megakaryocytes, and normoblasts were observed in greater than normal numbers in splenic imprints. The marrow smears revealed high ratios of myeloid to erythroid cells, frequently in the order of 20 : 1. A few of the liver sections showed distinct foci of immature granulocytic elements in the space of Disse (Fig. 1, A and B), as well as Kupffer cells containing hemosiderin. Overall, these features conformed with those described in the RF mice (*2*).

Other features seen in only a few of the bled mice were (i) chloroma tumors (Fig. 1D) in the liver, the area of the mandible, the thoracic cavity, and subcutaneous regions; (ii) myeloblasts containing Auer rods (Fig. 1E); and (iii) neutrophils with the "pseudo-Pelger" type nuclei. The latter two cell types, which were also noted in the rat (*1*), would lend additional support for the contention (*5*) that murine and human granulocytic leukemias have many features in common. In addition to all the features of ML detailed above, all mice with the disease consistently had elevated percentages in basophils (Fig. 1F) and myeloblasts in both blood and marrow (Table 1). Similar increases in basophil and myeloblast counts were also observed in rats induced to develop ML by phlebotomy after x-irradiation.

The minimal criteria for diagnosing ML in this study included premature death due to complications resulting from a combination of anemia, infection (generally pneumonia), and inanition, plus elevated amounts (at least 2.5 percent) of basophils or myeloblasts in either the blood or marrow. Three ML cases were diagnosed on the basis of blood values (one of these animals had chloroma), ten diagnoses were based on marrow values (two animals with chloroma), and thirteen diagnoses were based on both blood and marrow values (four animals with chloroma). All but one of the experimental mice developed ML; the exception developed thymic lymphoma (TL). Among the ten controls, one case of ML and two cases of TL were observed. The apparently preferential development of ML after bleeding in a strain known to be predisposed to development of ML and TL indicates that anemia is an effective triggering stress for the induction of ML (*6*) and also implies that a different stress is needed for inducing TL (*7*, *8*).

In summary, all members of the RF mouse population studied were naturally prone to ML development, and the anemia resulting from the removal of 50 percent of the blood volume of these animals was sufficient to trigger the overt manifestation of the disease. Overall, the results would tend to support the concept of a two-step mechanism of de novo induction of ML.

