Glycogenolytic Effect and Inhibition of Glycogen Phosphorolysis by 1,2,4-Dinitrophenol in Skeletal Muscle

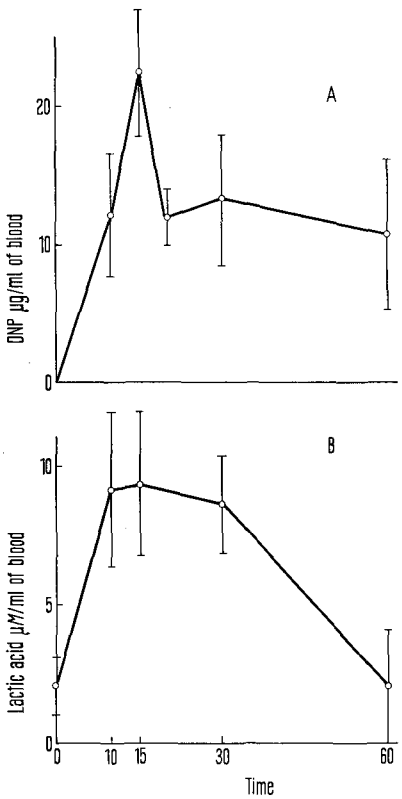
In previous publications[1,2] we have presented results concerning the anomalous effect of 2,4-dinitrophenol (DNP), on glycogenolysis, i.e. a strong inhibitor of glycogen phosphorolysis and at the same time a glycogenolytic agent. Working with muscle extracts and crystalline phosphorylases *a* and *b*, we observed that only phosphorylase *b* was inhibited by the drug, by competition with its coenzyme 5'-adenylic acid (AMP), and this observation brought some light to the phenomenon. Thus, if only phosphorylase *b* is inhibited by DNP, we have to consider the possibility that phosphorylase *a* is the active enzyme for the glycogenolysis in muscle, acting in tiny amounts[3], since we could not observe any inhibition of glycogenolysis in animals poisoned with DNP; on the contrary, glycogenolysis is even accelerated by the drug. However, it was important to know if the glycogenolytic effect as well as the inhibition of glycogen phosphorolysis caused by DNP occurred simultaneously in vivo.

For this purpose, samples of blood and muscle were taken simultaneously from the animals poisoned with DNP to perform the lactic acid determination, as well as the glycogen phosphorolysis.

In initial experiments a group of adult rabbits, weighing 2000–3000 g, was injected i.p. with DNP, 2.5 mg/100 g and samples of blood were taken at different times by cardiac punction. Aliquots of 1 ml were rapidly withdrawn into 1 ml of 6% perchloric acid. The acid supernatant (0.1 ml) was diluted to 3 ml with a solution containing 0.2 *M* glycine, 0.2 *M* semi-carbazide, 0.0025 *M* NAD, and 100 µg of lactic dehydrogenase crystallized from beef heart[4] pH 10.0. After 1 h of incubation at 37 °C, the lactic acid was determined by reading the optical density of the solution at 340 nm in a Beckman spectrophotometer. DNP was determined by diluting the blood acid supernatant conveniently, extracted with 2 volumes of ether, dried, dissolved in 0.75 *N* NH_4OH in ethanol and read at 360 nm, using 17,700 as molecular absorption coefficient. The Figure shows that in 10 min when the DNP concentration is high in blood, the lactic acid increases as a parallel event.

Experiments were made to verify whether the hematia metabolism could be responsible for the lactic acid increase in blood (Table I). As can be seen, we could not detect any significant increase of the lactic acid with prolonged incubation of the blood with DNP.

Once the time necessary to detect the DNP effect was established, 1 group of 5 rabbits was used to study the



DNP and lactic acid concentration in blood after injection of DNP. (A) Curve of DNP concentration in blood with the time. (B) Curve of lactic acid concentration in blood with the time. Each point represents the mean ± S.E.M. of the value of 10 adult rabbits.

