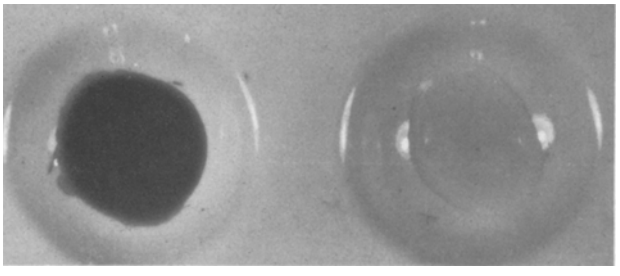
Iodotyrosine and methyl-para-tyrosine (tyrosine hydroxylase inhibitors) as well as diethyldithiocarbamate and phenylthiourea (phenoloxidase inhibitors) were tested for their ability to inhibit the melanization. The inhibitors of phenoloxidase at a concentration of 10^{-3} M were sufficient to inhibit darkening while the tyrosine hydroxylase inhibitors were ineffective. The diphenols dihydroxyphenylalanine, dopamine, norepinephrine, catechol and epinephrine proved to be effective substrates for the darkening reaction. These results confirm the effect reported by Hurst. The effects of the inhibitors and substrates provide evidence for the involvement of phenoloxidase in the darkening of hemolymph. An increased rate of darkening due to intoxication indicates an increased level of enzyme or enzyme activity. Phenoloxidase has been localized in the hemocytes of various orthopterans by histochemical methods[5,10,11]. Cell lysis and/or increased numbers of cells in the hemolymph of intoxicated individuals may account for the increased darkening. The release of catecholamines from the nervous system of crickets intoxicated by insecticides[12] may raise the redox potential of the hemolymph sufficiently to affect phenoloxidase activity[13]. The possibility that phenoloxidase serves a defensive function and is activated by insecticide produced stress is also under consideration.



Hemolymph from American cockroaches incubated with a 1% DOPA solution in Narahashi saline for 2 h. Left: hemolymph from cockroaches treated with DDVP vapor for 30 min and drawn 1 h later, right: hemolymph from untreated control roaches.

1 G. Fraenkel and K. Rudall, Proc. R. Soc. B. *134*, 111 (1947).
2 H. Mason, Adv. Enzymol. *16*, 105 (1955).
3 M. Pryor, J. exp. Biol. *32*, 468 (1954).
4 A. Sussman, Mycologia *43*, 338 (1951).
5 J.W. Preston and R.L. Taylor, J. Insect Physiol. *16*, 1729 (1970).
6 H. Hurst, Nature *156*, 194 (1945).
7 H. Shorey, J. econ. Ent. *54*, 1243 (1961).
8 T. Yamasaki and T. Narahashi, J. Insect Physiol. *3*, 146 (1959).
9 J. Sternberg and J.J. Corrigan, J. econ. Ent. *52*, 538 (1959).
10 J. Hoffman, A. Porte and P. Joly, C. r. hebd. Séanc. Acad. Sci., Paris *170*, 629 (1970).
11 R. Mills, S. Androuny and F.R. Fox, J. Insect Physiol. *16*, 1799 (1968).
12 F.E. Gardener and U.E. Brady, Pestic. Biochem. Physiol. *7*, 466 (1977).
13 F. Figge, J. cell. comp. Physiol. *15*, 233 (1940).

Orotic acid prevents changes in cardiac sarcolemmal glycoproteins and contractility associated with muscular dystrophy in hamsters[1]

L.E. Bailey

Department of Pharmacology, Faculty of Medicine, Sir Charles Tupper Medical Building, Dalhousie University, Halifax (Nova Scotia, Canada B3H 4H7), 10 April 1979

Summary. Orotic acid included in the diet of cardiomyopathic hamsters during the myolytic phase of the disease (30–60 days of age) prevented the reduction in cardiac sarcolemmal sialic acid, calcium binding, sialyltransferase activity and contractile activity associated with the cardiomyopathy.

The cardiomyopathy associated with Duchenne's muscular dystrophy in man is characterized by focal myolytic lesions and ultimately, development of congestive heart failure. The course of the myopathy in the dystrophic hamster model of this disease follows a similar pattern[2,3] and is associated with a deficiency in the incorporation of sialic acid into the sarcolemmal and interstitial glycoproteins of the myocardium[4]. This defect results in decreased contractility detectable as early as 30 days of age, and moreover is implicated in the pathogenesis of the disease[4]. Mortality is greatly increased by hypokalemia in dystrophic hamsters, however, only 2 potassium salts, orotate and aspartate effectively prevented ventricular hypertrophy and congestive heart failure[5]. I now report that orotic acid rather than potassium prevents the deficiencies in sarcolemmal glycoprotein composition associated with reduced contractility in the dystrophic hamster.