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References and Notes

1. J. K. Gong, *Science* **174**, 833 (1971).
2. A. C. Upton, F. F. Wolff, J. Furth, A. W. Kimball, *Cancer Res.* **18**, 842 (1958); D. F. Parson, A. C. Upton, M. A. Bender, V. R. Jenkins, E. S. Nelson, R. R. Johnson, *ibid.* **22**, 728 (1962); A. C. Upton, F. F. Wolff, E. P. Sniffen, *Proc. Soc. Exp. Biol. Med.* **168**, 464 (1961).
3. Animals were obtained from Jackson Laboratory, Bar Harbor, Maine 04609.
4. Earlier reports (*2*) showed that various groups of RF mice succumbing to ML after exposure to large single doses of x-rays (150 to 450 r) had average ages at death of 10.5 to 15.7 months. However, the survival time after irradiation of these leukemic populations (regardless of the dose of x-rays used or the age of the animals when they were irradiated) ranged from 9.3 to 11.7 months, or 9.9 ± 0.9 months (mean $\pm$ standard deviation). This time is similar to the average survival time of 9 months after phlebotomy found in our anemia-induced leukemic mice. It is thus possible to imagine that the primary leukemogenic effect of x-irradiation in the RF mice involved the triggering of the disease, possibly by an induced anemia. That most of the irradiated mice did not develop the disease could then be due to the absence of such an anemia in most of the exposed animals.
5. W. C. Moloney, A. D. Dorr, G. Dowd, A. E. Boschette, *Blood* **19**, 45 (1962); E. E. Varsa, E. S. Handler, A. S. Gordon, *ibid.* **26**, 309 (1965); D. F. Parsons, A. C. Upton, M. A. Bender, V. K. Jenkins, E. S. Nelson, R. R. Johnson, J. Furth, *ibid.* **9**, 688 (1945); T. B. Dunn, *J. Nat. Cancer Inst.* **14**, 1281 (1945).
6. It could be argued that anemia not severe enough to trigger ML could induce the development of TL. However, since the severity of radiation-induced anemia is dose-dependent, and increases in the incidence of TL in RF mice (*2*) occurred at an x-ray dose range higher than that needed to increase the incidence of ML, this argument would not appear valid.
7. Although the exact mechanism for TL induction is yet to be described, there is little doubt that the triggering of TL can be elicited by x-irradiation in the appropriate dose range, since incidence of both ML and TL was increased in RF mice that had been irradiated (*2*). That ML was found in nearly all of the bled RF mice, a strain in which many animals can develop TL, would indicate that proneness to both ML and TL probably coexisted in at least some individual animals. However, the finding of only TL among irradiated germ-free RF mice (*8*)—that is, when stresses for triggering both ML and TL were simultaneously applied—would suggest that proneness to both was not necessarily based on a single pathological entity. This observation on germ-free animals would also suggest that the "natural" proneness to ML seen in conventional mice was related to some exogenous factor.
8. H. E. Walburg, Jr., A. C. Upton, R. L. Tyndall, W. W. Harris, G. E. Cosgrove, *Proc. Soc. Exp. Biol. Med.* **118**, 11 (1965).
9. Smears were obtained from the bloody portion of a subcutaneous nodule several days before the death of the mouse. At the time of autopsy, the nodule was no longer bloody but was light green throughout. This color faded after exposure to air for 2 hours but reappeared within minutes after submergence in a 3 percent solution of hydrogen peroxide.
10. We thank Mary Byers for excellent technical assistance. This work was supported by grants FR-5330 and DE-001-67 from the U.S. Public Health Service.

23 March 1972

Terminal Saccharides on Sperm Plasma Membranes: Identification by Specific Agglutinins

Abstract. *Six specific agglutinins were used to identify the terminal sugar residues in the surface oligosaccharides of rabbit and hamster spermatozoa by specific agglutination. Species differences in epididymal sperm were found in the terminal residues, resembling* α-D-*mannose,* D-*galactose,* N-*acetyl*-D-*glucosamine, and* N-*acetyl*-D-*galactosamine. Species similarities were found in terminal residues, resembling* L-*fucose and* N-*acetylneuraminic acid. When ejaculated rabbit sperm were compared to epididymal sperm, the latter were more agglutinable with a specific agglutinin recognizing* N-*acetyl*-D-*glucosamine.*

Mammalian spermatozoa are complex haploid cells that are surrounded by a highly specialized continuous plasma membrane. Although no distinct morphological differences have been found among areas of the plasma membrane surrounding the sperm acrosomal, postacrosomal, midpiece, and tail regions (*1*), differences have been found in the distribution of charges on the membrane surface by the orientation of sperm in electric fields (*2*), the distribution of surface antigens (*3*), and the differential binding of colloidal iron to the plasma membrane surrounding the various sperm components (*4*). Also, the sperm plasma membrane undergoes ultrastructural changes in specific regions as the spermatozoon passes through the female genital tract and upon its penetration of the egg investments (*5*). These changes suggest that

there are local differences in the structure of the sperm plasma membrane that may be important in sperm recognition and ova fertilization.

In order to determine specific biochemical differences in the sperm plasma membrane, we have used purified agglutinins that recognize (almost exclusively) specific terminal saccharide residues in oligosaccharides to analyze distributional variations of plasma membrane saccharides. By observing which agglutinins specifically cause sperm agglutination and by observing which sperm regions are involved in the agglutination process, we have been able to identify differences in the plasma membrane saccharide distributions and also species differences between hamster and rabbit spermatozoa.

