hypertension are not in agreement with our clinical research(1,2) in which there was evidence that the biological half-life ($T_{½}$) of ^{22}Na often was prolonged in patients with uncomplicated essential hypertension. The present work on rats from 2 inbred strains having opposite genetic susceptibilities to hypertension induced by salt suggested the contrary: the biological $T_{½}$ of ^{22}Na was found to be *shorter* in the strain predisposed to hypertension, and did not appear to be related to elevations in blood pressure, *per se*. Tissue sodium was not found to be increased in animals with hypertension, nor was there evidence of potassium depletion. It is possible that the shorter biological $T_{½}$ of ^{22}Na in the group predisposed to hypertension was due to a slightly greater food (*i.e.*, NaCl) intake as compared with the animals resistant to the development of hypertension.

In sum, this evidence strongly suggests that however excess salt ingestion induces hypertension, it is not through gross accumulation of sodium, or depletion of potassium, in tissues. However, by the techniques used here, focal accumulation, *e.g.*, in arterioles(21), would not have been found.

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Binding of Sulfobromophthalein (BSP) Sodium and Indocyanine Green (ICG) by Plasma α_1 Lipoproteins.* (31299)

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Sulfobromophthalein (BSP) and the tricarbocyanine dye, indocyanine green (ICG), are water-soluble, anionic dyes. Both are excreted so efficiently by the liver in man and other animals that they may be employed to measure hepatic blood flow and to quantify hepatic function precisely under a wide variety of conditions(1,2). Although the data available on the biliary output of the 2 dyes in dog suggest that they may share, at least in part, a common excretory pathway, BSP and ICG are quite different in their physico-

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chemical properties. The studies presented here indicate that BSP is monomolecularly dispersed in water, isotonic saline and buffer solutions, and that ICG aggregates in these media. In circulating blood a fraction of BSP and most of the ICG is associated with the α_1 lipoproteins.

Materials. Blood was obtained from fasting patients on the wards of the Presbyterian Hospital and from fasting normal dogs. Free flowing samples of common duct bile were collected by catheter through a Thomas cannula in the same dogs. Normal human serum albumin was obtained from Cutter Labs.; rabbit anti-whole human serum from Berringweke Ag.; sodium taurocholate from Nutritional Biochem. Corp.; BSP and ICG from Hynson, Wescott and Dunning; $BS^{35}P$ from Nuclear-Chicago; Sephadex G-100 and G-200 from Pharmacia.

Methods and results. I. *Aggregation of ICG in solution.* Some difficulties in quantifying and "stabilizing" ICG led to the following studies on the dialysis, ultrafiltration and spectral absorption of the dye.

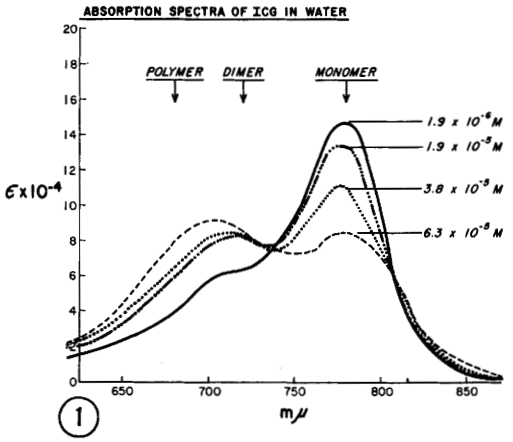
a. *Dialysis of ICG.* In 9 experiments, with duplicate measurements, 2 ml of dye at 5 to 8 mg% concentration in Millipore® filtered water, isotonic saline, phosphate, Tris or barbital buffers, pH range 6.0 to 8.6, were placed in cellophane sacs and rocked 24 to 42 hours against 6 ml of solvent. Not more than 4% of the theoretical equilibrium concentration of ICG was found in the pure water dialysates and less than 0.5% in saline or buffer dialysates. In the latter solutions, aggregation of the dye was clearly observed within the sacs.

