Changes in Glucose/Amino Acid/Calcium Uptake and Brush-Border Membrane-Associated Enzymes in Rat Small Intestine after the Administration of Embelin (Plant Benzoquinone), an Antifertility Agent

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(Received August 17, 1989)

Summary Administration of Embelin, an experimental antifertility agent, to male rats (20 mg/kg body wt/day, daily for 15 and 30 days), caused an elevation in the uptake of D-glucose, L-alanine, L-leucine, and calcium in the small intestinal segments. An increase was also noted in the intestinal brush border membrane (BBM)-associated enzymes, sucrase, lactase, maltase, alkaline phosphatase, and leucine aminopeptidase in both the intestinal homogenates and partially purified BBM preparations, particularly after 30-day administration of the drug. Embelin treatment also caused a significant increase in the microsomal glucose-6-phosphatase and the cytosolic enzyme, lactate dehydrogenase. In the Embelin-treated animals BBM-associated total lipids, phospholipids, cholesterol, triacylglycerol, unesterified fatty acids, ganglioside-sialic acids as well as the cholesterol/phospholipids molar ratio showed a considerable increase. All these changes in the Embelin-treated animals were restored back to the normal or near normal biochemical makeup when the drug therapy was withdrawn and the animals were allowed to recover for another 15 and 30 days, respectively.

Key Words Embelin, antifertility compound, intestinal uptake, glucose, amino acids, calcium, brush border membrane, hydrolases, membrane lipids

The plant-derived compound, Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) isolated from the dried berries of *Embelia ribes* *Burm.*, family: Myrisnaceae, has recently been reported to induce sterility in mice and rats (1–3), dogs (4), and also possesses antiimplantation (5) and post-coital antifertility activity (6) in rats. The isolation, chemical characterization, and bio-distribution of Embelin in different organs of rats have recently been reported from our labo-

ratory (7). We have also confirmed the potential of the compound as a reversible male antifertility agent with minimal side effects (8, 9). Evidence for antispermato-genic activity of the compound *in vitro* and *in vivo*, such as interference in spermatozoal motility, glycolysis and energy metabolism, fertility performance and ultrastructural aberrations have also been provided (10). However, detailed animal studies are needed before even accepting the compound as a potential antifertility agent and for possible clinical trial in future. We decided to look into the effects of the drug on the intestinal absorption of a number of end-product nutrients and also its interaction with the enzyme systems involved in the digestion which are localized in an extended part of the plasma membrane of the epithelial cells, called the brush border membrane (BBM). In addition, the effects of the drug on biochemical makeup of BBM are tested and the reversibility of the changes produced have also been studied.

MATERIALS AND METHODS

Drug treatment. Adult male rats weighing 150–200 g of Porton strain were obtained from the Central Animal House, Panjab University, and housed in individual cages under controlled light (7 AM to 7 PM) and temperature conditions (20–22°C). The animals were maintained on standard rat pellet (Lipton India, Bombay) and water *ad libitum*. All the animals were kept in quarantine for 20 days before drug treatment and grouped as follows:

Groups I and II. The animals were injected subcutaneously with Embelin, 20 mg/kg body wt/day, suspended in olive oil (0.2 ml vol.), for 15 days (Group I) or 30 days (Group II), respectively.

Groups III and IV. Drug treatment was withdrawn in half of the treated animals in Group II and they were kept for recovery for 15 and 30 days, which formed Groups III and IV, respectively. Normal controls, kept with each Embelin-treated group of animals, strictly received an equal volume of the carrier of the drug. All the groups (I–IV), drug-treated and recovery groups along with their corresponding controls, consisted of 3 animals each.

