BBA 4023

INSTANTANEOUS TISSUE HOMOGENIZATION

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(Received October 5th, 1962)

SUMMARY

Description is given of a steel block homogenizer, operating at temperatures below 0° utilizing the freezing-point depression occurring at high pressures. The advantages are: (a) low operating temperature; (b) no diluent required.

For the enzymes tested no destruction due to the treatment seems to take place. The effect of storage upon some enzymes, under various conditions, is examined.

The requirements of an ideal homogenizer are briefly discussed.

INTRODUCTION

The tissue homogenizing device probably most widely used to-day is the Potter–Elvehjem homogenizer. This extremely simple piece of equipment has definitely many advantages: It is cheap, it is easily repaired, it is reasonably well adjustable to the amount of material available, and it requires very little space to operate. Other techniques used in releasing intracellular components into solution or suspension include section, mincing and crushing. These techniques, as compared to the homogenization, are less rigid, and cellular organization is still retained to some extent. If total disruption of membranes is to be achieved normally the P.–E. homogenization is the method of choice.

There are several disadvantages in the P.–E. technique, some of which are listed below:

(a) The material to be homogenized is exposed to mechanical force for some length of time, particularly when tough tissues such as muscle tissue or connective tissue are treated.

(b) Some heat is developed due to friction, which supposedly is counterbalanced by cooling the tube, nevertheless the temperature between the pestle and the tube is unknown. This local temperature increase may be damaging to thermolabile components of the tissue.

Abbreviations: ALD, aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7); DPNH, nicotinamide-adenine dinucleotide, reduced form; G-6-PDH, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49); GOT, L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1, formerly known as glutamic oxalacetic transaminase; GPT, L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2, formerly known as glutamic pyruvic transaminase; I.U., International Units (μmoles of substrate converted per min); LDH, lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27); GPI, glucose phosphate isomerase (D-glucose-6-phosphate ketol isomerase, EC 5.3.1.9); P.–E., Potter–Elvehjem.

(c) In order to obtain a satisfactory homogenate there must be addition of liquid. If sufficient fluid is not available the viscosity of the suspension may be too high to permit the necessary shearing forces to develop. Following this dilution the homogenate is stored as a rather dilute suspension, with possible damage to the tissue components.

(d) One is committed to one and only one solvent in the subsequent analyses on the homogenate. This may be a limiting factor in the programming.

(e) Prolonged exposure to air may also occur with the P.–E. homogenizer. More elaborate equipment is necessary to obtain an aerosol-free homogenization.

Recently BONTING AND ROSENTHAL[1] published a report in which they compared different types of tissue preparation for enzyme determination using alkaline phosphatase (EC 3.1.3.1) and lactate dehydrogenase as indicators of the efficiency of the techniques. Storage problems were also investigated on the different types of preparation. The overall picture of this investigation is rather depressing. The author of the present article interprets the results in the mentioned paper to the effect that there is very little consistency between the enzyme levels remaining in the preparation, after different techniques have been applied. It also seems to the author that there are considerable storage problems.

One of the objectives of this laboratory is to measure enzyme activities in muscle tissue from normal and dystrophic human subjects. Because of the set-backs mentioned above, it was considered worthwhile to look for a new homogenization technique. The ideal requirements for such a new technique would include the following: (a) Duration of application of mechanical force should be minimal, (b) the temperature should be kept as low as possible, (c) no fluid should be added until after homogenization had taken place, (d) the homogenate should be storable, (e) exposure to air should be minimized, (f) operation should be simple, (g) costs should be reasonable.

In 1951 HUGHES[2] described a bacteria press working on the principle that the cell walls of bacteria will be disrupted when they are mixed with abrasives and exposed to a sudden increase in pressure. The simplicity of this device was obvious, and if it could be converted to use on tissue the objectives outlined above would be achieved. The present paper is dealing with the results obtained with such a press.

METHOD

General description of homogenizer

Figs. 1 and 2 show the homogenizer assembled and taken apart. A block of stainless steel was cut in half, and the two fresh surfaces lapped to within a few millionths of an inch. Short rounded guide pins and holes for same were fitted and the two halves reassembled. Three cylindrical holes of different sizes were drilled, and pistons fitting these holes to two to three thousandths of an inch were made. In one of the blocks a reservoir was cut around the bottom part of each cylinder. Four bolts with nuts and washers were used to assemble the unit.

