THE ADENOSINE TRIPHOSPHATE (ATP) DEPLETION TEST: COMPARISON WITH THE CAFFEINE CONTRACTURE TEST AS A METHOD OF DIAGNOSING MALIGNANT HYPERTHERMIA SUSCEPTIBILITY

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INTRODUCTION

HARRISON, *et al.*[1] using a Boehringer Kit[2] were the first to report that the ratio:

$$\frac{[\text{ATP}^*] \text{ in skeletal muscle equilibrated with carbogen and 4 per cent halothane for 45 minutes}}{[\text{ATP}^*] \text{ in skeletal muscle equilibrated with carbogen alone for 45 minutes}}$$

was less in muscle excised from MHS (malignant hyperthermia susceptible) Landrace swine than in muscle excised from normal swine. These investigators also measured ATP concentrations in muscle quick-frozen immediately following excision from MHS and normal pigs. The concentration of ATP in the former muscle was slightly less than in the latter but this difference was not statistically significant. Similar findings were later reported by Nelson, *et al.*[3] More recently, Isaacs and Heffron,[4,5] using the method of Lanprecht and Stein,[6] found that in three MHS humans the ATP content of skeletal muscle frozen immediately after excision was slightly, but nevertheless significantly, less than values observed in muscle obtained from five normal subjects.

A number of workers have shown that in MH patients, the increase in the resting tension (i.e. the contracture) of a skeletal muscle fascicle is greater than normal in the presence of caffeine, of caffeine plus halothane, or in some cases of halothane alone.[7–17] This test remains the most accepted method of diagnosing MH in human patients. It does, however, need some experience to carry it out, it requires specialized instrumentation often not available in a routine hospital laboratory and, at least at present, several hours of time for each sample to be examined.

In this paper we shall compare two tests for diagnosing malignant hyperthermia susceptibility in both normal and MHS human subjects: (1) the increase in resting tension of muscle fascicles equilibrated with caffeine in the presence and absence of halothane; and (2) the ATP ratio in muscle equilibrated with halothane plus carbogen, to muscle equilibrated with carbogen alone. The latter test offers substantial advantages since it requires the kind of experience and instrumentation which are available in the average hospital laboratory.

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*ATP – Adenosine triphosphate.

METHODS

Patient Selection

The patients selected for biopsy comprised the following groups:
(1) individuals with no systemic diseases and having no MHS relatives, who were undergoing orthopaedic procedures (for example, cup arthroplasty or Charnley hip replacement), thoracic operations such as transthoracic hiatus hernia repair, or abdominal operations such as elective cholecystectomies (Control patients);
(2) patients who had had previous malignant hyperthermic reactions with and without rigidity (MHS patients); and
(3) relatives of MHS patients (MHS relatives).

Anaesthetic Technique

The patients were premedicated with pantopon and diazepam. Anaesthesia was induced with Innovar and diazepam, and was maintained with nitrous oxide, oxygen and fentanyl. We found no interference from these agents in our *in vitro* studies.

Surgical Technique

The muscles biopsied were the vastus lateralis, the rectus abdominis or the intercostalis. Without employing cautery or excessive mechanical compression, all possible fat and connective tissues were removed from the surface of the muscle prior to severing its blood supply.

Effect of Caffeine (plus Halothane) on Isometric Skeletal Muscle Contracture

This study was carried out according to techniques which we have described in previous publications.[7,8,10] In brief, muscle fascicles were secured at each end with black silk sutures and then meticulously dissected free from the surrounding muscle, while maintaining constant tension on the sutures in such a manner as to preserve the whole cells free from contractures or excessive stretching. The fascicles were then immediately transported to the laboratory in ice-cold Ringer's solution. The time elapsed between excision and further processing in the laboratory was about ten minutes. The specimens were trimmed free of any irregularities or remaining fat and each was divided into two pieces to permit measurements in duplicate. The size of each muscle strip was approximately 3 × 5 × 20 mm, each weighing roughly 0.3 gm with a range from 0.2 to 0.4 gm. In trimming the muscle, care was taken to ensure that the direction of the long cut ran parallel to the fibres.