Measurement of intestinal uptake of nutrients. Overnight-fasted animals in all the groups were sacrificed under diethyl ether anaesthesia. Body weights, intestinal weights, and lengths were recorded. Starting from the ligament of Treitz, the entire small intestine was removed, immediately flushed with cold saline (0.9% NaCl in water), and everted using a thin stainless steel rod. A 25 cm jejunal segment, distal to the ligament, and another 25 cm ileal segment ending 5 cm from the caecum, were removed. Intestinal uptake of glucose or amino acids was measured in the jejunal portion by the tissue accumulation method of Crane and Mandelstam (11). Calcium uptake was similarly measured in the duodenum. Ileum portion was utilized for the preparation of BBM. These segments were cut into small rings (0.3–0.5 cm) and 4 randomly chosen rings were incubated in 5 ml of oxygenated Krebs ringer bicarbonate (KRB) buffer, pH 7.4, without fortifying with glucose but containing

5 mM D-glucose/L-leucine/L-alanine with trace amounts of the uniformly ^{14}C -labeled respective substrates. The incubation was carried out for 5 min at 37°C for glucose/amino acid uptake in a Dubnoff-type metabolic shaker at 150–160 oscillations per min to overcome the effects of unstirred water layers on the kinetics of nutrient uptake as suggested by Lherminier and Alvarado (12). Uptake of calcium was determined in the tissues in 5 ml of oxygenated Tris-HCl buffer, pH 6.9, containing 140 mM NaCl, 6 mM KCl, 4 mM Tris, and 1 mM CaCl_2 with a trace amount of $^{45}\text{CaCl}_2$. Incubations were carried out for 30 min at 37°C . At the end of the incubation period, the rings were removed from the incubation medium, repeatedly washed, gently blotted, and weighed. They were digested in 20% KOH (13), dissolved in a dioxan-based liquid scintillation cocktail, Bray's fluid (14), and the accumulated tissue radioactivity was measured in an LS 7900 Beckman's liquid scintillation counter with more than 90% counting efficiency for ^{14}C -isotopes. The tissue uptake was calculated and expressed as units/g tissue, where one unit represents $1\text{ }\mu\text{mol}$ of the substrate taken up per 5 min at 37°C . Extracellular space was separately measured by incubating the tissues in the presence of $[^3\text{H}]$ inulin and it amounted to about 5% of the external substrate concentration added in 5 min and 10% in 30 min of incubation time. The tissue uptake of nutrients was corrected for extracellular space following the method of Alvarado and Mahmood (15) and as described by us earlier (16).

Preparation of brush border membrane. BBM was prepared from the pooled intestinal homogenates following the method of Schmitz *et al.* (17) and as described by us earlier (16, 18). A 5% homogenate of the tissue was prepared in 1 mM Tris–50 mM mannitol buffer, pH 7.4, at 4°C with the help of a motor-driven homogenizer with Teflon pestle and glass tube. The homogenate was passed through two layers of cheese cloth; to the filtrate, anhydrous CaCl_2 was added in cold with constant stirring to a final concentration of 10 mM Ca^{2+} and centrifuged at $2,000 \times g$ for 10 min. The supernatant was centrifuged at $42,000 \times g$ for 20 min and the precipitate was suspended in 50 mM sodium maleate buffer, pH 6.5, containing 0.02% NaN_3 . The final membrane preparation was similar to the P_2 fraction of Schmitz *et al.* and was used as such for the enzyme assays and biochemical analysis. The membrane was essentially free from basolateral membrane, nuclei, mitochondria, and other organized subcellular structures as established by marker enzymes and reported earlier (16, 18). There was a 5-fold membrane purification as compared to the initial intestinal homogenates, using the marker enzyme sucrase, while lactase, maltase, alkaline phosphatase, and leucine aminopeptidase enrichment amounted to 7, 9, 7, and 3 times, respectively. Comparable results were obtained in rabbits, rats, and monkeys (19).

Enzyme assays. BBM-bound disaccharidases, such as sucrase, lactase, and maltase activities were measured following the method of Dahlqvist (20) by estimating the D-glucose formation with the glucose oxidase-peroxidase system. Alkaline phosphatase activity was assayed using the enzymatic hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol which was determined spectrophotometri-

cally at 420 nm (21). Leucine aminopeptidase activity was estimated according to Goldberg and Ruttenberg (22). Intestinal cytosolic enzyme, lactic dehydrogenase, and microsome-specific glucose-6-phosphate phosphohydrolase activities were estimated following the procedures of Wootton (23) and Freedland and Harper (24), respectively. Membrane-bound enzyme protein was estimated by the sodium dodecyl sulfate-Lowry procedure of Lees and Paxman (25) using bovine serum albumin as standard. Enzyme activities were expressed as μmol of the substrate hydrolyzed/min/g protein under standard assay conditions.

