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BIOCHEMICAL AND PHARMACOKINETIC STUDIES WITH ^{125}I-LABELED LEUCOTROFINA

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Leucotrofina (L), a partially purified calf-thymus extract, is effective in minimizing myelosuppression and in restoration of defective immunological function in a variety of diseases. However, no pharmacokinetic studies were made hitherto concerning the rate of influx and efflux of L-peptides at the level of different animal tissues. One of the possibilities to study these aspects is to prepare radioisotope labeled peptides and after introducing them into organism, to determine the rate of incorporation and of efflux. We prepared with the aid of gel-chromatography and affinity chromatography a fraction containing L-peptides labeled with ^{125}I with high specific radioactivity. After introduction of this fraction into a medium containing isolated rat hepatocytes, the ability of L-peptides to enter into cells and to bind to cell organelles and membranes was demonstrated. By introduction of L-peptides via i.p. route, high amounts of radioactivity were detected in bone marrow, kidney, liver, thymus, spleen and endocrine glands.

Thymus and its peptide hormones have crucial importance for the maturation of the T-lymphocyte series. Thymic influences may contribute to the sustenance of immunobiological resistance of animal organisms. Interest has been generally focussed on the use of thymic peptide extracts for the restoration of normal T cell function, especially in selected immunodeficiency disorders (1—5). A thymic preparation, Leucotrofina (Ellem Industria Farmaceutica Spa, Milan, Italy) has been effective in minimizing myelosuppression caused by chemotherapy used in the treatment of neoplastic disorders (6—9). However, no data were reported hitherto on the distribution of Leucotrofina in tissues, its affinity to tissues and cells and cell organelles, or on the mechanisms of action at cellular level. In our experiments we tried to prepare labeled thymic peptides with the aid of radioactive iodine isotopes (^{125}I), which could be bound to aromatic amino acids (thyrosine, phenylalanine and histidine) (10—11) and to study the distribution in tissues and cells.

MATERIAL AND METHODS

Preparation of radioactive iodine-labeled peptides: 40 μg Leucotrofina (solid lyophylised powder purchased from ELLEM Spa, Milan, Italy) were dissolved in 20 μl phosphate buffer (0.04 M; pH, 7.4), treated with 1 mCi aqueous solutions of $^{125}I_3^- Na^+$ and 20 μl chlor-

amine-T (80 μg) under continuous shaking with a micromagnetic stirrer for 30 seconds. After shaking (100 μl Na_2CO_3 (240 μg) and 200 μl $I_3^-K^+$ (2 mg) were added quickly to stop the oxidative reaction. Pipettings of 20—200 μl were made with Hamilton pipettes. For the first step a Sephadex G-15 column was used for purification of radioactive labeled peptides, prepared with the aid of diethylbarbiturate buffer (pH, 7.4). The effluent solution (15 drops) was collected with the aid of an automatic fraction collector (Medimpex, Hungary) and checked for radioactivity with a Gamma-type spectrometer (NZ-138 A). The first radioactive peak containing radioiodine labeled peptides was passed through a Sephadex B-50 column. Further purification was made by affinity chromatography using a SP-Sephadex C-25 column.

Intracellular distribution : The distribution of ^{125}I-labeled thymic peptides from Leucotrofina was studied after *in vitro* administration into a medium containing isolated rat hepatocytes prepared from 300 mg normal liver tissues of adult female rats (Wistar, albino strain). Liver cells were suspended in Krebs-Ringer phosphate buffer (pH, 7.4), containing 17 μM glucose and $2.25 \cdot 10^5$ CPM/ml radioactive peptide fraction. Incubation was made at 37.4°C in a Warburg apparatus. Cell organelles and cytosol were separated after homogenization of cells in 0.25 M sucrose-Tris buffer (pH, 7.4) with the aid of a Potter-Elvehjem homogenizer with teflon pestle. The separation of cell organelles and cytosol was made by ultracentrifugation using a Janetzky VAC-602 type device at 4°C as described in an earlier published work (12). Radioactivity of samples was counted in a scintillation liquid spectrometer (Betaszint BF-5003) at 10°C with 99 per cent efficacy for ^{125}I.

Pharmacokinetic study was carried out using 40—50 days old male or female Wistar rats injected intraperitoneally with $5.32.10^5$ CPM (in 0.5 ml solution) of ^{125}I-labeled Leucotrofina peptides (^{125}I-LP). Another group of animals received the same amount of radioactive anorganic iodine solution collected from the chromatography column. Animals were killed by cervical dislocation and exsanguination. Tissues, organs, blood and urine were examined for their content of radioactive materials depending on the time after administration.