Description of use

The block that contains the reservoirs is greased very lightly on the whole surface except for the dotted area shown in Fig. 3. The other block is not greased except for the area immediately opposite the reservoirs where a finger from which

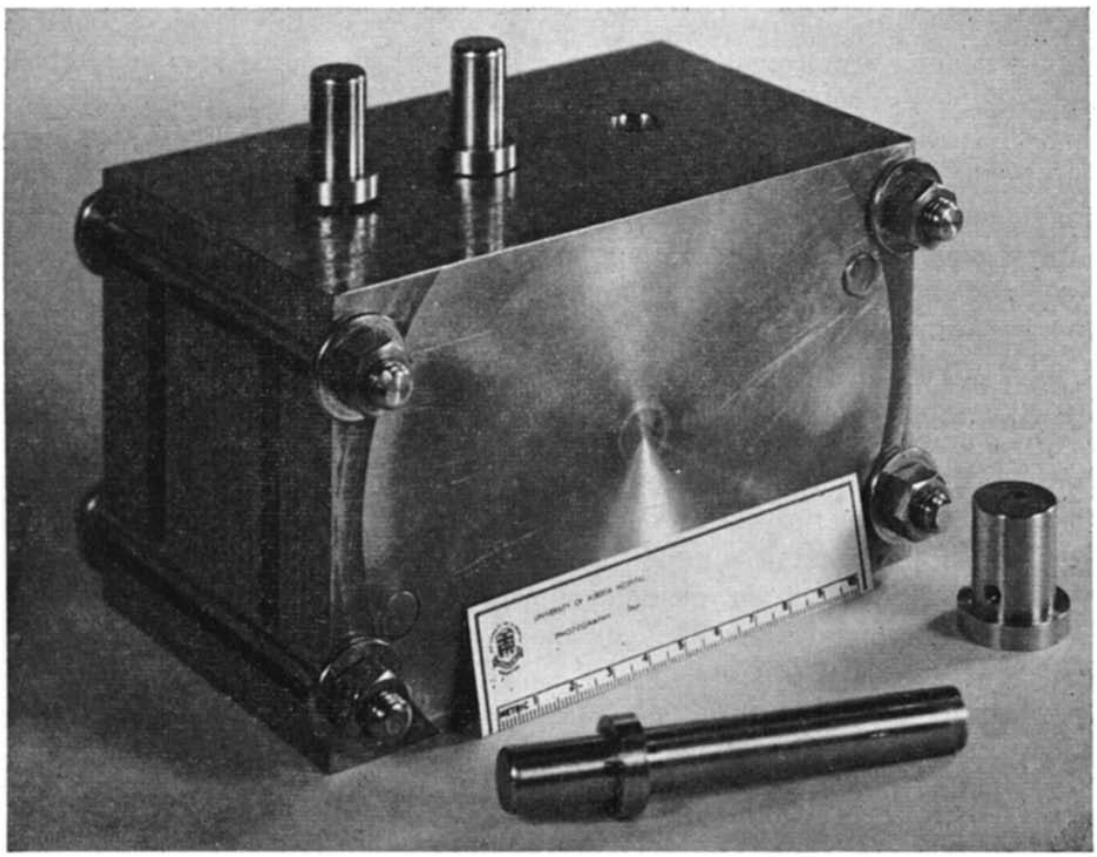

Fig. 1. Homogenizer, assembled. Adapter for fly press at right.

