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Intranuclear Binding of Hydrocortisone In Liver and Thymus Tissues[1] (38316)

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(Introduced by E. V. Morse)

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Glucocorticoids, a class of steroid hormones, are essential for the maintenance of a homeostatic state within the body. Numerous physiological effects of these hormones are well documented, however, their intracellular mode of action is unclear. Present evidence indicates the existence of protein receptor molecules in the cytosol which associate with the steroid (3, 5, 7, 9, 10, 13, 14). These complexes then migrate into the nucleus (2, 3, 8), where they alter RNA synthesis (5, 11, 12). The exact physical relationship of the cytoplasmic-receptor complex to the nuclear-receptor complex is unclear. It has recently been postulated by other investigators that the administration of steroids to adrenalectomized rats enhanced the movement of cytoplasmic receptor molecules into the nucleus (8).

Based on the above observations, studies were designed to determine the following: (1) if a migration from the cytosol to the nucleus occurred for hydrocortisone; (2) if pretreatment with hydrocortisone increased specific nuclear binding sites, and (3) to determine the specificity of cytosol and nuclear receptors for glucocorticoids.

Materials and Methods. Treatment of animals. Young Sprague–Dawley strain male rats (75–150 g) were bilaterally adrenalectomized 3–5 days prior to use and maintained on 0.1% sodium chloride drinking water. This time span was selected so that circulating levels of endogenous corticosteroids would be zero, and thus according to Kalimi *et al.* (8) this should increase the number of available cytosol receptor sites. The rats were divided into controls and experimentals. The experimentals were treated with either hydrocortisone, aldosterone or testosterone (1 mg/100 g body wt). Controls were injected with an equal volume of saline.

At varying intervals of time the rats were killed by cervical dislocation or decapitation, and the respective tissues to be used (either brain, heart, liver, or thymus) were removed. Organs which had a rich blood supply were perfused with cold heparinized 0.9% NaCl.

Distribution of ^{3}H-hydrocortisone between the cytosol and nuclear fractions of liver and hypothalamus. In this experiment five grams of perfused liver tissue was minced using a tissue press (Harvard Apparatus Co.) and mixed with 2 vol of Krebs Ringer Phosphate (KRP), pH 7.4. The suspension was gently homogenized using a loose-fitting pestle. Intact cells were settled by centrifuging at 400*g* for 10 min and resuspended in KRP buffer. The suspension was examined by a light microscope and found to consist of intact cells with few contaminants. Twenty ml of this suspension was transferred into a 50 ml flask, 0.5 μCi of 1,2,-^{3}H-hydrocortisone (40–60 Ci/mmole, New England Nuclear) was added, and the flask was incubated in a moving water bath at 37° for 40 min. At intervals of 1, 3, 5, 10, 20, and 40 min, 2 ml aliquots were taken and placed in centrifuge tubes containing cold KRP buffer, and centrifuged at 800*g* for 5 min. The resulting pellet was washed twice with buffer and resuspended in 9.5 ml of 1.5 m*M* $MgCl_2$ at 0°. The resuspension was homogenized in a chilled homogenizer and allowed to stand for 10 min. The homogenate was centrifuged at 800*g* for 10 min. The resulting supernatant (cytosol fraction) was saved and the pellet was washed in buffer, centrifuged, and the pellet (nuclear fraction) was saved. The amount of radioactivity in an aliquot of the cytosol fraction was determined by scintillation spectrometry. The nuclear pellet was resuspended in 2 ml of buffer and a 0.1 ml sample was counted. Chemical and color

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quenching were corrected by the external standard channel ratio technique. The amount of radioactivity in the total cytosol fraction and the total nuclear fraction was calculated.

The same procedure was followed for the hypothalamus, except for the fact that the entire hypothalamus was used.

Nuclear binding of hydrocortisone. The procedure for this experiment required two stages. First, the nuclei from each tissue were isolated. Second, the amount of nuclear binding was determined by a nuclear exchange assay.

