Purification of a liver DNA-synthesis promoter from plasma of partially hepatectomized rats

Juan J. DÍAZ-GIL,*|| Pedro ESCARTÍN,† Rafael GARCÍA-CAÑERO,* Carolina TRILLA,* Juan José VELOSO,* Gonzalo SÁNCHEZ,* Alberto MORENO-CAPARRÓS,‡, Carmen ENRIQUE DE SALAMANCA,‡ Rosa LOZANO,‡ José G. GAVILANES§ and Juan M. GARCÍA-SEGURA§

*Servicio de Bioquímica Experimental and †Servicio de Gastroenterología, Clínica Puerta de Hierro, San Martín de Porres 4, 28035 Madrid, Spain, ‡Departamento de Patología, Centro Ramón y Cajal, 28034 Madrid, Spain, and §Departamento de Bioquímica, Facultad de Ciencias, Universidad Complutense, 28040 Madrid, Spain

A protein was isolated from plasma of partially (70%) hepatectomized rats that, injected in mice, increases the uptake of [³H]thymidine by liver DNA by 200–300% over that by injected control saline. The purification procedure consists essentially of three chromatography steps, employing Sephadex G-75, DEAE-cellulose and hydroxyapatite. The hepatic promoter (HP) preparation shows a single band in SDS/polyacrylamide (15%)-gel electrophoresis (silver stained), with an M_r of 64000; its activity is suppressed by trypsin or pepsin and is unaffected by deoxyribonuclease or ribonuclease. On injection into mice (150 ng/mouse), it increases the mitotic index of the liver. It shows organ-specificity, acting on liver but not on spleen, kidney, lung or brain. In primary liver cultures, it produces an increase in uptake of [³H]thymidine into DNA in the range 1–10 ng/ml. In this system *in vitro*, it increases the uptake of ²²Na⁺ immediately after addition.

INTRODUCTION

The great capacity of regeneration exhibited by the liver of animals subjected to partial hepatectomy is very well known. Rats recover 66% of their liver in 8–10 days after partial hepatectomy (Bucher & Malt, 1971). Among the different theories proposed to explain this huge regeneration capacity of the liver is the appearance of serum factor(s) responsible for the extraordinary cell multiplication rate observed. Supporting this hypothesis, several authors have performed experiments, both *in vivo* and *in vitro*, showing the appearance of a liver-growth-factor activity in blood of animals subjected to partial hepatectomy. Paul *et al.* (1972) demonstrated the increase in [³H]thymidine uptake by fetal-rat liver cells in culture in the presence of serum of partially hepatectomized rats as compared with cultures incubated with normal rat serum. Moolten & Bucher (1967), with cross-circulation experiments between normal and partially hepatectomized rats, detected a stimulated incorporation of [¹⁴C]thymidine into hepatic DNA in the normal (partner) rat. Fisher *et al.* (1971) demonstrated that the humoral factor responsible for liver regeneration is found in portal blood and its concentration increases as a greater amount of liver is removed.

There have been many attempts to purify this growth factor. The initial materials used were serum (Morley & Kingdon, 1973), plasma (Goldberg *et al.*, 1980), cytoplasmic extract of liver of partially hepatectomized rats (Labrecque & Bachur, 1982) and, more recently, rat platelets (Russell *et al.*, 1984). In all cases, a semi-purified material was obtained.

We report below the purification (one single band in silver-stained SDS/polyacrylamide-gel electrophoresis)

of a hepatic DNA-synthesis promoter (HP) from plasma of partially hepatectomized rats that exhibits DNA-synthesis-promoting activity both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Chemicals were purchased from Sigma, Bio-Rad, Merck and Pharmacia, and [³H]thymidine (20 Ci/mmol) was from New England Nuclear.

Diethyl ether-anaesthetized Wistar rats (90–110 g) were 70% hepatectomized by the method of Higgins & Anderson (1931). The initial material for the purification procedure was plasma of partially hepatectomized rats (plasma PH), obtained by cardiac puncture 24 h after hepatectomy. The operation was carried out in all cases between 09:30 and 12:30 h, and the blood of at least ten rats per batch was pooled. The heparinized blood was centrifuged (27000 *g* for 15 min) and the supernatant collected, freeze-dried and kept at –20 °C until use. Before initiation of the purification, freeze-dried plasma was dissolved in 50 mM-Tris/HCl buffer, pH 7.6, centrifuged (4000 *g* for 10 min), and the clear supernatant kept for use.

