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Purification and Partial Characterization of a Liver Cell Proliferation Factor Called Hepatopoietin

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The purification and partial characterization of a liver cell proliferation factor called hepatopoietin are described. Hepatopoietin was isolated from remnant livers or blood plasma of partially hepatectomized rats and purified approximately 13,000-fold. The stokes radius was 2.65 ± 0.2 nm and the apparent molecular weight was calculated to be 38,000 ± 5,000 D. Hepatopoietin is a heat-stable glycoprotein and is organ specific but species nonspecific. In vivo it stimulates about three to four times the DNA synthesis as well as the mitotic rate of the liver of normal rats after i.p. injection. Hepatopoietin is inactivated upon incubation with galactosidase or trypsin-chymotrypsin.

Key words: rat, partial hepatectomy, liver cell proliferation, mitosis rate, characterization

Mammalian liver is characterized by two regulatory schemes: 1) the regulation of the average volume of the hepatocytes, which correlates with the body weight [1], and 2) the regulation of the numbers of hepatocytes. The number of hepatocytes is approximately the same in juvenile and adult animals. Rats, for example, have about 5×10^8 hepatocytes.

Both regulatory schemes are independent of each other [1]. After partial hepatectomy the low proliferation rate of hepatocytes (0–2 mitosis/10^4 cells) increases dramatically in two consecutive surges until the total number of cells is about the same as previously, after which the mitotic rate returns to the normal level. With this process the regenerating liver after partial hepatectomy is a useful model for the exploration of proliferation and growth.

This growth process is reproducible by surgical resection of two-thirds of the liver, causing a number of characteristic alterations in the remnant liver: The hepatocytes lose their content of glycogen and protein and concomitantly increase the cell volume by accumulation of water and lipids. Within 24 hr the turnover of protein and RNA begins to change in order to initiate DNA synthesis and mitotic activity of the quiescent liver cell. The restoration of the initial liver mass is obtained after approximately 2 weeks [2,3].

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There are numerous indications that the increase in proliferation of the hepatocytes after partial hepatectomy is caused by proliferation-active factors. Nevertheless, it cannot be excluded that the increase in proliferation is caused by disappearance of an inhibitor (chalone) existing in normal conditions [4,5].

Transfusion experiments between partially hepatectomized and normal animals were carried out about 15 years ago for the purpose of determining whether liver regeneration might be regulated by humoral regulation processes. Moolten and Bucher [6] and Grisham et al [7] demonstrated in parabiotic experiments that circulating blood of a partially hepatectomized rat stimulates DNA synthesis and mitosis in the liver of the normal rat. Other research groups investigated the influence of the pancreas on liver regeneration [8,9]. Bucher and Swaffield [10] showed that partially hepatectomized rats, with resected pancreas, underwent a normal liver regeneration after additional infusion of insulin and glucagon. The group of Starzl [11] tried to obtain information on the mechanism of liver regeneration by different eviscerations with and without hormonal substitutes.

As demonstrated in these previous investigations, a series of factors and influences exists that are responsible for the stimulation or inhibition of liver regeneration. The present paper describes the purification and partial characterization of a liver cell-proliferation factor called hepatopoietin from partially hepatectomized rats. The biochemical investigations of this factor may contribute to understanding of the control mechanisms of liver cell growth.

MATERIALS AND METHODS

Animals

Female Wistar rats (Institut für Strahlen- und Umweltforschung, Neuherberg, FRG) weighing 95–105 gm were used throughout the experiments. Food (Altromin 1324) and water were given ad libitum. Room lighting was controlled on a 12 hr dark:12 hr light regime. Partial hepatectomy was performed according to Higgins and Anderson [12]. All animals were sacrificed between 8:00 AM and 10:00 AM and the time of operation and injection were calculated accordingly.

