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**Induction of Hepatocyte Stimulating Activity by T<sub>3</sub> and Appearance of the Activity despite Inhibition of DNA Synthesis by Adriamycin\***A. Francavilla<sup>1</sup>, P. Ove<sup>2</sup>, D.H. Van Thiel<sup>3</sup>, Mona L. Coetzee<sup>2</sup>, S.-K. Wu<sup>4</sup>, A. DiLeo<sup>1</sup> and T.E. Starzl<sup>4</sup>Department of Gastroenterology, University of Bari, Bari, Italy<sup>1</sup>, Department of Anatomy<sup>2</sup>, Department of Gastroenterology<sup>3</sup>, Department of Surgery<sup>4</sup>, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A..**Summary**

A hepatocyte stimulating activity (HSA) has been extracted from rats that had received an injection of a pharmacological dose of T<sub>3</sub> 20 hours earlier. The injection of HSA from T<sub>3</sub>-treated rats into different recipient rats that had previously had 40% of their liver removed resulted in a significant increase in hepatic DNA synthesis. The injection of saline or HSA from normal rat liver had little or no effect on hepatic DNA synthesis in recipient rats. HSA from the T<sub>3</sub>-treated rats also stimulated DNA synthesis in Novikoff hepatoma cells and primary hepatocytes in culture, and in isolated normal rat liver nuclei in a nuclear incorporating system. In further experiments in which the increased DNA synthesis that follows partial hepatectomy was blocked by adriamycin, HSA appeared in these non-regenerating livers. This latter observation had indicated that the development of HSA is not merely an accompaniment of DNA synthesis.

**Key-Words:** Hepatocyte Stimulating Activity (HSA) – T<sub>3</sub> – Hepatic DNA Synthesis – Rat

**Introduction**

Extracts from livers which have increased hepatocyte proliferation contain a hepatocyte stimulatory activity (HSA) which can cause or amplify a regeneration-like response in third party test animals, and which can be demonstrated by tissue culture assays. HSA has been demonstrated in weanling rat livers (*LaBrecque* and *Bachur* 1982), and in the regenerating liver remnants of normal rats (*LaBrecque* and *Pesch* 1975; *Hatase*, *Fujii*, *Kuramitsu*, *Itano*, *Takahashi*, *Murakami* and *Nisida* 1979) and dogs (*Starzl*, *Terblanche*, *Porter*, *Jones*, *Usui* and *Mazzoni* 1979; *Terblanche*, *Porter*, *Starzl*, *Moore*, *Patzelt* and *Hayashida* 1980) after partial hepatectomy.

T<sub>3</sub> has been shown to cause a regeneration-like response in normal rat livers (*Klein*, *Chou*, *Short* and *Ove* 1981; *Short*, *Klein*, *Kibert* and *Ove* 1980). We report here evi-

dence that the livers of rats treated with this hormone contain HSA. In further experiments in which the increased DNA synthesis that follows partial hepatectomy was blocked by adriamycin, HSA appeared in these non-regenerating livers. This latter observation has indicated that the development of HSA is not merely an accompaniment of DNA synthesis.

**Materials and Methods****Animals**

Male Sprague Dawley rats received food and water ad libitum. They were kept in a temperature and light-controlled room. Rats weighing less than 100 g were considered weanling rats. Partial hepatectomy was performed according to *Higgins* and *Anderson* (1931). In sham-operated animals, the liver was manually manipulated and returned into the abdomen. Operations were performed between 7:30 and 9:00 AM.

**Materials**

Leibovitz L-15, Swimms S-77, Dulbecco's Modified Eagle's Medium (DME) Antibiotic/Antimycotic (penicillin, streptomycin, fungizone), and calf serum were purchased from GIBCO, Grand Island, New York. Fetal Bovine Serum was obtained from KC Biologicals Inc., Lenexa, Kansas. Collagenase Type 1A, trypsin inhibitor, T<sub>3</sub> (3,3',5'-triiodothyronine), and the unlabelled deoxyribonucleoside triphosphates were from Sigma Chemical Company, St. Louis, Missouri. [<sup>3</sup>H] thymidine and [<sup>3</sup>H] thymidine 5'-triphosphate were obtained from ICN Pharmaceuticals, Inc., Irvine, California.

