

Calcium Uptake in Frozen Muscle Biopsy Sections Compared with Other Predictors of Malignant Hyperthermia Susceptibility

K. Nagarajan, Ph.D.,* William N. Fishbein, M.D., Ph.D.,† Sheila M. Muldoon, M.D.,‡ G. Pezeshkpour, M.D.§

The frozen-section Ca^{45} uptake procedure of Mabuchi and Sreter has been applied to 51 frozen human muscle biopsies previously tested for halothane and for caffeine contracture responses. In preliminary tests with cat muscle, uptake varied with skeletal muscle fiber type, was absent from smooth muscle, and was blocked by agents which damage or extract sarcoplasmic reticulum. In the human biopsies, however, the calcium uptake values showed no significant correlation with any of the following parameters: (1) time of frozen-biopsy storage up to 5 yr; (2) fiber type distribution; (3) positive versus negative response in the halothane contracture test; (4) positive versus negative response in the caffeine contracture test; (5) positive versus negative response in the combined halothane/caffeine contracture test; and (6) presence of a documented clinical episode of malignant hyperthermia. In contrast, a positive halothane or caffeine contracture test each correlated highly with the presence of a prior clinical episode. We conclude that the evidence fails to support the use of the Ca^{45} uptake procedure in frozen sections of skeletal muscle biopsies as a valid indicator of malignant hyperthermia susceptibility. (Key words: Anesthetics, volatile: halothane. Malignant hyperthermia: caffeine; contracture tests; criteria; susceptibility. Muscle: contracture tests; frozen biopsy specimen; sarcoplasmic reticulum. Muscle, sarcoplasmic reticulum: calcium uptake.)

THE CURRENT DIAGNOSIS of malignant hyperthermia susceptibility (MHS) by contracture testing is complicated and cumbersome. It requires a physiology laboratory with trained staff at the ready, preferably with halothane perfusion and monitoring apparatus, for stimulation-contraction recording of muscle slips prepared within a few hours of biopsy. The availability of centers equipped for such testing and the attendant hospitalization is necessarily limited. It is hardly surprising, therefore, that less complex procedures using more accessible cells or tissue preparations are periodically introduced as substitutes, although none has, as yet, been accorded general acceptance. Of

particular interest to us was the Ca^{45} -uptake method of Mabuchi and Sreter on frozen sections of skeletal muscle,¹ which is claimed in preliminary reports^{2†} to provide accurate diagnosis of MHS, and is currently being used in clinical consultations.³ This method would provide a significant technical advance if it could be confirmed by other laboratories. It would circumvent the need for contracture testing on fresh muscle specimens at the time of biopsy, and also permit retrospective studies of stored frozen muscle biopsies.

The validity of this test, however, rests upon two pre-sumptions, neither of which has been proved: first, that frozen section calcium uptake accurately represents the sarcoplasmic reticulum uptake of calcium in skeletal muscle, and second, that the uptake of calcium by sarcoplasmic reticulum of skeletal muscle is abnormal in MHS. If the frozen section method is a valid prognosticator of MHS, then one would expect it to correlate closely with cases having a documented clinical episode, and with other predictors of MHS which have generally recognized validity, such as the halothane and the caffeine contracture tests.^{4,5} We were in a favorable position to evaluate such correlations, because we have carried out physiological contracture tests in suspect cases over a 5-yr period, and stored an additional quench-frozen muscle specimen. We compare here the calcium uptake results in 51 cases; a preliminary report of this study has been communicated.⁶

Methods

Calcium-45 was purchased from Amersham Company with a specific activity of 509 Ci/mole. Plastic microscope cover slips were obtained from Fisher Scientific Co. and Spectrofluor PPO-POPOP (Amersham, Arlington Heights, IL) scintillation fluid was used in wide-mouthed plastic vials for counting in a Searle Mark III 6880 scintillation spectrometer with appropriate window. All chemicals used were of reagent grade.

