

#1643

## CONFERENCE REPORT

# Further Steps of Hepatic Stimulatory Substance Purification

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*The hepatic stimulatory substance (HSS) extracted from weanling rat livers was purified 381,000-fold using chromatographic techniques including nondissociating polyacrylamide gel electrophoresis (nondenaturing PAGE). The activity of this highly purified HSS, named Acr-F<sub>4</sub>, was assessed in two in vivo models. In 40% hepatectomized rats, it produced a fivefold increase in the proliferative rate normally seen following this partial hepatectomy. In Eck fistula dogs, the level of base increase in hepatocyte renewal was amplified threefold by an infusion of Acr-F<sub>4</sub> (50 ng/kg/day). Acr-F<sub>4</sub> had no influence on the regenerative response of the kidney following a unilateral nephrectomy or of the bowel following a 40% resection of the small bowel. On the basis of these findings, it can be concluded that HSS (Acr-F<sub>4</sub>) has a high biological activity and is organ specific.*

**KEY WORDS:** hepatic stimulatory substance; purification; gel electrophoresis.

That liver regeneration could be augmented by extracts prepared from livers in an active proliferative state has been demonstrated by several investigators (1-4). However, all attempts focused on purification and characterization of the putative growth factor in these extracts have produced few and generally inconclusive results. A partially purified factor, hepatic stimulatory substance (HSS), capable of stimulating *in vivo* hepatocyte proliferation (5, 6) has been isolated from weanling rat liver homogenates.

In this paper, data concerning further purification and characterization of HSS is reported, made possible by the use of new laboratory techniques and the introduction of a very sensitive and reliable animal model for assessing HSS activity *in vivo*. Specifically, nondissociating polyacrylamide gel electrophoresis allowed us to obtain a 381,000-fold purification of HSS, eliminating almost all contaminating proteins in the preparation. In addition, the dog with a portacaval shunt coupled with the availability of specific antibodies against HSS made it possible to achieve this level of purification.

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## MATERIALS AND METHODS

**Chemicals and Materials.** Type-V neuraminidase, trypsin, aprotinin, and proteins used as molecular weight markers were purchased from Sigma Chemical Company, St. Louis, Missouri. [*methyl*-<sup>3</sup>H]Thymidine (50-80 Ci/mmol) was obtained from New England Nuclear, Boston, Massachusetts. L-1-Tosylamido-2-phenylethyl chloromethyltrypsin ketone was purchased from Worthington Biochemical Corporation, Boston, Massachusetts. Amicon ultrafiltration membrane filters were purchased from Amicon Corporation, Danvers, Massachusetts. The chemi-

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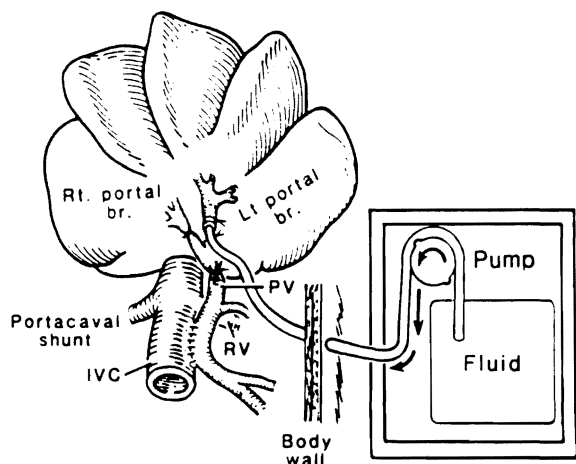


Fig 1. Portacaval shunt model in dogs.

cals required for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, California.

**Animals.** Adult male Fischer (F344) rats (180–200 g), weanling male rats (60–90 g), and male mongrel dogs (15–20 kg) were purchased from Hilltop Lab Animals, Scottsdale, Pennsylvania. They were maintained in temperature- and light- (6 AM to 6 PM) controlled rooms until used. They were given food and water *ad libitum*.