Table I. Lactic acid determination in rabbit blood cell

Time min	Lactic acid µmoles/ml blood	
	control	DNP 10^{-4} *M*
0	5.8	6.0
20	6.5	6.7
40	7.2	7.5
160	10.4	11.0

In 2 ml solution contained 1.95 ml of total blood, 2 mg of glucose (pH 7.4), at 37 °C. Aliquots of 0.3 ml were taken at different times and withdrawn in 0.60 ml of $HClO_4$ 6% for lactic acid determination as described.

Table II. Correspondence between the lactic acid increase and the inhibition of glycogen phosphorolysis

Treatment with DNP	Phosphorolysis mg Pi consumed in 10 min/100 g of muscle	Lactic acid mmoles/100 g of muscle	Lactic acid mmoles/100 g of blood
before	42.0 ± 5.5	1.79 ± 0.04	2.29 ± 1.3
after	11.4 ± 2.15	3.20 ± 0.16	9.14 ± 2.8

The determination indicated in the Table was made with anaesthetized rabbits before and after 10 min of the injection of 2.5 mg/100 g of DNP. One group of 5 animals was used.

[1] A. FOCESI JR. and J. MOURA GONÇALVES, Abstr. 6th Int. Congr. Biochem., New York (1964).
[2] J. MOURA GONÇALVES and A. FOCESI JR., Natn. Cancer Inst. Monogr. *27*, 71 (1967).
[3] E. HELMREICH and C. F. CORI, in *Advances in Enzyme Regulation* (Ed. G. WEBER; Pergamon Press, Oxford 1965), vol. 3, p. 91.
[4] G. W. SCHWERT, D. B. S. MILLAR and Y. TAKENAKA, J. biol. Chem. *237*, 2131 (1962).

glycogen phosphorylation in muscle and the production of lactic acid both in muscle and blood. Thus, the animals were anaesthetized with Nembutal 50 mg/kg and a sample was either homogenized with 6% perchloric acid for lactic acid determination or ground with sand in an ice-cold solution containing 3.8% sodium fluoride, 0.08*M* EDTA pH 7.0 (2 vol. of solution/g of muscle) for phosphorolysis assay. The pH 7.0 extract was centrifuged and 0.5 ml of the supernatant were incubated for 30 min at 37 °C with 0.5 ml of 1% glycogen and aliquots of 0.25 ml of the mixture were removed at different times and added to 4 ml of 10% trichloracetic acid. After centrifugation, glycogen phosphorolysis was determined assaying inorganic phosphorus in an aliquot of the supernatant, according to LOHMAN's and JENDRASSIK's method[5]. Aliquots of blood were also taken for lactic acid determination.

After removal of muscle sample and blood, the anaesthetized animals were injected with DNP 2.5 mg/100 g and were kept for 10 min to permit the drug action. New samples of muscle and blood were taken and treated as above.

Table II shows that when the amount of lactic acid was high in blood and muscle, the glycogen phosphorolysis was inhibited in muscle. These parallel events strongly support our previous observations[1,2].

Resumen. La fosforilase *b* del músculo esquelético es inhibida por el 2,4-dinitrofenol, de acuerdo con nuestras observaciones anteriores. La fosforilase *a* no es inhibida por la droga.

A. A. SIMÕES, A. FOCESI JR. and J. M. GONÇALVES

Departamento de Bioquímica, Faculdade de Medicina de Ribeirão Prêto (São Paulo, Brazil), 19 August 1968.

[5] K. LOHMAN and L. JENDRASSIK, Biochem. Z. *178*, 419 (1926).

Stimulation of Lymphocyte Transformation by 1-Fluoro-2,4-Dinitrobenzene

The addition of specific antigens to lymphocytes in vitro stimulates the appearance of transformed cells[1,2]. However, it is uncertain whether the transformation follows direct contact between the antigen and the lymphocyte or is the outcome of an intermediate step involving either antigen and some other cell such as the macrophage, or an antigen-antibody reaction.