Muscle biopsies from patients referred to the Anesthesiology Department, Uniformed Services University of the Health Sciences, for evaluation of MHS were quench-frozen in isopentane chilled to its freezing point over liquid nitrogen, and stored at -70°C , or, during the past

* Research Chemist, Toxicology Division, Armed Forces Institute of Pathology.

† Chief, Biochemistry Division, Armed Forces Institute of Pathology.

‡ Professor of Anesthesiology, Uniformed Services University of the Health Sciences.

§ Chief, Neuromuscular Pathology Division, Armed Forces Institute of Pathology.

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Address reprint requests to Dr. Fishbein: Biochemistry Division, Armed Forces Institute of Pathology, Washington, District of Columbia 20306.

† See also Allen PD, Ryan JF, Sreter FA, Mabuchi K: Rigid vs. non rigid MH, studies of Ca^{++} uptake and actomyosin ATPase. (Abstract) ANESTHESIOLOGY 53:S251, 1980.

2 yr, -130° C. Normal cat soleus and gracilis muscles were frozen in the same way, and used in preliminary experiments. All specimens were trimmed to a near square or rectangular shape prior to cutting $10\ \mu$ sections at -12 to -18° C for mounting on 22 mm square plastic coverslips which had just been air-dried from an ethanol dip. From 2–4 sections were mounted on each coverslip, and from 2–4 coverslips were used per biopsy. Separate initial and final sections were mounted on glass coverslips and stained with the alkaline ATPase stain⁷ and with hematoxylin and eosin (H&E), for microscopic evaluation and for quantitation of fiber typing and total section area by planimetry.

For incubation studies we followed the Mabuchi and Sreter procedure,¹ which is calculated to provide a free calcium ion concentration of 10 nM at 37° C in a solution containing 25 mM Tris-maleate, 50 mM Tris-oxalate, 100 μ M KCl, 100 μ M calcium chloride, 110 μ M EGTA, 5 mM MgCl_2 , 5 mM ATP, and 5 mM sodium azide, at pH 7.0. The radiolabelled calcium chloride was diluted and added in aliquots providing a final specific activity of approximately 0.4 Curie per mole of calcium chloride in the incubation mixture.

The only modification made was with regard to the mixing procedure. Mabuchi and Sreter employed a shaking device to mix the fluid about their coverslips, and had problems with section loss during incubation. They found it necessary to pre-dip their slips in weak hydrochloric acid before mounting, and to vacuum desiccate the mounted sections in order to obtain satisfactory adherence during incubation. We eliminated these problems by elevating the cover slips over small glass rods placed parallel at each side of a Columbia jar, with a magnetic micro-stirring bar in the center. Phenol red droplet tests confirmed that equilibration was complete within 5–10 s, and no section losses occurred during hour-long incubations. A preliminary dip in ethanol was necessary to eliminate surface charge on plastic coverslips, but no acid treatment or vacuum desiccation was used. Although glass slips provide a stronger adherence, we chose to use plastic slips to eliminate the danger of breakage during handling.

A plastic tray of water was placed over a multi-port magnetic stirrer in a 37° incubator and equilibrated for each experiment. Four to six covered Columbia jars, each containing four pair of back-to-back coverslips with freshly mounted specimens were then incubated for 1 h in the water tray, with continual stirring. Upon removal, the incubation mixture was aspirated and the slips were washed with a solution of 5 N acetic acid saturated with calcium oxalate to remove all soluble radioactive calcium. They were then incubator-dried, and transferred individually to vials containing 10 ml scintillation fluid for counting, along with separate samples of the radioisotope to provide the decay correction for that experiment. Cal-

culatation then provided the DPM per mmole calcium in the incubation fluid, and μ moles calcium per coverslip.

Projection planimetry was used to determine the square cm area of the H&E stained sections. Initial and final section areas did not differ by more than 16% in any case, and average values were used throughout. The tissue weight on each cover slip was then determined from the mean area, the section thickness (.001 cm), and the number of sections mounted, and corrected for the density of muscle, 1.06.⁸ All uptake values are reported as μ moles/gram muscle/h, and no corrections were made for collagen content, since microscopic examination of all specimens revealed none with increased connective tissue.

