Both CHO cells and human lymphocytes were treated with 0.075*M* KCl to spread the chromosomes and were fixed in a mixture of methanol and acetic acid (3:1). The cells were concentrated by centrifugation and drops of the concentrated fixed cells were placed on clean dry microslides, which were then stained for 15 minutes in 0.5 μg of Hoechst 33258 per milliliter in 0.067*M* Sorensen's buffer (*p*H 6.8), washed, mounted with the buffer and a cover glass, and exposed to approximately 30,000 J/m² of light from a Hanovia 100-watt mercury bulb (exposure, 30 seconds to 1 minute). The slides were then washed in distilled water and stained for 10 to 20 minutes in 3 percent Giemsa (Gurr's R66 in Sorensen's buffer, *p*H 6.8). One hundred differentially stained cells were scored at each point.

Because 5 percent saccharin was fed to the rats in the carcinogenicity test, this was the highest concentration used. In fact, no growth was observed either in CHO cells treated with more than 1 percent saccharin or in human lymphocytes treated with more than 0.5 percent saccharin (Tables 1 and 2). In no case did the saccharin change the *p*H of the medium as evidenced by its color. The experiments were highly repeatable. Four control experiments with CHO cells contained 8.45 to 8.75 SCE's per cell. This increased to 11.21 to 12.94 SCE's per cell after treatment with 1 percent saccharin.

In human lymphocytes the number of SCE's per cell increased from a control value of 9.5 to 9.8 to a value of 16.01 to 17.45 at 0.5 percent saccharin. In both cases the *P* values, as determined by the *t*-test of statistical significance, were far lower than .001. Lower concentrations of saccharin induced fewer SCE's.

The linear regressions of SCE's versus dose of saccharin are presented in Fig. 2; a greater increase was found in human lymphocytes than in CHO cells. It is not certain, however, whether this represents a difference in sensitivity between the two cell types, for, although the slopes of the curves differ by a factor of 4, the human lymphocytes were exposed to saccharin three times longer than were CHO cells. The increase with dose seen for both cell types stands in contrast to a recently published study with Chinese hamster cells from the DON line (*12*) in which the saccharin results were variable. Only a slight increase was found at all doses, leading the authors to conclude that saccharin "did not cause a pronounced increase in the frequency of SCE."

The present results show that saccharin can induce SCE's and that, in human and other mammalian cells, the highly sensitive short-term SCE test is capable of detecting cytogenetic effects of a weak carcinogen that does not induce reversions at the histidine locus in the *Salmonella* test (*13*).

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Myoadenylate Deaminase Deficiency: A New Disease of Muscle

Abstract. *Five cases of a new disease presented with muscular weakness or cramping after exercise; three of the cases also had an elevated serum creatine phosphokinase. Muscle biopsies were histologically normal but lacked adenylate deaminase by stain and solution assay, while the erythrocyte isozyme was normal. A clinical diagnostic test has been developed, and the human enzyme was separated by acrylamide-gel electrophoresis.*

Application of a new histoenzymatic stain (*1*) to frozen muscle biopsies has uncovered a new disease: myoadenylate deaminase deficiency. The occurrence of five cases in a series of 250 biopsies suggests that the disease must be fairly common, although it has not been recognized in the past. The main clinical and laboratory features are listed in Table 1. All were young males with a chief complaint, often since childhood, of muscle weakness or cramping after exercise (or both). Physical and neurological examinations were normal except for decreased muscle mass, hypotonia, and weakness in some cases. Skeletal growth and dentition were normal. Blood cell counts and blood chemistry, urinalysis, x-rays, and other laboratory data have regularly been normal, except for mildly elevated creatine phosphokinase values and nonspecifically abnormal electromyograms, which have generally been the reasons for the subsequent muscle biopsy.

Biopsies were evaluated at the Armed Forces Institute of Pathology with a series of histochemical stains: hematoxylin and eosin, periodic acid Schiff, crystal violet, modified Gomori trichrome, alkaline adenosinetriphosphatase (with and without prior incubation with acid), alkaline phosphatase, NADH (reduced nicotinamide adenine dinucleotide)-tetrazolium reductase, succinate dehydrogenase, phosphorylase, and nonspecific esterase on the fresh-frozen specimen; and electron microscopy on the clamped, glutaraldehyde-fixed specimen (*2*). Four of the cases had entirely normal histology and one had evidence of mild type 1 atrophy. Histograms were prepared (*2*) for cases 1, 2, and 3 where fiber orientation was suitable but showed no impressive abnormalities. Case 1 had a normal distribution and mean size of fiber types for his age and biopsy site but an increased coefficient of variation for type 2B fibers (32 percent). Case 3 had only 3 percent type 2B fibers, and 2 percent of the type 1 fibers were smaller than 40 μm in diameter. Case 2 had only 9 percent type 2B fibers, and 5 percent of the type 1 fibers were less than 40 μm in diameter. A normal complement of type 2B fibers appeared to be present in cases 4 and 5.

