Salt-Dependent Structural Changes of Neurohormones: Lithium Ions Induce Conformational Rearrangements of Ocytocin to a Vasopressin-like Structure†

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ABSTRACT: The preferred average conformation and structural subdomain interactions of the nonapeptide hormones vasopressin and ocytocin have been analyzed through the determination of their hydrodynamic volume and the thermal coefficient of the frictional resistance to rotation of their tyrosine residue. A spherical gross shape and an ellipsoidal gross shape were assessed respectively for ocytocin and vasopressin by fluorescence polarization analysis. Investigation of the thermal coefficient of viscosity and the critical temperature of both hormones and analogues indicated that strong interactions hold together the two structural subdomains of ocytocin (the flexible six-membered ring and the COOH-terminal tripeptide tail). An opposite situation was found in the case of vasopressin where such interactions could not be detected between the rigid ring and the flexible COOH-terminal tail. Lithium ions were shown to promote ocytocin binding to specific neurophysin sites restricted, under standard conditions, to vasopressin. In the presence of lithium, the gross conformational shape of ocytocin becomes similar to that of vasopressin but in the absence of salt. In addition, the ocytocin ring becomes more rigid in the presence of lithium while decreasing interactions between the ring and the COOH-terminal tail were detected. It is proposed that lithium ions induce specific conformational rearrangements of ocytocin toward a vasopressin-like structure, allowing recognition of this hormonal ligand by a specific vasopressin binding domain of neurophysins.

Neurophysins are a family of proteins from the hypothalamoneurohypophyseal tract that appears to be associated both with transport and biosynthesis of the peptide hormones ocytocin and vasopressin [for reviews see Pickering & Jones (1978), Breslow (1979, 1984), Cohen et al. (1979, 1983), Chaiken et al. (1983), Richter (1983), and Pickering & Swann (1984)]. The basic subunit of neurophysin consists in a single polypeptide chain (M_r 10 000), showing a marked tendency to form a dimer to which both hormones preferentially bind under physiological conditions (Nicolas et al., 1980). The dimeric form of neurophysin possesses one strong binding domain per protomer for both hormones and related peptides and exhibits a positive intradimeric cooperativity (Hope et al., 1975; Nicolas et al., 1978; Pearlmutter & Dalton, 1981; Tellman & Winzor, 1980).

The NH_2-terminal portion of neurohypophyseal hormones interacts with this site both the electrostatic and hydrophobic interactions involving residues 1–3 of the nonapeptide while the entire tripeptide COOH-terminal portion appears to be free of contact with the protein binding domain (Breslow, 1979; Cohen et al., 1979). Since both hormones bind to this site in a similar fashion and with comparable affinities, it is assumed that this process involves conformational requirements common to both ocytocin and vasopressin.

Investigations on the preferred average conformation of ocytocin and vasopressin in aqueous medium have not pointed out drastic differences except for the following features: (i) the tyrosine side chain in position 2 of ocytocin is fully exposed to the solvent and retains its rotational freedom while in vasopressin it is stacked by π–π interactions with the side chain of residue Phe,[3] and (ii) the vasopressin peptide tail exhibits a greater flexibility compared to ocytocin (Glickson et al., 1972; Deslauriers et al., 1974; Maxfield & Scheraga, 1977; Walter, 1977; Griffin et al., 1977; Convert et al., 1977).

A second, weaker, class of binding sites per neurophysin promoter is also detectable and was thought to be related to the duplicated domains of internal sequence homology lying on both sides of the unique central tyrosine in position 49 of the protein sequence (Camier et al., 1973; Wolff et al., 1975; Nicolas et al., 1978). However, the observation that this class of sites is restricted to vasopressin under standard conditions was unexpected on the basis of the parallelism in behavior of both hormones toward the first class of sites and the gross structural similarities between these ligands. This suggests that, although molecular requirements for binding to these sites are still unknown, subtle details of the hormone conformation might govern recognition and discrimination of ligands by this protein domain.

Lithium ions allow ocytocin binding to the sites of the second domain in a manner similar to that of vasopressin but in the absence of salt (Nicolas et al., 1978). This effect could be due to a salt-induced conformational rearrangement of ocytocin toward a vasopressin-like structure.

