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EXTRACTION OF COLLAGENASE FROM THE INVOLUTING RAT UTERUS

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Summary

Collagenase (EC 3.4.24.3) activity can be measured directly in homogenates of the involuting rat uterus. Latent forms of collagenase are activated by a brief exposure to trypsin; trypsin activity is then blocked with soybean trypsin inhibitor. Homogenizing conditions have been developed that permit 90—95% recovery of the total active and latent collagenase activity in a 6000 × *g* pellet, where it is presumably bound to its collagen substrate. This insoluble activity can then be extracted by heating to 60°C for 4 min in 0.04 M Tris · HCl buffer, pH 7.5, containing 0.1 M $CaCl_2$. Methods are presented for the estimation of the recovery of collagenase in the extracts; this approximates 65—70% of the total. Small amounts of activity can also be extracted from rat liver and kidney. This extraction procedure should be of use in purifying collagenase without culturing the enzyme-producing tissue and in the direct assay of tissue collagenase activity.

The activity extracted from rat uterus has been proven to be collagenase by its characteristic pattern of collagen breakdown products on disc electrophoresis and by the split of tropocollagen at interband 41 as shown by electron microscopy of reconstituted fragments. The activity is inhibited by EDTA, and this inhibition is not reversed by calcium or zinc ions.

Introduction

In a recent paper we have shown that the involuting rat uterus contains significant amounts of collagenase (EC 3.4.24.3) [1]. The enzyme is not readily soluble and is recovered in pellets produced by centrifuging uterine homogenates at 6000 × *g*. It is believed that the enzyme is in the pellet due to binding to its substrate: the insoluble collagen fibers. It is possible to measure this collagenase activity merely by incubating the pellet in buffer with added calcium and following the release of hydroxyproline-containing peptides from the endogenous

collagen [2]. However, this method is not ideal. Since it is not possible to vary the ratio of enzyme to substrate, it is difficult to tell if changes in enzyme activity under different physiological conditions are due to changes in enzyme levels, substrate susceptibility or inhibitor levels. Also, in characterizing the enzyme it is difficult to perform some of the usual tests such as following changes in optical rotation or the appearance of reconstitutable fragments of collagen [3]. For these reasons it would be desirable to bring the collagenase into solution.

Moreover, if one could extract collagenase from the tissue it might provide a useful way of preparing quantities of the enzyme. Almost all preparations of collagenase are obtained by tissue culture techniques that are tedious, time-consuming, and expensive. Eisen et al. [4] were able to extract small amounts of collagenase from skin. Harris et al. [5] extracted considerable amounts of collagenase from low-speed centrifuged pellets of homogenates of ascites carcinoma implanted in rabbit muscle. Nagai and Hori [6] and Sakamoto et al. [7] have developed extraction procedures useful for rat skin and embryonic chick bones, respectively. None of these methods has been completely successful when applied to the uterus, nor do any of the published procedures give any estimate of the collagenase in the tissue and the extent of recovery in the extracts. Several years ago we published [8], in abstract form, a method suitable for extracting collagenase from the rat uterus. The present paper gives full details of the method, including recent improvements, and presents data to prove that the extracted enzyme is collagenase.

Methods

Extraction of collagenase

Preliminary studies of the involuting rat uterus indicated that the maximum activity of collagenase was found at 1—2 days following parturition [9]. Accordingly uteri were removed from adult Sprague-Dawley rats at one day post partum, chilled, minced and homogenized in Ten-Broeck glass homogenizers with motor-driven pestles, using 10 ml fluid per g wet tissue. The homogenizing fluid was 0.01 M $CaCl_2$ containing 0.25% Triton X-100. The homogenates were centrifuged at 2° C for 20 min at 6000 × *g*. The supernatants were discarded and the pellets were resuspended by brief homogenization to their original volume in 0.05 M Tris buffer, pH 7.4, containing 0.1 M $CaCl_2$. The suspension was distributed in 50 ml stainless steel centrifuge tubes (15 ml/tube) and heated in a 60° C bath for 4 min with manual agitation. The tubes were then chilled in ice water and centrifuged in the cold at 10000 × *g* for 20 min. The supernatants were dialyzed overnight against 9 vols. 0.04 M Tris, pH 7.4, containing 0.15 M NaCl, to produce a final calcium concentration of 0.01 M. Any precipitate that formed was centrifuged off.