Purification procedure

It consists of three main steps, the first of which is chromatography on Sephadex G-75 (superfine grade; 88 cm × 5.0 cm column), with 50 mM-Tris/HCl buffer, pH 7.6. The total chromatogram was divided into different pools, each of which was freeze-dried. Portions of each pool (about 20 ml) were freeze-dried separately, then dissolved in 2 ml of saline (0.9% NaCl). Their protein content was determined, and then they were

Abbreviations used: HP, hepatic DNA-synthesis promoter; EGF, epidermal growth factor.

|| To whom correspondence and reprint requests should be addressed.

injected into mice in several doses to check activity. Samples exhibiting activity were dialysed (M_r cut-off 14000) against the same buffer to eliminate the excess of Tris. The second step of purification was chromatography on DEAE-cellulose (26 cm $\times$ 2.6 cm column), with 50 mM-Tris/HCl buffer, pH 7.6. Bound proteins were eluted by application of a linear gradient of 0–1.0 M-KCl (2 $\times$ 150 ml) in the same buffer. The peaks of proteins obtained in this step were dialysed against the same buffer to eliminate KCl, and freeze-dried, their activity being measured in samples, as indicated below. The peak that showed activity was subjected to the last step of purification, i.e. chromatography on hydroxyapatite (20 cm $\times$ 1.5 cm), in 10 mM-potassium phosphate buffer, pH 7.4. (Previously, samples were dissolved in phosphate buffer and dialysed to eliminate Tris buffer.) A linear gradient (2 $\times$ 100 ml) of 10–500 mM-phosphate buffer, pH 7.4, was applied to elute the proteins retained by hydroxyapatite. The protein peaks were dialysed against 10 mM-phosphate buffer to eliminate the excess of P_i , freeze-dried and dissolved in 4 ml of 10 mM-phosphate buffer, to measure their activity. Only one peak, F123, showed activity after the purification procedure. The concentrations of the gradients used in the last two steps were monitored by measuring the conductivity of each fraction and comparing it with that of previously prepared samples. All manipulations during the purification procedure were carried out at room temperature, but, with the final solution of our HP preparation (F123) it is convenient, after sterilization and protein determination, to divide it into batches and freeze-dry them to avoid any loss of activity. In all the experiments carried out with HP preparations, freeze-dried samples, dissolved in saline immediately beforehand, were used, and the rest of the solution was discarded (HP loses its activity at -20°C after about 3 weeks). Protein was determined by the method of Lowry *et al.* (1951).

Determination of HP activity as DNA-synthesis promoter

Swiss mice (25–29 g body wt.) were used for the experiments *in vivo*, receiving intraperitoneal injections (up to 200 μl) of HP or fractions from different stages of the purification procedure. Protein injected per mouse depends on each particular fraction; the amount is indicated in the Results section when necessary. Then 24 h later, [^3H]thymidine was injected (1 $\mu\text{Ci/g}$ body wt.), and 1 h thereafter, the mice were killed by cervical dislocation. ([^3H]Thymidine uptake reaches a maximum 24 h after injection of the 'active' fractions, decreasing later, this behaviour being similar in partial hepatectomy.) Liver was cut into cubes of about 0.4 cm each side and washed with 40 ml of saline on double gauze. Minced liver was dried on filter paper, homogenized by passage through a needle (Luer 100; 11 $\times$ 25 gauge) and weighed, after which 0.8 M-HClO₄ (4 ml/g) was added. For the extraction of DNA, the method of MacManus *et al.* (1972) was followed.

The activity of all fractions were monitored by measuring, in triplicate, in the supernatant of the DNA extraction, the radioactivity of the [^3H]thymidine incorporated (by β -counting, by standard procedures) and total DNA, by the method of Burton (1968). Specific incorporation of [^3H]thymidine into DNA (d.p.m./ μg of DNA) was compared in control mice (saline-injected) and those treated with different fractions, and the activity of our HP preparation was measured throughout the

purification procedure. All solutions injected in mice were previously sterilized by filtration through membranes of 0.45 μm pore size. The injection of fractions with HP activity into mice was performed between 09:30 and 10:45 h to avoid possible diurnal variations.

