Effect of Puromycin *in vitro* on the Phosphorylation of Precursor Protein in Lactating Rat Mammary-Gland Slices

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(Received 19 June 1967)

It is now well documented that free amino acids of blood are used for the synthesis of casein in the mammary tissue (Barry, 1961). It is also known that phosphorus that is bound by ester linkage to the hydroxyl group of the serine of casein (Lipmann, 1933; Burnett & Kennedy, 1954) is supplied by inorganic phosphorus of blood (Aten & Hevesy, 1938; Barry, 1952; Kleiber & Luick, 1956). However, the mechanism by which phosphorus is incorporated into the protein molecule in the mammary gland has not yet been clearly delineated. Phosphorus atoms can be introduced either in the preformed polypeptide chains, or at the amino acid level, i.e. serine can be phosphorylated and phosphoserine thus formed can subsequently be incorporated into casein molecule. The former can occur while polypeptide chains are still attached to ribosomes or after their release from the ribosomal surface.

Puromycin, which inhibits the biosynthesis of protein by acting as an analogue of esterified RNA (Yarmolinsky & de la Haba, 1959), is a very convenient and useful tool for studying the mode of incorporation of non-protein moieties into the conjugated proteins. For example, with the help of this antibiotic incorporation of iodine (Taurog & Howells, 1964; Soodak, Maloof & Sato, 1964; Tishler & Ingbar, 1965) and carbohydrates (Spiro & Spiro, 1966) into the thyroglobulin molecule, which is an iodinated glycoprotein, has been shown to occur after the formation of polypeptide chains. Thus we were prompted to use this antibiotic to study the mode of [^{32}P]P_i incorporation into phosphoprotein of lactating rat mammary-gland slices. Findings presented in this communication show that in rat mammary-gland cells there is a small pool of unphosphorylated or incompletely phosphorylated casein and that phosphorylation of casein takes place subsequent to polypeptide formation. [Among the proteins of milk synthesized by the mammary-gland tissue, since casein is the only phosphoprotein and constitutes the major fraction (about 80%), no attempt has been made in the present study to isolate casein. Incorporation of [^{32}P]P_i into phosphoprotein has been considered as phosphorylation of casein.]

Materials and methods. Lactating rats, 17–21

days after parturition, were stunned by a blow on the head and exsanguinated by decapitation. Mammary glands were quickly excised and dropped in ice-cold 0.9% NaCl. After washing two or three times with chilled 0.9% NaCl to remove adhering milk, mammary-gland tissue was sliced with a sharp razor blade by the free-hand technique of Deutsch (1936). Slices were incubated in Krebs–Ringer bicarbonate buffer (Deluca & Cohen, 1964) containing either DL-[U- ^{14}C]leucine or [^{32}P]P_i at 37° in O₂ + CO₂ (95:5). Other experimental details are given in Table 1.

Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949).

Treatment of ^{14}C protein was as follows. At the end of incubation, the reaction was stopped by adding 2 ml. of 10% (w/v) trichloroacetic acid. The tissue was homogenized and the homogenate spun at about 1000g for 10 min. The residue was washed free of acid-soluble compounds, nucleic acids and lipids (Singh, Raghupathy & Chaikoff, 1965). The dry protein powder thus obtained was dissolved in concentrated (88%) formic acid and samples were plated for ^{14}C counting in a windowless gas-flow counter with about 40% efficiency.

Preparation of ^{32}P samples for counting was as follows. The phosphoprotein phosphorus fraction was prepared for ^{32}P counting essentially according to the method of Schmidt & Thannhauser (1945) as modified by Schmidt & Davidson (1956). Phosphorus liberated during the alkaline hydrolysis of delipidated tissue was precipitated by adding magnesia mixture. Ammonium magnesium phosphate was recrystallized and dissolved in 0.1 N-HCl. Samples were used for estimation of inorganic phosphorus and ^{32}P counting.

Results and discussion. Data given in Table 1 show that, whereas 30 min. incorporation of leucine carbon into protein by rat mammary-gland slices was inhibited by puromycin by about 80%, under similar experimental conditions that of [^{32}P]P_i into phosphoprotein was practically unaffected. Puromycin is known to inhibit protein biosynthesis (Yarmolinsky & de la Haba, 1959). The antibiotic acts as substitute for aminoacyl-transfer-RNA and becomes attached to the incomplete polypeptide chains, thus causing its release from the ribosomes

Table 1. *Effect of puromycin on 30 min. incorporation of [32 P] P_i and DL-[U- 14 C]leucine into protein by lactating rat mammary-gland slices*

Mammary-gland slices weighing 15.0–20.0 mg. were suspended in Krebs–Ringer bicarbonate buffer, pH 7.4, in each Warburg flask with a side arm. The radioactive precursor (40 μ C of [32 P] P_i or 1 μ C of [14 C]leucine) was in the side arm. At the end of 5 min. preincubation at 37° the radioactive precursor was added to the rest of the incubation medium and flasks were incubated for 30 min. The gas phase was O₂+CO₂ (95:5). Samples for counting 14 C and 32 P were prepared according to the procedure described in the text. Results of 14 C incorporation studies are expressed as counts/min./mg. of protein and that of 32 P incorporation studies as counts./min./mg. of phospho-protein phosphorus.