(a) *Isolation of nuclei.* The methods used for isolating nuclei varied from tissue to tissue. Cerebral cortex nuclei were isolated according to the procedure reported by McEwen *et al.* (10). Liver and thymus nuclei were obtained by a modification of the procedure reported by Widnell and Tate (15). Five grams of perfused liver, or the entire thymus, were homogenized in 2 vol of 0.32 *M* sucrose–3m*M* $MgCl_2$ using a Potter–Elvehjem homogenizer. The homogenate was filtered through a double layer of cheesecloth and then rehomogenized in an all-glass Duall (Kantes Glass Co.) homogenizer. The homogenate was centrifuged at 800*g* for 3 min. Following centrifugation the crude nuclear pellet was resuspended in 20 ml of a buffer containing 2.4 *M* sucrose–1 m*M* $MgCl_2$ — 0.05 *M* Tris, pH 7.4, and centrifuged at 108,000*g* for 1 hr. The pellet obtained was washed and resuspended in 2 ml of buffer containing 10 m*M* Tris HCl–1.5 m*M* EDTA, pH 7.4. Microscopic examination of this suspension showed the nuclei to be intact and essentially free of cytoplasmic contaminants.

(b) *Determination of nuclear binding.* The amount of high-affinity nuclear binding of ^{3}H-hydrocortisone in control rats and rats previously treated with either hydrocortisone, aldosterone, or testosterone was determined by a modification of the exchange assay for estrogen binding reported by Anderson *et al.* (1). Four series of tubes, two tubes per series, were set up and labeled A, B, C, and D. To each tube in the series a 0.5 ml sample of nuclei was added. Series A received nuclei from control rats, series B from rats treated with hydrocortisone, series C from aldosterone-treated rats, and series D from rats pre-treated with testosterone. A 0.1 μCi aliquot of ^{3}H-hydrocortisone (30 Ci/mmole) was added to the first tube in each series. A 0.1 μCi aliquot of ^{3}H-hydrocortisone (30 Ci/mmole) containing a 100-fold excess of hydrocortisone was added to the second tube. All of the tubes were incubated for 5 min at 45° in a shaking water bath to allow the steroid-receptor complex to disassociate (3). After incubation the tubes were placed in an ice bath for 10 min. The purpose of this cooling was to allow the random reassociation of the receptors with the labeled and the free steroids (13). At this point 3 ml of ice cold 0.32 *M* sucrose—3 m*M* $MgCl_2$ was added to each sample and centrifuged at 39,000*g* for 30 min. The resulting pellets were then washed twice with EDTA buffer and centrifuged at 800*g* for 10 min to remove all of the excess unbound steroid. The pellet was then resuspended in 1 ml of EDTA buffer, and duplicate 0.1 ml aliquots were taken for protein determination and scintillation counting. The samples were placed in liquid scintillation vials and incubated at room temperature for 3 hr with 0.5 ml of Protosol (New England Nuclear). After this incubation 10 ml of a mixture of toluene and PPO (5 g PPO/liter toluene) was added, and the samples were placed in the dark for 12 hr before counting.

The specific nuclear binding was determined by subtracting the DPM's/mg protein in the tubes containing the 100-fold excess of hydrocortisone from the tubes in each series containing the high specific activity hydrocortisone.

This experimental procedure was used for all experiments designed to measure specific nuclear binding. A slight modification was made in some experiments designed to determine if aldosterone or testosterone could compete with hydrocortisone for the nuclear binding sites. The results from this procedure provided data indicating the specificity of the nuclear receptor sites for hydrocortisone. The only change was in the incubation series in which three tubes for each series were used. The first two tubes of the series contained the same material as in the original assay, but a third tube containing either 0.1 μCi of ^{3}H-hydrocortisone (30–60 Ci/mmole) plus a 100-fold excess of aldosterone or a 100-fold excess of testosterone was added.

Determination of optimal incubating concentration for ^{3}H-hydrocortisone. This experiment was conducted prior to the exchange assays in order to determine the concentration of ^{3}H-hydrocortisone need for optimal saturation of nuclear receptor sites. Nuclei were isolated from the liver of rats pretreated with 1 mg/100 g body

wt of hydrocortisone. The same procedures were followed for incubation but varying concentrations of ^{3}H-hydrocortisone (10^{-4}–10^{-10} *M*) were used.