The purified agglutinins we used in our study were the following: concanavalin A (Con A) (*6*), specific for terminal residues similar to α-D-glucose α-D-mannose; wheat germ agglutinin (WGA) (*7*) for residues similar to terminal *N*-acetyl-D-glucosamine; *Dolichos biflorus* agglutinin (DBA) (*8*) for terminal residues similar to *N*-acetyl-D-galactosamine; *Ricinus communis* agglutinin (RCA) (*9*) for residues similar to D-galactose and L-arabinose; *Ulex europeus* agglutinin (UEA) (*10*) for residues similar to L-fucose; and influenza virus (flu) (*11*) for identifying residues similar to *N*-acetylneuraminic acid residues.

Golden hamster and rabbit spermatozoa from the caudae epididymides and rabbit spermatozoa that were ejaculated were washed three times by centrifugation in 0.9 percent sodium chloride. The live spermatozoa were immediately used for agglutination experiments (Table 1). Controls were set up in parallel with inhibitor sugar present, and in the case of influenza virus, with neuraminidase-treated spermatozoa. The type of sperm agglutination was noted with a light or phase-contrast microscope as head-to-head, head-to-tail, or tail-to-tail.

The agglutination of spermatozoa with the purified agglutinins was specific. It only occurred when the agglutinin was present in the incubation medium (Figs. 1 and 3) and did not occur when inhibitory concentrations of a competitive sugar were present (Fig. 2) or when the terminal sugar was enzymatically removed (Fig. 4). Even at low magnification, differences in agglutination occurring primarily head-to-head (Fig. 3) could be distinguished from agglutination occurring with the entire surfaces of the spermatozoa (Fig. 1) or agglutination occurring primarily tail-to-tail (not shown). At higher magnifications, these differences were clearly observable. For example, primarily head-to-head agglutination (Figs. 5 and 6) is easily differentiated from other types of agglutination (Figs. 7 and 8).

Similarities and differences between the agglutinability of hamster epididymal, rabbit epididymal, and rabbit ejaculated spermatozoa were found when the specific agglutinins were used (Table 1). Species differences were

