The effect of chronic ethanol ingestion on synthesis and degradation of soluble, contractile and stromal protein fractions of skeletal muscles from immature and mature rats

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1. An investigation was carried out into the response of soluble, myofibrillar and stromal protein fractions of skeletal muscle to chronic ethanol feeding. Groups of male Wistar rats, of approx. 85 or 280 g body wt., were pair-fed on a nutritionally complete liquid diet containing glucose or a diet in which 36 % of the total energy was provided by ethanol. After 6 weeks, rates of protein synthesis were measured with a flooding dose of L-[4-³H]phenylalanine. 2. The protein contents of soluble, myofibrillar and stromal fractions in gastrocnemius muscle from small and large rats were decreased by ethanol feeding. Greater changes were observed in small than in large rats. 3. Fractional synthesis rates of soluble, myofibrillar and stromal proteins of gastrocnemius were all decreased by ethanol treatment. All fractions responded similarly, though percentage decreases in large rats were greater than in small rats. Absolute synthesis rates in gastrocnemius muscles were also decreased after ethanol treatment. All protein fractions responded similarly, and the magnitudes of the responses in large and small rats were also similar. 4. Fractional rates of breakdown, measured by the difference between fractional growth and synthesis rates, were apparently decreased, in both sets of rats, in all protein fractions. 5. It was concluded that chronic ethanol exposure causes perturbations in soluble, myofibrillar and stromal protein accretion by a mechanism involving unidirectional changes in protein synthesis and possibly breakdown.

INTRODUCTION

Chronic ethanol abuse in Man causes loss of skeletal-muscle mass, as determined by urinary creatinine excretion and mid-arm circumference measurements [1,2]. Biochemical analysis of biopsies from proximal muscle demonstrates a decrease in the protein/DNA ratio, suggesting that protein metabolism is altered [3]. This has been supported by metabolic studies in man which show that ethanol ingestion causes enhanced nitrogen excretion [4,5]. However, skeletal muscle is a heterogeneous mixture of proteins. It is possible that the content of certain labile proteins may be preferentially decreased compared with more stable proteins of skeletal muscle. This would be consistent with studies which show preferential decrease in the synthesis of proteins from the contractile apparatus relative to soluble proteins in starvation [6]. The objective of the present study was to investigate the pathological basis of the ethanol-induced abnormalities in skeletal muscle and to ascertain if myofibrillar protein fractions were also preferentially affected by chronic ethanol exposure. Experiments were therefore carried out in rats chronically fed with ethanol for 6 weeks and compared with appropriate controls.

METHODS

L-[4-³H]Phenylalanine was obtained from Amersham International (Amersham, Bucks., U.K.), and all other

chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Male Wistar rats obtained from the National Institute for Medical Research (Mill Hill, London N.W.7, U.K.), were of 80–90 g or 270–290 g body wt. at the commencement of liquid feeding. Animals were maintained in grid-bottomed cages in a temperature-controlled (20–24 °C) animal house on a 12 h-light/12 h-dark cycle commencing at 07:00 h. The rats initially selected were of approx. 100 g body wt. Animals of this size are particularly sensitive to nutritional disturbances. As sexually immature rats may not have a direct clinical equivalent, studies were also carried out in sexually mature, more slowly growing, rats. It is noteworthy that recent published data have shown that increasing numbers of teenagers regularly drink ethanol in quantities considered to be clinically prohibitive [7]. However, as the sexually immature rats were mature at the end of the feeding regime, we have also distinguished between both sets of animals by the terms 'small' and 'large' respectively.

For the first 3 days, rats were fed on solid laboratory chow. Rats were then paired on a weight basis and fed on either a nutritionally complete liquid diet containing ethanol or isoenergetic glucose [8,9]. The control diet comprised a commercial food drink supplemented with casein, glucose and vitamins [9]. The control diet consisted of fat, protein and carbohydrate, as 19, 15 and 66 % of total energy respectively. For ethanol feeding, 18 % of total energy as glucose was replaced with

Abbreviations used: S_i , S_p , S_B , specific radioactivity of phenylalanine in acid supernatants of tissue homogenate, of plasma and protein hydrolysates respectively; t , labelling period; k_s , fractional synthesis rate; k_g , fractional growth rate; k_d , fractional degradation rate; k_a , absolute synthesis rate.