b. *Ultrafiltration of ICG.* In 9 experiments, with duplicate measurements, half the volume of dye at 1.3 to 12.5 mg% concentration in Millipore filtered water, phosphate or Tris buffers, pH range 6.0 to 7.4 was filtered through cellophane tubing by one atmosphere negative pressure. Ultrafiltrates from water solutions of 1.3, 2.5 and 12.5 mg% ICG concentrations contained, respectively, 17, 10 and 3% of the original dye concentration. Not more than 0.5% of the original concentration of dye appeared in the ultrafiltrates of isotonic buffer solutions.

c. *Absorption spectrum of ICG.* The absorption spectrum of ICG was determined in a variety of solvents using the Beckman DU spectrophotometer (with 0.5 and 1.0 cm cells). Readings were made as quickly as possible after preparation of solutions in: distilled water (1.9×10^{-6} to 6.3×10^{-5} M); methanol (1.9×10^{-6} to 1.9×10^{-5} M); 0.001 to 1.0% sodium taurocholate (1.9×10^{-6} M) and 1.0% sodium taurocholate (2.0 to 4.0×10^{-6} M); isotonic saline (2.0×10^{-6} M); human serum (1.9 to 9.7×10^{-6} M); canine serum and canine bile (1.9 to 4.0×10^{-6} M). When molar absorptivities (extinction coefficients, ϵ), were plotted against wave length in mμ, a single absorption peak was found at 780 mμ for low concentrations of ICG in water. With increasing ICG concentrations in water this peak was progressively diminished and a second peak appeared at 720 to 700 mμ. In other solvents, the absorption spectrum of ICG showed a "shoulder" in the region of 720 to 740 mμ, but a second peak did not develop at measurable concentrations of ICG in methanol, undiluted bile, serum and sodium taurocholate (Fig. 2). A marked difference in the absorption peak and ϵ is noted for ICG above and below the critical micellar point (0.4 g%)(3) of the bile salt. The highest observed ϵ for ICG was in methanol.

In contrast to the observed behavior of ICG, BSP in aqueous solutions was found to be dialyzable, ultrafiltrable and have a single absorption peak (at pH>12) over a 30-fold concentration range. The ϵ for BSP was higher in distilled water than in any of the other solvent systems. Relative to the ϵ in water, the value of ϵ was 0.96 in 1% taurocholate, 0.92 in human serum, 0.88 in canine bile and 0.78 in methanol.

II. *Association of ICG and BSP with serum proteins.* ICG and BSP have been reported to be bound preponderantly to the albumin in plasma(1,4,5). However, radioactively tagged isotopes and new techniques for protein fractionation such as gel filtration, immunoelectrophoresis, ultracentrifugal flotation, and density gradient sedimentation have made possible a reexamination of the distri-



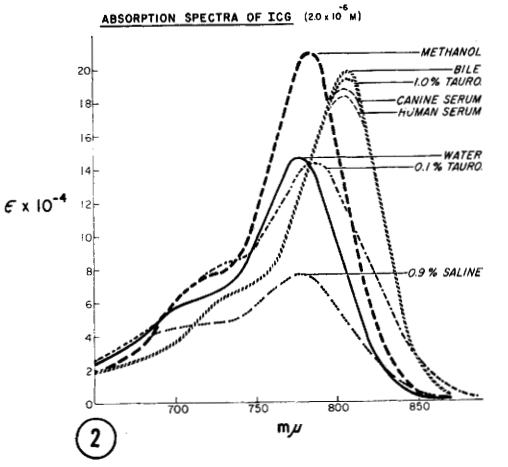


FIG. 1. Absorption spectra of ICG in water, at 4 different molar concentrations.
FIG. 2. Absorption spectra of ICG at 2×10^{-5} M in different solvents. ICG is shown in 2 concentrations of sodium taurocholate, one above the other below the critical micellar concentration (0.4 g%) of the bile salt.

bution of BSP and ICG among plasma proteins.