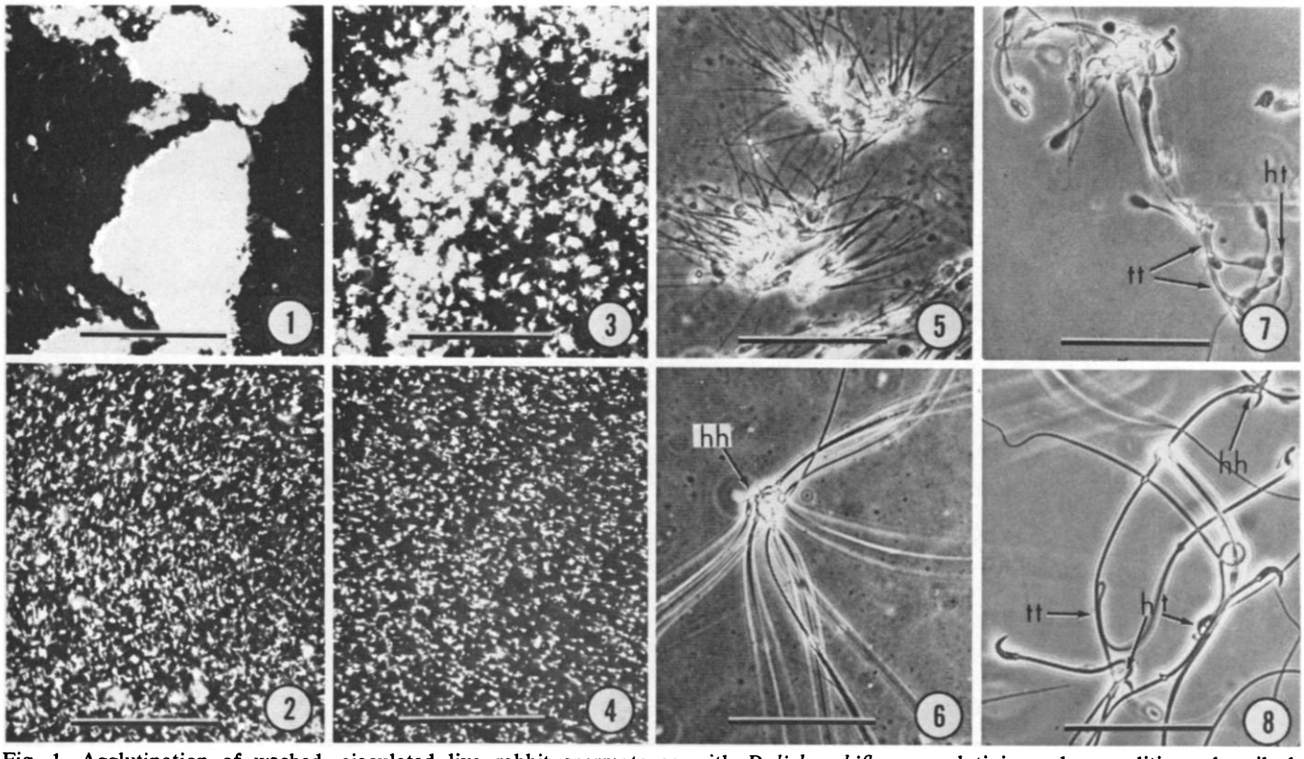

Fig. 1. Agglutination of washed, ejaculated live rabbit spermatozoa with *Dolichos biflorus* agglutinin under conditions described in Table 1. Magnification bar equals 500 μm for Figs. 1 to 4. Fig. 2. Control for experiment shown in Fig. 1. *N*-Acetyl-D-galactosamine, an inhibitor of the *D biflorus* agglutinin, was added to the rabbit spermatozoa before addition of the agglutinin. Fig. 3. Primarily head-to-head agglutination of washed, ejaculated live rabbit spermatozoa in the presence of influenza virus. Fig. 4. Control for the experiment shown in Fig. 3. The live rabbit spermatozoa were treated with neuraminidase prior to the agglutination assay. Fig. 5. Head-to-head agglutination of live rabbit spermatozoa in the presence of influenza virus, as in Fig. 3. Magnification bar equals 50 μm for Figs. 5 to 8. Fig. 6. The legend is the same as that for Fig. 5, except that live hamster spermatozoa were agglutinated with influenza virus. Agglutination is predominantly head-to-head (arrow, *hh*). Fig. 7. Agglutination of live rabbit spermatozoa with *D. biflorus* agglutinin. Agglutination is occurring tail-to-tail (arrow, *tt*) and head-to-tail (arrow, *ht*). Fig. 8. Agglutination of live hamster spermatozoa with *R. communis* agglutinin. Agglutination is occurring tail-to-tail (arrow, *tt*), head-to-tail (arrow, *ht*) and head-to-head (arrow, *hh*).

Table 1. Agglutination of spermatozoa by specific agglutinins. Freshly collected spermatozoa were washed three times by centrifugation (4 minutes each at 750 to 800*g*) in 0.9 percent NaCl and suspended in fresh 0.9 percent NaCl at concentrations of 0.7 to 1.2 $\times$ 10^7 sperms per milliliter (hamster) or 1.7 to 2.5 $\times$ 10^7 sperms per milliliter (rabbit). Agglutination experiments were performed by dropping equal volumes of the sperm suspension, 0.9 percent NaCl, and then the agglutinin solution onto glass slides and mixing by tilting gently for 3 to 5 minutes. Agglutination was observed with a light microscope and scored qualitatively on a scale of 0 to 4+ (no agglutination to virtually complete agglutination). The predominant sperm structures taking part in agglutination (that is, sperm head to sperm head, and so forth) were also noted (see Figs. 5 to 8). Simultaneous controls were run on the same slide with appropriate inhibitor saccharide solution substituted for the NaCl solution before the addition of agglutinin. No spontaneous agglutination of sperm in the absence of agglutinins occurred during the course of the experiments; hh, head-to-head; ht, head-to-tail; tt, tail-to-tail.

