References and Notes

1. B. R. Margulis, J. L. Ambrus, I. B. Mink, J. C. Stryker, *Am. J. Obstet. Gynecol.* **93**, 161 (1965).
2. L. Poller and M. Thompson, *Br. Med. J.* **2**, 23 (1966).
3. F. Lecompte and S. Renaud, *Thromb. Diath. Haemorrh.* **29**, 510 (1973).
4. S. Renaud and P. Gautheron, *ibid.* **30**, 299 (1973).
5. N. Shevde, J. Sussman, F. Rosner, *Am. J. Obstet. Gynecol.* **132**, 303 (1978).
6. L. McGregor, R. Morazain, S. Renaud, in *Haemostasis and Thrombosis* (Academic Press, New York, 1979), p. 565.
7. M. Ciavatti and S. Renaud, *Horm. Metab. Res.* **11**, 415 (1979).
8. M. Ciavatti, G. Michel, S. Renaud, *Biochim. Biophys. Acta*, in press.
9. The anticoagulant sodium nitrate, 3.8 percent, was used for the collection of the platelet-rich plasma; coagulation was then initiated by the addition of calcium chloride (see Fig. 1).
10. N. G. Ardlie, M. A. Packham, J. F. Mustard, *Br. J. Haematol.* **19**, 7 (1970).
11. S. Renaud and F. Lecompte. *Circ. Res.* **27**, 1003 (1970).
12. R. W. Colman, S. J. Shattil, J. S. Bennett. *Blood* **46**, 1033 (1975).
13. P. L. Yeagle, R. B. Martin, A. K. Lala, H. K. Lin, K. Bloch, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4924 (1977).

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Somatostatin: Occurrence in Urinary Bladder Epithelium and Renal Tubules of the Toad, *Bufo marinus*

Abstract. *Immunohistochemical techniques were used to detect immunoreactive somatostatin-like material in toad urinary bladder epithelium and in kidney distal tubules and collecting ducts. This material has immunological and chromatographic properties identical to those of synthetic cyclic somatostatin. The occurrence of somatostatin-like material in antidiuretic hormone-sensitive portions of the renal urinary system suggests a local regulatory or paracrine role for somatostatin.*

The recent demonstration that somatostatin inhibits the effects of vasopressin on water transport in toad urinary bladder (*1*) and that this tissue contains relatively large amounts of immunoreactive somatostatin led Forrest and his colleagues to propose that this peptide is an intrinsic regulator of epithelial transport in toad bladder (*1a*). Somatostatin, a tetradecapeptide originally identified as a hypothalamic hypophysiotropic hormone that inhibits secretion of growth hormone (*2*), is widely distributed in the extrahypothalamic central nervous system, the peripheral nervous system, various tissues of the gastroenteropancreatic system (*3, 4*), and thyroid tissue (*5*), but has not been consistently measured in rat kidney (*3, 4, 6*). The dual neuronal and gastrointestinal distribution of somatostatin has been demonstrated in all vertebrate classes (*7*).

In the gastrointestinal tract and pancreas, somatostatin-secreting cells have been found adjacent to other types of secretory cells whose function is influenced by somatostatin. This observation led to the postulation that such somatostatin-secreting cells outside the brain exert a local, "paracrine," effect on neighboring cells (*8*). In this study we demonstrated that somatostatin is present in the layer of toad urinary bladder epithelium that is responsive to antidiuretic hormone. Further, because somatostatin influences water resorption in mammalian kidney (*9*), we measured somatostatin in kidney by immunoassay and demonstrated its presence immunohistochemically in collecting ducts and distal tubules (but not in proximal tubules or glomeruli). The presence of this peptide modulator in renal tissue, when considered together with evidence that somatostatin impairs the hydroosmotic response to antidiuretic hormone in toad urinary bladder (*1*) and inhibits the response to this hormone in the mammalian kidney in vivo (*9*), suggests that somatostatin-like material may have a paracrine role in the renal-urinary tract.