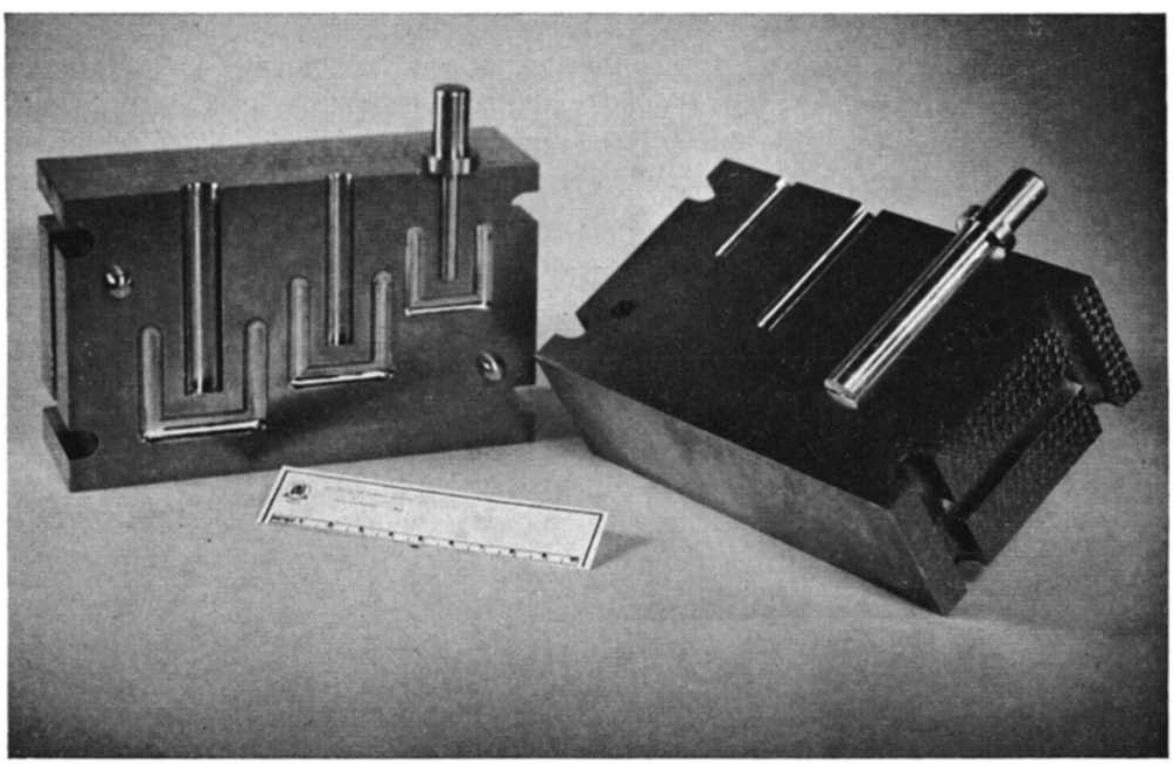

Fig. 2. Homogenizer, open.