**Determination of DNA synthesis in animals**

To test for HSA in an ethanol precipitate of liver extracts, partially (40%) hepatectomized rats were used. Six hours after the operation they were injected (I.P.) with 5 ml of HSA substance (5 mg/ml of an ethanol precipitate as described under "preparation of HSA"). Eighteen hours after HSA injection, the animals were injected (I.P.) with 10 µCi [<sup>3</sup>H] thymidine and killed 2 hours later. Livers were removed and citric acid nuclei prepared (*Coetzee*, *Short*, *Klein* and *Ove* 1982). DNA synthesis was determined as previously described (*Ove*, *Coetzee* and *Morris* 1971; *Ove*, *Coetzee* and *Morris* 1973). In some experiments, animals were not pulsed *in vivo*. Instead, the liver was removed and 400–600 mg liver slices were prepared and incubated in Krebs-Ringer solution pH 7.45 for 2 hours under 95% O<sub>2</sub>–5% CO<sub>2</sub> at 37° in the presence of 2 µCi [<sup>3</sup>H] thymidine. Slices were then homogenized in 0.1 M citric acid and citric acid nuclei were prepared followed by the determination of DNA synthesis as above.

**Preparations of HSA**

HSA was prepared as reported by *LaBrecque* and *Pesch* (1975). Livers or hepatoma tissue was homogenized in 35% (w/v) ice cold

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0.15 M NaCl. Homogenates were heated at 65° for 15 min and centrifuged at 30,000 xg for 20 min. Six volumes of cold ethanol were added to the resulting supernatant and the mixture was stirred for 2 hours in ice and again centrifuged at 37,000 xg for 20 min. The pellet was re-dissolved in H<sub>2</sub>O, 5 mg/ml final concentration, and frozen if not used immediately. Protein was determined by the method of Lowry (Lowry, Rosebrough, Farr and Randall 1951).

#### Isolation of hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats, 150–200 g, by the *in situ* 2 step collagenase perfusion technique, essentially as described by Seglen (1976). The cells were dispersed with gentle mincing and agitation in 100 ml Leibovitz (L-15) medium and centrifuged at 50 xg for 3 min. The cells were washed twice and resuspended in L-15 medium, 20 mM Hepes pH = 7.4, 10 uM dexamethasone, 1 uM insulin, 1 uM T<sub>3</sub>, 0.2% bovine serum albumin, 5% fetal calf serum, 0.15% glucose, and 1% antibiotic/antimycotic. Viability was determined by the trypan blue exclusion test and cell number with a hemocytometer. Only preparations with a viability of over 75% were used.

Cells were distributed at a cell density of 2–2.5 x 10<sup>6</sup> cells per 60 mm diameter dish in 4.0 ml medium. The medium was changed at 4 hr and the cells maintained at 37°C.

At 24 hr the medium was replaced with a serum-free L-15 medium and again at 48 hr, at which time the HSA fractions to be tested were added. Cells were exposed for 16 hr to 2 uCi [<sup>3</sup>H] thymidine per dish at 8 hr following fraction addition.

To determine DNA synthesis the cells were harvested by scraping the dishes and rinsing the dishes with 3.0 ml PBS. The rinse was combined with the cell suspension and centrifuged at 500 xg for 10 min. The pellet was dissolved in 1 ml 1 M NaOH and heated for 10 min at 80°C. Samples were cooled and trichloroacetic acid was added to a final concentration of 5% to precipitate the DNA. Incorporation of [<sup>3</sup>H] thymidine into DNA was determined as previously described (Ove, Coetzee and Morris 1973).