**Surgical Procedures.** Adult rats underwent either a 40% hepatectomy or a sham operation consisting of a laparotomy and manual manipulation of the liver between 7:30 and 9:30 AM using the method of Higgins and Anderson (7). Unilateral nephrectomy and 40% resections of the small bowel were performed as described previously (8).

In dogs, large side-to-side portacaval shunts were constructed with an excision of an ellipse of tissue from both the portal vein and the inferior vena cava and anastomosing the two vessels side to side (Figure 1). The shunts were made completely diverting by individually ligating the main right and left portal trunks distal to the anastomosis of the portal vein to the IVC (9). The tip of a small infusion catheter was placed into the ligated left portal branch within the liver and led through the body wall and

via a long subcutaneous tunnel to a small calibrated finger pump that was placed into a dog jacket (Figure 1).

**HSS Preparation and Purification.** The steps for HSS preparation and purification are summarized in Table 1. These methods (6) yielded an active fraction that has been identified as  $F_{150}$  because it elutes from the column with a 150 mM NaCl gradient.

**Nondissociating PAGE.** An aliquot of 0.6 mg lyophilized fraction  $F_{150}$  resuspended in Tris buffer 0.025 M, pH 8.3, underwent electrophoresis using nondissociating PAGE (10, 11) on 8% acrylamide. With this technique,  $F_{150}$  generates several distinct bands, and the gel can be divided in four zones from which its proteins can be eluted. The eluates, acrylamide fractions 1–4 (Acr  $F_1$ – $F_4$ ) are dialyzed against 150 mM ammonium acetate, lyophilized, and stored at  $-70^\circ\text{C}$  until being tested further.

**In Vivo Determination of Activity of HSS and Its Fractions.** In rats, 6 hr after a 40% partial hepatectomy, control rats were given intraperitoneal injections of 2 ml of 5 mM phosphate buffer, pH 7.4, whereas HSS-treated rats received either  $F_{150}$  or an acrylamide fraction dissolved in 2 ml phosphate buffer, 5 mM, pH 7.4, at the protein concentrations indicated in the tables. Seventeen hours later, 50  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine were injected intraperitoneally, and the animals were sacrificed 1 hr later. Six hours after surgery, the rats that had received a unilateral nephrectomy or 40% resection of the small bowel were treated as described above for the 40% hepatectomized rats.

[ $^3\text{H}$ ]Thymidine incorporation and mitotic index determinations were made as described previously (9, 12). An augmentation of all parameters, beyond the modest response that is present after sham surgery, was considered to be indicative of biologic activity of the liver extracts.

In dogs, the active electrophoretic fraction, Acr- $F_4$  was infused in the left hepatic lobes through the left portal branch as described in Table 2. At the time of sacrifice, liver tissue was obtained from the left and right hepatic lobes and shunt patency and catheter position were verified. The labeling index was determined as described earlier (9).

TABLE 1. PREPARATION OF HSS

Purification steps	Product
1. Remove the liver, immediately after killing by guillotine, between 7:00 and 8:00 AM	
2. Mince and then homogenize the liver in 150 mM sodium acetate buffer, pH 4.65 (35:100 w/v)	
3. Ultracentrifuge homogenate at 24,000 g for 30 min at 4° C	Cytosol fraction (Cyt-F)
4. Heat at 65° C for 15 min	
5. Centrifuge at 30,000 g for 20 min at 4° C, collect supernatant and add to it 6 vol of cold ethanol (1:6, v/v)	
6. Stir at 2–8° C for 2 hr	
7. Centrifuge 30,000 g for 20 min at 4° C	
8. Resuspend precipitate in 0.150 M ammonium acetate, pH = 6	Alcohol fraction (OH-F)
9. Filter OH-F through an Amicon membrane with a molecular weight cutoff of 30,000 Da	
10. Collect the filtrate and concentrate it by a 500-Da cutoff Amicon membrane	Mr 30,000 fraction (30 kDa-F)
11. Lyophilize 30 kDa-F	
12. Resuspend lyophilized 30 kDa-F in phosphate buffer 5 mM, pH 6, and perform chromatography using mono Q HR 5/5 column with a linear 0–200 mM NaCl gradient in phosphate buffer	
13. Collect the chromatographic peak at 150 mM NaCl gradient	150 fraction ( $F_{150}$ )

