Short Communications

Abolition by Actinomycin D Pretreatment *in vivo* of the Stimulating Effect of Serum Proteins on the Protein Synthesis of Yoshida Ascites Hepatoma *in vitro*

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Previous work from this Laboratory has shown that the addition of normal rabbit serum globulins to the incubation medium produces a strong stimulation of the incorporation *in vitro* of radioactive glycine into protein of Yoshida ascites-hepatoma cells (Marsilii & Chiarugi, 1965). To obtain some information on the mechanisms of this stimulation it seemed desirable to investigate whether the effect of serum globulin was in some way related to the metabolism of nucleic acids. It appeared therefore worth while at the onset to establish whether the effect of globulin was influenced by actinomycin D, a compound known to inhibit DNA-dependent synthesis of RNA (Collins, 1965; Reich, 1964).

The present paper records the results of experiments in which the effects of rabbit-serum globulins and albumins on the incorporation *in vitro* of labelled glycine into protein were studied with hepatoma cells derived from rats pretreated with actinomycin D or untreated.

Materials and methods. Six male Wistar rats, inoculated 7 days earlier with Yoshida ascites hepatoma (AH 130), were used for each experiment. Three of these rats were given intraperitoneally 50 µg. of actinomycin D/100g. body wt. 12hr. before the experiment, and three served as controls. At the time of the experiment the tumours from each group of three animals were collected and pooled, and the cells separated by centrifugation at 500 g for 5min. at room temperature. The sedimented cells were washed once with 3vol. of a basal medium containing NaCl (0.12M), KCl (13mm), MgSO₄ (0.65mm) and sodium phosphate buffer, pH 7.4 (10mm). The same medium was used for the final suspension of washed cells.

A 2.5ml. portion of cell suspension (10–12mg. dry wt. of cells) and 0.3ml. of basal medium including, where indicated, 10mg. of serum proteins, were pipetted into a 20ml. conical flask and shaken in air at 38° for 15min. in a Dubnoff metabolic shaking incubator. After this preincubation, the incorporation experiment was started by adding 2 µC of [1-¹⁴C]glycine (0.26 µmole dissolved in 0.2ml. of water) and the incubation continued for 60min. under the conditions indicated

above. To remove the excess of serum proteins added, at the end of the incubation the entire contents of a Dubnoff vessel were poured into a centrifuge tube kept in cracked ice and containing 3vol. of ice-cold basal medium. After being chilled, the cells were sedimented by centrifugation and the supernatant was discarded. The inside of the tube was then washed with ice-cold basal medium without resuspending the well-packed cells. Samples without serum proteins were treated in the same manner.

The washed cells were suspended in 1% (w/v) trichloroacetic acid to precipitate proteins; these were purified by the method of Rabinovitz, Olson & Greenberg (1954), plated on 5cm.² aluminium disks (1mg. of protein/disk) and counted dry at infinite thinness in a thin-window gas-flow counter (FDW-1; Tracerlab, Waltham, Mass., U.S.A.). The efficiency of counting [1-¹⁴C]glycine at infinite thinness under the described conditions was 30%. Enough counts were taken to keep the counting error below 2% and corrections were applied for background and dead-time.

γ-Globulins (6.5s or IgG) and albumins (4s) were prepared from normal rabbit serum by gel filtration on Sephadex G-200 (Flodin & Killander, 1962), followed by dialysis against water and freeze-drying. The [1-¹⁴C]glycine used in these experiments had a specific activity of 7.9mc/m-mole and was obtained from The Radiochemical Centre, Amersham, Bucks. Actinomycin D was a gift from the Merck, Sharp & Dohme Research Laboratories, West Port, Pa., U.S.A.

The significance of the differences between the means was tested by the analysis of variance.

Results and discussion. As indicated above, in the present experiments the labelled amino acid was added 15min. after the addition of the γ-globulin. Preliminary experiments had, in fact, indicated that, when both the globulin and the labelled amino acid were added at the same time, the stimulating effect of globulin on the incorporation became evident 10–15min. after the addition. This probably corresponds to the time required for the entry of protein into the cells.

The results summarized in Table 1 show that

Table 1. *Effect of rabbit serum proteins on the incorporation in vitro of [1-¹⁴C]glycine into protein of Yoshida ascites-hepatoma cells from actinomycin D-treated and untreated rats*

The results are given as means $\pm$ S.E.M. of five experiments.

Rats	Incorporation (counts/min./mg. of protein)		
	Control	γ -Globulin	Albumin
Untreated	858 $\pm$ 115	3524 $\pm$ 608	2525 $\pm$ 671
Actinomycin D-treated	729 $\pm$ 58	983 $\pm$ 64	914 $\pm$ 23

both globulins and albumins from normal rabbit serum greatly stimulate the incorporation *in vitro* of radioactive glycine into protein of hepatoma cells ($P < 0.01$), globulins appearing to be more effective than albumins in producing the stimulation. The results also show that pretreatment of tumour-bearing rats with actinomycin D does not significantly affect the incorporation in the basal medium ($P > 0.3$), whereas it abolishes almost completely the stimulating effect of serum proteins ($P < 0.01$).

The study of the kinetics of the incorporation has shown that, in cells from both actinomycin D-treated rats and controls, the rate of incorporation proceeded as a linear function of the time from the beginning up to at least 1 hr., both in the presence and absence of serum globulins.

To conclude, the results presented show clearly that the stimulating effect of serum proteins on the incorporation of labelled glycine into protein of Yoshida ascites-hepatoma cells is abolished by pretreatment of tumour-bearing rats with a dose of actinomycin D that practically does not affect the basal level of incorporation. Thus, on the basis of what is known on the mechanism of action of actinomycin D (Collins, 1965; Reich, 1964), it appears reasonable to conclude that serum proteins affect the transcription reactions in some way.

Two main problems arise at this point. The first one is whether the stimulation of the incorporation brought about by serum proteins is due to the protein molecule itself or to the products of its degradation. That such an effect might be due to simple molecules such as amino acids was excluded by separate experiments showing that a mixture of amino acids reproducing the composition of normal rabbit serum γ -globulin (except tryptophan) (Porter, 1959) actually depressed the glycine incorporation, most probably because of the dilution of the marker. On the other hand, the possibility cannot be ruled out that the stimulation of protein synthesis by rabbit serum proteins is due to peptides arising from the degradation of these proteins. There are several examples of peptides, hormonal and otherwise, having a stimulatory effect on protein synthesis.

The second problem is how rabbit serum proteins, or peptides derived from their degradation, bring about the stimulation of protein synthesis. This problem is now under investigation in this Laboratory from several points of view, and it has been shown (G. Marsilii & V. Chiarugi, unpublished work) that the addition of normal rabbit serum globulins to hepatoma-cell suspensions is followed by an enhanced nuclear synthesis of rapidly labelled RNA.

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The Effects of Sugars on Incorporation of [³H]Leucine into Insulins

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Increasing the glucose content of the medium in which slices of ox pancreas are incubated greatly stimulates the incorporation of labelled leucine

into ox insulin (Taylor, Parry & Smith, 1964). This effect appears to be specific for insulin, and alterations in the sugar content of media in which