#### Novikoff hepatoma cell cultures

Stock cultures of Novikoff N 15167 hepatoma cells were maintained in 100 mm diameter tissue culture dishes. The medium, DME, was supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin, and 5% calf serum. The atmosphere of the incubator was maintained at 5% CO<sub>2</sub>.

Cells for assays were set up in 60 mm diameter culture dishes at a cell density of 5 x 10<sup>5</sup> per dish in 4.0 ml DME-5% calf serum. When cells had grown to a density of 2 x 10<sup>6</sup> per dish, the medium was replaced with DME-0.1% calf serum. Twenty-four hr later medium was again changed (0.1% calf serum-DME) and the HSA fractions added. Cells were exposed for 2 hr to 0.25 uCi [<sup>3</sup>H] thymidine at 20–22 hr. Cells were harvested and thymidine incorporation determined as described for hepatocytes.

#### Isolation of nuclei and incorporation of [<sup>3</sup>H] TTP

Nuclei were isolated from rat liver as previously described (Ove, Coetzee and Morris 1973). The nuclei were washed once with 0.3 M sucrose and were immediately used for assay. An aliquot of the nuclear suspension was removed for determination of DNA (Burton 1968).

The reaction mixture contained (0.5 ml final volume) 50 umol Tris-HCl, pH 7.5; 2 umol MgCl<sub>2</sub>, 4 umol 2-mercaptoethanol; 80 umol KCl, 1 umol ATP; 0.04 umol each dATP, dCTP and dGTP and 0.02 umol [<sup>3</sup>H] TTP (50 uCi/umol). The reaction was stopped at times indicated by the addition of 1 ml 1 M NaOH and radioactivity determined as previously described (Ove, Coetzee and Morris 1973).

## Results

### DNA Synthesis in Donors of Extracts

The baseline DNA synthesis in weanling rats was more than double that in normal control animals that had received saline injections (Table 1). Synthesis was increased more than 8 fold by T<sub>3</sub> injection in normal female rats, and 6 fold in normal males. The response to T<sub>3</sub> was intermediate between that in controls and that after 70% hepatectomy.

The injection of adriamycin immediately after hepatectomy completely blocked the DNA response, and in high doses even suppressed the natural level of DNA synthesis (Table 1).

### In Vitro Testing of Extracts

DNA synthesis twenty-four hours after 40% hepatectomy was double that found in normal rats. This elevated baseline was augmented slightly by liver extracts from sham-operated donors as shown in Table 2. DNA synthesis was increased 5 to 6 times by extracts from weanling or regenerating livers, and by extracts of livers from T<sub>3</sub>-injected rats. Even though DNA synthesis had been inhibited by adriamycin following hepatectomy in extract donors (Table 1), HSA was found in these non-regenerating livers. The results in Figure 1 show that DNA synthesis reaches a maximum around 24 hours after the injection of HSA. Maximal incorporation of [<sup>3</sup>H] thymidine into DNA also occurs around 24 hr following partial hepatectomy or the injection of T<sub>3</sub>.

### In Vitro Testing of Extracts

Extracts from normal liver did not affect thymidine incorporation in Novikoff hepatoma cells indicating the absence or low levels of HSA. In contrast, HSA was present in the livers of weanling rats and liver remnants of animals which had been prepared with a 70% hepatectomy or an injection of T<sub>3</sub> as shown in Table 3.

A partial fractionation of weanling liver HSA could be achieved by a stepwise ethanol precipitation. The fractions were prepared by adding 1 volume of ethanol to the 65° heated supernatant followed by stirring for 2 hours in ice and centrifugation at 37,000 xg for 20 min. This procedure was repeated 5 more times. The resulting precipitates were designated fractions I–VI. The activity was recovered in fractions V and VI with no significant stimulatory activity in fractions I–IV. For this procedure 20 mg protein of the 65° supernatant were precipitated with successive volumes of ethanol and 2.75 mg, 7.18 mg, 4.12 mg, 1.62 mg, 0.76 mg and 0.83 mg protein were recovered in fractions I–VI respectively.

