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# Distribution of a negative regulator of haematopoietic stem cell proliferation (AcSDKP) and thymosin β4 in mouse tissues

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A competitive enzyme immunoassay using acetylcholinesterase as tracer for thymosin β4, has been developed. Using this assay and a previously described EIA for AcSDKP, a negative regulator of pluripotent haematopoietic stem cell proliferation, the levels of these two peptides were determined in mouse tissue extracts. The combination of EIAs with different HPLC procedures validated these methods and clearly demonstrated the ubiquity of these peptides in mouse tissues. Similar results are reported for rabbit thymus which suggest different hypotheses for AcSDKP biosynthesis.

Negative haematopoietic regulator; AcSDKP tetrapeptide; Thymosin β4; Enzyme immunoassay; Acetylcholinesterase; Mouse tissue

## 1. INTRODUCTION

*n*-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) is a tetrapeptide first isolated from fetal calf bone marrow in 1977 [1] and subsequently purified and identified [2]. It is a potent inhibitor of pluripotent haematopoietic stem cells (CFU-S), and hence reduces mortality in mice after administration of lethal doses of cytosine arabinoside (Ara-C) [3]. In the mouse, AcSDKP is produced in the bone marrow [4] and was shown to be a physiological regulator of CFU-S [5]. It also inhibits the entry into cycle of fetal or adult rat hepatocytes [6]. It has no effect in GM-CFC in the mouse [7]. However, in humans, this inhibition occurs in the haematopoietic progenitors of normal bone marrow but not in the equivalent leukaemic cells [8]. Using a specific enzyme immunoassay (EIA), we have recently shown that this peptide is present in human white blood cells [9]. The peptide sequence Ac-S-D-K-P- exists as the N-terminal part of thymosin β4 (mol. wt. 4963), a thymic polypeptide active in the regulation and differentiation of thymus-dependent lymphocytes (for a review see [10] (Fig. 1), and appears to be an ubiquitous peptide with other unidentified basic cellular functions [11,12]. Recently, it was shown that AcSDKP can be generated from thymosin β4 by a one-step enzymatic cleavage in vitro and in vivo [13].

A recent report has shown that a polyclonal antiserum obtained by coupling an analog (AcSDKPY) to the antigenic carrier was able to neutralize the biological activity of AcSDKP in mice [14]. To obtain more specific antibodies for AcSDKP, we have developed two different strategies for immunogen preparation. One involves linkage of the peptide to the antigenic carrier via its lysine side chain [9], and the other presented here uses linkage of a C terminal elongated peptide (AcSDKPDC) via its cysteine side chain. The latter immunogen has been used to obtain antibodies which specifically recognize the N-terminal part of thymosin β4 but not AcSDKP. In the light of these findings, we have performed EIA of thymosin β4 using acetylcholinesterase (AChE) as a tracer [15].

We describe here the assay and the distribution of AcSDKP and thymosin β4 in mouse tissues and validation studies using high-performance liquid chromatography (HPLC) combined with EIA measurements.

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## 2. MATERIALS AND METHODS

### 2.1. *Reagents*

AcSDKP (mol. wt. 487) was kindly provided by Biomeasure (Hopkinton, MA). Bovine thymosin β4 and human thymosin β10 were purchased from Peninsula Lab (Merseyside, UK) and thymosin α1 from Bachem (Bubendorf, Switzerland). All other synthetic peptides were synthesized in our laboratory by solid-phase peptide technology [9]. Oxidation of the thymosin β4 form was achieved by treatment with $H_2O_2$ for 18 h. Pure AChE (EC 3.1.1.7) was prepared from the electric organs of the electric eel *Electrophorus electricus* and its activity was measured as previously described [16]. Unless otherwise stated, all reagents were from Sigma (St. Louis, MO) and solvents from Prolabo (Paris, France). EIA buffer was a 0.1 M phosphate buffer pH 7.4 containing 0.9% NaCl, $10^{-3}$ M EDTA, 0.1% bovine serum albumin and 0.01% sodium azide.

