Bloomfield, V. A. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 421.
Brenner, S. L., & Korn, E. D. (1980) *J. Biol. Chem. 255*, 841.
Carlson, F. D., & Fraser, A. B. (1974) *J. Mol. Biol. 89*, 273.
Clarke, M., & Spudich, J. A. (1977) *Annu. Rev. Biochem. 46*, 797.
Dancker, P., & Low, I. (1979) *Z. Naturforsch., Biosci. 34C*, 555.
Flory, P. J. (1953) *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, NY.
Fujime, S. (1970) *J. Phys. Soc. Jpn. 29*, 751.
Fujime, S., & Ishiwata, S. (1971) *J. Mol. Biol. 62*, 251.
Garcia de la Torre, J., & Bloomfield, V. A. (1981) *Q. Rev. Biophys. 14*, 81.
Gethner, J. S., & Gaskin, F. (1978) *Biophys. J. 24*, 505.
Hanson, J., & Lowy, J. (1963) *J. Mol. Biol. 6*, 46.
Hartwig, J. H., & Stossel, T. P. (1979) *J. Mol. Biol. 134*, 539.
Howard, T. H., & Lin, S. (1979) *J. Supramol. Struct. 11*, 283.
Icenogle, R. D. (1981) Ph.D. Dissertation, Cornell University, Ithaca, NY.
Kawamura, M., & Maruyama, K. (1970) *J. Biochem. (Tokyo) 67*, 437.
Koppel, D. E., Axelrod, D., Schlessinger, J., Elson, E. L., & Webb, W. W. (1976) *Biophys. J. 16*, 1315.
Lanni, F., Taylor, D. L., & Ware, B. R. (1981) *Biophys. J. 35*, 351.
Lee, W. I., Schmitz, K. S., Lin, S.-C., & Schurr, J. M. (1977) *Biopolymers 16*, 583.
MacLean-Fletcher, S. D., & Pollard, T. D. (1980a) *J. Cell Biol. 85*, 414.
MacLean-Fletcher, S., & Pollard, T. D. (1980b) *Biochem. Biophys. Res. Commun. 96*, 18.
MacLean-Fletcher, S., & Pollard, T. D. (1980c) *Cell (Cambridge, Mass.) 20*, 329.
Maeda, T., & Fujime, S. (1977) *J. Phys. Soc. Jpn. 42*, 1983.
Maruyama, K. (1964) *J. Biochem. (Tokyo) 55*, 277.
Maruyama, K., Kaibara, M., & Fukada, E. (1974) *Biochim. Biophys. Acta 371*, 20.
Maruyama, K., Hartwig, J. H., & Stossel, T. P. (1980) *Biochim. Biophys. Acta 626*, 494.
Mihashi, K. (1964) *Arch. Biochem. Biophys. 107*, 441.
Newman, J., & Carlson, F. D. (1980) *Biophys. J. 29*, 37.
Newman, J., Swinney, H. L., & Day, L. A. (1977) *J. Mol. Biol. 116*, 593.
Oosawa, F. (1980) *Biophys. Chem. 11*, 443.
Oosawa, F., & Asakura, S. (1975) *Thermodynamics of the Polymerization of Protein*, Academic Press, New York.
Pollard, T. D., & Mooseker, M. S. (1981) *J. Cell Biol. 88*, 654.
Schliwa, M. (1981) *Cell (Cambridge, Mass.) 25*, 587.
Schurr, J. M. (1976) *Q. Rev. Biophys. 9*, 109.
Tait, J. F., & Frieden, C. (1982) *Arch. Biochem. Biophys. 216*, 133.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.
Tellam, R., & Frieden, C. (1982) *Biochemistry 21*, 3207.

Raman Spectroscopic Study of the Interaction between Sulfate Anion and an Imidazolium Ring in Ribonuclease A†

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ABSTRACT: Raman spectra of ribonuclease A in D_2O solution at various pD values have been studied with special attention to the N-deuterated imidazolium ring vibration at 1408 cm^{-1}, the SO_4^{2-} symmetric stretching vibration at 984 cm^{-1}, the amide I′ band, and the tyrosine doublet. Concomitant decrease and increase in the intensities of the 1408- and 984-cm^{-1} bands in the pD range between 5 and 7 indicate that a sulfate anion is actually hydrogen bonded to an imidazolium ring of a histidine residue located in the interior of the molecule. The mechanism of the sulfate desorption has been compared with that on heat denaturation.

