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Solubilization of Serotonin and Related Compounds in Benzene-*n*-Butanol in Presence of Human Plasma.* (26802)

GRACE P. KERBY AND S. M. TAYLOR
Department of Medicine, Duke University Medical Center, Durham, N. C.

Recent studies in this laboratory(1) have suggested that, in the presence of a nondialyzable factor present in human plasma, serotonin is taken up *in vitro* by human platelets as a complex with divalent cations. Uptake of serotonin by intact platelets was considerably enhanced by presence of plasma. The enhancement of uptake was partially prevented by addition of disodium EDTA or of Mg^{++} but not by addition of disodium calcium EDTA; it was blocked by simultaneous addition of disodium EDTA and Mg^{++}. Serotonin was shown further to be capable of solubilizing calcium phosphate, indicating some form of complexing with Ca^{++}.

In the present study the extent of solubilization of serotonin and related compounds in fat solvents was explored further in presence and absence of human plasma. An *in vitro* system was used, patterned after that of Woolley(2) who has extracted a lipid from animal tissues capable of solubilizing serotonin in benzene-butanol in the presence of Ca^{++}. This extract has been postulated to contain the nonenzymic portion of serotonin receptors. Woolley later demonstrated(3) increased transport of radioactive Ca^{++} into benzene-butanol *in vitro* in the combined presence of added receptor substance and serotonin.

Human plasma was found capable of solubilizing serotonin and a number of other substances in benzene-butanol. However only serotonin and tryptamine of the substances tested showed reversal of this effect in the combined presence of divalent cations and chelators. The overall findings supported the hypothesis that divalent cations bound to a portering substance or substances present in human plasma could form a complex with serotonin or tryptamine to solubilize these substances in benzene-butanol in the absence of competing chelators.

Methods. In experiments patterned after those of Woolley(2), one ml of physiologic saline (with or without added Ca^{++} or Mg^{++}, 270 μM, and/or disodium EDTA,† pH 7.4, or disodium calcium EDTA,† pH 6.9, 270 μM) was added to 6.0 ml of platelet-free plasma (from venous blood, using disodium EDTA and processing as in earlier studies (4) but discarding platelets) either nondialyzed or dialyzed (revolving against a total of 75 volumes in 5 increments of balanced

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† As Endrate Disodium or Endrate Disodium Calcium, kindly made available by Abbott Laboratories.

TABLE I. Solubilization of Serotonin in Benzene-Butanol in Presence of (a) Non-dialyzed and (b) Dialyzed Human Plasma.*

	Serotonin (0.25 μM) added to:*	Plasma added*	Serotonin (μM $\times$ 10^{-2}) recovered from benzene-butanol	Duplicate recoveries, % variation
(a)	Balanced buffered salt solution	None	.86	2.2
	Idem + disodium EDTA	"	.67	0
	Physiologic saline	Non-dialyzed	2.45	1.5
	Disodium EDTA + saline	"	1.83	4.1
	" calcium EDTA + saline	"	2.37	3.1
	$MgSO_4$	"	1.74	1.1
	Disodium EDTA + $MgSO_4$	"	.61	7.6
	" calcium EDTA + $MgSO_4$	"	1.56	5.4
(b)	Physiologic saline	Dialyzed	.74	9.8
	Disodium EDTA + saline	"	.71	6.9
	" calcium EDTA + saline	"	.74	8.3
	$MgSO_4$	"	.81	7.5
	Disodium EDTA + $MgSO_4$	"	.81	3.1
	" calcium EDTA + $MgSO_4$	"	.89	4.1

* See *Methods* for details.

