The Specific Labeling in Hypertonic Medium of a Spleen Protein

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The biosynthesis of a 12000 dalton protein species released into the medium by mouse spleen was resistant to the inhibitory effect of hypertonic medium on protein synthesis. This protein fraction comprised 10% or more of the total radioactive protein in the medium but could hardly be detected in the spleens. It was labeled very often to a higher extent in diseased animals. It is not related antigenically to either β-microglobin, mouse-interferon, or to mouse immuno-globulins. A preferential uptake into cells of certain organs, which, when themselves incubated in organ cultures did not synthesize and release the protein, was observed.

Introduction

Cellular protein synthesis in tissue culture is reversibly sensitive to medium hypertonicity [1–3]. Since viral protein synthesis very often is less sensitive to hypertonic medium conditions, this technique has been used to label preferentially the proteins of DNA-[4] as well as RNA viruses [2, 5, 6]. We intended to use this technique to investigate the protein synthesis of spleens from mice inoculated with the agent of the scrapie disease. This agent causes a so-called slow virus disease of the nervous system in sheep and goats as well as in the experimental mouse system [7, 8]. Although we have not detected any scrapie agent-specific protein synthesis, we made the observation that medium hypertonicity did not block the biosynthesis of a relatively small spleenic protein secreted into the medium. These experiments are described in this communication.

Materials and Methods

Animals

Mice of the inbred strain STU [9] were used. Animals were inoculated [10] either intracerebrally or intraperitoneally at 4–6 weeks of age with normal or scrapie brain dilution of 10^{-2}–10^{-3}. The scrapie agent was obtained from Dr. R. Kimberlin and colleagues at the Institute for Research on Animal Diseases, Compton, England. The material was passaged at least twice in STU-mice before use.

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Organ culture

Organ cultures were performed using Medium 199 [11] with Hank's salt solution [12] from which leucine had been omitted. To the medium were added 0.030 M HEPES and antibiotics. Hypertonic medium was prepared by addition of 8 g NaCl/l to the above [3].

Two spleens (150–280 mg) per sample – removed 10–50 days after inoculation – were incubated in a 25 ml-Erlenmeyer flask containing 3 ml of medium. Radioactive leucine (Amersham-Buchler, Braunschweig, Germany) was added (25 µCi/ml), and the sealed flasks were kept at 37 °C on a rocking table for periods of 36–70 h. The medium was then removed for protein isolation. The spleens were again incubated twice for 1–2 h with Dulbeccos modification of Eagles minimum essential medium [13] to effectively eliminate free radioactive leucine. This medium was discarded. The spleens were stored at −20 °C until use.

Isolation of proteins from the culture medium and gel electrophoresis

The incubation medium was centrifuged at $500 \times g$ for 10 min to remove particulate material. Protein was determined in 0.1 ml of the supernatants. The supernatants were then passed through a column (30 × 1.5 cm) of P10 (Bio-Rad, München, Germany) to separate the high molecular weight protein fraction from free [^{3}H]leucine. Gel electrophoresis was performed according to Laemmli [14] at a concentration of 12.5% polyacrylamide of the separating gel on 25–50 µl samples out of 10 ml of the high molecular weight fraction. After electrophoresis the gels were frozen at −10 °C, and cut into 1 mm slices. These were incubated in 0.2 ml Soluene 300 (Packard Instruments, Frankfurt/Main, Germany) at 50 °C for 2 h and were then counted in 2 ml of a toluene based scintillator solution.

Protein determination

Protein concentrations were determined with Coomassie Brilliant Blue G-250 (Sigma, Heidelberg, Germany) according to Bradford [15] using bovine serum albumin as a standard.