Other analytical procedures. BBM-bound lipids were extracted in 20 vol. of chloroform-methanol (2:1, v/v), following the method of Folch *et al.* (26), phase-separated with 0.9% KCl, lower organic phase washed several times with the theoretical upper phase of Folch (chloroform: methanol: KCl, 3:48:47, v/v), and finally dried under vacuum in a rotary evaporator and under a stream of N_2 . The dried lower phase was redissolved in a known volume of chloroform-methanol (2:1, v/v) and lipid classes were analyzed by colorimetric procedures. The extracted total lipid was quantitated by the method of Fringes and Dunn, using olive oil as the standard (27). Lipid-bound phosphorus was digested with HClO_4 , quantitated following the procedures of Bartlett (28) and computed for mass weight of phospholipids, taking into account the fact that the average molecular weight of phospholipids was 775. Cholesterol was determined by the ferric chloride-acetic acid procedure of Zlatkis *et al.* (29). Triacylglycerol was quantitated by glycerol analysis after hydrolysis with alcoholic KOH (30). Unesterified fatty acids were determined by the method of Lowry and Tinsley (31) using palmitic acid ($\text{C}_{16:0}$) as the standard. Lipid-bound ganglioside-sialic acid was determined in the extracted Folch upper phase of BBM lipids by the thiobarbituric acid method of Warren (32) after hydrolyzing with 0.1 N H_2SO_4 for 1 h at 60°C .

Statistical analysis. The differences between the means $\pm$ SEM for animals in the control and experimental groups were examined by using the Student's *t*-test for paired data. Statistical differences at least at 5% level ($p < 0.05$) were accepted as significant.

Materials. Embelin was procured from Bio Organics, Madras, with label purity of 99%. Purity of compound was, however, further established in our laboratory as reported earlier (7-10). All chemicals, solvents, and standards were of the highest purity available commercially. Glucose oxidase peroxidase and L-leucyl- β -naphthylamide were obtained from Sigma Chemical, St. Louis, Mo. Radioisotopes employed in the present study, [^{14}C]D-glucose (Sp. Ac. 255 mCi/mmol), [^{14}C]L-leucine (Sp. Ac. 288 mCi/mmol), [^{14}C]L-alanine (Sp. Ac. 78 mCi/mmol), and [^{45}Ca]CaCl₂ (Sp. Ac. 6 mCi/g) were obtained from the Radioisotope Division, The Bhaba Atomic Research Center, Trombay, Bombay.

RESULTS AND DISCUSSION

A marginal increase in the body weight and intestinal weight and length was

caused by the Embelin administration (Table 1) reflecting that the effect of the drug may be at metabolic level (through hormonal control or otherwise) on the growth of intestines and possibly the number and divisions of epithelial cells. A significant increase was observed in the uptake of end-product nutrients, such as glucose, alanine, and leucine in intestinal segments after Embelin treatment (accounted for 19–21% stimulation), which, however, recovered back to normal or near normal level after the drug treatment was stopped (Table 1). Embelin administration caused 20.46% stimulation in Ca^{2+} uptake which also recovered to normal values after the termination of the drug.

BBM-associated enzymes, sucrase, lactase, maltase, and alkaline phosphatase activity showed a considerable increase in Embelin-treated rats (Table 2) in the partially purified BBM, suggesting further that the functions of intestine such as the absorption and digestion of the nutrients were stimulated. There was also an increase in the BBM-bound enzymes of intestinal homogenates (Table 3). The presence of these enzymes in greater amount showed considerable purification of BBM in our isolation procedure, while the other subcellular marker enzymes such as glucose-6-phosphatase (microsomal) and lactate dehydrogenase (cytosolic) were present in much less amount in BBM as compared to the homogenate. Interestingly, however, glucose-6-phosphatase, which is the terminal hydrolytic enzyme for many important pathways, and lactate dehydrogenase, which represents a key step in glycolysis and intermediary metabolism (33), also showed considerable degree of stimulation in both the intestinal homogenates as well as the isolated membrane fraction in Embelin-treated rats.

