Table I

No. of animals	Days after operation	Weight of muscles (g)		Lipid content (mg/g of wet wt.)		Iodine number (mg/100 g of lipid)	
		Control	Denervated	Control	Denervated	Control	Denervated
5	8	6.722	4.444	14.3	16.2	180	131
4	18	5.335	3.025	17.2	20.0	202	143
4	10	4.430	2.915	14.7	17.5	244	186
4	13	4.917	3.160	13.2	16.7	195	167
		Control	Tenotomized	Control	Tenotomized	Control	Tenotomized
3	8	3.625	2.990	17.1	15.3	216	210

The total cholesterol is significantly increased after denervation but not after tenotomy. The mean value for normal muscle (expressed as μg/g of wet wt.) was 1.119 ± 0.095 S.E.M. and for denervated muscle 1.420 ± 0.132 from 6 experiments (18 animals). A strong increase in cholesterol content was found when the microsomal fractions instead of whole denervated muscles were used (Table II). No significant change in phospholipid content per mg of microsomal protein has been found in denervated muscle.

The results point to the existence of differences in the lipid constituents of membranes of normal and denervated muscles. Preliminary observations by means of thin-layer chromatography reveal[10] that the difference in fatty acid saturation involves the phospholipids of whole muscles and of their subcellular fractions. Variations in the chemical structure of the paraffinic chains of the phospholipids have significant consequences in the physicochemical state of the lipids in membranes. The unsaturated bonds appear to prevent a highly condensed state of lipid molecules[4,5].

It is noteworthy that the so-termed condensing effect of cholesterol in mixed-films depends on the nature of hydrocarbon tails of the phospholipid in the system. For instance, cholesterol present in a monolayer with a phospholipid containing *cis*-unsaturated chains produces a condensing effect on the monolayer[11].

Research is now in progress to elucidate some qualitative change in fatty acid composition of normal and denervated muscles and in the dynamic processes of phospholipids[12].

Table II

No. of animals	Weight of muscles (g)		Cholesterol μg/mg of microsomal protein[a]	
	Control	Denervated	Control	Denervated
3	3.290	2.320	14.67	35.70
3	3.700	2.460	14.41	42.00
3	3.824	2.668	15.45	37.90
3	3.403	2.181	16.00	40.50
3	4.010	2.080	11.80	19.55
	Control	Tenotomized	Control	Tenotomized
3	3.431	2.990	15.40	17.00

[a] The microsomal fractions were obtained in a Beckman Spinco L-2 centrifuge at 150,000 *g* in 0.3 *M* sucrose with 10 μ*M* histidine, pH 7.45, after separation of residues and mitochondria (20 min 800 *g*; 10 min 6000 *g*; 20 min 20,000 *g*).

Riassunto. In numero di iodio dei grassi estratti da muscoli denervati è diminuito e il contenuto di colesterolo risulta aumentato. L'aumento è assai spiccato se si confrontano gli estratti lipidici delle frazioni microsomali.

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Effect of 2,4-Dinitrophenol on the Phosphorylases System of the Skeletal Muscle in vivo

The system phosphorylase A/phosphorylase B has an important role in the regulation of glycogenolysis in muscle. Evidence has been accumulating that phosphorylase A is a rate-limiting enzymic step in that metabolic pathway. Thus glycogenolytic agents as adrenalin, glucagon[1] and electrical stimulus[2], for instance, increase the breakdown of glycogen in muscle and accumulate hexosemonophosphates and lactic acid, with concomitant increasing of phosphorylase A. Among the glycogenolytic agents, 2,4-dinitrophenol (DNP) has been considered an exception[3,4].

The phosphorylases are inhibited by the drug in experiments either of glycogen phosphorolysis or of glycogen synthesis from glucose-1-phosphate, performed

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with normal extracts and isolated muscle. A recent paper[5] also shows that the glycogen phosphorolysis is inhibited in poisoned animals. Studies of the inhibition of the phosphorylases in crude extracts recently prepared and in the crystalline enzymes showed that only phosphorylase B was inhibited by DNP, by competition with its co-enzyme, adenylic acid (AMP)[6]. Despite the inhibition of phosphorylase B, the muscles are still able to carry out activities even at a higher level. These studies, however, could not explain the glycogenolytic effect of DNP.

It is well known that DNP has other effects, such as the activation of ATPase and the inhibition of aged phosphorylase A; and we believe that if these collateral effects could be avoided, at least during the enzymatic determinations, by using fluoride and by performing the assays immediately after the sacrifice of the animals, the actual content of phosphorylase A and total (A plus B), as well as of phosphorylase B kinase in muscle could be detected. In the present communication is shown the content of these enzymes in skeletal muscle of rats injected with the drug.

