Riassunto

È stato studiato il contenuto di ATP nella cartilagine epifisaria di giovani ratti diabetici per allossana. Il deficit insulinico da diabete allossanico provoca, oltre ad arresto dell'accrescimento scheletrico, una diminuzione della concentrazione di ATP nella cartilagine di coniugazione. Il significato biologico di questo risultato è messo in relazione col meccanismo d'azione dell'insulina e con l'importanza dell'ATP nel processo di ossificazione encondrale.

Some Relationships Between the Pancreatic β-Cells and the Metabolism of the Epiphyseal Cartilage

II. *Cartilage Cocarboxylase Activity of Young Alloxan Diabetic Rats*

ZAMBOTTI[1] first discovered the presence of cocarboxylase in the epiphyseal cartilage. It was also established by ZAMBOTTI that this enzyme is strictly related to the intensity of the osteogenic process: in fact he showed that the cocarboxylase activity in the epiphyseal cartilage of young rabbits is inversely proportional to their age. I have reached identical conclusions in my researches in which the participation of cocarboxylase in various phases of evolution of the normal fracture callus[2] and also the behaviour of the cartilage cocarboxylase during growth in conditions (avitaminosis C) of reduced osteogenic power were studied[2]. These relationships, shown first by ZAMBOTTI, explain the histological and histochemical alterations of the epiphyseal cartilage demonstrated by ROASENDA and CAMURATTI[3] in deficiency of a particular vitamin which has the cocarboxylase as its active form, i.e. vitamin B_1.

I have studied the behaviour of the cocarboxylase in the epiphyseal cartilage of alloxan diabetic rats for the following reasons:

(1) Insulin deficiency is accompanied by an arrest of growth and alterations of osteogenic power of ossifiable cartilage[4].

(2) There is a strict relationship between the intensity of the osteogenic process and the amount of cocarboxylase activity[5].

(3) The diminution of the osteogenic power provoked by insulin deficiency is accompanied by a reduced concentration of ATP in the epiphyseal cartilage[6] in the alloxan diabetic rats. It is known that ATP is in large measure synthesized by means of the oxidation of two α-ketoacids (pyruvic and α-ketoglutaric)[7] for the oxidative utilization of which the cocarboxylase is essential.

(4) There is also a strict relationship between ATP and cocarboxylase regarding their metabolism. In fact, the biosynthesis of cocarboxylase from vitamin B_1 comes about by means of the reaction (B_1 + ATP→cocarboxylase + AMP)[8] catalyzed by the thiaminkinase, an endoergonic reaction which, since it receives the energy liberated by the demolition of ATP, should occur with minor intensity when the concentration of ATP is diminished.

Albino male rats, of Italicus strain, 50 days old, were divided into two groups. In one of these groups alloxan diabetes was provoked (200 mg of Merck alloxan, intraperitoneally). The control group was not treated. Ten days after the alloxan treatment, the body weight and blood sugar were controlled and then the diabetic rats (blood glucose after 12 h of fasting = 2·8–4·9 g/1000 cm^3) were controlled for another 20 days, with periodical examinations (control of body weight and of blood sugar every 5th day). 30 days after the alloxan treatment all the rats, diabetic and normal were killed, their body weight and blood sugar being controlled. The limbs were removed and placed in ice water. The cocarboxylase of the epiphyseal cartilages was extracted and measured according to the OCHOA and PETER's method[9], as modified by SILIPRANDI[10].

In the epiphyseal cartilages of the diabetic rats (which presented an arrest of skeletal growth identical to that previously described[6]) the cocarboxylase activity was reduced in respect to that in the control group (normal rats = γ 6·2/g wet weight; alloxan diabetic rats = γ 3·45/g wet weight). Results identical to those obtained in the normal rats were also demonstrated in diabetic rats treated with insulin (daily subcutaneous injection of Zn-insulin protaminate, 4 U.I./kg), from the 10th to the 13th day after the alloxan injection. It is therefore concluded that the cocarboxylase content decrease in the epiphyseal cartilage accompanying the arrest of the skeletal growth can be attributed in the alloxan diabetic rats to the insulin deficiency.

Coordinating my results on ATP and cocarboxylase with those obtained by other authors[11], it seems logical to deduce that a deficient production of insulin induces an arrest of osteogenesis provoking a reduced synthesis of ATP by damaging those reactions in which cocarboxylase is interested. On the other hand, the analogous behaviour of ATP and cocarboxylase in insulin deficiency leads us to suppose that in epiphyseal cartilage the metabolism of these two substance is interdependent: the insulin by means of the energy furnished by the ATP supports the transformation of thiamin into diphosphothiamin; the latter, in its turn, participates in the formation of ATP by means of the pyruvate and α-ketoglutarate oxidative decarboxylation. This hypothesis on the mechanism of the participation of the insulin in the osteogenic process by means of ATP and cocarboxylase is very probable, other than for my present results (osteogenesis normalization in the diabetic rats simultaneously with normalization of the amount of ATP and of cocarboxylase in the epiphyseal cartilage, after insulin treatment) also for my other experimental data: alloxan diabetes induces in the epiphyseal cartilage a diminished pyruvate and α-ketoglutarate oxidation; this process which is in strict relationship with the ATP synthesis[7], is also normalized by insulin treatment.

[1] V. ZAMBOTTI, Il Farmaco, ed. scient. *10*, 1017 (1955).

[2] E. BARBIERI, unpublished data.

[3] F. ROASENDA and C. CAMURATTI, Minerva Ortopedica *3*, 170 (1952).