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isoenergetic ethanol, for the first 3 days. Thereafter, for a further 6 weeks the proportion of glucose replaced by ethanol was increased to 36 % of total energy. Control rats received identical amounts of diet on a paired basis, as described previously [10]. Both control and ethanol-containing diets were isonitrogenous, isolipidic and isovolumetric as well as containing identical amounts of vitamins and micro-nutrients [10]. At the end of the study, rates of muscle protein synthesis were measured as described below.

Feeding patterns

Inherent in the feeding techniques above are a number of nutritional facets that merit brief mention, as feeding is a major determinant of skeletal-muscle protein synthesis. Compared with solid-laboratory-chow-fed rats (who generally consume their diet nocturnally), ethanol-fed rats consume their diet in a relatively more uniform manner throughout the day. As ethanol-fed rats consume their diet in amounts which are insufficient to maintain maximal growth rates, the nature of the feeding method also implies that control rats are similarly given these sub-optimal quantities of diet and they immediately consume large quantities of their restricted diet when they are presented with it. Owing to the adverse reactions to ethanol withdrawal, we decided to carry out measurements when both groups were in the 'fed' state. By the time synthesis measurements were completed, ethanol-fed rats had consumed approx. 25 % of their total daily intake, whereas the corresponding values for control rats was 50 % of their daily intake (V. R. Preedy, J. S. Marway & T. J. Peters, unpublished work). However, throughout the period of synthesis measurements, both groups of rats continually consumed their diets.

Measurement of muscle protein synthesis

The method of McNurlan *et al.* [11] was used to measure muscle protein synthesis, as modified by Garlick *et al.* [12] for [$4\text{-}^3\text{H}$]phenylalanine. Control and ethanol-treated rats were given their liquid diets between 08:00 and 08:30 h on the morning of the experiment and injected intraperitoneally with radiolabel (150 mCi; 0.1 Ci/mol; 20 ml/kg body wt.) between 11:00 and 15:00 h. Rats were killed 15 min later by decapitation. Blood was collected into ice-cold heparinized tubes for isolation of plasma. Gastrocnemius muscles were dissected, blotted, weighed and stored at -70°C for subsequent analysis.

Muscles and plasma were processed for phenylalanine specific radioactivities and for protein as described previously [12,13]. Fractional rates of protein synthesis, defined as the percentage of tissue protein renewed each day (i.e. k_s , %/day), were calculated from the formula

$$k_s = 100 \times \frac{S_B}{S_i \times t}$$

where S_B is the specific radioactivity of protein-bound phenylalanine, S_i the specific radioactivity of free phenylalanine in acid supernatants of muscle homogenates, and t the time of incorporation between injection of label and dissection of muscle into iced water [12]. Consideration was taken of the small rise of S_i to plateau during measurement [14], though S_p was considered to attain plateau instantaneously [14].

The above formula assumed that the precursor phenylalanine was incorporated into tissue protein via

intracellular compartments. As there was no direct evidence for this, k_s was also calculated by substituting S_p , the specific radioactivity of free phenylalanine in plasma (representing extracellular compartments), for S_i in the formula. The results of these k_s values were essentially the same as those calculated by using S_i and are therefore not presented.

Fractional rates of protein breakdown, defined as the percentage of tissue protein degraded each day, was calculated from the formula:

$$k_d = k_s - k_g \text{ (%/day)}$$

where k_g was the fractional growth rate (defined as the mean increment in body weight, i.e. %/day). k_g was measured for each animal on the three consecutive days before injection of labelled phenylalanine. The mean percentage growth rate for each group was then calculated. Values for k_s for each fraction were measured as described above. This formula assumes that the fractional rate of growth reflected the fractional rate of protein accretion during the 3-day period.

k_a , defined as the absolute rate of protein synthesis (mg/day), was obtained by the formula:

$$k_a = \frac{P}{100} \times k_s \text{ (mg/day)}$$

where P was the total protein content (mg/muscle) and k_s the fractional synthesis rate calculated from S_i or S_p .