Preparation of Hepatopoietin Extract From Remnant Livers After Partial Hepatectomy

Partially hepatectomized rats were killed 12 hr after operation under ether narcosis. The remaining liver (PHL) was rinsed with saline (0.9%). The livers were homogenized with fourfold water (w/v), acidified to pH 5.5 with 0.1 N HCl and denatured by heating at 95°C for 20 min. After centrifugation (15 min, 4.000g) the supernatant was removed and stored. The precipitate was diluted to the original volume with distilled water and heat denaturation and centrifugation were repeated twice. The three supernatants were pooled and lyophilized. The lyophilisate (PHL) was stored at −20°C for later use. Control livers of normal rats were treated in the same way (NL).

Preparation of Hepatopoietin Extract From Blood Plasma After Partial Hepatectomy

Blood from partially hepatectomized rats was removed under ether narcosis 12 hr after operation with a heparinized syringe. The blood from the rats was pooled and centrifuged (15 min, 4.000g). The plasma of the partially hepatectomized rats (PHP) was acidified and heat denaturated as described above. The resulting lyophilized

supernatants were dissolved in distilled water, brought to pH 5.5 with 0.1 N HCl, and incubated for 1 hr at 37°C with 0.5 U neuraminidase (Behringwerke, Marburg, FRG). The excess neuraminidase was inactivated (95°C, 30 min), the mixture centrifuged, and the supernatant lyophilized. The lyophilisate (PHPNeu) was stored at −20°C for later use. Control plasma from normal rats was treated in the same way (NPNeu).

Purification of the Crude Hepatopoietin Extract From Livers (PHL) or Plasma (PHPNeu) of Partially Hepatectomized Rats

Affinity chromatography. Crude extract (2 ml) derived from PHL or PHPNeu was chromatographed on a wheat germ lectin Sepharose 6 MB column (1 × 15 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with 50 mM TRIS buffer pH 7.6. Elution was carried out with 50 mM Tris buffer (10% N-acetyl-glucosamine, pH 7.6) at a constant flow rate of 12.5 ml/hr. Adsorbance at 280 nm was monitored continuously by using an Uvicord (LKB, Bromma, Sweden) photometer. Portions of 2.5 ml were collected for in vivo assay and were subsequently pooled in two fractions followed by lyophilization.

Gel filtration. The lyophilized wheat germ lectin fraction 2 (WGL2) was gel filtered on an Ultrogel AcA 44 column (1.5 × 71 cm, LKB, Bromma, Sweden). Elution was carried out with 25 mM Tris buffer (pH 7.6) at a flow rate of 12.5 ml/hr. Optical density at 280 nm was monitored as described above. The eluate collected in 2.5 ml portions was finally pooled in three fractions, which were lyophilized and stored at −20°C.

Hydrophobic chromatography. The lyophilized Ultrogel fraction 3 (U3) was chromatographed on an Octyl-Sepharose CL-4B column (1 × 9 cm, Pharmacia) previously equilibrated with 1.5 M Tris buffer (pH 7.6). Elution was first carried out with the same buffer (fourfold column volume) at a flow rate of 15 ml/hr. The second step was an elution with a gradient (1.5 M Tris buffer, pH 7.6, and 20% glycerine in distilled water) followed by a step of 20% glycerine (in distilled water). The eluate, collected in 1.25 ml portions, was pooled in three separate fractions, which were desalted (Sephadex G 10, batch technique), lyophilized, and stored at −20°C.

Enzymatic Treatment of the Purified Hepatopoietin Extract

Trypsin-chymotrypsin treatment. The purified hepatopoietin extract (peak O2 after hydrophobic chromatography) was dissolved in distilled water, brought to pH 7.6 with 0.1 N NaOH, and incubated for 2 hr at 30°C with 80 U trypsin and 90 U chymotrypsin (both pure grade; Serva, Heidelberg, FRG). The remaining enzyme activities were destroyed by incubation at 95°C for 30 min. The solution was centrifuged and lyophilized. The lyophilisate was dissolved in 2 ml 0.9% NaCl solution and injected i.p. into normal rats.

β-Galactosidase treatment. The purified hepatopoietin extract was dissolved in distilled water, brought to pH 7.3, and incubated at 37°C for 1 hr with 5 U β-galactosidase (Sigma, München, FRG). The remaining enzyme activity was destroyed by heating (95°C, 30 min). After centrifugation the solution was lyophilized, dissolved in 2 ml saline, and injected i.p. into normal rats.