Both the uptake of glucose/amino acid and the disaccharidases/aminopeptidase activities were similarly affected in the Embelin-treated animals, suggesting that the two systems are possibly controlled by a similar or common mechanism, such as the induction of the membrane-bound carrier molecules and enzymes, their increased synthesis, or increase in the binding and solubilization of the substrate to the proteins (substrate affinity constant) as shown by us earlier (16, 18). Such kinetic effects may also explain the increase in Ca^{2+} uptake due to interaction of the drug with the Ca^{2+} channel forming proteins or $\text{Na}^+/\text{Ca}^{2+}$ antiport. Increase in Ca^{2+} uptake results in stimulation in a number of intracellular events mediated by calcium (34).

A marked change in the chemical compositions of the isolated BBM fraction was caused by Embelin treatment. Analysis of phospholipids and cholesterol showed an increase in these important membrane constituents but hardly showing any change in phospholipid/cholesterol molar ratio (Table 4), indicating that possibly there may not be any alteration in membrane fluidity. The changes in membrane fluidity, however, have further contributed in the functioning of the membrane-bound protein molecules (35). Increase in total lipids and other classes such as triacylglycerol, free fatty acids and lipid-bound ganglioside-sialic acid level is also highly visible in Embelin treatment. Intestine has been known to be an important lipid-synthesizing tissue and therefore, molecular mechanism by which

Table 1. Effects of Embelin and subsequent withdrawal of the drug on the intestinal uptake of certain end-product nutrients in male albino rats.

Parameters studied	20 mg/kg body weight Embelin/day						Recovery					
	15-day treatment			30-day treatment			15-day			30-day		
	Contl.	Exptl.		Contl.	Exptl.		Contl.	Exptl.		Contl.	Exptl.	
Body weight gain (g)	15.16 ±0.43	15.83 ±0.52		21.66 ±1.15	22.83 ±1.18		25.00 ±0.80	25.83 ±0.65		29.33 ±0.54	30.00 ±0.63	
Intestinal weight (g)	4.93 ±0.28	5.15 ±0.19		4.96 ±0.17	5.25 ±0.16		4.90 ±0.12	5.18 ±0.17		4.91 ±0.14	5.03 ±0.13	
Intestinal length (cm)	95.00 ±1.26	95.66 ±1.46		96.50 ±1.93	97.50 ±2.13		95.16 ±1.48	97.00 ±2.19		94.83 ±1.42	95.83 ±1.86	
Substrate uptaken ($\mu\text{mol}/5 \text{ min incubation/g tissue}$)												
D-Glucose	8.59 ±0.18	9.51 ±0.12*		8.38 ±0.07	10.05 ±0.29**		8.31 ±0.08	9.37 ±0.40		8.31 ±0.04	8.68 ±0.14	
L-Alanine	7.26 ±0.24	8.27 ±0.28		7.28 ±0.10	8.80 ±0.12		7.07 ±0.02	8.30 ±0.23		7.19 ±0.09	7.44 ±0.10	
L-Leucine	4.25 ±0.06	4.64 ±0.06		4.27 ±0.07	5.14 ±0.08		4.28 ±0.06	4.67 ±0.05		4.26 ±0.03	4.44 ±0.04	
Calcium ($\mu\text{mol}/h \text{ incubation/g tissue}$)	2.63 ±0.10	2.93 ±0.03*		2.59 ±0.05	3.12 ±0.06		2.60 ±0.01	2.99 ±0.03		2.61 ±0.03	2.68 ±0.02	

Values are mean $\pm$ SEM of 3 independent experiments, analyzed in duplicates. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are statistically different from the respective control group.

Table 2. Effects of Embelin and subsequent recovery on certain BBM-bound hydrolases and other enzymes in partially purified small intestinal BBM of albino rat.