Muscle fractionation

All steps were performed between 0 and 4°C . Gastrocnemius muscles were quantitatively fractionated into 'soluble' (33000 g-supernatant or sarcoplasmic proteins), 'myofibrillar' (contractile-apparatus proteins) and 'stromal' (collagen, elastin and unextractable contractile protein) as described by Smith & Sugden [15] and Preedy & Peters [13]. Briefly, muscles were homogenized in water, and a sample containing 200–400 mg of tissue was rapidly transferred to 20 ml of low-ionic-strength buffer, of composition 10 mM-imidazole, 60 mM-KCl, 0.5 mM-EGTA, 4 mM-MgCl₂, 1 mM-Na₂S₂O₄, 1 mM-dithiothreitol and 0.5 % (v/v) Triton X-100, pH 7.0 [15]. After centrifugation (33000 g, 10 min) supernatants were decanted ('sarcoplasmic' fraction). Re-homogenization, extraction and centrifugation of the precipitate showed that the amount of soluble protein in the precipitate (stromal plus myofibrillar fraction) is approx. 1–2 % (V. R. Preedy & P. H. Sugden, unpublished work). The pellet was then disrupted in a ground-glass homogenizer with 10 ml of high-ionic-strength buffer, of composition 100 mM-KH₂PO₄, 50 mM-K₂HPO₄, 300 mM-KCl, 1 mM-EDTA, 5 mM-ATP, pH 6.3 [15]. After centrifugation (33000 g, 10 min), supernatants containing solubilized myofibrillar protein were decanted. Pellets were resuspended in an additional 10 ml of high-ionic-strength buffer, disrupted and centrifuged. Myofibrillar extracts were combined. The final pellet formed the stromal fraction [15].

We determined the extent to which proteins were lost during the various stages of processing. This was calculated by measuring the amount of protein in the mixed skeletal-muscle homogenate (i.e. 100 %) and comparing this with the amount of protein in the individual protein fractions after the various extraction procedures. The results indicated losses were negligible. Recoveries (means $\pm$ S.E.M. of seven to nine observations)

were: gastrocnemius from small control and ethanol-fed rats, $97 \pm 1\%$ and $97 \pm 1\%$ respectively; gastrocnemius from large control and ethanol-fed rats, $99 \pm 1\%$ and $97 \pm 1\%$ respectively.

After an initial precipitation with 0.33 M-trichloroacetic acid, pellets were washed in 2×12 –14 ml of 0.2 M-HClO₄. Protein pellets were then digested in 10 ml of 0.3 M-NaOH, and a sample was removed for protein determination. After re-precipitation of the protein with 2 M-HClO₄, pellets were washed repeatedly (8 times, 12–14 ml each) in 0.2 M-HClO₄ for determination of specific radioactivities as described previously [12,13].

Statistical analysis

All data are presented as means $\pm$ S.E.M. for seven to nine pairs of observations. As individual rats were pair-matched for the feeding regime, differences between means were assessed by paired *t* test. Relative sensitivities of individual fractions were assessed by calculating data as ratios in individual animals, i.e. soluble/myofibrillar, soluble/stromal and myofibrillar/stromal. These values are only presented when statistical significance was attained. Significance was indicated at $P < 0.05$.

RESULTS AND DISCUSSION

Other studies have shown that proteins from the different skeletal-muscle protein fractions may respond differently to patho-physiological interventions [6,16]. However, apart from the studies by Preedy & Sugden [17] and Preedy & Peters [13], there are limited other data in which a reliable and accurate technique has been used to measure protein synthesis in an experiment where proteins have also been quantitatively extracted. In practical terms this is a very important point, as measurement of protein synthesis in individual fractions may not relate to protein loss. Another limitation of other studies has been that protein synthesis in the fractions discarded during processing may be physiologically important. For the present study virtually 100% recoveries were obtained. The extraction procedures were also reproducible, as judged by the fact that

coefficients of variation for protein concentrations were no greater than 8%, in control and ethanol-fed rats, whether small or large groups. An accurate and proven technique was used to measure the fractional synthesis rates of individual protein fractions, i.e. the flooding-dose method [11,12,18–20].