Sulfate anion is known to locate immediately adjacent to His-12 and His-119 (Wyckoff et al., 1970; Richards & Wyckoff, 1971) and stabilize the native structure of ribonuclease (Ginsberg & Carroll, 1965; Von Hippel & Wong, 1965; Winchester et al., 1970). Commercial ribonuclease A (RNase A) often contains an excess amount of sulfate anion, and the Raman spectrum of its aqueous solution gives a strong and sharp band at 984 cm^{-1} due to the symmetric stretching vibration of the anion (Lord & Yu, 1970). When the solution is heated, the intensity of the 984-cm^{-1} band increases. If the heat-denatured material is kept for a long time at elevated temperature (>70 °C) and is then cooled to room temperature, the intensity of the sulfate band remains essentially constant. If, however, the cooling process is begun shortly after the system reaches 70 °C, the sulfate band intensity decreases reversibly (Chen & Lord, 1976). Chen and Lord interpreted these results in the following way. In the course of binding to one of the histidines in RNase, the sulfate anion is converted in effect to HSO_4^- which does not give a Raman band at 984 cm^{-1} but gives two weaker bands at 895 and 1053 cm^{-1}. When the enzyme is heated, some of the bound sulfate anion is released, and the 984-cm^{-1} band increases in intensity. Irreversible denaturation by heat impairs the ability of the protein to bind sulfate anion strongly.

In order to test this proposal, it is useful to study the pH dependence of the sulfate band and that of a vibration due to imidazolium ring at the same time. Deprotonation of the

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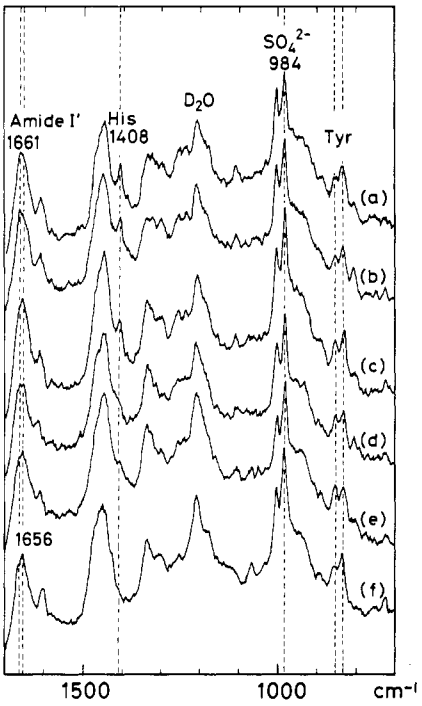

FIGURE 1: Raman spectra of 12% RNase A in D_2O at various pD values: (a) pD 4.3; (b) pD 5.2; (c) pD 6.4; (d) pD 6.9; (e) pD 7.6; (f) pD 10.5.