Stokes Radius and Molecular Weight Determination

Analytical gel filtration (Ultrogel AcA 44 column, Bio-Rad, München, FRG) was carried out for the determination of the stokes radius [13,14] and the determination of the apparent molecular weight [13]. The gel was equilibrated at 4°C with 0.8

N KCl or 1% Triton WR1339 in 50 mM Tris buffer, pH 7.6. Fractions of 2.5 ml were collected at a constant flow rate of 12 ml/hr. The following calibration proteins were used: Stokes radius (SR): lipoxidase from soy beans (SR 3.85 nm), bovine serum albumin (SR 3.53 nm), ovalbumin (SR 2.73 nm), and chymotrypsin from pancreas (SR 2.24 nm); molecular weight (MW): alcohol dehydrogenase (MW 84,000), bovine serum albumin (MW 67,000), β-amylase (MW 57,000), ovalbumin (MW 45,000), and chymotrypsinogen (MW 25,000). For each calibration protein two column runs were carried out.

Protein Determination

Protein concentration was determined by the method of Lowry et al [15] for all steps of the purification.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gels, 5–20%, were prepared and developed according to the method of Laemmli [16]. Protein bands were visualized by Coomassie blue R250 according to Weber and Osborn [17].

In Vivo Assay

Measurement of ^{3}H-thymidine incorporation into DNA. The lyophilized hepatopoietin extracts or the different fractions of purification, redissolved in 2 ml of saline, were injected i.p. into normal rats in the period between 13:00 PM and 14:00 PM. Nineteen hours after injection the rats were injected i.p.with 50 μCi 6- ^{3}H-thymidine (specific activity 25 Ci/mmol). One hour later the rats were killed and the livers were resected and frozen in liquid nitrogen. The isolation of liver cell nuclei was carried out according to Widnell and Tata [18]. DNA in the resultant pellet was extracted according to Schmidt and Tanhauser [19]. DNA was quantitatively determined by Burton's technique [20] and the radioactivity was measured with a liquid scintillation counter and the results expressed as specific activity in dpm/μg DNA.

Mitosis determination. Twenty-four hours after injection of the purified hepatopoietin extract the rats were sacrificed and the livers removed. For histological preparation a part of the lobus caudatus was used.. The tissue samples were fixed with a solution according to Davidson [21]. After fixation, the tissue samples were dehydrated by ethyl alcohol. The samples were treated with methylbenzoate-Celloidin according to the method of Peterfi [22], washed in benzene, and embedded in paraffin. With a microtome (Jung, Heidelberg, RFG) 5 μm slices were made and treated for nuclear staining with a solution according to Weigert [23]. For preservation and examination, the stained slices were fixed in resin (Entellan, Merck, Darmstadt, FRG). The mitosis rates of the liver slices were determined by counting 10^4 cells/slice with a light microscope considering all phases of mitosis.

Dose response. Different amounts of PHL and PHPNeu extracts were injected i.p. into normal rats. After labelling with ^{3}H-thymidine, the specific activity was determined as described above.

One unit is the amount of activity obtained from three livers or 7 ml blood plasma of partially hepatectomized rats. The following amounts were used: ⅓, ⅔, 1, 2, and 3 units, corresponding to about 28, 56, 84, 168, and 252 mg protein for PHL, and 12, 23, 35, 70, and 105 mg protein for PHPNeu.

RESULTS

Purification and Evaluation of Hepatopoietin From Liver or Plasma of Partially Hepatectomized Rats

A summary of all purification steps is shown in Table I. All the steps were carried out, if not mentioned otherwise, at 0–4°C.

Control values of normal rats after saline injections showed a specific activity of about 100 dpm/μg DNA. Fractions of the different purification steps having a specific activity above 240 dpm/μg DNA were defined as positive.

The factor of enrichment from this positive-negative in vivo assay is expressed as the decrease of protein/active unit.