The results with Novikoff tumor cell cultures were confirmed in normal hepatocytes, although there were variations in the degrees of response (Table 4) and again, the preparation, especially from weanling rats, was more effective at a lower than a higher concentration.

**Table 1** Hepatic DNA synthesis in weanling, T<sub>3</sub>-injected and partially hepatectomized rats

Treatment or Condition of Rat	Incorporation	[ <sup>3</sup> H] thymidine cpm/mg DNA
Weanling rats (100 grams)	(7)	2630 ± 420
Normal rats-saline injection-(180–220 grams)	(21)	1260 ± 340
200 µg T <sub>3</sub> injection ♀ rats	(12)	10310 ± 560
200 µg T <sub>3</sub> injection ♂ rats	(12)	7450 ± 380
70% hepatectomy + 0	(21)	17420 ± 1020
70% hepatectomy + 4 mg adriamycin	(4)	1190 ± 200
70% hepatectomy + 6 mg adriamycin	(4)	340 ± 110
70% hepatectomy + 8 mg adriamycin	(4)	90 ± 20

The partially hepatectomized rats were injected with 10 µCi [<sup>3</sup>H] thymidine at 21 hours after the operation.

T<sub>3</sub> injected rats were injected with the [<sup>3</sup>H] thymidine 23 hours after T<sub>3</sub> injection.

T<sub>3</sub> and adriamycin were injected IP.

Adriamycin was injected following partial hepatectomy.

[<sup>3</sup>H] thymidine was injected into the tail vein and animals were killed 1 hour later.

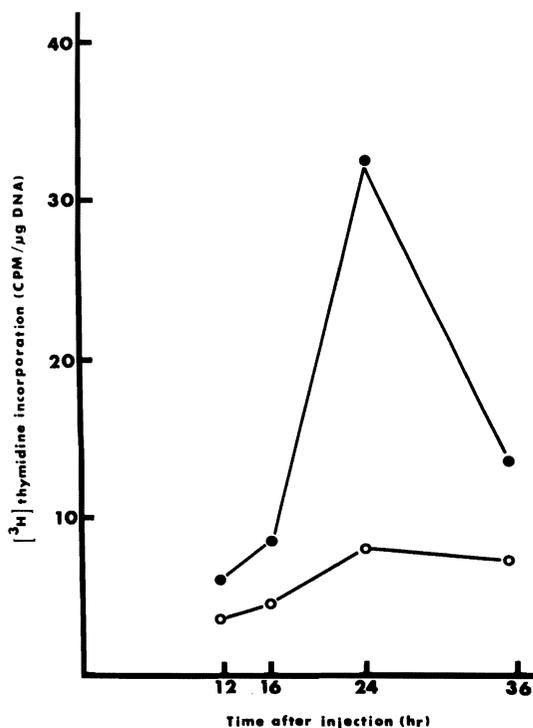
The numbers are the averages of the number of rats shown in parentheses ± SD.

**Table 2** Stimulation of hepatic DNA synthesis by different liver extracts

Source of HSA	[ <sup>3</sup> H] thymidine incorporation cpm/mg DNA
Normal rat + Saline	(60) 2480 ± 380*
Sham operated	(8) 4040 ± 690
Weanling rat	(16) 13160 ± 1680
70% hepatectomy	(14) 12480 ± 2240
T <sub>3</sub> injected rats	(8) 11900 ± 1510
70% hepatectomy + 6 mg adriamycin	(4) 7280 ± 520

\* This level of DNA synthesis is double that found in normal rats (see Normal rats, Table 1), and represents the response to 40% hepatectomy.