Urinary bladders and kidneys were quickly excised from decapitated *Bufo marinus* (*10*), rinsed in 200 m*M* NaCl, and frozen on dry ice for immunofluorescence processing or fixed for 2 to 4 hours in 10 percent neutral buffered formaldehyde for peroxidase-antiperoxidase (PAP) immunohistochemistry (*11-13*). Acetic acid extracts of tissue homogenates were lyophilized and reextracted with acetone-petroleum ether (*14*). Procedures for Biogel P4 chromatography, protein determination, and somatostatin radioimmunoassay have been described (*15*).

Immunoreactive somatostatin was readily demonstrated by immunofluorescence in the epithelial and subepithelial layer of urinary bladder (Fig. 1a). Concentrations of antibody to somatostatin ranged from 1:50 to 1:200. Fluorescence was most intense in the epithelial layers, where it was present in virtually all cells, and was less intense in the subepithelial layer. Immunoreactivity was eliminated by preabsorption of the antiserum with synthetic somatostatin. In the kidney, a distinct pattern of immunoreactive somatostatin was observed in many tubules (Fig. 1b). As in the bladder, immunofluorescence was eliminated by preabsorption with synthetic somatostatin.

Immunohistochemical preparations with the PAP technique confirmed that immunoreactive somatostatin was distributed in the urinary bladder epithelium. In the kidney, positive reaction product was concentrated in most collecting ducts and many distal tubules (Fig. 1c). Positive staining was observed at antibody to somatostatin titers between 1:200 and 1:1000, but staining was negative at an antibody dilution of

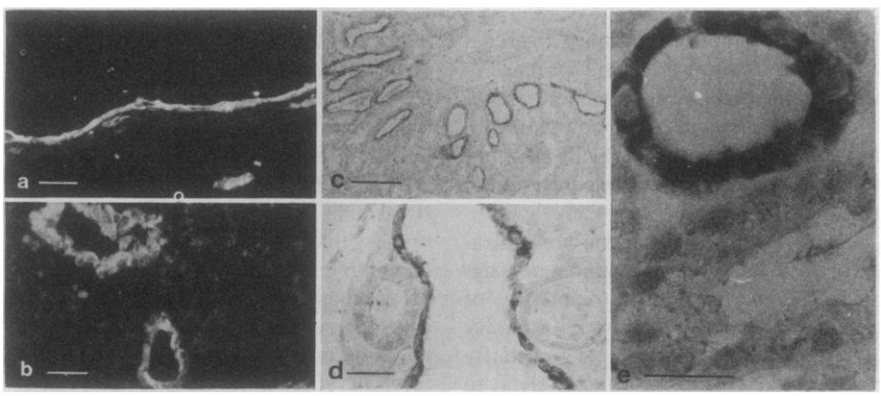

Fig. 1. (a and b) Fluorescence micrographs of toad urinary bladder (a) showing immunoreactive somatostatin in the epithelial layer and of toad kidney (b) showing somatostatin in two tubules, with many nonfluorescing tubules. (c, d, and e) Peroxidase-antiperoxidase visualization of somatostatin in kidney distal tubules and collecting ducts, with many somatostatin-negative proximal tubules; (d) shows somatostation-positive collecting duct and somatostatin-negative proximal tubule (left of duct) and glomerulus (right of duct); and (e) shows a tubule with dense cytoplasmic immunoreactivity (upper part of photo) in contrast to a complete absence of immunoreactivity in the proximal tubule (lower part of photo). Scale bars: 25 μm in (a), (b), (d), and (e); 100 μm in (c).