a. *Gel filtration* of sera containing ICG and BSP. Using the method of Flodin(6), 35 by 400 mm columns were packed with Sephadex G-100 or G-200 and were eluted with Tris-HCl 0.1 M-NaCl 0.2 M; pH 7.4 at 6°C. Two ml of human or canine serum were layered at the gel buffer interface and eluted in 40 to 70 fractions of 1.5 to 3.0 ml volume. The protein concentration of each filtered fraction was determined by spectral absorption at 280 mμ, the ICG at 810 mμ and BSP at 580 mμ (at a pH>12) after addition of 20% KOH. Filtration of the sera of both species resulted in 2 protein peaks (I, II, in order of elution) when G-100 was used, and 3 peaks (I, II, III) with G-200, as described by Flodin. G-100 filtration of 14 canine sera or plasmas and 11 human sera gave the following mean distribution of protein: for canine sera, 58% of the protein was in peak I (globulin) and 42% in peak II (albumin) (S.D. ± 2.8); for human sera 51% was in peak I and 49% in peak II (S.D. ± 2.2). G-200 filtration of 2 canine and 2 human sera gave the following mean distribution of protein: for canine sera, 10% of the protein was in peak I, 45% in peak II, 45% in peak III; for human sera, 16% in peak I, 37% in peak II and 47% in peak III. The presence of dyes did not appear to alter these distributions. Nine canine and 9 human sera containing BSP or ICG and 3 canine sera containing both dyes were filtered on G-100. Table I shows the percentage of dye in peaks I and II. In canine sera, 60% of the BSP and 95% of the ICG was in peak I, while in human serum 30% of the BSP and 80% of the ICG was similarly associated. For comparison with the above findings 2 human sera with elevated bilirubin levels (5 and 30 mg%), 1 human serum pool to which Evan's blue was added and 3 canine sera to which "Risa" (albumin I^{131} tagged) was added, showed approximately 10% (range 6-14%) of these substances in peak I. Because of slow filtration rates through G-200, and resultant dye deterioration, only 4 sera containing dye were separated on this gel. The dye distribution in canine serum was: for ICG, peak I, 22%; peak II, 68%; peak III, 10%; and for BSP, peak I, 3%; peak II, 70%; peak III, 27%. In human serum the dye distribution was: for ICG, peak I, 33%; peak II, 53%; peak III, 14%; and for BSP, peak I, 10%; peak II, 25%; peak III, 65%.

Eight solutions of human albumin (Cutter, 25%), which had been diluted with phosphate-chloride buffer, (μ 0.16, pH 7.4), to a final concentration of 4.1 to 5.0 g%, were filtered through G-100 gel. A mean of 10% of the protein was found in peak I. Filtration of BSP in 4 albumin solutions at BSP/alb

TABLE I. Distribution* of Dyes in Two Serum Protein Peaks† Following Sephadex G-100 Filtration.

BSP			ICG			BSP			ICG		
Conc, mg %	Peak I	Peak II	Conc, mg %	Peak I	Peak II	Conc, mg %	Peak I	Peak II	Conc, mg %	Peak I	Peak II
Canine serum (dyes added *in vitro*)						Human serum (dyes added *in vitro*)					
2.9	60	40									
4.7	72	28	‡4.7	94	6	3.9	34	66	8.3	85	15
7.2	80	20									
7.4	75	25	6.9	94	6	9.1	38	62	8.2	82	18
20.0	55	45									
Mean	68	32		94	6		36	64		84	16
Canine serum (dyes present *in vivo*)						Human serum (dyes present *in vivo*)					
‡3.0	57	43				4.4	27	73			
3.0	64	36	‡8.1	95	5	10.0	23	77	.6	82	18
3.0	70	30				13.0	34	66			
‡3.7	58	42	‡8.6	95	5	23.0	27	73			
16.0	52	48									
Mean	60	40		95	6		28	72			

* Distribution expressed as % of total dye.
† Peak I, proteins having mol wt >100,000; Peak II, proteins having mol wt <100,000.
‡ Serum in which both dyes are present.

molar ratios of 0.14 to 4.0, showed a mean of 4% of the dye in peak I. Three similar filtrations of ICG-albumin solutions showed a mean of 19% of the dye in peak I.

b. *Paper and immunoelectrophoretic* analysis of peak I and peak II fractions following G-100 filtration. Fractions collected from filtration of 4 canine and 4 human sera were concentrated by ultrafiltration, pipetted on sheets of 3 MM Whatman paper and subjected to 18-hour electrophoresis with phosphate buffer 0.038 M, pH 7.9, at 6°C. The resultant patterns were dyed with bromphenol blue for protein positions and sudan black for lipid. Peak I fractions were shown to contain an α_2 globulin and β lipoprotein at the protein front, α_1 lipoproteins, β and γ globulins at the summit and traces of albumin appearing thereafter. Peak II fractions contained albumin, an α_1 and a β globulin in the transferrin position. BSP or ICG, when present, were clearly observed by day or ultraviolet light in a position corresponding to the α_1 lipoprotein of peak I and, in the case of BSP, to the albumin of peak II. At high dye levels in serum (above 8 mg% ICG or 15 mg% BSP) both dyes were also faintly detectable in the β lipoprotein position of peak I.