Cardiomyopathic (BIO 14.6; Trenton Experimental Laboratory Animal Co., Bar Harbour, ME) and age-matched normal (BIO.RB) Syrian hamsters, 30 days of age, were segregated into 12 groups of 5 animals each. Each group received ad libitum ground laboratory chow or ground laboratory chow containing either 10% sodium or 10% potassium orotate (Sigma Chemical Corp., St. Louis, MO) for either 30 or 60 days. Neither the intake of food nor body weight differed between groups during the treatment period. Statistical analysis indicated that tissue water, sialic acid content and sialyltransferase activity were not affected by duration of treatment or by the different salts of orotic acid. The results from these groups were pooled to yield comparisons between treated and untreated normal and myopathic animals. The hearts were removed and the ventricles homogenized to produce 1-5 cell fragments. These fragments were subjected to hypoosmotic shock and salt extraction to produce sarcolemmal 'ghosts'[6]. Enzymatic digestion was not used and all media contained a protease inhibitor (aprotinin, 1000 units $\cdot$ l^{-1}). A probability level of 0.05 was preselected as the criterion of statistical significance.

The activity of sialyltransferase in sarcolemma from untreated myopathic hamster hearts was significantly less than that of normal animals (table 1). The activity of this enzyme in sarcolemma from myopathic hearts after treatment with orotic acid did not differ from untreated or treated normal hearts. As reported previously, there was a significant decrease in the sialic acid content of sarcolemma from hearts of dystrophic hamsters[4] (table 1). 10% orotic acid in the diet of the myopathic animals restored the membrane content of sialic acid to levels not different from normal. ATP-independent calcium binding to neuramini-

Table 1. The effect of dietary orotic acid on sialyltransferase activity and sialic acid content of cardiac sarcolemma

	Sialyltransferase activity[a] (pmoles · $mg^{-1} \cdot h^{-1}$)		Sialic acid content[b] (nmoles · mg^{-1})	
	Normal	Myopathic	Normal	Myopathic
Control	81 ± 15 (10)	43 ± 9 (7)	120 ± 15 (8)	90 ± 7 (8)
Orotic acid	79 ± 12 (16)	80 ± 14 (16)	110 ± 15 (16)	115 ± 12 (16)

Mean ± SEM of the number of groups of hearts indicated in parentheses. Each group contained 5 hearts. [a] Sialyltransferase activity was determined with excess asialofetuin as sialic acid acceptor[11]. The incubation medium contained 0.5 mg sarcolemmal protein, 10 mM Tris (pH 7.4), 0.2 mMEGTA, 10 mM $MnCl_2$, 10 mM Mg Cl_2, 1.2 mM UDP, 0.1% Triton X-100, 0.5 asialofetuin and 0.5 mM CMP · ^{14}C sialic acid. Although the kinetics of enzyme activation were not established for hamster sarcolemmal sialyltransferase, the concentration of substrate used in these experiments was 50 times the estimated K_m for sialyltransferase activity in rat liver[11] to ensure enzyme activity was measured at maximum initial velocities. The mixture was incubated at 37 °C for 30 min[9]. [b] Sialic acid was hydrolyzed from 100 µg of the sarcolemmal preparation by incubation in 2 ml 0.1 M H_2SO_4 for 1 h at 80 °C. The concentration of the thiobarbiturate derivative was determined spectrofluorometrically at 570 nm with excitation at 550 nm[12].

Table 2. The effect of dietary orotic acid on calcium binding to neuraminidase-sensitive sites on cardiac sarcolemma

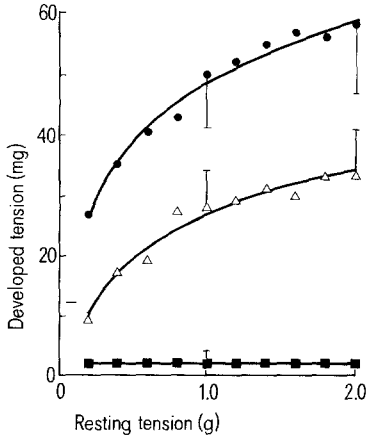
	Normal		Myopathic	
	K_D (mM)	Capacity (nmoles · mg^{-1})	K_D (mM)	Capacity (nmoles · mg^{-1})
Control	1.1	252 (2)	1.0	126 (2)
Orotic acid	1.7	260 (4)	1.4	274 (4)

