amount of ^{32}P liberated from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Hydroxylamine (0.16M) is more effective than 0.5mM- K^+ in this regard. This effect of K^+ has been reported many times previously (e.g. Charnock & Post, 1963a; Whittam, Wheeler & Blake, 1964; Post, Sen & Rosenthal, 1965; Gibbs *et al.* 1965) and is generally thought to represent a greatly increased rate of dephosphorylation of the phosphoprotein intermediate under the influence of K^+ .

The presence of 0.1mM-ouabain in addition to either K^+ or hydroxylamine again decreases the liberation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and increases the amount of ^{32}P -labelled phosphoprotein complex formed. This finding supports the view that hydroxylamine functions as a cation and can replace K^+ in this system.

To examine this further, hydroxylamine (0.36M) was substituted for K^+ (20mM) in three assays of ATPase activity with a standard chemical assay system for the liberation of inorganic phosphate from non-radioactive ATP (2mM) in the presence of 80mM- Na^+ (Charnock & Post, 1963b). In these experiments the presence of K^+ increased the phosphate released from 53 to 277 μmoles of phosphate/mg. of protein N/hr. whereas addition of hydroxylamine increased the release of phosphate to 93 μmoles of phosphate/mg. of protein N/hr. (values are the means of three experiments, assays in duplicate). In every experiment addition of 0.1mM-ouabain decreased the liberation of phosphate to that found with Na^+ alone.

Thus hydroxylamine appears to function as a K^+ -like cation in the reaction mechanism of $(\text{Na}^+ + \text{K}^+)\text{-activated ATPase}$. The conclusion of Schoner *et al.* (1966) that the ^{32}P -labelled phospho-

protein complex formed ($\text{E} \sim \text{P}$) from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ cannot be an intermediate in the overall reaction mechanism of this enzyme is therefore no longer valid. Whether hydroxylamine acts directly as a cation, or indirectly by the liberation of NH_4^+ ions, is not resolved by these experiments.

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Effects of Serum Proteins *in vitro* on the Synthesis of Rapidly Labelled Ribonucleic Acid and on the Polysome Pattern of Yoshida Ascites-Hepatoma Cells

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Normal rabbit serum globulins strongly stimulate the incorporation *in vitro* of radioactive glycine into protein of Yoshida ascites-hepatoma cells (Marsilii & Chiarugi, 1965) and this stimulation is abolished by actinomycin D pretreatment of tumour-bearing rats (Marsilii & Chiarugi, 1966).

The present experiments were carried out to study the effects of normal rabbit globulins *in vitro* on the synthesis of rapidly labelled RNA and on the polysome pattern of Yoshida ascites-hepatoma cells.

Material and methods. Yoshida ascites hepatoma

(AH130) was taken from rats inoculated 7 days earlier. The cells were separated by centrifugation at 500g for 5min. at room temperature and washed twice with 3vol. of a medium containing NaCl (0.12M), KCl (13mM), MgSO_4 (0.65mM) and sodium phosphate buffer, pH 7.4 (10mM). Washed cells were resuspended in the same medium.

A 10ml. sample of cell suspension (approx. 30mg. dry wt. of cells) was pipetted into a 50ml. conical flask containing, when indicated, 30mg. of freeze-dried γ -globulins (6.5s globulin or immunoglobulin G) prepared from normal rabbit serum by the method of Flodin & Killander (1962). The flask was then shaken at 38° in a Dubnoff metabolic shaking incubator. After 15min. of incubation $10\mu\text{C}$ of [$\text{G-}^3\text{H}$]uridine (4.6m μ moles dissolved in 0.1ml. of water) was added into the flask and the incubation continued for a further 30min.

At the end of the incubation the flask was immediately cooled in NaCl-ice mixture and then its entire contents were poured into a centrifuge tube kept in ice and containing 3vol. of ice-cold medium. The cells were then sedimented by centrifugation at 500g for 5min. at 0° and the supernatant was discarded. The sedimented cells were subjected to osmotic 'shock' by resuspending them in ice-cold medium diluted 1:3 with water, and then separated by centrifugation in the cold as indicated above. The supernatant was poured off and the swollen packed cells were homogenized, with a Potter-Elvehjem glass homogenizer with a Teflon pestle, in 10ml. of 0.25M-sucrose containing CaCl_2 (3mM) and tris buffer, pH 7.4 (50mM).