Collagenase assays

Collagenase activity in the 6000 × *g* uterine pellets before extraction, or in the 10000 × *g* pellet after extraction procedures that did not involve heating, were assayed by the method of Ryan and Woessner [2]. The pellets were resuspended in their original volume of 0.04 M Tris · HCl buffer, pH 7.4, containing

0.01 M calcium chloride, 0.15 M NaCl, and antibiotics. 2 ml of suspension was distributed to each assay tube. At this point latent collagenase was activated by adding 0.1 ml buffer containing 100 μg crystalline trypsin (Worthington, Freehold, N.J.). The samples were incubated at 37° C for 3 min, then a 5-fold excess of soybean trypsin inhibitor was added (360 μg in 0.1 ml buffer). Incubation was then continued for 18 h at 37° C in a Dubnoff metabolic shaker. Blanks contained 0.01 M EDTA in place of calcium. The digests were chilled and centrifuged at 30000 × *g* for 30 min. Both supernatants and pellets were hydrolyzed in 6 M HCl and assayed for hydroxyproline [10]. The hydroxyproline in the supernatant, after subtraction of the blank value, was taken as a measure of collagen digestion. In this assay the only substrate present is that collagen that is present in the pellets (about 800 μg hydroxyproline per pellet).

Collagenase in extracts of uterine pellets was assayed by release of radioactive peptides from [^{14}C]glycine-labeled collagen gels. This assay followed the micro-tube method of Eisen et al. [11] and employed radioactive collagen purified from salt extracts of guinea pig skin by the method of Gross [12]. Blanks were run with 0.01 M EDTA in place of calcium. After centrifugation of the digests for 10 min at 50000 × *g* at room temperature, the supernatants (2 ml) were removed to low-potassium scintillation vials. Modified Bray's [13] solution (14 ml) was added and the samples were counted in a TriCarb 574 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). The counts were corrected to 100% efficiency by the use of an external standard.

The recovery of collagenase in extracts was estimated by determining how much enzyme was present in a uterine pellet and then measuring the amount extracted by adding the extract to a second pellet and measuring the increment of collagen digestion. A 6000 × *g* pellet was prepared from one uterus and divided into 3 parts. One portion (A) was assayed for its endogenous collagenase activity. A second portion (B) was extracted at 60° C to solubilize the collagenase. This extract was added to a third portion (C) which was then assayed to give the sum of collagenase from the pellet plus the extract. When the collagenase activity of (A) was subtracted from that of (C) the difference was due to the activity of the extract. After making a small correction for soluble hydroxyproline carried along in the extract of (B), the recovery of collagenase in the extract was calculated. This procedure was repeated using 5 different uteri. Both pellets and extracts were activated by brief exposure to trypsin as described above. The linearity of this assay method was proven by the use of concentrated extracts, diluted to different concentrations.

Results

Trypsin activation of uterine collagenase

For several years we have employed the direct method of collagenase assay using 6000 × *g* pellets [2]. In this method the supernatant from the first centrifugation was discarded because it was inhibitory to collagenase activity. However, there was always concern that we might be discarding collagenase activity with this supernatant. Recent work by Sakamoto et al. [14] suggested the use of trypsin to bind trypsin inhibitors such as α-2-macroglobulin, which may also inhibit collagenase [15]. An excess of soybean trypsin inhibitor can

then be added to block unreacted trypsin so that it does not attack collagenase or the collagen substrate. This principle was developed into a method of measuring collagenolytic activity in uterine supernatants.

Homogenates of 1-day post partum rat uteri were prepared in 10 vols. 0.05 M Tris · HCl buffer, pH 7.5, with 0.15 M NaCl and 0.01 M $CaCl_2$. These homogenates were centrifuged at 6000 × *g* and the pellets were assayed in the usual manner [2]. If the supernates were added to the pellets, they inhibited the collagenase activity by 72%. However, if the supernatants were treated with trypsin, they lost this inhibitory effect. It was found that the activity in the pellets was also enhanced 33% by treatment with trypsin.