Each muscle strip, secured by a silk suture to an electrode housing, was immersed in 30 ml of a Krebs Ringer's solution at pH 7.4 and 23° C adapted for human tissues (Table I). The upper end of the muscle was connected by a second silk suture to a Grass force displacement transducer (ST-10-DC). Isometric tension was recorded with a Grass polygraph. The initial tension was set at 0.5 or 1.0 gm (the magnitude of the contracture was not altered by changing the baseline tension from 0.5 to 1.0 gm). In order to assess viability the muscle was stimulated every five seconds through platinum electrodes connected to a square wave Grass stimulator which was set to deliver 8 volt impulses of 20 milliseconds duration. Oxygen containing 5 per cent carbon dioxide (carbogen) was bubbled through the bath at 1.0 litre

TABLE I

HUMAN KREBS RINGER'S SOLUTION

118.4 mM NaCl
3.3 mM KCl
0.9 mM $MgSO_4$
1.1 mM KH_2PO_4
11.1 mM Glucose
24.9 mM $NaHCO_3$
2.5 mM $CaCl_2$
pH of medium adjusted to 7.4 at 25° C

TABLE II

CLASSIFICATION OF SUBJECTS BASED ON THE CAFFEINE-INDUCED MUSCLE CONTRACTURE TEST

	Caffeine Specific Concentration (mM)					
Halothane Absent (1.0 vol%) Present	<8.5 <1.30	<8.5 >1.30	>8.5 <1.30	>8.5 >1.30	Halothane absent	Halothane present
Normals	—	—	1†	13	14.3 (1.3)‡	2.46 (0.26)‡
Non-Rigid relatives	—	—	—	7	26.3 (2.4)	2.52 (0.20)
Non-Rigid patient	—	—	2	5	22.3 (2.2)	2.19 (0.33)
Rigid relatives	13	1	15	13	11.0 (0.6)	1.17 (0.08)
Rigid patients	15	1	1	—	4.86 (0.68)	0.63 (0.08)

*The concentration required to raise the resting skeletal muscle tension by one gram.
†Number of Subjects.
‡Average caffeine specific concentration (in mM), with its standard error between parentheses.

per minute. As desired, 1.0 vol % halothane was added to the carbogen. Caffeine in concentrations varying from 0.25 to 32.0 mM was added directly to the bath. Each caffeine dose was left in the bath for five minutes and then removed by washing. The parameter measured was the contracture expressed as grams of tension increase produced by graded concentration of caffeine within four minutes, once in the presence and once in the absence of halothane 1 vol % in the gas phase.

From the concentration response curves we calculated the concentration of caffeine in mM required to raise the resting tension of the isometric skeletal muscle preparation by one gram (Table II). Hereafter, this value will be known as the "caffeine specific concentration."

Effect of Halothane on the Depletion of ATP in Isolated Skeletal Muscle

For the ATP study two muscle slices were removed from the patient. One 100 to 300 mg specimen was immediately placed in a large brass clamp which had been pre-cooled in liquid nitrogen. (Brass was used because of its hardness and high thermal conductivity.) The pressure exerted by the clamp pancaked the muscle to extreme thinness. Freezing throughout all parts of the sample was thus fast and uniform so that the rate of ATP hydrolysis in the sample was kept to a minimum during the freezing period. This specimen was then stored in liquid nitrogen until required for ATP assay. A second specimen of 200 to 600 mg was rapidly divided into two equal halves. One half was placed in a bath of Krebs Ringer solution (Table I) at 37° C and bubbled with carbogen (95 per cent oxygen and 5 per cent CO_2). The other half was placed in a second bath similar in all ways to the first, except that halothane 4 per cent was added to the carbogen. Equilibration of each

sample was continued for 30 minutes. This time period was 15 minutes less than that recommended by Harrison[1] but we found during preliminary experiments that this shorter equilibration had no significant effect on the final ATP depletion ratio. At the termination of the equilibration the two muscle specimens were simultaneously removed from their baths and placed in liquid nitrogen where they were stored until needed for ATP measurement.