Isolation of virtually pure nuclei and preparation of nuclear RNA were carried out as described by Di Girolamo, Henshaw & Hiatt (1964). Sucrose-gradient centrifugation of purified RNA was carried out as described by Hiatt (1962). After centrifugation the bottom of the tube was punctured with a hypodermic needle and the distribution of RNA in the sucrose gradient was monitored by means of a 1cm. flow cell, in conjunction with a GME ultra-violet attachment (Gilsom Medical Electronics, Middleton, Wis., U.S.A.) connected to a linear strip recorder. The system was standardized against a Uvispek spectrophotometer (Hilger and Watts) with different concentrations of yeast RNA. After measurement of E_{265} , 1ml. fractions of the gradient were collected, mixed with 10ml. of Brei's solution and counted in a liquid-scintillation counter (mark 1; Nuclear-Chicago Corp.) with a counting efficiency of approx. 35% for ^3H . Corrections were applied for quenching changes due to different sucrose concentrations and for background.

Polysome sedimentation pattern analysis of liver and hepatoma cells was carried out according to Webb, Blobel & Potter (1964). Cell preparation,

homogenization and incubation conditions were the same as indicated above, except that [$\text{G-}^3\text{H}$]uridine was replaced by 2.5 μC of [$1\text{-}^{14}\text{C}$]glycine (0.32 μmole dissolved in 0.25ml. of water). Radioactivity of the gradient fractions (1.0ml.) was measured as indicated above, with a counting efficiency of approx. 40% for ^{14}C .

[$\text{G-}^3\text{H}$]Uridine and [$1\text{-}^{14}\text{C}$]glycine were obtained from The Radiochemical Centre, Amersham, Bucks. Deoxyribonuclease and ribonuclease (once recrystallized) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Results and discussion. Fig. 1(a) shows that the radioactivity of all fractions of nuclear RNA separated on the sucrose gradient is greater in cells incubated in the presence of immunoglobulin G than in cells incubated in saline alone. The difference of radioactivity is particularly evident in the peak corresponding to 38s.

The sedimentation pattern and the radioactivity of polysomes isolated from hepatoma cells incubated in the presence or absence of immunoglobulin G is shown in Fig. 1(b). It is apparent that during the incubation in saline there is a degradation of heavy polysomes and that this degradation is decreased if the medium is supplemented with immunoglobulin G. The polysome pattern of fresh (non-incubated) hepatoma cells (see Fig. 1c) is practically the same as the pattern found after incubation in the presence of immunoglobulin G. Fig. 1(b) also shows that the radioactivity of all fractions of polysomes is greater after incubation in the presence of immunoglobulin G than after incubation in saline alone.

A comparison of polysome pattern of hepatoma cells with that of rat liver is made in Fig. 1(c), which also shows the pattern obtained with ribonuclease-treated liver polysomes.

The results reported indicate that the stimulation of protein synthesis *in vitro* induced in hepatoma cells by immunoglobulin G (Marsilii & Chiarugi, 1965) is accompanied by an increased synthesis of rapidly labelled RNA. It is noteworthy that the addition of immunoglobulin G to a hepatoma cell-free amino acid-incorporating system prepared by the method of Trakatellis, Axelrod & Montjar (1964) does not modify appreciably the incorporation of labelled amino acids into protein (G. Marsilii & V. Chiarugi, unpublished work).

To conclude, the results of the present investigation and the previously described effects of actinomycin D (Marsilii & Chiarugi, 1966) support the hypothesis that rabbit immunoglobulin G enhances protein synthesis of intact hepatoma cells by stimulating nuclear transcription. However, the possibility cannot be excluded that both nuclear transcription and protein synthesis appear to be stimulated by immunoglobulin G only because these

Fig. 1. Centrifugation patterns in sucrose density gradients of nuclear RNA and polysomes from hepatoma cells incubated in the presence or absence of immunoglobulin G. (a) Nuclear RNA; —, E_{265} for both conditions (presence or absence of immunoglobulin G); ● and ○, radioactivity in the presence or absence of immunoglobulin G respectively. (b) — and ●, Sedimentation pattern and radioactivity respectively of polysomes from cells incubated in the presence of immunoglobulin G; ---- and ○, same in the absence of immunoglobulin G. (c) Polysome pattern of fresh non-incubated hepatoma cells (—) as compared with that of normal rat liver (----); ·····, ribonuclease-treated rat liver polysomes (ribonuclease treatment as described by Wettstein, Staehelin & Noll, 1963).