RESULTS AND DISCUSSIONS

The data concerning the intracellular distribution of ^{125}I-LP separated by gel-filtration (fig. 1), administered *in vitro* into a medium containing rat hepatocytes, revealed their ability to enter into cells (table 1). After a 10 minute lasting incubation, 62.61 per cent of total incorporated radioactivity was detected in the nuclear fraction, especially in non-specific bound form. After treatment of nuclear fraction with Triton X-100 solution (1 %), 90 per cent of radioactivity was eluated suggesting that the ^{125}I-labeled peptides were bound tightly to the external nuclear membranes (12) and perhaps only a small amount of radioactivity could enter into nucleus. A continuous decrease of radioactivity of nuclear extract was noticed depending on time. After 10—30 minutes the redistribution of radioactivity suggests the dissociation of peptides from nuclear membrane and incorporation into ribosomal, mitochondrial and cytosol fractions.

Pharmacokinetic studies with *in vivo* administered ^{125}I-LP showed a rapid uptake into liver, kidney and bone marrow (figs. 2—3). The specific radioactivity was the highest in the case of bone marrow, followed by kidney, liver, thymus and blood serum. At the level of bone marrow, the radioactivity remained at a high value after 24 hours too. Other organs and tissues, e.g. : spleen, endocrine glands, striated muscle contained only small amounts of radioactive compounds. Brain tissue incorporated a very small amount of ^{125}I-LP, suggesting little affinity of central nervous system for thymic peptides or perhaps the blood-brain barrier opposed influx of these types of peptides. A great percentage of radioactivity was eliminated into urine in the first hour after adminis-

tration (16555 CPM; 23.68 per cent), between 1 – 23 hours 69.16 per cent (44862) and between 23 – 24 hours only a little amount (8505 CPM), suggesting a relatively high turnover time.

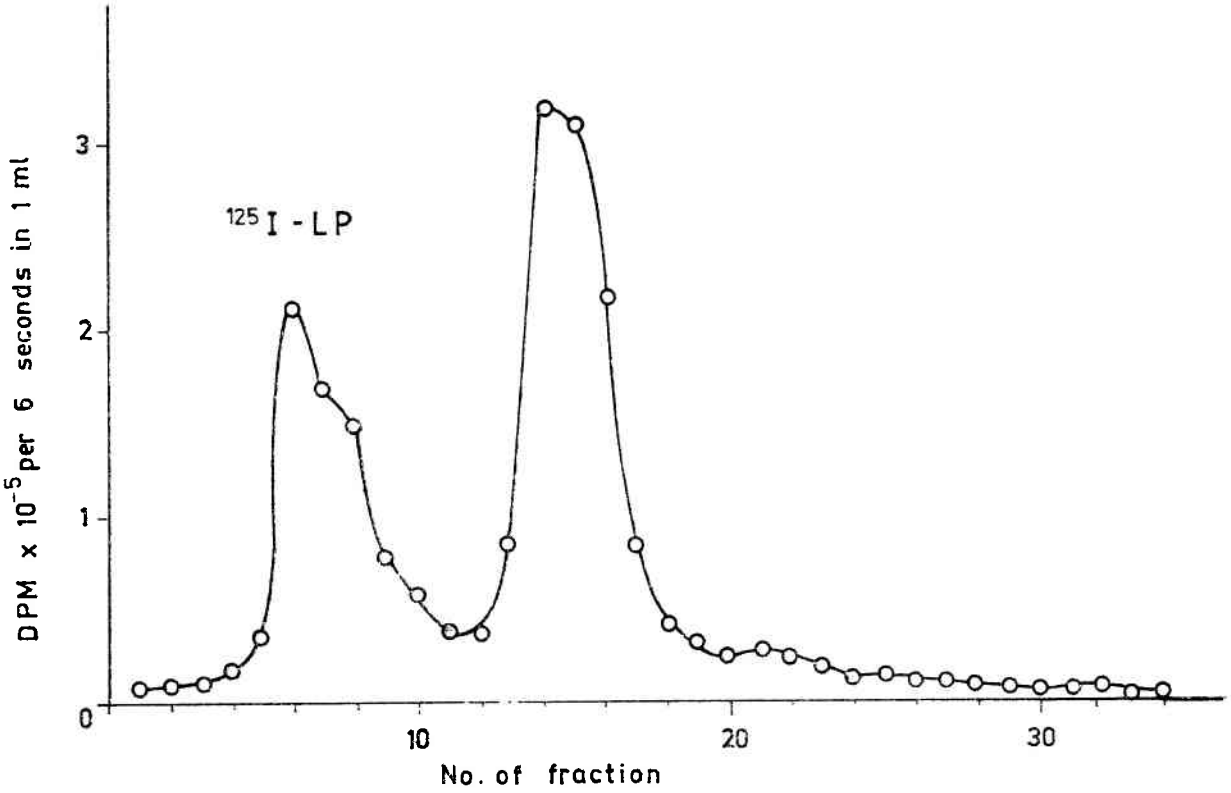

Fig. 1. – Gel-chromtography of ^{125}I-LP on Sephadex G-15 column.

