Differences between Liver and Hepatoma Cells in Their Complements of Adenosine 3',5'-Monophosphate-binding Proteins and Protein Kinases*

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SUMMARY

The soluble fractions from both rat liver and an established hepatoma cell line (HTC cells) contain protein kinases and proteins which bind adenosine 3',5'-monophosphate (cyclic AMP). Liver cytosol exhibits somewhat more binding activity and less protein kinase activity than HTC cells. Protein kinase activity from both sources is stimulated by cyclic AMP.

Scatchard plots for the binding of cyclic AMP have been compared at pH 4 and 6.5. At both pH values, liver shows nonlinear plots which are increased in curvature and apparent affinities by prior dialysis of the samples, mostly because of the removal of endogenous cyclic AMP. For HTC cells the Scatchard plot at pH 4 is also nonlinear and exhibits high binding affinity (KD < 0.5 nM), but prior dialysis is unnecessary and the shape of the curve differs from that of liver. At pH 6.5, the binding affinity observed in HTC extracts (KD > 10 nM) is lower than seen with liver and is insensitive to dialysis for a day or 2. However, this binding can be partially converted to higher affinity by aging or by overnight treatment at pH 4. The binding activity present in liver extracts is considerably stable than that from HTC cells at pH 4 in the absence of cyclic AMP. Increasing the pH and adding cyclic AMP stabilize the binding activities from both sources. Much of the nonlinearity observed in the Scatchard binding plots is in the opposite direction from that caused by the instability of binding components at low concentrations of cyclic AMP.

Chromatography on DEAE-cellulose shows that HTC extracts lack one of two major cyclic AMP-stimulated protein kinase fractions which are present in liver. The deficiency seems to be due primarily to the absence of a cyclic AMP-binding fraction. Chromatographically analogous protein kinase peaks are present in both HTC and liver extracts, although their proportions differ. The cyclic AMP-binding fraction which is present only in liver is very labile at pH 4 and accounts for the greater instability of binding activity in liver extracts compared with HTC samples. HTC cells and liver also differ in the properties of the cyclic AMP-stimulated protein kinase fraction which is common to both. The activity of this chromatographic fraction from HTC cells is less responsive to cyclic AMP and has a lower binding affinity than that from liver. This lower HTC binding affinity can be increased by the same treatments which are effective with unfractionated extracts.

The HTC cell line was established in tissue culture by Thompson et al. (1) from a chemically induced rat hepatoma and still express some, though not all, functions characteristic of liver. The cells resemble liver in having an inducible tyrosine aminotransferase but differ in the hormonal inducers which are active. Whereas either glucocorticoids (2), insulin (3), glucagon (3), or catecholamines (4) can induce tyrosine aminotransferase in liver, only glucocorticoids and insulin have been found effective in HTC cells (1, 5-7). In liver, glucagon and catecholamines stimulate adenylate cyclase (8) and thereby cause an increase in the concentration of cyclic AMP (9). Cyclic AMP, in turn, brings about the intracellular effects of these hormones (10), including the induction of tyrosine aminotransferase (4, 11). The very low adenylate cyclase activity detected in HTC cells (6, 12) may account for their failure to respond to glucagon and catecholamines.

We have been investigating whether HTC cells also differ from liver in other aspects of the cyclic AMP system. The cell line has apparently retained at least some of the components needed to respond to cyclic AMP, since Stellwagen (7) has demonstrated that its tyrosine aminotransferase can be induced by exogenously added N6,O2'-dibutyryl cyclic AMP (but not by cyclic AMP itself). However, the response requires rather high concentrations of the cyclic AMP derivative and is smaller than the induction in liver, which suggests possible differences in the cellular machinery mediating the effect. Since it is not known how cyclic AMP induces enzymes in eukaryotes, we

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The abbreviations used are: HTC, hepatoma tissue culture; cyclic AMP, adenosine 3',5'-monophosphate.
have compared liver and HTC cells with the idea that differences between them might provide insight into the mechanism of this process.

In this comparison, we have emphasized cyclic AMP-binding proteins since they would seem to be essential for mediating the actions of cyclic AMP. One function of such proteins is clearly to serve as regulatory subunits for cyclic AMP-stimulated protein kinases (13-19). Consequently, we have also examined protein kinases in HTC cells and liver. Whether the kinases are involved in enzyme induction is not known, although this has been postulated (14, 20). The idea is supported by the recent finding that lymphoma cell mutants which are deficient in cyclic AMP-stimulated protein kinase also fail to show the usual induction of phosphodiesterase by cyclic AMP (21). A function has also been proposed for cyclic AMP-binding protein apart from protein kinases in the release of newly synthesized tyrosine aminotransferase from neonatal rat liver polysomes (22). By analogy with the results in bacteria (23), there may be still other functions for cyclic AMP-binding proteins.

