Adenine and Adenosine Are Toxic to Human Lymphoblast Mutants Defective in Purine Salvage Enzymes

Abstract. *Mutants deficient in adenosine kinase or adenine phosphoribosyltransferase activities were selected from the WI-L2 line of human lymphoblasts. The adenosine kinase–deficient mutant was still as sensitive as its parent to growth inhibition caused by adenosine when adenosine deaminase was inhibited. Similarly, the adenine phosphoribosyltransferase mutant remained sensitive to growth inhibition caused by adenine. Thus, the toxicity of adenine and adenosine to human lymphoblasts is not mediated by nucleotides to which they may be converted.*

The purine adenine and its sugar derivative adenosine inhibit the growth of both bacteria (*1*) and animal cells in culture (*2–5*). Selective toxicity of adenosine to dividing lymphoid cells (*6–9*) is thought to be the basis for a type of severe combined immunodeficiency disease (*10*) characterized by absence of the activity of adenosine deaminase (ADA, E.C.3.5.4.4), which converts adenosine to inosine. In ADA deficiency, unrestricted phosphorylation of adenosine to adenosine monophosphate by adenosine kinase (AK; E.C.3.7.1.20) and subsequent effects of adenine nucleotides on lymphocyte metabolism, in particular, decreased pyrimidine nucleotide synthesis (*11*) has been proposed as the mechanism for adenosine toxicity (*4, 6*). Evidence that the toxic effects of adenosine require its phosphorylation derive primarily from reports that mouse fibroblast (*4*) and Chinese hamster (*5*) cells with varying degrees of diminished AK activity are resistant to growth inhibition by exogenous adenosine. Adenosine deaminase activity was present in these lines and could have affected the measurement of residual AK activity in cell extracts, as well as resistance to growth inhibition caused by adenosine.

In studying the regulation of purine biosynthesis in the WI-L2 long-term human splenic lymphoblast line (*12*), we isolated mutants deficient in AK and adenine phosphoribosyltransferase (APRT, E.C.2.4.2.7) activities (*13*). We report here that, despite high-level resistance to the purine analogs used in their selection, the APRT$^-$ and AK$^-$ mutants remain sensitive to the cytostatic effects of adenine and adenosine, respectively. Adenine is not metabolized in APRT$^-$ mutants, and the studies with adenosine were performed under conditions in which its deamination, as well as phosphorylation, was blocked.

AK$^-$ and APRT$^-$ mutants were selected in mass culture for resistance to analogs of adenosine, 6-methylthioinosine (also referred to as 6-methylmercaptopurine riboside), 2 μM; or of adenine (2,6,diaminopurine), 20 μM, respectively. Survivors, designated MTI^r (for methylthioinosine resistant) and DAP^r (for diaminopurine resistant), respectively, were then cloned in agarose (*14*). Their phenotypes and biochemical characteristics have been stable for 2 years and 1 year in the absence of selective conditions, respectively. No mutagen was used in the selection of the DAP^r mutants or the MTI^r mutant U3. In selecting the MTI^r mutant 107, parental cells were first treated with ethylmethane sulfonate (1 μM for 45 minutes at 37°C). A double mutant deficient in both AK and hypoxanthine guanine phosphoribosyltransferase (HGPRT, E.C.2.4.2.8) was derived from MTI^r 107 by a second selection (without further mutagenesis) for resistance to 10 μM thioguanine and was designated MTI-TG (for methylthioinosine and thioguanine resistant).

The specific activities of AK, APRT, and HGPRT in extracts of WI-L2 and MTI^r lymphoblasts are shown in Table 1. Deficiency of AK activity was further demonstrated by the inability to detect phosphorylation of either [^{14}C]MTI (*15*) or [^{14}C]adenosine in cell extracts after fractionation by glycerol gradient ultracentrifugation (not shown). Both activities cosedimented in the case of WI-L2, but were undetectable in gradients of equivalent amounts of an extract of MTI^r 107. This experiment was necessary since a mutant in an Ehrlich ascites tumor line has been reported which lacked the ability to phosphorylate MTI but retained some activity with adenosine as substrate (*16*). In the assay for phosphorylation of [^{14}C]adenosine, 5 μM erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was present to inhibit ADA activity by > 95 percent (*8, 17*). The inhibitor is not required in studies with MTI, which is neither deaminated nor phosphorylized (*15*) in mammalian cells. AK assays have been done in tris-maleic acid, potassium succinate, potassium phosphate, and tris-HCl buffers (0.1*M*) at *p*H ranging from 4.6 to 8.0 (optimum at *p*H 5.6 in succinate or maleate buffers), and at a wide variety of $MgCl_2$, adenosine triphosphate (ATP), and adenosine concentrations in an attempt to detect greater residual activity in the mutant, but at most 0.1 percent of parental activity was found.