In each of the human cases, contracture testing had been carried out on fresh vastus lateralis muscle strips within 2 h of biopsy. Muscle strips of 60–100 mg weight were dissected, mounted, and equilibrated in an organ bath with carbogen-gassed Krebs-Ringer solution at 37° C, pH 7.4. One end of the strip was attached by silk thread to a Grass FT03 force-displacement transducer for continuous isometric tension recording. Basal tension was set at 2.0–2.5 g, which evokes optimal twitch responses in muscle strips of this size. Contracture testing in the presence of halothane and/or caffeine was carried out after a 10-min period of direct electrical stimulation (0.1 Hz, 2 msec, 15 V) to verify viability. Criteria employed in determining a positive response were an increase in basal tension of at least 0.5 g with 1–2% halothane, at least 1 g increase with 4 mM (or less) caffeine, and at least 1 g increase with 1 mM caffeine and 1% halothane together.

The methods used for determining the area (or volume) contribution of type 1 and type 2 fibers on alkaline ATPase stains have been described previously.⁹ Standard statistical methods were used for data analysis: Student's *t* test for comparing two sample means, the Wilcoxon Rank Sum test for two-sample rankings, and the Chi-square distribution for contingency table analysis.

Results

PRELIMINARY EXPERIMENTS WITH CAT MUSCLES

These experiments were done to develop a standardized technique, evaluate optimal conditions, and determine the specificity of the reaction. Sections stored at -130° C overnight retained 85–90% of the Ca^{45} uptake of freshly mounted sections, while those stored overnight at 4° had only 20% uptake. To ensure maximum uptake activity, we carried out all further experiments with freshly mounted sections. Using 20-min increments of incubation time, we found that uptake increased linearly with time ($\pm 10\%$) for 60–80 min. The 60-min incubation period employed by Mabuchi and Sreter¹ was therefore considered appropriate for all further experiments, and

TABLE 1. Effect of Different Treatments and Muscle Types on Calcium Uptake

Muscle	Treatment	$\mu\text{m Ca}^{45}/\text{gm/h}$, Mean \pm SD (N)	% Uptake
Cat gracilis	None	19.8 \pm 1.9 (11)	100.0
Cat gracilis	1% Triton-X-100	2.2 \pm 0.2 (4)	10.9
Cat gracilis	0.1% Triton-X-100	10.3 \pm 0.9 (4)	51.2
Cat gracilis	10% Formalin	0.3 \pm 0.04 (4)	1.6
Cat gracilis	Omit ATP	0.7 \pm 0.1 (4)	3.7
Cat soleus	None	11.1 \pm 2.0 (4)	56.1
Human myometrium	None	0.8 \pm 0.7 (2)	4.0

Formalin and Triton treatments were for 5 min, followed by two water and two buffer rinses before incubation for the usual 60 min.

should discriminate any kinetic differences among samples.

Next we carried out a survey to see whether the Ca^{45} uptake fulfilled minimum requirements for a sarcoplasmic reticulum function. These results are summarized in table 1. We could confirm Mabuchi and Sreter's observation that omission of ATP resulted in a complete lack of uptake.¹ In addition, uptake was eliminated by a 5-min pretreatment with 10% formalin, which inactivates virtually all cell enzymes, or with 1% Triton X-100, which is well known to extract sarcoplasmic reticulum. A 10-fold lower dose of Triton, which completely blocks calcium uptake in fragmented SR,¹⁰ inhibited uptake about 50% in this system. Human uterine myometrium, a good representative of smooth muscle tissue, had essentially no calcium