Results. *^{3}H-hydrocortisone uptake by the cytosol and nuclear fractions of the liver and hypothalamus.* The rate of disappearance of ^{3}H-hydrocortisone from the cytosol and the subsequent migration of ^{3}H-hydrocortisone into cell nuclei of the liver and hypothalamus are shown in Figs. 1 and 2. The amounts of radioactivity in both the cytosol and nuclear fractions are shown at 1, 3, 5, 10, 20, and 40 min intervals of incubation time after the addition of ^{3}H-hydrocortisone to intact cells. These results indicate a migration of the radioactivity from the cytosol into the nucleus.

The amount of ^{3}H-hydrocortisone present in the liver cytosol decreased during the first 5–10 min. Simultaneously an increase was observed in the amount of ^{3}H-hydrocortisone in liver nuclei (Fig. 1). The total amount of radioactivity lost from the cytosol was slightly over 10,000 DPM's, and the amount gained by the nuclei was about 7300 DPM's. That essentially 72% of the radioactivity lost from the cytosol was accounted for by an increase in the nuclear fraction is apparent from these data. The same type of migration pattern as found in the liver was observed in the hypothalamus. A comparison of the counts lost from the cytosol to the counts gained by the nuclei revealed that approximately 78% of the radioactivity lost from the cytosol shifted into the nuclear fraction (Fig. 2).

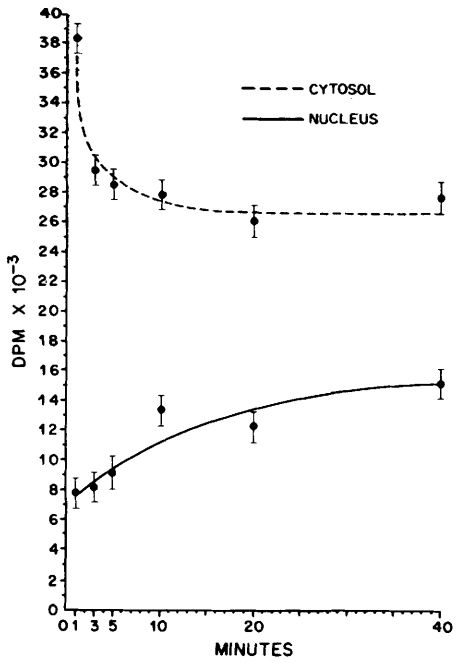

FIG. 1. Radioactivity present in the cytosol and nuclear fraction of liver cells at various intervals of incubation time after the addition of ^{3}H-hydrocortisone to intact cells. Each point represents the average of five experiments ± SE of the mean.

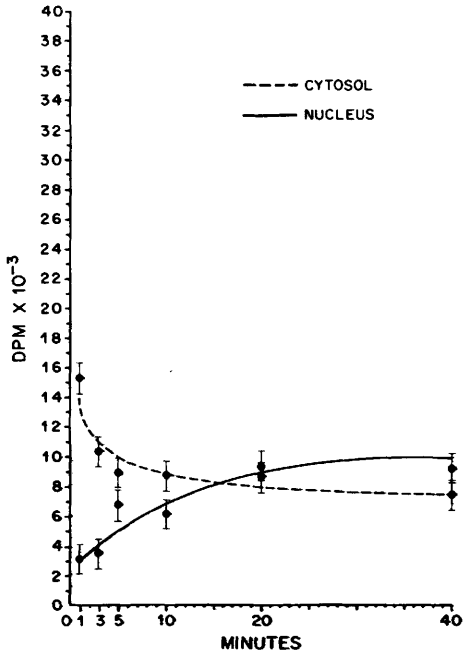

FIG. 2. Radioactivity present in the cytosol and nuclear fractions of hypothalamic cells at various intervals of incubation time after the addition of ^{3}H-hydrocortisone to intact cells. Each point represents the average of five experiments ± SE of the mean.

The fate of the remaining 28% in the liver and 22% in the hypothalamus is only a conjecture, but perhaps before all of the complexes were able to migrate into the nucleus, some dissociated, and the steroid was lost in the washing procedure.

Optimal incubating concentration of ^{3}H-hydrocortisone. The molar concentration of ^{3}H-hydrocortisone which would provide for maximum nuclear binding was determined in isolated liver nuclei incubated with varying concentrations (10^{-4}–10^{-10} *M*) of hydrocortisone. Each sample contained 0.01 μCi of ^{3}H-hydrocortisone.