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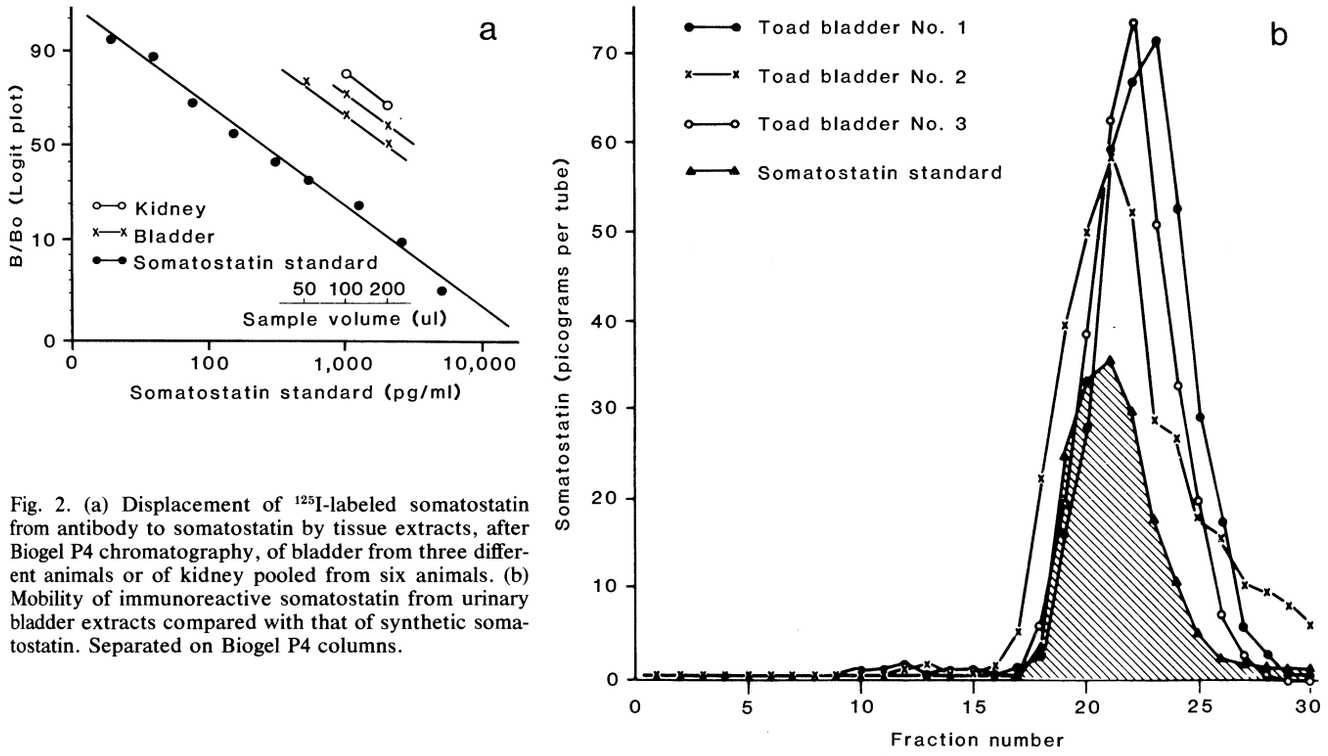

Fig. 2. (a) Displacement of ^{125}I-labeled somatostatin from antibody to somatostatin by tissue extracts, after Biogel P4 chromatography, of bladder from three different animals or of kidney pooled from six animals. (b) Mobility of immunoreactive somatostatin from urinary bladder extracts compared with that of synthetic somatostatin. Separated on Biogel P4 columns.

1:5000 or when antibody was preabsorbed with synthetic somatostatin. Preabsorption of the antibody with arginine vasopressin or arginine vasotocin ($2 \times 10^{-4}M$) did not alter positive immunoreactivity. The intense staining was demonstrated throughout the cell (Fig. 1e) in collecting ducts and distal tubules (Fig. 1, c to e, upper tubule), but not in the cells of proximal tubules (Fig. 1, d and e, lower tubule) nor in glomeruli (Fig. 1d).

Extracts of the toad bladder (N = 46) contained 4.5 ± 0.3 pg of somatostatin per microgram of protein; kidney (N = 4), 0.015 ± 0.002 pg per microgram of protein. The immunoreactivity of samples from both the bladder and kidney showed parallel displacement in the radioimmunoassay when compared with standard cyclic somatostatin (Fig. 2a), and bladder extracts eluted from Biogel P4 with the same mobility as standard somatostatin (Fig. 2b). Because the concentrations of somatostatin in toad kidney were low, Biogel P4 chromatography of kidney extracts was not satisfactory.

Since intense positive immunofluorescence and PAP staining were observed throughout the epithelial cell layer of the urinary bladder, we conclude that somatostatin is present in both principal cell types of this layer, the granular cells and mitochondria-rich cells. Although the glomeruli and proximal renal tubules were immunohistochemically negative, many distal tubules and most collecting ducts stained positively for somatostatin. This heterogeneity of positive somatostatin immunoreactivity in the distal nephron suggests that different segments of the distal nephron are functionally heterogeneous.