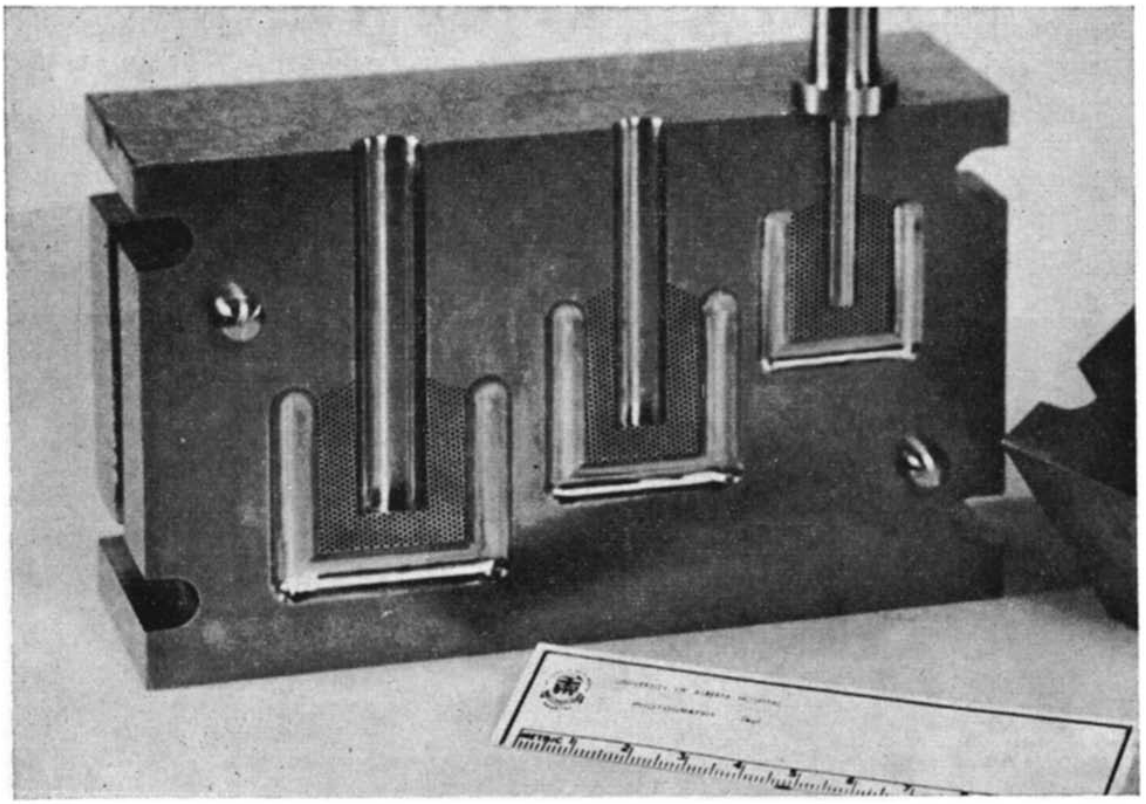

Fig. 3. Close up of homogenizer. Grease-free area indicated with dots.

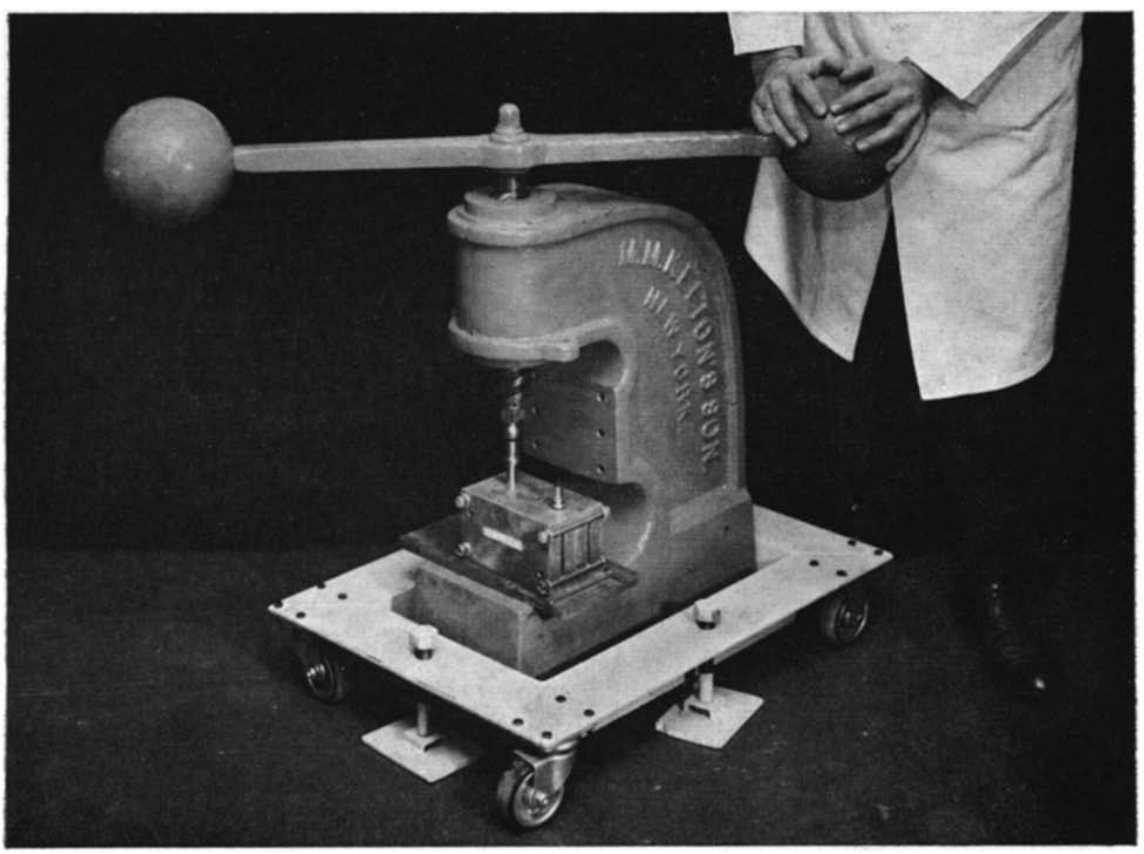

Fig. 4. Homogenizer in position for the blow.

lubricant has been wiped off, is applied. The two blocks are assembled, the pistons inserted and the four bolts and nuts tightened as much as possible. The unit is then set in a deep-freezer.