We are investigating the action of antigen in hypersensitivity using 1-fluoro-2,4-dinitrobenzene (FDNB) because, in addition to being a well-established inducer of contact hypersensitivity, this substance reacts rapidly with proteins to form stable compounds[3]. The identification of the cellular localization of these compounds and their chemical composition may uncover the steps in the development of hypersensitivity, and in particular of delayed hypersensitivity.

We have found that the addition of FDNB to cultures of lymphocytes, obtained from guinea-pigs sensitized to the dinitrophenyl moiety, is followed by the appearance of transformed cells.

The test animals were outbred female, albino guinea-pigs weighing 600–800 g. Sensitization was induced by injection into the nuchal skin of 40 µg 1-chloro-2,4-dinitrobenzene dissolved in distilled water, i.e. a modification of the method of CHASE[4]. 11 days later, the animals were bled under aseptic conditions by severing the jugular vein and collecting blood from each animal into separate, sterile bottles containing glass beads, 3–5 mm in diameter. After gentle shaking for 5–10 min, the defibrinated blood was transferred to fresh bottles and to it was added 1% Methocel (USP Grade, Dow Chemicals Co.) in 0.2*M* phosphate buffer, pH 7.2 (0.3 ml/ml blood). After mixing, the blood was allowed to settle for 45 min at 37 °C. Sedimentation resulted in the separation of a layer of leucocyte-enriched serum. The upper $^2/_3$ of this leucocyte layer was centrifuged for 5 min at 200 *g*, the supernatant removed and the cells washed twice with Hanks solution, with centrifugation between washings. The resultant preparation of leucocytes consisted of 97–100% lymphocytes. The proportion of erythrocytes to leucocytes in the preparation was about 1:1.

Erythrocytes and serum were obtained from the residual defibrinated and sedimented blood by centrifugation for 10 min at 200 *g*. The serum was harvested, the remaining leucocytes discarded and the red cells washed twice with Hanks solution.

From the blood of each of 11 sensitized animals, 3 sets of duplicate leucocyte cultures were then prepared. The first set contained leucocytes conjugated with FDNB; the second, conjugated erythrocytes and unconjugated leucocytes; the third, unconjugated leucocytes and serum to which FDNB had been added.

The white and red cells were separately conjugated with FDNB by suspension for 30 min in sodium phosphate buffer 0.2*M* pH 8.5, adjusted to contain 10 µg FDNB per $1.0–1.3 \times 10^6$ cells/ml. After incubation, the cells were centrifuged for 5 min at 200 *g*, the supernatant discarded and the cells washed thrice with Hanks solution. The leucocytes were then suspended (approximately 10^6 cells) in a culture medium consisting of 2 ml Medium 199 (Commonwealth Serum Laboratories, Melbourne) and 1 ml autologous serum. Cultures of leucocytes from unsensitized guinea-pigs were similarly prepared. The conjugated erythrocytes were mixed with an approximately equal number of unconjugated, autologous leucocytes before being suspended in culture medium.

Conjugated serum proteins were prepared by adding sodium phosphate buffer, 0.2*M*, pH 8.5, containing 10 µg/ml FDNB to each ml of serum and incubating the mixture for 30 min at 37 °C. This mixture was then added to Medium 199 in a proportion of 1:2 and used to suspend unconjugated, autologous leucocytes.

The Table summarizes the percentage of transformed cells appearing in the various cultures. Small numbers

[1] G. PERMAIN, R. R. LYCETTE and P. H. FITZGERALD, Lancet *1*, 637 (1963).
[2] M. W. ELVES, S. ROATH and M. C. G. ISRAËLS, Lancet *1*, 806 (1963).
[3] H. N. EISEN, L. ORRIS and S. BELMAN, J. exp. Med. *95*, 473 (1952).
[4] M. W. CHASE, Int. Archs Allergy appl. Immun. *5*, 163 (1954).