Optimum activity was found if both pellets and supernatants were treated for 3 min at 37°C with 100 μg trypsin for each 200 mg wet tissue used for the homogenates. A 5-fold excess of soybean trypsin inhibitor blocked any further action of trypsin, as shown by appropriate blanks. It was shown by this approach that the supernatant contained 20% of all detectable activity and the pellet contained 80%.

Initial homogenization procedure

Since the trypsin experiment revealed that appreciable amounts of collagenase were present in the supernatant, homogenization techniques were sought that would maximize the recovery of collagenase in the 6000 × *g* pellet produced immediately after homogenization. It was not desirable to extract the collagenase directly in the first homogenization step since the insolubility of the bulk of the collagenase offered the possibility of extensive purification by discarding all soluble proteins. Various homogenizing fluids were tested to see which would result in the highest collagenase recovery in the insoluble pellet as measured by the pellet assay with trypsin activation. The fluids tested were distilled H_2O, 0.25% Triton X-100, 0.15 M NaCl, 0.25 M sucrose, 0.04 M Tris (pH 7.5), 0.04 M Tris containing 0.01 M $CaCl_2$ and 0.15 M NaCl, and 0.01 M $CaCl_2$ containing 0.25% Triton X-100. The last named solution proved to be the best. An average of 95% of the total activity detectable in the whole homogenate was recovered in the pellet using this fluid. It is thought that calcium, which is a required cofactor for collagenase, either facilitates the binding of some collagenase that is not yet attached to its substrate or else reduces elution of bound enzyme by the homogenizing fluid. The sucrose experiment and experiments with Triton X-100 addition indicate that the collagenase is probably not contained in lysosomes or similar particulates.

Extraction of collagenase from uterine pellets

Pellets were prepared by homogenizing uteri in the Triton/calcium solution as described in Methods. The pellets were then extracted with various solutions at 4°C and 37°C. The residue that remained insoluble was assayed by the pellet assay method to determine how much activity had been lost in comparison to unextracted pellets. None of the following treatments removed more than 10—25% of the collagenase from the pellet: triple extraction with 0.15 M NaCl, extraction with 0.1% Triton X-100 for 3 h, and 2 M NaCl/0.1% Triton X-100.

Lack of success in extracting collagenase in the cold or at 37°C prompted a study at higher temperatures. Since rat collagen has a shrinkage temperature of

about 58—62°C [16], it was decided to heat the collagen to its shrinkage temperature. The enzyme might be more readily extracted if its substrate changed conformation. Excess calcium was also added in the belief that if calcium bound the enzyme to its substrate, excess calcium might displace the enzyme from the substrate. In these experiments the collagen substrate was partly denatured and could no longer serve for enzyme assay. It was necessary to assay the extracts on radioactive collagen gels as described in Methods.

In a study of the time required for maximum extraction of collagenase at 60°C, it was found that 4 min gave the maximum yield. Denaturation of the enzyme presumably outweighed further extraction beyond this time. Varying the concentration of $CaCl_2$ indicated that optimal release of enzyme was obtained between 0.1 and 0.25 M $CaCl_2$ (Fig. 2). NaCl at the same ionic strength was not an effective extractant. Calcium acetate was equally effective as calcium chloride, indicating the anion is not critical.

Using 0.25 M $CaCl_2$ and a 4-min time interval, the effects of temperature were studied (Fig. 3). Optimum extraction was obtained between 50 and 60°C. At the higher temperature some protein denaturation probably occurred, resulting in extracts of higher specific activity. For this reason 60°C was finally selected as the optimum temperature. Above 60°C collagenase begins to be lost by denaturation. The extraction volume has been optimized: smaller volumes gave less complete extraction and larger volumes gave greater dilution.

