incubated for 30 min at 37°C, with occasional agitation. At the end of the incubation period, the fibrils and mitochondria were precipitated by addition[1] 2 ml 20% trichloroacetic acid. After centrifuging, the inorganic P content of the clear supernatant fluid was determined with a BECKMAN Spectrophotometer by the method of FISKE and SUBBAROW[2], on 1 ml aliquot. To establish the influence upon enzymatic activity of progesterone and desoxycorticosterone, similar systems were prepared in which 0·35 ml borate buffer was replaced by either 0·35 ml of desoxycorticosterone glucoside 1×10^{-3} M solution or 0·35 ml of 1×10^{-3} M suspension of progesterone, which is not soluble in water. Appropriate blank determinations were carried out on mixtures from which either the mitochondria and fibrils or the substrate had been omitted. The P amounts obtained were substracted. Corrected values were expressed as a ration γ P/mgN.

The total nitrogen content was determined by usual micro Kjeldahl method.

The results of experiments are summarized in the Table.

As shown in the Table, desoxycorticosterone, as well as progesterone, produced a strong inhibitory action on ATPase activity of both cardiac and skeletal muscle fibrils and mitochondria. Such an inhibition was about 30% for heart mitochondria and 20% for muscle mitochondria. Similar inhibition values were obtained with both cardiac and skeletal muscle fibrils. Higher activities were shown by fibrils prepared with the first method, including tryptic digestion, than by those isolated without trypsin. This fact agrees with PERRY's observation on fresh fibrils.

On the grounds of the results of the present experiments, the inhibition on ATPase activity by both steroid hormones may be responsible for the inhibition of the contraction of myofibrils which was previously described.

Since no considerable differences between mitochondria and fibrils ATPase activities were observed, the question arises whether muscular granules are derived from the disrupture of myofibrils.

Further researches on this problem are in progress.

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Riassunto

L'autrice ha studiato l'attività ATPasica dei mitocondri e miofibrille isolate da muscolo e cuore di cavia ed ha trovato: 1) Il progesterone e il desossicorticosterone esercitano una azione inibente su tale attività. 2) Le miofibrille preparate con tripsina hanno una attività maggiore di quelle preparate con saccarosio. 3) Non esiste notevole differenza tra attività ATPasica dei mitochondri e quella delle miofibrille.

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Action of Papain and of Trypsin on the Morphology and some Enzymatic Activities of Isolated Mitochondria

It was shown in previous experiments that some enzymatic principles present in liver homogenates exert an inactivating influence on succinoxidase of isolated mitochondria. Although it was reported by MEYER[1], and more recently by ZOLLINGER[2], that mitochondria show a great resistance against proteases (pepsin in acid, trypsin in alcaline medium), the action of some purified proteases, namely papain and trypsin, on the morphology and on the enzymatic activities of isolated mitochondria was studied in this work in order to see whether the inactivating influence of homogenates might be connected with their protease activity.

Papain used in this work was a crude preparation from the latex of *Carica papaya*. Trypsin was a crystalline preparation from pancreas, isolated according to KUNITZ[3]. Protease activity of both enzymes was tested by titrating with 0·01 N NaOH the acidic groups liberated from a denaturated hemoglobin 2% solution after an incubation time of 30 min at 38°C with 1 mg of the enzyme material. Both papain and trypsin were found to be very active, and devoid of desoxyribonuclease, ribonuclease, lecithinase and lipase activities, as tested respectively with the methods of LASKOWSKI and SEIDEL[4], KUNITZ[5], MACFARLANE[6], WILLSTÄTTER[7].

Mitochondria were isolated from 1 g liver with the technique of HOGEBOOM *et al.*[8], by centrifugation of 0·25 *M* sucrose 10% homogenates, albino rats from a selected strain being used as experimental animals. After a previous centrifugation at 1500 g for 15 min, which produced sedimentation of tissue débris, unbroken cells, red cells, nuclei and of some mitochondria, the main portion of mitochondria was collected by a second centrifugation at 12,000 g in the SS-I SERVALL Centrifuge, in the cold room at a maximal temperature of 5°C. Washings were made by resuspension with 0·25 *M* sucrose and sedimentation at 12,000 g for 30 min. Morphological observations were made with the ZEISS-WINKEL phase contrast microscope. Turbidimetric readings were made with a BECKMAN Mod. DU spectrophotometer, by measuring the extinction at 750 mμ.

Enzymatic activities were studied at 38°C in a WARBURG conventional apparatus, the experiments lasting 1 h. After the final sedimentation of mitochondria, the pellet on the bottom of the centrifuge tube was suspended into 6 ml of 0·067 *M* phosphate buffer solution (pH 7·4) and the homogeneous suspension was distributed into 2 WARBURG vessels, 3 ml for each of them. 0·2 ml of sodium succinate, or of 0·2 *M* dl-alanine or 1 *M* sodium lactate were added respectively for the study of succinic acid oxidase, d-aminoacid oxidase and lactic acid oxidase, final concentration of substrates being 0·0125 *M* for succinate and dl-alanine and 0·0625 *M* for lactate. 0·20 ml KOH 30% were placed in the central well. Gaseous environment was air.

