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STUDY OF GEROVITAL H_3 ACTION ON MITOCHONDRIAL FRACTION IN RAT LIVER AND BRAIN

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Summary. The present paper discusses mitochondrial fraction in rat liver and brain by studying the following functional aspects: GH_3 action on oxidative phosphorylation, binding of sodium ions and of procaine hydrochloride to the mitochondrial membrane and the effect on its structural integrity at various concentrations.

The supplementary release of protons caused by the presence of 2.10^{-5} and 8.10^{-5}M procaine hydrochloride per mg protein, dependent on the ionic strengths, was at a maximum at 80 mM NaCl. The procaine molecule, positively charged at pH 6.5, is able to form ionic bonds with the proteins or phospholipids of the mitochondrial membrane. An important increase of oxygen and phosphorus consumption was produced at the lower GH_3 concentrations of $1.5.10^{-5}$ and 3.10^{-5}M per mg mitochondrial protein. Higher GH_3 concentrations (10^{-3}M per mg protein) produced an inhibitory effect, blocking ADP phosphorylation to ATP.

It is possible that GH_3 in lower concentrations of 10^{-5}M per mg mitochondrial protein, produces the activation of the respiratory chain enzymes.

As clinical researches and pharmacologic investigations were undertaken with a view of detecting the mechanism of action of the therapy based on procaine, the studies carried out on beer yeast, as well as on rat liver homogenate with the Warburg method [1, 2, 3], showed that the presence of procaine in the test tubes determined an increase of the oxygen consumption due to the activation by procaine of the processes of oxidative phosphorylation.

In 1972, Hrachovec published the results of comparative researches which showed that Gerovital H_3 (GH_3), (procaine hydrochloride 2%, benzoic acid 0.12%, potassium metabisulphite 0.10%, and disodium phosphate 0.01%) had a more pronounced inhibitive action than procaine hydrochloride, upon the monoaminoxidase in the brain, liver and heart of rats [4]. These researches were carried out both upon homogenates *in vitro* and upon mitochondrial fractions, after the animals had been injected intraperitoneally. In contrast to the properties of other classical MAO inhibitors, it was demonstrated that GH_3 is a weak, reversible and competitive inhibitor of MAO [5, 6].

We have used the mitochondria to assess the effects of GH_3 on oxidative phosphorylation, binding of sodium ions and procaine hydrochloride to mitochondrial membrane and the effect on its structural integrity.

MATERIALS AND METHODS

We used female Wistar rats, the groups being divided as follows: young rats (2—3 months), adults (6—8 months) and old rats (22—24 months).

Liver and brain mitochondria were isolated essentially as described by Schneider and Hogeboom [7] in 0.25 M sucrose, 0.05 M TRIS-HCl, pH 7.4.

Oxidative phosphorylation was measured by classical Warburg manometric methods at 38°C [8]. The main compartment reaction mixture (the final pH 7.5) consisted of the following components in the Warburg flask: 1.2 ml of a solution containing 0.02 M TRIS, 0.06 M potassium phosphate, 0.5 M sucrose; 0.15 ml of a 5% solution of bovine serum albumine (BSA); 0.6 ml of 0.05 M $MgCl_2$ solution; 0.30 ml of a solution of hexokinase (12.5 mg/ml); 0.6 ml of the mitochondrial suspension (8—10 mg protein) and 0.1 ml of various GH_3 (procaine) concentrations, ranging from $1.5.10^{-5}$ to 1.10^{-3} M/mg protein. The side arm contained: 0.15 ml of a solution of 0.45 M glucose, 0.15 ml of 0.05 M ADP solution and 0.15 ml of 0.1 succinate. The central well contained a small wick of filter paper and 0.2 ml of 20% KOH. The reaction was stopped by the addition of 0.65 ml of 10% trichloroacetic acid. Inorganic phosphate was determined by the method of Fiske-Subbarow [9].