TABLE 2. PROTOCOL FOR Acr-F<sub>4</sub> INFUSION AND % OF LABELED NUCLEI DETERMINATION IN ECK FISTULA DOGS

Beginning of infusion	6 hr after surgery
Duration of infusion	4 days
Infused material	Acr-F <sub>4</sub> (50 ng/kg body wt/day) in saline solution containing NH <sub>4</sub> acetate 5 mM and bovine serum albumin 0.5 mg/100 ml
Volume infused	25 ml/day
Treatment for labelling nuclei	200 μCi of [ <sup>3</sup> H]thymidine (82.2 Ci/mmol)/kg body wt injected IV two hours before killing

**Determination of DNA Synthesis in Organs Other Than Liver.** DNA synthesis in kidney and small intestine was determined as described previously (8).

**Analyses of Physical and Chemical Properties.** F<sub>150</sub> and Acr-F<sub>4</sub> were tested for trypsin and chymotrypsin sensitivity (13), heat stability, and neuroaminidase sensitivity (14).

**SDS-PAGE.** SDS-polyacrylamide gradient slab gel, using 7.5–20% gel with a 5% stacking gel, was prepared and developed according to the method of Laemmli (15). Both F<sub>150</sub> and Acr-F<sub>4</sub> undergo electrophoresis under these conditions. Protein bands were visualized using Coomassie blue R250 according to the method of Weber and Osborn (16).

**Monoclonal Antibody (Ab).** Murine monoclonal antibodies against Acr-F<sub>4</sub> were raised using PHC 43 and PHC 67 cells (17). The cells were cultured in serum-free medium and the monoclonal Abs were separated by protein-A chromatography. Activity was assessed by ELISA and found to be in the IgG fraction.

**Protein Determination.** Protein content was determined by the method of Lowry et al (18). Submicrogram

quantities were measured using the method of McKnight (19).

**Statistical Analysis.** The unpaired Student's *t* test was used for the statistical analysis of all data.

## RESULTS

In earlier investigations (6), the greatest degree of purification of HSS was obtained using fast protein liquid chromatography. By this technique, F<sub>150</sub> (Figure 2), which was the most active fraction in 40% hepatectomized rats, was prepared. The use of nondissociating PAGE made it possible to further purify F<sub>150</sub> (Figure 3).

The activity of F<sub>150</sub> and its PAGE fractions were compared using 40% hepatectomized rats (Figure 4). The administration of F<sub>150</sub> (3 μg/rat) produced results as previously reported (6). When fractions of