In small rats there were small but variable (2–4%) changes in the concentrations of individual fractions in gastrocnemius muscles in response to ethanol treatment (Table 1). However, the total amounts of soluble, myofibrillar and stromal protein in small rats were significantly decreased, by 17–23%. Table 1 also shows that the decline in the stromal fraction was greater than in the myofibrillar fraction, as the ratio total protein in myofibrillar fraction/total protein in stromal fraction increased from 1.37 ± 0.05 to 1.41 ± 0.06 , in control and ethanol-fed rats respectively ($P < 0.05$). Examination of other ratios did not reveal any statistically significant differences. Smaller decreases in total protein content were observed in the larger group of rats, but statistical significance was only attained for the soluble fractions. Similar qualitative changes were observed when data for protein contents were calculated relative to body weight. However, the magnitude of the response was smaller (Table 1).

The above changes in protein content must be explained by alterations in protein synthesis and/or degradation [21]. Table 2 shows that ethanol treatment caused specific radioactivities of muscle proteins in gastrocnemius from both small and large rats to decrease by 15–22%. When data in small rats were expressed as synthesis rates, as calculated from S_i , k_s in soluble, myofibrillar and stromal fractions decreased by 11–12%. This was only statistically significant for the stromal fraction (Table 2). However, when k_s was calculated from S_p , significance was attained for soluble and stromal fractions ($P < 0.025$ and $P < 0.01$ respectively; results not shown). Inspection of the individual ratios did not demonstrate that any one fraction was particularly more sensitive. In large rats, k_s calculated from S_i was decreased in soluble myofibrillar and stromal fractions, though this was not statistically significant for the stromal

Table 1. Effect of chronic ethanol feeding on protein composition of gastrocnemius muscle

Male Wistar rats weighing 80–90 g (immature rats) or 270–290 g (mature rats) initial body weights were fed on a control or ethanol-containing liquid diet for 6 weeks as described in the Methods section. Soluble, myofibrillar and stromal proteins were separated on the basis of differential solubility. All data are presented as means $\pm$ S.E.M. for 7–9 observations. Negative and positive prefixes signify decreases and increases respectively. Differences between control and ethanol-fed rats were assessed by Student's *t* test for paired samples: $\dagger P > 0.05$ (not significant); $* P < 0.05$, $*** P < 0.01$, $**** P < 0.001$.

Parameter	Protein fraction	Small rats			Large rats		
		Control	Ethanol	Difference (%)	Control	Ethanol	Difference (%)
Protein concn. (mg/g wet wt.)	Soluble	57.5 ± 1.5	56.6 ± 1.1	–2†	61.8 ± 0.3	59.7 ± 0.9	–3*
	Myofibrillar	73.9 ± 1.3	76.7 ± 1.6	+4†	71.5 ± 1.0	72.2 ± 0.7	+1†
	Stromal	46.0 ± 1.1	44.5 ± 0.9	–3†	52.8 ± 1.7	51.4 ± 1.3	–2†
Total protein (mg)	Soluble	143 ± 5	112 ± 4	–22****	257 ± 4	234 ± 7	–9*
	Myofibrillar	184 ± 5	152 ± 5	–17****	297 ± 4	284 ± 7	–4†
	Stromal	114 ± 4	88 ± 3	–23***	220 ± 10	202 ± 6	–8†
Total protein/body wt. (mg/kg)	Soluble	637 ± 20	567 ± 12	–11***	660 ± 12	649 ± 6	–2†
	Myofibrillar	817 ± 14	769 ± 23	–6*	763 ± 9	787 ± 13	+3†
	Stromal	510 ± 15	446 ± 13	–12***	564 ± 22	560 ± 12	–1†

Table 2. Effect of chronic ethanol feeding on specific radioactivities and synthesis rates in gastrocnemius protein fractions

Rates of protein synthesis were measured by injection of a large concentration of L-[4-³H]phenylalanine, at the end of 6 weeks liquid feeding, as described in the Methods section. All data are presented as means $\pm$ S.E.M. for 6–7 observations. Negative prefixes indicate a decrease. Differences between means were assessed by Student's *t* test for paired samples: †*P* > 0.05 (not significant); **P* < 0.05; ***P* < 0.025; ****P* < 0.01; *****P* < 0.001.