### 2.2. *Apparatus*

Solid-phase EIA was performed using automatic Titertek microtitration equipment (washer, dispenser and reader) from Labsystems

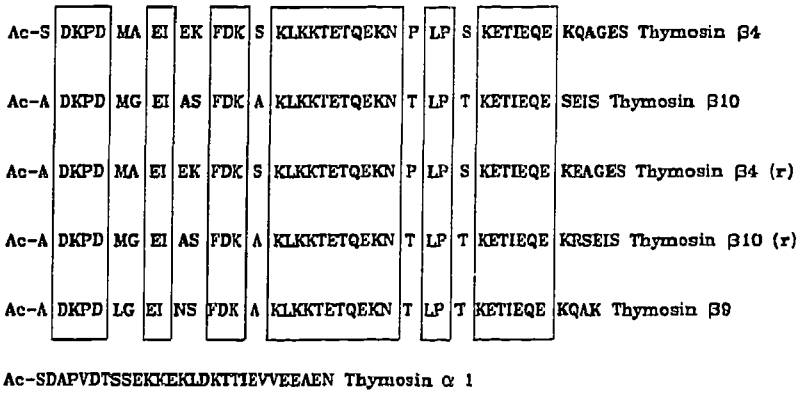


Fig. 1. Amino acid sequences of thymosin $\beta 4$ and thymosin-related peptides (r = rabbit). The regions of homology are boxed.

Oy (Helsinki, Finland) and microtiter plates Maxisorb from Nunc (Roskilde, Denmark). HPLC experiments were performed with a 2249 LC LKB gradient pump (Bromma, Sweden) and Nucleosil C18 (SFCC, France) and $\mu$ Bondapack C18 (Waters, USA) columns for AcSDKP and thymosin $\beta 4$ separations, respectively. Tissue extracts were concentrated by adsorption onto a reverse-phase C18 Baker column (Phillipsburg, USA) and by a SpeedVac concentrator SVC 200 (Savant Instruments Inc., Farmingdale, NY).

### 2.3. *Antiserum production*

The preparation of AcSDKP antiserum for these studies has been described elsewhere [9]. For thymosin $\beta 4$ antibody production, AcSDKPDC was linked to the maleimidated keyhole limpet hemocyanin (KLH) used as antigenic carrier. KLH was maleimidated following a procedure described for bovine serum albumin (BSA) [17]. Briefly, to 50 mg of KLH in 5 ml of phosphate buffer 0.1 M pH7, were added 50 $\mu$mol of *N*-ethyl maleimide in order to block any thiol group. The reaction was allowed to proceed for 30 min at 22°C and then 15 mg (0.5 ml) of *N*-succimidyl-4(*N*-maleimido methyl) cyclohexane-1-carboxylate (SMCC) in anydrous dimethylformamide (DMF) was added. After 30 min, the mixture was filtered through a G25 column (Pharmacia, Sweden) with phosphate buffer (0.1 M pH 6) as eluting solvent and stored at −80°C until use. For immunogen preparation, 20 $\mu$mol of AcSDKPDC and 10 $\mu$mol of maleimidated KLH were left to react in 5 ml of phosphate buffer 0.1 M, pH 6 for 1 h at 30°C and 18 h at 4°C. The mixture was dialysed against 0.9% NaCl. Five white rabbits were immunized and boostered with 0.8 mg each of immunogen following the procedure described by Vaïtukaitis et al. [18] using complete Freund's adjuvant (Difco, Detroit MI) and subcutaneous injections. The rabbits were bled on a weekly basis after the first booster and after the boosters given every month. One bleeding (L831 S5) was selected after the fourth booster and kept at 4°C after the addition of sodium azide (0.02% final).

### 2.4. *Preparation of enzymatic tracer*

Maleimidated G4 form of AChE was used to couple AChE to AcSDKPDC following a procedure previously described [19]. To 0.25 nmol of maleimidated AChE (1 ml) was added 2.5 nmol of AcSDKPDC (5 ml), both in phosphate buffer 0.1 M pH 6. The reaction was allowed to proceed for 1 h at 30°C and 24 h at 4°C. The enzymatic conjugate was purified by molecular sieve chromatography [16] and stored at −80°C for several months without loss of enzyme activity or immunoreactivity.