The binding of Na^+. Mitochondrial fraction from the liver of adult rat was suspended in 0.25 M sucrose at 50 mg protein per ml. The suspension was kept at 0°C and used within 4 hours. The pH changes in the mitochondrial suspensions were followed with a combination glass electrode linked to an Orion pH-meter. From the initial and final pH values the amount of H^+ produced in medium was evaluated. In this experiment pH units were transformed in concentration of hydrogen ions by logarithmic calculation. The binding of Na^+ was measured according to the procedure of Gear [10]. Mitochondrial suspension containing 2.5 mg protein per ml in 0.25 M sucrose, was mixed with different NaCl solutions (80 mM, 160 mM, 240 mM, 320 mM) at three pH values: 6.5,7 and 8. A constant osmolarity of 160 mosM was maintained with sucrose except for the final 320 mM NaCl.

Binding of procaine molecule. Rat liver mitochondria were added (5 mg protein) to make a total volume of 2 ml containing increasing NaCl concentrations from 80 to 320 mM.at pH 6.5. The net ejection of H^+ was monitored. In another experiment the medium contains 2.10^{-5} and 8.10^{-5} M procaine hydrochloric per mg protein. Results are expressed as nanomoles of H^+ ejected per mg mitochondrial protein. Mitochondrial protein was determined by the Lowry procedure [11].

The influence of different concentrations of GH_3 on mitochondrial protein concentration in supernatant was studied at six levels of GH_3 (M/mg protein) added to 5 ml aliquots of mitochondria: $1.5.10^{-5}$, 3.10^{-5}, $1.5.10^{-4}$, 3.10^{-4}, 1.10^{-3}, 2.10^{-3}. These were incubated at 25°C for 10 minutes and then centrifuged at 15000 g for 10 minutes. The protein content was estimated in the supernatant and the variations were expressed in per cent.

RESULTS

Mitochondrial fractions of rat liver suspended in salt-free isotonic sucrose with buffered media containing NaCl of various concentrations, release H^+ ions into the medium. The amounts of H^+ released increase with pH and with the salt concentrations. At pH 6.5, 7 and 8 the increase of salt concentrations is accompanied by a large release of H^+ into the medium (Table 1).

Measurement of proton release by the mixture of mitochondria with different NaCl concentrations (80 mM, 160 mM, 240 mM, 320 mM) is thus a sensitive

Table 1

H+ ejection during procaine binding to rat liver mitochondria

	NaCl, mM											
	pH 6.5				pH 7				pH 8			
	80 mM	160 mM	240 mM	320 mM	80 mM	160 mM	240 mM	320 mM	80 mM	160 mM	240 mM	320 mM
Procaine 2.10^{-5}M per mg prot.	16	17	21	24	–	–	–	–	–	–	–	–
Procaine 8.10^{-5}M per mg prot.	15	16	20	22	–	–	–	–	–	–	–	–
Without Procaine	13	16	20	23	19	23	26	30	25	30	31	36

means of following a cation binding. Procaine-hydrochloride was therefore added to a sodium-containing medium at pH 6.5 and the proton ejection monitored during one minute. The supplementary release of proton caused by the presence of 8.10^{-5} M and 2.10^{-5} M procaine hydrochloride per mg protein, dependent on ionic strengths, was maximum at 80 mM NaCl.

Since GH_3 appeared to be bound to mitochondria, it was expected that the effects would be more closely related to the ratio of drug: mitochondrial protein, than to the initial molarity of drug in the incubation medium. The results revealed the variation between 1% and 70% of protein concentrations in supernatants with the GH_3 concentration comprehended between $1.5.10^{-5}$ M and 2.10^{-3} M