Several lines of evidence suggest that both the immunoreaction product detected by immunofluorescence and PAP staining and the material immunoassayed in the toad bladder and kidney are true somatostatin. First, positive immunohistochemical staining was eliminated by preabsorption of antibody to somatostatin with synthetic somatostatin. Second, the material extracted from both the bladder and the kidney displaced ^{125}I-labeled somatostatin from antibody to somatostatin in a manner identical to that of synthetic somatostatin, and urinary bladder extracts displayed the same chromatographic behavior on Biogel P4 columns as did the authentic tetradecapeptide (Fig. 2).

In conclusion, this study indicates that somatostatin may serve a local regulatory or paracrine function in the antidiuretic hormone-sensitive portions of the toad renal-urinary system. The relevance of this finding to mammalian renal function is uncertain, since data concerning the presence of somatostatin in the mammalian renal-urinary system are conflicting. We previously reported that we could not detect this peptide by radioimmunoassay in extracts of rat kidney (*6*), nor were Vale *et al.* (*3*) able to do so, although Kronheim *et al.* (*16*) found immunoreactive material in small amounts in this tissue. Since infusions of somatostatin increase urine flow and decrease urine osmolality during hormonally induced antidiuresis in dogs (*9*), somatostatin may play a role in the regulation of mammalian renal function.

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References and Notes

1. J. N. Forrest, Jr., and D. B. P. Goodman, *Clin. Res.* **27**, 414 (Abstr.) (1979); J. W. Strandhoy and T. E. Zook, *Res. Commun. Chem. Pathol. Pharmacol.* **24**, 599 (1979); J. N. Forrest, Jr., J. L. Bolaffi, S. Reichlin, D. B. P. Goodman, *Clin. Res.* **28**, 533 (Abstr.) (1980); J. N. Forrest, Jr., S. Reichlin, D. B. P. Goodman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4984 (1980).
1a. J. N. Forrest, Jr., J. L. Bolaffi, S. Reichlin, D. B. P. Goodman, *Clin. Res.* **28** (Abstr.) (1980); J. L. Bolaffi, S. Reichlin, I. M. D. Jackson, J. Forrest, Jr., D. B. P. Goodman, *J. Histochem. Cytochem.* **28**, 612 (Abstr.) (1980); J. N. Forrest, Jr., S. Reichlin, D. B. P. Goodman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4984 (1980).
2. P. Brazeau, W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier, R. Guillemin, *Science* **179**, 77 (1973).
3. W. Vale *et al.*, *Recent Prog. Horm. Res.* **31**, 365 (1975).
4. S. Reichlin, R. Saperstein, I. M. D. Jackson, A. E. Boyd, III, Y. Patel, *Annu. Rev. Physiol.* **38**, 389 (1976).