### 2.5. *Tissue extraction*

Four-week-old female Balb/c mice were killed and the tissues of 5 animals were removed, immediately weighed, pooled and frozen in liquid nitrogen. Extraction was performed as previously described [20]. Organs were homogenized at 2–4°C in 5 ml of 6 M guanidinium chloride using an Ultra-Turrax T18 homogenizer (Janke and Kunkel, Germany). The homogenates were diluted with 5 ml of 0.1% TFA and centrifuged at 18 000 × *g* for 1 h (L5-50 Beckman, Palo Alto, CA). The floating lipid layer was removed and the supernatant passed through a Baker C18 column. The column had been washed with 5 ml methanol followed by 5 ml TFA 0.1% prior to loading of the biological extracts. After washing with 5 ml 0.1% TFA, the material was eluted with 5 ml of acetonitrile containing 0.05% TFA, evaporated to dryness and resuspended in EIA buffer or 0.1% TFA for immunological or HPLC analysis, respectively.

### 2.6. *Competitive EIA procedure*

EIA using acetylcholinesterase for AcSDKP analysis has been described elsewhere [9]. A similar procedure was used for thymosin $\beta 4$ measurements. Briefly, the assay was performed using microtiter plates coated with mouse monoclonal anti-rabbit immunoglobulins. Enzymatic tracer (0.3 Ellman units/ml [16]), diluted antiserum (L831 S5 1/40 000) and standard or sample all in 50 $\mu$l EIA buffer, were added to each well. After 18-h incubation at 4°C and washing, 200 $\mu$l of enzymatic substrate (Ellman reagent [16]) were added. One hour later the yellow color was automatically measured at 414 nm in each well.

### 2.7. *HPLC analysis of immunoreactive material*

In order to validate the nature of the immunoreactivity present in the biological samples, HPLC was combined with EIA as described for AcSDKP analysis in human blood cells and plasma [9]. For thymosin $\beta 4$, a minor modification of the HPLC procedure previously described [20] was used. The tissue extracts were eluted from the column using different gradients (reservoir A = 0.1% TFA, reservoir B = acetonitrile, TFA 0.05%). Peptides were eluted using successively 4% B for 30 min (flow rate 0.5 ml/min), then 4% B to 45% B for 55 min and 45% B to 100% B for 10 min (flow rate 1.5 ml/min each, respectively). Fractions were collected each minute, evaporated to dryness and resuspended in 0.25 ml EIA buffer for assay.

## 3. RESULTS AND DISCUSSION

### 3.1. *Sensitivities and specificities of EIAs*

We have previously described the development of a specific and sensitive EIA for AcSDKP [9]. We report here the standard curve (Fig. 2) and a specificity study using thymosin-like peptides (Table I). As expected in the light of the previous data [9], the specificity of AcSDKP antibodies is very high since no cross-reactivity (CR) with thymosin $\beta 4$-like peptides was observed, in spite of the DKP sequence in their N-terminal region (Fig. 1).

The hetero-bifunctional SMCC reagent is now commonly used to link peptides that contain a cysteine resi-

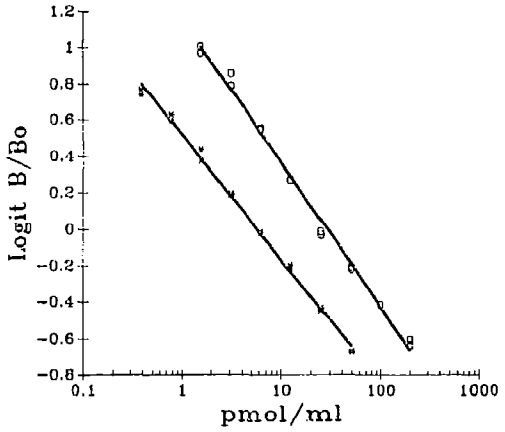


Fig. 2. Representative Logit-Log representation [21] of routine EIA standard curves for AcSDKP (x-x) and Thymosin $\beta$4 (0-0) assays, respectively. B and $B_0$ represent the bound enzyme activity measured in the presence or absence of competitor, respectively. (Logit $B/B_0$ = $Log(B/B_0/1-B/B_0)$.