In order to test for this hypothesis and to gain further information about the preferred average conformation of both ocytocin and vasopressin, we have used herein a fluorescence polarization method to analyze both the fast and slow motions of chromophores in peptides and proteins. This was done through the determination of the thermal coefficient of the frictional resistance to rotation of fluorophores (Weber et al., 1984; Rholam et al., 1984; Scarlata et al., 1984) and analysis of the hydrodynamic volume at molecules (Perrin, 1926; Weber, 1953). Using this technique, we report refined data on the conformation of both ocytocin and vasopressin in aqueous medium and on neurophysin–hormone interactions at the molecular level.

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Materials and Methods

Neurophysins were obtained in a homogeneous form by isoelectric focusing from an acetone powder made from fresh pituitaries (Camier et al., 1973). The hormones (ocytocin, vasopressin, vasotocin, and isotocin) were purchased from Sigma. The tripeptide analogue of the NH_2-terminal sequence of ocytocin was from Bachem. The purity of hormones and analogue was assessed by thin-layer chromatography on cellulose plates run in two different solvent systems. Tritiated hormones were prepared and purified by affinity chromatography as already described (Pradelles et al., 1972). They were tested as reported previously (Camier et al., 1973). Spectral quality glycerol was from Aldrich.

Binding Measurements. Ocytocin and vasopressin binding to neurophysin was analyzed by equilibrium dialysis as described previously (Nicolas et al., 1980) either in the absence or in the presence of variable LiCl. The saturation ratio, $\langle\bar{\nu}\rangle = C_b/C_0$, was expressed as bound ligand (C_b) per total protein concentration (C_0) considering the molecular weight of monomeric neurophysin equal to 10000. Protein concentration was evaluated by absorption measurements on a Cary 118C spectrophotometer, assuming a molar absorbance of 3400 cm^{-1} M^{-1} at 260 nm. Nonlinear iterative fitting of the binding isotherms was performed as described previously (Nicolas et al., 1980).

Fluorescence Spectroscopy. Measurements of the fluorescence polarization were made with the photon counting instrument of Jameson et al. (1978). Corrections were introduced for the counts present as solvent background. Each value is the average of three independent determinations. Fluorescence lifetimes were obtained by the cross-correlation phase method (Spencer & Weber, 1969) with an SLM 4800 instrument (SLM Instrument Inc., Urbana, IL). Each value of fluorescence lifetime is the average of a set of five series of measurements. For lifetime and polarization studies, excitation was by 280-nm light isolated by a combination of both a monochromator and a Corning 7-54 filter. Filters 0-53 were used to isolate the fluorescence emission. The viscosity values for glycerol–water mixtures were taken from Miner & Dalton (1953). The polarization and lifetime of neurophysin and hormones, in 80% glycerol–20% phosphate buffer (0.05 M, pH 7.0), were determined in the interval −40 to 20 °C. The data were analyzed by using the relation established in a previous paper (Weber et al., 1984):

$$Y = \ln [A(0)/A(T) - 1] - \ln (RT\langle\tau\rangle/V) = b(T - T_0) - \ln [\eta(0)]$$

where $A(0)$ is the limiting anisotropy, $A(T)$ is the value of the anisotropy at the kelvin temperature T, R is the gas constant, $\langle\tau\rangle$ is the lifetime of the excited state, V is the effective volume of the fluorophore, $\eta(0)$ is the viscosity at temperature $T(0)$, and b, the thermal coefficient of the "local" viscosity, characterizes the resistance that the environment offers to the rotation of fluorophores.

In the general case (Rholam et al., 1984; Scarlata et al., 1984), the plot of Y vs. the temperature $t = T - T_0$ yields two slopes corresponding to two regions in which the rotational motions of the fluorescent residue are limited either by the external solvent [$b(S)$] or by the peptide environment ($b(U)$], respectively.