On one occasion a specimen of human serum containing 8.3 mg% ICG (added *in vitro*) was separated by G-100 filtration and a total of 82% of the dye was found in peak I eluate fractions. Fractions corresponding to the summit of peak I, containing 51% of the dye in the original serum, were concentrated by ultrafiltration and applied to an immunoelectrophoretic plate (2.5% Noble agar, 0.038 M phosphate buffer, pH 7.9). Following electrophoresis, the dye position was traced, before it had faded. The plate was developed overnight with antiserum and then placed over the dye tracing. The clearly defined dye area lay in the α_1 position, but a faint trace was discernible in the β position.

c. *Paper electrophoresis of sera containing* $BS^{35}P$. $BS^{35}P$ was added *in vitro* to 4 canine and 4 human serum pools with final concentrations of 0.1, 0.2 and 20 mg% BSP. Aliquots (0.4 ml) of each were applied to lines 4 cm long on wide sheets of 3 MM Whatman paper, and electrophoresed for 18 hours at 6°C, using barbital buffer .075 μ and pH 8.6. The resultant protein patterns were outlined under ultraviolet light. One centimeter strips were cut along the outside edge of each pattern; one strip being dyed with bromphenol blue, the other with sudan black. The undyed center strip was cut into zones according to the protein pattern. Each zone was eluted with 0.5 ml 0.1 N NH_4OH and

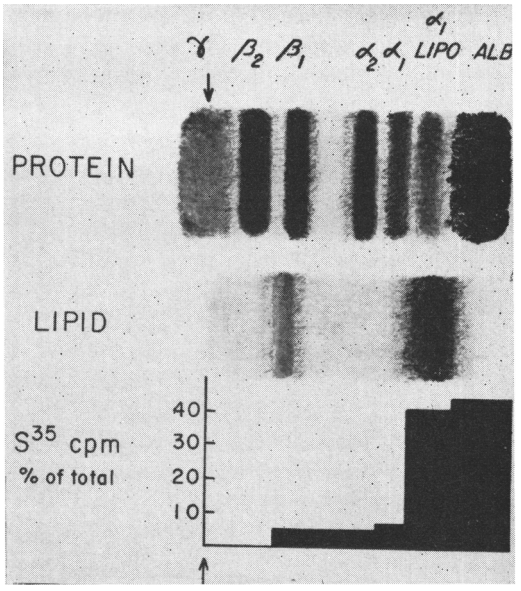


FIG. 3. Distribution of $BS^{35}P$ (0.2 mg %) in canine serum proteins following paper electrophoresis in barbital buffer, 0.075 μ, pH 8.6. (tracing)

counted in Bray's medium in a tri-carb scintillation spectrometer (Packard model #3003). Fig. 3 shows the patterns obtained from the canine sera having 0.2 mg% BSP levels and the distribution of the S^{35} labelling in those samples. Table II summarizes the results on $BS^{35}P$ distribution in 4 canine and 4 human sera. An equal distribution of dye between the a_1 lipoproteins and albumin was found in canine serum. In human serum the two proteins did not separate under these conditions.

d. *Density gradient sedimentation*(7) of sera containing ICG and BSP. Two canine sera containing ICG and BSP drawn after separate but simultaneous injections of equimolar solutions of the two dyes, were layered on a 10 to 40% sucrose gradient, centrifuged at 35,000 rpm for 16 hours in an SW 39 rotor of a Spinco Model L preparative ultracentrifuge at 10 to 15°C, and fractions were collected dropwise in 23 tubes. In Fig. 4 are plotted the measured optical densities of protein(8), BSP and ICG in each tube. Both dyes showed concentration peaks closer to the top of the centrifuge tube than the albumin peak of serum protein. This suggested that the dyes were associated with lipoproteins which have specific density less than albumin.