Electrophoresis

SDS/polyacrylamide (15%)-gel electrophoresis was performed by the method of Laemmli (1970). M_r markers from Pharmacia were used. Proteins were revealed by a silver-stain technique (Oakley *et al.*, 1980).

For microscopic observations, mouse livers (controls and HP-injected) were minced and kept in 10% (v/v) formalin until use. They were processed by standard histological techniques and cut into 3 μm portions, stained with haematoxylin/eosin and observed microscopically. For mitotic-index determination after HP injection, the method of Morley & Boyer (1977) was used.

Method of isolation of hepatocytes

Hepatocytes from 150 g Wistar rats were isolated by a collagenase perfusion technique (Bonney *et al.*, 1974). Isolated hepatocytes were immediately washed and suspended as stock in Waymouth medium with 10 munits of insulin/ml, 10% (v/v) fetal-calf serum and antibiotics. Viability was assessed by the Trypan Blue exclusion method. Our preparations showed at least 80% viability, and were seeded on Costar 12-well trays previously coated with u.v.-sterilized collagen, at a density of 20000 cells/cm² in 2 ml of Waymouth medium. Cultures were left for 24 h at 37 $^\circ\text{C}$ in air/CO₂ (19:1) before any manipulations were carried out.

The effect of HP on DNA synthesis was studied by using [^3H]thymidine (1 $\mu\text{Ci/ml}$) in a previously washed culture lacking insulin and in which fetal-calf serum was replaced by 0.2% bovine serum albumin. Freeze-dried HP samples were dissolved in the culture medium immediately before use, and portions added to the different wells and made up with Waymouth medium to 2 ml volume in every experiment. After 24 h of culture in the presence of different concentrations of HP, or 10% fetal-calf serum or saline as controls, cultures were washed with 2 $\times$ 2 ml of 5% (v/v) trichloroacetic acid at 3 $^\circ\text{C}$ during 10 min to eliminate soluble radioactivity, and the DNA-incorporated radioactivity was extracted with 0.8 M-HClO₄ at 70 $^\circ\text{C}$ for 30 min. A sample was used to measure radioactivity, and the precipitate was used to measure protein by the Lowry method. Specific incorporation is expressed as d.p.m./mg of protein. Each data point is the mean of four separate determinations.

For $^{22}\text{Na}^+$ -uptake measurements, hepatocyte cultures were washed with 2 $\times$ 2 ml of culture medium lacking insulin and fetal-calf serum, and 1 ml portions of medium (prewarmed to 37 $^\circ\text{C}$) containing 3 μCi of $^{22}\text{Na}^+$ (4.5×10^6 d.p.m.)/ml plus 1 mM-ouabain were added, and uptake was measured over 10 min with additions of HP, fetal-calf serum and amiloride as necessary. After this period, cultures were washed with 2 ml portions of cold 250 mM-sucrose, pH 6.0. Under these circumstances, the radioactivity detected represents the internalization of $^{22}\text{Na}^+$ (not due to externally bound $^{22}\text{Na}^+$) (Sanui & Rubin, 1979). Radioactivity was extracted with 1 ml of 5% trichloroacetic acid during 90 min at room temperature. Samples of the supernatant were used to measure radioactivity by β -counting by standard procedures, and the protein attached was measured by the Lowry method.

Fig. 1. Chromatography on Sephadex G-75 (superfine grade; 88 cm $\times$ 5.0 cm column) of plasma PH (18 ml)

Elution was with 50 mM-Tris/HCl buffer, pH 7.6, at a flow rate of 60 ml/h; 7.0 ml fractions were collected. The entire chromatogram was divided into pools (V_0 , F1 etc.), each of which was freeze-dried.

The results are expressed as d.p.m./mg of protein, and are means of four separate determinations.

RESULTS

In Fig. 1, we present the elution pattern of 18 ml of plasma from partially hepatectomized rats, 24 h after hepatectomy (plasma PH), on Sephadex G-75 (superfine grade). The method for measuring the activity of the hepatic promoter (HP) was delineated in the Materials and methods section. The peaks marked as V_0 , F1, F2 and F3 showed activity (incorporation of [3 H]thymidine into mouse liver DNA as compared with saline-injected controls) and were subjected to the second step of the purification scheme.