All five cases showed no reaction in the adenylate deaminase stain, and this was confirmed quantitatively by solution assay of homogenized microtome sections (*3*) (Table 2) compared to nine controls. To verify tissue integrity, two other highly soluble enzymes were also assayed: adenosine deaminase (*3*), which is very low in muscle, and creatine phos-

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phokinase (*4*), which is very high. Cases 1 to 5 had less than 1 percent of the mean adenylate deaminase activity of the controls, but had normal levels of both adenosine deaminase and creatine phosphokinase. Equivolume mixtures of deficient and normal homogenates showed no evidence of inhibition.

The major protein bands in all 14 homogenates were quantitatively similar in acrylamide gel electrophoresis, and the enzyme could be identified by catalytic staining in the control cases (Fig. 1). Human adenylate deaminase in the crude homogenate of case 12 migrated about 40 percent farther than purified rabbit muscle enzyme, at two gel strengths. This indicates a molecular weight equivalent to that of the rabbit muscle enzyme, that is, 280,000 (*5*). Although band resolution is compromised by the requirement for moderate concentrations of salt, this is the first demonstration of the migration and in situ identification of adenylate deaminase activity in gel electrophoresis.

The only previously reported instance of myoadenylate deaminase deficiency was in a patient with periodic paralysis (*6*). However, in the same report three other patients with that disease had normal enzyme levels, and no additional reports have issued since, although a number of other patients with periodic paralysis have been tested (*6*). The relation of that patient to our series is obscure; none of the five cases studied here had a history at all suggestive of periodic paralysis, nor any of the histopathologic features associated with that disease, and in all the potassium concentration in the serum was normal.

Blood specimens were obtained from four of the cases, and the adenylate deaminase and adenosine deaminase in the red cells of these patients were within the normal range as compared to nine control specimens (Table 3). If the disease is presumed to be hereditary, then there would be two distinct genetic loci controlling the erythrocyte and muscle isozymes. Such a hypothesis is in accord with available evidence, which indicates that myoadenylate deaminase is antigenically unique to muscle (*7*), and that the isozyme from human red cells has distinctive kinetic properties (*8*). Table 3 also shows that two of the patients had mildy elevated plasma creatine phosphokinase levels (*4*).

Adenylate deaminase catalyzes the irreversible deamination of adenosine monophosphate (AMP) to inosine monophosphate (IMP). Resynthesis of AMP occurs by the sequential action of adenylosuccinate synthetase [in a step re-

Table 1. Main features of five cases of myoadenylate deaminase deficiency. Abbreviations: EMG, electromyogram; CPK, serum creatine phosphokinase.

Case	Age	History; physical; laboratory data	Muscle histology
1	12	Late motor development; muscle weakness; frequent childhood infections and allergies; marked decrease in static motor strength; hypotonia; normal EMG and CPK	Normal
2	19	Muscle cramping since age 4; difficulty in controlling contraction-relaxation; small pectorals, rare fasciculations; elevated CPK and aldolase; complex polyphasic units and runs of positive waves in EMG	Mild type 1 atrophy
3	23	Chest muscle cramping; elevated CPK	Normal
4	32	Frequent childhood infections and allergies, rapid muscle fatigue and postexercise cramping; elevated CPK, abnormal EMG	Normal
5	40	Muscles stiff, sore, and weak; difficulty in grasping for 9 months; also numbness and paresthesias; hypoactive reflexes, stiffness in gait and movement; normal CPK, EMG, and spinal fluid	Normal

Table 2. Adenylate deaminase (AMPDA), adenosine deaminase (ADA), and creatine phosphokinase (CPK) in frozen muscle biopsies from five cases of myoadenylate deaminase deficiency and nine controls. Mixtures of normal and deficient homogenates were held for 30 minutes before they were assayed, and the result was calculated on the basis of the normal contribution only. Storage was at −65°C; the assay was done at 25°C. Abbreviations for the biopsy site are as follows: Q, quadriceps; T, triceps; G, gastrocnemius; D, deltoid; B, biceps humerus; ?, unknown. All assays were done in duplicate over a twofold range of concentrations that checked within 10 percent.