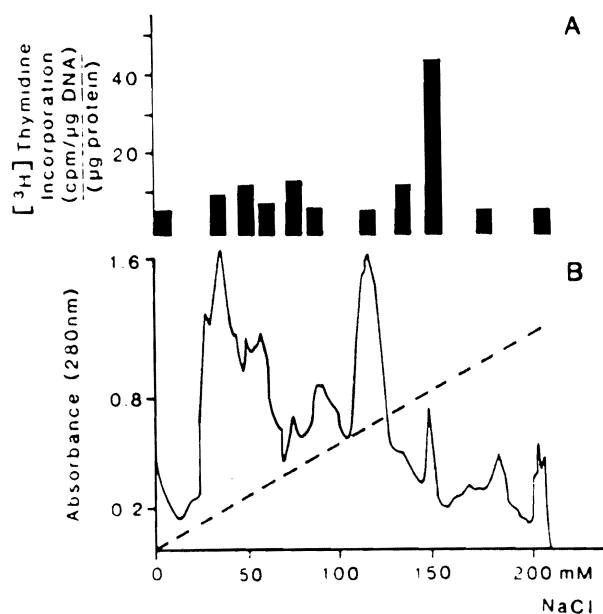


Fig 2. Elution and activity profile of HSS from FPLC. Stimulatory activity of FPLC fractions (A). Elution profile of 30 kDa (B). The amount of protein injected for each fraction was 3 μg. Each bar expresses [<sup>3</sup>H]thymidine average values from six rats. The statistical analysis shows that only the value of F<sub>150</sub> was significant (*P* < 0.0001).

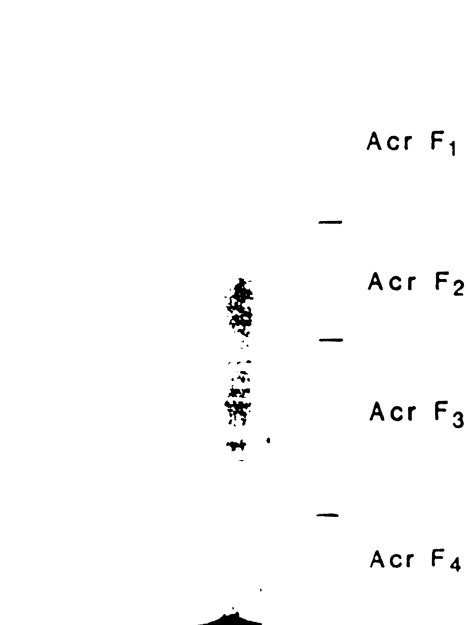
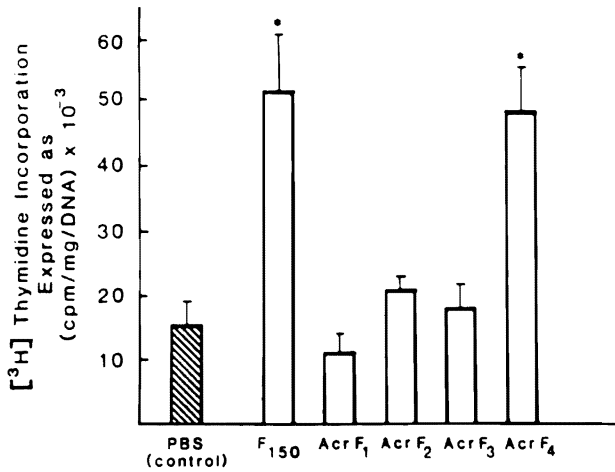


Fig 3. Nondissociating PAGE of F<sub>150</sub>. For our nondissociating continuous system, we used Tris HCl 0.375 M as resolving gel buffer and Tris HCl 0.025 M + glycine 0.192 M as reservoir buffer. The acrylamide gel concentration was 8%. The material used for this run was obtained as pool of ten F<sub>150</sub>.