All the following preparatory steps were performed in the cold room at 2° C with the muscle lying on ice. Each frozen muscle specimen was sliced very thin. The still frozen slices were transferred to a pre-weighed and pre-cooled Thomas flask containing 2 ml of 12 per cent trichloroacetic acid. After reweighing the flask, the weight of the muscle was calculated by subtraction. The muscle in its acid was homogenized for one minute by a motor driven Teflon pestle cooled by an ice jacket. The homogenate was centrifuged in a refrigerated International centrifuge model B20A using an 870 Fixed Angle Rotor head at 4200 g for 20 minutes. The remaining steps were performed at 23° C. The pH of the supernatant was adjusted to 7.2 with KOH. The ATP concentration of the neutralized supernatant was determined by a Sigma kit #366-uv. This technique was based on the following equations:

$$\text{ATP} + \text{3-diphosphoglyceric acid} + \text{NADH} \xrightarrow[\text{phosphokinase}]{\text{phosphoglyceric}} \text{ATP} + \text{1,3-diphosphoglyceric acid}$$

$$\text{1,3-diphosphoglyceric acid} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{3-phosphoglyceraldehyde}} \text{3-phosphoglyceraldehyde} + \text{NAD} + H_3PO_4$$

The disappearance of NADH was observed at 340 nm in a Beckman 25 spectrophotometer. The values were expressed as μM of ATP per gram of wet frozen muscle.

Results

Classification of Subjects Based on Caffeine-Induced Skeletal Muscle Contracture

As demonstrated elsewhere [7,8,10] and indicated in Table II, the susceptibility for malignant hyperthermia can be diagnosed quite effectively in patients whose MH episode is, or would be characterized by rigidity ("rigid patients"). In all of these subjects the caffeine specific concentration (the concentration required to raise the resting skeletal muscle tension by one gram) was either less than 8.5 mM in the absence of halothane, or less than 1.30 mM in the presence of 1.0 vol % halothane. In fact, neither of those two critical specific concentrations was reached in 15 of the 17 patients.

In contrast, both critical caffeine concentrations were reached or exceeded in all but one of the normal subjects. Even the single exception showed caffeine specific concentrations of 12.5 mM and 1.20 mM in the absence and presence of halothane, respectively. Thus, MHS of potentially rigid patients can indeed be discussed on the basis of their caffeine specific concentrations.

As may be expected from genetic considerations which will be discussed in

TABLE III

DISTRIBUTION OF ATP DEPLETION RATIOS IN MHS PATIENTS, THEIR RELATIVES AND IN NORMAL SUBJECTS

Category / Range*	ATP Depletion Ratio 0.0–0.2	0.2–0.4	0.4–0.6	0.6–0.8	0.8–1.0	1.0–1.2	Mean	(S.E.)	t†
Uncorrected for Age									
Normals	–	1	2	4	7	7	0.875	0.049	–
Non-rigid relatives	–	–	–	1	2	3	0.947	0.073	0.63 n.s.
Non-rigid patients	–	–	–	3	1	–	0.805	0.068	0.53 n.s.
Rigid N-relatives	–	–	–	2	3	2	0.937	0.066	0.58 n.s.
Rigid I-relatives	2	2	4	5	–	2	0.602	0.081	3.31§
Rigid R-relatives	2	1	4	6	1	–	0.520	0.068	4.12§
Rigid patients	1	1	4	4	1	–	0.557	0.078	3.50§
Corrected for Age‡									
Normals	–	–	1	3	12	3	0.886	0.034	–
Non-rigid relatives	–	1	–	2	3	–	0.765	0.093	0.97 n.s.
Non-rigid patients	–	–	3	–	1	–	0.615	0.094	1.84 n.s.
Rigid N-relatives	–	–	1	2	4	–	0.799	0.044	0.74 n.s.
Rigid I-relatives	2	4	5	2	–	2	0.488	0.091	4.31§
Rigid R-relatives	2	3	2	5	1	–	0.468	0.083	4.34§
Rigid patients	2	4	3	–	2	–	0.385	0.096	5.94§

*Range of ATP depletion ratios including the lower, but excluding the upper limit.

†Observed t-values based on analyses of variance involving all subjects, and comparing the mean values of the various groups with that of the controls. The significance levels of the t-statistics are given by C.W. Dunnett, Biometrics, *20*: 482 (1964).

‡Corrected to a reference age of 45 years.

n.s. Not significant, $P \geqslant 0.05$.