BBM-bound enzymes ($\mu\text{mol}/\text{min}/\text{g}$ protein)	20 mg/kg body weight Embelin/day				Recovery			
	51-day treatment		30-day treatment		15-day		30-day	
	Contl.	Exptl.	Contl.	Exptl.	Contl.	Exptl.	Contl.	Exptl.
Sucrase	1,924.06 ± 47.12	2,093.89 ± 48.38	1,940.87 ± 47.54	2,191.57 $\pm 25.08^{**}$	1,929.95 ± 41.35	2,130.97 $\pm 43.05^{*}$	1,932.36 ± 23.66	1,993.09 ± 24.40
Lactase	634.94 ± 40.81	770.39 ± 41.90	640.48 ± 41.17	860.24 $\pm 43.44^{*}$	643.37 ± 41.35	791.50 ± 43.04	637.68 ± 40.99	657.71 ± 42.28
Maltase	8,658.30 ± 81.63	8,968.38 $\pm 48.17^{*}$	8,695.10 ± 47.54	9,421.74 $\pm 50.16^{***}$	8,694.56 ± 47.75	9,173.34 $\pm 35.15^{**}$	8,657.00 ± 47.33	8,769.62 ± 48.81
Alkaline phosphatase	5,961.11 ± 112.05	6,400.22 ± 45.71	5,992.00 ± 68.60	6,926.66 $\pm 136.82^{**}$	5,997.23 ± 78.13	6,553.44 $\pm 71.74^{**}$	5,944.66 ± 44.71	6,196.71 $\pm 46.12^{*}$
Leucine aminopeptidase	1,382.01 ± 48.86	1,525.28 ± 25.08	1,394.08 ± 24.64	1,655.07 $\pm 26.00^{**}$	1,400.16 ± 24.74	1,567.06 $\pm 25.77^{**}$	1,387.97 ± 49.07	1,431.59 ± 25.30
Glucose-6-phosphatase (Microsomal enzyme)	41.10 ± 2.48	45.36 ± 1.13	40.93 ± 1.92	48.96 ± 2.03	40.20 ± 2.24	46.61 ± 1.16	40.75 ± 1.91	42.03 ± 1.98
Lactate dehydrogenase (Cytosolic enzyme)	52.77 ± 4.60	65.46 ± 2.76	51.01 ± 2.71	74.90 $\pm 2.86^{**}$	51.24 ± 2.73	67.26 $\pm 2.84^{*}$	52.99 ± 4.68	59.22 ± 2.79

Values are mean $\pm$ SEM of 3 independent experiments, analyzed in duplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ are statistically different from the respective control group.

Table 3. Effects of Embelin and subsequent recovery on certain BBM-bound hydrolases and other enzymes in the intestinal homogenates of male albino rats.

BBM-bound enzymes ($\mu\text{mol}/\text{min}/\text{g}$ protein)	20 mg/kg body weight Embelin/day				Recovery			
	15-day treatment		30-day treatment		15-day		30-day	
	Contl.	Exptl.	Contl.	Exptl.	Contl.	Exptl.	Contl.	Exptl.
Sucrase	438.48 ± 24.40	537.48 $\pm 25.31^*$	440.47 ± 24.51	641.25 $\pm 27.08^{**}$	444.47 ± 24.74	566.44 ± 26.68	444.47 ± 24.74	462.55 ± 24.63
Lactase	99.65 ± 24.40	144.70 ± 25.31	99.52 ± 24.16	176.89 ± 27.08	101.01 ± 24.74	152.49 ± 26.68	101.01 ± 24.74	120.66 ± 42.66
Maltase	956.68 ± 42.27	1,054.30 ± 43.85	961.02 ± 42.47	1,149.84 $\pm 27.08^*$	949.55 ± 24.74	1,089.32 $\pm 26.68^*$	949.55 ± 24.74	985.45 ± 24.62
Alkaline phosphatase	847.97 ± 46.12	947.17 $\pm 47.83^*$	862.73 ± 26.75	1,049.33 $\pm 25.88^{**}$	848.54 ± 14.55	986.32 $\pm 14.55^{**}$	837.52 ± 13.49	855.64 ± 23.28
Leucine aminopeptidase	393.68 ± 25.30	482.58 ± 26.25	383.48 ± 29.35	541.04 $\pm 44.76^*$	410.44 ± 38.35	495.54 ± 15.97	398.41 ± 43.62	433.36 ± 25.53
Glucose-6-phosphatase (Microsomal enzyme)	99.96 ± 3.02	107.56 ± 2.05	100.41 ± 1.14	118.16 $\pm 2.19^{**}$	100.38 ± 1.16	110.29 $\pm 2.16^*$	99.43 ± 2.01	103.69 ± 1.15
Lactate dehydrogenase (Cytosolic enzyme)	164.00 ± 9.66	182.38 ± 12.28	160.16 ± 15.60	227.44 ± 10.71	161.62 ± 5.65	209.14 $\pm 10.56^*$	163.93 ± 7.48	179.27 ± 9.75

Values are mean $\pm$ SEM of 3 independent experiments, analyzed in duplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are statistically different from respective control group.