Fig. 2 shows DEAE-cellulose chromatography of peak F3, the only one that retained activity after this second step of purification. (We usually carried out two or three Sephadex G-75 chromatographies, making a pool of two

or three F3 peaks, in order to have enough protein for the following steps.) A linear gradient of KCl dissolved in the same buffer (50 mM-Tris/HCl pH 7.6) was applied to the column, and the peaks of proteins were checked for activity. Peak F23 (eluted at about 180 mM-KCl) showed activity, and therefore was subjected to the remaining step in the purification procedure.

Fig. 3 shows the protein pattern of peak F23 chromatographed on hydroxyapatite, in 10 mM-phosphate buffer, pH 7.4. A linear gradient of phosphate buffer (10–500 mM) was applied and the protein peaks were analysed for activity. At about 40 mM-phosphate an activity-retaining protein peak was eluted. We will refer to this peak of protein as our hepatic promoter preparation (HP). When this purification schedule was followed with plasma from normal, sham-operated (not hepatectomized), rats, no activity was detected.

Table 1 shows the extent of enrichment at each step for

Fig. 2. Chromatography on DEAE-cellulose (26 cm $\times$ 2.6 cm) of peak F3 (from Sephadex G-75)

Elution was with 50 mM-Tris/HCl buffer, pH 7.6, at a flow rate of 30 ml/h; 4.0 ml fractions were collected. Further elution was carried out with a gradient of KCl (0–1.0 M) in the same buffer (2×150 ml).

Fig. 3. Chromatography on hydroxyapatite (20 cm $\times$ 1.5 cm) of peak F23 (from DEAE-cellulose)

Elution was with 10 mM- P_i buffer, pH 7.4, at a flow rate of 15 ml/h; 3.0 ml fractions were collected. Further elution was carried out with a gradient of P_i buffer, 10–500 mM (2×100 ml).

Table 1. Enrichment of HP activity throughout the purification procedure

For details see the Materials and methods section. Specific activity is expressed in d.p.m./ μ g of DNA per mg of protein injected.

Fraction	Total protein (mg)	Recovery (protein) (%)	Specific-activity range	Total activity	Yield (% of specific activity)	Purification (fold)
Plasma PH (36 ml)	2898	100	3.65–56.0	Unknown	—	—
F2 (Sephadex G-75) (49 ml)	2.499	0.086	8627	21 558	100	1
F23 (DEAE-cellulose) (12 ml)	0.138	0.00476	50 000	6900	32	6
F123 (hydroxyapatite) (4 ml)	0.006	0.00021	720 000	4320	20	83

a typical preparation. Specific activity (d.p.m./ μ g of DNA per mg of protein injected) for plasma PH exhibits a great deal of variation, some batches producing even lower incorporation of [3 H]thymidine than in saline-injected control mice. Here we indicate a range which, in any case, is underestimated. The reason for this could be the simultaneous presence of stimulator and inhibitor factors acting together. For this reason (lack of a real value for basal activity in plasma), we have calculated the purification (fold), considering 100% to be that exhibited by peak F3, obtained in the first step in the purification procedure. With this strategy, we do not consider the first step, possibly the main one, in the calculation of the purification (fold). The recovery of HP in terms of protein exhibits some variations, depending on different batches.

SDS/polyacrylamide (15%) gel electrophoresis (silver-stained) of our HP preparation gave the result shown in Fig. 4. Our HP preparation gave a single band with an apparent M_r of 64000.