Parameter	Protein fraction	Small rats			Large rats		
		Control	Ethanol	Difference (%)	Control	Ethanol	Difference (%)
Specific radioactivity (d.p.m./nmol)	Soluble	0.139 $\pm$ 0.003	0.115 $\pm$ 0.005	–17**	0.092 $\pm$ 0.004	0.078 $\pm$ 0.003	–15***
	Myofibrillar	0.098 $\pm$ 0.003	0.082 $\pm$ 0.004	–16*	0.060 $\pm$ 0.004	0.047 $\pm$ 0.002	–22†
	Stromal	0.122 $\pm$ 0.005	0.104 $\pm$ 0.004	–15**	0.079 $\pm$ 0.003	0.067 $\pm$ 0.004	–15†
<i>k_s</i> , calculated from <i>S_i</i> (%/day)	Soluble	8.14 $\pm$ 0.19	7.15 $\pm$ 0.32	–12†	6.19 $\pm$ 0.18	5.23 $\pm$ 0.17	–16****
	Myofibrillar	5.73 $\pm$ 0.20	5.09 $\pm$ 0.28	–11†	4.06 $\pm$ 0.27	3.16 $\pm$ 0.14	–22*
	Stromal	7.17 $\pm$ 0.30	6.41 $\pm$ 0.25	–11*	5.35 $\pm$ 0.18	4.52 $\pm$ 0.25	–16†
<i>k_a</i> , calculated from <i>S_i</i> (mg/day)	Soluble	11.40 $\pm$ 0.60	8.05 $\pm$ 0.49	–29**	15.93 $\pm$ 0.49	12.27 $\pm$ 0.48	–23***
	Myofibrillar	10.37 $\pm$ 0.46	7.67 $\pm$ 0.55	–26**	12.05 $\pm$ 0.72	8.95 $\pm$ 0.37	–26**
	Stromal	8.35 $\pm$ 0.67	5.76 $\pm$ 0.45	–31***	11.77 $\pm$ 0.66	9.19 $\pm$ 0.66	–22†

Table 3. Effect of chronic ethanol feeding on indirectly measured rates of protein breakdown in muscle fractions

Fractional degradation rates (*k_a*) were calculated indirectly, from the difference between fractional synthesis and growth rates, as described in the text. Negative prefixes indicate a decline. As values for *k_a* were not obtained directly, the assumptions used in their calculation are described in depth in the Results and discussion section.

Protein fraction	<i>k_a</i> , derived via <i>k_s</i> calculated from <i>S_i</i> (%/day)					
	Small rats			Large rats		
	Control	Ethanol	Difference (%)	Control	Ethanol	Difference (%)
Soluble	5.78	5.11	–12	5.68	4.86	–14
Myofibrillar	3.37	3.05	–10	3.55	2.79	–21
Stromal	4.81	4.37	–9	4.84	4.15	–14

fraction. Similar decreases in *k_s* were obtained when calculated from *S_p* (results not shown). Inspection of the ratios of synthesis rates in individual fractions showed that no fraction was more susceptible to ethanol feeding.

Although the changes in *k_s* in small rats were small, it was possible that an effect on whole-body muscle protein synthesis occurred in response to ethanol feeding. The results in Table 2 also showed that, for small rats, *k_s* in all fractions fell significantly, by 26–31%, whether calculated from *S_i* or *S_p* (results not shown). The magnitude of the effects was decreased when *k_s* was expressed relative to body weight (17–25% decline; results not shown). Analysis of ratios did not reveal any greater susceptibility to ethanol in any of the three fractions. Similar data were obtained for the large rats.

Although the decline in synthesis in small rats was relatively small, consideration should be given to the fact that even small perturbations in synthesis may have contributed to the diminished protein mass via its accumulative effect over 6 weeks of ethanol feeding. In both small and large rats, protein synthesis was decreased similarly in all three fractions. This contrasts with other catabolic states such as starvation, in which synthesis of

myofibrillar fractions is more responsive [6]. As rats were fed on isonitrogenous diets, decreased muscle protein content must be due to the efficiency or way in which amino acids were metabolized by either skeletal muscle or other tissue(s). Significant decreases were also observed for *k_a* in skeletal muscle, and again all fractions responded similarly. As skeletal-muscle mass comprises 40% of whole-body weight and approx. 25% of whole-body protein synthesis [21], these results indicate that ethanol feeding caused disturbances in whole-body nitrogen metabolism.