5. S. Van Noorden, J. M. Polak, A. G. E. Pearse, *Histochemistry* **53**, 243 (1973); T. Hokfelt, S. Efendic, G. Hellerstrom, D. Johansson, R. Luft, A. Arimura, *Acta Endocrinol. (Copenhagen) Suppl.* **200**, 5 (1975).
6. Y. Patel and S. Reichlin, *Endocrinology* **102**, 523 (1978).
7. W. Vale, N. Ling, J. Rivier, J. Villareal, C. Rivier, C. Douglas, M. Brown, *Metab. Clin. Exp.* **25** (Suppl.), 1491 (1976); S. Falkmer, R. P. Elde, C. Hellerstrom, B. Peterson, *ibid.* **27**, 1193 (1978); B. Shapiro, M. Sheppard, S. Kronheim, B. L. Pimstone, *J. Endocrinol.* **80**, 407 (1979); P. Giraud, P. Gillioz, B. Conte-Devolx, C. Oliver, *C.R. Acad. Sci.* **228**, 127 (1979); J. A. King and R. P. Millar, *Endocrinology* **105**, 1322 (1979).
8. A. G. E. Pearse, in *Molecular Endocrinology*, I. MacIntyre and M. Szelke, Eds. (Elsevier/North-Holland, New York, 1977), p. 309; W. G. Forssman, V. Helmstaedter, J. Metz, G. Muhlmann, G. E. Feurie, *Metab. Clin. Exp.* **27** (Suppl.), 1179 (1978); L.-I. Larsson, N. Goltermann, L. de Magistris, J. F. Rehfeld, T. W. Schwartz, *Science* **205**, 1393 (1979).
9. I. A. Reid and J. C. Rose, *Endocrinology* **100**, 782 (1977); N. Brautbar, B. S. Levine, J. W. Coburn, C. R. Kleeman, *Am. J. Physiol.* **237**, E428 (1979).
10. Adult female *B. marinus*, native to the Dominican Republic, were obtained from Connecticut Valley Biological Supply, Southhampton, Mass., or National Reagents, Bridgeport, Conn., and were maintained, unfed, on moist bedding at room temperature until decapitation.
11. We produced antibody (655) to somatostatin by injecting rabbits with somatostatin coupled to methylated bovine serum albumin. Goat antibody to rabbit gamma globulin (G-ARGG), rabbit PAP, and fluorescein isothiocyanate–linked goat antibody to rabbit gamma globulin (FG-ARGG) were obtained from Cappel Laboratories; normal goat serum was obtained from Miles Laboratories; and normal rabbit serum was obtained from nonimmunized (control) rabbits. Cyclic synthetic somatostatin was obtained from Peninsula Laboratories, synthetic arginine vasopressin and 3,3′-diaminobenzidine (DAB) were obtained from Sigma Chemical, and synthetic arginine vasotocin was obtained from Calbiochem.
12. Freshly frozen sections (8 to 10 μm) were cut in a cryostat, mounted on gelatinized slides, dried in air overnight, postfixed in 10 percent neutral buffered formaldehyde for 10 minutes, and processed by immunofluorescence techniques. [A. H. Coons, in *General Cytochemical Methods*, Y. F. Danielli, Ed. (Academic Press, New York, 1958)]. The sections were washed twice in 10 m*M* phosphate-buffered saline (PBS) (*p*H 7.4) for 20 minutes, incubated in normal goat serum (1:20) for 30 minutes, in antibody to somatostatin for 24 hours at 4°C at titers of 1:50, 1:100, 1:200, and in FG-ARGG (1:10) for 30 minutes. Then they were thoroughly washed in PBS, mounted on glass slides under PBS and glycerin (1:1), and examined for immunofluorescence with a Zeiss microscope. Adjacent sections were incubated overnight with antibody to somatostatin [preabsorbed with somatostatin (200 μg/ml)].
13. Formaldehyde-fixed tissues were embedded in paraffin, cut in 4-μm sections, mounted on albumin-coated slides, and treated according to the PAP methods of L. A. Sternberger, P. H. Hardy, J. J. Cuculis, and H. G. Meyer [*J. Histochem. Cytochem.* **18**, 315 (1970)]. The sections were then treated with methanol and H_2O_2 (9:1) for 30 minutes to remove endogenous peroxidase activity, washed in PBS for 20 minutes and in normal goat serum (1:20) for 30 minutes, and incubated with antibody to somatostatin for 24 hours at 4°C at titers of 1:750, 1:1000, and 1:5000. Next, the sections were incubated with G-ARGG (1:50) for 60 minutes, PAP (1:50) for 30 minutes, and DAB-H_2O_2 [7.5 mg of DAB per 10 ml of 0.2*M* tris-HCL (*p*H 7.4) plus three drops of 3 percent H_2O_2] for 5 to 10 minutes. Then the sections were counterstained with methyl green, dehydrated through xylene, mounted with Permount, and examined for brown reaction product. Adjacent sections were incubated with antibody to somatostatin [preabsorbed with somatostatin (200 μg/ml)] overnight at 4°C. Sections were also exposed to somatostatin similarly preabsorbed with arginine vasopressin (200 μg/ml) and arginine vasotocin (200 μg/ml).
14. A. Arimura *et al.*, *Metab. Clin. Exp.* **27** (Suppl.), 1139 (1978).
15. Y. Patel and S. Reichlin, in *Methods of Hormone Radioimmunoassay*, B. M. Jaffe and H. R. Behrman, Eds.) (Academic Press, ed. 2, New York, 1979).
16. S. Kronheim, M. Berelowitz, B. L. Pimstone, *Clin. Endocrinol.* **5**, 619 (1976).
17. We thank J. Bollinger for expert technical assistance and A. Duflot-Vignery for her excellent photographs. This work was performed during J.L.B.'s tenure as a National Research Service Fellow (IF 32 AM 06018) and J.N.F.'s tenure as an established investigator of the American Heart Association (77-228); D.B.P.G. is an established investigator of the American Heart Association (77-173). Supported by NIH grants AM 16684, AM 17433, AM 19813; American Heart Association grant 77-103; and National Research Service award IF 32 AM 06018.