buffered salt solution‡ plus original disodium EDTA concentration of 2.69 $\times$ 10^{-3}M over a 48-hour period at 5°C). Control tubes with 6.0 ml of balanced buffered salt solution (pH 7.6) instead of plasma were included in every experiment. Test substance (0.25 μM) was added in 1.0 ml of balanced buffered salt solution or saline as indicated in tables, with or without added Ca^{++} or Mg^{++}, 270 μM. The mixture was shaken vigorously in glass-stoppered tubes with 10 ml benzene-*n*-butanol (1:1) for 5 minutes at room temperature and then centrifuged at 100 g to completely clear the benzene-butanol layer. Five ml of the clear top organic layer was transferred to a clean glass-stoppered tube containing 2 ml 0.1N HCl. The tube was shaken and centrifuged as before; the organic solvent was suctioned off and discarded, and 10 ml benzene were added to the aqueous layer. After re-shaking and re-centrifuging, the acid aqueous layer was transferred with a Pasteur pipette to a quartz cuvette for analysis of spectrum and measurement of test substance, using an Aminco-Bowman Spectrophotofluorometer. The test substances used, and the activation/fluorescent wave length used for measurement, were as follows: "L" Tryptophan (N.B.C.) at 270 mμ/330 mμ, tryptamine HCl (N.B.C.) at 270 mμ/355 mμ; serotonin creatinine sulfate (kindly made available by Upjohn Co.) at 295 mμ/335 mμ; 5-hydroxyindole-3-acetic acid (5HIAA; Calif. Found. Bioch. Research) at 285 mμ/335 mμ; 1-benzyl-2-methyl-5-methoxytryptamine (BAS; kindly made available by Merck, Sharp and Dohme) at 290 mμ/360 mμ; tyramine monohydrochloride (N.B.C.) at 270 mμ/330 mμ; norepinephrine (Levophed Bitartrate, Winthrop) at 270 mμ/320 mμ. A 3-point standard curve of test substance in appropriate amounts in 0.1N HCl was measured simultaneously in each instance. The nature of the experiment precluded subjecting the standards to the entire extraction procedure, since untreated standards were insoluble in benzene-*n*-butanol. However, as noted, controls of 0.25 μM of test substance in 7 ml of balanced buffered salt solution (pH 7.6) were carried through the entire procedure in each experiment so that the amount of test substance carried over non-specifically in each extraction could be assessed. All tests were run in duplicate. Experimental variation for each determination is indicated in the accompanying tables. With 3 exceptions noted in Table III, all determinations checked within 10%.

Results. Results are summarized in Tables I-III. In the presence of human plasma, sero-

‡ Ten ml stock solution (7.5% NaCl, 0.75% KCl, 0.1% Na_2HPO_4, 0.12% KH_2PO_4, 0.05% K_2HPO_4) and 7 ml 1% Na_2HPO_4 per 100 ml, aqueous (total phosphate concentration, 6.7 x 10^{-3}M).

TABLE II. Effect of Divalent Cations on Solubilization of Serotonin in Benzene-Butanol.*

Serotonin (0.25 μM) added to:*	Plasma added*	Serotonin ($\mu M \times 10^{-2}$) recovered from benzene-butanol	Duplicate recoveries, % variation
(a) Balanced buffered salt solution	None	.86	2.2
Physiologic saline	Non-dialyzed	2.45	1.5
$CaCl_2$	"	1.35	4.8
Disodium EDTA + $CaCl_2$	"	.70	7.8
$MgSO_4$	"	1.74	1.1
Disodium EDTA + $MgSO_4$	"	.61	7.6
(b) Physiologic saline + $CaCl_2$	None	.56	4.9
$CaCl_2$	Non-dialyzed	1.35	4.8
PO_4 buffer (0.25M) + $CaCl_2$	None	.25	7.3

* See *Methods* for details. (a) and (b) refer to comments in text (see *Results* and *Discussion*).

tonin was partially solubilized in benzene-butanol (Table I-a). In testing the effect of dialyzed plasma in the system, the original plasma concentration of added disodium EDTA was maintained in order to take advantage of the tighter chelating action of EDTA over that of the plasma proteins. Thus both originally bound and free divalent cations were dialyzed out of the plasma, to the extent achievable by use of 75 volumes of dialyzing balanced buffered salt solution with added disodium EDTA over a period of 48 hours. As noted in Table I-b, solubilization of additional serotonin into benzene-butanol in the presence of plasma was completely suppressed by prior plasma dialysis which, among other things, was sufficient to remove virtually all originally bound and free Ca^{++} and Mg^{++} from the plasma.