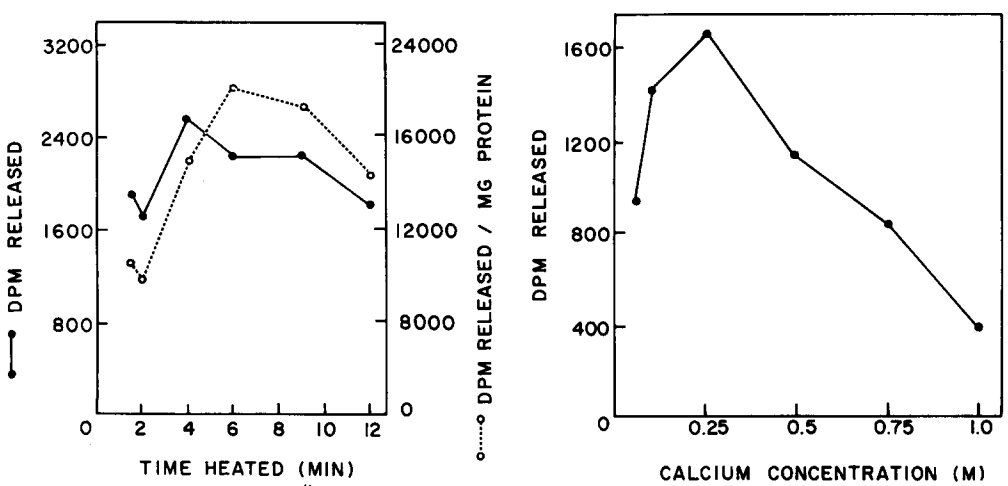

Fig. 1. Effect of heating at 60°C for various periods of time on the extraction of collagenase from rat uterine pellets. The 6000 X *g* pellets were resuspended in 0.04 M Tris buffer, pH 7.4, containing 0.25 M $CaCl_2$. The suspensions were placed in metal centrifuge tubes and placed in the 60°C bath for the indicated time. They were then chilled, centrifuged at 10000 X *g* for 20 min, and 200 µl portions of the supernatant were assayed on [^{14}C]collagen gels (10410 dpm/gel) according to the method of Eisen et al. [4]. The graph shows the counts released after 48-h incubation at 37°C. Protein was estimated by the absorption at 280 nm.

Fig. 2. Effect of calcium concentration on the extraction of collagenase activity from uterine pellets at 60°C. Pellets were suspended in 0.04 M Tris buffer, pH 7.5, containing the indicated concentration of calcium chloride. After heating to 60°C for 4 min, the suspensions were chilled and centrifuged 20 min, 10000 X *g*, and 200 µl of supernatant were assayed on [^{14}C]collagen gels (5600 dpm/gel). Incubation was at 37°C for 68 h.

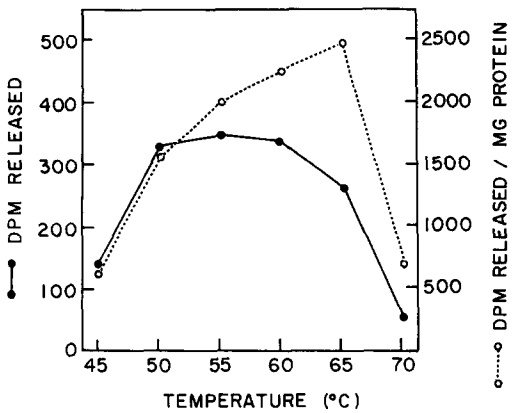

Fig. 3. Effect of temperature on collagenase extraction from uterine pellets. Pellets were suspended in 0.04 M Tris buffer, pH 7.4, and 0.25 M $CaCl_2$. The suspensions were heated in metal tubes to the indicated temperatures for 4 min and then chilled and centrifuged 20 min, 6000 × *g*. The supernatants were dialyzed against Tris buffer containing 0.01 M $CaCl_2$ to restore the calcium to a uniform level. At this point a protein precipitate usually formed and was removed by centrifugation. Protein was estimated by the absorption at 280 nm. The supernatant was assayed as in Fig. 2. The gels contained 2210 dpm [^{14}C]-collagen.

Other features of the extraction procedure as given under Methods have also been explored. The uterine tissue may be stored up to 1 month in the freezer with little change in extractible collagenase. Uteri at 1 day post partum have the same enzyme concentration as 2-day uteri, but they weigh twice as much. Homogenizing time is not critical, but the fluid volume is. Centrifugation below 6000 × *g* gives reduced yields, above 6000 × *g* the yield is constant, but extraneous protein content increases. The pH of extraction is not critical, it may lie anywhere between 6.8 and 8.9.