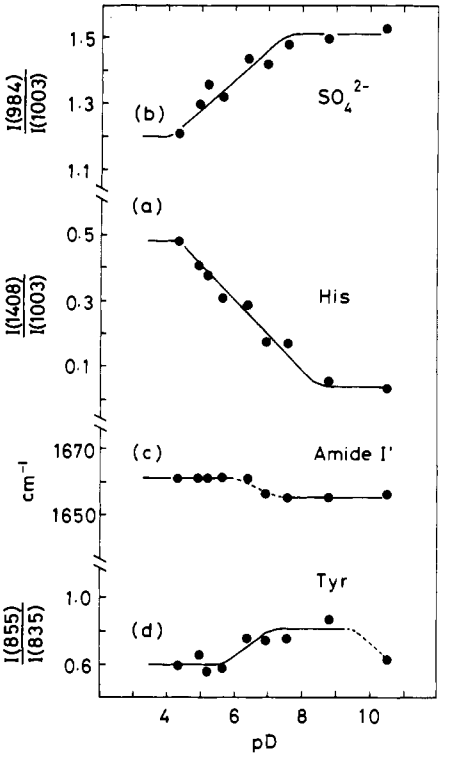

FIGURE 2: pD-dependence curves of 12% RNase A in D_2O as monitored by (a) the imidazolium ring vibration at 1408 cm^{-1}, (b) SO_4^{2-} stretching at 984 cm^{-1}, (c) the amide I' vibration at 1661–1656 cm^{-1}, and (d) the tyrosine doublet. The base lines used in obtaining the peak intensities of the bands are the following: 1408-cm^{-1} band, a straight segment connecting the intensities at 1420 and 1380 cm^{-1}; 1003- and 984-cm^{-1} bands, a straight segment connecting the intensities at 1025 and 970 cm^{-1}; 855- and 835-cm^{-1} bands, a straight segment connecting the intensities at 870 and 785 cm^{-1}. The phenylalanyl ring breathing band at 1003 cm^{-1} is chosen as the internal intensity standard.

imidazolium ring at a higher value of pH must necessarily release the sulfate anion if the anion is hydrogen bonded to the imidazolium ring. However, RNase A in H_2O solution gives no strong histidyl band to be monitored. On the other hand, a sharp band at 1408 cm^{-1} in the Raman spectrum of RNase A in D_2O solution has been assigned to a vibration of the N-deuterated imidazolium ring of the histidine residue (Lord & Yu, 1970), and this assignment has recently been confirmed on the basis of a detailed study on model compounds (Tasumi et al., 1982).

We have studied the Raman spectra of RNase A in D_2O solution at various pDs by monitoring the bands at 984 and 1408 cm^{-1} as well as the amide I' and the tyrosine doublet. On the basis of these results we discuss the binding site and nature of the interaction and compare the mechanism of sulfate desorption due to pH changes with that produced by heat denaturation.

Experimental Procedures

Bovine pancreatic RNase A was purchased from Sigma Chemical Co. as a "salt-free" sample and was used without further treatment. D_2O, DCl, and NaOD were purchased from Merck Sharp &Dohme, Canada Ltd. *N*-Acetyl-L-phenylalanine methylamide was supplied by courtesy of Professor T. Miyazawa of Tokyo University.

The "pH" (pD) of the solution was adjusted with DCl and NaOD and measured on a Hitachi-Horiba F-7 pH meter with a micro combination electrode (Nisshin Rika type CE103C). The direct readings on the meter were assumed as the pD values without correction.

The Raman spectra were obtained on a JEOL JRS-400D spectrophotometer with the 488.0-nm line of a Coherent Radiation CR3 argon ion laser as the exciting line (spectral slit width 7 cm^{-1}).

The Raman spectra of a mixed aqueous solution of *N*-acetyl-L-phenylalanine methylamide (0.016 M) and Na_2SO_4 (0.012 M) were recorded at pH 4.3, 6.0, 8.0, and 10.0. The peak intensity of the sulfate band at 984 cm^{-1} relative to that of the phenylalanyl ring-breathing band at 1003 cm^{-1} was constant in the pH region.

Results and Discussion

pD Dependence of the Spectrum of RNase A in D_2O. The Raman spectra of D_2O solutions at various pDs are shown in Figure 1. The 1408-cm^{-1} band has been assigned to a mode in which the N_1–C_2–N_3 symmetric stretching and N–D bending vibrations of the N-deuterated imidazolium ring are mixed (Tasumi et al., 1982). A model study shows that the intensity of this band in L-histidine is 0.4 times that of the 1003-cm^{-1} band in L-phenylalanine with the 488.0-nm excitation (Takamatsu, 1979). In the case of RNase A at pD 4.3 the intensity of the 1408-cm^{-1} band relative to that of the 1003-cm^{-1} band is about 0.5. If we assume that the model ratio is applicable to RNase A, the four histidine residues in the molecule are all deuteronated at this pD value.