Terminal saccharides recognized	Inhibitor	Inhibitor concentration	Sperm agglutination								
			Rabbit ejaculated			Rabbit epididymal			Hamster epididymal		
			hh	ht	tt	hh	ht	tt	hh	ht	tt
Concanavalin A (500 μg/ml)											
α-D-Glucose	None		4+	3+	1+	4+	3+	1+	2+	3+	4+
α-D-Mannose	Sucrose	0.2*M*	0	0	0	0	0	0	0	0	0
*Influenza virus (1000 hemagglutinating units)**											
N-Acetylneuraminic acid	None		4+†	0	0	4+	0	0	4+	0	0
	Neuraminidase‡	200 unit/ml	0	0	0	0	0	0	0	0	0
Dolichos biflorus *agglutinin (250 μg/ml)*											
N-Acetyl-D-galactosamine	None		2+	3+	4+	2+	3+	4+	0	0	0
	N-Acetyl-D-galactosamine	0.2*M*	0	0	0	0	0	0	0	0	0
*Wheat germ agglutinin (4000 hemagglutinating units)**											
N-Acetyl-D-glucosamine	None		1+	1+	0	4+	2+	0	0	2+	4+
	N-Acetyl-D-glucosamine	0.2*M*	0	0	0	0	0	0	0	0	0
Ricinus communis *agglutinin (250 μg/ml)*											
D-Galactose	None		3+	2+	0	3+	2+	0	3+	4+	4+
L-Arabinose	D-Galactose	0.2*M*	0	0	0	0	0	0	0	0	0
Ulex europeus *agglutinin (300 hemagglutinating units)*§											
L-Fucose	None		0	0	0	0	0	0	0	0	0
	L-Fucose	0.2*M*	0	0	0	0	0	0	0	0	0

* Hemagglutination units were determined with rabbit erythrocytes (*6, 10*). † Spermatozoa were agglutinated almost exclusively acrosomal area to acrosomal area; sluggish or motionless sperm became very active upon contact with virus. ‡ Sperm suspension was incubated for 5 to 10 minutes at room temperature with an equal volume of *Vibrio cholerae* neuraminadase solution (500 unit/ml; Sigma) prior to the agglutination assay. § Hemagglutination units were determined with human type O erythrocytes (*9*).

found with Con A, DBA, WGA, and RCA. The DBA did not agglutinate hamster spermatozoa, an indication that little or no terminal *N*-acetate-D-galactosamine was present on the hamster epididymal sperm plasma membrane. Con A, WGA, and RCA agglutinate hamster epididymal spermatozoa more strongly tail-to-tail as compared with rabbit epididymal spermatozoa, signifying the presence of more terminal α-D-mannose or α-D-glucose, D-galactose, and *N*-acetyl-D-glucosamine on hamster epididymal sperm tails. These same sugars appear to be more prevalent on rabbit epididymal sperm heads, than tails, as Con A, WGA, and RCA caused strong head-to-head agglutination of rabbit epididymal spermatozoa. Species similarities were found with influenza virus and UEA. Both rabbit and hamster spermatozoa were agglutinated head-to-head with influenza virus, and neither was agglutinated with UEA.

This lattter finding indicates that L-fucose is not a major terminal saccharide residue on the spermatozoa used in our study. Differences in the plasma membrane saccharides between epididymal and ejaculated rabbit spermatozoa were seen with WGA. Apparently the amount of terminal *N*-acetyl-D-glucosamine decreases or its distribution changes after contact of the spermatozoa with seminal plasma. Since it is known that seminal plasma components adsorb to epididymal spermatozoa (*12*), this change may reflect masking of *N*-acetyl-D-glucosamine sites on the sperm surface.