due to proteins [22]. We have previously used this procedure to obtain an immunoreactive rat atriopeptin-AChE conjugate which has allowed the development of a highly sensitive immunoassay [19]. This approach led us to synthesize the AcSDKPDC peptide in order to prepare an immunogen and enzymatic conjugate. Thymosin $\beta$4 was fully recognized by the antibodies resulting in a standard curve (Fig. 1) with a relative high but acceptable detection limit of 5 pmol/ml (B/Bo 80%), in comparison with 0.5 pmol/ml recently described for another thymosin $\beta$4 EIA [23], where the antiserum was obtained by immunization of rabbits with complete thymosin $\beta$4. Table I shows the specificity of this assay analyzed with various peptides. The structural homology between AcSDKPDC and the N-terminal part of thymosin $\beta$4 (AcSDKPDM...) (Fig. 1) strongly suggests that the latter could be the epitope. Nevertheless, epitope size remains a critical factor forthe affinity, since a shorter peptide such as AcSDKP is not able to compete with AcSDKPDC tracer for the antibody (CR = 0.01%). Also, a discrete change in primary structure (alanine instead of serine in the N-terminal part of thymosin $\beta$10) can dramatically influence antibody recognition (CR = 13%). Since oxidized thymosin $\beta$4 is fully recognized, the methionyl residue cannot be implicated in binding with antibody sites.

Table I

Cross-reactivity study

| Peptide sequence | Cross-reactivity (%)* | |
|---|---|---|
| | AcSDKP assay | Thymosin $\beta$4 assay |
| Ac-SDKP | 100 | 0.01 |
| Ac-SDRP | 100 | 0.8 |
| Ac-ADKP | <0.01 | 0.3 |
| Ac-SDK | <0.01 | <0.01 |
| SDKP | <0.01 | <0.01 |
| Ac-SDKPDC | <0.01 | 166 |
| Thymosin $\beta$4 | 0.01 | 100 |
| Thymosin $\beta$4 [o]** | <0.01 | 100 |
| Thymosin $\beta$10 | <0.01 | 13 |
| Thymosin $\alpha$1 | <0.01 | <0.01 |

*Cross-reactivity was determined by comparing the molar concentration at $B/B_0$ 50% of each peptide to that of AcSDKP and expressing it as a percentage of AcSDKP immunoreactivity. **oxidized form

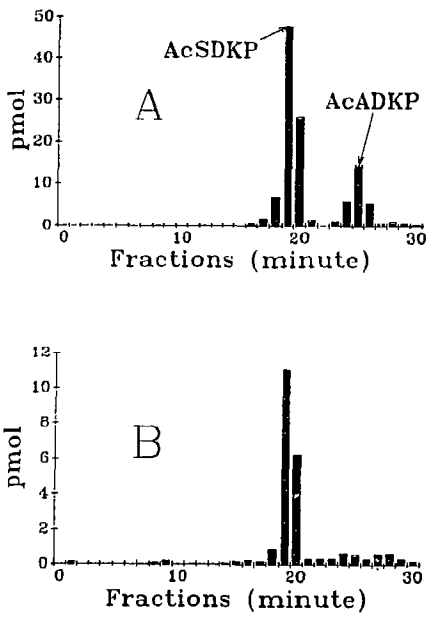


Fig. 3. HPLC profiles of immunoreactive synthetic AcSDKP and AcADKP peptides (A) and mouse thymus extract (B).

### 3.2. *Immunoreactive material in mouse tissues*

Using AcSDKP and thymosin $\beta$4 assays, we have measured the immunoreactive material present in different mouse tissues (Table II). In each case, the dilution

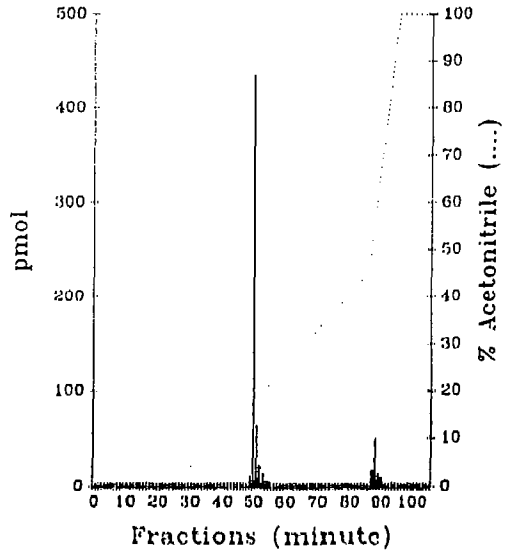


Fig. 4. HPLC profile of thymosin $\beta$4-like material in mouse thymus extract. Bovine thymosin $\beta$4 has the same retention time (48 min).