For the determination of the content of phosphorylase A and total[7], adult rats were either injected or not i.p. with DNP 2.5 mg/100 g. One hour after injection they were anaesthetized with ether and a sample of skeletal muscle was rapidly removed, weighed and ground in ice-cold solution containing $0.001M$ EDTA and $0.02M$ sodium fluoride, pH 6.8, in the proportion of 50 ml/g of muscle. 1 ml of the centrifuged homogenate was incubated with glycogen during 20 min and an aliquot of 0.4 ml was then treated with glucose-1-phosphate and with either water or AMP. The final concentrations were: 1% glycogen, $0.016M$ glucose-1-phosphate, $0.001M$ AMP in a total volume of 0.8 ml. Following a reaction period of 7 and 15 min, aliquots of 0.2 ml were taken and added to 7 ml of $5N/70$ sulphuric acid. Inorganic phosphate liberated from glucose-1-phosphate was determined by the LOHMANN and JENDRASSIK method[8].

The same animals used to determine the activities of phosphorylase A and total, were also used for lactic acid determination[9]. Thus samples of blood were taken from normals and DNP-poisoned rats and rapidly withdrawn into 3% perchloric acid in the proportion of 3 ml/1 ml of blood. An aliquot of 0.2 ml of each acid supernatant was diluted to 3 ml with solution containing (final concentration): $0.2M$ glycine buffer, pH 10.0, $0.2M$ semi-cabazide, $0.0025M$ NAD and 50 µg of lactic dehydrogenase crystallized from beef heart[10]. After $^1/_2$ h incubation at 30 °C the optical density was read at 340 nm in Beckman spectrophotometer model DB.

Table I shows the content of phosphorylase A and total in muscle and of the lactic acid in blood of control and poisoned rats with DNP. The solution of the extract has the advantage that the AMP is not found in concentration high enough to activate phosphorylase B, thus it is possible to admit that the phosphorylase activity found without adding AMP is due to phosphorylase A. On the other hand, the dilution of the extract also prevents the inhibition of phosphorylase B by DNP, once is necessary, in crude extract concentration of about $10^{-3}M$, to obtain 50% inhibition of the enzyme[6]. The presence of EDTA and fluoride show the actual content of phosphorylase A and total, once they inhibit the interconversion between the 2 enzymes. The Table shows that the content of phosphorylase A and of total phosphorylases is higher in poisoned rats. It can be seen, however, that the activity of phosphorylase A presents an increase of about 3 times while the total phosphorylases activity is only 1.3 times. This could suggest that in poisoned rats there is an activation of the phosphorylase B kinase, the enzyme that catalyzes the reaction phosphorylase B → phosphorylase A, could also be an activation of the phosphorylase A only. In order to verify this hypothesis, experiments were done to detect the activity of the phosphorylase B kinase[11]. For which, male rats were either injected or not with DNP 2.5 mg/100 g. After 1 h they were anaesthetized with ether and the blood was drained from the jugular vein. A sample of muscle of each rat, from the lower extremities, was cooled for 30 min in crushed ice, then weighed and ground in sand with ice-cold solution of $2 \times 10^{-3}M$ EDTA, pH 7.0, in proportion of 2 ml/g of muscle. The homogenate was then centrifuged at $4000 \times g$ for 30 min and the supernatant was filtered through glass wool and used

Table I. Content of phosphorylases and lactic acid in normal and poisoned rats by DNP

Determinations	Normal	DNP poisoned
Phosphorylase A	84 ± 17[a]	240 ± 42
Total phosphorylase (+AMP)	284 ± 90	528 ± 110
Ratio $\frac{\text{Phosporylase A}}{\text{Total phosporylase}} \times 100$	21.9 ± 6.0	45.5 ± 12.0
Lactic acid	1.7 ± 0.9	7.0 ± 2.0

[a] S.E.M. The content of phosphorylases is expressed in units according to CORI et al.[7] in 15 min of reaction and per g of muscle. The lactic acid is expressed in μM/ml of blood. 2 groups of 20 rats were used.

Table II. Effect of DNP on phosphorylase B kinase in vivo and in vitro

Muscle extracts and addictions	Kinase activity
Control (16 rats)	1440 ± 300[a]
DNP poisoned (16 rats)	3840 ± 850
Pooled extract of normal rats	1460
The same + DNP $10^{-4}M$	1920

[a] S.E.M. The activity of phosphorylase B kinase in muscle extracts is expressed in units of phosphorylase A formed in 5 min/g of muscle, from phosphorylase B according to the FISCHER and KREBS[11] method.