The tissue to be homogenized is cut into pieces of sufficiently small size to fit into the hole selected for use, depending on the amount of tissue available. The tissue is then frozen in liquid nitrogen. The unit is removed from the freezer, the tissue pieces transferred to the proper cylinder and the piston reinserted. On top of the piston is

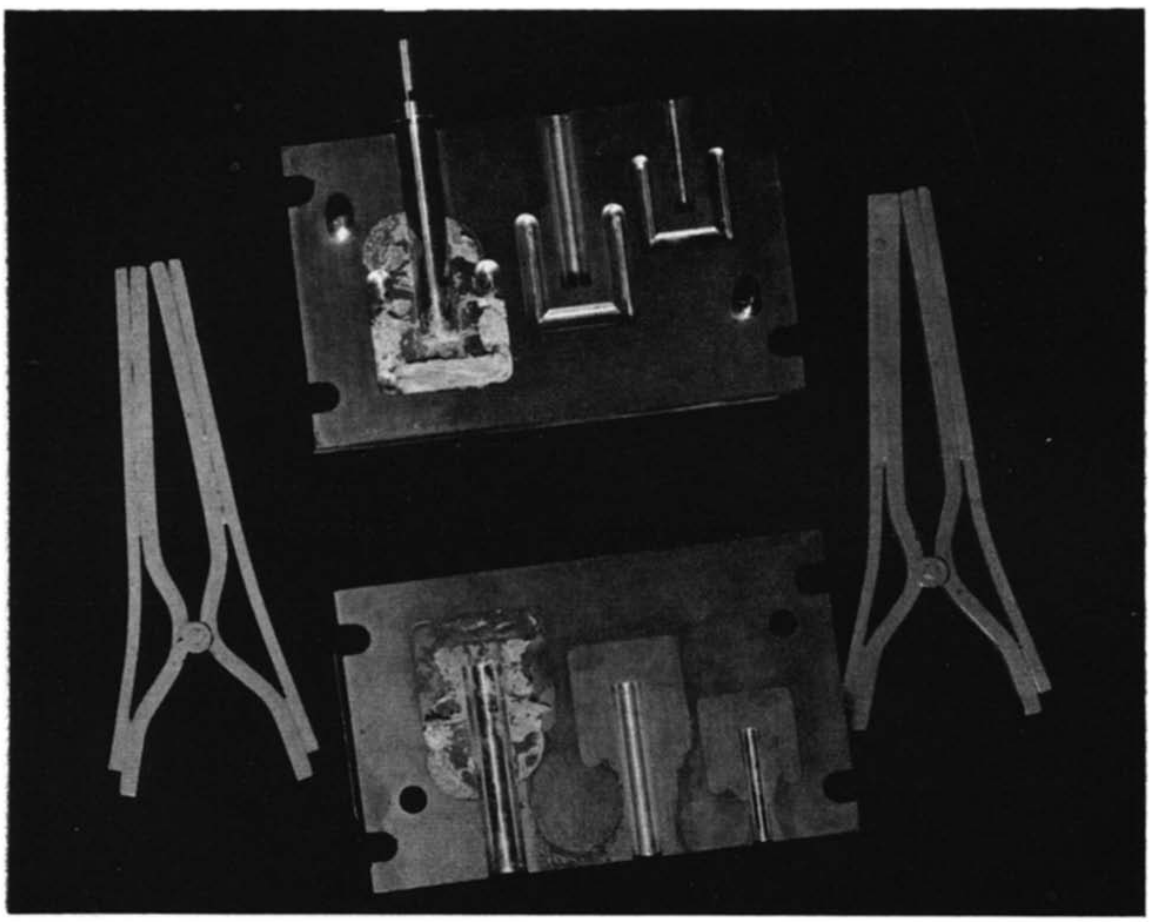

Fig. 5. Homogenizer disassembled after operation. Also the prying tongs.