In studies with intact cells, MTI^r clones phosphorylated [^{14}C]MTI at < 0.1 percent of the maximal rate of WI-L2. The inability to phosphorylate adenosine was demonstrated by comparing intracellular nucleotide formation from [^{14}C]-adenosine by the AK$^-$–HGPRT$^-$ double mutant with that by a derivative of WI-

Table 1. Specific activities of adenosine kinase (AK), hypoxanthine guanine phosphoribosyltransferase (HGPRT), and adenine phosphoribosyltransferase (APRT) in lymphoblast extracts. Cells (6×10^5 to 10×10^5 cell/ml) were grown to the mid-log phase in AutoPow minimal essential medium (Gibco) plus 10 percent fetal calf serum (*14*) and collected by centrifugation; they were then washed with Dulbecco's phosphate-buffered saline containing bovine serum albumin (2.5 mg/ml). After complete aspiration of washing buffer, the cells were resuspended in 0.01*M* tris-HCl, *p*H 7.4, at a density of 2×10^7 cell/ml, and lysed by freezing and thawing three times in liquid nitrogen. After centrifugation for 45 minutes at 27,000*g* at 4°C, the supernatants were dialyzed for 20 hours against 2000 volumes of 0.025*M* tris-HCl, *p*H 7.4, 0.025*M* KCl at 4°C. Enzyme activities were determined in reaction mixtures (0.05 ml, final volume) containing 10 μl of dialyzed extract diluted in dialysis buffer, and the following: (i) for AK, 0.1*M* tris-maleic acid buffer, *p*H 5.5; 4 m*M* ATP (neutralized to *p*H 7.0 with NaOH); 1.5 m*M* $MgCl_2$; 5 μM EHNA; and 50 μM [8-^{14}C]adenosine (Amersham/Searle, 59 μc/μmole). (ii) For HGPRT: 0.05*M* tris-HCl, *p*H 7.4; 5 m*M* $MgCl_2$; 4 m*M* PP-ribose-P, magnesium salt (P-L Biochemicals); and 100 μM [8-^{14}C]hypoxanthine (59 μc/μmole, Amersham/Searle). (iii) For APRT as for HGPRT except that 200 μM [8-^{14}C]adenine (59 μc/μmole, Amersham/Searle) replaced [^{14}C]hypoxanthine. All assays were for 20 minutes at 37°C and were terminated by applying 25 μl to squares (2 by 2 cm) of DE 81 paper (Reeve Angel) and washing four times for 15 to 30 minutes in 1 m*M* ammonium formate. In the case of adenosine kinase, activity was negligible in the pellet after the centrifugation at 27,000*g*. The conditions used were optimized for *p*H, $MgCl_2$, ATP, and buffer. Results are from the linear (with extract and time) portion of curves, although much more concentrated extracts of mutant cells were also tested. Mixing of normal and mutant extracts gave results expected from the degree of dilution of the normal extract. In addition to using the DEAE paper technique, we have also chromatographed reaction products, and measured labeling of appropriate nucleosides and bases to be certain that differences in rates of product nucleotide degradation were not responsible for differences in activities in normal and mutant cell extracts.

Cell line	Specific activity (nmole min^{-1} mg^{-1})		
	AK	HGPRT	APRT
WI-L2	2.63	7.99	8.89
MTI^r-107	< 0.02	5.67	9.43
MTI^r-U3	< 0.02	6.14	9.22
MTI-TG	0.03	0.07	11.12