The results presented here on the head-to-head agglutination of rabbit and hamster spermatozoa with influenza virus and those of Ericsson *et al.* (*13*) on the head-to-head agglutination of rabbit spermatozoa with Sendai virus indicate that the tail receptors for colloidal iron at low *p*H (*4*) may not all be sialic acid. Both of these viruses have sialic (*N*-acetylneuraminic) acid as important components of their cell surface receptors (*11, 14*), and treating sperm with neuraminidase renders the sperm nonagglutinable with influenza virus. Thus, sialic acid must be present on rabbit and hamster sperm heads, but the tail membrane anionic groups that bind colloidal iron and increase in strength during sperm maturation (*4*) may not be sialic acid. This finding is also supported by the observation that neuraminidase treatment of rabbit and hamster sperm does not prevent binding of colloidal iron to sperm tails at low *p*H (R. Yanagimachi, unpublished data). Neuraminidase-insensitive acidic groups such as sulfate or *N*-acetyl-*O*-diacetylneuraminic acid (*15*) may be responsible for some tail acidic groups.

After this report was submitted for publication, Edelman and Millette (*16*) reported that Con A agglutinates mouse sperm (isolated from *vas deferens*), and the distribution of fluorescent-labeled Con A and ^{125}I-labeled Con A indicated that Con A receptors are more prevalent on mouse sperm heads than on tails. The exact location of these membrane oligosaccharides may be determined with the use of ferritin-conjugated plant agglutinins (*17*).

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References and Notes

1. D. W. Fawcett, *Int. Rev. Cytol.* **7**, 195 (1958); J. L. Hancock, *Advan. Reprod. Physiol.* **1**, 125 (1966); D. W. Fawcett and D. M. Phillips, *J. Reprod. Fertil.* **6** (Suppl.), 405 (1969); L. Piko, in *Fertilization*, C. B. Metz and A. Monroy, Eds. (Academic Press, New York, 1969), vol. 2, p. 325; D. W. Fawcett, *Biol. Reprod.* **2** (Suppl.), 90 (1970).