placed an adapter and the whole unit is lined up on a fly press immediately under the pressure head, as shown in Fig. 4. With a gentle pressure the material is squeezed into a solid block in the bottom of the cylinder, the handle turned back a quarter of a full turn and with great force pushed forward again. In view of the weight of the balls and the bar it is advisable to make the final blow a two-man operation. The operator of the fly press will all of a sudden feel a decrease in resistance, during which phase the piston is travelling smoothly downwards. A stopper is built into the fly press in such a way that the piston will not touch the bottom of the cylinder.

The unit is now removed from the fly press and placed with the reservoir side down, upon a support; the bolts are removed and with a pair of prying tongs the two blocks are separated.

Fig. 5 shows the appearance of the two blocks after a successful homogenization. The contents of the reservoir is a solid mass from which the desired amount can be

scooped out and weighed into the tube of a P.–E. homogenizer. The pestle is inserted and manually turned a few times which is sufficient to overcome the stickiness. Then appropriate medium is added in the amount necessary to obtain the desired concentration, and still manually the contents of the tubes are mixed with the pestle.

Mode of action

When the tissue is frozen in liquid nitrogen the material becomes very brittle. During the subsequent initial crushing in the cylinder a substantial amount of the cell membranes is consequently disrupted. The main effect, however, in the author's opinion is the tremendous force that is applied and which causes the freezing point to be depressed so that the whole mass is liquefied. Since there is no grease in the area between cylinder and reservoir whereas the rest of the inner surfaces are kept apart by the thickness of the lubricant layer there is a narrow space through which the liquid under pressure can escape from the cylinder and enter the reservoir, where it will solidify immediately due to the lower pressure and the low temperature of the homogenizer itself.

Enzyme determinations

The following enzymes have been determined on muscle homogenates obtained with this technique: ALD, with a new DPNH technique[3]; GOT, according to KARMEN[4]; GPT, as suggested by Sigma Chemical Co.[5]; LDH, with a modification of HILL's procedure[6]; G-6-PDH, with a TPN method[7]; GPI, with a modification of BODANSKY's technique[8].

Reagents

Silicon grease.

RPS: Ringer, Phosphate, Serum. Ringer solution, 0.1 M phosphate buffer (pH 7.4), and heat-inactivated serum are mixed in the proportion 6:1:1, and used as suspending medium for all dilutions, except where G-6-PDH is to be determined.

Glycyl–glycine buffer: 0.25 M glycyl–glycine buffer (pH 7.5) is used for suspension of the homogenate prior to G-6-PDH analysis.

All results are given as I.U./l.

RESULTS

Appearance

Macroscopically a cross-section of the contents of the reservoir looks like a folded sheet, the end of which will often be found on the area of the block between the cylinder and the reservoir itself. This confirms the belief that the mass is temporarily liquefied during the passage from the cylinder to the reservoir.

Examination under the microscope reveals that freezing in N_2 causes a swelling of the fibers, although the cellular organization is maintained.

The pellet in the bottom of the cylinder contains some cells, none of them intact, and the remainder consists of an unorganized mass. In the reservoir the outline of a greatly distorted cell can be found occasionally, and intact cells are totally absent.

Homogeneity

It might very well be that some cell components would leave the mass in the cylinder more readily than others and that consequently the composition of the distal

part of the reservoir contents might be different from those in the proximal part. In order to check this, experiments of the following kind were performed. A piece of muscle was treated with the standard technique, an imaginary line drawn through the middle of the reservoir thus dividing it in an inner and an outer portion, each of these were again divided in a left and a right side. Comparison of these four parts of the homogenate was done by estimation of ALD, GPI, GOT, GPT and LDH. The results are listed in Table I, which represents a typical experiment of this kind. It is readily seen that the deviations between the different areas are well within the experimental error.

TABLE I

ENZYME ACTIVITIES IN HOMOGENATES OBTAINED FROM VARIOUS PARTS OF THE HOMOGENIZER

Units: I.U. per g of wet muscle.