Table 1

In vitro incorporation of ^{125}I-L-peptide fraction into isolated rat liver cell fractions

Cell fraction	Time (min)	Total DPM*	Per cent of total administered	Per cent of total cell DPM
Nuclear fraction (1000 × g sediment)	10	6440	2.862	62.61
	30	4056	1.802	47.77
	60	1053	0.468	25.06
Mitochondrial fraction (10.000 × g sediment)	10	165	0.073	1.60
	30	369	0.164	4.35
	60	227	0.101	5.40
Ribosomal fraction (105.000 × g sediment)	10	184	0.082	1.79
	30	154	0.068	1.81
	60	256	0.114	6.09
Cytosol fraction (105.000 × g supernatant)	10	3496	1.553	33.99
	30	3912	1.739	46.07
	60	2666	1.185	63.44

* Total administered radioactivity : 225.000 DPM

All data presented are the arithmetical media of three determinations.

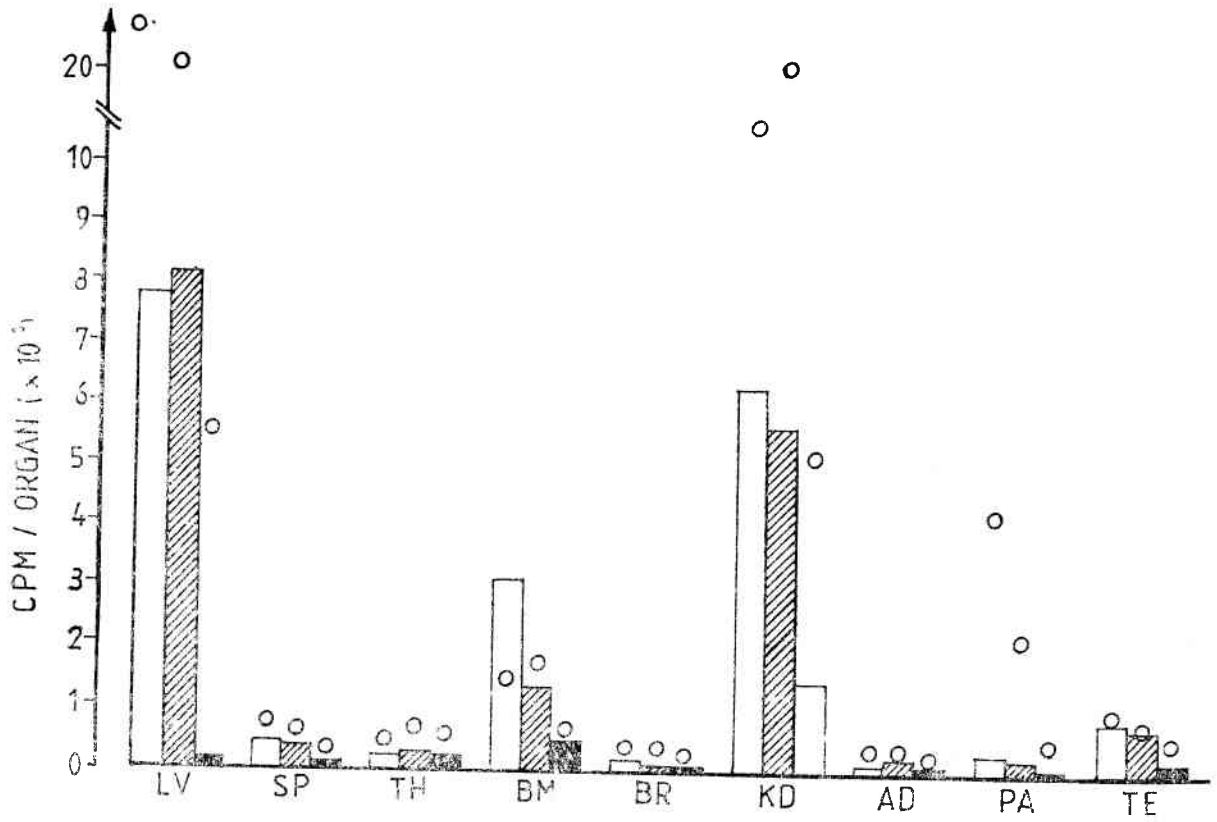

Fig. 2. — ^{125}I-Radioactivity of whole rat organs. (LV = liver, Sp = spleen, TH = thymus, BM = bone marrow, BR = brain, KD = kidney, AD = adrenals, PA = pancreas, TE = = testicles, O = anorganic iodine, □ = 10 minutes, ▨ 60 minutes, ■ = 24 hours after administration).