Case	Age	Sex	Site	Storage (months)	Diagnosis	Enzyme (nmole/min) per milligram of phosphorus			AMPDA/ ADA
						AMPDA	ADA	CPK	
					Patients				
1	11	M	Q	<1	Normal	4.73	0.634	16,656	7.5
2	19	M	G	<1	Mild 1 atrophy	2.11		42,600	
3	23	M	D	<1	Normal	3.01		28,960	
4	32	M	D	18	Normal	0.46	1.715	12,810	0.3
5	40	M	T	<1	Normal	1.63	1.282	19,751	1.3
					Controls				
6	42	M	G	<1	Mild atrophy	1001.0	0.884	25,591	1133.0
7	35	M	?	4	Normal	370.7	0.545		680.0
8	27	M	Q	2	Normal	210.4	1.641	15,060	128.2
9	18	M	D	1	Normal	232.2	0.697	16,650	333.3
10	14	F	Q	1	Mild atrophy (+ equal volume No. 5 = 488.6)	523.1	0.662	12,090	790.4
11	43	F	D	<1	Denervation (+ equal volume No. 3 = 469.2)	503.4		17,870	
12	19	M	Q	<1	Normal (+ equal volume No. 2 = 722.1)	694.3		33,660	
13	32	M	B	15	Normal (+ equal volume No. 4 = 239.3)	248.2		32,270	
14	29	F	?	4	Normal (+ equal volume No. 1 = 671.0)	705.8	1.760	28,550	401.0
Control means:						498.8	1.032	22,717	577.7
95 percent confidence interval:						212.8	0.558	6,960	380.5

quiring guanosine triphosphate (GTP) and aspartate] and adenylosuccinase, the three-enzyme sequence constituting the purine nucleotide cycle (*9*). Although the exact role of adenylate deaminase in muscle physiology remains controversial, the following facts seem to be clearly established: (i) the enzyme level in skeletal muscle is more than tenfold higher than in any other tissue, although substantial levels also occur in erythrocytes, kidney, and brain; (ii) it is the major enzyme catalyzing muscle production of ammonia, which increases concomitantly with contraction; (iii) it is not essential for muscular contraction; and (iv) it is primarily cytoplasmic and soluble, and exists in multiple forms with complex allosteric properties that vary with the mode of isolation, the tissue source, and the exact composition and conditions of the assay (*8–10*).

Of the many possible roles this enzyme might play in muscle metabolism, the most favored, currently, is the maintenance of a high ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) during strenuous muscular activity. A more inclusive parameter has been introduced by Atkinson (*11*), namely, the energy charge, which we may define as the fractional saturation of adenylates with phosphoanhydride bonds. Muscle is the prime example of a tissue that can outrun its ATP supply despite large depot sources (such as creatine phosphate) and a good oxygen supply. If we assume an ample activity of myokinase, which interconverts AMP plus ATP with 2 ADP, the only way that a high ratio of ATP to ADP (or energy charge) can be maintained is by continual removal of the low energy component, AMP. The energy charge (or ATP/ADP ratio) may in turn regulate glycolysis by its control of key regulatory enzymes, particularly phosphofructokinase (*12*). The most sophisticated interpretation is that of Tornheim and Lowenstein, who have shown that the purine nucleotide cycle and glycolysis may interdigitate in muscle extracts to produce coupled cyclic oscillations in the levels of intermediates (*13*). However, no clear picture has been presented as to the significance of these oscillations, or the deficit to be expected in their absence. Study of patients with myoadenylate deaminase deficiency may ultimately provide a solution to this problem.

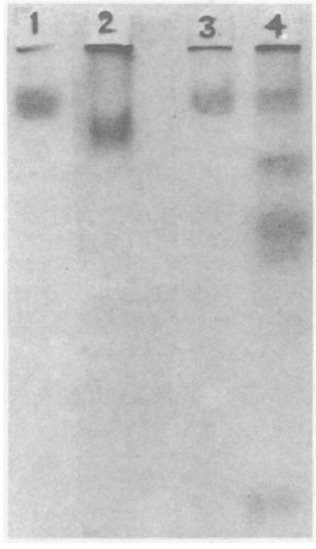

Fig. 1. Acrylamide-gel electrophoresis of human and rabbit muscle adenylate deaminase. Samples were run for 13 hours at 45 V (90 mA) in 5 percent gels in 0.1*M* KCl and tris buffer, *p*H 8.5. Slots 2 and 4 contain muscle homogenate (*3*) from case 12, developed in catalytic stain (*1*) and in Coomassie blue (*17*), respectively. Slots 1 and 3 show the corresponding stains of purified rabbit muscle adenylate deaminase (Sigma Chemical).