Reservoir area	*Enzyme:*	*ALD*	*GPI*	*GOT*	*GPT*	*LDH*
Outer edge, left		34.1	208	42.2	2.67	90.6
Outer edge, right		32.1	216	38.5	2.53	80.3
Inner edge, left		35.0	218	41.9	2.72	81.9
Inner edge, right		33.5	185	41.9	2.82	76.8

TABLE II

ENZYME ACTIVITIES IN SUPERNATANT AFTER VARIOUS DEGREES OF CENTRIFUGATION

Units: I.U. per g of wet muscle.

Treatment	*Enzyme:*	*ALD*	*GPI*	*GOT*	*GPT*	*LDH*
No centrifugation		34.1	208	42.2	2.67	90.6
100 × *g*, 5 min		34.2		44.4		93.7
1000 × *g*, 15 min		33.6	223	42.7	2.77	87.1

It is evident that neither with this device nor with any other tissue processing machine is a homogeneous solution obtained, taking the word homogeneous in its chemical sense. After the manual treatment of the reservoir contents, diluted with an appropriate fluid, in the P.–E. homogenizer, we have a suspension that contains particulate matter. It is possible that the tissue components which are to be assayed, may adhere to or be contained in these particles. To clarify this, experiments were carried out in the following manner.

An "homogenate" is prepared, and diluted in the P.–E. homogenizer with RSP to a concentration of approx. 80 mg of wet muscle per ml. This mixture is highly viscous and contains a lot of particulate matter. After thorough mixing the suspension is split into three parts; one is diluted at once to the appropriate dilutions necessary for the various enzymes to be assayed, the second part is centrifuged for 5 min at 100 × *g*, and the third portion is centrifuged for 15 min at 1000 × *g*. Supernatants from the second and third portion are then compared in enzyme analysis with the first untreated specimen. Results of such an experiment are given in Table II.

Again in this case the variation is within experimental error.

Enzyme stability

As stated in the introduction the reason for developing this new technique was to circumvent the rough treatment the material to be analyzed was given in P.–E. and other homogenizers. Yet it can hardly be considered gentle treatment to freeze to a temperature of —196°, or to apply pressures above 1500 atm, although it should be remembered that the exposure is of a rather short duration. In order to check probable detrimental effects upon the enzymes in the technique, the following experiments were carried out.

Serum was divided into 3 portions: A, B and C. Portion A was assayed for various enzymes directly, Portion B was frozen in liquid nitrogen, allowed to melt by standing at room temperature, and Portion C was frozen in liquid nitrogen, the tube was broken and the resulting column of frozen serum introduced into the homogenizer. Pressure was applied whereby the serum was forced into the reservoir from where it was scooped up, transferred to a tube and allowed to melt. Enzyme analyses were carried out on all three specimens. Results of these experiments are given in Table III. Again no deviation from experimental error is demonstrable.

TABLE III

EFFECT OF FREEZING AND PRESSURE ON SERUM

Units: I.U. per l of serum.

Portion	*Treatment*	*Enzyme:*	*ALD*	*GOT*	*GPT*	*LDH*
A	No treatment		6.79	25.7	12.1	279
B	Frozen in liquid N_2		6.73	25.4	12.7	278
C	Frozen in liquid N_2, then pressurized		6.38	27.6	11.5	275

A similar experiment was done with G-6-PDH. A small amount of a solution of this enzyme was injected into the center of a lump of muscle. The muscle was then frozen in nitrogen, transferred to the homogenizer and smashed. G-6-PDH determination was carried out on the homogenate. Due to the fact that the muscle specimen to be homogenized did not have a uniform distribution of the enzyme, the results were not sufficiently satisfactory to be tabulated. The recoveries were in the range 90–110 %, from which it can be tentatively concluded that no major deterioration has taken place.

Storage

Stability of the enzymes during storage varies greatly with the conditions under which the preparations are stored. It is beyond the scope of this report to list the effect of any possible combination of temperatures, dilutions, absence or presence of particulate matter, concentrations of raw material in the dilution, for a variety of enzymes. Results from a few of the possible combinations are listed in Tables IV–VII.