The resulting curve indicated that the maximum amount of binding occurred at a concentration of 10^{-6} *M* (Fig. 3). Therefore a 10^{-6} *M* concentration was used in each of the exchange assay experiments.

Effects of previous treatment with hydrocortisone on the number of specific nuclear receptor sites. The amount of nuclear binding was determined in cell nuclei isolated from the cerebral cortex, heart, liver, and thymus of adrenalectomized control rats and adrenalectomized rats previously injected with hydrocortisone (1 mg/100 g body wt). The appropriate tissue was removed 1 hr after treatment, and the amount of high-affinity specific nuclear binding was determined by the exchange assay.

Previous treatment with hydrocortisone significantly increased the high-affinity specific nuclear binding in liver ($P < 0.01$) and thymus ($P < 0.05$) (Fig. 4). No effects of treatment were observed in nuclei isolated from the cerebral cortex or the heart.

Specificity of cytoplasmic and nuclear receptors for hydrocortisone. A control group of rats and three experimental groups were used to determine if treatment with nonglucocorticoids altered nuclear receptors and nuclear binding of hydrocortisone. Rats were treated with either hydrocortisone, aldosterone, or testosterone (1 mg/100 g body wt) 1 hr before killing. Pretreatment with aldosterone or testosterone did not alter the amount of nuclear binding as compared to that of control rats (Fig. 5), but pretreatment with hydrocortisone did significantly ($P < 0.01$) increase the high-affinity nuclear receptors in the liver and thymus. Thus, nonglucocorticoid steroids were unable to increase nuclear binding.

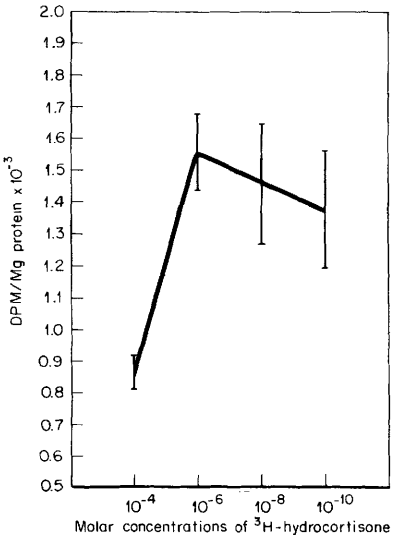

FIG. 3. Specific nuclear binding of ^{3}H-hydrocortisone in nuclei incubated with a constant amount of radioactive hydrocortisone (0.01 μCi), but varying amounts of nonradioactive hydrocortisone. Each point represents the average of five experiments.

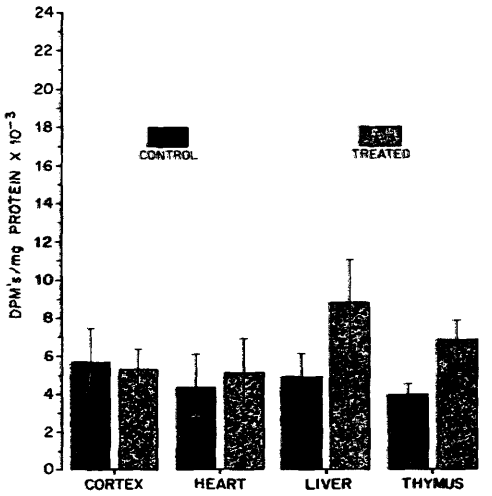

FIG. 4. Specific nuclear binding of ^{3}H-hydrocortisone in different tissues of control rats and rats treated with 1 mg of hydrocortisone/100 g body wt. Cell nuclei were isolated, and the amount of nuclear specific binding was determined by the exchange assay given in Materials and Methods. Each bar represents the average of 3–5 determinations ± SE of the mean.

The specificity of the nuclear receptors for hydrocortisone was determined in liver and thymus nuclei isolated from rats previously treated with hydrocortisone. Pretreated rats were used, since this increased the number of nuclear receptors. To measure the specificity of the nuclear receptors, isolated nuclei were incubated with 0.1 μCi of ^{3}H-hydrocortisone + 100-fold excess of aldosterone, with 0.1 μCi of ^{3}H-hydrocortisone + 100-fold excess of testosterone or with 0.1 μCi of ^{3}H-hydrocortisone + 100-fold excess of unlabeled hydrocortisone. The amount of nuclear binding was determined by the exchange assay.