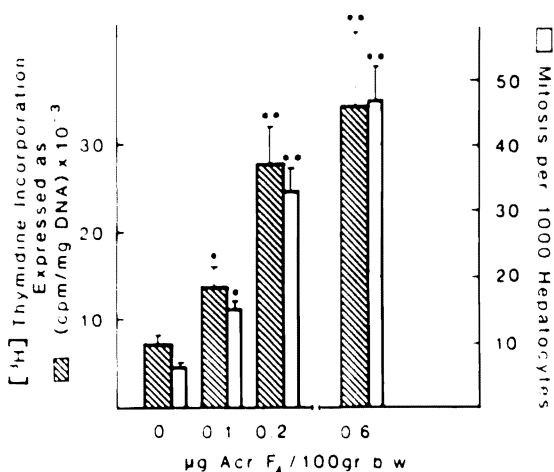
## HSS PURIFICATION



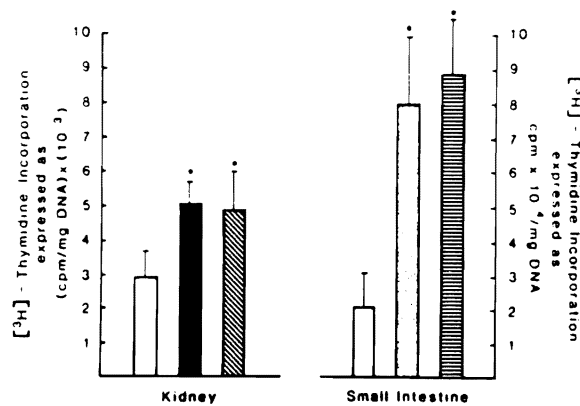
**Fig 4.** Effect of  $F_{150}$  and PAGE fractions (Acr-F<sub>1</sub>-F<sub>4</sub>) on DNA synthesis in 40% hepatectomized rats. The experimental conditions are described in Materials and Methods. The values expressed by the bars are averages  $\pm$  SD from no less than 15 rats. All HSS fraction were dissolved in 2 ml PBS at the following concentrations:  $F_{150}$  1.5  $\mu$ g/ml; Acr-F<sub>1</sub> 0.05  $\mu$ g/ml; and Acr-F<sub>2</sub>-F<sub>4</sub> 0.15  $\mu$ g/ml. \* $P < 0.01$  versus control.

Acr-F<sub>1</sub>-F<sub>4</sub> were tested, the only fraction with stimulatory activity similar to that of  $F_{150}$  was found in Acr-F<sub>4</sub>.

The results shown in Figure 5 demonstrate a dose-effect relation between the amount of Acr-F<sub>4</sub> injected and the resultant increase in hepatocyte DNA synthesis and the number of mitoses enumerated. In addition, with the highest dose of Acr-F<sub>4</sub> (0.6  $\mu$ g/100 g body wt), the activity achieved was fivefold greater than the background response in control animals.



**Fig 5.** [<sup>3</sup>H]Thymidine incorporation and mitotic index in 40% hepatectomized rats treated with different doses of Acr-F<sub>4</sub>. The experimental conditions are described in Materials and Methods. The values expressed by the bars are averages  $\pm$  SD from no less than 15 rats. Acr-F<sub>4</sub> doses were dissolved in 2 ml PBS.



**Fig 6.** [<sup>3</sup>H]Thymidine incorporation in kidney and small intestine from normal rats and Acr-F<sub>4</sub>-treated or untreated rats with unilateral nephrectomy or 40% resected small intestine. The values represented by the different bars ( $\square$  controls,  $\blacksquare$  unilateral nephrectomy,  $\square$  partial enterectomy,  $\square$  Acr-F<sub>4</sub> treatment + partial enterectomy), are the means  $\pm$  SD from 10 rats.

To evaluate the organ specificity of Acr-F<sub>4</sub>, rats with either a unilateral nephrectomy or a 40% resection of the small bowel were tested. No increase in DNA synthesis was found in the contralateral residual kidney or in the remaining small intestine (Figure 6).

The experiments performed in dogs demonstrate that when Acr-F<sub>4</sub> was administered as a continuous infusion beginning 6 hr after portacaval shunt in the left portal vein, the mitotic rate tripled in the left liver lobe while no effect was seen in the right side of the liver. This effect was completely eliminated with the addition of anti-Acr-F<sub>4</sub> monoclonal antibody to the infusion fluid (Table 3). The monoclonal antibody vehicle was inert when tested alone.

