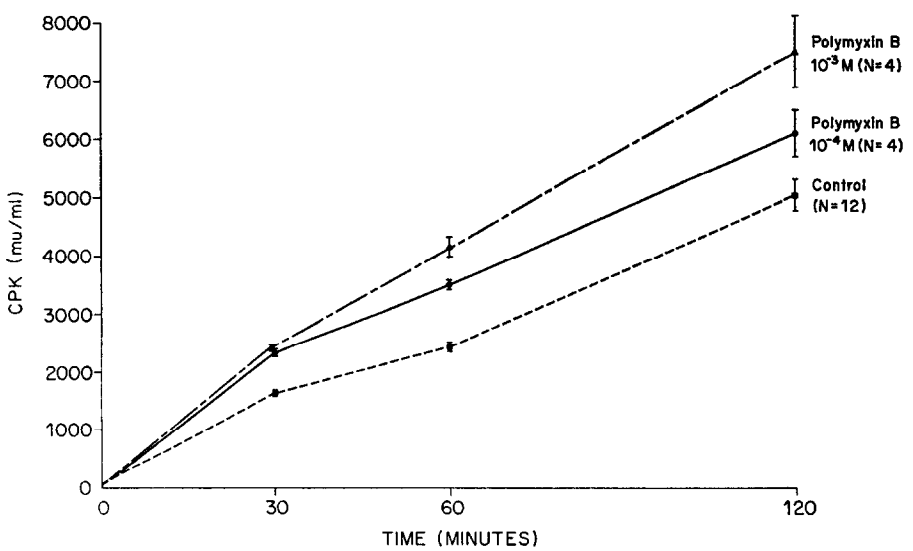

FIG. 2. Effect of polymyxin B on efflux of CPK from rat extensor digitorum longus *in vitro*. Extensor digitorum longus muscles from male, 150-g, Sprague–Dawley rats were dissected, tied to supports via the tendons and incubated with or without polymyxin B for 120 min as previously described.[10] CPK activity in the medium was analyzed immediately.[8] The number of muscles tested is given in parentheses. The significance of the differences in the efflux of CPK in the presence and absence of polymyxin B was analyzed by means of the unpaired Student's *t*-test (one-tailed): $P < 0.001$ at 30 and 60 min, $P < 0.05$ at 120 min.

Effect of ambient temperature on plasma CPK activity after polymyxin B and 5-HT. Polymyxin B and 5-HT both decrease body temperature in the rat.[12,16] Hypothermia is a very potent factor in increasing PCPK activity in the rat.[17] The increase in PCPK levels in the rat after chlorpromazine (Cpz) treatment has been related to its capacity to lower body temperature.[10] The increase in rat PCPK levels following Cpz administration is blocked by keeping the animal at an ambient temperature of 31°, which prevents hypothermia from developing.[10] Following polymyxin B, rectal temperature fell slightly but significantly (mean fall in rectal temperature at 90 min, 1·4° ± 0·2° (S.D.) and those rats whose plasma CPK activity did not increase after polymyxin B for compound 48/80) did not have any fall in rectal temperature. However, at an ambient temperature of 31° there was no significant change in rectal temperature following polymyxin B, but the mean increase in plasma CPK activity (616 mU/ml ± S.D. 462 mU/ml; range 104–1440 mU/ml) was not significantly different from the increase in plasma CPK activity which occurs at an ambient temperature of 22°. The increase in PCPK activity following 5-HT, 20 mg/kg, s.c., is also not blocked by an ambient temperature of 28°, which prevented the hypothermia following 5-HT.

Muscle incubation experiments. As indicated in Fig. 2, polymyxin B, at 10^{-3} M or 10^{-4} M, significantly increased the CPK levels in the incubation medium ($P < 0.001$ at 30 and 60 min, $P < 0.05$ at 120 min using one-tailed *t*-tests). Compound 48/80 at 1·0 mg/ml had similar effects on the efflux of CPK but dextran at 10^{-3} M did not increase the efflux of CPK (data not presented).

DISCUSSION

Administration of polymyxin B and compound 48/80 increased PCPK activity in five-sixths of the rats to whom it was administered. Rats which had no rise in PCPK activity following i.p. polymyxin B or compound 48/80 also failed to show a fall in body temperature or the signs of "anaphylactoid shock" demonstrated by the other animals. This suggests that these animals did not absorb the drugs. Differences in extent of drug absorption from the peritoneal cavity may also explain part of the large variation in PCPK levels in those animals who did respond to both drugs.

The isoenzyme studies suggest that the source of the additional PCPK activity following polymyxin B or compound 48/80 is increased release of CPK from skeletal muscle. No evidence of type II (cardiac muscle) CPK was found in plasma after polymyxin B or compound 48/80. The significantly greater increase in PCPK levels as well as the more consistent occurrence of an increase in PCPK levels following i.m. injection of polymyxin B supports the hypothesis that increased efflux of CPK from muscle is the cause of the increase in PCPK levels. Since polymyxin B can promote increased transfer of albumin from blood into liver and kidneys and possible other organs as well,[18] it is likely that only a portion of the CPK released from skeletal muscle was retained in the circulation, the rest entering other organs. The normal means of clearance of CPK from plasma, providing they are operative during anaphylactoid shock, would also remove from the circulation some CPK released by polymyxin B or compound 48/80.