We performed a radioimmunoassay of HP with anti-insulin and anti-glucagon sera, from guinea pig and rabbit respectively (detection limits of 0.25 ng/ml and 30 ng/ml respectively), using 1 μ g of HP of our preparation; this excluded contamination by any of the hormones. The absence of EGF from our preparation is assumed on the basis of the following facts. (a) EGF can be associated in a 74000- M_r form, being stable from pH 5 to pH 8 (James St. Hilaire & Jones, 1982); our SDS/polyacrylamide-gel electrophoresis was carried out at pH 8.8, and no band of low M_r (M_r of EGF = 6045) was detected. (b) EGF exists in plasma at a concentration of 1 ng/ml (James St. Hilaire & Jones, 1982), at least 100 times more dilute than our HP. (c) In an experiment *in vitro* with Swiss 3T3K cells, HP, at a concentration of 100 ng/ml, failed to stimulate DNA synthesis, either alone or with insulin; because EGF stimulates DNA synthesis in this system between 0.5 and 5.0 ng/ml (Dicker & Rozengurt, 1978), this means that our HP preparation has less than 0.5% contamination with EGF, if any. The presence of a class of somatomedin, non-suppressible insulin-like activity (N-SILA) or EGF-like factors in our preparation is also improbable, since their M_r is lower than 10000 in every case (Von Wyk & Underwood, 1978). With respect to the presence of the other possible hepatotrophic factors (growth hormone and parathyroid hormone), no band was detected by SDS/polyacrylamide-gel electrophoresis in our preparations, and calcitonin, Ca^{2+} , tri-iodothyronine, heparin and prostaglandins have M_r values below 14000 (cut-off point of the dialysis tube used). The concentration of some of these factors in our preparation,

Fig. 4. SDS/polyacrylamide (15%) gel electrophoresis of the HP preparation (0.05 μ g)

Proteins used as M_r markers (kit from Pharmacia) were: phosphorylase *b* (M_r 94000); albumin (bovine plasma) (67000); egg albumin (ovalbumin) (43000); carbonic anhydrase (bovine erythrocyte) (30000); trypsin inhibitor (20100); β -lactalbumin (14400). The marker kit was dissolved in 100 μ l of buffer, and 15 μ l of a 1/100 dilution (from the stock solution) was loaded on the gel. Amounts of markers ranged between 0.10 and 0.22 μ g.

after four steps of dialysis, must be negligible, if present at all.

Further checking the purity of the HP preparation, we passed peak F123 through Sephadex G-150 (superfine grade). The single peak of protein obtained was injected into mice, showing specific activity identical with that of F123.

We studied the action of several degrading enzymes (*in vitro*) on the activity of our HP preparation *in vivo*. In this experiment, the HP (80 ng/mouse) was incubated with 500 μ g of trypsin/ml, 500 μ g of pepsin/ml, 100 μ g of deoxyribonuclease/ml or 230 mg of ribonuclease/ml (Morley & Kingdon, 1973) separately for 30 min at 37 °C. After this incubation period, our HP preparation was injected intraperitoneally into mice (in triplicate) and specific incorporation was measured as usual. To obviate

Table 2. Action of several degradative enzymes on the activity of the HP preparation

For details see the text. The values presented are the means ($\pm$ S.D.) for three injected mice.

	Incorporation	
	(d.p.m./ μ g of DNA)	(%)
Saline	38.2 $\pm$ 16.5	100 $\pm$ 43
HP	71.8 $\pm$ 11.4	188 $\pm$ 29
HP (incubation 37 °C, 30 min)	59.0 $\pm$ 1.8	154 $\pm$ 4
HP + trypsin	39.1 $\pm$ 15.3	102 $\pm$ 40
HP + pepsin	33.0 $\pm$ 7.9	86 $\pm$ 20
HP + deoxyribonuclease	59.7 $\pm$ 13.3	156 $\pm$ 34
HP + ribonuclease	62.8 $\pm$ 5.2	164 $\pm$ 13

Fig. 5. Dose-dependence curve of the HP preparation *in vivo*

Results (d.p.m. of [3 H]thymidine/ μ g of liver DNA are means $\pm$ S.D. for three injected mice, assayed in duplicate. See the results section for fuller details.

any loss of activity of the HP during the incubation period, samples of HP, without enzymes, were included. The result is shown in Table 2. The HP in this experiment (80 ng/mouse) increases the uptake of [3 H]thymidine to 188% of the controls, and a loss of activity was observed

after the incubation period. With respect to the actions of the enzymes, it seems that neither deoxyribonuclease nor ribonuclease has any influence on the activity of HP, but that trypsin and pepsin suppress it. (Injections of the enzymes alone do not exert any influence on DNA synthesis in saline controls.) This result corroborates the protein nature of HP.

