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IN VITRO DEGRADATION AND STABILIZATION OF THE GLUCOCORTICOID BINDING COMPONENT FROM MOUSE FIBROBLASTS*

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SUMMARY

The stability characteristics of both the triamcinolone acetonide-bound and unbound forms of the glucocorticoid binding component (receptor) found in mouse fibroblasts was examined *in vitro*. Degradation is markedly temperature-dependent, and the bound form is more stable than the unbound form. No evidence could be obtained suggesting enzyme degradation occurs, and we believe it is a physical process. High concentrations of glycerol or glucose (more than 10%) stabilize the bound form of the receptor, but have no effect on the thermal stability of the unbound form. Low concentrations (1 mM) of certain glucose metabolites (glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, and ribose-5-phosphate) stabilize fresh preparations of the unbound receptor, but have no effect on the bound form. Evidence has been obtained showing that the unbound receptor changes with time in its thermal stability properties when kept *in vitro* at 0°, even though it retains much of its ability to bind steroids. The bound form of the receptor, kept *in vitro* at 0°, does not undergo any change in its thermal stability properties.

INTRODUCTION

FIBROBLASTS are target cells for the antianabolic action of glucocorticoid steroids. A profound reduction in the rate of replication of strain L929 mouse fibroblasts growing in tissue culture is observed after administration of triamcinolone acetonide (TA), a potent synthetic glucocorticoid.‡ Binding of radioactive TA to a macromolecule found in the cytosol and 7000*g* pellet is specific for the growth inhibitory effect as inferred from the requirements of binding both in the intact cell[1] and in the 105,000 *g* supernatant fraction[2].

Some of the factors which maintain the steady-state level of glucocorticoid binding in sensitive and resistant L cells have been examined[3]. The resistant cells are a cloned resistant subline in which growth is not inhibited by concentrations of TA 1000-fold greater than that capable of maximal growth inhibition in the sensitive strain. Steroid binding occurs initially in the cytosol to form what appears to be an essentially irreversibly bound complex. There is an active turnover or recycling of the complex in the intact cell. The steady-state level of cytoplasmic

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‡Triamcinolone acetonide is 9α-fluoro-11β, 16α, 17α, 21-tetrahydroxy - 1,4 - pregnadiene - 3,20 - dione-16,17-acetonide. 11α-Cortisol and 11β-cortisol refer to 11α, 17α, 21-trihydroxy-4-pregnene- 3,20-dione and 11β, 17α, 21-trihydroxy-4-pregnene-3,20-dione, respectively.

complex in whole cells is maintained at 37° by a process which does not require simultaneous protein synthesis. The complex binds to the low speed particulate fraction (presumably the nucleus) at 37° but not at 0°. The cyclical release of the unbound receptor available for new binding from the particulate fraction is temperature dependent, requires glucose metabolism, and is inhibited by cyanide or dinitrophenol. The addition of excess unlabeled glucocorticoid will result in a rapid decrease in bound radioactive steroid in the cytosol, but this chase effect is apparently not due to exchange. Rather, it is due to competition for re-binding as the binding component cycles through the particulate fraction.

The glucocorticoid binding protein isolated from the 105,000 g soluble fraction has an apparent molecular weight of 620,000 as determined by filtration through Sephadex G-200 and has been purified 2100-fold[4]. Unfortunately, less than microgram quantities of bound material are recovered, making further work difficult. The unbound macromolecule degrades more rapidly *in vitro* than the steroid-bound form[2]. The problem of degradation poses a major obstacle to isolation and purification in this and in other glucocorticoid binding systems. For example, in lymphosarcoma, the unbound TA binding component was estimated to degrade 80 times faster than the bound form[5]. And in the rat thymocyte system attempts to estimate the molecular weight of the corticosterone binding component were hindered by the relatively rapid rate of degradation of the bound complex during filtration[6]. These reports, and the relative paucity of investigations concerning isolation and purification of glucocorticoid binding components, indicate that *in vitro* degradation poses a major obstacle, although there is no evidence yet that this kind of degradation occurs in the intact cell. In this study we have examined some conditions for the *in vitro* stabilization of both the bound and unbound forms of the binding component against degradation. Degradation, as that term is used in this paper, signifies either loss of bound steroid or loss of capacity to bind steroid.