The rats used in these assays had 40% of their liver removed at 9 A.M. HSA from the indicated sources was injected (25 mg protein containing HSA in 5 ml H<sub>2</sub>O) IP six hours after the operation. At 9 A.M. the next day, animals were injected with 10 µCi [<sup>3</sup>H] thymidine and killed 1 hour later. All animals weighed between 200 and 250 g. The numbers are the averages of the number of rats indicated in parentheses ± SD.



**Fig. 1** DNA synthesis at different times after HSA injection. Animals were injected with 25 mg protein containing HSA at zero time. At 10, 14, 22 and 34 hours, animals were injected with 10 µCi [<sup>3</sup>H] thymidine and killed 2 hours later. Incorporation of [<sup>3</sup>H] thymidine into hepatocytes was determined as described in "Methods". Each point on the graph represents the average of two determinations. ●—●, injection of HSA prepared from 70% hepatectomized rats. ○—○, injection of saline.

The hepatocyte nuclei isolated from normal unoperated rats which were given an intraperitoneal saline injection 24 hours prior to sacrifice had low incorporation of [<sup>3</sup>H] TTP (Figure 2A). Incorporation was increased moderately ( $P \leq 0.10$ ) when the pretreatment was with weanling extract, and markedly ( $P \leq 0.05$ ) when the treatment was with T<sub>3</sub>. P values were determined by the Student's t-test.

In rats that were preconditioned by 40% hepatectomy a day before isolation of nuclei, the baseline level was higher (Figure 2B). Further increments in [<sup>3</sup>H] TTP incorporation were seen in the rats given injections of extracts from weanling ( $P \leq 0.05$ ) and T<sub>3</sub> injected rats ( $P \leq 0.05$ ).

**Table 3** Stimulation of DNA synthesis in Novikoff hepatoma cells by HSA from various sources

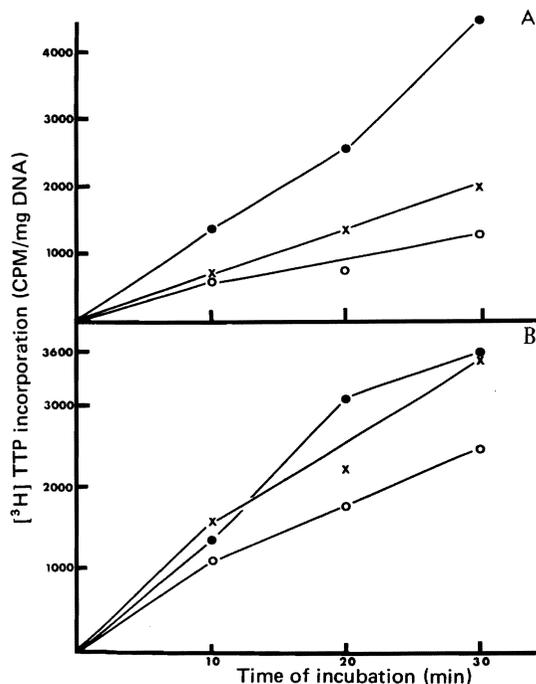
Source of Extract	µg protein of HSA		
	50	100	200
None		640 ± 110	
Sham operated rats	683 ± 84	627 ± 27	397 ± 62
Weanling rats	966 ± 151	1222 ± 185	857 ± 45
70% hepatectomy	1402 ± 210	1856 ± 150	1229 ± 160
T <sub>3</sub> injected rats	774 ± 53	1030 ± 120	1325 ± 82
EtOH fractions from weanling rats	V	928 ± 105	1990 ± 270
	VI	986 ± 75	1594 ± 110

The assay has been described in "Materials and Methods". Cell density was between 1.5 and 2 x 10<sup>6</sup> at termination of experiment. Cells were exposed to 0.5 µCi [<sup>3</sup>H] thymidine per dish for 2 hours, 20 hours after addition of HSA in a total volume of 4 ml medium containing 0.1% calf serum. Numbers are the averages of 3 or more individual dishes ± S.D.