Expt. no.	Radioactive precursor	Incorporation of 14 C or 32 P		
		Puromycin absent	Puromycin (0.1 μ mole/ml.) added	Inhibition by puromycin (%)
1	DL-[U- 14 C]Leucine	116	22	81
2	DL-[U- 14 C]Leucine	142	36	75
3	[32 P] P_i	43.6×10^4	40.0×10^4	8
4	[32 P] P_i	49.5×10^4	47.7×10^4	4
5	[32 P] P_i	33.8×10^4	26.4×10^4	22

Fig. 1. Effect of puromycin on the incorporation of DL-[U- 14 C]leucine (●) and [32 P] P_i (□) into protein by rat mammary-gland slices as a function of time. Experimental conditions were the same as given in Table 1, except that incubations were done for 30, 60 and 120 min. Radioactivity incorporated in the presence of puromycin has been expressed as percentage of that incorporated in the control experiments where puromycin was not added.

A study of the incorporation *in vitro* of [32 P] P_i into phosphoprotein of mammary-gland tissue in the presence of puromycin as a function of time revealed that puromycin did not affect appreciably 32 P incorporation during a 30 min. incubation but did inhibit during longer intervals (1 and 2 hr.) (Fig. 1). This suggests that there is perhaps a small pool of unphosphorylated or incompletely phosphorylated casein in the mammary-gland cells that can sustain the phosphorylation reaction even when the synthesis of new protein is blocked by puromycin, but when incubation period exceeds 30 min. availability of non-phosphorylated precursor becomes a limiting factor and hence 32 P incorporation into phosphoprotein is lowered in the absence of the formation of new protein.

The authors gratefully acknowledge Mrs Malathy Singh's valuable help in conducting the experiments. S.S.D. thanks the Ministry of Health, Government of India, for financial assistance.

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(Allen & Zamecnik, 1962; Nathans, 1964). It is therefore to be expected that, if phosphorylation of serine precedes the incorporation of the amino acid into the casein molecule, puromycin will block the incorporation of [32 P] P_i into the protein. Thus our present findings, showing no inhibition of 32 P incorporation by puromycin, indicate that phosphorus atoms are introduced into the casein molecule after the formation of polypeptide chains. While this communication was in preparation a paper wherein authors have arrived at a similar conclusion with mouse mammary-gland explants has been published (Turkington & Topper, 1966).

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Biochem. J. (1967) **104**, 50 c

Characteristics of the Binding of Rotenone in the Respiratory Chain and the Inhibition Sites of Amytal and Piericidin A

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(Received 19 June 1967)

There has been little agreement on the site of inhibition of Amytal and rotenone in the respiratory chain. Originally Chance (1956), on the basis of 'cross-over' experiments, proposed that Amytal inhibited NADH oxidation between substrate and flavoprotein. This conclusion was first widely accepted and was extended to rotenone by Öberg (1961) and Ernster & Lee (1964), but Burgos & Redfearn (1965) and workers in our Laboratory (Minakami, Cremona, Ringler & Singer, 1963; Singer, 1966) reported that both of these agents inhibit between NADH dehydrogenase and ubiquinone in phosphorylating as well as non-phosphorylating preparations and pointed out that contrary observations could be traced to fallacies inherent in the 'cross-over' technique as applied to flavoproteins. Conclusive evidence that rotenone acts on the O₂ side of the dehydrogenase has been presented by Horgan & Singer (1967). On complete extraction of NADH dehydrogenase (also of NADH-ubiquinone reductase) from particles inhibited by [⁶a-¹⁴C]rotenone, all the label remained attached to the residue containing the electron-transport system.

The availability of labelled rotenone prompted the extension of these studies to the determination of the reaction site of barbiturates and of the new antibiotic piericidin A (Hall, Wu, Crane & Folkers, 1966) in the NADH oxidase chain and to an investigation of the specificity, stoichiometry and reversibility of rotenone binding. All methods were as in previous work (Horgan & Singer, 1967). [¹⁴C]-

Rotenone (2.36 mc/m-mole) and piericidin A were gifts from Dr John E. Casida, University of California, and Dr Karl Folkers, Stanford Research Institute, respectively.

Fig. 1(a) shows that, although on titration of ETP* with rotenone the inhibition rises in a hyperbolic curve, as also found by Burgos & Redfearn (1965), rotenone binding is quite linear, even though five times as much rotenone is added as that required for maximal inhibition. When the titration was performed in the presence of 2% (w/v) bovine serum albumin, however, a biphasic binding curve was obtained, showing a rapid initial rise, corresponding to the specific binding site in the NADH dehydrogenase segment of the chain, and a slower secondary rise, representing binding at other points (Fig. 1b, curve A). The more the rotenone-treated particles were washed with 2% bovine serum albumin, the more evident became the biphasic nature of the curve. That the initial, rapidly rising, portion of the binding curve represents combination at the site involved in the inhibition of NADH oxidation and the linear part is due to unspecific binding is shown in curve B in Fig. 1(b). Here the particles were first inhibited (98%) with unlabelled rotenone and then titrated with [¹⁴C]-rotenone. The initial phase of the curve is thereby almost completely abolished, but the slope of the linear portion remains essentially unaffected. Exactly the same effect may be observed if the particles are first inhibited with piericidin A, which

* Abbreviation: ETP, electron-transport particles.