MATERIALS AND METHODS

Materials. [1,2,4-^{3}H]-Triamcinolone acetonide was purchased from Schwarz Bioresearch, Inc., Orangeburg, New York. Two separate lots were employed in these experiments, one with a specific activity of 20 Ci per mmole and the other at 9·2 Ci per mmol. Radioactive steroids were added in a solution of 10% ethanol at 1/100 of the incubation volume. α-D-Glucose-1-phosphate (G-1-P) was obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents were the best grades commercially available.

All of the tested compounds were prepared as 10^{-2}M solutions in 250 mM HEPES buffer,* pH 7·35, and introduced at 10% of the final incubation volume. The controls received an equivalent amount of vehicle.

Cell culture. Suspension cultures of L929 cells were maintained in basal medium[7] modified as previously described[8]. Cultures were maintained with constant stirring in an atmosphere of humidified air. The cells were harvested by centrifugation at 600 g for 10 min in a refrigerated centrifuge.

Incubation of intact cells. After harvest, the cell pellet was gently resuspended in growth medium without serum, with 25 mM HEPES buffer at pH 7·35 and with 10^{-8}M ^{3}H-triamcinolone acetonide (^{3}H-TA), then incubated at 37° in those experiments involving prelabeled binding component.

*HEPES buffer is N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.

Cell homogenization procedure. The cell suspension was harvested by centrifugation at 600 g and the medium was removed. All subsequent procedures were carried out at 0–4°. The cell pellet was suspended in 1·5 volumes of hypotonic solution of 0·01M Tris buffer at pH 7·35 and 0·1 mM EDTA for 5 min, and homogenized with 15 strokes of a tight-fitting pestle in a Dounce-type glass homogenizer. After homogenization, 0·15 volume of hypertonic buffer (1·43M NaCl, 0·11M KCl, 33 mM $MgCl_2$, and 0·11M Tris, pH 7·35) was added to bring the broken cell suspension to isotonicity.

Preparation of the 10,000 *g and* 105,000 *g supernatants.* The broken cell suspension was centrifuged at 600 g for 10 min and the supernatant was then centrifuged at 10,000 g for 30 min. The 10,000 g supernatant was diluted 1:1 with isotonic buffer. This diluted preparation defines the 10,000 g supernatant fraction as that term is employed in this paper.

The 10,000 g supernatant prior to dilution was centrifuged at 105,000 g for 1 h then the supernatant was diluted 1:1 with isotonic buffer. This preparation defines the 105,000 g supernatant fraction.

In vitro incubation and assay of binding capacity. In those experiments in which degradation of the unbound component was under examination, either the 10,000 g or 105,000 g supernatant was incubated at the temperatures indicated in the figure legends with 10^{-3}M compounds or vehicle and 25 mM HEPES buffer, pH 7·35. Incubations were terminated by rapid immersion into ice. The binding capacity remaining in the incubations was assayed after incubating 1·0 ml of the samples with 10^{-8}M ^{3}H-TA and 1 mM G-1-P at 0° for 20 h at which time maximal binding is attained.

Binding assay. The bound steroid was separated from the free by passage through 1 × 25 cm columns of Sephadex G-25 with an elution buffer of 0·01M Tris-0·04M KCl, pH 7·35. The elution period is 6–8 min from the time a 1·0 ml sample is placed on the column until the bound macromolecular fraction comes off. All assays were carried out at 4°. Approximately 1 ml fractions were collected from the columns, and the macromolecular peak was identified by optical density. The amount of bound steroid was assayed by combining the macromolecular peak fractions and determining radioactivity content.