Cytochrome oxidase activity was studied by the method of STOTZ *et al.*[9]. 0·2 ml of a mitochondrial suspension prepared by diluting the particles isolated from 1 g of tissue to 5 ml with 0·067 *M* phosphate buffer were used as enzyme source. Concentration of added cytochrome *c* was $2{\cdot}4 \times 10^{-4}$ *M* and that of hydroquinone was 7 mg pro vessel. Controls for autoxidation

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Table I

Effect of proteases on enzymatic activities of isolated mitochondria. (The data represent mm^3 O_2 consumed in 1 h at 38°C with air as gaseous environment).

Enzymes	Experiment No.	Protease added	Amount of protease added mg	Control without protease added	In the presence of protease	Inactivation %
Succino-oxidase	1	Papain	1	311·48	200·40	35·7
	2	Papain	1	322·47	206·56	35·9
	3	Papain	2	311·48	132·72	54·18
	4	Papain	2	322·47	137·26	57·4
	5	Papain	5	322·47	77·63	75·9
	6	Papain	8	311·48	82·30	73·6
	7	Papain	10	315·98	64·80	79·5
	8	Trypsin	0·1	321·03	70·51	78·04
	9	Trypsin	1	321·03	51·97	83·8
	10	Trypsin	1	313·78	51·05	83·7
	11	Trypsin	2	313·78	47·03	85·02
	12	Trypsin	2	321·03	48·09	85·02
	13	Trypsin	5	313·78	34·18	88·9
	14	Trypsin	10	310·40	27·12	91·3
Cytochrome oxidase	15	Papain	0·5	21·07	7·12	66·2
	16	Papain	1	14·57	5·04	65·4
	17	Papain	5	18·33	3·93	78·6
	18	Papain	10	15·30	1·90	87·6
	19	Trypsin	0·5	21·07	1·2	94·4
	20	Trypsin	1	14·57	0·7	95·2
	21	Trypsin	5	18·33	0	100·0
	22	Trypsin	10	15·30	0	100·0
D-aminoacid oxidase	23	Papain	1	24·43	22·69	7·2
	24	Papain	10	26·12	12·40	52·6
	25	Trypsin	1	25·87	10·42	59·8
	26	Trypsin	10	23·6	8·4	64·5
Lactic acid oxidase	27	Papain	1	44·42	29·23	34·2
	28	Papain	10	47·52	20·12	57·7
	29	Trypsin	1	44·42	19·94	55·2
	30	Trypsin	10	49·12	11·72	76·2

of hydroquinone in presence of boiled mitochondria and for endogenous O_2-uptake of mitochondria plus cytochrome *c* were performed. Cytochrome *c* was prepared from horse heart according to KEILIN and HARTREE[1] and was found to contain 0·37% Iron.

As shown in Table I, succinoxidase and cytochrome oxidase activities of isolated mitochondria are practically completely inactivated by both papain and trypsin at a very low concentration. Trypsin seemed to possess a more drastic inactivating influence than papain. Inactivation was not so strong on d-amino acid and on lactic acid oxidases. It was seen also that the turbidity of mitochondrial suspensions is very strongly reduced as a consequence of treatment with papain and with trypsin (Table II). In the attempt to study whether this fact is due to a disruption of mitochondria, mitochondrial counts were made following the general procedure outlined by ALLARD *et al.*[2]. It is difficult to make very exact counts with this method, owing to the small diameter of the particles, to their non-constant size and to their great tendency to conglutinate. Mitochondrial counts thus give only approximate values and only very large differences can be regarded as significant. The counts were repeated 5–6 times for each experiment and the average value was taken. Mitochondria from 1 g of liver were suspended in 10 ml of 0·067 *M* phosphate buffer and the homogeneous suspension was divided into 2 fractions, each of 5 ml. 2 mg of trypsin or of papain were added to the second one and both fractions were incubated at 38°C for 1 h. Counts were then made under the phase contrast microscope, with Ph2 objective, by using a common BÜRKER apparatus for blood elements counts. A very strong decrease of the number of mitochondria as a consequence of the treatment with proteases was found with this procedure. In fact, the control without papain gave the value of $24{\cdot}2 \pm 5{\cdot}1 \times 10^{10}$ mitochondria pro gram of fresh tissue, while the papain-treated sample contained only $6{\cdot}4 \pm 1{\cdot}2 \times 10^{-10}$ mitochondria. The values for trypsin were respectively $25{\cdot}0 \pm 4{\cdot}6 \times 10^{10}$ and $6{\cdot}2 \pm 1{\cdot}6 \times 10^{10}$. Residual mitochondria appeared to be deeply damaged. The action of small amounts of papain or of trypsin on the morphology of mitochondria was then studied and followed at various periods of time from the beginning of the experiment. Very large changes are produced by both enzymes. Normal mitochondria appear as vesicles with a diameter of 1–3 μ and have a more dense shadowy rind resembling a new moon at one side of the sphere. A few minutes after the addition of the protease preparation, many mitochondria begin to appear more rifrangent than the normal ones. The process seems to start in the centre of the particle, which is soon transformed into a rifrangent sphere surrounded by a dense peripheral layer. 20–30 min after the beginning of the experiment some images can be seen which result probably from the coalescence of many damaged elements. These

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images resemble strongly those reported by ZOLLINGER[1] to be produced by treatment with crystalline desoxyribonuclease, but desoxyribonuclease activity was absent in the protease preparations used in this work. The number of mitochondria then began to decrease. After many hours, only a few mitochondria could be seen in the treated preparations.