The addition of 100-fold excess of aldosterone or a 100-fold excess of testosterone to the incubation procedure had no significant effect in either tissue (Fig. 6), but the addition of a 100-fold excess of hydrocortisone competed for the nuclear receptors and significantly ($P < 0.01$) decreased the binding of

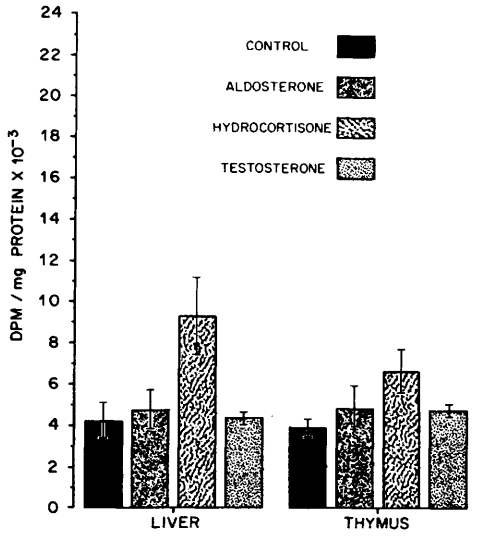

FIG. 5. High-affinity nuclear receptors of the liver and thymus in control rats vs rats pretreated with hydrocortisone (1 mg/100 g body wt), aldosterone (1 mg/100 g body wt), or testosterone (1 mg/100 g body wt). The amount of nuclear specific binding was determined by the exchange assay given in Materials and Methods. Points are based on the average of five experiments ± SE of the mean.

$p < 0.01$ when the hydrocortisone treated group is compared to either the control, aldosterone, or testosterone treated groups.

^{3}H-hydrocortisone (Fig. 6).

Discussion. The results of a time study on the uptake of ^{3}H-hydrocortisone by the cytosol and nuclear fractions of the liver and hypothalamus (Figs. 1 and 2) indicated a rapid movement of the steroid from the cytosol into the nucleus. This increased nuclear concentration may result from an initial association of the steroid with cytoplasmic receptors followed by alteration in the configuration of the receptor which enhances its migration into the nucleus. Beato *et al.* (4) reported that one can increase the incorporation of ^{3}H-hydrocortisone in a liver nuclei fraction by incubating that fraction with a homologous cytosol fraction.

An optimal binding concentration of 10^{-6} *M* was chosen since this concentration saturated nuclear binding sites (Fig. 3). It is important to note that a concentration of 10^{-6} *M* is adequate to cause maximum physiological effect of hydrocortisone.

Experiments were conducted in order to determine if previous treatment with hydrocortisone altered the amount of specific nuclear binding in the cerebral cortex, heart, liver, and thymus. These tissues were selected since the liver and thymus responds greatly to glucocorticoids, whereas, the cerebral cortex and heart do not respond greatly to these steroids. Following a 1 hr pretreatment with hydrocortisone (1 mg/100 g body wt), an increase in amount of high-affinity specific nuclear binding in the liver and thymus was observed (Fig. 4). However, there was no significant change in either the cerebral cortex or the heart. This seemed to indicate a relationship between nuclear binding and physiological effects, since the liver and thymus are known to respond greatly to glucocorticoids. The cerebral cortex and heart showed no significant increase in the number of nuclear receptors following hydrocortisone treatment. The absence of cytoplasmic receptors capable of migrating into the nucleus may be the reason for a lack of significant differences between control and experimental groups in the cortex and heart. Previous studies with estrogens revealed that pretreatment of animals with estradiol-17B resulted in increased specific nuclear binding of estradiol in uterine nuclei (1, 6). The uterus is a