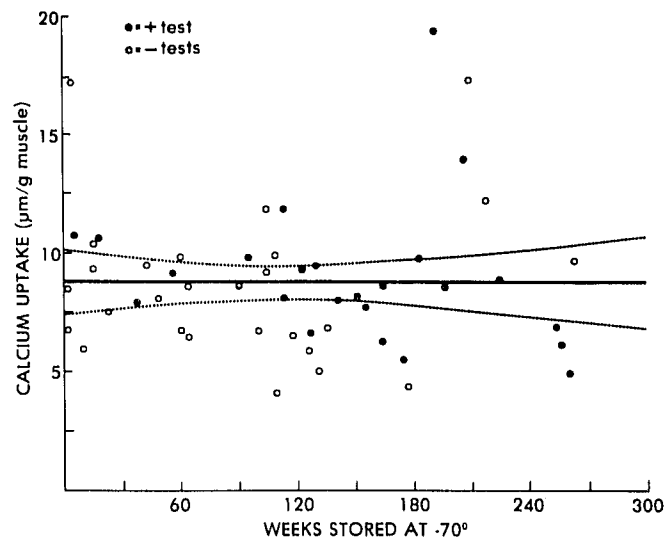


FIG. 1. Scatterplot of Ca^{45} uptake levels in 51 muscle biopsies relative to the time specimens were stored at -70°C or below. Cases with a positive halothane or a positive caffeine contracture test are denoted by solid circles; those with both tests negative are denoted by open circles. The best-fit regression line had a slope of -0.000158 ; its 90% confidence limits (dotted curves) show that it does not differ significantly from horizontal (correlation coefficient 0.0038).