Number in parentheses indicate the number of groups of 5 hearts from normal and myopathic animals. Orotic acid was given for 60 days. Calcium binding was assayed in 50 µg sarcolemmal protein at free calcium concentrations from 0.01 to 10 mM with ^{45}Ca as the tracer. The ^{45}Ca bound to the sarcolemma was determined by equilibrium centrifugation, and the neuraminidasesensitive component determined by resolution of a Scatchard plot by least squares analysis[3].

dase-sensitive sites on sarcolemma from myopathic hearts was reduced (table 2). Orotic acid restored the calcium binding capacity of this pool to normal without significant effect on the K_D for calcium binding. The length-tension relationship for 3 electrically driven, left atria from normal hamsters differed significantly from that of atria from untreated myopathic animals in which developed tension remained at less than 5 mg at all resting tensions from 0.2 to 2.0 g (figure). Developed tension in atria removed from myopathic animals treated for 60 days with sodium orotate was significantly greater than in atria from untreated myopathic animals but less than that of atria from normal animals.

I conclude that inclusion of 10% orotic acid for at least 30 days in the diet prevents the changes in sialyltransferase activity, sialic acid content and calcium binding associated with reduced contractility and the development of congestive heart failure in dystrophic hamsters.

Decreased sialyltransferase activity has been measured in hearts from cardiomyopathic hamsters before the development of the focal lesions at 30 days of age, and at all subsequent stages of the disease until congestive heart failure develops at 250–300 days of age[4,7]. This effect was associated with reduced sialic acid content and reduced neuraminidase-sensitive calcium binding on the sarcolemma in vitro, and reduced contractility and superficially bound calcium in the intact heart[4]. The consequences of a



Length-tension curves for left atria obtained from 3 untreated normal hearts (●), 3 untreated myopathic hearts (■), and 3 myopathic hamsters hearts after 60 days treatment with sodium orotate (△). Atria were bathed in oxygenated Krebs-Henseleit solution at 30 °C, pH 7.4, and were driven electrically at 2 beats · sec^{-1} by platinum field electrodes.

deficiency of sarcolemmal sialic acid are 2-fold; first, the number of binding sites for the calcium involved in excitation-contraction coupling is reduced[4]; and second the permeability of cardiac cells to calcium is increased[8]. This deficiency in sarcolemmal glycoproteins causes decreased contractility and increased calcium influx, both of which may ultimately contribute to the focal calcareous lesions characteristic of the cardiomyopathy[4]. Orotic acid, unlike other interventions which retard development of the cardiomyopathy[9], did not compromise myocardial contractility. Restoration of sialyltransferase activity to normal levels by orotic acid thus appears to interrupt the sequence of events leading to reduced contractile activity and perhaps to the development of the cardiomyopathy. The mechanism by which orotic acid produces this effect is unknown; however, the substance is a precursor for the de novo synthesis of pyrimidine nucleotides and may thus affect synthesis of RNA.

Although the histological changes in cardiac muscle of dystrophic hamsters resemble closely those of the cardiomyopathy in Duchenne's muscular dystrophy of man[2], defective incorporation of sialic acid into the sarcolemmal glycoproteins of the heart has not been reported; nevertheless, changes in conformation and structure of the plasma membrane of skeletal muscle[10] and other tissues[11] have been consistently observed in patients with this disease.

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2 J.K. Perloff, A.C. de Leon, Jr, and D. O'Doherty, Circulation Res. *41*, 702 (1977).
3 E. Bajusz, Am. Heart J. *77*, 686 (1969).
4 T.S. Ma, J.C. Baker and L.E. Bailey, Cardiovasc. Res., in press (1979).
5 E. Bajusz, F. Homburger, J.R. Baker and P. Bogdonoff, Ann. N.Y. Acad. Sci. *156*, 396 (1969).
6 M. Tada, J.O. Finney, Jr, H.H. Schwartz and A.M. Katz, J. molec. cell. Cardiol. *4*, 417 (1972).
7 C.J. Limas and C. Limas, Biochim. biophys. Acta *540*, 543 (1978).
8 J.S. Frank, E.A. Langer, L.M. Nudd and K. Seraydarian, Circulation Res. *41*, 702 (1977).
9 G. Jasmin and B. Solymoss, Proc. Soc. exp. Biol. Med. *149*, 193 (1975).
10 B. Mokri and A.G. Engel, Neurology *25*, 1111 (1975).
11 N.A. Pickard, H.O. Gruemer, H.L. Verrill, E.R. Isaacs, M. Robinow, W.E. Nance, E.C. Myers and B. Goldsmith, New Engl. J. Med. *299*, 841 (1978).
12 R.J. Bernacki, Eur. J. Biochem. *58*, 477 (1975).
13 K.S. Hammond and D.S. Papermaster, Analyt. Biochem. *74*, 292 (1976).