e. *Ultracentrifugal flotation*(9) of lipoproteins in sera containing BSP. Four human sera and one canine serum, drawn 5 minutes after intravenous injection of BSP, were diluted to a final density of 1.21 by addition of an appropriate volume of a NaCl-KBr solution of 1.350 density. The mixtures were centrifuged 16.5 hours at 40,000 rpm in a 40.3 rotor of a Spinco Model L preparative ultracentrifuge at 10 to 15°C. The plastic centrifuge tubes, which contained the serum solutions, were then cut in the center of the clear zone between the supernatant lipoproteins and the rest of the serum proteins. The supernatant volume was about 1/5 of the total. Analysis of the supernatant and infranatant solutions for total protein, albumin (I^{131} tagged) and BSP, showed the following per cent of those components in the lipoprotein

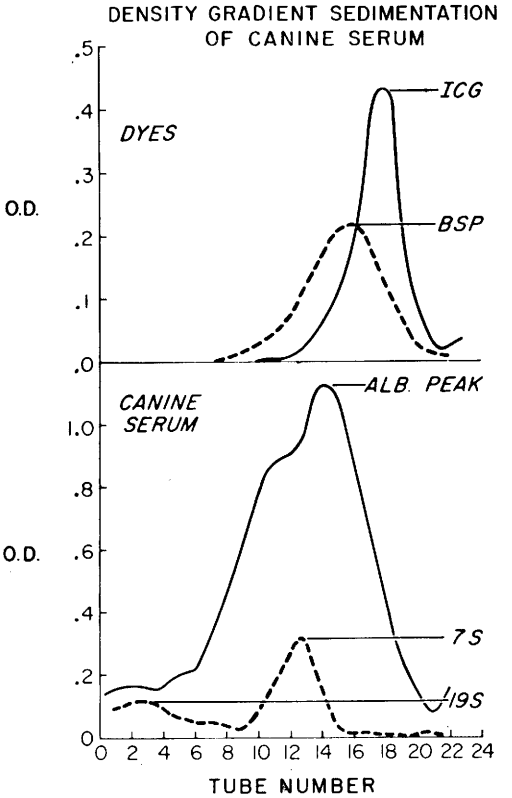


FIG. 4. Concentrations of proteins and dyes in fractions collected after density gradient sedimentation of an *in vivo* specimen of canine serum, ICG (13 mg%), BSP (10 mg%). The 7 S marker was human fraction II; the 19 S marker was porcine thyroglobulin.

TABLE II. Distribution* of $BS^{35}P$ Following Paper Electrophoresis† of Serum.

Conc, mg %	Alb	α_1 lipo	α_1 glyco	α_2	β	γ	Total glob
			$BS^{35}P$ in canine serum				
0.1	44	41	7	4	4	0	56
0.1	42	35	13	7	4	0	59
0.2	39	36	12	8	5	0	61
20.0	56	25	7	6	6	0	44
			$BS^{35}P$ in human serum				
0.1	80	6	4	3	5	0	20
0.1	81	11	2	2	4	0	19
0.2	80	6	6	0	8	0	20
20.0	64	17	5	3	7	4	36

* Distribution expressed as % of total $BS^{35}P$.
† Electrophoresis in barbital buffer, 0.075 μ, pH 8.6. The space between alb and α_1 glyco in human serum shows no protein stain.

fraction: in human sera a mean of 9% of the protein (range 6-11%), 2% of the albumin and 44% of the BSP (range 38-49%) were present in the supernatant solution and in canine serum 13% of the protein and 84% of the BSP were similarly associated.

Discussion. Aggregation of ICG. The ease with which BSP in water and electrolyte solutions passed through cellophane membranes indicated monomolecular dispersion of this solute. Under the same conditions, ICG in water penetrated membranes minimally and further evidence of aggregation of this dye was deduced from its spectral behavior in a variety of solvents. Whereas BSP in water showed a single absorption peak over a wide concentration range, ICG in water showed a single absorption peak (monomer) for dilute solutions, but a gradual diminution of that peak and a new absorption peak (dimer, polymer) as ICG concentrations were raised. Detailed analysis of similar spectral behavior has served as evidence for the polymerization of acridine orange(10) and thiacyanine dyes (11).