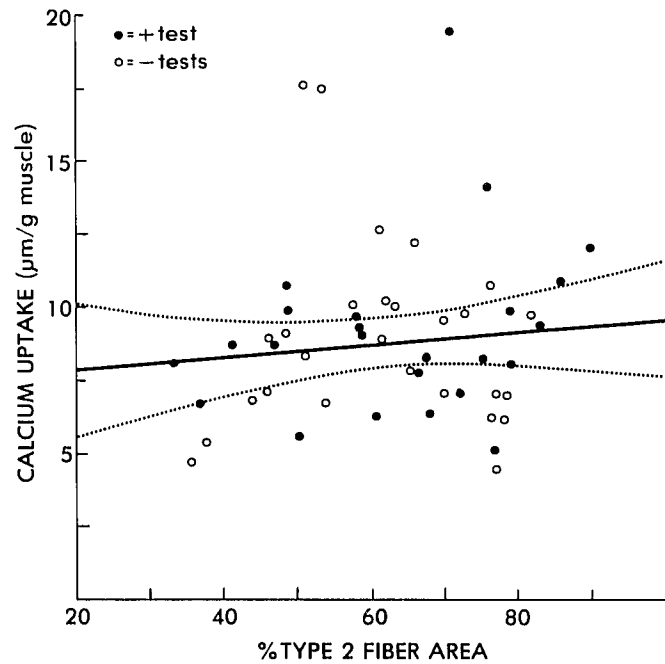


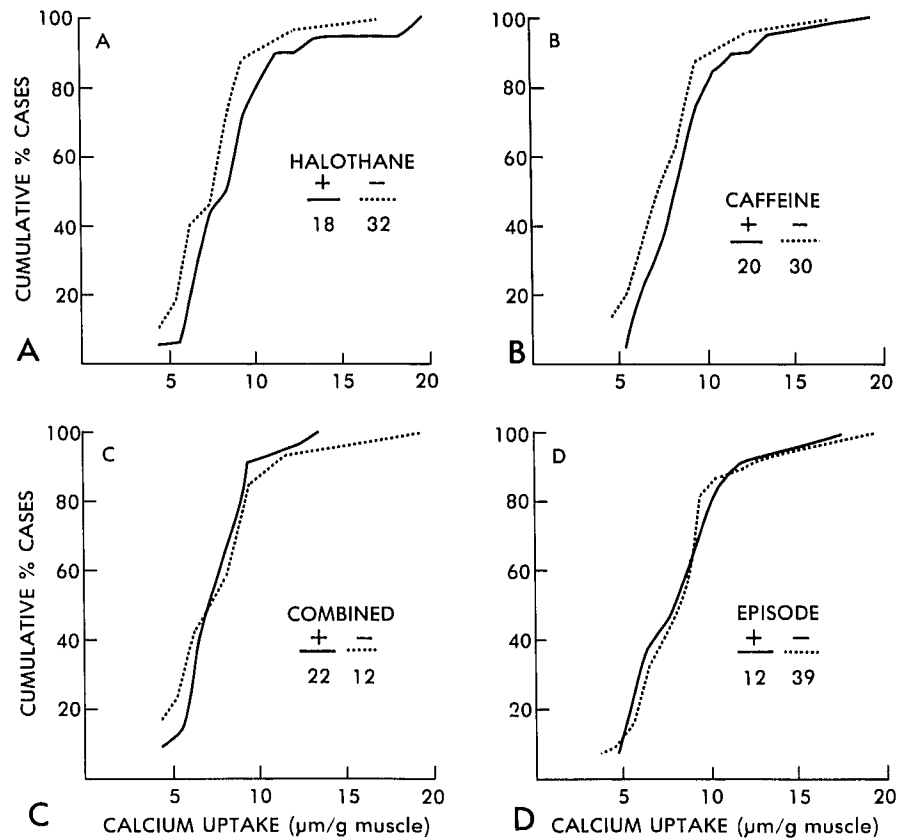
FIG. 2. Scatterplot of Ca^{45} uptake levels in 51 muscle biopsies relative to their type 2 fiber area contribution. Cases with a positive halothane or a positive caffeine contracture test are denoted by solid circles; those with both tests negative are denoted by open circles. The best-fit regression line had a slope of $+0.0223$; its 90% confidence limits (dotted curves) show that it does not differ significantly from horizontal (correlation coefficient 0.11).

uptake; and cat soleus, a type 1 muscle, had about half of the uptake of cat gracilis, a type 2 muscle. From these studies we can conclude that the procedure does fulfill these minimal requirements for a bona fide sarcoplasmic reticulum function.

HUMAN BIOPSY STUDIES

A total of 51 biopsies was quantitated for calcium uptake. Figure 1 shows all uptake data plotted *versus* the time of storage of the specimens in the deep freeze. Over the past 100 weeks, the storage has been at -130°C , but was at -70°C prior to that time. The filled circles denote cases which had a positive halothane or caffeine contracture test at the time of biopsy, while open circles denote cases in which both tests were negative. Four of the specimens were tested within 1 month of biopsy, and nine within 6 months of biopsy; these recent specimens show no peculiarities in distribution in figure 1. The least-squares fitted regression line is almost exactly horizontal, indicating no variation with storage time, and the 90% confidence limits of the line are shown by the dotted curves. The calcium uptake values cover a five-fold range, so that a significant change with storage time would be readily detectable. The positive and negative cases are scattered throughout the storage intervals, and there is

FIG. 3. Cumulative distribution frequency (cdf) graphs of the Ca^{45} uptake levels for positive (solid line) versus negative (dotted line) responders in four predictors of MHS: the halothane contracture test (A); the caffeine contracture test (B); the combined test (C); and prior episode of MH (D). The statistical analyses of these data are listed in table 2.



no clustering that might distort interpretation. This graph suggests that one may do calcium uptake studies on 5-yr stored specimens without fear of losses in activity.

Figure 2 displays the calcium uptake data relative to the percent type 2 fiber area in the specimen. The least squares fitted regression line has a slight positive slope; but the 90% confidence limits (which are the least strin-

gent limits that one could accept) clearly show that the line does not differ significantly from horizontal, indicating no correlation with fiber type distribution. The positive and negative cases (with regard to contracture tests) are scattered throughout the fiber-area range, and there is no clustering that might vitiate correlation analysis.