Table 4 summarizes the physicochemical characteristics of Acr-F<sub>4</sub>. It contains one major protein band with a molecular weight of about 14,000 (Fig-

**TABLE 3.** EFFECT IN DOGS OF LEFT PORTAL BRANCH INFUSION FOR 4 DAYS OF Acr-F<sub>4</sub>, BEGINNING 6 HR AFTER PORTACAVAL SHUNT

Substance	No. animals	Labeled nuclei 1000 hepatocytes	
		Right lobe	Left lobe
Vehicle	3	4.4 $\pm$ 0.6	4.8 $\pm$ 0.4
Acr-F <sub>4</sub>	3	4.4 $\pm$ 0.5	12.2 $\pm$ 1.0†
Acr-F <sub>4</sub> + Monoclonal Ab*		4.1 $\pm$ 0.2	4.3 $\pm$ 0.3

\*A mixture of active IgG(s), adding up to 150  $\mu$ g of protein daily, was added to the vehicle containing Acr-F<sub>4</sub> and incubated for 2 hr at 37 °C before infusion.

†Significantly different from control ( $P < 0.001$  compared to all other groups).

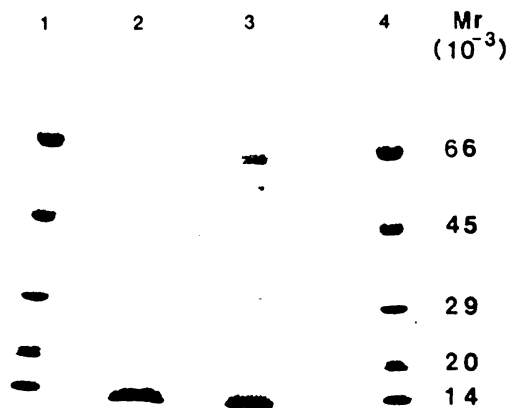


Fig 7. SDS-PAGE of  $F_{150}$  and Acr- $F_4$ . The gel preparation is described in Materials and Methods. Lanes 1 and 4 = standard mixture (molecular weights are multiplied by  $10^{-3}$ ); slot 2 = Acr- $F_4$ ; slot 3 =  $F_{150}$ .

ure 7). Experiments conducted in 40% hepatectomized rats demonstrated that Acr- $F_4$  is heat-resistant and is not digested by neuroaminidase, whereas it is sensitive to proteolytic enzymes (data not shown).

## DISCUSSION

The idea of a specific intrinsic liver growth factor was conceived almost 40 years ago when Teir and Ravanti (20) and Blomqvist (21) first reported a growth stimulatory activity in crude mesh extracts of weanling and regenerating rat liver but not in extracts from normal adult rat liver. Since then, a large number of studies (1-6) have suggested that regenerating liver is a source of a growth stimulator which is specific for the liver (Table 5).

Among the substances proposed and studied, HSS has been studied most extensively by LaBrecque and coworkers (1, 2), Starzl et al (3), and Francavilla et al (5, 6).

Data reported previously (6) and the new data in this paper regarding Acr- $F_4$  are summarized in Table 6. A 381,000-fold increase in activity over the original material was achieved using the 40% hepatectomized rat model as the test system. The activity present in this fraction (Acr- $F_4$ ) is not species-specific, as demonstrated by the results obtained in dogs as well as rats and produced a dose-response that was specific for the liver (Figure 6).

The HSS found in weanling rat liver also has a powerful regenerating or growth effect on dog liver as assessed by the Eck fistula model. The degree of stimulation achieved with 50 ng/kg/day was as potent as gram quantities of crude cytosol (3, 4) and

was as pronounced as the most potent well-recognized hepatotrophic substance currently available: insulin (9). In common with insulin (9) and crude cytosol (4), purified HSS affects only the directly infused liver tissue with little spillover to the uninfused liver. This suggests that it is largely degraded or consumed within a single pass through the liver, leaving little or none available to effect the contralateral hepatic lobes.