Results and Discussion

Binding of Ocytocin to Neurophysin II. A concentration of 1.4 M in lithium chloride has been previously shown to reveal a second, weaker, binding domain of neurophysin,

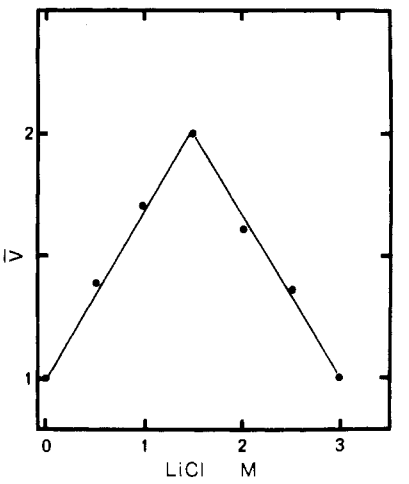

FIGURE 1: Effects of lithium ions on ocytocin binding to neurophysin. Saturation ratio obtained from Scatchard plot analysis (acetate buffer, ph 5.6) where it is expressed as the bound ligand concentration per protein molar concentration, considering the molecular weight of native neurophysin is 10000.

Table I: Effect of Salt on the Binding of Ocytocin to Neurophysin

cosolvent ($mol \cdot L^{-1}$)	$\langle\bar{\nu}\rangle$	$\langle\bar{\nu}\rangle/c_f$ ($\times 10^{-5}$) (mol^{-1})
acetate (0.1)	1	1.94
LiCl (0.5)	1.4	1.14
LiCl (1.0)	1.7	0.65
LiCl (1.5)	2	0.44
LiCl (2.0)	1.7	0.35
LiCl (3.0)	1	0.27

making it available for ocytocin under these conditions (Nicolas et al., 1978). Isotherms according to Scatchard were obtained for the binding of ocytocin (0.001–1 mM) to neurophysin II (0.05 mM, pH 5.6) in the presence of various concentrations (0–3 M) of LiCl (Nicolas, 1979). When further analyzed by plotting the apparent saturation ratio $\langle\bar{\nu}\rangle$ vs. concentration of LiCl, a bell-shaped curve was obtained (Figure 1), whose maximum is reached for a salt concentration of approximating 1.5 M. Under these conditions, two ocytocin molecules are bound per neurophysin protomer. Raising the concentration of LiCl up to 3 M leads to a reversal of this phenomenon with the value of the saturation ratio decreasing from 2 to 1. Similar experiments, conducted with vasopressin, indicated that two molecules of peptides were bound per protomer whatever the concentration of LiCl was (results not shown).

In addition, while the affinity of vasopressin toward the second class of binding sites remained nearly identical either in the absence or in the presence of salt, there was a continuous decrease in its affinity for the first common class of sites as the concentration of LiCl increased (Table I). This was also found for ocytocin binding, but both peptides were bound with a similar affinity at two thermodynamically identical sites in the presence of 1.5 M LiCl. This has been shown to reflect a reduction of the electrostatic component of the interactions between the NH_2-terminal portion of the hormones and a carboxylate group located at the high-affinity binding domain of neurophysin for a high ionic strength (Nicolas et al., 1978; Nicolas, 1979).

Therefore, except for the electrostatic contribution, both classes of sites could possess common structural hydrophobic moieties. However, lithium ions are known to have many abnormal properties in aqueous solution and to dissociate multimeric systems and/or to induce conformational changes in certain domains of macromolecules (Isemura & Goto, 1964;

Table II: Effect of Salt on the Hydrodynamic Parameters of Hormones

hormone	cosolvent (mol·L^{-1})	ρ_h/ρ_o	$p = a/b$[a]
ocytocin	acetate (0.1)	0.96	1
	LiCl (1.5)	1.56	3
	LiCl (3.0)	1.2	1.2
	NaCl (1.5)	1.1	1
vasopressin	acetate (0.1)	1.7	3.5
	LiCl (1.5)	1.5	3

[a] $p = a/b$: axial ratio of prolate ellipsoid.

Goto & Isemura, 1964; Von Hippel & Schleich, 1969). Thereby, it is tempting to suggest that lithium ions either promote conformational changes in neurophysins or alter the conformation of ocytocin to resemble vasopressin.