Figure 2a gives a pD plot of the intensity of the 1408-cm^{-1} band. The intensity decreases in the course of the pD change from 5 to 7, but a weak shoulder remains at pD values higher than 7.6 (Figures 1 and 2a). This weak band is not due to the N-deuterated imidazolium ring or to the imidazole ring but is assigned to the symmetric stretching vibration of the carboxylate anion (Tasumi et al., 1982). The contribution from this band has been subtracted in the evaluation of the above-mentioned intensity of the 1408-cm^{-1} band at pD 4.3. Accordingly, the dedeuteronation takes place in the pD region between 5 and 7 for all four histidine residues. This result is consistent with an NMR titration study (Markley, 1975).

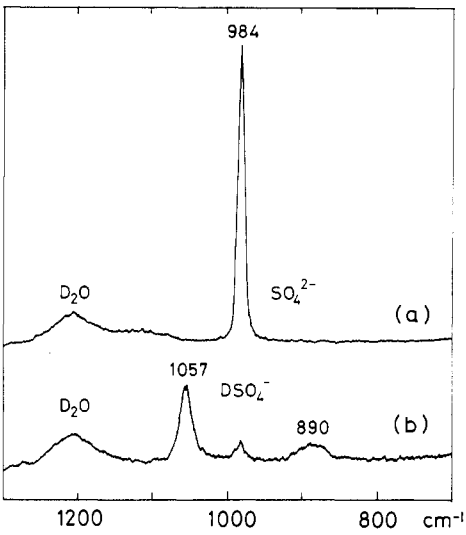

FIGURE 3: Raman spectra of Na_2SO_4 in D_2O (0.2 M) at (a) pD 6.9 and (b) pD 0.5.

The sulfate band at 984 cm^{-1} increases in intensity when the pD value is raised. Figure 2b gives a pD plot of this band. On the basis of the pH independence of the peak intensity of the 984-cm^{-1} band relative to that of the 1003-cm^{-1} band mentioned above, the increase in the relative intensity of the 984-cm^{-1} band is reasonably ascribed to the increase in the amount of the SO_4^{2-} species in the solution. In an equimolar solution of Na_2SO_4 and L-phenylalanine, the intensity of the 984-cm^{-1} band is about the same as that of the 1003-cm^{-1} band of L-phenylalanine (Takamatsu, 1979). When this ratio is applied to the spectrum of RNase A at pD 4.3 and 8.8, the numbers of sulfate anions per RNase A molecule are estimated to be about three and four, respectively. Hence, the number of desorbed sulfate anions per RNase molecule is one. As is clear from Figure 2a,b the respective increase and decrease in intensities of the 984- and 1408-cm^{-1} bands occur concomitantly. Clearly, dedeuteronation of the imidazolium ring and desorption of sulfate anion take place over the same pD range.

The band of the sulfate anion bound to the active site of the RNase A molecule is not observed in the Raman spectra. This is readily understood if the sulfate anion exists in the form of DSO_4^-, since the intensity of the strongest band of DSO_4^- anion at 1057 cm^{-1} is only 0.25 times that of the 984-cm^{-1} band of SO_4^{2-} in D_2O (Figure 3); i.e., the intensity of the former in RNase A solution at pD 4.3 (Figure 1a) would be less than 0.1 times that of the sulfate band. The above discussion gives strong support to the proposal by Chen & Lord (1976) that a sulfate anion is hydrogen bonded to an N-H of the imidazolium ring of one of the histidine residues.

It is of interest to note that there is a just detectable shift in the frequency of the amide I′ peak (Figures 1 and 2c) about pD 6.9. Although no conformational change in the peptide backbone is expected near pH 7 at room temperature, it may be that a minor alteration of the tertiary structure of the enzyme accompanies the elimination of SO_4^{2-} from the active site.