§$P < 0.01$.

detail in another paper,[8] relatives of rigid patients do not follow a homogeneous pattern but could be classified into at least three groups[8] (Table II). The caffeine specific concentrations of the first group ("Rigid-N relatives") correspond to those of the normal subjects: not less than 8.5 and 1.30 mM caffeine in the absence and presence of 1.0 vol % halothane, respectively. The caffeine specific concentrations of a second group ("Rigid-R relatives") are, conversely, similar to those of the rigid patients: less than 8.5 and 1.30 mM caffeine in the absence and presence of 1.0 vol % halothane, respectively.

The third group among the relatives of rigid patients ("Rigid-I relatives") exhibited mixed or intermediate characteristics: the caffeine specific concentration was greater than 8.5 mM in the absence of halothane, but it did not exceed 1.30 mM in the presence of 1.0 vol % halothane. In all groups, the degree of proximity of relatives to the probands had no effect on the caffeine specific concentration.

Only one relative indicated the possibility of the fourth conceivable combination of caffeine specific concentrations. While a normal 1.90 mM caffeine was required to induce the standard muscle contracture in the presence of halothane, in the absence of the anaesthetic 8.9 mM caffeine already gave rise to this response. However, this is not far from the critical specific concentration of 8.5 mM. By taking into consideration the experimental errors,[8] this individual may be considered to be a rigid-N relative.

For patients whose MH episode is not characterized by rigidity ("non-rigid patients") and their relatives ("non-rigid relatives") the caffeine-induced muscle contracture test does not yet provide a useful diagnostic tool[8] (Table II).

Effect of Halothane on the Depletion of ATP in Isolated Skeletal Muscle (ATP Depletion Ratio)

Preliminary evaluation of the ATP concentration measurements under various conditions suggested that the best discrimination between MHS patients and normal subjects is provided by the ATP depletion ratio defined by:

$$\text{ATP-ratio} = \frac{[\text{ATP}] \text{ in muscle equilibrated with carbogen plus halothane}}{[\text{ATP}] \text{ in muscle equilibrated with carbogen alone}}$$

Indeed, the distributions of ATP ratios given in Table III demonstrate that they are substantially, consistently and statistically significantly lower in rigid patients and their RR and IR relatives (but not in the C relatives) than in control subjects. This implies that characteristics of the ATP depletion ratio are similar to those of the caffeine-induced muscle contracture test in the presence (but not in the absence) of halothane. Non-rigid patients and their relatives did not show a shift from the control values.

The distributions of ATP ratios were further separated (Table III) when it was noted that, in control subjects, the ratios tended to decrease with advancing ages (Figure 1) and, therefore, they were standardized to the approximately average age of 45 years. If it was further assumed that the age correction among patients and their relatives was the same as among the controls (0.010 units of ATP ratio decrease per year); then, since our patients and their relatives were, on the average, substantially younger than our sample of normals, the corrected ratios of