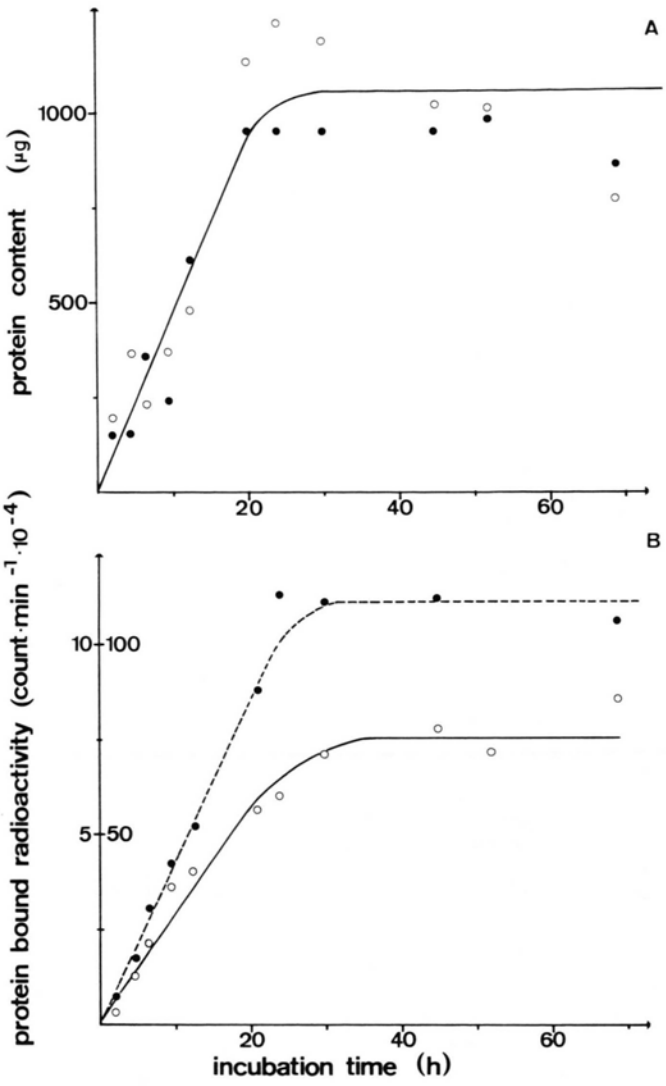

Fig. 1. Release of total protein (A) and protein-bound [^{3}H]leucine (B) into the culture medium under normal (○) and hypertonic (● and left ordinate in B) growth conditions. Two spleens with 185 mg were grown in 4 ml of radioactive medium. At indicated times the protein content was measured in 0.1 ml aliquots of the medium. Protein-bound radioactivity was measured in 0.1 ml medium after precipitation with 100 µl of 10% trichloracetic acid, containing an excess of non-radioactive leucine, 2 washes with 5% trichloracetic acid and resolution of the protein in 0.5 ml 0.1 N NaOH for 1 h at 70 °C (single determinations).

Results

The release of protein and protein-bound [^{3}H]leucine from mouse spleens into the medium

When mouse spleens were incubated in either isotonic or hypertonic serum-free medium they constantly released protein into the medium for a period of about 20–30 h (Fig. 1). Independent of the osmolarity, the protein released by 100 mg of spleen reached a final amount of about 1 mg of protein per 3 ml of incubation medium. In contrast, the total amount of protein-bound radioactive leucine released into the hypertonic medium was only about 16% of that present in normal medium.

In six independent experiments hypertonic medium always resulted in a marked inhibition of [^{3}H]leucine incorporation into spleen-associated, as well as into released proteins. Unexpectedly, at hypertonic conditions [^{3}H]leucine incorporation was reduced to about 5% in spleen associated proteins but only to about 10% in the medium released proteins. This indicated that the synthesis of proteins which were released into the medium was less sensitive to high osmolarity than the synthesis of proteins that remained associated with spleen tissue.

Analysis of the radioactive proteins released into the medium

The proteins released from spleen cells into the medium were analyzed by SDS-gelelectrophoresis. When normal spleens had been incubated in isotonic medium, peaks of radioactivity were observed at positions corresponding to proteins with the MW of 60000, 25000, 12000 and 7000 (Fig. 2a) as deduced from reference substances. Experiments with spleens from animals which had been inoculated with normal or scrapie brain homogenates, yielded almost identical electrophoresis patterns. However, the level of radioactivity at the 12000 MW position in most experiments was higher in diseased animals.

Gel electrophoresis of radioactive proteins from hypertonic media revealed an almost complete inhibition of the ^{3}H-incorporation into the higher molecular weight proteins whereas the 12000-molecular weight species was still synthesized (Fig. 2b). Thus the continued biosynthesis of spleen released proteins in hypertonic medium seems to be restricted to the 12000 MW protein species.