Table II

Extinction at 750 mμ as a measure of the turbidity of normal and protease treated mitochondrial suspension. (Blank with distilled water; values for extinction of the added amount of enzymes subtracted)

Protease added	Amount of protease added mg	Extinction of the control without protease added	Extinction of the sample in the presence of protease
Papain	1	1·175	0·530
Papain	3	0·960	0·330
Trypsin	1	1·120	0·500
Trypsin	3	1·070	0·430

In other experiments, the action of proteases on the release of N and of nucleic acids P from mitochondria into the suspension fluid was investigated. Mitochondria from 1 g liver were diluted to 5 ml with 0·067 *M* phosphate buffer and the homogeneous suspension was divided into 2 fractions, to the second of which 3 mg of papain or of trypsin were added. After 1 h of incubation at 38°C, mitochondria were centrifuged down from the suspensions at 15,000 g for 30 min and the supernatant fluids were analyzed for N and nucleic acids P content. N was determined by microkjeldahl technique. Nucleic acids P was determined, following the technique of SCHMIDT and THANNHAUSER[2]. Controls for N and nucleic acids P of the enzyme material added were subtracted.

As shown in Table III, large amounts of N are released in the supernate as a consequence of the action of proteases. The amounts of nucleic acids P released in the supernate were also higher for the treated samples than for the untreated ones.

Chromatographic analysis of the amino acids released in the supernatant fluid was made according to the two directions capillary ascension method of WILLIAMS and KIRBY[3], mixtures of (1) 80 ml isobutanol, 20 ml acetic acid, 20 ml distilled water and of (2) 50 ml isobutanol, 50 ml pyridine beeing used as solvents. The spots were developed at 70°C for 30 min with ninhydrin 0·5% in isobutanol. It was found that both trypsin and papain produce release of amino acids from mitochondria into the supernate. Only a very slight amount of amino acids are released from untreated mitochondria, and only very faint spots of glycine and glutamic acid could be observed. The spots were larger after action of the proteases, and were particularly strong after treatment with trypsine. The following amino acids were identified from papain-treated chromatograms: lysine, arginine, hystidine, glycine, glutamic acid, alanine, tryptophan. The following amino acids were identified in the trypsin-treated chromatograms: leucine, phenylalanine, tyrosine, tryptophane, valine, methionine, proline, alanine, threonine, glutamic acid, aspartic acid, glycine, serine, hydroxyproline, arginine, lysine, histidine, namely all amino acids which were found to be present in mitochondria hydrolysates (DIANZANI[1]).

From the experiments described in this paper, evidence is given that mitochondria are digested by proteases. The presence in mitochondria of a semipermeable membrane, probably phospholipin in nature, has been suggested by many authors (CLAUDE and FULLAM[2], DALTON *et al.*[3], de DUVE *et al.*[4], ZOLLINGER[5]). Since neither lecithinase nor lipase activities were found to be present in the protease preparations used in this work, it suggests that a protein also may be contained in the mitochondrial membrane.

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Résumé

L'auteur a étudié l'action de la papaïne et de la trypsine sur la morphologie et sur quelques activités enzymatiques des mitochondries isolées du foie de rat. La succinoxidase et la cytochrome-oxidase sont fortement inhibées par les deux protéases, tandis que l'inhibition de la d-aminoacidoxidase et de la lacticoxidase est plus faible. Le nombre des mitochondries est notablement réduit à la suite du traitement avec les deux protéases et une forte quantité de N et d'acides aminés sont aussi libérés dans la liqueur surnageante. L'auteur montre aussi les modifications de l'image en contraste de phase des mitochondries après traitement avec la papaïne et la trypsine.

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Table III

Release of Nitrogen (N) and of nucleic acids Phosphorus (P) from mitochondria as a consequence of treatment with proteases (N and P contents of proteases added were substracted)

Protease added	Amount of protease added mg	mg N of Control without protease	mg N In the presence of protease	γ of nucleic acids P	
				Control without protease	In the presence of protease
Papain	1	11·7	21·5	2·6	5·76
Papain	2	11·7	21·7	2·6	6·34
Papain	3	11·6	22·5	2·3	6·62
Trypsin	1	11·7	27·7	2·3	6·34
Trypsin	2	12·0	28·5	2·3	6·62
Trypsin	3	11·8	29·2	2·01	7·2