Table 3. Adenylate deaminase (AMPDA), adenosine deaminase (ADA), and creatine phosphokinase (CPK) in blood cells and plasma in four cases of myoadenylate deaminase deficiency and nine normal controls. Sonicated red cells were centrifuged, and the supernatant was assayed at 25°C in duplicate over a twofold range of concentrations which checked within 15 percent.

Item	Enzyme per gram of hemoglobin (μmole/min)		AMPDA/ ADA	CPK per milliliter of plasma (nmole/min)
	AMPDA	ADA		
Case 1	3.50	0.382	9.2	58.4
Case 3	5.33	0.343	15.5	108.7
Case 4	3.58	0.566	6.3	191.0
Case 5	4.01	0.607	6.6	59.6
Mean ± S.E. (9)	4.24 ± 0.68	0.429 ± 0.16	11.9 ± 7.4	38.9 ± 4.0
Range	3.42 to 5.62	0.158 to 0.723	6.2 to 29.3	27.3 to 60.3

Although definitive diagnosis requires enzymatic assay on a fresh or frozen muscle biopsy, a simple clinical blood test with which to evaluate suspect cases would be useful. Ammonia determinations are relatively simple, and most of the ammonia formed in muscle does transfer readily into venous blood. We have therefore devised a simple sponge-squeezing procedure with a sphygmomanometer inflated to mean arterial pressure, and have used plasma lactate to measure glycolytic activity, and plasma ammonia to estimate myoadenylate deaminase activity (*14*). The increases in each of these metabolites produced by exercise, compared to the values obtained in a resting blood sample, are plotted (Fig. 2) for four controls and three patients. The controls show a linear response of ammonia to lactate increase (with a molar ratio of about 1:30), as might be expected if myoadenylate deaminase were, indeed, keyed to glycolytic activity. The patients' ratios fell far below the normal range, suggesting that this procedure should provide an effective diagnostic test. Failure of plasma (or blood) ammonia to rise could be used alone as a simpler, but less decisive test, since there would be no independent estimate of muscular work. Two of the patients did show an ammonia increase, although it was small relative to that of lactate. The source of this rise could be erythrocyte adenylate deaminase, which might serve a crucial salvage function in this disease, or the sequential action of 5′-nucleotidase (or other phosphatase) and adenosine deaminase. Creatine phosphokinase levels did not increase significantly after exercise in any of the patients' plasma samples or those of the controls.

A decade or so ago the clinical diagnosis of benign congenital hypotonia was commonly made in patients with symptoms similar to those presented here (*15*). That entity fell out of favor with the advent of techniques for the histochemical study of frozen sections of muscle biopsies and the absence of abnormalities in such cases. In retrospect, it appears likely that many of those cases may have had myoadenylate deaminase

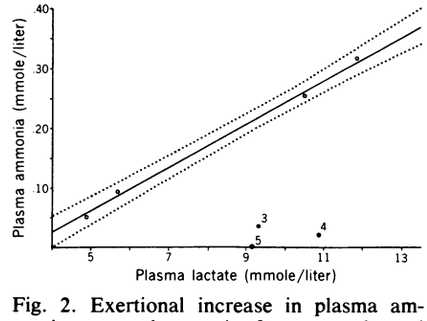

Fig. 2. Exertional increase in plasma ammonia versus lactate in four controls and three patients with myoadenylate deaminase deficiency. The least-squares regression line and 95 percent confidence limits are shown for the controls.

deficiency. The same may also apply to many current cases of nonprogressive congenital myopathy and the limp-infant or floppy-baby syndrome, where minor or no histopathologic changes are encountered (*16*).