Table 2. Nucleotide formation from [^{14}C]adenosine by intact lymphoblasts. Portions (0.1 ml) of AG^{r}9 clone 35-1 (HGPRT$^-$) (*14*) and MTI-TG (AK$^-$–HGPRT$^-$) (approximately 1.2×10^6 cells) in growth medium containing 10 percent fetal calf serum that had been heated for 5 hours at 62°C (to inactivate ADA >98 percent) and dialyzed against 0.9 percent NaCl for 5 days were incubated with [^{14}C]adenosine (90.1 count/min per picomole; 125 μM, final concentration) for 30 or 60 minutes at 37°C. The tubes were then placed on ice, 5 ml of ice-cold medium was added, and the cells were centrifuged at 200*g* for 2½ minutes at 4°C. The medium was aspirated completely, and the cell pellet was extracted with 2 ml of 1*N* HCl for 30 minutes at 0°C. After centrifugation (10 minutes at 800*g* at 4°C) the supernatant was passed over a column of Dowex-50 (H^+), and the column was washed with 3 ml of 1*N* HCl. The flow-through and wash were combined (soluble nucleotides) and hydrolyzed (100°C for 60 minutes) to release free purine bases. After lyophilization and resuspension in 75 μl of water, a portion was chromatographed with adenine, guanine, and hypoxanthine markers on cellulose thin-layer plates; the marker areas were identified under ultraviolet light and cut out, and the radioactivity was counted (*13*). Controls for each cell line were run in which [^{14}C]adenosine was added to cells at 0° instead of 37°C. The separation of purine nucleotides from nucleosides and bases by the Dowex-50 column was demonstrated with appropriate radioactive compounds.

Incubation time (minutes)	Incorporation of [^{14}C]adenosine into soluble nucleotides containing (pmole/10^6 cell)		
	Hypoxanthine	Guanine	Adenine
	HGPRT cells		
30	176	73	1288
60	370	156	2191
	AK$^-$–HGPRT$^-$ cells		
30	55	13	49
60	107	22	99

L2 lacking HGPRT activity alone (*14*). Labeled hypoxanthine may be derived from [^{14}C]adenosine by successive deamination and phosphorolysis, but, in these lines, nucleotide formation from hypoxanthine is blocked by ~98 percent [Table 1 (*14*)]. Thus labeled adenine nucleotides derived from [^{14}C]adenosine in HGPRT$^-$ cells principally represents AK activity. At an extracellular concentration of 125 μM, [^{14}C]adenosine was converted to intracellular adenine nucleotides by AK$^-$–HGPRT$^-$ at about 4 percent of the rate observed with HGPRT$^-$ cells (Table 2). A portion of this low rate of nucleotide synthesis in AK$^-$–HGPRT$^-$ cells probably results from the 2 percent residual HGPRT activity in this mutant (Table 1), as at 125 μM most of the adenosine is deaminated by these cells. In experiments not shown, at lower concentrations of adenosine, saturating for its phosphorylation but not for deamination, nucleotide formation from [^{14}C]adenosine by the AK$^-$–HGPRT$^-$ mutant was < 1 percent that of WI-L2 or the HGPRT$^-$ mutant.

Because adenosine is rapidly deaminated to inosine by ADA in cells or serum, its effects on growth of cultured cells have been studied in the presence of inhibitors of ADA such as coformycin or EHNA (*7, 8*), and with horse or heated calf serums lacking this activity to greater or lesser degree (*4, 8, 9*). In the experiments shown in Tables 3 and 4, horse and heated calf serums were used and 5 μM EHNA was present, an amount sufficient to inhibit deamination of adenosine by lymphoblast cultures by > 95 percent (*8, 9*). This concentration of EHNA had little effect on cell growth, but enhanced the sensitivity of WI-L2 to growth inhibition by adenosine (Table 3). Combinations of 5 μM EHNA plus up to 50 μM adenosine were equally effective in inhibiting growth of WI-L2 and AK$^-$ mutants (Table 4). In contrast, the mutant clones grew normally in 1 mM MTI while WI-L2 was inhibited completely at 0.5 μM. In other studies, we found that uridine partially reversed the toxicity of adenosine (plus EHNA) to WI-L2 lymphoblasts but not to its AK$^-$ derivatives (*18*).