The study reported here delineates the differences we have observed between liver and HTC cells in both cyclic AMP-binding proteins and protein kinases. In making this comparison, we have also observed some generally unrecognized properties of cyclic AMP-binding proteins. These include non-linear Scatchard (24) binding plots and extremely high apparent affinities for cyclic AMP. A more detailed examination of these properties and their potential significance is presented in an accompanying paper (25).

**EXPERIMENTAL PROCEDURES**

**Materials**—Cyclic AMP was purchased from Calbiochem. The cyclic AMP(H) (22.1 to 24.1 Ci per mmole), [3H]ATP (5 to 10 Ci per mmole), and Omnifluor were obtained from New England Nuclear Corp. Bovine serum albumin and DEAE-cellulose (sodium mesh, 0.89 meq per g) were from Sigma. Calf thymus histones (unfractionated) were from Schars-Mann, and AG 50W-X4 resin (100 to 200 mesh) was purchased from Bio-Rad Laboratories. Sera and powdered medium for tissue culture were obtained from Dounce homogenizer. In most of the experiments described here, serum and medium were prepared in 10 mM Tris.HCl-0.5 mM dithiothreitol, pH 7.6, with a flow rate of 40 ml per hour, and 5-ml fractions were collected. After at least 60 ml had been collected, a linear gradient of 0 to 1 M KCl was applied in the eluting buffer (total volume = 290 ml). The salt concentration in the effluent was measured by conductivity.

**Binding Assays**—The binding of cyclic AMP to proteins was measured by a modification of the membrane filtration method of Gilman (28) as described in detail in the accompanying paper (25). For routine assays, the binding was allowed to take place for at least 2 hours at 0°C, pH 4.0, and 175 nm cyclic AMP. Data for Scatchard binding plots were obtained at either pH 4.0 or 6.5 with binding times of 16 to 24 hours and cyclic AMP concentrations ranging from 0.1 to 175 nm. Blank values were measured by omitting protein (25). For Scatchard plots, the amount of cyclic AMP bound was corrected for this blank and the amount free was corrected for the presence of some radioactivity which was not cyclic AMP (about 6% of the total radioactivity added).

**Protein Kinase Assays**—Protein kinase was assayed in a final volume of 150 µl which contained 50 mM sodium glycerol phosphate buffer (pH 6.0), 10 mM magnesium acetate, 0.3 mM EGTA, 2 mM theophylline, 30 µg of histones (unfractionated), and either 2.5 or 100 µM [γ-32P]ATP (0.1 to 0.7 µCi per assay). Cyclic AMP (when present) was 5 µM, and enzyme was added as 50 µl of the total volume. The reaction was initiated by adding the [γ-32P]ATP and cyclic AMP. After the reaction was complete (when present), the reaction was stopped by transferring 100 µl to a numbered filter paper disc (Whatman No. 3MM filter paper, 2.3 cm) and immersing the disc in a beaker of cold 20% trichloroacetic acid. When all the paper discs from an experiment had been collected in the beaker, they were washed three times with 5 M trichloroacetic acid, heated 15 min at 90°C in 50% trichloroacetic acid, washed twice more with cold 20% trichloroacetic acid, washed twice with ethanol, and finally with ether. The paper discs were then dried and counted in 5 ml of 0.4% (w/v) Omnifluor in toluene. The counting efficiency using a Beckman LS-245 liquid scintillation spectrometer was greater than 95%. The assay was used under conditions of approximate linearity with respect to time and enzyme concentration.

**Phosphodiesterase Assays and Measurements of Cyclic AMP Breakdown**—Phosphodiesterase was assayed at 37°C in 40 mM Tris-HCl buffer, pH 8.0, containing 2 mM MgCl₂ and 0.1 mM cyclic [H]AMP (0.5 µCi) in a final volume of 100 µl. The reaction was stopped by adding 100 µl of cold 10% trichloroacetic acid. After centrifugation for 5 min at 1000 X g, an aliquot (about 20 µl) of the supernatant fraction was applied to Whatman No. 1 filter paper with carrier amounts of cyclic AMP, 5'-AMP, and adenosine. Ascending chromatography was performed at room temperature in a solvent composed of 30% (v/v) 1 M ammonium acetate and 70% (v/v) ethanol. Spots were detected by ultraviolet light, cut out, and counted as described elsewhere (25) for membrane filters.

With all HTC extracts and with the chromatographic fractions obtained from the major products, the products of breakdown were identified by paper chromatography in which they were negative. However, crude liver extracts produced mainly [H]adenosine, presumably because of phosphatase action on 5'-[H]AMP. In all cases, the reaction was linear with both time and enzyme concentration when the two breakdown products were summed.