Preparation and test of the hepatopoietin extract from liver or plasma of partially hepatectomized rats. Crude hepatopoietin extract was produced from the remaining liver or from the blood plasma at the same time from three partially hepatectomized rats. The extracts were dissolved in 2 ml saline and injected i.p. into normal rats. The results of ^{3}H-thymidine incorporation are shown in Table II. The extract of normal liver NL as well as of neuraminidase-treated normal plasma NPNeu showed the same values of incorporation activity as the control. The extracts of

TABLE I. Partial Purification of the Liver Cell-Proliferation Factor Hepatopoietin*

	Volume (ml)		Protein (mg/ml)		Total protein (mg)		Factor of enrichment	
Steps of purification	A	B	A	B	A	B	A	B
Liver/blood plasma	31.5	7.0	33.2	70.0	1046	490	1	1
Boiled extract	38.0	38.5	2.2	0.91	83.6	35.15	13	14
Wheat germ lectin–Sepharose 6 MB (WGL2)	20.0	20.0	0.4	0.2	8.1	4.1	129	120
Ultrogel AcA 44 (U3)	24.0	17.5	0.04	0.02	1.0	0.43	1038	1121
Octyl-Sepharose (02)	1.5	2.0	0.05	0.02	0.08	0.04	12913	11667

*The values refer to 7 ml of plasma or three remnant livers from partially hepatectomized rats. The enrichment was determined from the samples which are used for the following purification steps.
A: Purification of hepatopoietin from liver of partially hepatectomized rats.
B: Purification of hepatopoietin from blood plasma of partially hepatectomized rats.

TABLE II. ^{3}H-Thymidine Incorporation of Different Extracts Injected Into Normal Rats

Extract	NaCl[a]	Sham[b]	NL[c]	NPNeu[d]	PHL[e]	PHPNeu[f]
Spec. activity (dpm/μg DNA)	104 ± 22	99 ± 32	100 ± 40	128 ± 31	344 ± 124	462 ± 142
n	8	6	7	7	9	9

[a]Physiological saline as control
[b]Liver extract from sham operated rats
[c]Liver extract from normal rats
[d]Neuraminidase treated plasma extract from normal rats
[e]Liver extract from partially hepatectomized rats
[f]Neuraminidase treated plasma extract from partially hepatectomized rats.

TABLE III. ^{3}H-Thymidine Incorporation After Injection of the Pooled Fractions of the Ultrogel AcA 44 column

Peak	U1	U2	U3
Spec. activity (dpm/μg DNA)	148 ± 11	140 ± 8	296 ± 18
n	6	6	6

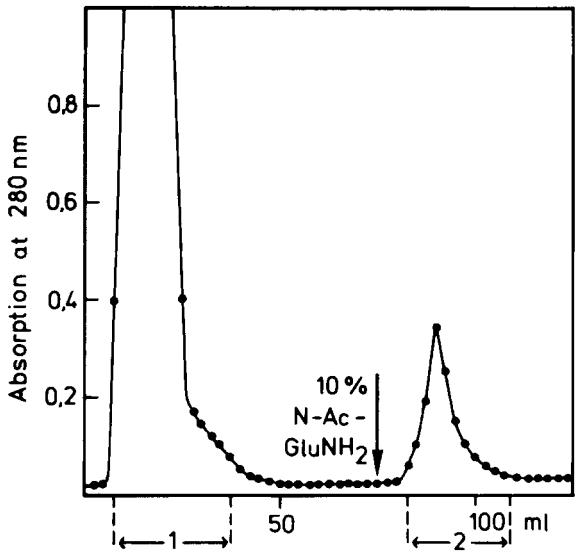

Fig. 1. Affinity chromatography of PHL with wheat germ lectin-Sepharose 6MB.

partially hepatectomized liver PHL and of the neuraminidase-treated plasma of these rats PHPNeu showed a three- to fourfold increase.

PHL and PHPNeu were used for further purification of hepatopoietin.