The tyrosyl doublet at 850 and 830 cm^{-1} is known to arise from Fermi resonance between a fundamental of one phenyl ring vibration and the first overtone of another. The intensity ratio of the doublet correlates with the strength of the hydrogen bond of the phenolic hydroxyl group, and the standard values of the ratio for "normal", "strongly H-bonded", and ionized tyrosines have been proposed (Siamwiza et al., 1975). These standard values are not necessarily applicable to the D_2O solution of systems containing tyrosyl residues since even a slight change in frequencies of the two vibrations caused by the O-deuteration could give a large effect on the intensity ratio. This point must be clarified, and the standard values for deuterated systems must be determined for quantitative arguments. For the time being, however, the ratio is plotted in Figure 2d. The ratio starts to change at a pD value higher than 6 and reaches the highest value at 8.8 and then drops at 10.5. The change between 6 and 8 is ascribed to the conversion of one or more of the strongly hydrogen bonded tyrosines (25, 97, and 73) to weaker hydrogen bonding (Gilbert et al., 1982). Since this change occurs over about the same pD range as the slight shift in the amide I′ frequency, it may likewise be due to alteration in the tertiary structure as a result of removal of the SO_4^{2-} anion. The decreased value of the ratio at still higher pD is due to the ionization of most of the phenols.

Nature of the Interaction between Sulfate Anion and Histidine. It is evident that there exists a specific interaction between sulfate anion and an imidazolium ring in RNase A. On the other hand, the intensity of the sulfate band in the spectrum of an equimolar D_2O solution of Na_2SO_4 and *N*-acetyl-L-histidine methylamide is independent of pD change; i.e., no such interaction as observed for RNase A exists in aqueous solution between the sulfate anion and the imidazolium ring of histidine (Takamatsu, 1979). Accordingly, histidine residues exposed on the surface of the RNase A molecule are not expected to bind strongly to sulfate anion. One of the two histidine residues, His-12 and His-119, buried near the active site of RNase A probably participates in the strong interaction.

In crystals of RNase A grown in an alcohol–water mixture (in the presence of phosphate buffer), a phosphate anion is bound in the vicinity of His-12 and His-119 (Kartha et al., 1976). Existence of a sulfate anion in a similar position in RNase S has also been suggested (Wyckoff et al., 1970). A low-temperature X-ray diffraction study on RNase A has verified that a sulfate anion does exist in crystals grown in an acidic solution at a position about 3 Å separated from the imidazolium ring of His-12 but not in those in alkaline solution (pH >9) (G. A. Petsko, personal communication). This observation strongly supports the above inference from the Raman study. Since it has been suggested in an NMR study on modified RNase A that the proton on N_1 in His-12 is strongly hydrogen bonded (Tanokura, 1979), it is probable that hydrogen bonding to the sulfate anion is provided by the proton (deuteron) on N_3 in His-12.

It is of interest to compare the mechanisms of desorption due to pD increase and that caused by temperature increase. The "melting temperature" of the protein indicated by the intensity change of the sulfate band is the same as that found by other Raman indicators such as the amide III, the 937-cm^{-1} band, tyrosine doublet, and S–S and C–S stretching bands (Chen & Lord, 1976). On the other hand, a preliminary study on temperature dependence of the Raman spectrum of D_2O solution shows that the intensity of the 1408-cm^{-1} band does not change in the region of the melting temperature (Takamatsu, 1979). These facts lead to the conclusion that the desorption of sulfate anion on elevation in temperature is due to the conformational change of the main chain which forces the sulfate anion out of the optimum site for the particular hydrogen bonding with the imidazolium ring of His-12.

In the case of pD dependence, desorption of sulfate anion occurs at the same pD range as that of the dedeuteronation of imidazolium ring. While it differs slightly from that of the conformational change in the main-chain structure and that of tyrosine H bonding, the latter two are sufficiently close on

the pD scale to the loss of SO_4^{2-} ion that they may well be a consequence of this loss. Thus we conclude that (1) a sulfate anion is strongly hydrogen bonded to the imidazolium N-H(D) of His-12 located in the active site in a specific way, (2) deprotonation (dedeuteronation) of the imidazolium ring results in the desorption of the sulfate anion, and (3) the surrounding structure in the active site plays an important role in maintaining the sulfate anion at the optimum position for such hydrogen bonding and desorption of the sulfate anion results in a slight relaxation of this structure as shown by its effect on the amide I′ band and the tyrosine doublet.