Alternatively, we also employed the charcoal technique for the assay of binding[9]. An aliquot (0·6 ml) of a supernatant incubation was removed and mixed with a 0·3 ml charcoal suspension (3·75% Norit A and 0·375% Dextran T70 in isotonic buffer). The mixture was centrifuged for 10 min at 10,000 and a 0·10 ml aliquot was removed for scintillation counting. Under the conditions used in this study, there was no noticeable difference in binding between the two assay methods.

Assays for radioactivity and protein. The aliquot to be assayed was added to 10 ml of scintillation solution prepared according to Bray[10] and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3310. Protein determinations were made according to the method of Oyama and Eagle[11].

RESULTS

If 10^{-8}M radioactive TA is incubated with the supernatant fraction of L cells at 0°, binding attains a maximum at about 20 h. Loss of binding at this temperature is extremely slow. The amount bound to the macromolecular fraction, assayed by passage over Sephadex G-25, is specific for glucocorticoids, based on the observa-

tion that more than 90% of the binding can be competed for by 11β-cortisol and other active glucocorticoids such as dexamethasone or fluocinolone acetonide,but the binding of ^{3}H–TA cannot be blocked by 10^{-5}M 11α-cortisol which has no measurable glucocorticoid activity[2]. The dose response curves of competition for ^{3}H–TA binding by the various steroids parallel their potency as glucocorticoids and ^{3}H–TA binding saturates at the expected concentration. The concentration of ^{3}H–TA employed in this paper is such that near saturation of available binding component occurs while non-specific binding is less than 10% of the initial total binding. A more complete account of the conditions for *in vitro* binding is given by Pratt and Ishii[].

The temperature-dependent degradation rate of the bound complex. If the 105,000 *g* supernatant fraction is prebound to saturation at 0° with ^{3}H–TA and then incubated at various temperatures in the presence or absence of a 1000-fold excess of unlabeled TA, the bound ^{3}H–TA is lost as a first-order function in a strongly temperature-dependent manner (Fig. 1, part A). The rate of loss in the presence of excess cold chase is the same as in its absence, indicating that exchange occurs at an immeasurably slow rate even at higher temperatures. This finding is in accord with a large number of other observations, all of which indicate exchange *in vitro* occurs at a negligible rate. The Ahrrenius plot of the rate of loss of binding as a function of temperature (Fig. 1, part B) allows us to calculate the activation energy for loss of binding as about 40 cal per mole binding component. One possible explanation for such a high energy of activation is multiple bond breakage prior to release of bound steroid. Attempts to resolve possible smaller fragments of the macromolecule still bound to radioactive steroid by filtration over Sephadex G-200 after incubation at 37° for 30 min were not successful. It has previously been demonstrated by extraction with organic solvents followed by thin-layer chromatography that the steroid is not covalently bound to the binding component, nor is it metabolized.