Figure 2b shows furthermore that media from spleens of animals which had been inoculated with either normal or scrapie brain suspensions contain more radioactive 12 K protein than that of the control. No matter which medium had been taken, this protein did not change its position on the gel when either heating or mercapto-ethanol were omitted prior to the electrophoresis, indicating that disulfide linkages did not affect the molecular weight determination of this protein. Treatment of the tritiated protein with 10 µg of either trypsin, chymotrypsin or pronase for 1 h at 37 °C always resulted in conversion of the 12000 dalton molecule into small fragments. When samples of [^{14}C]12 K protein obtained from normal spleens and [^{3}H]12 K protein from scrapie-infected spleens or vice versa were run on the same gel the radoiactive material occupied exactly the same position indicating that we probably deal with the same protein in both cases.

After the detection of the 12K protein in the incubation medium, we investigated spleen homogenates for the presence of this protein. We could detect only small amounts of 12K protein in a sedimentable fraction obtained by high speed centrifugation (100000 × g for 3 h) and hot extraction with a buffer containing SDS. In contrast to the amount of radioactivity incorporated into medium-released 12K protein, only about 0.1% of the total protein-bound radioactivity was found as 12K protein in the homogenate.

A protein with a molecular weight of about 11600–11800 [16, 17] known to be synthesized by lymphocytes [18–21], and which partly is released into the medium [20] is β-microglobulin. It was, therefore, attempted to precipitate 12K protein with an antiserum directed against human β-microglobulin. Cross reactivity between human and murine β-microglobulin has been reported [22].

No preferential precipitation of 12K protein with this serum was observed. 12K protein and β-microglobulin, therefore, seem to be different proteins. An antiserum directed against mouse-interferon (generously supplied by Dr. J. Gresser, Institute de Recherches Scientifiques sur le Cancer, Villejuif, France) or against the immunoglobulin fraction of STU-mice also did not precipitate 12K protein. Thus no antigenic relationship can be demonstrated between 12K protein and these proteins.

Injection of labeled 12K protein into mice

Since the 12K protein is released or secreted from the spleen *in vitro* it is likely that this occurs also *in vivo*. We, therefore, injected into the tail vein of mice specifically labeled [^{3}H]12K protein prepared using hypertonic medium. Radioactive proteins from isotonic medium, comprising all released proteins, served as controls. Animals were sacrified 4 or 24 h after injection and the radioactivity in the various tissues was determined, after solubilization in Soluene 300 and decolourization with hydrogenperoxide.

The tritium levels detected 4 and 24 h after intravenous injection of either [^{3}H]12K protein or total [^{3}H]protein are listed in Table I. Four hours after injection we find 630 dpm/0.1 ml of serum for [^{3}H]-12K protein. In comparison we can group the various organs into three categories. Group I such as red blood cells, muscle, heart, brain, eye, bone etc. contain definitely less radioactive 12K protein than serum. Group II organs such as thymus, lymph-nodes, spleen, lung etc. contain about the same level as serum 4 or 24 h after injection. Group III organs contain definitely higher levels of [^{3}H]12K protein after 4 h and even after 24 h. In this latter group we find kidney, liver, small intestine etc.