Affinity chromatography with wheat germ lectin. PHL or PHPNeu were loaded on a wheat germ lectin-Sepharose column. The incorporation activity was found after elution with N-acetyl-glucosamine buffer in the second peak WGL2 (Fig. 1). The specific activity of WGL2 in the in vivo assay was 375 ± 82 dpm/μg DNA (n = 6), whereas WGL1 corresponded with 137 ± 31 dpm/μg DNA to the control values.

Ultorgel AcA 44 gel chromatography. The lyophilized peak WGL2 was loaded to an Ultrogel AcA 44 column. After elution the pooled fractions were tested in normal rats (Table III) An increased incorporation rate was only found in peak U3 (Fig. 2), which indicates a molecular weight below 50,000 D. The enrichment compared to the original material was about 1.100-fold.

Hydrophobic chromatography. The lyophilized peak U3 was loaded on an octyl Sepharose CL-4B column and the gradient followed by a subsequent elution step was applied. Three peaks were obtained and the increased ^{3}H-thymidine incorporation was found in peak O2 (Fig. 3, Table IV). The enrichment compared to the

TABLE IV. ^{3}H-Thymidine Incorporation After Injection of the Pooled Fractions of the Octyl-Sepharose CL-4B Column

Peak	01	02	03
Spec. activity (dpm/μg DNA)	117 ± 12	274 ± 24	121 ± 16
n	5	5	5

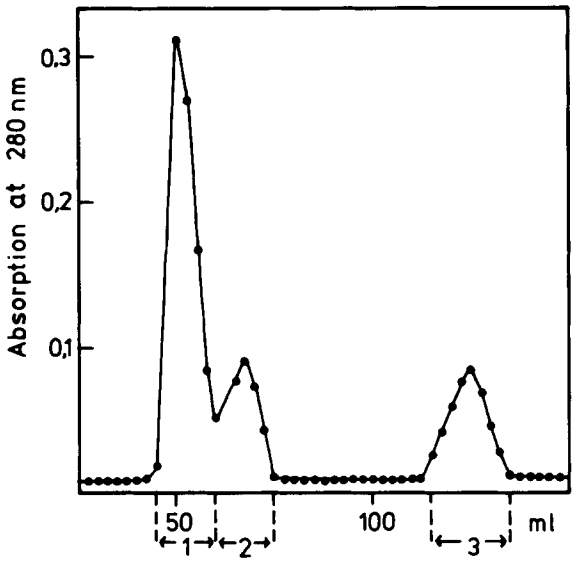

Fig. 2. Gel chromatography of WGL2 with Ultrogel AcA 44.

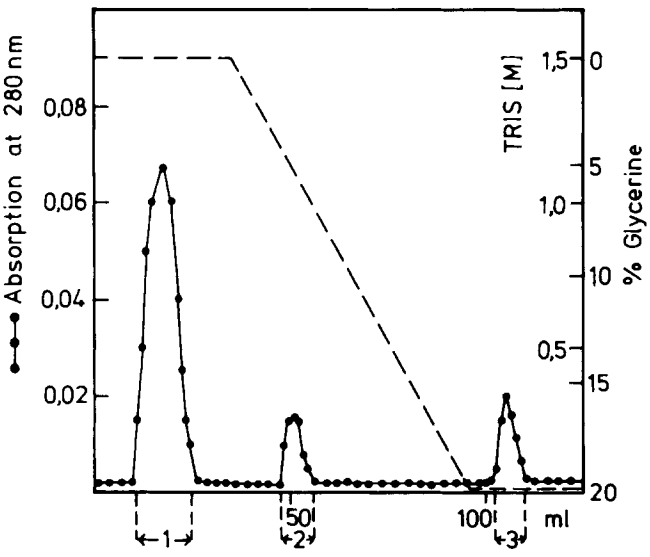

Fig. 3. Hydrophobic chromatography of U3 with Octyl-Sepharose CL-4B.

original material was about 13,000-fold measured by protein concentration/active unit, which was 80 μg of purified PHL.