Growth of WI-L2 lymphoblasts is inhibited completely above ~10 μM DAP (Table 5, experiment A). A mutant resistant to 200 μM DAP (Table 5, experiment A) had < 0.5 percent of parental APRT activity in extracts (< 0.02 nmole/min per milligram of protein when assayed as in Table 1). At an extracellular concentration of 450 μM [^{14}C]adenine, intact mutant cells converted <1 pmole/min per 10^6 cells to intracellular nucleotides. The maximal rate for the parent strain was approximately 130 pmole/min per 10^6 cells, with a half-maximal velocity achieved at about 2 to 4 μM [^{14}C]adenine. Despite virtually complete absence of APRT activity, growth rates of both mutant and parent were inhibited to similar degrees by various concentrations of adenine (Table 5, experiment B) (*19*). In contrast to previously reported results with mouse L cells (*2*) neither exogenous uridine nor uridine plus the four purine and pyrimidine deoxyribonucleosides corrected the adenine-mediated growth inhibition (Table 5, experiments C and D). Thus, it is unlikely that either diminished pyrimidine nucleotide synthesis or ribonucleoside reduction are alone responsible for the toxicity of adenine to human lymphoblasts (*20*). In other experiments, we found no significant conversion of [^{14}C]adenosine to [^{14}C]adenine in the presence of phosphate, either in the presence or absence of EHNA, by medium in which DAP^r or MTI^r mutants had grown to high density, or by extracts of concentrated suspensions (2×10^7 to 5×10^7 cell/ml) of these cells. Thus, such an activity, attributable either to mycoplasma contamination (*21*) or to adenosine phosphorylase reported to be present in some mammalian cells (*22*), does not appear responsible for the continued toxicity to these mutants of adenine or adenosine, respectively.

Table 3. Growth inhibition by adenosine and its potentiation by EHNA. WI-L2 lymphoblasts were grown from an initial density of 1×10^5 cells per milliliter for 72 hours in Auto-Pow minimal essential medium containing 10 percent horse serum plus the indicated additions. The batch of horse serum used had virtually undetectable ADA activity.

Adenosine (μM)	Relative growth* EHNA (μM)	
	0	5
0	1.00	0.81
1	1.00	0.75
10	0.66	0.39
50	0.50	0.11
100	0.41	
500	0.10	

*Relative to the cell density achieved by the control (no additions) after 72 hours of growth = 1.00. Doubling time was 16 to 20 hours in this medium.

In contrast to our results with human lymphoblasts, AK deficiency is reported to have increased the resistance of 3T3 mouse fibroblasts to the toxic effects of adenosine by approximately 70-fold (*4*). In addition to differences in cell type and species, the mutant studied by Ischii and Green (*4*) apparently contained two mutations. The line was derived by selecting first for a 2000-fold increase in resistance to MTI. However, the mutant so selected had "only slight reduction" in AK activity and was then subjected to further selection for resistance to a second adenosine analog, tubericidin. Resistance to adenosine was studied only after the second round of selection; therefore it is possible [as discussed (*23*)] that it was the first mutation that resulted in resistance to adenosine, rather than AK deficiency (which was determined without inhibiting ADA activity and was not demonstrated in intact cells). That resistance to adenosine may occur in cells which retain significant AK activity is indicated by studies of two adenosine-resistant Chinese hamster cell mutants (*5*). In one, AK activity was normal, and the basis for resistance was not determined. The second had about 63 percent of parental AK activity in extracts, but

this appeared to differ from normal enzyme in substrate dependence and *p*H optimum. Again, ADA activity present in crude extracts can interfere with accurate characterization of residual AK activity.

In summary, our results, and the conclusions which seem consistent with them, include the following: (i) Mutational loss of at least 95 percent of the ability to phosphorylate adenosine to intracellular nucleotides is not accompanied by a significant increase in resistance to the growth inhibitory effects of adenosine. Therefore adenosine itself, rather than a nucleotide to which it is converted, may be directly toxic to human lymphoblasts in which ADA is inhibited. (ii) Adenine toxicity is unaltered in lymphoblasts which have lost > 99 percent of their APRT activity, suggesting the base itself is toxic, analagous to the case with adenosine. (iii) The modes of toxicity of adenine and adenosine differ, since pyrimidines ameliorate the growth inhibition of normal human lymphoblasts caused by adenosine (*4, 9*) but not by adenine (Table 5). If their toxicities were only due to intracellular nucleotides to which they are converted, we might expect similar modes of action since they are both converted to AMP initially. Although our studies demonstrate that adenosine and adenine may be directly toxic to cells, we understand that different metabolic consequences, perhaps contributing to or altering their toxicity, may occur in cells that are capable of converting them directly to nucleotides. Such differences might be a consequence of the differences in their cosubstrates for AMP formation, 5-phosphoribosyl pyrophosphate (PP-ribose-P) for the APRT, and ATP for the AK reaction. Furthermore, a recent report suggests that adenine and adenosine may contribute to different intracellular ATP pools (*24*). Nevertheless, our results raise the possibility that increases in adenine nucleotide concentration observed in erythrocytes (*25*) and lymphocytes (*26*) of ADA-deficient children may not necessarily be the cause of their immune deficiency. We have also found (*18, 27*) that, while complete growth inhibition of normal lymphoblasts caused by adenosine was accompanied by expansion of adenine nucleotide pools, no such expansion was detectable in AK^- mutants inhibited to the same degree.