[4] P. DE MOOR, Exper. Diab. (Ed. Masson, Paris 1953). – R. MARTINETTI and G. ANDREANI, Boll. Soc. ital. Biol. sper. *23*, 582 (1947).

[5] V. ZAMBOTTI, Il Farmaco, ed. scient. *10*, 1017 (1955). – E. BARBIERI, unpublished data.

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[10] D. SILIPRANDI and N. SILIPRANDI, Acta Vitaminol. *5*, 3 (1951).

[11] R. MARTINETTI and G. ANDREANI, Boll. Soc. ital. Biol. sper. *23*, 582 (1947). – E. BARBIERI, Exper., previous communication.

These results indirectly confirm the significance of the ATP, demonstrated by CARTIER[12], and of the cocarboxylase, demonstrated by ZAMBOTTI[1], for a normal behaviour in the osteogenic process.

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Riassunto

Studiando il meccanismo biochimico con cui la insulina controlla il processo di osteogenesi, si è stabilito che in condizioni di deficit insulinico (diabete allossanico) esiste nel ratto accanto ad un arresto dell'accrescimento scheletrico, una notevole riduzione dell'attività cocarbossilasica nella cartilagine epifisaria. Si conclude che nella cartilagine di coniugazione ATP e cocarbossilasi sono strettamente interdipendenti per quanto riguarda la loro biosintesi; inoltre che il loro normale metabolismo è indispensabile al normale svolgersi del processo di osteogenesi.

[12] P. CARTIER, Exposés Ann. Biochim. Méd. *14*, 73 (1952).

Influence of Post Irradiation Administration of Methionine on the Excretion of Creatine, Creatinine and N-Methyl Nicotinamide in Urine of Rats

Earlier observations from this laboratory have demonstrated a precipitous fall in the levels of free-methionine and choline in rat livers[1] and an enhanced excretion of methylated end-products such as creatine, creatinine and N'-methyl-nicotinamide in urine of rats given 600 *r* total body X-ray irradiation[2]. This implied that radiation exposure probably disrupted the methyl economy and suggested that there may be a cause-and-effect relationship between radiolability of methionine[3] and the levels of the metabolites studied. These considerations prompted us to study how far replacement therapy of methionine would have beneficial effects in modifying the reported biochemical lesions of radiation exposures. In this communication, the influence of post-irradiation administration of methionine on the excretion of creatine, creatinine, N'-methyl-nicotinamide and nitrogen in urine is reported. The influence of giving methionine half an hour before irradiation has also been investigated.

Experimental.—1½ to 2 month old wister rats, inbred in our colony, were used in this investigation. The animals were divided into 5 groups of 6 rats each, as follows. The first group served as controls and the animals did not receive any treatment at all. The second group of animals were not irradiated but received injection of methionine at a dose rate of 5 mg/kg of body weight. The animals in the 3rd group were irradiated with 600 *r* X-rays. In the 4th group, animals were irradiated and received methionine ½ h after irradiation. The last group was also irradiated but received methionine before irradiation.

All animals, control as well as irradiated, were fasted for 24 h during the collection of urine. Drinking water was, however, made available to them *ad libitum*. Animals were housed in metabolic cages in pairs to facilitate the collection of urine.

For the estimation of creatine and creatinine, the urine samples were first treated with iodine-iodide reagent as described by TAUSSKY[4] to remove the interfering substances, followed by extraction of the excess of iodine with chloroform. Urine samples were then adjusted to pH 2·5 and taken for the estimation of creatine and creatinine. The conversion of creatine to creatinine was carried out by autoclaving the urine samples (pH 2·5) as recommended by CLARK *et al.*[5]. Preformed creatine and total creatinine was then estimated colorimetrically[4] by adding 1 ml of 0·04 *N* picric acid and 1 ml of 0·75 *N* NaOH. After 20 min of stabilization, the colour intensity was measured in Klett Summerson colorimeter with green filter.

For the estimation of N'-methyl-nicotinamide, the urine samples were treated with acetone and 6 *N* NaOH, followed by addition of 6 *N* HCl to produce stable fluorescent condensation product as described in detail by HUFF and PERLZWEIG[6]. The fluorescence was measured in Pfattz and Baur instrument with the combination of green and yellow filters.

Nitrogen in urine samples was estimated colorimetrically by nesslerization procedure as outlined by UMBREIT *et al.*[7].

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[5] L. C. CLARK and H. L. THOMPSON, Anal. Chem. *21*, 1218 (1949).

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Influence of administration of methionine (5 mg/kg of body weight) on the excretion levels of creatine, creatinine, N'-methyl nicotinamide and nitrogen in irradiated animals.

No. of rats	Group	Nitrogen mg/day/rat	Creatine mg/day/rat	Creatinine mg/day/rat	N'MeNA mg/day/rat
4	Control	56·5 ± 0·5	0·54 ± 0·089	1·96 ± 0·28	0·236 ± 0·004
6	Control + Methionine	52·6 ± 1·52	0·57 ± 0·07	1·8 ± 0·037	0·198 ± 0·003
6	*Irradiated*	92·8 ± 4·3	1·19 ± 0·11	3·87 ± 0·24	0·425 ± 0·009
6	*Irradiated* + Methionine given *before* irradiation	85·63 ± 4·8	1·3 ± 0·2	3·42 ± 0·38	0·215 ± 0·005
6	*Irradiated* + Methionine given *after* irradiation	85·8 ± 3·39	0·463 ± 0·001	3·08 ± 0·12	0·212 ± 0·006

Standard errors have been calculated using the formula $(\Sigma d^2/n(n\text{-}1)^{\frac{1}{2}}$.