In the absence of competing chelator, overall solubilization of serotonin was somewhat decreased by addition of Ca^{++} or Mg^{++} in the presence of nondialyzed plasma (Table II-a). Addition of either Ca^{++} or Mg^{++} and disodium EDTA with its tighter chelating action than plasma-binding-substances completely suppressed the enhanced solubilization of serotonin in benzene-butanol in the presence of plasma (Table II-a). Addition of disodium calcium EDTA had little suppressive effect in the presence of Mg^{++} (Table I-a).

Substitution of hyperphysiologic phosphate buffer (0.25M, pH 7.0) for plasma in the *in vitro* system to which serotonin and Ca^{++} were added resulted in suppression of solubilization of serotonin in benzene-butanol (Table II-b).

As shown in Table III, serotonin was not alone among substances tested in being solubilized in benzene-butanol by a factor or factors present in human plasma. The degree of solubilization on addition of plasma was proportionally greatest with tyramine and least with the serotonin analogue, BAS. However, only serotonin and tryptamine exhibited reversal of enhanced solubilization in benzene-butanol by human plasma when disodium EDTA and Mg^{++} were added. Only tryptamine (Table III-b) of the substances tested showed a pattern of change to all test additions (*i.e.*, disodium EDTA, disodium calcium EDTA, Mg^{++} and combinations thereof) which was identical to the pattern exhibited by serotonin (Table I-a).

In the studies reported in Table III, the fluorescent spectra of the substances extracted from benzene-butanol solution into 0.1N HCl were identical with those of the original substance used, with 3 exceptions: Tyramine and norepinephrine showed a slight but definite shift of fluorescent spectrum toward the visible end of the spectrum whenever plasma had been used in the *in vitro* system. It was thought possible that the test substance in each instance might have displaced some naturally occurring substance from its plasma binding site and that this substance was then solubilized in benzene-butanol and extracted. However, this could never be established, if so, and the substances measured were definitely shown not to be

TABLE III. Solubilization of Related (to Serotonin) Compounds in Benzene-Butanol.*

Compound added (.25 μM) to:*	Plasma added*	Recovery of added compound ($\mu M \times 10^{-2}$) from benzene-butanol	Duplicate recoveries, % difference
(a) *Tryptophan**			
Balanced buffered salt solution	None	1.24	0
Idem + disodium EDTA	"	1.17	5.7
Physiologic saline	Non-dialyzed	2.42	2.8
Disodium EDTA + saline	"	2.43	1.4
" calcium EDTA + saline	"	2.43	1.4
$MgSO_4$	"	2.43	1.4
Disodium EDTA + $MgSO_4$	"	2.52	0
" calcium EDTA + $MgSO_4$	"	2.45	0
(b) *Tryptamine**			
Balanced buffered salt solution	None	4.21	9.7
Idem + disodium EDTA	"	3.92	3.7
Physiologic saline	Non-dialyzed	8.35	1.4
Disodium EDTA + saline	"	6.69	1.7
" calcium EDTA + saline	"	7.49	1.6
$MgSO_4$	"	6.66	2.6
Disodium EDTA + $MgSO_4$	"	3.46	1.7
" calcium EDTA + $MgSO_4$	"	6.58	1.8
(c) *Tyramine**			
Balanced buffered salt solution	None	1.39	6.1
Idem + disodium EDTA	"	1.31	6.5
Physiologic saline	Non-dialyzed	7.69	6.6
Disodium EDTA + saline	"	7.39	19.4
" calcium EDTA + saline	"	8.79	7.7
$MgSO_4$	"	7.69	6.6
Disodium EDTA + $MgSO_4$	"	6.33	8.0
" calcium EDTA + $MgSO_4$	"	7.69	6.6
(d) *BAS**			
Balanced buffered salt solution	None	.84	8.6
Idem + disodium EDTA	"	.99	9.8
Physiologic saline	Non-dialyzed	1.34	0.9
Disodium EDTA + saline	"	1.28	1.9
" calcium EDTA + saline	"	1.12	10.8
$MgSO_4$	"	1.26	5.7
Disodium EDTA + $MgSO_4$	"	1.31	5.5
" calcium EDTA + $MgSO_4$	"	1.31	5.5
(e) *5HIAA**			
Balanced buffered salt solution	None	.15	25.0
Idem + disodium EDTA	"	.15	0
Physiologic saline	Non-dialyzed	.57	9.8
Disodium EDTA + saline	"	.56	6.7
" calcium EDTA + saline	"	.56	6.7
$MgSO_4$	"	.61	3.1
Disodium EDTA + $MgSO_4$	"	.60	0
" calcium EDTA + $MgSO_4$	"	.61	3.1
(f) *Norepinephrine**			
Balanced buffered salt solution	None	.32	8.7
Idem + disodium EDTA	"	.31	0
Physiologic saline	Non-dialyzed	1.34	2.1
Disodium EDTA + saline	"	1.22	2.2
" calcium EDTA + saline	"	1.32	4.2
$MgSO_4$	"	1.22	2.2
Disodium EDTA + $MgSO_4$	"	1.06	2.6
" calcium EDTA + $MgSO_4$	"	1.20	2.3