Enzymatic Treatment of Purified Hepatopoietin

Trypsin-chymotrypsin digestion. The treatment of the purified hepatopoietin (peak O2) with trypsin-chymotrypsin resulted in inactivity, as shown by a decrease of the incorporation rate. The average from five experiments was 97 $\pm$ 40 dpm/μg DNA, which compared to control values (104 $\pm$ 22 dpm/μg DNA).

β-Galactosidase digestion. The digestion of hepatopoietin with β-galactosidase resulted in a diminution of the incorporation rate (103 $\pm$ 14 dpm/μg DNA), which is comparable to control values.

Stokes Radius and Molecular Weight Determination

Stokes radius. The stokes radius was determined by aid of gel filtration (Fig. 4). For the purified hepatopoietin the Stokes radius was 2.65 $\pm$ 0.2 nm (n = 3).

Molecular weight. Purified hepatopoietin was used for gel filtration (Fig. 5), resulting in an apparent molecular weight of 38,000 $\pm$ 5,000 D. As described in Materials and Methods, an SDS gel electrophoresis of the different steps of the hepatopoietin purification was carried out. As can be seen in Figure 6, only a single stained band resulted from the last step O2 of the purification. In comparison to the used marker proteins, this band indicated a molecular weight of about 40,000 D. This value corresponds well with the apparent molecular weight determination above.

In Vivo Assay

DNA synthesis. As is shown in Table II, the extracts from liver (PHL) and from neuraminidase-treated plasma (PHPNeu) of partially hepatectomized rats caused a significant three- to fourfold increase of the ^{3}H-thymidine incorporation into liver

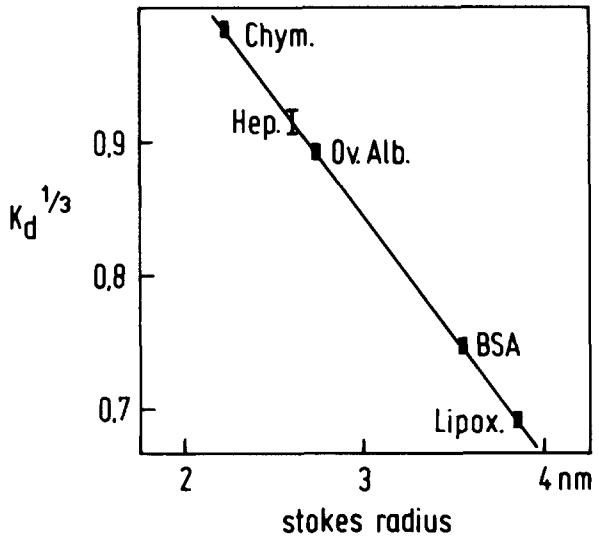

Fig. 4. Stokes radius of purified hepatopoietin (Hep.). Marker proteins: Chym., chymotrypsin from pancreas; Ov.Alb., ovalbumin; BSA, bovine serum albumin; Lipox., Lipoxidase.

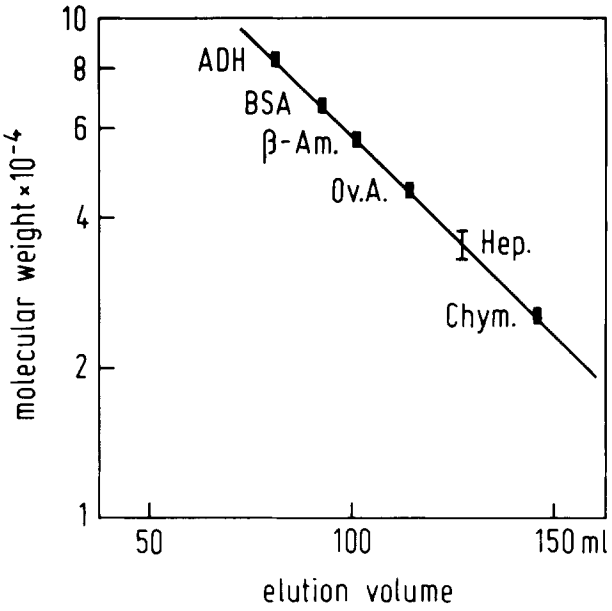

Fig. 5. Apparent molecular weight of purified hepatopoietin (Hep.). Marker proteins: ADH, alcohol dehydrogenase from liver; BSA, bovine serum albumin; β-Am., β-amylase from barley; Ov. A., ovalbumin; Chym., chymotrypsinogen from pancreas.

cell nuclei. The extracts from normal or sham-operated rats corresponded to saline control.