Hydrodynamic Volume of Hormones. The polarization of the fluorescence in proteins, determined by the overall rotation of the protein molecules as well as by rotations of individual residues, is based on Perrin's equation (Perrin, 1926; Weber, 1953):

$$A_0/\langle A\rangle = 1 + \langle\tau\rangle RT/(V\eta)$$

where $\langle A\rangle$ is the average anisotropy, A_0 is the limiting anisotropy, $\langle\tau\rangle$ is the limiting anisotropy, $\langle\tau\rangle$ is the fluorescence mean lifetime, and η is the viscosity.

At high values of the ratio T/η, the slope of the Perrin plot describes the slow rotational motions of the protein as a whole. If the fluorescence lifetime is independent of the viscosity, the dependence of $\langle A\rangle^{-1}$ vs. T/η, by monitoring Tyr^2 fluorescence of both ocytocin and vasopressin either in the absence or in the presence of LiCl, reflects then changes in the hydrodynamic volume of the hormones. The axial ratio of the peptides, (a/b), was calculated from the equation:

$$(\rho_h/\rho_o) = f(a/b)$$

where ρ_o is the relaxation time of an equivalent sphere (Weber, 1952, 1953; Jablonski, 1961), ρ_h is the harmonic mean relaxation time, and $f(a/b)$ is a function of the axial ratio, which may be calculated from Perrin's equations (Perrin, 1936).

The data obtained for ocytocin and vasopressin, either in the absence or in the presence of LiCl, are summarized in Table II. Through these calculated hydrodynamic parameters, it is readily apparent that LiCl has no effect on the shape of vasopressin (ellipsoid of revolution of axial ratio $a/b = 3$). Furthermore, we attribute the change in limiting anisotropy of vasopressin with LiCl to be purely electrostatic in character. In the absence of salt, the shape of ocytocin is pseudospherical. This finding is in agreement with previously proposed structures for this hormone (Urry & Walter, 1971; Kotelchuck et al., 1972). In 1.5 M LiCl, the shape of ocytocin undergoes profound changes, becoming ellipsoidal with an axial ratio similar to that of vasopressin. For increasing concentrations of LiCl (from 1.5 to 3 M), the shape of ocytocin reverses to its initial form. Under the same conditions, it was also found that lithium chloride has no effect on both the shape and the dimeric structure of neurophysin II (results not shown). Therefore, lithium ions appear to alter the ocytocin conformation in a way that allows its recognition by the weak vasopressin binding sites.

Local Motions of Residues in Hormones. To get more information on the structural changes induced by LiCl, we focused on the fast motions of tyrosine in both neurophysin and hormones using the assumption that, in a medium of sufficiently high viscosity, the fluorescence depolarization is attributed solely to the local motions of residues (Wahl & Weber, 1967; Munro et al., 1977; Lakowicz & Wber, 1980). The results, obtained for these motions in 80% aqueous glycerol, are summarized in Table III.

As in the preceding experiments, the conformation of ocytocin is greatly affected by LiCl, whereas vasopressin structure remains apparently unaltered. We noted that the parameter values of the two hormones differ under the standard experimental conditions although they become closer in 1.5 M LiCl.

As a control experiment, we tested the NH_2-terminal tripeptide part of ocytocin to determine whether a rigid tertiary structure is necessary for LiCl-induced changes. The results obtained indicate that lithium chloride has no significant effect on the rotational motions of the tripeptide Tyr^2. This is supported by the binding of the tripeptide exclusively to the strong sites of neurophysins (Nicolas et al., 1978). Thus, according to the above observations, a specific conformation, similar to that of ocytocin, is needed to be affected by lithium chloride.