Recovery of the enzyme activity in extracts

The only method currently available for the estimation of collagenase in the insoluble pellet of uterine tissue is the direct incubation of the pellet with added calcium, followed by the measurement of released hydroxyproline-containing peptides [2]. In order to estimate recovery of extracted enzyme it was decided to use the same assay in which the uterine collagen fibers serve as substrate. Since these fibers contain endogenous collagenase, it is necessary to measure the incremental increase in collagen breakdown when exogenous collagenase is added. The details are given under Methods. Trypsin activation was used for every sample. The results are presented in Table I. An average recovery of 68% was obtained. The remaining activity is presumably still in the pellet. However, the pellet collagen is no longer a suitable substrate for assay of this residual activity since it has become susceptible to nonspecific proteolytic digestion following the heating step. Further extraction by repeating the original procedure brings out additional activity. About 10—15% of the total activity is obtained in a second extract and lesser amounts in subsequent steps.

Comparison to other procedures for collagenase extraction

Since our original report of collagenase extraction appeared [8], several

TABLE I

ESTIMATION OF THE RECOVERY OF COLLAGENASE AFTER EXTRACTION

Pellets were prepared from the uteri of 5 rats and extracted with 0.1 M $CaCl_2$ in 0.05 M Tris buffer, pH 7.5, at 60°C for 4 min. Collagenase recovery in the extracts was determined by adding the extracts to uterine pellets as detailed under Methods. Results are given as mean ± S.D.

	μg Hydroxyproline released/uterus	% Initial activity
Endogenous activity of pellet	601 ± 82	100
Increment due to added extract	407 ± 62	68 ± 6

workers have developed methods for extracting collagenase from various tissues. These methods have all been applied to the 6000 × *g* uterine pellets, and comparison is made to results with the present method (set equal to 100%). Extraction with 1 M NaCl at 37°C for 48 h [17] extracted only 50% as much as the present method. Other variations proposed by Wirl [17] yielded only 10—15%. The method of Nagai and Hori [6] yielded 40% and two other methods [7,18] yielded less than 10%. Since the best methods only approached

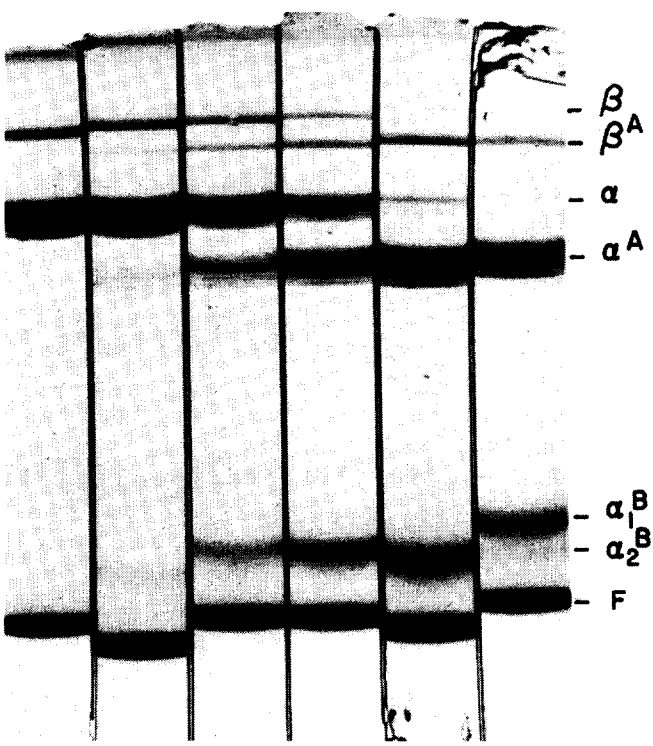

Fig. 4. Disc electrophoresis patterns of collagenase-degraded collagen. Guinea-pig skin collagen was digested at 28°C with uterine collagenase, it was then heat-denatured and subjected to disc electrophoresis by the method of Nagai et al. [19]. Incubation time increases from left to right (0, 2, 4.5, 7, 10, 29 h, respectively). The viscosity loss at each incubation time was 0, 5, 18, 35, 48 and 52%, respectively. The α and β components of collagen were split into 3/4- and 1/4-length pieces designated A and B. F indicates the tracking-dye front.

40—50% of the activity extracted by the present method and since our method extracts only 65—70% of the enzyme, several of the methods are of minimal value when applied to the uterus.