Our results are consistent with the existence of purine receptors that recognize adenine or adenosine (or both) and that are involved in mediating their effects on cells. Alternatively, these purines may directly interfere with an intracellular enzyme (or enzymes) and interrupt one or more pathways essential for growth.

Table 4. Effect of adenosine plus inhibitor of ADA on growth of normal and mutant lymphoblasts. Cell lines were grown in AutoPow minimal essential medium containing either 10 percent heated (5 hours, 62°C) fetal calf serum or 10 percent horse serum, plus the indicated additions, from an initial cell density of 0.5 to 1.0 × 10^5 cell/ml. The heated fetal calf serum (FCS) had less than 1 percent of the ADA activity present in unheated serum.

EHNA (μM)	Adenosine (μM)	Serum	Relative growth*		
			WI-L2	MTI^r-U3	MTI-TG
0	0	Heated FCS	1.00	1.00	1.00
5	0	Heated FCS	0.98	0.95	0.93
5	10	Heated FCS	0.73	0.51	0.53
5	25	Heated FCS	0.41	0.30	0.33
5	50	Heated FCS	0.25	0.20	0.29
5	50	Horse		0.08	0.11

*As defined in footnote to Table 3.

Table 5. Growth inhibition by adenine in the presence and absence of uridine and the four deoxy nucleosides. WI-L2 and a DAP^r clone containing < 0.5 percent residual APRT activity were grown in AutoPow minimal essential medium (*18*) containing 10 percent dialyzed fetal calf serum and the indicated additions, at initial cell densities of 1 to 1.7 × 10^5 cell/ml. Both lines doubled in 16 to 20 hours in the absence of additions.

Addition	Relative growth*	
	WI-L2	DAP^r
Experiment A		
0.01 m*M* 2,6-diaminopurine	0.15	
0.02 m*M* 2,6-diaminopurine	0.08	
0.2 m*M* 2,6-diaminopurine	0.06	0.82–0.98
Experiment B		
0.1 m*M* adenine	1.0	0.70
0.4 m*M* adenine	0.67	0.52
0.6 m*M* adenine	0.48	0.40
0.8 m*M* adenine	0.36	0.31
1.0 m*M* adenine	0.08	0.11
Experiment C		
1.0 m*M* adenine + 0.5 m*M* uridine	0.11	0.13
0.5 m*M* uridine	0.92	1.0
Experiment D		
0.5 m*M* adenine	0.31	
0.5 m*M* adenine + 0.2 m*M* uridine + 0.02 m*M* each dCyd, dThd, dGuo, dAdo†	0.34	
0.02 m*M* uridine + 0.02 m*M* each dCyd, dThd, dGuo, dAdo†	1.13	

*Relative to control in each series (no additions) at 72 hours = 1.0. †Deoxycytidine, deoxythymidine, deoxyguanosine, deoxyadenosine, respectively.

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A Method for Detecting 8-Methoxypsoralen in the Ocular Lens

Abstract. *The use of 8-methoxypsoralen for treating psoriasis could prove hazardous if this photosensitizing agent enters the ocular lens. Phosphorescence spectra of intact rat lenses reveal concentrations of 8-methoxypsoralen on the order of 10^{-5}M after intraperitoneal injection of 8-methoxypsoralen. There is evidence that this drug can function as a photosensitizing agent, enhancing ultraviolet-induced changes within the lens.*

Ambient ultraviolet radiation has been implicated as a factor in the increased fluorescence and pigmentation that develops in the nucleus of the aging human lens, and in the pathogenesis of the senile nuclear (brown) cataract (*1–3*). There is also the possibility that ultraviolet-induced lenticular change may be enhanced in the presence of photosensitizing drugs within this organ (*4–6*). The photochemical reactions of furocoumarin derivatives, particularly 8-methoxypsoralen (8-MOP) have recently received widespread attention (*7, 8*).

Since the introduction of psoralens as a method for treating vitiligo in 1947 (*9*) and since their recent use for treating psoriasis (*10*), there has been sporadic interest concerning the potential relation between psoralens and cataract formation. Some reports have suggested the experimental production of cataracts in animals given very large doses of 8-MOP and exposed to long-wave ultraviolet radiation (*4*). However, the presence of 8-MOP has never been demonstrated in the lenses of experimental animals or in human lenses.