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3. Thirty 16-μm sections were homogenized in microtubes with 0.1 ml of 15 percent sucrose, 10 m*M* tris, *p*H 7, and centrifuged; 2 to 10 μl of the supernatant was assayed by absorption at 555 nm, in a 1-cm cell, at 25°C (*p*H 6.8) in a 1-ml solution containing 0.144 m*M* phenol red, 3.3 m*M* triethanolamine, 300 m*M* KCl, and 1 m*M* AMP. This assay can be used interchangeably for muscle extracts and hemolyzates, and for adenosine deaminase by replacing AMP with adenosine, and decreasing the salt and buffer content threefold. Substrate cross-reactivity was less than 1:2,000 for adenylate deaminase and less than 1:50,000 for adenosine deaminase.
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14. Blood was drawn with minimal stasis from one arm, then from the other arm after 100 to 150 squeezes in 1 to 2 minutes, and centrifuged at once. Plasma lactate was assayed by the method of P. A. Drewes [in *Clinical Chemistry, Principles and Techniques*, R. J. Henry, D. C. Cannon, J. W. Winkelman, Eds. (Harper & Row, New York, ed. 2, 1974), pp. 1330–1334] and plasma ammonia was assayed by the method of A. Mondzac, G. E. Ehrlich, and J. E. Seegmiller [*J. Lab. Clin. Med.* **66**, 526 (1965)].
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Cytochrome c Oxidase as the Receptor Molecule for Chemoaccumulation (Chemotaxis) of *Euglena* Toward Oxygen

Abstract. *Chemoaccumulation of* Euglena gracilis *toward oxygen was selectively inhibited, without concomitant effects on cell motility, by cyanide (10^{-6} to 10^{-4} molar) and carbon monoxide (5×10^{-5} to 5×10^{-4} molar). Above these concentrations, motility of the cell was impaired and the chemosensory response was inhibited. Azide did not affect chemoaccumulation even at 5×10^{-3} molar. It is concluded that cytochrome a_3 serves as the chemoreceptor molecule for oxygen-mediated behavioral responses in* Euglena.

Chemoaccumulation of bacteria toward oxygen was discovered almost a hundred years ago (*1*). Although understanding of this response has been advanced (*2, 3*), identification of the binding or receptor protein for oxygen has not been reported. Multiple functions of receptor proteins have been demonstrated in bacteria for the galactose-binding protein (*4*) as well as the ribose-binding protein (*5*). These proteins have also been shown to be the stimulus receptors for the respective chemotactic movements.

We recently discovered that the unicellular alga *Euglena gracilis* exhibits shock responses to spatial gradients of oxygen (*6*). These "chemophobic responses" are expressed as changes of the cell's direction of movement encountering a too high or too low oxygen concentration, and serve to trap the cell in regions of presumably favorable concentrations of oxygen.

Since in eukaryotes, cytochrome c oxidase acts as the terminal oxygen acceptor of the respiratory chain, we reasoned that it might be the receptor molecule for the chemosensory response. This hypothesis was tested by studying the effects of cytochrome inhibitors on chemoresponses.

Euglena gracilis, strain Z, was grown in Bloomington medium (*7*) supplemented with vitamin B_1 (0.5 mg/ml) and vitamin B_{12} (4 μg/ml). Cultures were grown in an incubator at 25°C under constant cool-fluorescent illumination (1.0 W/m^2), and were used for experiments 5 to 7 days after inoculation. At this age, cells were centrifuged, and suspended in supernatant to a density of approximately 10^6 cell/ml. They were observed in a Neubauer chamber (0.1 mm thickness) under an inverted Nikon type M microscope, with constant yellow illumination (interference filter, 577 nm). This wavelength cannot be perceived by the cell's photosensory system, and does not support photosynthesis at the intensity of the experiment. Light intensities were measured with a model 68 Kettering radiant power meter.

The behavioral response of the cells was determined 10 minutes after addition of the chemicals. As an assay for the chemoresponse toward oxygen, we used the spontaneous formation of an expanding ring within 5 minutes after the cells were introduced into the chamber (*6*). This response to oxygen is qualitatively similar to that of motile bacteria (*3*).

Various inhibitors of cytochrome c oxidase were added. There is evidence for the existence of a complex of cytochrome a in *Euglena* (*8, 9*) even though its structure and mechanism of action have not been established. If the two cytochromes a and a_3 could be separated, the cytochrome a_3 moiety would have to be considered the terminal oxidase, since it alone is rapidly autoxidizable (*10*).

Sodium cyanide had no effect at $10^{-7}M$, but at concentrations ranging from $10^{-6}M$ to $10^{-4}M$ it specifically inhibited ring formation in the *Euglena* suspensions. At these concentrations, swimming rates (defined as the time required by the cell to swim its own length) as well as the ability of the cells to respond to photic stimuli (*11*) were not significantly different from those of controls. At $10^{-3}M$ cyanide and above, motility of the cells was impaired and ring formation was inhibited.

Carbon monoxide was ineffective at $5 \times 10^{-6}M$, but at concentrations of $5 \times 10^{-5}M$ and $5 \times 10^{-4}M$ it was found to specifically inhibit ring formation. No ef-

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