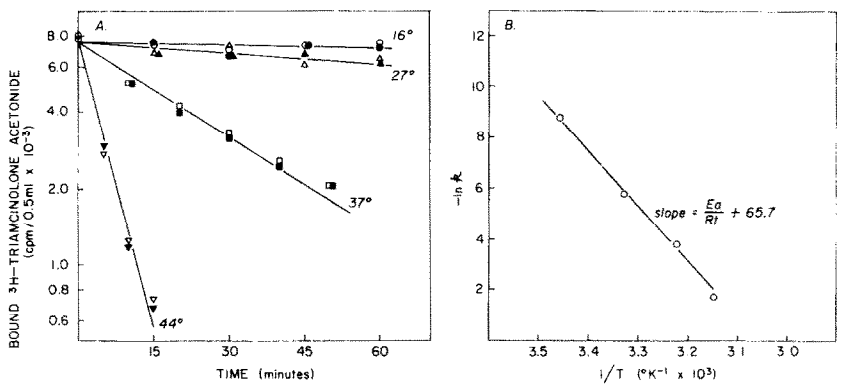

Fig. 1. The temperature dependent rate of loss of [^{3}H]-triamcinolone acetonide binding in the 105,000 *g* supernatant and the Ahrrenius plot. *Part A*: The 105,000 *g* supernatant (796 μg protein-N/ml) prebound to saturation at 0° for 20 h with 10^{-8}M ^{3}H–TA (20 Ci/mmol) was divided and incubated at various temperatures, as labeled in the figure, in the presence (closed symbols) or absence (open symbols) of 10^{-5}M unlabeled TA. At various times, a 0·5 ml aliquot was removed and filtered through Sephadex G-25. The amount of radioactivity bound to the pooled macromolecular fractions was determined as explained in the Methods section. *Part B*: The data presented in Part A are replotted using the Ahrrenius equation (15) where k is the rate of loss of ^{3}H–TA binding, R is the gas constant and E_a is the activation energy.

The effect of some non-specific agents on the rate of loss of bound steroid and on the rate of loss of binding capacity of the unbound component. The presence of high glycerol concentrations was found to protect against loss of binding when the prebound 105,00 *g* supernatant fraction was incubated for 4 h at 33° (Fig. 2). Similar protection can be afforded by high glucose concentrations (not shown). No significant differences in the rate of loss of binding could be detected in the presence of high salt, dithiothreitol, EDTA, or anaerobic conditions, but thiol reagents such as p-chloromercuribenzoate and N-ethylmaleimide increased the rate of loss (Fig. 3). This latter finding shows that the binding component itself is sensitive to these reagents, as are steroid receptors in other tissues [12, 13], and is contrary to an expected decreased rate of loss had these agents inhibited a degradative enzyme. Addition of boiled supernatant (as a cofactor control) or supernatant incubated for 2 h at 37° to remove binding component (but presumably retaining a hypothetical degrading enzyme) had no effect on the rate of loss of prebound steroid measured at 37°. Such experiments should have detected enzyme or cofactor activity if either were present in rate-limiting amount, and thus these results also fail to implicate an enzymic degradation process.

On the other hand, when the unbound component was incubated at 37° for 10 min in the presence of high glycerol concentrations, no protection against loss of binding capacity was observed (Fig. 4). Similar results are observed in both a 10,000 *g* and 105,000 *g* supernatant. In fact, retention of binding capacity decreased from 17% to about 6% (approximate level of non-specific binding) as the glycerol concentration was increased from 10 to 30% v/v. The presence of G-1-P in the absence of glycerol stabilized the unbound component so that only 52% of the binding capacity was lost, as compared to 83% loss if G-1-P is not present. But at glycerol concentrations over the range from 10 to 30%, retention of binding

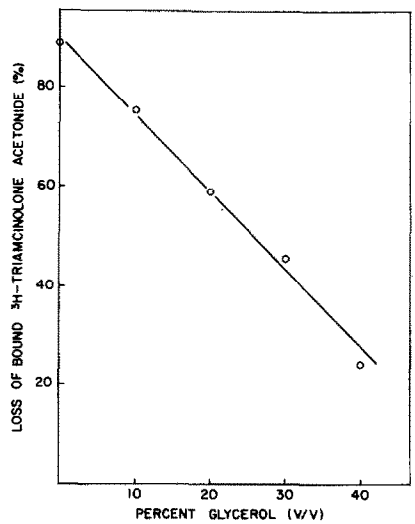

Fig. 2. The effect of glycerol on the loss of bound [^{3}H]-triamcinolone acetonide. The 105,000 *g* supernatant was preincubated for 20 h with 10^{-8}M ^{3}H–TA (20 Ci per mmole) at 0°C, then unlabeled TA (10^{-5}M) and HEPES buffer (25 mM) was added. The steroid-bound supernatant was then divided and incubated at 33°C for 4 h in the presence of various concentrations of glycerol. The incubations contained 838 μg protein-N/ml. The figure presents the per cent loss of bound ^{3}H–TA at 4 h relative to a 0°C control without glycerol containing 20×10^3 c.p.m./ml.