* See *Methods* for details.

serotonin by analysis of spectrum. 5HIAA showed a slight but definite shift of fluorescent spectrum toward the visible end of the spectrum in all samples following extraction, with or without addition of plasma to the *in vitro* system. These were consistent and reproducible observations.

Discussion. Enhanced solubilization of serotonin in benzene-butanol in presence of human plasma was suppressed when virtually all originally-bound and free divalent cations were dialyzed out of the plasma prior to testing (Table I-a). When Ca^{++} or Mg^{++} was added in molecular excess in the presence of plasma, however, solubilization was somewhat decreased below the level attained with nondialyzed plasma alone (Table II-a). If solubilization of serotonin in benzene-butanol depended simply on complexing with free divalent cations, unchanged or increased solubilization might have been expected in the circumstances, and presence of plasma should not have been necessary. Such not being the case, the observations suggested that plasma bound divalent cations were important in the solubilization and that addition of an excess of free Ca^{++} or Mg^{++} caused a shift in equilibrium of complexing of serotonin with bound and free divalent cations.

The complete suppression of the solubilization of serotonin seen in the presence of plasma in benzene-butanol on addition of either Ca^{++} or Mg^{++} and disodium EDTA to the *in vitro* system (Table II-a) was expected from earlier data, in view of the tighter chelating action of disodium EDTA over that of plasma binding substances. The order of addition of reactants to the system, prior to extracting with benzene-butanol, placed serotonin last in the system. Thus serotonin apparently complexed with divalent cations bound either to plasma components or to chelator. No fat solubilization could be demonstrated with EDTA-chelated complexes, however. Addition of disodium calcium EDTA had little suppressive effect on solubilization of serotonin benzene-butanol in the presence of Mg^{++} (Table I-a), in contrast. Since the molar concentration of chelated magnesium far exceeded the molar concentration of either calcium or serotonin, this suggested preferential (and perhaps sole) binding of serotonin with Ca^{++} rather than by Mg^{++}. Where suppression of fat solubilization of serotonin was seen in the presence of excess Mg^{++} and disodium EDTA, it is probable that the higher stability constant of Ca^{++} chelates over Mg^{++} chelates resulted in considerable transfer of bound calcium from plasma components to the tighter binding of the added chelator before the added Mg^{++} was chelated.