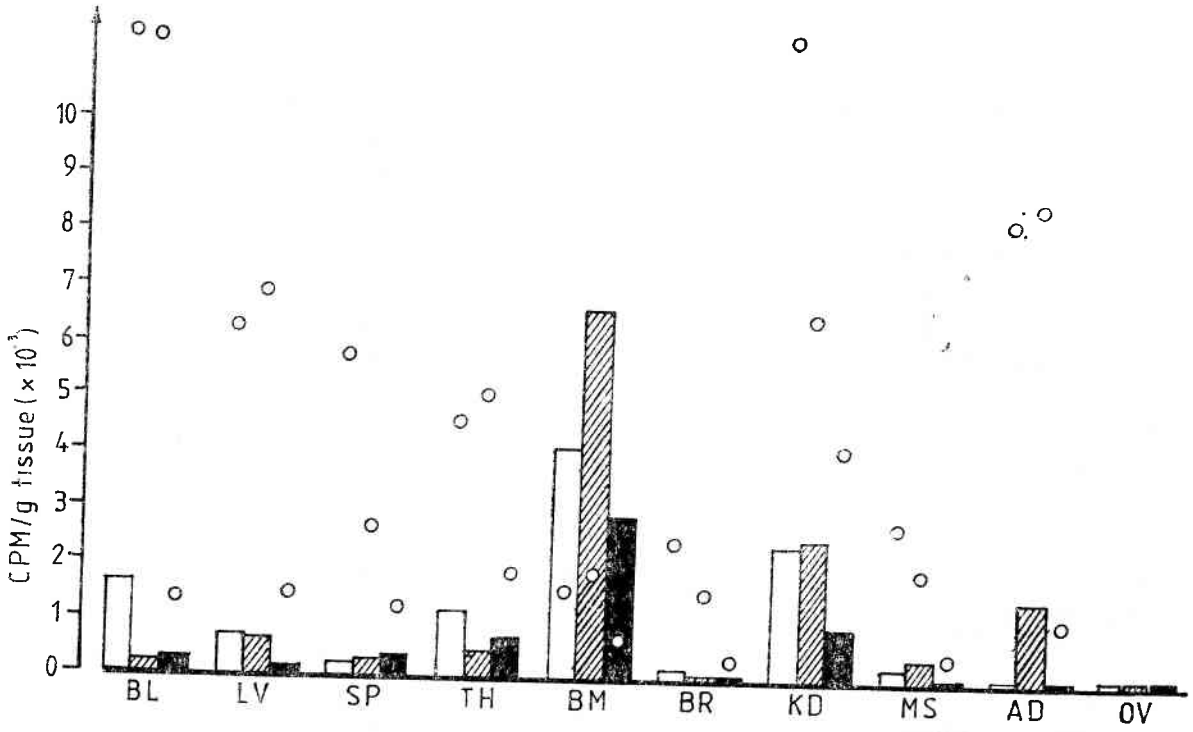

Fig. 3. — Specific affinity of different tissues and organs of rats injected i.p. with ^{125}I-LP. (MS = striated muscle, OV = ovary).

These results suggest the preferential uptake of Leucotrofina-peptides into the bone marrow, which is in good agreement with the hypothesis that this organ is a "target" one for thymic peptides. Our earlier results showed that Leucotrofina administered *in vivo* enhanced the rate of (1—^{14}C) leucine incorporation into proteins of gamma-irradiated rat bone marrow, spleen, liver and thymus (13). This observation is perhaps not accidental, because the induction of protein synthesis in these organs could be in relation with their affinity to thymic peptides. It must be mentioned that the dynamic changes of anorganic ^{125}I were completely different from the influx and efflux of ^{125}I-LP (figs 1 and 2) validating our hypothesis concerning the affinity of some organs for Leucotrofina-peptides.

These studies represent a good evidence that therapeutically administered thymic peptides enter into cells and show high affinity for their target organs, involved in the maintenance of immunological capacity of animal organism. Kouttab and Twomey (14) demonstrated that Thymomodulin (the active ingredient of Leucotrofina), survives *in vivo* administration (oral or parenteral) and modulates the maturation of T cells. We believe that ^{125}I-LP has similar or identical chemical composition with Thymomodulin, and its binding to bone marrow, thymus, spleen, liver and ohter cells represents the first step in the mechanism of action of this drug.

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