Lowell *et al.* [22] have provided evidence to support the possibility that the degradation of myofibrillar protein may be regulated independently from the remaining muscle protein. It was therefore important to ascertain whether a similar mechanism occurred in the present experiments. However, estimation of myofibrillar protein breakdown *in vivo*, from urinary *N*⁷-methylhistidine, is complicated by the fact that *N*⁷-methylhistidine is also derived from non-muscle sources [23]. For this reason we used an alternative approach to measure *k_a* in myofibrillar proteins, based on the difference between *k_s* and *k_g*.

Table 3 shows that chronic ethanol feeding was associated with an apparent decrease in k_d . The effect in small rats was similar to that in large rats. Similar qualitative changes in k_d (i.e. a decrease owing to ethanol feeding) were obtained when k_s was calculated from S_p (results not shown). However, these values for k_d were obtained by indirect measurements, and are discussed in greater depth below. Our results were different from those of Lowell *et al.* [22], in that k_d decreased and all fractions responded similarly. However, our results were similar to those of Martin & Peters [24], who proposed that ethanol-induced myopathy in Man may also be associated with a decrease in myofibrillar protein degradation.

It is important to investigate the strengths or limitations of the assumptions used in the measurement of protein degradation, as applied to the protein fractions. The formula $k_d = k_s - k_g$ is based on the assumption that the fractional rate of animal growth represents the fractional rate of protein accretion or loss of the individual protein fractions [21]. This formula may not be applicable in acute studies, where rapid changes in body weight may not necessarily reflect changes in muscle protein content. For example, acute starvation (i.e. 2 days) may induce changes in body weight via diminished gut content, dehydration, or loss of fat or glycogen. Ideally, the protein content between two time intervals should be measured. However, on a practical basis it is difficult to measure small changes in protein mass between two short time intervals, owing to limitations in assay sensitivity and the relatively slow turnover of muscle protein. For example, over a 3-day period, the minimum change in muscle protein content will be 1.2% (large rats, ethanol group) and maximum 7.1% (small rats, control group). Alternatively, one could measure the amount of protein at the start and the end of the experiment. This has the major limitation in assuming that rates of protein turnover during these periods are similar. The last option is to measure growth of the rat over the shorter period and assume that changes in protein mass reflect these changes. This technique has been used in other studies to measure k_d in both normal control and treated animals [14,25].

The problems used in the assumption that k_g for body weight reflects k_g for muscle protein should be directly addressed. The fact that ethanol treatment causes changes in the amount of muscle mass per unit body weight suggests that our experimental treatment influences the rate at which non-muscle substances may be accreted or lost. This may be the deposition or loss of fat or other tissue components. The degree of tissue hydration or changes in the mass of other tissues may also alter muscle mass per unit body weight. It is therefore possible that this may impart some errors on our estimation of k_d . However, it should be remembered that, although we measure k_g over 3 days, the changes in the amount of muscle mass per unit body weight occur over 6 weeks. In the alcoholic rat model it is highly improbable that ethanol treatment induces a dramatic change in the last 3 days of 6 weeks treatment. We therefore believe that these errors over 3 days will be small. Rats were weighed at the same time each day, thus excluding variability owing to diurnal changes. However, if mean growth rates in large rats were in error by 20% (taking an extreme example of increasing mean growth rates of control rats and decreasing mean growth rates in alcoholic rats each

by 20%), then k_d of all three fractions would still decrease, by 11–17%.

Other assumptions used for the calculation of k_d pertain to the measurement of protein synthesis. The first is that the diurnal changes in muscle protein synthesis are small. This has been validated experimentally by Reeds *et al.* [26], who showed that the maximum error incurred by measurement of k_s at various time points was 6%. Another assumption relates to the synthesis rate at the end of treatment reflecting synthesis rates in the 3-day period of growth measurements. We think this is a valid proposal for a chronically treated animal.

The above work provides information on the qualitative changes in k_d rather than quantitative measurements. Overall, we consider this approach to be useful in providing an experimental framework in which to assess further the involvement of degradative pathways. Together with the observations on synthesis, our data seem to suggest that chronic ethanol exposure causes a general decrease in metabolic processes, such as protein synthesis and possibly protein degradation. This may be due to its direct effect on muscle, or via a mechanism that in general does not appear to distinguish between the different protein compartments.

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