The introduction and increasing use of 8-MOP as a major therapeutic drug for psoriasis (*10*) could prove to be a significant hazard if this drug enters the lens, and an even more significant hazard if it accumulates with repeated dosage. There is some evidence that 8-MOP and other photosensitizing agents may bind to specific macromolecules such as DNA and certain proteins, thereby enhancing their photosensitizing action (*7, 11*). It should be noted that, because the lens is completely encapsulated and never sheds any of its cells, the possibility exists that 8-MOP may not only enter the lens but also may become bound to one or more of the macromolecules within the lens and thus accumulate with repeated therapy. Hence, 8-MOP could then accumulate and exert its photosensitizing effect over a long period of time.

It is generally accepted that the photochemical action of psoralen in the skin involves cyclo addition to pyrimidine bases in DNA and light-induced interstrand cross-linking of DNA molecules (*7, 11, 12*). A similar mechanism has been proposed for damage by 8-MOP to the photosensitized lens (*6*). We now describe a method for detecting 8-MOP in the lenses of experimental rats and demonstrate that this drug does enter the lens when administered in doses approximating those given to patients with psoriasis.

We used our own laboratory bred strain of Sprague-Dawley rats, which were put in the dark after 4 weeks of age and maintained in these conditions throughout the experimental period. These animals were given a single (0.1 ml) intraperitoneal injection of 8-MOP in dimethyl sulfoxide (DMSO) (4 to 6 mg per kilogram of body weight) or a recrystallized suspension (0.2 ml) of 8-MOP in

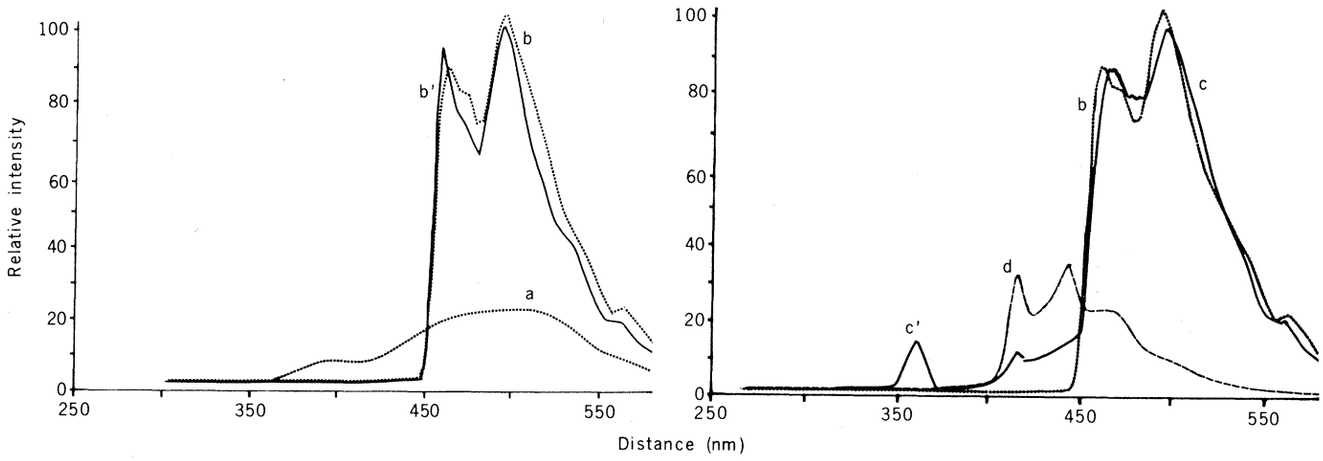

Fig. 1 (left). Fluorescence spectrum (a, ·····) of 8-MOP at 25°C. Instrument sensitivity 10×. Phosphorescence spectra of $10^{-3}M$ 8-MOP in ethylene glycol (b, ·····) and in ethanol (b′, ———) at 77°K. Instrument sensitivity 1×. Fig. 2 (right). Phosphorescence spectra of $10^{-3}M$ 8-MOP in ethylene glycol (b, ·····) and whole rat lens showing 8-MOP (c, ———) and tryptophan (d, -------) phosphorescence at 77°K. Excitation peak at 360 nm (c′). The instrument sensitivity for 8-MOP phosphorescence in lens (c) was 100× as compared to 1× sensitivity for $10^{-3}M$ 8-MOP in ethylene glycol (b). The lens tryptophan phosphorescence (d) was also obtained with 1× sensitivity and 290 nm excitation.