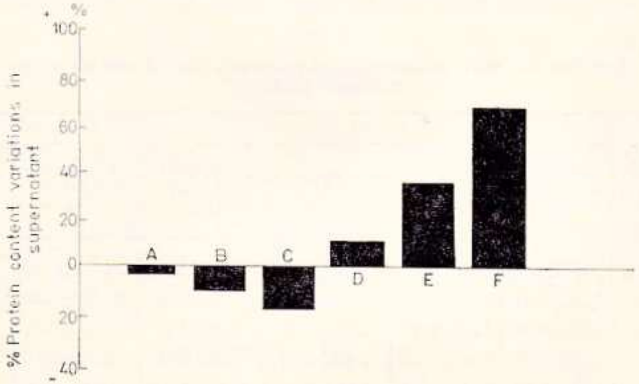

Fig. 1. — The influence of different concentrations of GH_3 on mitochondrial protein concentrations at six levels of GH_3 (M/mg protein) added to 5 ml aliquots of mitochondria: $1.5.10^{-5}$ (A), 3.10^{-5} (B), $1.5.10^{-4}$ (C), 3.10^{-4} (D), 1.10^{-3} (E), 2.10^{-3} (F).

per mg of mitochondrial protein (Fig. 1). Having in view this aspect, the effect was studied of the treatment with GH_3 *in vitro*, at various concentrations, on oxidative phosphorylation. For this purpose the determinations were performed on rat liver and brain mitochondrial preparations.

The effects of GH_3 on oxygen consumption and P/O ratio are shown in Tables 2, 3 and 4. The results demonstrate that the treatment with GH_3 *in vitro* at two levels of concentration ($1.5.10^{-5}$ M and 3.10^{-5} M per mg of mitochondrial

Table 2

Effect of GH_3 *in vitro* on oxidative phosphorylation and respiration in liver and brain mitochondria of young rat (μatoms/mg protein/30')

GH_3 concentration (M/mg protein)	Liver			Brain		
	Oxygen consumption	Phosphorus consumption	P/O	Oxygen consumption	Phosphorus consumption	P/O
—	2.08±0.08	3.84±0.11	1.84±0.08	2.34±0.08	4.40±0.25	1.85±0.04
$1.5.10^{-5}$	6.71±0.16	13.50±0.13	2.00±0.10	7.06±0.10	13.90±0.21	2.03±0.06
3.10^{-5}	3.88±0.08	7.55±0.09	1.94±0.06	4.09±0.08	7.89±0.16	1.91±0.08
$4.5.10^{-5}$	2.50±0.09	4.58±0.10	1.81±0.04	2.80±0.12	6.04±0.14	1.80±0.04
6.10^{-5}	1.88±0.06	3.46±0.18	1.84±0.02	2.04±0.08	3.55±0.10	1.74±0.03

* Each value given is an average by five animals (mean ± S.E.M.)

Table 3

Effect of GH_3 *in vitro* on oxidative phosphorylation and respiration in liver and brain mitochondria of adult rat (μatoms/mg protein/30')

GH_3 concentration (M/mg protein)	Liver			Brain		
	Oxygen consumption	Phosphorus consumption	P/O	Oxygen consumption	Phosphorus consumption	P/O
—	2.05±0.08	3.89±0.25	1.87±0.04	2.40±0.09	4.58±0.18	1.87±0.09
$1.5.10^{-5}$	6.39±0.16	12.64±0.24	1.98±0.07	7.10±0.18	13.63±0.25	1.92±0.06
3.10^{-5}	4.10±0.05	7.96±0.19	1.94±0.10	5.38±0.12	9.68±0.22	1.80±0.04
$4.5.10^{-5}$	2.79±0.08	3.26±0.17	1.80±0.10	3.24±0.06	6.76±0.16	1.78±0.08
6.10^{-5}	1.81±0.10	3.12±0.23	1.74±0.08	2.16±0.04	4.18±0.10	1.83±0.10
1.10^{-4}	1.80±0.09	2.92±0.14	1.64±0.07	1.80±0.11	3.00±0.06	1.60±0.07
1.10^{-3}	0.90±0.08	—	—	0.45±0.03	—	—

* Each value is an average by five animals (mean ± S.E.M.)