Table 4. Effects of Embelin and subsequent recovery on lipid compositions of intestinal BBM of male albino rats.

BBM-lipid classes ($\mu\text{g}/\text{mg}$ protein)	20 mg/kg body weight Embelin/day						Recovery			
	15-day treatment			30-day treatment			15-day		30-day	
	Contl.	Exptl.		Contl.	Exptl.		Contl.	Exptl.	Contl.	Exptl.
Total lipids	323.23 ± 14.14	379.26 ± 14.51		326.06 ± 14.26	430.12 $\pm 15.05^{**}$		327.50 ± 14.32	401.84 ± 25.83	324.63 ± 14.19	346.79 ± 14.64
Phospholipids	141.41 ± 1.76	148.15 ± 1.81		141.19 ± 1.77	155.10 $\pm 1.88^{**}$		141.73 ± 1.67	152.21 ± 1.86	140.57 ± 1.77	144.99 ± 1.82
Cholesterol	83.69 ± 1.76	91.85 ± 1.81		84.22 ± 1.77	99.84 $\pm 1.88^{**}$		84.79 ± 1.79	94.36 $\pm 1.86^*$	84.05 ± 1.77	86.69 ± 1.82
Triacylglycerol	60.02 ± 5.65	80.59 ± 5.80		60.55 ± 5.70	98.30 ± 6.01		60.82 ± 5.73	92.54 $\pm 5.96^*$	60.28 ± 5.67	66.96 ± 5.85
Unesterified fatty acid	11.54 ± 2.82	16.59 ± 2.90		11.64 ± 2.85	20.89 $\pm 1.50^*$		11.69 ± 2.86	17.27 ± 1.42	11.59 ± 1.42	13.15 ± 1.46
Ganglioside sialic acid	43.63 ± 2.24	53.09 $\pm 0.76^*$		44.24 ± 1.24	63.40 $\pm 0.51^{***}$		43.27 ± 0.57	56.73 $\pm 0.78^{***}$	44.05 ± 1.23	46.15 ± 0.77
Cholesterol/phospholipid molar ratio	0.591	0.619		0.597	0.643		0.598	0.619	0.593	0.597

Values are mean $\pm$ SEM of 3 independent experiments, analyzed in duplicates. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are statistically different from the control group.

this lipophilic drug could possibly induce lipid biosynthesis (turnover of intestinal lipid biosynthesizing enzymes) remains a subject of speculation. As compared to the present results, in earlier studies (16, 18) we have shown that the intestinal functions are highly inhibited by gossypol, an established antifertility agent of plant origin. The difference in the mechanism of action of the two compounds may derive from the fact that their chemical structures are different: gossypol is a polyphenolic compound while Embelin is known to be a benzoquinone. Also, gossypol has also been shown by us to bind to membrane compounds, change the fluidity, and thereby may restrict the expression of nutrient-transporter molecules and membrane-bound enzymes, while the present results may only reflect an overall general effect of Embelin on metabolism.

The intestinal enzymic activities are thought to be, in normal circumstances, under the control of hormonal, nervous, and/or intraluminal factors (36). The effect of Embelin administration on BBM-enzymic activities and nutrient transporter-molecules has to be carefully investigated in relation to the well-known modifications of gonadal and other hormonal secretions by Embelin (37, 38). The hormones may influence the intestinal function directly (primary action) on cryptic mitosis, differentiation of the immature cells, specific transfer mechanisms, and enzyme synthesis or it can exert secondary effects at organ level by gastric emptying, intestinal motility, appetite of the animal, digestive secretions, or changes in the luminal or blood milieu of the mucosal cells (36). It is important to note, however, that changes in intestinal functional constituents are reversible or at least reach near normal makeup during recovery period once the Embelin treatment is withdrawn, indicating perhaps that the drug reasonably fulfills the criteria of an ideal contraceptive chemical, *i.e.*, full reversibility.

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