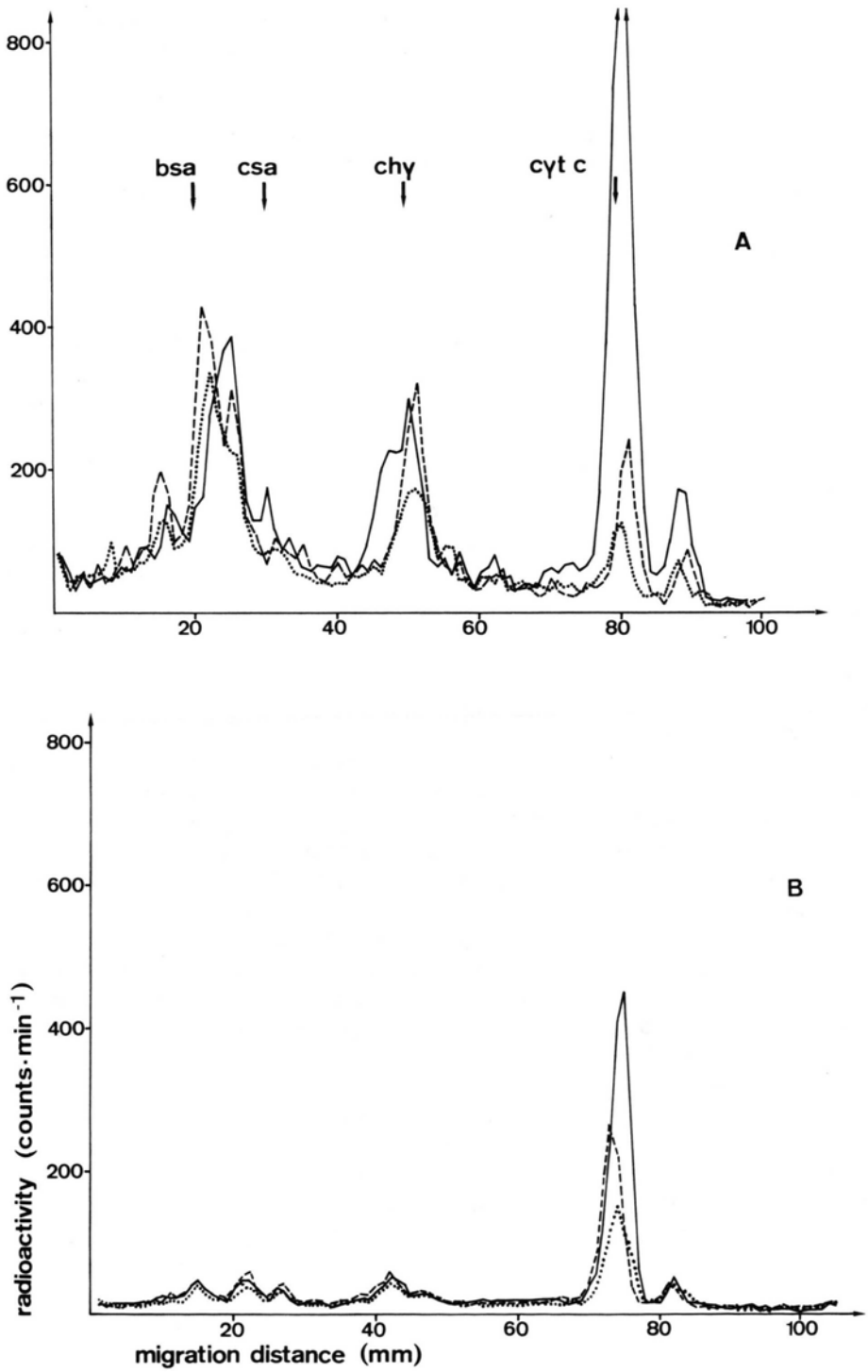

Fig. 2. SDS-gelelectrophoresis of radioactive proteins released into the medium during a 42 h incubation period. A) 25 µl out of 10 ml high molecular weight fraction of an isotonic incubation. B) 50 µl out of 10 ml of a hypertonic incubation. · · · control, - - - normal brain injection, —— scrapie brain injection. Arrows indicate positions of reference substances. Reference substances and their molecular weights bsa: bovine serum albumin 67000, csa: Chicken serum albumin 45000, chy: chymotrypsinogen 25000, cyt c: cytochrome c 12400.

Table I: Distribution of radiolabeled high molecular weight fraction proteins from media of cultured spleens in various mouse organs after intravenous infusion.

Group	Tissue	12 K protein [a]		Total protein [b]	
		4 h [d]	24 h	4 h	24 h
I	red blood cells	50	– [e]	–	–
	heart	350	360	210	330
	muscle	160	280	100	140
	oesophagus	350	500	310	640
	brain	250	250	125	250
	eye	220	290	140	180
	blood vessel wall	280	400	250	270
	bone	260	370	160	260
	skin	60	120	–	–
II	thymus	280	620	200	340
	lymphnodes	330	420	290	380
	serum	630	480	640	600
	spleen	750	640	350	640
	lung	700	510	400	400
	ovaries	650	560	290	570
	adrenal gland	690	760	430	910
	stomach	820	750	400	600
	rectum	740	750	300	600
III	colon	1200	1500	340	710
	small intestine	1100	990	530	1000
	liver	1700	1000	600	830
	kidney	1100	780	620	740
	salivary gland	850	570	420	490

[a] Salt independent proteins isolated from medium of spleens incubated at hypertonic conditions and labeled with [^{3}H]leucine.
[b] Proteins isolated from medium of spleens incubated under isotonic conditions and labeled with [^{3}H]leucine.
[c] Hours after injection of labeled material.
[d] Mean values of two experiments. The deviation between the values was generally between 50 – 200 DPM.
[e] Not determined.