Table 4

Effect of GH_3 *in vitro* on oxidative phosphorylation and respiration in liver and brain mitochondria of old rat (μ atoms/mg protein/30')

GH_3 concentration (M/mg protein)	Liver			Brain		
	Oxygen consumption	Phosphorus consumption	P/O	Oxygen consumption	Phosphorus consumption	P/O
–	2.01±0.09	3.78±0.19	1.89±0.08	2.38±0.11	4.42±0.24	1.85±0.05
$1.5.10^{-5}$	6.45±0.12	13.11±0.20	2.03±0.07	6.08±0.18	12.30±0.22	2.04±0.07
3.10^{-5}	4.06±0.13	7.99±0.18	1.97±0.04	5.90±0.10	11.32±0.31	1.92±0.02
$4.5.10^{-5}$	3.10±0.14	5.60±0.22	1.80±0.12	3.10±0.08	5.58±0.20	1.80±0.05
6.10^{-5}	1.80±0.11	3.21±0.16	1.78±0.07	2.08±0.13	3.75±0.18	1.78±0.04

Each value is an average for five animals (mean ± S.E.M.)

protein) produce an increase of oxygen and phosphorus consumption with a slight modification of the P/O ratio. GH_3 concentration at 6.10^{-5} M/mg protein slightly decreases oxygen consumption and ADP phosphorylation to ATP. Phosphorylation activity and mitochondrial respiration in the brain were increased in young, adult and old rats at two levels of GH_3 concentration: $1.5.10^{-5}$ M and 3.10^{-5} M per mg of mitochondrial protein. These effects decrease at the concentration of $4.5.10^{-5}$ M GH_3. 6.10^{-5} M GH_3 per mg of mitochondrial protein slightly inhibits oxygen consumption and decreases the P/O ratio. Studies of oxidative phosphorylation were performed on mitochondrial preparations from the liver and brain of adult rats in the presence of GH_3 at the ratio of 1.10^{-4}M per mg of protein and 1.10^{-3} M per mg of protein. It was observed that 1.10^{-4} GH_3 mg of mitochondrial protein inhibits oxygen and phosphorus consumption with a change of the P/O ratios, and 1.10^{-3} M GH_3 per mg of protein blocking ADP phosphorylation to ATP.

DISCUSSION

The release of H^+ ions in the medium by the mixture of mitochondria with different salts solutions represents a sensitive means of following this effect with procaine hydrochloride added to the medium. The process might be caused by displacement of H^+ from protonated anionic groups of the mitochondria by the binding of Na^+ in good agreement with the data reported by Gear and Lehninger [10]. Procaine hydrochloride in forming the salt, in aqueous solutions, yields the positively charged quaternary amine ion, $R \equiv NH^+$ for short. Dissolved in water, the cation is in dissociation equilibrum with the base according to the following:

$$R \equiv NH^+ \rightleftarrows H^+ + R \equiv N$$

The direction of this dissociation depends on the prevailing concentration of hydrogen ions. At pH 6.5 procaine hydrochloride ($pK_a = 8.9$) is positively charged and releases H^+ ion on binding. The supplementary release of protons caused by the presence of 8.10^{-5} M and 2.10^{-5} M procaine hydrochloride per mg

protein dependent on ionic strengths, being at a maximum at 80 mM NaCl, suggests an analogy between the Na^+ behaviour and that of quaternary amine specific to the procaine molecule at pH 6.5.

In many respects mitochondria suspended in sucrose solutions behave like particles of a cation exchange resin in the protonated form, which can release H^+ to the media in exchange with a variety of different cations, like quaternary amine of the procaine molecule.

Water lysed rat liver mitochondria which have lost about 50% of the total mitochondrial protein in soluble forms, were found to release H^+ when mixed with NaCl medium [12]. In agreement with the data reported by Gear [9], dissociating groups could be contributed by either the protein or phospholipids of the membrane, the latter source appears more likely.