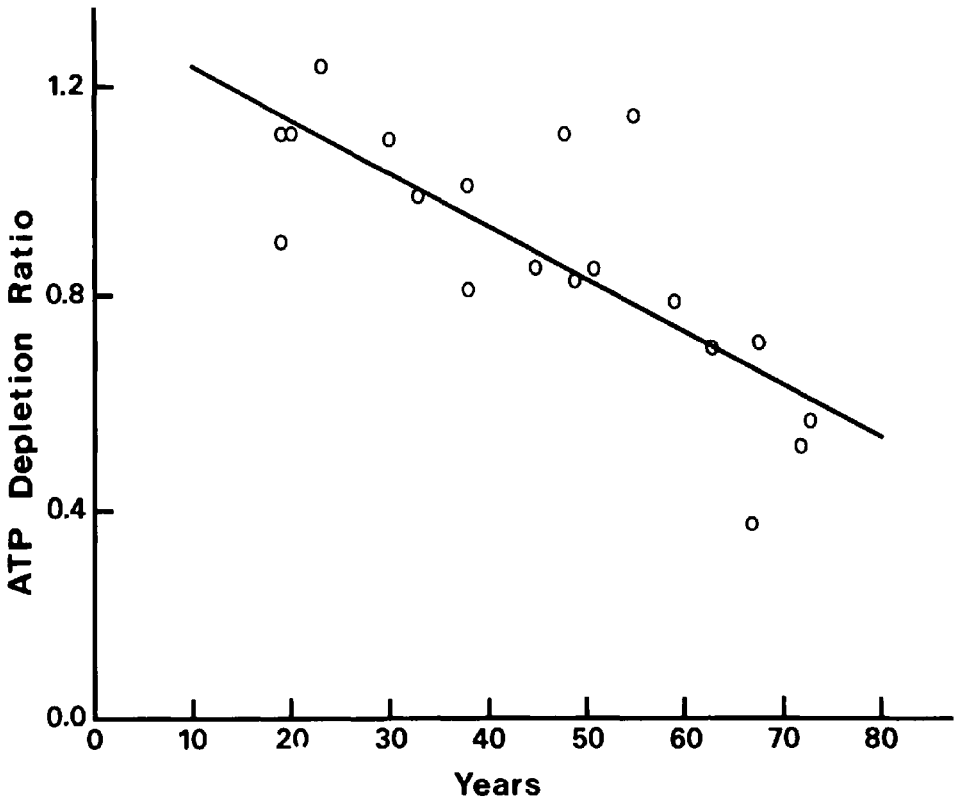

FIGURE 1. Relationship between ATP depletion ratio and the age of control subjects. ATP depletion ratio = ([ATP] in muscle equilibrated with carbogen plus halothane)/([ATP] in muscle equilibrated with carbogen alone).

rigid patients and of their rigid-R and rigid-I relatives were shifted more clearly away from those of the controls. Still, while the average difference between these groups was impressive, the separation of individual values for diagnostic purposes was not as clear-cut as were the caffeine-induced muscle contractures (comparison of Tables II and III).

It was investigated further whether the separation between the ATP-ratios of rigid patients (and their rigid-R and rigid-I relatives) and of controls was due to differences in the numerators or in the denominators. Initial analysis of the age effects in normal subjects revealed that $[ATP]_{carb}$ was independent of age (correlation coefficient, $r = -0.05$). In contrast, $[ATP]_{carb + hal}$, the ATP concentration in normal muscles equilibrated with carbogen plus halothane, decreased ($r = -0.50$, $P < 0.05$) by about 0.023 units per year. Therefore, age-corrected $[ATP]_{carb + hal}$ but uncorrected $[ATP]_{carb}$ measurements are listed in Table IV. ATP concentrations in those specimens which were frozen immediately after excision were similar in all categories of patients (Table IV).

In muscles equilibrated with carbogen only, statistically significant deviations from the control ATP concentrations could not be detected. Still, the measurements in non-rigid patients and their relatives tended to be enhanced by about 50 per cent (Table IV).

TABLE IV

ATP CONCENTRATIONS* IN MUSCLES OF MHS PATIENTS, THEIR RELATIVES AND IN NORMAL SUBJECTS

	Equilibrated with carbogen + halothane			Equilibrated with carbogen			Quick-frozen		
Category†	Mean‡	S.E.	t§	Mean	S.E.	t§	Mean	S.E.	t§
Normals	1.57	0.17	—	1.86	0.24	—	3.32	0.20	—
Non-rigid relatives	1.97	0.37	1.10	2.76	0.74	1.85	4.38	0.68	2.07
Non-rigid patients	1.62	0.38	0.12	2.65	0.36	1.37	4.43	0.54	1.84
Rigid N-relatives	1.70	0.19	0.38	2.15	0.23	0.63	4.33	0.34	2.11
Rigid I-relatives	0.94	0.23	2.28	1.86	0.25	0.01	3.98	0.26	1.77
Rigid R-relatives	1.03	0.23	1.89	1.96	0.19	0.28	4.15	0.32	2.14
Rigid patients	0.61	0.23	3.19‖	1.71	0.36	0.38	3.60	0.37	0.68

*In μM/gm of wet muscle.

†Categories are based on caffeine-induced skeletal muscle contracture measurements.

‡Corrected to a reference age of 45 years.

§Observed t-values based on analyses of variance involving all subjects, and comparing the mean values of the various groups with that of the controls. The significance levels of the t-statistics are given by C.W. Dunnett, Biometrics, *20*: 482 (1964).

‖$P < 0.05$.

In the presence of carbogen and halothane, the standardized ATP muscle concentrations were substantially and significantly lower in rigid patients than in controls and almost significantly lower in the rigid-R and rigid-I relatives of rigid patients. The same conclusions are reached in the absence of age correction. Thus it is the equilibration with halothane which is responsible for the reduction of the ATP depletion ratio on rigid patients and some of their relatives. No change from the control values was noted in non-rigid patients and their relatives.