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immediately. The reaction mixture contained: 0.1 ml of 0.21 *M* *tris*-0.21 *M* glycerolphosphate buffer, pH 8.6; 0.1 ml of solution of phosphorylase B (crystallized with 10^{-4} *M* AMP, centrifuged and dissolved in 0.015 *M* cystein, pH 7.0, to 25 mg/ml concentration); 0.05 ml of phosphorylase B kinase (muscle extract); 0.05 ml of 0.1 *M*/mg acetate-0.03 *M* ATP, pH 7.0, and 0.02 ml of 1 *M* NaF. After 5 min of reaction at 30 °C an aliquot of 0.1 ml was removed and diluted 1:20 in 0.02 *M* glycerolphosphate-0.03 *M* cystein buffer, pH 6.8, and the phosphorylase activity was assayed in the absence of AMP, according to CORI et al.[7].

The use of fluoride during the procedure was intended to prevent the activation of ATPase[12] caused by DNP and also to prevent the reaction phosphorylase A to B by inhibition of the PR enzyme.

Table II shows that, actually, the drug activates the in vivo phosphorylase B kinase. Experiments to verify the in vitro effect were, up to date, unsuccessful, the little activation observed in the kinase could not explain the results in vivo. Experiments performed in vitro by several authors in order to verify the activation of the phosphorylases, were also unsuccessful, so we suppose that DNP has, in fact, an indirect effect on the glycogenolysis, only observed when it is injected into the animal.

Our results show that DNP in vivo increases the content of phosphorylase A from phosphorylase B by activation of the phosphorylase B kinase and, at the same time, also increases the content of lactic acid. This strongly suggests that DNP actuates as do other glycogenolytic agents, and corroborates the results of other authors who consider phosphorylase B kinase as one of the most important enzymes in the control of the glycogenolysis in skeletal muscle.

Résumé. Des rats empoisonnés par le DNF ont une teneur de phosphorilase A trois fois plus grande que les animaux normaux. Cette augmentation est due à l'augmentation de la quinase de la phosphorilase B. Nous n'avons pas décelé d'action directe in vitro du DNF sur cette quinase, de sorte que l'action in vivo paraît plutôt indirecte. Les résultats suggèrent que le DNF a une action semblable à celles des autres agents glycogènolytiques.

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Intracellular pH of the Peritoneal Macrophage Effects by Silica and by Drugs Affecting Cell Metabolism

Silica is thought to damage the phagocytes by rupture or permeation of the phagolysosomal membranes due to the powerful hydrogen-bonding activity of surface SiOH groups[1]. Silica has been shown to cause the extrusion of lysosomal content into the extracellular space[2], and this might follow a previous discharge into the cytoplasm. In this case cell acidification[3,4] and inhibition of cell metabolism might occur with initiation of autolytic processes.

In an attempt to verify this possibility, the changes intervening in the pH of phagocytes under such conditions were investigated. Cell pH was calculated by the distribution between extra- and intra-cellular water of the weak acid 5,5-dimethyl-2,4-oxazolidinedione-2-^{14}C (DMO)[5,6]. The effects of metabolic poisons and uncouplers were also analyzed.

Peritoneal macrophages were obtained and handled as previously indicated[2] and the experiments carried out at 37 °C in a Warburg apparatus as indicated in the Tables. DMO-2-^{14}C (New England Nuclear, Boston, Mass.) specific activity 7.76 mc/m*M* was used in amount of

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Table I. Intracellular pH of peritoneal macrophages incubated in Ringer *tris*-HCl buffer initial pH 7.23 ± 0.017

	Incubation (min)	O_2 uptake (μl/mg dry wt.)	Inhibition (%)	Extracellular pH	Intracellular pH
Control (5)	0–30	9.7 ± 1.23	–	7.16 ± 0.045	6.85 ± 0.031
	90–120	6.4 ± 0.66	32.5 ± 4.99	7.09 ± 0.035	6.79 ± 0.068
Silica (6)	0–30	9.0 ± 0.97	–	7.17 ± 0.037	6.98 ± 0.067
	90–120	5.1 ± 0.53	41.8 ± 5.98	7.13 ± 0.033	6.55 ± 0.083

Figures represent means and the standard errors. O_2 uptake measured by the WARBURG direct method in air as gas phase and CO_2 trapped by KOH. Silica (tridymite batch 67-70-72M[2]) used in amount of 2.0 mg/10^7 cells. The figures in parentheses indicate the number of observations.