To determine the nonspecific part of the salt effects, the rotational motions of ocytocin were analyzed in 1.5 M NaCl. We found that the ionic strength is the main contributor responsible for the increase in $b(U)$ whereas the change in t_c seems to be specific to LiCl. Since the thermal coefficient was interpreted as being a function of how coupled the motions of the chromophore are with the peptide environment (Rholam et al., 1984; Scarlata et al., 1984), an increase in $b(U)$ is thus attributable to a decrease in interactions between the two subdomains (the six-membered ring and the tripeptide tail)

Table III: Salt Effects on the Characteristic Parameters of Neurophysin and Peptide Hormones

	cosolvent (mol·L^{-1})	$b(U)$ (×100) (°C^{-1})	t_C (°C)	Amp (deg)[a]
neurophysin II	acetate (0.1)	3.5	−11	13
	LiCl (1.5)	4.5	−10	14
	LiCl (3.0)	4.0	−14	12.5
tripeptide [Cys(*S*-methyl)-Tyr-Ile-NH_2]	acetate (0.1)	6.6	−10	14
	LiCl (1.5)	5.5	−6	16
ocytoxin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2)	acetate (0.1)	3.5	−16	12
	LiCl (1.5)	5.0	−2	19
	LiCl (3.0)	4.0	−14	12.5
	NaCl (1.5)	5.0	−15	12.5
vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2)	acetate (0.1)	5.0	+7	26
	LiCl (1.5)	5.5	+8	26
vasotocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys-Gly-NH_2)	acetate (0.1)	4.8	−16	12
	LiCl (1.5)	5.5	−3	18.5
	LiCl (3.0)	4.6	−14	12.5
isotocin (Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-Gly-NH_2)	acetate (0.1)	4.6	−15	12.5
	LiCl (1.5)	4.8	−6	16

[a] The amplitude (Amp) was calculated from the equation determined in a previous paper (Scarlata et al., 1984): $\langle\cos\phi\rangle^2 = [2A(t_c)/A(0) + 1]/3$.

constituting the ocytocin structure.

In order to provide further support to this proposal, we carried out, under the same experimental conditions, a study of vasotocin in which both the NH_2-terminal and COOH-terminal parts are respectively analogous to those of ocytocin and vasopressin. Through the results presented in Table III, we noted that ocytocin and vasotocin have the same motional amplitude (or the same t_c) whereas the same $b(U)$ is obtained for vasopressin and vasotocin. Therefore, the cyclic subdomain of vasotocin undergoes a similar conformational change as that seen with ocytocin. A similar change is also observed for isotocin which differs from ocytocin by two amino acids (Ser^4 and Ile^8). These data indicate clearly that the NH_2-terminal part of ocytocin, vasotocin, and isotocin is specifically perturbed by lithium ions. When the concentration of LiCl is increased to 3.0 M, the change in the parameters (essentially the critical temperature) of ocytocin, vasotocin, and isotocin are reversed.

Conclusion

The present study clearly indicates that recognition of the weak neurophysin binding site is under the control of conformational clues specifically restricted to the vasopressin molecule. This is supported by (i) differences in gross shape (hydrodynamic volume) and fine structural details (local motions of Tyr^2) of hormones in the absence of ions, (ii) the absence of conformational perturbation of neurophysin under high lithium concentration compared to standard conditions, and (iii) the good correlation observed between changes in the binding properties, the hormonal shape, and critical temperature values of their Tyr^2 as the lithium concentration is increased. In particular, in the presence of 1.5 M LiCl, conditions under which ocytocin binds to both classes of sites as vasopressin does but in the absence of salt, the gross conformation of both hormones becomes closely similar.

Detailed analysis of the characteristic parameters of hormones and analogues indicated that lithium ions act through two distinct effects: (i) an unspecific ionic strength effect which causes the COOH-terminal tripeptide tail of ocytocin to weaken its interactions with the six-membered ring at high salt concentration (this is the unique effect observed when sodium ions are used as the primary salt in place of lithium) and (ii) a specific effect on the cyclic backbone conformation of ocytocin generated by selective, although yet uncharacterized, interactions between lithium ions and some residues of ocytocin.

A rationale is to assume that discrimination between both hormones by the weaker neurophysin binding domain is based on both fine and gross conformational details between these hormonal ligands. Moreover, as ocytocin covalent structure differs from the vasopressin one by only two amino acid residues (in positions 3 and 8), it is reasonable to assume that these conformational differences originate from these particular residues.