Discussion

The statistically similar values observed for ATP concentrations in fresh frozen skeletal muscle obtained from normal and MHS human patients and relatives agree with values reported for MHS and normal porcine muscle by Harrison, *et al.*[1] and by Nelson, *et al.*[3] but are slightly different from those described by Isaacs, *et al.*[4,5]. Isaacs' mean values were, however, based on a very small sample size.

These results show that the "ATP depletion ratio" provides a useful complement to the caffeine contracture study in the diagnosis of NH susceptibility in human patients. However, the detection of two patients with normal ATP depletion ratios but abnormal caffeine contracture tests suggests that the ATP depletion ratio may not be quite so sensitive a diagnostic indicator of susceptibility to the rigid form of MH as is the caffeine contracture test. Nevertheless, when presented with a patient suspected of having rigid MH in a medical centre which lacks sufficient equipment, expertise or time, determination of the ATP depletion ratio may be an acceptable alternative to the more difficult caffeine contracture test, especially when carried out in conjunction with examination of other parameters, for example: microscopic appearance, histochemistry, paraffin sections and electron micrographs,[4,5,10,12,18–24] assessment of clinical muscle abnormalities,[10,12,25–34] serum CPK[12,22,25,26,35–42] and neurophysiological studies.[20,21,26,33,34,43] The "ATP depletion ratio" has the additional advantage of requiring somewhat less surgical skill during excision than does the caffeine contracture test. It must be remembered, however, that the ATP depletion ratio cannot be employed to determine susceptibility in patients belonging to non-rigid families. The caffeine contracture test, therefore, remains the preferred diagnostic tool.

The age dependence of the ATP depletion ratio and its numerator has been shown only in the normal subjects. In contrast, a similar relationship could not be demonstrated for MHS patients or their relatives. The age range of the MHS individuals is less than that of the normal patients and so in them a possible relationship between age and the ATP depletion ratio would be more difficult to detect. Other explanations for this lack of age difference in the MHS subjects could be attributed to the small number of individuals in these groups or to their pathological heterogeneity. Furthermore, it may have been that in MHS muscle the pathological depletion of ATP by halothane is great enough to mask normal age differences. Finally, the incidence of reactions decreases with advancing age.[29,44] This apparent clinical improvement may be due to an amelioration of the biochemical muscle defect with, therefore, relatively higher ATP ratios in older MHS subjects. The reactions of the caffeine contracture test appear to be independent of age and sex.[8]

The reason for the lower ATP depletion ratios in rigid MH than in normal muscle is not elucidated by our experiments. Previously published data have proposed that the reduction is due to accelerated utilization because of increased activation of myosin ATPase by calcium and, therefore, an enhanced rate of hydrolysis of ATP. Decreased production of ATP because of uncoupling of oxidative phosphorylation by calcium[45–51] may also play a role in the reduction of muscle ATP.

SUMMARY

The adenosine triphosphate (ATP depletion ratio, which is the ratio

$$\frac{\text{[ATP] in skeletal muscle equilibrated with carbogen and 4\% halothane for 30 minutes}}{\text{[ATP] in skeletal muscle equilibrated with carbogen alone for 30 minutes}}$$

is less than normal in most but not in all rigid MHS patients. The ratio is normal in non-rigid MHS patients. This diagnostic tool is, therefore, useful in the diagnosis of rigid MH. It is not, however, such a sensitive diagnostic parameter as the caffeine contracture test.

RÉSUMÉ

Le rapport de la déplétion en adénosine triphosphate, qui est le rapport entre :

$$\frac{\text{(ATP) dans le muscle squelettique mis en équilibre avec du carbogène et 4\% d'Halothane durant 30 minutes}}{\text{(ATP) dans le muscle squelettique mis en équilibre avec du carbogène durant 30 minutes}}$$

est plus petit que normalement chez la plupart des patients qui ont une susceptibilité à la forme rigide de l'hyperthermie maligne, mais non chez tous. Ce rapport est normal dans la forme non rigide. Il s'agit donc d'un examen utile dans le diagnostic de la forme rigide de l'hyperthermie maligne. Cet examen n'est toutefois pas aussi sensible que celui de la contraction musculaire à la caféine.

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