Quaternary amine of the procaine molecule positively charged is able to form ionic bond with oppositely charged (COO^-, PO_4^{3-}). After Feinstein [13], procaine molecules compete with calcium for charged regions on the polar tails of phospholipid molecule. In Fig. 2 is represented a model of bridge complex formation between one procaine molecule and two phospholipid molecules. The polar aromatic amine ($+\delta$) and aliphatic amine groups are shown oriented toward the oppositely charged phosphate groups. Procaine molecule could displace Ca^{2+} bound to a variety of phospholipids, in agreement with the relative nonspecificity of salts in promoting H^+ release from mitochondria.

GH_3 with 2% procaine hydrochloride was tested *in vitro* for its effects on oxidative phosphorylation at various concentrations. The increase of oxygen and phosphorus consumption was produced at the lower GH_3 concentrations of $1.5.10^{-5}$ M per mg protein and 3.10^{-5} M per mg protein. Higher GH_3 concentrations (10^{-3} M per mg protein) produced inhibitory effects and damaged membrane integrity.

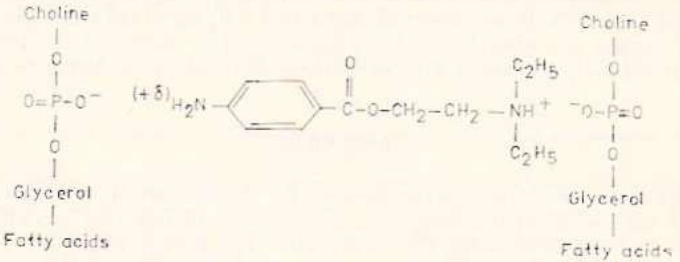

Fig. 2. — Proposed model of bridge complex formation between one procaine molecule and two phospholipid molecules.

These results agree with the studies carried out on beer yeast as well as upon rat liver homogenate by the Warburg method [1]. Research on yeast suspension shows that, depending on the dose, the action of procaine upon respiration was threefold: small doses (0.001%) had a stimulating effect, large doses (1%) had an inhibitory effect and average doses (0.01%) did not affect the oxygen consumption.

Previously, we have reported [14] the effect of GH_3 on succinate dehydrogenase activity in liver and brain mitochondria. An important increase of enzyme

activity at lower GH_3 concentrations was found (1.10^{-5} M per mg protein), especially in the nervous tissue. The brain mitochondria are in this respect much more sensitive than those extracted from the liver. It is possible that GH_3 in lower concentrations stabilises the mitochondrial membrane through fixation on it, preserving its integrity and, at the same time, influencing the membrane transport process and the activity of the respiratory chain enzymes.

Résumé. Les recherches ont été effectuées sur la fraction mitochondriale du foie et du cerveau de rat, en abordant les aspects fonctionnels suivants: l'action du Gérovital H_3 sur la phosphorylation oxydative, le rattachement des ions de sodium et de la procaïne hydrochlorique à la membrane mitochondriale, ainsi que l'action exercée par le Gérovital H_3 sur l'intégrité structurale mitochondriale aux diverses concentrations.

La libération supplémentaire de protons, produite par $2.10^{-5} - 8.10^{-5}$ M procaïne hydrochlorique par mg de protéine, dépend de la teneur ionique, en présentant un maximum à 80 m M NaCl. La molécule de procaïne chargée positivement à pH = 6,5 est en mesure de former des liaisons ioniques avec les protéines ou les phospholipides de la membrane mitochondriale. Gérovital H_3, à de petites concentrations, de l'ordre de $1{,}5.10^{-5} - 3{,}10^{-5}$M par mg. de protéine mitochondriale, détermine une consommation augmentée d'oxygène et de phosphore.

Les concentrations plus grandes de Gérovital H_3, de l'ordre de 10^{-3}M par mg de protéine, ont un effet inhibiteur, en bloquant la phosphorylation de l'ADP à l'ATP.

Il est possible que le Gérovital H_3, en concentration de l'ordre de 10^{-5} M par mg de protéine mitochondriale, produise une activation des enzymes de la chaîne respiratoire.

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