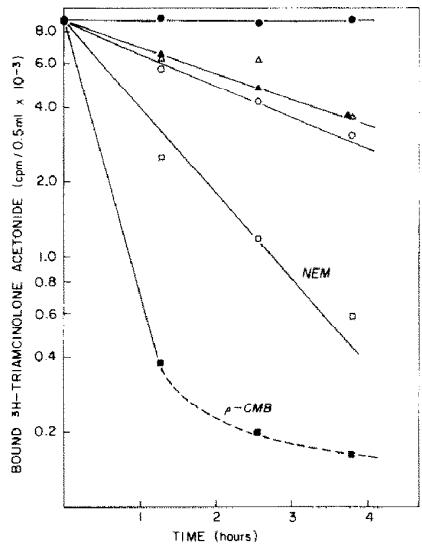

Fig. 3. The effect of p-chloromercuribenzoate (p-CMB), N-ethylmaleimide (NEM), and "aged" or boiled supernatant on the rate of loss of bound ^{3}H–TA as explained in the legend to Fig. 1. A second portion was either incubated for 2 h at 37° ("aged") or boiled for 5 minutes, then both samples were centrifuged and the supernatant retained. The ^{3}H–TA bound supernatant was divided then incubated at 37°C with the following additions: □, 10^{-4}M NEM; ■, 10^{-4}M p-CMB; △, an equal volume of boiled supernatant; ▲, an equal volume of "aged" supernatant; ○, control; ●, 0°C control.

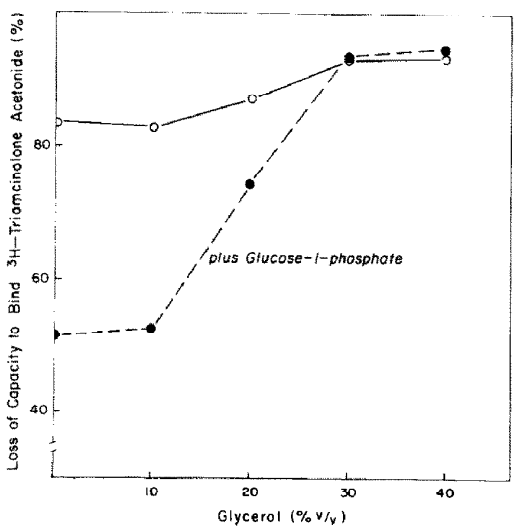

Fig. 4. The effect of glycerol concentration in the presence and absence of glucose-1-phosphate on the binding capacity of the unoccupied glucocorticoid receptor. The 10,000 g supernatant fraction was isolated from mouse fibroblasts. The superantant was incubated at 37° for 10 min in the presence or absence of 1 mM glucose-1-phosphate and increasing concentrations of glycerol. The incubations were stopped in ice and the binding capacity remaining was determined as explained in the Methods section. Parallel incubations were conducted at 0° (data not shown) and glycerol did not interfere with the assay for binding capacity. The figure presents the per cent loss of binding capacity of the 37° incubations relative to corresponding 0° controls.

capacity was reduced to non-specific levels. The reason for the use of G-1-P and the nature of its stabilizing influence on the unbound component will be subsequently discussed in greater detail. Incubation of the unbound component with 30% glucose also did not lead to stabilization (data not shown).