More detailed analysis of the specific interactions of lithium with neurohypophyseal hormones are needed in order to establish a preferred model of action of lithium at a molecular level. However, the fact that tripeptide analogues of the NH_2-terminal sequence of either ocytocin or vasopressin are unable to bind to the weak sites, even in the presence of lithium, indicates that the overall conformation of hormones is the main contributor to recognition by the weak sites. In contrast such conformational requirements do not seem to be necessary for the recognition of both hormones by the high-affinity binding domain.

Although molecular interactions governing the binding to these weak sites are still unknown, it appeared from thermodynamic parameters deduced from direct binding measurements (Cohen et al., 1979; Nicolas et al., 1980) that hydrophobic interactions play a major role in this binding domain. This is compatible with the hypothesis that peptide conformation determines the specificity of access to these sites and that both residues −3 and −8 of vasopressin participate in a critical way to the proper conformation adopted by this hormone rather than established by specific molecular interactions with some unknown neurophysin residues in the weak sites.

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Proteolysis-Associated Deglycosylation of β_1-Adrenergic Receptor in Turkey Erythrocytes and Membranes†

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ABSTRACT: A protease that can be inhibited by glutathione, dithiothreitol, and *o*-phenanthroline but not by ethylenediaminetetraacetic acid converts the 50-kilodalton β-adrenergic receptor in turkey erythrocyte membranes to a 40-kDa polypeptide which retains the specific ligand binding site. This conversion is attenuated in intact erythrocytes. The large 50-kDa peptide contains N-linked, complex carbohydrates and is retained on wheat germ agglutinin–Sepharose. The 40-kDa product of proteolysis does not bind to the wheat germ agglutinin and can thus be separated from the 50-kDa polypeptide by lectin chromatography. However, the large difference in molecular weights of the two receptor peptides cannot be accounted for solely by the different extent of glycosylation.

With different radioactive photoaffinity labels specific for the ligand binding site of β-adrenergic receptors, two β_1-adrenergic receptor polypeptides differing in mass by about 10 kilodaltons (kDa)[1] have been found in turkey erythrocyte membranes [cf. Burgermeister et al. (1982) and Lavin et al. (1982)]. Similarly, two forms of the β-receptors with 65- and 55-kDa masses were observed in cultured S49 lymphoma cells (Rashidbaigi et al., 1983), and β_1- and β_2-receptors with two forms each of 62 and 55 kDa were detected in mammalian and frog ventricular myocardial membranes (Stiles et al., 1983). Recently, we have presented evidence indicating that the larger 50-kDa form (P50) of the turkey erythrocyte β-receptor is converted in the membrane in a time- and temperature-dependent reaction to the smaller 40-kDa form (P40) [see Figure 4 in Hekman et al. (1984)]. We now show, on the basis of inhibition characteristics, that this proteolytic reaction is catalyzed in the membrane by an enzyme which is probably a metalloprotease and that the reaction is greatly attenuated in intact erythrocytes. Moreover, we have subjected β_1-receptor polypeptides purified about 5000-fold by affinity chromatography (Feder et al., 1984) to chromatography on wheat germ agglutinin-Sepharose and shown that of the two polypeptides the P50 form was bound preferentially to the lectin. Endoglycosidase F treatment of these polypeptides showed that the P50 and not the P40 form was a substrate for this enzyme. These findings lead us to conclude that restricted proteolysis in the membrane removes a peptide fragment from the P50 β_1-receptor polypeptide to which are joined N-linked complex carbohydrate chains, presumably containing mannose and sialic acid (Goldstein & Hayes, 1978; Monsigny et al., 1980; Elder & Alexander, 1982). Moreover, the differences in molecular weights between the two forms

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; [^{3}H]DHA, [^{3}H]dihydroalprenolol; WGA, wheat germ agglutinin; GlcNAc, *N*-acetylglucosamine; OMeGlcNAc, *O*-methyl-*N*-acetyl-D-glucosamine; [^{125}I]ICYP, [^{125}I]iodocyanopindolol; ICYP azide-2, 1-(4-azidobenzoyl)-3,3-dimethyl-6-hydroxy-7-(2-cyano-3-iodoindol-4-yloxy)-1,4-diazaheptane; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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