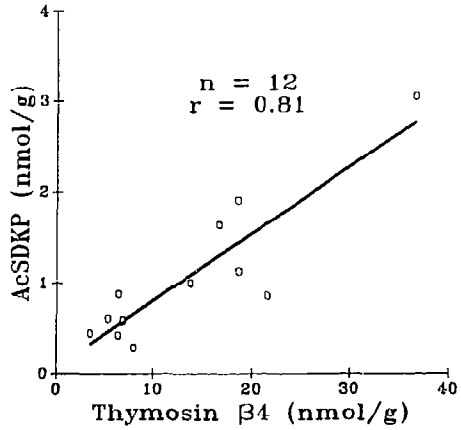


Fig. 5. Correlation between the AcSDKP and thymosin $\beta$4 levels in mouse tissue extracts ($r$ = 0.81).

curves of biological samples were closely parallel to the standard curve (data not shown). The results show clearly that the AcSDKP-like material is present in all tissues, with a higher concentration in the spleen. This is the first evidence of the ubiquity of this peptide in mammalian tissues. The same observation has been reported for thymosin $\beta$4 in rat or mouse tissues using different analytical procedures such as HPLC [25], RIA [26] and RNA analysis [12]. When the immunoreactive materials assayed in each tissue were analyzed by HPLC, we observed one immunoreactive peak which strictly co-eluted with standards. Fig. 3 and Fig. 4 present the patterns obtained with thymic extract. With different AcSDKP and thymosin $\beta$4 levels, the other tissues presented the same HPLC/EIA profiles. In thymosin $\beta$4 analysis, a tiny peak was eluted at 100% corresponding to an unknown more hydrophobic material (Fig. 4). Taking into account the high specificity of EIA and the performance of HPLC separation procedures [9,27], we assume that the EIAs measure the true AcSDKP and thymosin $\beta$4 peptides, respectively. In each assay, the level of these two peptides were poorly correlated ($r$ = 0.81) (Fig. 5). These results suggest that AcSDKP is synthesized or liberated by pathways or cellular events other than those of thymosin $\beta$4. This hypothesis is presently under investigation in our laboratory. Among the animal species studied, the rabbit possesses a thymosin $\beta$4 which differs only in the first amino acid (alanine instead of serine) [28]. We found in the rabbit thymus 2 and 29.2 nmol/g of AcSDKP and thymosin $\beta$4-like material, respectively. The immunochromatograms were exactly the same as those presented in Fig. 3 and Fig. 4 for mouse tissue. Since synthetic AcSDKP is well separated from AcADKP (Fig. 3A), we assume that the AcSDKP-like material in rabbit thymus corresponds to the true AcSDKP molecule. For thymosin-like material, if we take into account the structural homology of thymosin $\beta$10 (human) and thymosin $\beta$4 (rabbit) (Fig. 1), we can expect a cross-reactivity near to 10% for the latter. Consequently, the thymosin $\beta$4

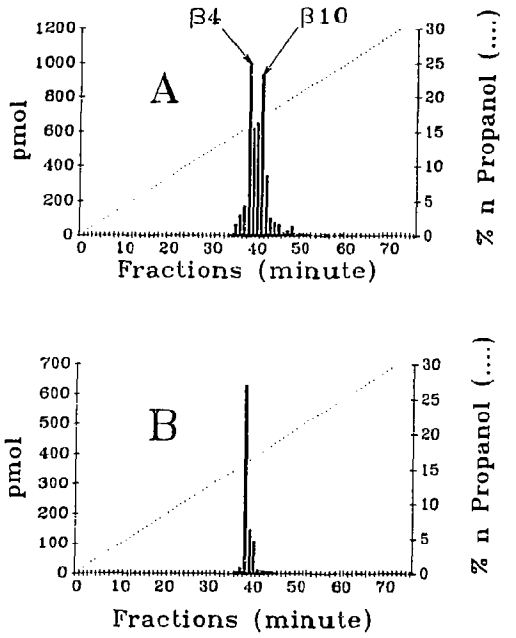


Fig. 6. HPLC profiles of immunoreactive synthetic thymosin $\beta$4 and thymosin $\beta$10 peptides (A) and rabbit thymus extract (B). The HPLC procedure is described in ref. 29 (C18 column, flow 0.65 ml/min, reservoir A: 1 M formic acid/0.2 M pyridine pH 3, reservoir B: $n$-propanol).

