Enhanced 5-Nucleotidase Activity Associated with White Muscle Disease.* (25910)

J. R. Schubert, O. H. Muth, J. E. Oldfield and L. F. Remmert
(Introduced by J. S. Butts)
Oregon Agric. Exp. Station, Corvallis, Oregon

Histochemically it has been shown that the proliferation of connective tissue characteristic of human muscular dystrophy is accompanied by an appreciably greater 5-nucleotidase reaction than is present normally(1). This observation prompted an investigation of the status of this enzymatic aberration in tissues from lambs previously characterized relative to affectation by white muscle disease (WMD)(2). Results of such studies are reported here.

Materials and Methods. The assay procedure used includes experimentally indicated departures from conditions specified for other tissues(3). Three g samples of minced semitendinosus muscle were taken in duplicate from polyethylene packaged tissues which had been stored frozen up to 18 months beyond necropsy. The tissue was comminuted in 0.05 M NaCl-0.005 M Tris buffer, pH 8.3 using a glass mortar and pestle and a Potter-Elvehjem homogenizer. The supernatant fraction from 15 minutes centrifugation at 5,000 $\times$ g was diluted with buffer to 10 times the weight of the tissue. One-half of this extract was dialyzed 20 hours against 25 volumes of buffer which was changed once. After recentrifugation, the supernatant fraction of the dialyzate was diluted with buffer to 20 volumes relative to the original sample weight. All steps were performed at 0-5°C. Aliquots representing 100 mg tissue equivalent of the freshly prepared or dialyzed extracts were assayed separately. The nitrogen contents of each enzyme preparation and of the residue from initial centrifugation were determined by the Kjeldahl procedure. Three incubation mixtures were used in assaying the hydrolytic activity in each enzyme preparation, one containing no substrate, one containing adenosine-5'-monophosphate (AMP) and one with p-nitro phenyl phosphate (NPP). Incubation was carried out in 45 ml glass-stoppered centrifuge tubes containing 0.051 M Tris buffer, pH 8.3, 0.01 M NaCl, 29 μmoles substrate (if any) and enzyme in a total volume of 10 ml. Two ml aliquots of brei were withdrawn and added to 8 ml 10% trichloroacetic acid (TCA) immediately after adding enzyme and again after a one hour incubation at 37°C with shaking. Phosphorus contents of TCA filtrates were determined by the Fiske-Subbarow procedure. Net amount of phosphorus liberated from each substrate was taken as the final less the initial values, further corrected for amount released in absence of substrate. The latter values were consistently negligible.

Results. Under the conditions described, hydrolysis of AMP was linear during the incubation period and was proportional to enzyme concentration. The enzyme was saturated at concentration of AMP used. A rather broad pH optimum was found for AMP hydrolysis between 8.8 and 7.8, but the activity decreased markedly towards pH 7.5. Activity against NPP, however, was minimal at pH 8.3. Dialysis resulted in virtual elimination of NPP hydrolysis while consistently enhancing that of AMP.

On a tissue equivalent basis, the full activity of homogenates was retained in supernatant fractions up to 25,000 $\times$ g. Prior autolysis of several homogenates for 3 days at 70°F under chloroform(3) resulted in substantially reduced activity. Rehomogenization of initial tissue residues did not materially change the ratios of activities obtained with diseased and normal tissues.

The 5-nucleotidase activities found in tissues from normal and WMD lambs necropsied from 2 to 6 weeks of age, and in tissues from their twins sacrificed at less than one week of age(2), are shown in Table I.

Compared to the notably constant 5-nucleotidase activity in tissues from control lambs,

* Technical Paper No. 1304, Oregon Agric. Exp. Station.

TABLE I. 5-Nucleotidase Activity of Semitendinosus Muscle of Normal and White Muscle Diseased Lambs.