Stabilization of the unbound component against loss of binding capacity. Steroid-bound and unbound 10,000 *g* supernatant was prepared from equivalent suspensions of L cells. The unbound supernatant is susceptible to complete loss of binding capacity within 10 min incubation at 37° (Fig. 5). The bound component is partially stabilized against degradation, as previously mentioned. When the unbound component is incubated in the presence of 1 mM G-1-P, there is an initial rapid loss of about a third of the total binding capacity followed by a decreased rate of loss of binding capacity at a rate similar to that shown by the steroid-bound component. The biphasic nature of the loss of binding capacity in the presence of G-1-P is presented in more detail in the insert to the figure. There is an initial lag of about 1 or 2 min, possibly due to temperature equilibration, followed by loss of binding capacity at the same rate as the control. After 4 or 5 min, there is a sharp transition to the stabilized rate. The zero-time values indicate the assay of binding capacity is complete relative to the values measured in the prebound supernatant, and is not affected by the presence of G-1-P. Samples maintained at 0° show that

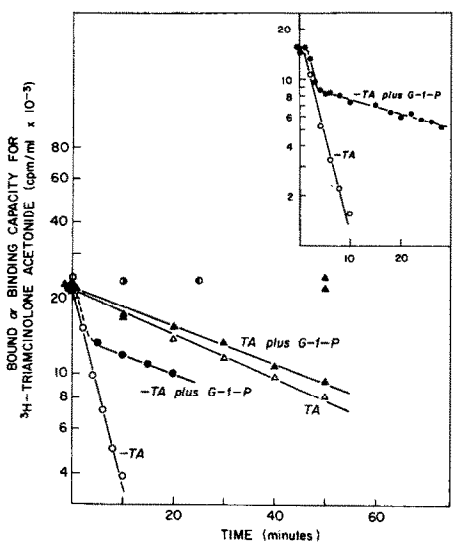

Fig. 5. The effect of glucose-1-phosphate on the rate of loss of bound [³H]-triamcinolone acetonide and on the rate of loss of binding capacity of the unbound component. Prebound or unbound 10,000 *g* supernatant fractions were prepared from equivalent amounts of L cells. The supernatant fractions were divided and incubated at either 0 or 37° in the presence or absence of 1 mM glucose-1-phosphate. At various times, one ml aliquots were removed into ice. The bound form was immediately filtered through Sephadex G-25 but the unbound was assayed as explained in the Methods section for remaining capacity to bind. The insert presents a separate experiment with the unbound 10,000 *g* supernatant fraction only in which the early time events were more closely examined. The incubation conditions were as follows: –○–, unbound 37° control (–TA); –◑–, unbound 0° control; –●–, unbound 37° plus 1 mM G-1-P; –◐–, unbound 0° plus 1 mM G-1-P; –△–, bound 37° control; –▲–, bound 0° control; –▲–, bound 37° plus 1 mM G-1-P; and –▲–, bound 0° plus 1 mM G-1-P. The specific activity of TA was 20 Ci per mmol.

no changes occurred during the incubation period in the presence or absence of G-1-P. The results are the same if performed with the isolated 105,000 *g* supernatant fraction.

Other components were tested in order to study the specificity of the G-1-P stabilizing effect (Table 1). Other active compounds were glucose-6-phosphate, fructose-1,6-diphosphate, and ribose-5-phosphate, all of which also gave the same biphasic response. ATP and phosphoenolpyruvate were partially active. Inorganic phosphate ion was inactive. The inactivity of glucose, deoxyglucose and deoxyglucose-6-phosphate implies that further metabolism is required. Phosphoenolpyruvate is an excellent source of ATP and was a partially active compound, but glyceraldehyde-3-phosphate was inactive, implying that its rate of metabolism was insufficient to provide a high level of ATP. The ATP effect is possibly due to phosphorylation of endogenous sugars, but a mixture of ATP and glucose did not potentiate the ATP effect. These observations support the notion that a sugar phosphate or an early metabolite of sugar phosphates is responsible for the observed stabilization of the unbound component.