levels in rabbits will be around 300 nmol/g, compared to 15.6 nmol/g found by HPLC analysis [29]. This discrepancy suggests that our assay may measure other immunoreactive peptides not detected by our HPLC procedure. Rabbit thymosin $\beta$10 (Fig. 1) may be a likely candidate, since this peptide is slightly different from thymosin $\beta$10 [29]. We have here used an HPLC procedure [29] which allows the separation of thymosin $\beta$4 and thymosin $\beta$10 in rabbit tissue extracts, and synthetic thymosin $\beta$4 and thymosin $\beta$10 were also separated (Fig. 6A). When the rabbit thymus extract was analyz-

Table II

Content of AcSDKP and thymosin $\beta$4 in mouse tissues

| Tissues | AcSDKP* | Thymosin $\beta$4* |
|---|---|---|
| Thymus | 1.64 ± 0.68 | 16.8 ± 1.8 |
| Spleen | 3.05 ± 1.50 | 36.6 ± 7.5 |
| Muscle | 0.45 ± 0.07 | 3.5 ± 0.1 |
| Brain | 0.29 ± 0.09 | 8 ± 0.1 |
| Skin | 0.59 ± 0.11 | 6.9 ± 0.4 |
| Bone marrow ** | 1 ± 0.4 | 13.8 ± 0.5 |
| Lung | 0.86 ± 0.25 | 21.6 ± 1.3 |
| Liver | 0.61 ± 0.04 | 5.4 ± 1 |
| Intestine | 1.91 ± 0.83 | 18.6 ± 7 |
| Stomach | 1.13 ± 0.24 | 18.7 ± 1.9 |
| Kidney | 0.43 ± 0.02 | 6.4 ± 0.2 |
| Heart | 0.88 ± 0.36 | 6.4 ± 2.1 |

*Concentration (nmol/g) of AcSDKP and thymosin $\beta$4. Means of 3 experments (5 animals/experiment) **pmol/$10^6$ cells.

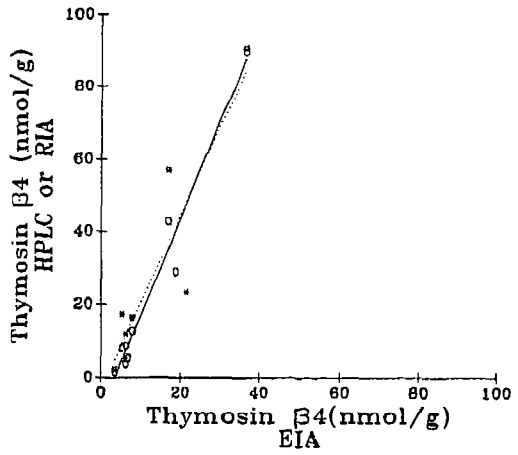


Fig. 7. Correlation between the thymosin $\beta 4$ levels found in mouse tissues by EIA and in rat tissues by HPLC (*-*) ($r = 0.90$) and RIA (0-0) ($r = 0.98$), respectively.

ed, we observed only one peak which co-eluted with standard thymosin $\beta 4$ (Fig. 6B). These data have led us to conclude that AcSDKP is also present in the rabbit thymus and that if thymosin $\beta 4$ is involved in the biosynthesis of AcSDKP as recently described [13] for mouse, another precursor could be present in rabbit tissues. Our EIA for thymosin $\beta 4$ combined with HPLC analysis seems very specific, but a discrepancy is observed between the thymosin $\beta 4$ levels found with our assay and those described in the literature for rat tissues using HPLC [25] or RIA [26] analysis, even if the correlation coefficients are high ($r = 0.90$ ($n = 8$) and $r = 0.98$ ($n = 8$), respectively) (Fig. 7). These differences might be due to other parameters such as the animal species, age or physiological state.

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