2. A. C. Nevo, I. Michaeli, H. Schildler, *Exp. Cell Res.* **23**, 69 (19→ A. D. Bangham, *Proc. Roy. Soc. London Ser. B* **155**, 292 (1961); J. M. Bedford, *Nature* **200**, 1178 (1963).
3. W. Henle, G. Henle, L. A. Chambers, *J. Exp. Med.* **68**, 335 (1938).
4. G. W. Cooper and J. M. Bedford, *Anat. Rec.* **169**, 300 (1971); R. Yanagimachi and Y. D. Noda, *Proc. Annu. Meet. Amer. Soc. Biol. 11th* (1971), abstr., p. 660.
5. L. Piko and A. Tyler, *Proc. Int. Congr. Anim. Reprod. 5th* **2**, 372 (1964); C. Barros, J. M. Bedford, L. E. Franklin, C. R. Austin, *J. Cell Biol.* **34**, C1 (1967); C. R. Austin, *The Ultrastructure of Fertilization* (Holt, Rinehart & Winston, New York, 1968); J. M. Bedford, *Biol. Reprod.* **2** (Suppl.), 128 (197→ R. Yanagimachi and Y. D. Noda, *J. Ultrastruct. Res.* **31**, 465 (1970).
6. B. B. L. Agrawal and I. J. Goldstein, *Biochim. Biophys. Acta* **147**, 262 (1967); R. D. Pretz and I. J. Goldstein, *Biochemistry* **9**, 2870 (1970). Concanavalin A was obtained as a chromatographically pure agglutinin from Miles-Yeda (Kankakee, Ill.).
7. Wheat germ agglutinin was purified according→ M. M. Burger and A. R. Goldberg [*Proc. Nat. Acad. Sci. U.S.A.* **57**, 359 (1967)].
8. *Dolichos biflorus* agglutinin was a gift from Dr. M. E. Etzler and was prepared according to the method of M. E. Etzler and E. A. Kabat [*Biochemistry* **9**, 869 (1970)].
9. R. G. Drysdale, P. R. Herrik, D. Franks, *Vox Sang.* **15**, 194 (1968). *Ricinus communis* agglutinin was prepared by the procedure of G. L. Nicolson and J. Blaustein [*Biochim. Biophys. Acta* **266**, 543 (1972)]. Local wild *R. communis* beans were ground and saline was extracted. The agglutinin was precipitated in the 60 percent ammonium sulfate fraction. After dialyzing the precipitate against 0.05*M* sodium phosphate–0.2*M* sodium chloride buffer, *p*H 7.4, the dialyzate was applied to an affinity column and washed until absorbance at 280 nm was below 0.5. The *R. communis* agglutinin was eluted by a step gradient of 0.2*M* D-galactose and was extensively dialyzed to remove the bound galactose.
10. *Ulex europeus* agglutinin was prepared from pulverized seeds according to a modification of the procedure of I. Matsumoto and T. Osawa [*Biochim. Biophys. Acta* **194**, 180 (1969); *Arch. Biochem. Biophys.* **140**, 484 (1970)]. A crude saline extract of *U. europeus* agglutinin was precipitated in the 40 percent ammonium sulfate fraction. After dialysis, the agglutinin was further purified by chromatography on Sephadex G-100 and the agglutinin peak was concentrated fourfold. The concentrated agglutinin solution had a titer of 600 against human type O erythrocytes. It was specifically inhibited by L-fucose and did not contain any chitobiose activity.
11. E. Klenk and H. Lempfrid, *Z. Physiol. Chem.* **307**, 278 (1957); B. Tess and E. Kempf, *J. Bacteriol.* **86**, 239 (1963); *Arch. Ges. Virusforsch.* **19**, 70 (1966). Influenza virus was purified according to J. J. Holland and E. D. Kiehn [*Science* **167**, 202 (1970)] and was used as described→ G. L. Nicolson, V. T. Marchesi, and S. J. Singer [*J. Cell Biol.* **51**, 265 (1971)].
12. A. J. Weil and A. E. Finkler, *Proc. Soc. Exp. Biol. Med.* **98**, 794 (1958); A. J. Weil, *Science* **131**, 1040 (1960); ——— and J. M. Rodenburg, *Proc. Soc. Exp. Biol. Med.* **109**, 567 (1962).
13. R. J. Ericsson, D. A. Buthala, J. F. Norland, *Science* **173**, 54 (1971).
14. C. Scholtissek, R. Drzeniek, R. Rott, in *The Biochemistry of Viruses*, H. B. Leog, Ed. (Dekker, New York, 1969), p. 232.
15. R. A. Gibbons, *Biochem. J.* **89**, 380 (1963); C. Ravetto, *J. Histochem. Cytochem.* **16**, 663 (1968).
→ G. M. Edelman and C. F. Millette, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 2436 (1971).
→ G. L. Nicolson and S. J. Singer, *ibid.* p. 942; *Proc. Electron Microsc. Soc. Amer.* **29**, 536 (1971); *Ann. N.Y. Acad. Sci.*, in press; G. L. Nicolson, *Nature* **233**, 244 (1971); *ibid.*, in press.
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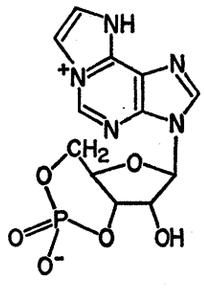
Fluorescent Modification of Adenosine 3′,5′-Monophosphate: Spectroscopic Properties and Activity in Enzyme Systems

Abstract. *The synthesis of a highly fluorescent analog of adenosine 3′,5′-monophosphate, namely, 1,*N^6*-ethenoadenosine 3′,5′-monophosphate, has provided a powerful probe for systems involving adenosine 3′,5′-monophosphate. The potential utility of this analog is indicated by its long fluorescent lifetime, detectability at low concentration, and relatively long wavelength of excitation (300 nanometers). In protein kinase systems it is a highly acceptable substitute for adenosine 3′,5′-monophosphate.*