Calculation basis	Enzyme preparation	Substrate	μmoles P released/hr* Lot 1—Normal control† Age 2-6 wk [10]	<1 wk [8]	Lot 2—WMD basal Age 2-6 wk [9]	<1 wk [6]
100 mg tissue	Dialyzed	AMP	1.2 ±.06 (.9-1.5)	1.6 ±.2 (.9-2.3)	6.4 ± 1.4‡ (2.0-14.3)	1.7 ±.2 (1.0-2.1)
Idem	Fresh	"	1.0 ±.1 (.4-1.4)	1.5 ±.2 (.9-1.9)	5.8 ± 1.3‡ (1.0-13.3)	1.5 ±.1 (.9-1.7)
"	" §	NPP	.8 ±.06 (.5-1.1)	.6 ±.1 (.4-1.1)	.4 ± .1 (.1- .9)	.7 ±.2 (.1-1.7)
mg of extract N	Dialyzed	AMP	2.6 ±.2 (1.9-3.6)	3.9 ±.5 (2.2-5.7)	14.8 ± 3.4‡ (3.8-32.2)	4.8 ±.5 (2.9-6.1)

* Mean ± stand. error. Ranges among lambs in parentheses.
† Lots 1 and 2 as reported previously(2). No. of lambs in brackets.
‡ Significantly greater than Lot 1, 2-6 wk ($P < .01$).
§ Hydrolysis of NPP by dialyzed preparations uniformly <.1 μmole P/hr.

WMD tissues exhibited a range in values up to 12-fold the normal average, probably related to stage of the pathological process(4). The differences were no less when expressed on an enzyme nitrogen basis, or when related to the dry matter or nitrogen contents of the tissues. There was little difference in activities in tissues from the twins of normal and WMD animals, sacrificed at a few days of age.

Nonspecific phosphatase activity against AMP, as measured by NPP hydrolysis(3), could be virtually restored in dialyzed enzyme preparations by addition of 1 x 10^{-3} M Mg^{++}. An apparent Mg^{++} stimulation of 5-nucleotidase activity in nondialyzed preparations could be accounted for by increases in NPP hydrolysis, as could additional stimulation in combination with 1 x 10^{-4} M ethylenediamine tetracetic acid. The latter alone, however, inhibited both hydrolyses severely. Calcium ions added up to 0.1 M were without effect on enzyme preparations from either normal or WMD tissues.

These results lend credence to the earlier postulate of the possible importance of the 5-nucleotidase reaction in soft tissue calcification(5). Considering WMD in part as a growth aberration(6), the reaction could conceivably assume a role in deranged nucleoprotein and protein syntheses. Levels of 5-nucleotidase activity in tissues from lambs variously responding to selenium and vitamin E therapy(2,6), are currently under study. Similar studies are also being made of analogous conditions with respect to rat liver necrosis(7).

Summary. Suitable conditions for assay for 5-nucleotidase activity in lamb skeletal muscle tissue are described. The activity of this enzyme was significantly greater in tissues from lambs affected by white muscle disease than in tissues from normal lambs.

The authors acknowledge efficient technical assistance of Pauline Fleetwood.

1. Beckett, E. B., Bourne, G. H., *Nature,* 1957, v179, 771.

2. Muth, O. H., Oldfield, J. E., Remmert, L. F., Schubert, J. R., *Science,* 1958, v128, 1090.

3. Ahmed, Z., Reis, J. L., *Biochem. J.,* 1958, v69, 386.

4. Muth, O. H., *J. Am. Vet. Med. Assn.,* 1955, v126, 355.

5. Reis, J. L., *Biochem. J.,* 1951, v48, 548.

6. Oldfield, J. E., Muth, O. H., Schubert, J. R., PROC. SOC. EXP. BIOL. AND MED., in press.

7. Schwarz, K., *Symposium on Liver Function, Am. Inst. Biol. Sci.,* 1958, Publ. #4, 509.

Received May 23, 1960. P.S.E.B.M., 1960, v104.