The capacity to disrupt mast cells does not seem to play a role in the effect of polymyxin B and compound 48/80 on PCPK activity since other mast cell disrupters: dextran,[19] ovomucoid,[20] *d*-tubocurarine,[21] and the antihistamines[22] did not increase PCPK activity. The lack of effect of these latter mast cell disrupters on PCPK activity

suggests that the mast cells themselves are not the source of increased CPK activity. No evidence could be found that the following constituents of rat mast cells: histamine, serotonin, or heparin were the mediators of the increased PCPK activity following polymyxin B or compound 48/80. Aminoguanidine, an inhibitor of diamine oxidase,[11] which is a major pathway of histamine catabolism in male rats,[23] and the only important pathway in female rats,[24] did not enhance the effect of polymyxin B on PCPK activity, nor did it modify the absence of an increase in PCPK levels following administration of exogenous histamine. It is of interest that despite the profound effects of histamine on capillary permeability[25] and other aspects of the rat's circulation[26] it has no net effect on the levels of CPK activity in plasma. However, the flux of CPK in and out of the circulation might be greatly altered by histamine with no or slight change in the equilibrium level of CPK activity. The lack of effect of histamine on PCPK activity in the rat might indicate that muscle is not the source of the increase in LDH activity in rabbit plasma following histamine administration as noted by Wells.[5]

5-HT is not likely to be the cause of the increase in PCPK activity following polymyxin B treatment because, as previously mentioned, polymyxin B is a poor releaser of 5-HT from rat mast cells.[12] The lack of effect of reserpine on PCPK levels, the failure of reserpine, PCPA, or methysergide pretreatment to modify the effect of polymyxin B on PCPK levels, and the inhibition by methysergide of the effect of exogenous 5-HT on PCPK levels, also suggests that 5-HT is not involved in the effects of polymyxin B and compound 48/80 on PCPK levels.

Heparin did not increase PCPK activity, although the same dose of heparin employed in this investigation increases plasma diamine oxidase activity, possibly due to release of this enzyme from the intestinal mucosa.[27]

Since dextran and ovomucoid produced anaphylactoid shock without increasing PCPK levels, it is unlikely that the anaphylactoid-like shock produced by polymyxin B and compound 48/80 is the cause of the increased CPK efflux from muscle.

High initial absorbance would have been noted if large amounts of ATP from mast cells or other tissues were present in plasma from polymyxin B-treated rats but was not observed. The absence of CPK activity following the assay of plasma from polymyxin B-treated rats with the entire coupled enzyme system used to detect CPK activity, but with the substrate CrP omitted, also indicates that there was essentially no contribution to the PCPK activity from preformed ATP.

Succinylcholine and *d*-tubocurarine, both more potent neuromuscular blocking agents than polymyxin B and compound 48/80,[28] had no effect on rat PCPK activity, although in man and cats these neuromuscular blocking agents, in combination with the anesthetic agent halothane, do increase PCPK levels.[29,30] However, the mechanism of the neuromuscular blockade produced by polymyxin B and compound 48/80 is not identical to either that produced by succinylcholine or *d*-tubocurane.[28]

The incubation studies *in vitro* provide further indication that polymyxin B and compound 48/80 can increase the rate of release of CPK from skeletal muscle. The relatively high concentrations of polymyxin B and compound 48/80 necessary to augment release of CPK *in vitro* may be misleading because diffusion is the only way for the drugs to reach the interior of the intact muscle, and for CPK to reach the incubation medium. The rate of diffusion of dextran, because of its size, might be less than that of polymyxin B and compound 48/80.

The increased efflux of CPK from muscle cells *in vivo* and *in vitro* after exposure to polymyxin B or compound 48/80 could result from a functional change in cell membrane permeability, selective damage to the cell membrane, or as part of more generalized muscle cell injury. We have found extensive skeletal muscle pathology in rats following a single injection of polymyxin B, 5·0 mg/kg, i.p.* Polymyxin B is believed to be lethal to gram-negative bacteria by increasing the permeability of the cell envelope, which includes the cell wall and basement membrane, and causing leakage of cytoplasm.[31] Comparable damage to muscle might be responsible for the release of CPK.

Polymyxin B is administered to man, i.m., in doses of 1·5 to 2·5 mg/kg/day in three divided doses.[32] These doses are in the range which produce increased PCPK activity in rats. Severe, aching pain which begins 40–60 min following a single i.m. injection of polymyxin in man is, in fact, a frequent occurrence.[32] It seems likely that polymyxin B is likely to have myopathic effects in man as well as in rats.

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