Under our usual conditions of assay (Fig. 5), freshly prepared cytosol solution was kept at 0° for perhaps an hour or less before starting the studies of rates of degradation at elevated temperatures. The effect of time at 0° was explored more carefully (Fig. 6), and two results emerged. First, the ability of G-1-P to stabilize the unbound receptor decreased the longer the unbound receptor was held at 0°. This occurred despite the fact that the total initial binding capacity of the system changed only very slightly over the time period involved. It did not matter whether G-1-P was present throughout the period of preincubation at 0°, or added only at the conclusion of this preincubation. A second finding that emerged (Fig.

Table 1. The activity of sugar phosphates and other compounds at 1 mM concentrations as stabilizers against loss of capacity to bind ^{3}H-TA[a]

Substance	Activity[b]
D-Glucose-1-phosphate (G-1-P)	yes
D-Glucose-6-phosphate (G-6-P)	yes
Fructose-1,6-diphosphate (FPP)	yes
D-Ribose-5-phosphate (R5P)	yes
Adenosine-5′-triphosphate (ATP)	partial
Phosphoenolpyruvate (PEP)	partial
Inorganic phosphate	no
D-Glucose	no
2-Deoxy-D-glucose (DG)	no
2-Deoxy-D-glucose-6-phosphate (DG-6-P)	no
DL-Glyceraldehyde-3-phosphate	no
Adenosine-5′-diphosphate (ADP)	no
Adenosine-5′-monophosphate (AMP)	no
Adenosine-3′:5′-cyclic monophosphate (c-AMP)	no
β-Diphosphopyridine nucleotide (DPN)	no
5-Phosphorylribose-1-pyrophosphate (PRPP)	no
Uridine-5′-diphosphogalactose (UDP-gal)	no

[a]The experimental conditions were the same as that explained in the legend to Fig. 4.

[b]The activity of various substances are presented relative to the activity of G-1-P.

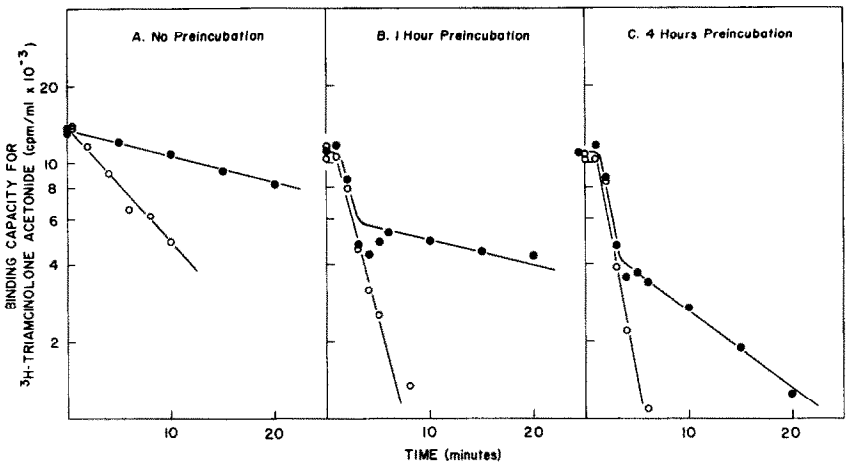

Fig. 6. The effect of preincubation on the stabilization of the unbound component by glucose-1-phosphate. The 10,000 g supernatant fraction was divided and 1 mM glucose-1-phosphate was added to one portion. One sample from each portion was incubated immediately at 37° (A), while the other samples were kept in ice for 1 h (B) and 4 h (C) before starting the 37° incubation. At various times 1·0 ml aliquots were removed and the binding capacity assayed as explained in the Methods section. The incubations were as follows: –●–, plus 1 mM glucose-1-phosphate; –○–, control. The specific activity of TA was 9·2 Ci per mmol.