**Table 4** Stimulation of DNA synthesis in primary cultures of hepatocytes by liver extracts

Source of HSA	Protein Added (µg)	[ <sup>3</sup> H] thymidine Incorporation CPM/10 <sup>6</sup> cells	% Increase
None	0	1630 ± 95	—
Sham hepatectomy	50	1862 ± 167	14
	100	1741 ± 220	7
Weanling rat	50	3717 ± 960	128
	100	2633 ± 325	62
Adult 70% hepatectomy	50	1891 ± 41	16
	100	2266 ± 196	39
T <sub>3</sub> injected rat	50	2103 ± 253	29
	100	2543 ± 211	56

Hepatocytes were plated at a cell density of 2 x 10<sup>6</sup> cells per 60 mm dish in 4 ml medium. Medium was changed at 4 and 24 hours. Additions were made as indicated at 24 hours and cells were pulsed for 16 hours with 2 µCi [<sup>3</sup>H] thymidine per dish. Label was added 8 hours after HSA additions. Numbers are the averages of 3 determinations ± S.D.



**Fig. 2** Stimulation of DNA synthesis in nuclei isolated from rats injected with HSA. Animals were injected with HSA, T<sub>3</sub> or saline and nuclei were isolated 20 hours later. [<sup>3</sup>H] TTP incorporation was determined as described in "Methods".

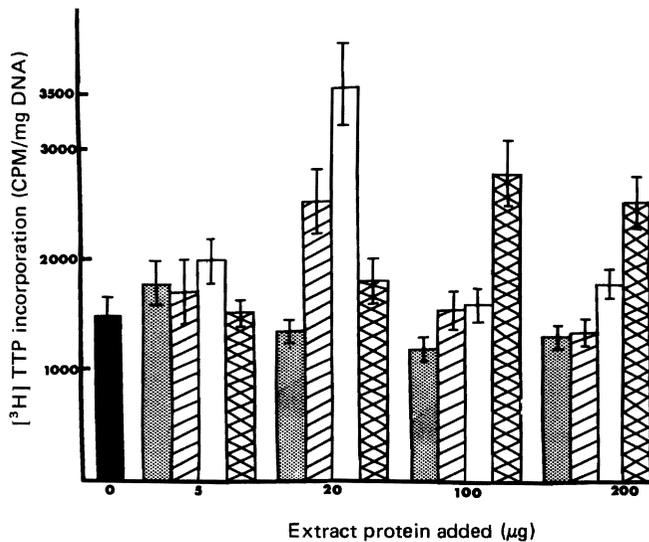
**2A** Injections into normal rats: ●—●, injection of T<sub>3</sub>, 200 µg/100 g; x—x, HSA from weanling rats; o—o, saline.

**2B** Injections were made into animals that had 40% of their liver removed 4 hours before the injection. ●—●, HSA from T<sub>3</sub>-injected rat; x—x, HSA from weanling rats; o—o, saline.

Nuclei were isolated from normal uninjected rats and were incubated with [<sup>3</sup>H] TTP in the presence or absence of HSA. As shown in Figure 3 incorporation was increased with extracts from weanling, 70% hepatectomy and T<sub>3</sub>-treated donors with no stimulation due to extracts from normal sham operated animals.

### Discussion

Evidence is provided that at least some events associated with liver regeneration are influenced by hormones (Starzl,



**Fig. 3** Stimulation of DNA synthesis in isolated nuclei by liver extracts. The assay procedure has been described in "Materials and Methods". Control value is the average of 21 determinations and the values with extracts added are from 3 determinations each.