Under the conditions of the present study, while solubilization of several substances in benzene-butanol was demonstrable in the presence of plasma (Table III), only tryptamine of the substances tested shared with serotonin the characteristics which suggested a possible role of plasma bound calcium in its solubilization in benzene-butanol. Collier(5) has summarized fairly recently many biologic actions shared by tryptamine and serotonin. Whether the present findings will ever be shown to be related to biologic events in the sense of indicating a role in the transport of bound calcium through lipoidal cellular walls is a matter of conjecture at the moment. It is of interest that the *in vitro* conditions imposed in the present studies duplicated those of certain of the platelet uptake studies reported earlier(1). Where this was true, some parallelism was evident between degree of solubilization of serotonin in benzene-butanol in the present studies and degree of uptake of serotonin by intact platelets in the earlier studies. A chief difference between the present and former studies where *in vitro* conditions were otherwise duplicated was in the use of 75% platelet-free plasma in the present experiments rather than platelets in 4% plasma as in the prior(1) experiments. The effect of varying plasma concentration has not yet been tested in the present system, nor has plasma fractionation been done as yet to try to determine wherein plasma activity lies as regards solubilization of the test substances in benzene-butanol. Both studies are planned.

Summary. Using an *in vitro* system patterned after that of Woolley(2), serotonin, tryptamine, tyramine and several related test substances were solubilized in benzene-bu-

tanol in varying degree in the presence of human plasma. Only serotonin and tryptamine of the substances tested were significantly influenced in their fat solubilization in the presence of plasma by addition of an excess of Ca^{++} or Mg^{++} and/or chelators to the *in vitro* system. Solubilization of both serotonin and tryptamine in benzene-butanol in presence of plasma was decreased somewhat by addition of Ca^{++} or Mg^{++} or disodium EDTA in excess to the system. It was eliminated by addition of disodium EDTA (but not disodium calcium EDTA) and either Ca^{++} or Mg^{++} to the system, also by prolonged prior dialysis of the plasma against balanced buffered salt solution plus disodium EDTA. The overall findings supported the hypothesis that divalent cations bound to a substance or substances present in human plasma formed a complex with serotonin or tryptamine to solubilize these usually lipid-insoluble materials in benzene-butanol, in the absence of competing chelators.

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Influence of Epinephrine and Fasting on Free Fatty Acid Mobilization in Goldthioglucose-Induced Obesity. (26803)

NORMAN B. MARSHALL (Introduced by R. O. Stafford)
Department of Nutrition and Metabolic Diseases, The Upjohn Co., Kalamazoo, Mich.

Regulatory and metabolic types of obesity in mice have been described by Mayer(1). In the former, accelerated rates of lipogenesis are considered to be a consequence of hyperphagia resulting from hypothalamic damage. In the latter, it is suggested that hyperphagia is secondary to hyperlipogenesis arising from an inherent metabolic defect.

Recently it was reported that obese-hyperglycemic mice (O-H), representing metabolic obesity, exhibit an impairment in their ability to mobilize free fatty acids (FFA) *in vitro*, from adipose tissue in response to fasting and epinephrine stimulation(2).

To determine whether the defect is related to the etiology of the obese-hyperglycemic syndrome, or if it is a nonspecific consequence of obesity *per se*, the influence of fasting and epinephrine stimulation was investigated in goldthioglucose† obese (GTG) mice, an example of regulatory obesity.

Materials and methods. 50-60 mg portions of epididymal adipose tissue were obtained from goldthioglucose-induced obese CBA mice in the dynamic phase of obesity and from lean controls. Obese mice were approximately 5 months old, weighed 42-50 g, had been obese for 2 months, and were still actively gaining weight. Lean mice were the same age and weighed 27-37 g. Tissues were incubated in a Krebs-Ringer bicarbonate medium containing 0.1% glucose and 2% bovine serum albumin. FFA content of adipose tissue and release into the medium were measured 3 hours following addition of either epinephrine* or, as in the case of controls, water. Analytical methods used are identical with those reported earlier(2).

Results and discussion. Adipose tissue from fed goldthioglucose obese mice and lean controls produced equivalent amounts of FFA during incubation without hormonal stimulation (Fig. 1). In the lean group, epinephrine induced an increase in FFA content of both tissue and medium which amounted to a 6-fold increase in total FFA production ($p<.001$). Similarly, epinephrine

* Adrenalin, Parke Davis Co., Detroit, Mich.

† Goldthioglucose, courtesy Schering Corp., Bloomfield, N. J.