processes are depressed in cells incubated in saline alone, possibly because of a release of ribonuclease from lysosomes. The decreased degradation of polysomes observed in the presence of immunoglobulin G (cf. Figs. 1b and 1c) and the ineffectiveness of immunoglobulin G in cell-free systems (see above) are of interest in this connexion. On the other hand, if the stimulating effect of immunoglobulin G is due to a better preservation of cell structures during the incubation, then the abolition by actinomycin D pretreatment *in vivo* of the stimulating action of immunoglobulin G on protein

synthesis (Marsili & Chiarugi, 1966) should be interpreted in the sense that structures (or mechanisms) to be protected by immunoglobulin G are already altered in cells derived from actinomycin D-treated animals.

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Conversion of Reduced Nicotinamide-Adenine Dinucleotide Dehydrogenase into Reduced Nicotinamide-Adenine Dinucleotide-Ubiquinone Reductase

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Two types of soluble purified preparations of the NADH dehydrogenase component of the respiratory chain have been described in the literature. One is a high-molecular-weight form (mol.wt. $5 \times 10^5 - 8 \times 10^5$) extracted with phospholipase A at low temperature (Ringler, Minakami & Singer, 1963). Its properties parallel those of particle-bound NADH dehydrogenase except that it does not catalyse the Amytal- and rotenone-sensitive reduction of ubiquinone (Machinist & Singer, 1965a). The reduction of external ubiquinone in submitochondrial particles, however, is phospholipid-dependent and may occur by way of endogenous ubiquinone-10 (where the numeral indicates the number of isoprene units in the side chain) rather than by direct reaction with the dehydrogenase (Machinist & Singer, 1965b).

The other type is of lower molecular weight and is extracted by acid-ethanol or phospholipase A at elevated temperatures, which leads to fragmentation of the enzyme and to major changes in its properties (Watari, Kearney & Singer, 1963). Such preparations show high levels of Amytal- and rotenone-insensitive cytochrome *c* reductase, indophenol reductase and ubiquinone-1 reductase activities not seen in more native preparations. These emerge as a result of protein modification during extraction.

It has been reported (Pharo & Sanadi, 1964; Pharo, Sordahl, Vyas & Sanadi, 1966) that, when the heat-acid-ethanol procedure is applied to phosphorylating particles, a flavoprotein is ex-

tracted that reduces long-chain ubiquinone homologues in an Amytal- and rotenone-sensitive reaction. This NADH-ubiquinone reductase was reported to be extractable only from phosphorylating particles (ETP_H*) but not from non-phosphorylating ones. The apparent retention of Amytal- and rotenone-sensitive ubiquinone reductase activity has led to the interpretation that the physiological ubiquinone reaction site has been retained in this preparation (Sanadi, Pharo & Sordahl, 1965).

Machinist & Singer (1965a,b) have extracted NADH-ubiquinone reductase from ETP_H and NADH-cytochrome reductase from the non-phosphorylating particle ETP by the procedure of Pharo & Sanadi (1964). No appreciable difference was found in reactivity with ubiquinone-1, ubiquinone-6, cytochrome *c* or menadione, in absorption spectrum, in chromatographic properties, in sedimentation coefficient, in substrate specificity or in iron, labile sulphur or FMN content. The development of the assay method used by Pharo *et al.* (1966) for ubiquinone-6 and ubiquinone-10 reduction has enabled us to complete the comparison and to demonstrate the conversion of the high-molecular-weight form of the enzyme into a product indistinguishable from NADH-ubiquinone reductase obtained by the heat-acid-ethanol procedure.

Heat-acid-ethanol extracts prepared from ETP and from phosphorylating particles (ETP_H or

*Abbreviations: ETP, electron-transport particle; ETP_H, phosphorylating electron-transport particle; SP_{HL}, ETP_H 'sonicated' without additives (Pharo *et al.* 1966).