Acknowledgments

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References

Chen, M. C., & Lord, R. C. (1976) *Biochemistry 15*, 1889–1897.
Gilbert, W. A., Lord, R. C., Petsko, G. A., & Thamann, T. J. (1982) *J. Raman Spectrosc. 12*, 173–179.
Ginsberg, A., & Carroll, W. R. (1965) *Biochemistry 4*, 2159–2174.
Kartha, G., Bello, J., & Harker, D. (1976) *Nature (London) 213*, 862–865.
Lord, R. C., & Yu, N.-T. (1970) *J. Mol. Biol. 51*, 203–213.
Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–80.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647–785.
Siamwiza, M. N., Lord, R. C., Chen, M. C., Takamatsu, T., Harada, I., Matsuura, H., & Shimanouchi, T. (1975) *Biochemistry 14*, 4870–4876.
Takamatsu, T. (1979) Ph.D. Thesis, The University of Tokyo.
Tanokura, M. (1979) Ph.D. Thesis, The University of Tokyo.
Tasumi, M., Harada, I., Takamatsu, T., & Takahashi, S. (1982) *J. Raman Spectrosc. 12*, 149–151.
Von Hippel, P. H., & Wong, K. Y. (1965) *J. Biol. Chem. 240*, 3909–3923.
Winchester, B. G., Mathias, A. P., & Rabin, B. R. (1970) *Biochem. J. 117*, 299–307.
Wyckoff, H. W., Tsernoglu, D., Hanson, A. W., Knox, J. R., Lee, B., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 305–328.

Soluble 5′-Nucleotidase: Purification and Reversible Binding to Photoreceptor Membranes†

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ABSTRACT: Bovine rod outer segment membranes contain two types of 5′-nucleotidase, an integral enzyme and a peripheral enzyme. The peripheral enzyme, which accounts for about 25% of the total 5′-nucleotidase activity, was extracted with ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) buffer in the dark. The soluble enzyme was purified to apparent homogeneity by column chromatography on Blue Sepharose, 5′-AMP–Sepharose, and Sephacryl S-200. The purified enzyme had a molecular weight of about 67 000 and an isoelectric point of 6.5 and did not seem to consist of subunits. The enzyme hydrolyzed both 5′-AMP (K_m = 1.3 μM) and 5′-GMP (K_m = 2.3 μM). Adenosine 5′-(α,β-methylenediphosphate) (K_i = 4.2 μM), adenosine 5′-diphosphate (ADP) (K_i = 0.4 μM), adenosine 5′-triphosphate (ATP) (K_i = 3.3 μM), and rabbit G-actin were competitive inhibitors. Concanavalin A (K_i = 4.6 μg/mL) inhibited the enzyme noncompetitively. EGTA extracted 5–10 times as much enzyme in the dark as in the light. $CaCl_2$ and $MgCl_2$ at 5 mM inhibited the extraction of enzyme both in the dark and in the light. $CaCl_2$ (5 mM) facilitated the rebinding of enzyme to the membrane. Light had little effect on the rebinding of enzyme whether in the presence or absence of Ca^{2+}. The enzyme rebound to the bleached membrane in the presence of Ca^{2+} became readily extractable again in the dark if the chromophore of rhodopsin was regenerated and excess Ca^{2+} was removed. The amount of enzyme bound at Ca^{2+}/rhodopsin ≃ 20 in the dark was about 50% of the total peripheral enzyme associated with intact rod membranes. The binding of 5′-nucleotidase to rod membranes was specific; malate dehydrogenase and cytochrome *c* were not bound to the membranes at any Ca^{2+} concentration tested. In the presence of Ca^{2+} the enzyme was bound to membranes that had been treated with urea but not to rhodopsin–phosphatidylcholine vesicles. Hence, the nature of the membrane component involved in the binding of peripheral 5′-nucleotidase remains unknown.

A rapid decrease in 3′,5′-cGMP[1] concentration occurs during the light-induced hyperpolarization of vertebrate rod photoreceptors [see, for a review, Hubbell & Bownds (1979)]. This is catalyzed by a light-activated cyclic nucleotide phosphodiesterase (Bitensky et al., 1978). There is electrophysiological evidence implicating a role for 3′,5′-cGMP in the depolarization of photoreceptor membranes (Nicol & Miller, 1978). In spite of the rapid hydrolysis of 3′,5′-cGMP, guanylic

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[1] Abbreviations: 3′,5′-cGMP, guanosine cyclic 3′,5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 5′-AMP, adenosine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate.

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