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Liquid-Phase Dehydration of Aqueous Ethanol-Gasoline Mixtures

Abstract. *Two-phase mixtures of gasoline, water, and ethanol were dehydrated with both starch and saponified starch-g-polyacrylonitrile (HSPAN). Whereas starch absorbed ethanol as well as water, HSPAN selectively absorbed the water component, allowing ethanol to dissolve in the gasoline phase.*

Ladisch and Dyck (*1*) have shown that the ratio of the combustion energy of ethanol to the distillation energy as a function of the percentage of ethanol in the distillate drops rapidly above about 92 percent ethanol. As one approaches 95 percent ethanol, the input of energy begins to approach the amount of energy gained through combustion. Azeotropic distillation to produce the nearly anhydrous ethanol that is needed for blending with gasoline (to avoid phase separation) is a further detriment to the energy balance. The water tolerance of a blend of 10 percent ethanol in gasoline is about 0.3 percent (*2*). Ladisch and Dyck (*1*) successfully dehydrated aqueous ethanol by passing the vapor through a column packed with a number of inexpensive dehydrating agents, such as starch, cracked corn, cellulose, and carboxymethyl cellulose. The use of saponified starch-*g*-polyacrylonitrile (*3*) (HSPAN, sometimes referred to as super slurper) was also suggested.

In this report, we describe a method for the dehydration of ethanol in the liquid phase, in which an absorbent polymer, particularly HSPAN, is contacted at room temperature with a two-phase mixture of aqueous ethanol and unleaded gasoline (Table 1). The HSPAN used was a commercial sample (SGP 502S, Henkel Corporation) (*4*) that was screened to isolate the fraction passing 20-mesh but held by 40-mesh. It contained 10 percent water. The aqueous ethanol solutions contained 95, 90, and 80 percent ethanol (by volume) [93.7, 86.7, and 75.9 percent (by weight)], and the ratios of aqueous ethanol to gasoline were chosen to give typical gasohol blends of 10:90 by volume [11.1 to 11.3 percent ethanol (by weight)], after removal of water. The weight of polymer added was slightly in excess of that needed to absorb all of the lower, water-containing layer.

Gas chromatographic analysis for ethanol was made difficult by the numerous components present in gasoline; however, acceptable results were obtained on a Bendix 2500 gas chromatograph with a flame ionization detector (2 m by 0.32 cm glass column packed with 15 percent Carbowax 20*M* on Gas-Chrom Q). The column temperature of 65° to 190°C was programmed at 15°C per minute. We constructed a standard curve of detector response versus the percentage of eth-

Table 1. Dehydration of aqueous ethanol-gasoline mixtures: analysis of the gasoline phase. Gasoline (90 ml) was mixed with sufficient aqueous ethanol to give 90:10 (by volume) gasohol, after removal of water. Polymer was added and the mixture shaken overnight at room temperature. An ethanol content in gasoline of 10 percent (by volume) corresponds to 11.1 percent ethanol (by weight) because of the higher density of ethanol. For this calculation, the density of gasoline was equated with the density of octane.

Aqueous ethanol used, % by volume (% by weight)	Controls, no polymer added		Cornstarch* added			HSPAN† added		
	Water (% by weight)	Ethanol (% by weight)	Amount (g)	Water (% by weight)	Ethanol (% by weight)	Amount (g)	Water (% by weight)	Ethanol (% by weight)
95 (93.7)	0.51	10.5	2	0.60	10.6	0.5	0.50	11.1
90 (86.7)	0.39	7.4	8	0.43	8.6	5	0.41	10.8
80 (75.9)	0.20	5.0	10	0.18	5.2	10	0.41	10.6

*Water content, 12 percent. †Water content, 10 percent.

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