Figure 3 shows the cumulative distribution frequency

TABLE 2. Correlation of Calcium Uptake with Four Other Predictors of Malignant Hyperthermia Susceptibility

Predictor	$\mu\text{m Ca}^{45}$ Uptake/g		Statistical tests		
	N	Mean \pm SD	Student's <i>t</i>	<i>P</i>	Rank Sum <i>P</i>
Halothane					
Pos.	18	9.28 \pm 3.3			
Neg.	32	8.17 \pm 2.6	1.22	0.23	>0.10
Caffeine					
Pos.	20	9.21 \pm 3.2			
Neg.	30	8.14 \pm 2.7	1.24	0.22	>0.10
Halo. + caff.					
Pos.	22	7.92 \pm 2.3			
Neg.	12	8.78 \pm 4.0	0.68	0.50	>0.10
MH episode					
Yes	12	8.93 \pm 3.3			
No	39	8.70 \pm 3.1	0.03	0.90	>0.10
Total cases	51	8.74 \pm 3.1			

TABLE 3. Contingency Table of the Outcome of Halothane and Caffeine Contracture Tests Compared with the Incidence of Prior MH Episodes

Contracture Test	Clinical Episode		Total	χ^2	<i>P</i>
	Yes	No			
Halothane					
Pos.	9 (4.4)	11 (15.6)	20		
Neg.	2 (6.6)	28 (23.4)	30		
Total	11	39	50	8.16	<.01
Caffeine					
Pos.	11 (4.8)	12 (15.2)	23		
Neg.	0 (7.2)	27 (22.8)	27		
Total	11	39	50	13.9	<.01

The numbers in parentheses show the expected values for chance association; the Chi-square (χ^2) values include a continuity correction.¹⁵

TABLE 4. Essential Features in 12 Patients with Clinical Episodes

Case	Episodes	Trigger	Fever	Acidosis	CPK Units	Other Events	Hal	Caff	Ca ⁴⁵ Uptake
18 M	1	Hal + sux	106° F	pH 6.9	45,000	Arrhythmia	+	+	4.81
23 M	1	Hal + sux	104° F	P _{CO₂} 115	10,000	iv Dantrolene	+	+	9.17
18 M	1	Isoflurane	104° F	pH 6.9	423	iv Dantrolene	+	+	7.80
49 M	3	Hal	105° F				+	+	11.81
13 M	1	Hal + sux			540,000	4+ myoglobinuria	-	+	10.44
10 M	1	Hal + sux	105° F			Arrhythmia/rigidity	+	+	9.68
16 M	1	Hal	105° F			Arrhythmia	+	+	6.06
6 M	1	Hal		P _{CO₂} 200	13,700	Arrhythmia/iv dantrolene	+	+	6.49
10 M	1	Hal + sux	100° F	pH 7.1	24,000	Rigidity/iv dantrolene	-	+	8.82
53 F	3	Anesthetics	105° F			Rigidity/packed in ice	ND	ND	17.28
42 M	4	Exercise	104° F		800	Hospital documented	+	+	6.81
22 M	3	Exercise	105° F		370	Hospital documented	+	+	8.04

Criteria for inclusion involve the three quantified parameters: fever, acidosis, and serum creatine phosphokinase (CPK) elevation. One of the following three sets of criteria must be satisfied: (a) single episode with extreme change in one parameter (>104° F, pH < 7.1 or P_{CO₂} > 80 mmHg, CPK > 10,000 iu), (b) single episode with marked change in two parameters (>103° F, pH < 7.2 or P_{CO₂} > 65 mmHg, CPK

> 1,000 iu), or (c) multiple documented episodes with significant change in two parameters (>102° F, pH < 7.3 or P_{CO₂} > 50 mmHg, CPK > 350 iu.). Ca⁴⁵ expressed as μ moles/g muscle/h; Hal = Halothane Contracture Test; Caff = Caffeine Contracture Test; Sux = Succinylcholine; iv = Intravenous; ND = not done