Mitosis determination. Counting 10^4 cells/slice of liver tissue, treated as described in Materials and Methods, showed that there is not only an increase in ^{3}H-thymidine incorporation after hepatopoietin injection but also a real increase of the proliferation rate of liver cells. The mitosis rate in hepatopoietin-treated rat liver was 5–7 mitosis/10^4 cells (n = 3), in contrast to 0–2 mitosis/10^4 cells in saline controls. Injections of extracts from normal rats resulted also in control values.

Dose response. As can be seen in Table V the dose response for PHL and PHPNeu extracts resulted in a minimum trigger level of ⅔ units corresponding to 56 and 23 mg protein, respectively (for definition see Materials and Methods). Below this amount the incorporation rate corresponded to control values. Maximum incorporation was obtained with 1 unit, and higher amounts of hepatopoietin did not further increase the incorporation rate. Analogous concentrations of NL extracts were ineffective and achieved control values (Table V).

DISCUSSION

The results presented show that the remnant liver and the blood plasma of partially hepatectomized rats contain a liver cell-proliferation factor, which we called hepatopoietin. The factor from these two sources differs only in the content of neuraminic acid. Hepatopoietin is found as an inactive prefactor in the blood plasma of partially hepatectomized rats and could be activated after neuraminidase treatment (PHPNeu). Hepatopoietin from the remnant liver as well as from the blood plasma is heat stable, organ specific, and species nonspecific [24,25]. PHL and PHPNeu also

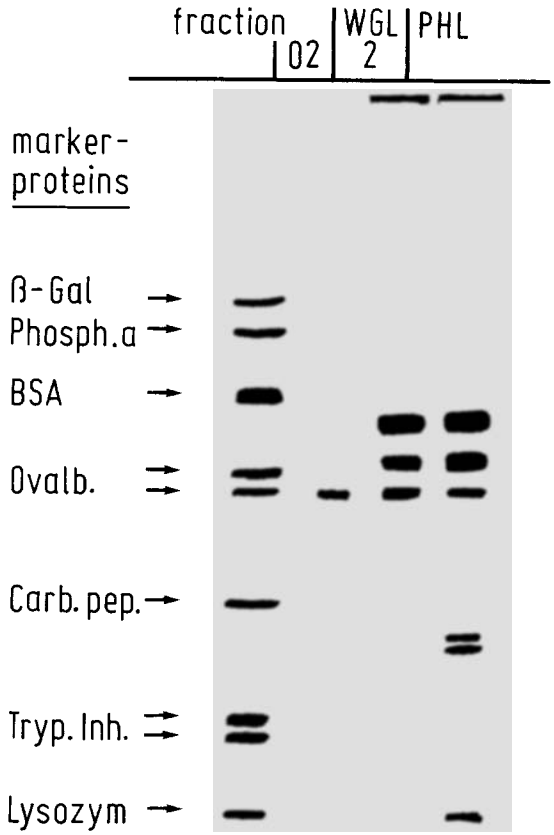

Fig. 6. SDS gel electrophoresis of different purification steps of hepatopoietin. PHL, boiled extract of partially hepatectomized liver; WGL2, after wheat germ lectin column, twofold amount of initial material; O2, after Octyl-Sepharose column, tenfold amount of initial material. Marker proteins: β-Gal, β-galactosidase from Escherichia coli; Phosp.a, phosphorylase from rabbit muscle; BSA, bovine serum albumin; Ovalb., ovalbumin; Carb.pep., carboxipeptidase from pancreas; Tryp.Inh., trypsin inhibitor from soya beans; Lysozym, lysozyme from white of chicken egg.