Fig. 5 shows a typical curve of dose-dependence of our HP preparation *in vivo*. Mice were injected with increasing quantities of HP, after which liver DNA was extracted and specific incorporation measured. This analysis was carried out with all the preparations of HP, so far producing very similar results. We have consistently observed the same dose-dependence profile with different preparations, with a maximum activity at about 150 ng of HP/mouse, which decreases at higher doses. This effect is discussed below.

The effect of HP injection on mitotic index (MI) of rat liver was studied. Mice were injected with saline (controls) or 150 ng of HP/mouse (three mice per group). After 24 h, they were treated with 0.2 mg of colchicine/100 g body wt, and killed 6 h later, and liver slices were processed as described in the Materials and methods section. Some 10000 cells/group were examined in different fields under oil immersion magnification ($\times 1000$) to calculate the percentage of cells in mitosis. The number of mitotic cells in control (saline-injected) mice was 2 (0.02%), and the corresponding value in HP-injected mice was 82 (0.82%). Hepatocytes in control (saline-injected) mice show an extremely low MI, which clearly increases after HP action.

Table 3 reflects organ specificity. In this experiment, mice were injected with saline (controls) or with HP (110 ng/mouse). [3 H]Thymidine (1 μ Ci/g body wt.) was given at 24 h; and 1 h later mice were killed by cervical dislocation, and the liver, spleen, kidney, lung and brain of each mouse was cut into roughly 3 mm cubes, washed with saline and homogenized. DNA was extracted, and radioactivity and total DNA were measured in each case. Specific incorporation was compared between control samples and those injected with HP, the other values being expressed as a percentage of this baseline. Standard deviations are included (mean of four mice, assayed in duplicate). No action of the HP was detected in spleen, kidney, lung or brain. By contrast, the activity in liver was stimulated by about 160% over that of the controls.

Fig. 6 depicts the activity of the HP preparation in a primary liver cell culture. HP was added 24 h after seeding, at increasing concentrations in the range 1–80 ng/ml. At the same time 1 μ Ci of [3 H]thymidine/ml was added, and 24 h later cells were harvested and the

Table 3. Organ specificity of the HP

Mice were injected with HP (110 ng/mouse) and then with [3 H]thymidine as usual. DNA was isolated from liver, spleen, kidney, lung and brain, and specific incorporation measured. Data are expressed in terms of d.p.m./ μ g of DNA and as a percentage of controls (in parentheses) in the same organ, in saline-injected control mice. S.D. values are included ($n = 4$).

	Liver	Spleen	Kidney	Lung	Brain
Control (saline)	38.2 $\pm$ 16.5 (100 $\pm$ 43%)	160.0 $\pm$ 94.7 (100 $\pm$ 59%)	16.8 $\pm$ 6.1 (100 $\pm$ 36%)	35.5 $\pm$ 3.4 (100 $\pm$ 9%)	4.6 $\pm$ 1.7 (100 $\pm$ 36%)
+ HP	97.0 $\pm$ 6.6 (254 $\pm$ 17%)	182.3 $\pm$ 50.4 (114 $\pm$ 31%)	17.7 $\pm$ 4.4 (105 $\pm$ 26%)	31.4 $\pm$ 6.6 (89 $\pm$ 19%)	3.8 $\pm$ 0.8 (83 $\pm$ 17%)

Fig. 6. Dose-dependence of HP *in vitro*

Results are means $\pm$ S.D. ($n = 3$). See the results section for complete details.

DNA was extracted as indicated in the Materials and methods section. Ordinates represent the specific incorporation in terms of d.p.m. ($\times 10^{-3}$)/mg of protein. A high sensitivity to the concentration of HP was observed (increase in the uptake of [^3H]thymidine was detected with addition of as little as 1 ng of HP/ml), reaching a maximum at about 10 ng of HP/ml. With concentrations of HP higher than 15–20 ng/ml, a decrease in the action of HP is observed, to essentially the same values as the controls between 30 and 80 ng HP/ml.