In a further study we considered the possibility that there might still be collagenase in the original discarded supernatant that was escaping detection even after trypsin activation. We followed the same procedure used by Eisen et al. [4] for extracts of human skin, fractionating the supernatant on a Sephadex G-150 column and testing the resulting fractions before and after KCNS treatment [3] to reverse possible α-2-macroglobulin inhibition. Only slight traces of activity were found by these methods.

Evidence that extracted enzyme is a collagenase

The enzyme extracted from the 1-day post-partum uterus has a broad pH optimum centered on 7.5 and requires calcium ion for activity. It digests soluble collagen to give the disc gel patterns shown in Fig. 4. The two fragments labeled A and B in these patterns represent the 3/4- and 1/4-length pieces of tropocollagen as shown by electron microscopy of reconstituted segment long-spacing crystallites: guinea pig skin collagen was split at interband 41 as is typical for known collagenases [20]. Fragments shorter than the TC$\frac{A}{3}$ fragment were not observed. The enzyme was not inhibited by 0.01 M cysteine, 1 mM iPr_2P-F, 10^{-5} M ovomucoid, or soybean trypsin inhibitor. Inhibition by 0.01 M EDTA was complete and was not reversed by dialysis against Ca^{2+} or Zn^{2+}.

Discussion

A method is presented for the extraction of collagenase from the involuting rat uterus, a tissue rich in this enzyme. In the first step of extraction, an homogenizing fluid has been sought which causes the maximum retention of the enzyme in the insoluble 6000 × *g* pellet. The best fluid, yielding about 95% of the enzyme in the pellet, is 0.25% Triton X-100/0.01 M $CaCl_2$. This result indicates that collagenase is unlikely to be a lysosomal or membrane-bound enzyme. In the second step the enzyme is solubilized by extracting the pellet with 0.1 M $CaCl_2$ at 60°C. Both the heat and high calcium concentration are required for optimum extraction. The results are consistent with, but do not prove, the hypothesis that the enzyme is associated with its collagenous substrate. In the third step of the method trypsin treatment for 3 min is applied to reveal occult activity in the extracts. The results have been interpreted as suggesting the presence of protease inhibitors such as α-2-macroglobulin in the extracts. These inhibitors would react with trypsin more rapidly than with collagenase, so that they could not subsequently inhibit collagenase during the incubation with collagen substrates. This method has been particularly useful in estimating the total collagenase activity in the initial uncentrifuged homogenates where inhibitors seem to be abundant. However, our latest data suggest that the trypsin activation of the final extracts is not due to the blocking of inhibition since inhibitor levels are low at this stage, but rather to the proteolytic activation of a proenzyme form of collagenase [21].

While several methods have been proposed in recent years for the extraction of collagenase from various tissues, these methods do not appear to be optimal

for the uterus. The method of Sakamoto et al. [7] and the cold salt extraction of Wirl [17] bring out only a small part of the uterine enzyme. Methods involving heat at either 37° C for 48 h [16] or 45° C for 2 h [6] are much better, but still only about 50% as effective as the present method. The present study offers a distinct advance over previous work in that it has been possible to get a reasonable estimate of the total collagenase content of the tissue by means of the digestion of endogenous collagen in the pellet assay. This in turn permits an estimate of the recovery by noting the increment in digestion when extracts are added to unextracted pellets. The present method appears to recover 65—70% of the initial activity. No other papers on collagenase extraction have offered any evidence of the effectiveness of the extraction.

Once the collagenase has been extracted it is in a convenient form for characterization. In the present study this characterization has been carried far enough to show that the enzyme is a true collagenase, cleaving the molecule of collagen into 75 : 25 proportions at interband 41. Other properties, especially inhibition patterns, match those reported for collagenase measured directly in whole homogenates of rat uterus [1] and collagenase obtained from cultures of rat uteri [3] in all respects except we did not observe the smaller collagen fragments of 62 and 67% produced by the enzyme from culture.

The development of direct tissue assays for collagenase and of extraction methods to solubilize collagenase opens the way to more exact quantitation of this enzyme in tissues, especially during changing physiological states. The methods detect that activity which is bound to the insoluble fraction of the tissue and is often missed by other assay methods. Finally, it offers an attractive alternative to the culturing of tissue for the assessment of physiological changes in the enzyme.

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