- = Control
- ▨ = Sham operated adult rat extract
- ▩ = 70% partially hepatectomized rat HSA
- ◼ = T<sub>3</sub> injected rat HSA
- ▤ = Weanling HSA

Francavilla, Porter and Benichou 1978; Francavilla, Porter, Benichou, Jones and Starzl 1978). It has been shown before that a single injection of a pharmacological dose of T<sub>3</sub> resulted in a dramatic increase in DNA synthesis in the liver (Klein et al. 1981; Short et al. 1980). Maximal DNA synthesis occurred between 20 and 24 hours after the injection, a time course similar to DNA synthesis induced by the removal of 70% of the liver. The mechanism of this stimulation has not been resolved. From our present data it seems that one of the mechanisms by which T<sub>3</sub> induces hepatic DNA synthesis might be the induction or activation of a humoral factor (HSA) which in turn stimulates hepatic DNA synthesis.

Humoral and cytoplasmic factors similar to HSA have been reported from a number of laboratories (LaBrecque and Bachur 1982; LaBrecque and Pesch 1975; Hatase et al. 1979; Starzl et al. 1979; Terblanche et al. 1980; Starzl and Terblanche 1979; Moolten and Bucher 1967). All of these have in common that they can be extracted only or in greater quantity from proliferating liver or from the serum of rats in which the liver cells are proliferating. Our results indicate that HSA is not just a byproduct of DNA synthesis. When DNA synthesis following partial hepatectomy was completely inhibited by the injection of adriamycin, HSA could still be extracted from liver remnants. Furthermore, HSA could be extracted from liver remnants of 70% hepatectomized animals 16 hours after the operation. These data are not shown in the results. An increased DNA syn-

thesis is barely detectable at 16 hours and it is not until 20 hours after the operation that substantial DNA synthesis occurs.

At present we do not know whether HSA is newly synthesized in response to removal of part of the liver or in response to T<sub>3</sub> injections, or whether activation of a dormant molecule is involved. Since identical extraction procedures resulted in stimulatory activity whether regenerating liver or liver from T<sub>3</sub> injected animals were used, it appears that the same substance in both preparations causes the stimulations. This can only be confirmed once HSA has been substantially purified. Another possibility is that T<sub>3</sub> causes a release of HSA from storage. Attempts to inject T<sub>3</sub>-treated or 70% hepatectomized rats with a dose of cyclohexamide that would inhibit protein synthesis, resulted in death of the animals before the time that we normally prepare the extracts. HSA does seem to be associated with a protein, however. Treatment of the ethanol precipitate containing HSA with protease or trypsin resulted in the loss of HSA.

In many instances increased [<sup>3</sup>H] thymidine incorporation was not proportional to the amount of HSA added. There are many reports indicating that the liver contains inhibitors of DNA synthesis and cell proliferation (Lieberman and Ove 1960; Kuramitsu, Matsui, Tokuda and Hatase 1982; Klein, Coetzee, Madhav and Ove 1979; McMahon and Iype 1980), one of which has been shown to be arginase (Lieberman and Ove 1960, Klein et al. 1979), others to be inhibitors of thymidine kinase (Klein et al. 1979). It is most likely that our HSA fractions contain, in addition to the stimulating activity, some inhibitors. Indeed our results with a stepwise ethanol precipitation indicate that it might be possible to remove such inhibitors from the stimulating activity. A better dose response could be shown with fractions V and VI than with most of the HSA preparations.

It is too early to speculate on the mechanism of action of HSA, but the results of the experiments with isolated nuclei suggest that it has possibly a direct effect on chromatin. T<sub>3</sub> added to such nuclei does not stimulate [<sup>3</sup>H] TTP incorporation, nor does T<sub>3</sub> stimulate cell proliferation if added *in vitro*.

The use of T<sub>3</sub> to produce HSA in normal or adult rats seems superior to other methods that involve hepatic injury such as partial hepatectomy or administration of CC1<sub>4</sub>. We are now attempting the further purification of HSA. Only after considerable purification will we be able to identify the active component and can design experiments that will give us some insight into its mechanism and site of action.

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