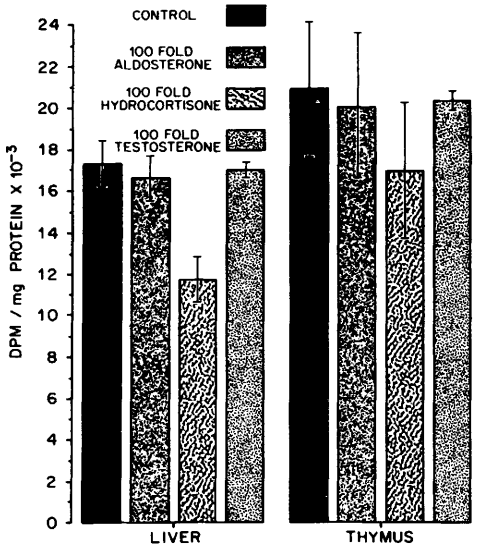

FIG. 6. Specificity of nuclear binding sites for hydrocortisone. Liver and thymus nuclei were incubated with (a) 0.1 μCi of ^{3}H-hydrocortisone (F), (b) 0.1 μCi of ^{3}H-F + 100-fold excess of aldosterone, (c) 0.1 μCi ^{3}H-F + 100-fold excess of unlabeled hydrocortisone, or (d) 0.1 μCi ^{3}H-F + 100-fold excess of testosterone. The amount of nuclear binding was determined by the exchange assay. Each value represents the average of 5 experiments ± SE of the mean. $p < 0.01$ for (c) vs (a), (b), or (d).

known target organ for estrogens, and investigations for intracellular binding of these hormones have been very fruitful.

The liver and thymus from rats pretreated with nonglucocorticoids, aldosterone or testosterone, showed no significant difference from the control rats in the number of specific nuclear binding sites. However, hydrocortisone-treated rats showed a significant increase in liver and thymus specific nuclear binding sites when compared to the controls (Fig. 5). The inability of aldosterone or testosterone to increase nuclear binding is probably due to the inability of these steroids to interact with cytoplasmic receptors and enhance their movement into the nucleus. In particular, even though aldosterone, a mineralocorticoid, is similar in origin to glucocorticoids, its three-dimensional structure differs enough from that of hydrocortisone that the cytosol receptors of the liver and thymus will not interact with it. These receptors are therefore apparently quite selective and may be abundant only in tissues actively responding to glucocorticoids.

The specificity of the nuclear receptors for hydrocortisone is shown in Fig. 6. There was no significant difference in liver or thymus nuclear binding, if one compared control nuclei with nuclei incubated with either 0.1 μCi of ^{3}H-hydrocortisone plus a 100-fold excess of aldosterone or a 100-fold excess of testosterone. Neither aldosterone nor testosterone competed for the nuclear receptors driven in by hydrocortisone. Only when the samples were incubated with 0.1 μCi of ^{3}H-hydrocortisone + 100-fold excess of hydrocortisone did a significant difference in nuclear binding occur. The presence of the unlabeled hydrocortisone lowered the amount of nuclear binding due to active competition for the nuclear receptor sites. Consequently, these receptors remain specific throughout their interactions with hydrocortisone.

Summary. Intranuclear uptake and binding of hydrocortisone was determined in several tissues of the rat. The first experiment was a time-uptake study designed to determine the relative distribution of ^{3}H-hydrocortisone between the cytosol and nuclear fractions of liver and hypothalamus tissue. Maximum uptake was observed in each tissue as early as 1 min after the addition of the isotope. This was followed by a rapid migration of the radioactivity into the nucleus. Maximum nuclear uptake had occurred by 20 min. The increased nuclear uptake was accompanied by a decreased cytoplasmic radioactivity.

The second experiment was designed to determine if pretreatment of rats with hydrocortisone increased specific nuclear binding in the cerebral cortex, heart, liver, and thymus. These tissues were selected because the liver and thymus are known to respond greatly to glucocorticoids, whereas, the cerebral cortex and heart have now shown such a response. It was observed that pretreatment with hydrocortisone increased nuclear binding in the liver and thymus, but hydrocortisone treatment had no effect on the cortex or the heart.

The inability of nonglucocorticoids to drive receptors into the nucleus was apparent since pretreatment with either aldosterone or testosterone did not increase the number of specific nuclear binding sites in either the liver or the thymus.

The nuclear receptors in the liver and thymus were shown to be specific for hydrocortisone by the ability of nonradioactive hydrocortisone to compete for the receptor sites, whereas, aldosterone or testosterone offered no competition.

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