TABLE V. ^{3}H-Thymidine Incorporation of Different Concentrations of NL and PHL Extracts

Extract	⅓ Unit	⅔ Unit	1 Unit	2 Units	3 Units
PHL extract	125 ± 34	240 ± 47	461 ± 139	446 ± 65	434 ± 33
	n = 4	n = 4	n = 7	n = 4	n = 3
NL extract		107 ± 20	147 ± 35	153 ± 27	
		n = 3	n = 8	n = 3	

increase the incorporation rate of ^{3}H-thymidine in primary liver cell cultures [26]. Behavior of PHL and PHPNeu is similar during the chromatographic procedures and they appear in the same peaks. Enzymatic treatment with trypsin-chymotrypsin or β-galactosidase inactivates both PHL and PHPNeu.

The apparent molecular weight of PHL and PHPNeu was identical 38,000 ± 5,000 D. The determination of the Stokes radius showed also correspondence.

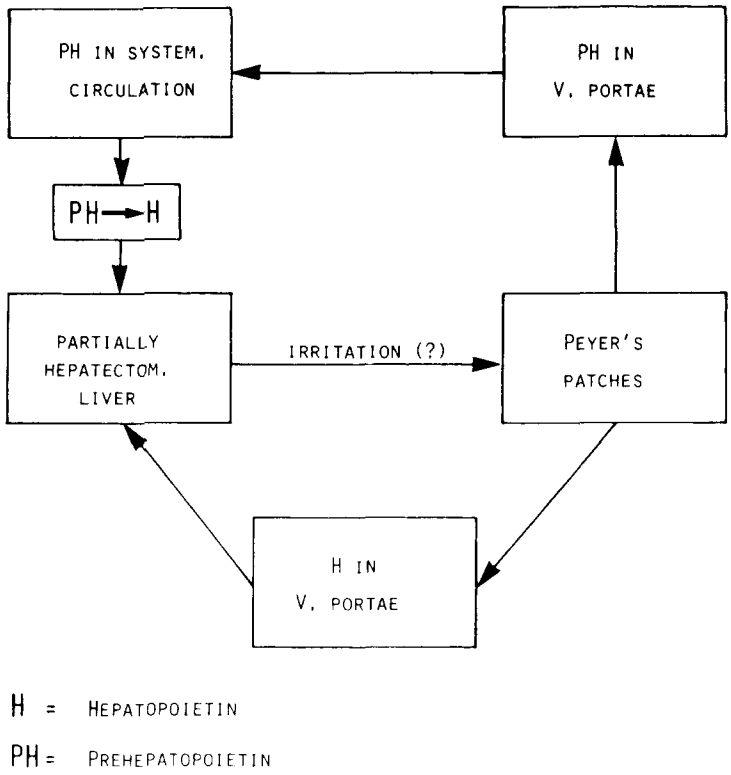

Fig. 7. Scheme for a regulatory system of liver cell proliferation.

These results suggest that hepatopoietin from PHL and PHPNeu is the same factor. It is a glycoprotein with carbohydrate terminal galactose and has a molecular weight of about 38,000 D. On the basis of the agreement between the properties of PHL and PHPNeu and of further results from our group that showed that the Peyer's patches are the origin of hepatopoietin [27], and that DNA synthesis in partially hepatectomized rats seemed to be depressed by simultaneous repeated injections of galactose-containing carbohydrates [unpublished data], we propose the following scheme for a regulatory system of liver cell proliferation [28] (Fig. 7).

In response to partial hepatectomy an irritation appears, secreting hepatopoietin as well as the inactive prehepatopoietin from the Peyer's patches. Hepatopoietin reaches the liver by V. portae and attaches with its carbohydrate terminal galactose to the β-galactose receptors of the hepatocytes, causing their proliferation. Prehepatopoietin, by comparison, circulates in the blood protected by its carbohydrate terminal neuraminic acid. Prehepatopoietin is desialinated and activated by the enzymes neuraminidase or sialyltransferase, which probably originate from the liver cell membranes, causing the second surge of hepatocyte proliferation after partial hepatectomy.

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