6) was that the rate of loss of binding capacity at 37° of the unbound receptor changed, the longer the unbound receptor was kept at 0°. The unbound receptor seemed to become less stable the longer it was kept at 0°. Similar experiments were done with pre-bound receptor material, obtained from whole cells incubated with ^{3}H–TA. The results (data not shown) were entirely different, and incubating the bound receptor at 0° had no effect whatever on the rate of degradation of the receptor complex at 37°.

It was possible that the supernatant solutions aged by preincubation in ice lost the capacity to metabolize G-1-P, and this was responsible for the time-dependent loss of the stabilizing effect on unbound receptor. This was explored by preparing fresh supernatant and supernatant aged for 8 h in ice. We reasoned that if aged supernatant was deficient in its capacity to metabolize G-1-P, the addition of fresh supernatant ought to partially or completely restore the response to G-1-P. The results (Table 2) indicate that the mixture of fresh and aged supernatant did not restore the binding capacity of the aged supernatant. The result is more nearly the simple sum of the fresh and aged supernatant binding capacity values. This result, as the previous results also indicated, suggests that there is an alteration in the properties of the unbound binding component with time at 0°.

DISCUSSION

The unbound form of the cytoplasmic glucocorticoid binding component is extremely unstable at 37°. It can be stabilized against loss of binding capacity by hexose or pentose phosphates or an early metabolite of these compounds (Table 1). Although both hexose and pentose phosphates are active, lack of specificity may not be concluded due to the possibility of interconversions of these compounds. The failure of deoxyglucose-6-phosphate, a non-metabolizable substrate, to stabilize the component indicates metabolism is required. Alternatively, this

Table 2. Effect of combining fresh supernatant and supernatant preincubated for 8 h at 0°C on stabilization by 1 mM glucose-1-phosphate[a]

Incubation composition			Binding capacity for [^{3}H]-triamcinolone acetonide (c.p.m./ml × 10^{-3})	
Supernatant		Isotonic buffer		
Fresh	Aged		0 Min.	15 Min.
1 vol	—	1 vol	12·2	8·2
			12·3	8·4
—	1 vol	1 vol	12·0	1·7
			11·7	1·5
1 vol	1 vol	—	20·5	8·0
			22·0	8·2

[a]A suspension of L cells was divided into two equal portions. The 15,000 *g* supernatant was prepared from one portion and preincubated at 0°C for 8 h ("aged" supernatant). The other portion of cells was maintained intact in complete growth medium at 4°C for 8 h, then the 15,000 *g* supernatant was prepared ("fresh" supernatant). The incubations of composition indicated in the table were conducted at 37°C for 15 min in the presence of 1 mM glucose-1-phosphate. The binding capacity remaining was determined in replicate by the charcoal technique described in the Methods.

observation may be evidence for more stringent structural constraints on a directly-acting substrate.

The binding component appears to be continuously decreasing in responsiveness to the stabilizing influence of G-1-P, although the initial steroid binding capacity is not significantly diminished, when it is kept at 0° (Fig. 6) due to an alteration in its properties (Table 2). That the stability properties of the unbound binding component change with time, even at 0°, is also shown by the increasing degradation rate observed at 37°, following incubation for several hours at 0°, which is observed in the absence of G-1-P (Fig. 6). In contrast, there is no change in the thermal stability of the bound form of the binding component after incubation for several hours at 0°.

Other than in response to hexose and pentose phosphates, the steroid bound and unbound forms of the glucocorticoid binding component are distinctly different by at least two other criteria. The bound form degrades much more slowly than the unbound receptor, and the bound form can be further stabilized by high glycerol or glucose (greater than 10%) concentrations. Unbound receptor is not stabilized against loss of binding capacity under similar conditions. These three observations may be indirect evidence of a change in conformation of the binding component upon association with glucocorticoids. In this respect, the binding component associated with anti-glucocorticoids might be expected to show altered physical properties if the conformation is altered. It will be of interest, then, to determine the effect of cortexolone[14] on the thermal stability properties of the receptor.

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