(cdf) plots of calcium uptake in cases positive and negative with regard to four different predictors of MHS: a documented clinical episode of MH, the halothane contracture test, the caffeine contracture test, and the combined halothane-caffeine contracture test. The combined test is no longer used in our laboratory, because it is so often positive (about 2/3 of cases), but the results are shown for the first 34 cases studied, where it was performed. Each graph shows the percentage of cases accumulating at progressively higher calcium uptake values for positive responders (solid line) versus negative responders (dotted line). The curves are very close together in all four graphs, indicating that there is little difference in the calcium uptake of positive versus negative responders. This conclusion is supported by statistical analysis of the data, as shown in table 2. The Wilcoxon Rank Sum test and Student's *t* test each show no significant difference in calcium uptake between positive and negative responders by any of the four criteria; the means and standard deviations are listed in the table for each group.

In contrast to the lack of correlation of the calcium uptake with any of the MHS predictors, we can verify that the halothane and the caffeine contracture test each correlate quite strongly with cases having a documented episode of MH. Table 3 shows this data for all cases in which the contracture tests were performed. Of the 11 cases with a clinical episode, nine had a positive halothane test and 11 had a positive caffeine test, whereas of the 39 remaining cases, only 11 and 12, respectively, had positive tests. For each of the tests, Chi-square analysis indicates that the association between a positive test and a clinical episode is significant at the 1% level, even though we are

comparing these cases, not with normal subjects, but with other referral patients, who may well include some MHS cases. Essential features in the patients who were considered to have had one or more clinical episodes are collected in table 4, along with their calcium uptake values and contracture test responses.

Discussion

We are not among those who claim any diagnostic procedure as pathognomonic for MHS. This reservation applies as well to a documented clinical episode, since some of these patients have had prior anesthesia without incident, and might therefore do so again. In addition, there is no uniformly accepted set of criteria for the "documented episode," which may be diagnosed in the absence of fever and rigidity.** However, almost all investigators in the field acknowledge a high sensitivity of the halothane and caffeine contracture tests and of prior clinical episodes as reliable predictors of future risk. It is only reasonable and prudent to require any new procedure to show good correlation with these generally accepted and widely used techniques before it can be considered valid.

Because of its relative ease and its applicability to stored specimens, the frozen-section Ca⁴⁵ uptake procedure is very appealing, despite the fact that it rests upon the entirely unproven assumptions noted in the introduction. It would indeed be an immense gain if all frozen muscle

** Nelson TE, Flewellen EH: Malignant hyperthermia: Diagnosis, treatment and investigations of a skeletal muscle lesion. *Texas Reports on Biology and Medicine* 38:105-120, 1979

biopsies could be analyzed for MHS, and this is why we undertook the present study. Unfortunately, we found no significant correlation of calcium uptake with any of the accepted prognosticators of MHS. We therefore conclude that the frozen muscle section calcium uptake has no significant predictive value for the diagnosis of MHS.

We found no significant decline in calcium uptake in biopsy specimens stored up to 5 yr at -70°C . Since simple enzymes may decay markedly in that time period,¹¹ the procedure measures a stable phenomenon, which may yet prove useful in some other context. The uptake also did not vary significantly with fiber type in human biopsies, despite the wide range of type 2 fiber contribution sampled (33–90%). Although type 2 fibers have a more elaborate sarcoplasmic reticulum than do type 1 fibers, the uptake values vary widely from species to species. Mabuchi and Sreter found a five-fold greater uptake in type 2 fibers of the rabbit,¹ and we found a two-fold greater uptake in the cat, but these data are from pure-fiber-type muscles, and the mixed muscles of man may behave quite differently. The volume of SR in human type 2 muscle is only about 50% greater than that in type 1 muscle,¹² which, therefore, does not call for a markedly greater calcium uptake. Most important, however, is the reservation that calcium uptake determined in this frozen section, whole-muscle method, may also fail to correlate with that obtained in studies using fragmented (and purified) sarcoplasmic reticulum.

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