Table 4 shows the effect of HP on $^{22}\text{Na}^+$ uptake in rat liver cell culture. Amiloride was added at 0.4 mM when necessary. This concentration is sufficient to inhibit the uptake of Na^+ by the cell in situations of stimulated uptake by a mitogen (Moolenaar *et al.*, 1982). Ouabain was always present at 1 mM to stop $\text{Na}^+ + \text{K}^+$ -dependent ATPase (Moolenaar *et al.*, 1982). At 24 h after seeding, medium was changed and saline, fetal-calf serum or HP added, together with $^{22}\text{Na}^+$ and amiloride if necessary. The uptake was stopped after 10 min, and radioactivity was extracted and counted as indicated in the Materials and methods section. Both the increase of $^{22}\text{Na}^+$ uptake by HP and its partial inhibition by amiloride are indications that HP is a mitogen for hepatocytes.

Table 4. Effect of HP on $^{22}\text{Na}^+$ uptake in rat liver cell culture

See the Results section for details. Results are means $\pm$ S.D. ($n = 4$).

	$^{22}\text{Na}^+$ uptake	
	(d.p.m./mg of protein)	(% of control)
Saline	2347 $\pm$ 377	100 $\pm$ 16
+ amiloride	2376 $\pm$ 240	101 $\pm$ 10
FCS (10%)	2118 $\pm$ 40	90 $\pm$ 2
+ amiloride	1914 $\pm$ 95	82 $\pm$ 4
HP (10 ng/ml)	16677 $\pm$ 3735	710 $\pm$ 159
+ amiloride	5188 $\pm$ 1920	221 $\pm$ 82

DISCUSSION

The existence of liver growth factor(s) has been postulated by many authors with different approaches. Some of them, using cross-circulation experiments, have shown an increase in liver DNA synthesis of normal rats perfused with blood from partially hepatectomized partners (Moolten & Bucher, 1967; Sakai, 1970; Fisher *et al.*, 1971; Short *et al.*, 1972).

The method that we have used for the purification of our HP preparation includes the detection of its DNA-synthesis-promoting activity by injection into mice of the fractions suspected of being active.

The fact that pools V_0 , F1, F2 and F3 (Fig. 1) show activity in most batches and that this activity was lost (in peaks V_0 , F1 and F2) during the purification process reinforces the difficulty in reaching clear conclusions with respect to the real activity of plasma PH as the initial material.

In Fig. 5, there are several notable points. The stimulation of DNA synthesis is reached by injection of a few nanograms of our HP preparation per mouse. The maximal stimulation (at about 150 ng of HP/mouse) is 2–3 times that of controls. Increases are identical with those found by other authors for injections of semi-purified preparations of other DNA-synthesis promoters (Morley & Kingdon, 1973; Goldberg *et al.*, 1980), lending support to the idea that the maximal increase in DNA synthesis by injection *in vivo* of a hepatotrophic factor could be very close to this order. We have already pointed out above the constant specific activity of HP among different batches. The effect of HP at higher doses (more than 150 ng/mouse) has been consistently observed with different preparations. Although it is difficult to explain from a theoretical point of view, in the literature there exist some experiments performed by different authors, resulting in similar paradoxes (Leffert & Koch, 1978; Leffert *et al.*, 1976). Levitzki *et al.* (1975) have proposed a theoretical model, applied to hormone–receptor systems, in which ligand binding is negatively co-operative, thus predicting that maximal signals can be generated by low ambient hormone concentrations. The interaction of the proposed liver growth factor with its supposed receptor might be explained by this model.

With respect to the organ-specificity of our HP preparation, it seems to be clearly established in regard to the organs studied, but the possibility that other organs could be responsive to the action of HP has not been ruled out.

When HP action in DNA synthesis is studied *in vitro* (Fig. 6), the results resemble the situation *in vivo* rather well (Fig. 5). There is a visible increase in [^3H]thymidine accumulation into DNA, by up to 10 times the control values, with 1–10 ng of HP/ml, the same range of activity of some known growth factors (Barnes & Sato, 1980) in cell culture. We have no explanation for the decrease in the activity of HP from 15 to 30 ng/ml, although the pattern closely resembles again the situation *in vivo* for [^3H]thymidine incorporation (Fig. 5).