When total ^{3}H-labeled spleen released proteins were given to the animals and the levels of radioactivity in the various organs were compared with serum 4 h after injection, we found a group of organs that contained less radioactivity. This group coincided with group I of the [^{3}H]12 K protein experiments. We did not find, however, any organ that contained more radioactive material than serum after 4 h.

Using the same experimental procedure as described for spleen we tested the synthesis of 12 K protein in liver, kidney, salivary gland and lung (organs which had accumulated this protein) to determine if these organs themselves synthesize the protein. None of these organs synthesized and released 12 K protein.

Discussion

Cultivation of mouse spleen under hypertonic medium conditions yields a specifically labeled protein species which is released into the medium. We assume that the 12 K protein might be a protein with a yet unknown biological function. It cannot be a breakdown product of other proteins, since the synthesis of the other proteins is almost immediately shut off at the moment of changing the osmolarity of medium [2, 3]. It is rather unlikely that catabolism of various molecular species of radioactive high molecular weight proteins gives rise to a single radioactive protein. Furthermore, other organs cultivated did not produce and release 12 K protein into the medium. The question then arises of whether this protein is a product of a latent murine virus. Considering that no murine virus is known to induce release of a single protein of 12000 daltons, this is rather unlikely. Moreover, we find 12 K protein also in a different mouse strain (unpublished observations) obtained from a different breeder and housed in a different environment. Therefore, it seems to be likely that 12 K protein is a protein originating from an as yet unidentified cell species of the spleen.

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[1] E. Robbins, T. Pederson, and P. Klein, J. Cell Biol. **44,** 400 (1970).
[2] J. L. Saborio, S.-S. Pong, and G. Koch, J. Mol. Biol. **85,** 195 (1974).
[3] G. Wengler and G. Wengler, Europ. J. Biochem. **27,** 162 (1972).
[4] J. M. England, M. K. Howett, and K. B. Tan, J. Virol. **16,** 1101 (1975).
[5] H. P. Madore and J. M. England, J. Virol. **16,** 1351 (1975).
[6] D. L. Nuss, H. Oppermann, and G. Koch, Proc. Nat. Acad. Sci. USA **72,** 1258 (1975).
[7] A. G. Dickinson, Slow virus diseases of animals and man, ed. R. H. Kimberlin), p. 209. North-Holland Publishing Company, Amsterdam-Oxford 1976.
[8] R. H. Kimberlin, Sci. Prog. Oxf. **63,** 461 (1977).
[9] W. Schäfer, Z. Naturforsch. **34 c,** 306 (1979).
[10] R. H. Kimberlin, J. Neurochem. **19,** 2767 (1971).
[11] R. C. Parker, Methods in tissue culture, 3rd edition Harper and Row, Publ. Comp. 1962.
[12] J. H. Hanks and R. E. Wallace, Proc. Soc. Exp. Biol. Med. **71,** 196 (1949).
[13] R. Dulbecco and G. Freemann, Virology **8,** 396 (1959).
[14] U. K. Laemmli, Nature **227,** 680 (1970).
[15] M. M. Bradford, Anal. Biochem. **72,** 248 (1976).
[16] I. Berggård and A. G. Bearn, J. Biol. Chem. **243,** 4095 (1968).
[17] B. A. Cunningham and I. Berggård, Transplant. Rev. **21,** 3 (1974).
[18] K. Nilsson, P.-E. Evrin, I. Berggård, and J. Pontén, Nature New Biol. **244,** 44 (1973).
[19] K. Nilsson, P.-E. Evrin, and K. I. Welsh, Transplant. Rev. **21,** 53 (1974).
[20] M. D. Poulik and A. D. Bloom, J. Immunol. **110,** 1430 (1973).
[21] N. Tanigaki and D. Pressman, Transplant. Rev. **21,** 15 (1974).
[22] M. Katagiri, T. Natori, N. Tanagaki, V. P Kreiter, and D. Pressman, Transplantation **19,** 230 (1975).