Adenosine 3′,5′-monophosphate (cyclic AMP) is a key regulatory agent in most mammalian tissues (*1*). The value of a fluorescent derivative of cyclic AMP for gaining additional information concerning its many roles has been recognized (*2*). In view of the interesting enzymatic and fluorescent properties of 1,N^6-ethenoadenosine triphosphate or εATP (*3*) it was deemed worthwhile to study the influence of a similar structural modification of cyclic AMP on enzyme systems. Accordingly, we have synthesized 1,N^6-ethenoadenosine 3′,5′-monophosphate (**1**), abbreviated "cyclic εAMP" (*4*), by reaction of chloroacetaldehyde with cyclic AMP under general conditions described earlier (*5*) and have demonstrated its behavior with representative enymes.



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More specifically, an aqueous solution of chloroacetaldehyde was prepared as follows. One hundred grams of chloroacetaldehyde dimethylacetal (Aldrich) in 500 ml of 50 percent H_2SO_4 (weight per volume) was refluxed gently for 45 minutes and the mixture was evaporated under vacuum while the receiving vessel was cooled. The upper layer of the distillate was adjusted to *p*H 4.5 with 1*N* NaOH and distilled as before. The recovered distillate, ~1.0 to 1.6*M* in chloroacetaldehyde, was used in 20-fold excess to convert cyclic AMP to the derivative (**1**), with a 1,N^6-etheno bridge, $C_{12}H_{12}N_5O_6P \cdot H_2O$ (melting at 267–8°C, with decomposition), by stirring at *p*H 4.0 to 4.5 at 37°C for 24 hours, decolorization with charcoal, and evaporation to dryness. Reprecipitation from aqueous ethanol followed by a washing in ethanol yielded pure product. Complete conversion of cyclic AMP to cyclic εAMP was monitored by thin-layer chromatography and by ultraviolet absorption (*5*). Both cyclic AMP and the etheno-bridged product **1** are stable under the conditions of synthesis, and no hydrolysis was detected by a thin-layer chromatographic comparison with 3′εAMP and 5′εAMP prepared in analogous fashion from 3′AMP and 5′AMP, respectively. The R_F values observed for these compounds on Eastman Chromagram cellulose sheets without fluorescent indicator, with the solvent system isobutyric acid : ammonium hydroxide : water (75 : 1 : 24, by volume), were as follows: cyclic εAMP, 0.27; 3′εAMP, 0.25; 5′εAMP, 0.19, compared with 0.40 for cyclic AMP. The reaction mixture resulting from the treatment of cyclic AMP with chloroacetaldehyde showed only one bluish fluorescent spot. The chloroacetaldehyde-modified compounds are readily detectable under an ultraviolet lamp in amounts of 0.5 μg. The ultraviolet spectra of **1** and εATP (or other reported analogs) (*3*, *6*) are similar in the shape and relative heights of the individual maxima. Thus, structure **1** is suitable for studies in the presence of nucleic acids or proteins. The ultraviolet absorption band of lowest energy lies at longer wavelength (≃300 nm in *p*H 7 buffer) than that of the normally occurring bases or aromatic amino acids, and it is thus possible to excite this fluorescent analog of cyclic AMP (**1**) selectively. Moreover, it is fluorescent at neutral *p*H (maximum near 410 nm) and in ionic environments (*6*). The long fluorescent lifetime (close to 20 nsec) and the possibility of detection at very low concentration (~ $1 \times 10^{-8}M$) provide the opportunity for using more detailed fluorescence techniques.

There is one well-documented biochemical mechanism by which cyclic AMP regulates at least certain differentiated functions of eukaryotic cells. Cyclic AMP stimulates the activity of cell