Serum protein binding of BSP and ICG. Filtration of sera containing BSP or ICG through Sephadex G-100 showed that, in canine serum, 90% of the ICG and 60% of the BSP appeared in the globulin peak. In human serum that peak contained 80% of the ICG and 30% of the BSP. These findings could not be attributed to dimerization or dimensional alteration of albumin molecules by the two dyes, inasmuch as the dyes were found in the albumin peak when pure dye-albumin solutions were gel filtered. Therefore efforts were made to identify the globulins with which the dyes were associated in whole serum, and the techniques of paper and immunoelectrophoresis, ultracentrifugal flotation and density gradient sedimentation were employed. The electrophoretic positions of the dyes relative to those of protein and lipid in canine serum, and the knowledge(12) that the concentration of α_1 lipoproteins found in canine serum is twice that of human serum, all suggested an association with this globulin group. Electrophoretic analysis of gel eluates and centrifugal techniques applied to whole serum also supported this hypothesis. It is possible that the percentage of dye found in the lipoprotein fraction by the ultracentrifugal flotation method may be exaggerated by some dye displacement at the high salt concentrations employed(13). For this reason the values obtained from gel filtration probably are more accurate. Although the role of the α_1 lipoproteins in endogenous triglyceride transport has recently been pointed out by Fredrickson *et al*(14), the part played by those proteins in dye transport to the liver has not been considered previously. Whether the liver removes the portion of the dyes associated with the α_1 lipoproteins in a different manner or at a different rate from the albumin bound moiety, cannot be stated at present.

Summary. The colloidal nature of ICG in aqueous solutions has been characterized by dialysis, ultrafiltration and spectral absorption measurements. Using gel filtration (Sephadex G-100 and G-200), ultracentrifugal flotation, density gradient sedimentation, immuno- and paper-electrophoretic methods, evidence has been accumulated to show that 90% of the ICG in canine serum and 80% of the dye in human serum is associated with globulins. Comparable values for BSP are 60% and 30%. The data suggest that the α_1 group of lipoproteins has a higher affinity for the dyes than albumin.

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Water Permeability of Normal and Pathological Lake Trout Corneas.* (31300)

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Allison(1-3) reported that lake trout are susceptible to sunburning and that they also develop corneal and lenticular lesions when reared in a hatchery environment. Hoffert and Fromm(4) have described a corneal lesion which occurs in lake trout from the Harrietta, Mich., hatchery and have categorized the symptoms of the lesion into stages. The purpose of this paper is to present data on the water permeabilities of lake trout corneas representative of the stages as described by Hoffert and Fromm(4). We also investigated the roles of the endothelial and epithelial layers of the cornea in the maintenance of the permeability characteristic of normal or non-pathological corneas.

Materials and methods. The 2- to 4-year-old lake trout *(Salvelinus namaycush)* used in these experiments were provided by the Michigan Conservation Department from their hatchery at Harrietta, Mich. In the laboratory they were kept in fiberglass lined tanks at 9°C under conditions of 15 hours light and 9 hours darkness each day.

A technique for holding trout corneas *in vitro* between 2 chambers in such a manner as to allow the fluid bathing each surface to be regulated has been described(5). In all experiments chemically defined tissue culture media (TC 199, Difco Laboratories, Detroit, Mich.) was used to bathe the endothelial surface of corneas and tap water was used to bathe the epithelial surfaces. Depending upon which direction water movement was to be investigated, 0.2 ml of tritiated water (0.1 mc HTO/ml) was added to either the 3 ml of TC 199 or the 3 ml of tap water. Four hours later, 20 lambda samples of the 2 media were taken using disposable micropipettes (Drummond Microcaps), and transferred to glass vials containing 15 ml of a liquid scintillation counting solution. That part of each cornea which was exposed to the 2 media was dissected free and also placed in 15 ml of counting solution. This solution contained a primary scintillator (5 g PPO; 2,5-diphenyloxazole), a secondary scintillator (50 mg α-naphthylphenyloxazole), and a solvent system of 80 g naphthalene plus dioxane to make 1 liter. Since dioxane is a good dehydrant it

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