As was suggested by Koch & Leffert (1979), an amiloride-sensitive Na^+ influx is necessary to initiate hepatocyte proliferation. This action is clearly shown in Table 4, in which not only is there a net uptake of $^{22}\text{Na}^+$ in response to HP, but this uptake is blocked by the presence of amiloride in the same concentration range used by Koch & Leffert (1979). This event occurs in a

short enough time to suggest that it is a consequence of the addition of HP. The results shown in Table 4 deal with HP working as a mitogen and clearly resemble those obtained with a combination of some known hepatotrophic factors (Richmans *et al.*, 1976).

We thank Dr. Isabel Valverde (Department of Metabolism, Nutrition and Hormones, Clínica de la Concepción, Madrid, Spain) for the radioimmunoassays of glucagon and insulin performed in her laboratory, Dr. Abelardo López-Rivas (Department of Microbiology, Centro de Biología Molecular, Universidad Autónoma, Madrid, Spain) for the 3T3K experiment carried out in his laboratory, and Ms. Martha Messman for her help in the preparation and typing of this manuscript. This work was supported by Grants 83/0626 and 83/0629 from the Fondo de Investigaciones Sanitarias (F.I.S.S.), Spain.

REFERENCES

- Barnes, D. & Sato, G. (1980) *Cell* **22**, 649–655
- Bonney, R. J., Becker, J. E., Walker, P. R. & Potter, V. R. (1974) *In Vitro* **9**, 399–413
- Bucher, N. L. R. & Malt, R. A. (1971) *Regeneration of the Liver and Kidney*, Little, Brown, Boston
- Burton, K. (1968) *Methods Enzymol.* **12**, 163–166
- Dicker, P. & Rozengurt, E. (1978) *Nature (London)* **276**, 723–726
- Fisher, B., Szuch, P., Levine, M. & Fisher, E. R. (1971) *Science* **171**, 575–577
- Goldberg, M., Strecker, W., Feeny, D. & Rubenstroth-Bauer, G. (1980) *Horm. Metab. Res.* **12**, 94–96
- Higgins, G. M. & Anderson, R. M. (1931) *Arch. Pathol.* **12**, 186–202
- James St. Hilaire, R. & Jones, A. L. (1982) *Hepatology* **2**, 601–613
- Koch, K. S. & Leffert, H. L. (1979) *Cell* **18**, 153–163
- Labrecque, D. R. & Bachur, N. R. (1982) *Am. J. Physiol.* **242**, G281–G288
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leffert, H. L. & Koch, K. S. (1978) *Hepatotropic Factors CIBA Found. Symp.* **55**, 61–94
- Leffert, H. L., Koch, K. S. & Rubalcava, B. (1976) *Cancer Res.* **36**, 4250–4255
- Levitzki, A., Segal, L. A. & Steer, M. L. (1975) *J. Mol. Biol.* **91**, 125–130
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacManus, J. P., Franks, D. J., Youdale, T. & Braceland, B. M. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1201–1207
- Moolenaar, W. H., Yorden, Y., de Laat, S. W. & Schlessinger, J. (1982) *J. Biol. Chem.* **257**, 8502–8506
- Moolten, F. L. & Bucher, N. L. R. (1967) *Science* **158**, 272–274
- Morley, C. G. D. & Boyer, J. L. (1977) *Biochim. Biophys. Acta* **477**, 165–176
- Morley, C. G. D. & Kingdon, H. S. (1973) *Biochim. Biophys. Acta* **308**, 260–275
- Oakley, B. R., Kisch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363
- Paul, D., Leffert, H., Sato, G. & Holley, W. R. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 374–377
- Richmans, R. N., Claus, T. H., Pilkis, S. J. & Friedman, D. L. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3589–3593
- Russell, W. E., McGowan, J. A. & Bucher, N. L. R. (1984) *J. Cell Physiol.* **119**, 183–192
- Sakai, A. (1970) *Nature (London)* **228**, 1186–1187
- Sanui, H. & Rubin, H. (1979) *J. Cell. Physiol.* **100**, 215–226
- Short, J., Brown, R. F., Husakova, A., Gilbertson, J. R., Zemel, R. & Lieberman, I. (1972) *J. Biol. Chem.* **247**, 1757–1766
- Von Wyk, J. J. & Underwood, L. E. (1978) *Biochem. Actions Horm.* **5**, 101–148

Received 2 August 1985/7 October 1985; accepted 20 November 1985