It is readily seen that no overall rule can be applied to the problem of proper storage, when highly diluted specimens are kept, whereas concentrated suspensions, and the homogenate itself, exhibit much better stability.

TABLE IV

EFFECT OF STORAGE CONDITIONS ON DILUTE SUPERNATANT

Units: I.U. per g of wet muscle.

Stored in:		*Refrigerator*			*Freezer*		
Concentration	*(µg wet muscle/ml)*	*233*	*456*	*456*	*233*	*456*	*456*
Days	*Enzyme:*	*ALD*	*GOT*	*LDH*	*ALD*	*GOT*	*LDH*
0		33.6	42.7	87.1	33.6	42.7	87.1
1		34.8	43.0	77.4	29.8	39.1	3.9
5		10.4	41.5	78.8	29.6	34.7	0
7		—	45.8	76.7	28.3	31.0	0

TABLE V

EFFECT OF STORAGE CONDITIONS ON NON-CENTRIFUGED DILUTED HOMOGENATE

Units: I.U. per g of wet muscle.

Stored in:		*Refrigerator*			*Freezer*		
Concentration	*(µg wet muscle/ml)*	*240*	*479*	*479*	*240*	*479*	*479*
Days	*Enzyme:*	*ALD*	*GOT*	*LDH*	*ALD*	*GOT*	*LDH*
0		34.1	42.2	90.6	34.1	42.2	90.6
1		32.6	44.9	73.6	32.7	39.0	0
5		29.9	44.1	64.8	29.4	33.4	0
7		19.4	41.1	81.2	29.8	31.1	0

TABLE VI

EFFECT OF STORAGE CONDITIONS ON CONCENTRATED HOMOGENATE SUSPENSION

Units: I.U. per g of wet muscle.

Stored in:	*Refrigerator*				*Freezer*				
Concentration:	*56.7 mg wet muscle/ml*								
Days Enzyme:	*ALD*	*GPI*	*GOT*	*LDH*	*ALD*	*GPI*	*GOT*	*GPT*	*LDH*
0	28.2	291	6.6	114	28.2	291	6.6	0.61	114
9	8.92	293	7.3	101	21.7	239	4.6	0.51	29

TABLE VII

EFFECT OF STORAGE CONDITIONS ON UNDILUTED HOMOGENATE

Units: I.U. per g of wet muscle.

Stored in:	*Refrigerator*					*Freezer*				
Days Enzym:	*ALD*	*GPI*	*GOT*	*GPT*	*LDH*	*ALD*	*GPI*	*GOT*	*GPT*	*LDH*
0	35.0	278	37.8	3.26	146	35.0	278	37.8	3.26	146
1	28.7	283	31.5	2.98	147	31.6	251	37.0	3.65	125
6	16.2	277	24.5	3.10	124	30.7	256	35.6	2.98	125

Durability of homogenizer itself

HUGHES discusses the need for regrinding or repolishing of the homogenizer quite frequently. It is to be remembered that he used abrasives to a large extent to disrupt the cell walls of his bacteria. In our technique no hard particles are present, and we have had excellent service from the homogenizer without need for repair, until alignment was lost due to imprudent overloading of the cylinder beyond the capacity of the reservoir. Nevertheless the cost of repair was minimal since it was only a matter of re-reaming the inner surface of the cylinder, and the manufacture of a new piston.

DISCUSSION

As stated in the INTRODUCTION the objective was to develop a new homogenization technique that would exclude some of the inherent defects of the P.–E. homogenizer. This objective seems to be achieved. However, it is much too early to say whether the presented technique is applicable in all the cases where the P.–E. homogenizer has rendered good service. So far no attempts have been made at preparation of mitochondria, microsomes, or other intracellular constituents.

There remains a considerable amount of work to do, to determine the best use to which this piece of apparatus may be put. Only time can tell whether this technique will be supplementary or complementary to previous methods. At the present it can be stated that for the enzymes mentioned in this report, and for muscle tissue, the technique is adequate.

ACKNOWLEDGEMENTS

The Neuro-Muscular Research Laboratory is supported by the Muscular Dystrophy Association of Canada.

The invaluable help from Mr. R. MCDONALD and the staff of the University of Alberta Machine Shop is gratefully acknowledged.

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