In an earlier report (6), it was shown that HSS prepared under these conditions loses its *in vitro* activity while retaining its *in vivo* activity. Thus it has not been possible to compare the HSS purified by LaBrecque et al (22) and Fleig and Hoss (23), which remained active *in vitro* with Acr- $F_4$ . The explanation for the disparities between *in vivo* and *in vitro* growth stimulation seen with Acr- $F_4$  and these other fractions will not be resolvable until these substances are known.

In conclusion, the retention of *in vivo* activity of a highly purified HSS fraction, the ability to abolish the stimulatory activity of this fraction with specific monoclonal antibodies, and the organ specificity of Acr- $F_4$  suggests that its complete identification should be close at hand.

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TABLE 4. PHYSICOCHEMICAL CHARACTERISTICS OF Acr- $F_4$

Mol wt (Da)	14,000
Heat resistance	+
pH stability	4.5-7.5
Alcohol stability	+
Resistance to	
Trypsin	-
Chymotrypsin	-
Neuroaminidase	+

HSS PURIFICATION

TABLE 5. SUMMARY OF THE LITERATURE

Investigator	Time period	Biological source	Name of substance	Biological assay system	Resistance to				Response organ/ species	Purification (%)	M <sub>r</sub> *
					Heat	Try- psin	Chymo- trypsin	Neuro- amidase			
Blomqvist	1957	NbReL†		<i>in vivo</i> : NR <i>in vitro</i> : HTC cells							
LaBrecque	1975-1987	WRL-ReRL	HSS	<i>in vivo</i> : 40% HeR <i>in vitro</i> : L-929 fibroblast	+	-	-	+	Yes/No	110,000	14,000-15,000
Hatase	1979	NL		<i>in vivo</i> : N and 34% HeR <i>in vivo</i> : dog with portacaval shunt	-	-					30,000
Starzl	1979	ReL from 70% He dog	Hepato-						Yes		
Goldberg	1980-1985	ReL from 70% He rat	poinetin	<i>in vivo</i> : NR	+	-	-	+	Yes/No	13,000	38,000
Terblanche	1980	Re dog L		<i>in vivo</i> : 40% HeR	+	-	-	+	Yes/No	38,000	15,000-50,000
Francavilla	1984-1985	WRL	HSS	<i>in vivo</i> : 34% He female R and WR <i>in vitro</i> : hepatocyte cells	+				No		14,000-25,000
Schwarz	1985	ReL from 70% He R and pig		<i>in vitro</i> : NR-6 line fibroblast	-	+	-	-			
Lieberman	1984	Mouse plasma membrane		<i>in vivo</i> : NR	-						
Fleig	1986	ReL from 60% He rabbit									

\*Determined by SDS-PAGE.

†L, liver; N, normal; Nb, newborn; R, rat; Re, regenerating; He, hepatectomized; W, weanling.

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TABLE 6. STEPS OF PURIFICATION AND BIOLOGICAL ACTIVITY OF F<sub>150</sub> AND Acr-F<sub>4</sub> FROM WEANLING RAT LIVER\*

Maternal injected	Protein (µg/rat)	DNA synthesis (cpm/mg DNA)	Specific activity (units/mg protein)	Purification-fold
Cytosol	7.5 × 10 <sup>4</sup>	43,350 ± 8,820	0.02	
OH-F	1.0 × 10 <sup>4</sup>	66,350 ± 11,350	0.30	15
30 kDa-F	0.27 ± 10 <sup>4</sup>	63,520 ± 13,220	1.05	52
F <sub>150</sub>	3	54,380 ± 10,200	762	38,100
Acr-F <sub>4</sub>	3 × 10 <sup>-1</sup>	49,350 ± 7,084	7620	381,000

\*All data, with the exception of the ones regarding Acr-F<sub>4</sub> (see figure 4), are from our previous publication (6). The purification scheme of HSS has been described in Table 1. The [<sup>3</sup>H]thymidine incorporation in a 40% hepatectomized rat given an injection of PBS was 16,550 ± 3000 cpm/mg DNA. The numbers are averages from no less than 20 different rats ± SD.

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