To measure the breakdown of cyclic [H]AMP during binding assays, protein was precipitated with 0.1 volume of 55% trichloroacetic acid. This treatment was shown to release protein-bound radioactivity into the supernatant fraction. An aliquot of the supernatant fraction was then analyzed by the chromatographic procedure described above for phosphodiesterase assays.

**Assay of Cyclic AMP**—Cyclic AMP was assayed essentially by the method of Gilman (28), as modified by Manganelli and Vaughan (29). Cyclic AMP was extracted with 1.5 ml of 5% trichloroacetic acid, diluted to 3 ml after addition of cyclic [H]AMP tracer plus 150 µM of 1 x HCl, and applied to an AG 50W-X4 column (6 x 0.6 cm) equilibrated with 0.05 M sodium phosphate buffer at 4°C. The cyclic AMP was eluted with 5 ml of water, lyophilized, and reconstituted with 0.5 ml of 100 mM sodium phosphate buffer, pH 6.4. To 100 µl of the unknown was added 100 µl of Tris-dithiothreitol buffer containing 0.5 µg of protein kinase (Sigma), 5 µg of protein kinase inhibitor (Sigma), and 2 nm cyclic [H]AMP to give a final pH of 6.5. Binding was determined after overnight incubation at 0°C as described elsewhere (25).
RESULTS

Comparison of Amounts of Activity in Unpurified Extracts—Extracts from both liver and HTC cells contain components which bind cyclic AMP. As shown in Table I, the specific activity is somewhat higher in liver although the difference is not large. In both cases the binding is extremely specific for cyclic AMP, as is shown in the accompanying paper (25). Protein kinases are also present in both liver and HTC cells, and in this case HTC cells exhibit the higher specific activity (Table I). Stimulation by cyclic AMP in these unpurified extracts is comparable in the two cases. The measurements of binding and protein kinase activities were made at saturating concentrations of cyclic AMP and ATP, respectively. The specific activities in both low and high speed supernatant fractions are fairly similar (Table I). Although the binding activity is quite stable in the refrigerator for several days, the protein kinase activity has some tendency to increase above the values shown in Table I during storage, especially with HTC extracts.

Comparison of Scatchard Plots for Cyclic AMP Binding—To compare the binding components in liver and HTC cells more thoroughly, we measured the binding as a function of the cyclic AMP concentration and used the data to construct Scatchard plots (24). We have employed a wide range of cyclic AMP concentrations in order to look for potential heterogeneity in the binding and have examined unpurified postmitochondrial supernatant fractions to minimize the possible loss of components. The binding reaction was allowed to take place overnight, which we found to be adequate for all the cyclic AMP concentrations used (25). The presence of phosphodiesterase activity in the extracts restricted the pH values which could be employed in the absence of high concentrations of theophylline. Even though the assays were carried out at 0°, a high percentage of the cyclic AMP was broken down during overnight incubations at pH values between 7 and 8. The problem was circumvented by lowering the pH. We have selected pH values of 4.0 and physiological values and yet the cyclic AMP breakdown (in the absence of added Mg2+) is still sufficiently small (1 to 5% per day) to allow binding to be determined in the absence of theophylline.

When examining binding in unpurified preparations, the effect of endogenous cyclic AMP must also be considered.

Table I
Amounts of cyclic AMP-binding and protein kinase activities in extracts from liver and HTC cells

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>Supernatant fraction used</th>
<th>Protein kinase activity</th>
<th>Binding activity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- Cyclic AMP</td>
<td>+ Cyclic AMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gmoles/mg</td>
<td>gmoles/mg</td>
</tr>
<tr>
<td>Liver</td>
<td>27,000 X g</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>Liver</td>
<td>105,000 X g</td>
<td>23</td>
<td>44</td>
</tr>
<tr>
<td>HTC</td>
<td>27,000 X g</td>
<td>75</td>
<td>130</td>
</tr>
<tr>
<td>HTC</td>
<td>105,000 X g</td>
<td>48</td>
<td>97</td>
</tr>
</tbody>
</table>

Such unlabeled cyclic AMP would dilute the radioactive compound used to detect binding and might also mask some binding sites. For this reason, Scatchard plots were determined after dialysis of the extracts for various times. It was anticipated that prolonged dialysis might be needed, since others have reported difficulty in completely removing bound cyclic AMP (22, 30, 31) and we have found very slow release rates for some of the binding in HTC cells and liver (25).

The results of our binding measurements are shown in Fig. 1. For liver, the Scatchard plots at both pH 4 and 6.5 are markedly affected by prior dialysis (Fig. 1, A and C). In each case, dialysis increases the curvature and the apparent affinities (negative slopes) of the nonlinear plots obtained. The changes produced by the first day of dialysis are consistent with the removal of endogenous cyclic AMP amounting to 1 to 2 nm in the final binding assay. This is a reasonable level to expect since the reported cyclic AMP concentration in unstimulated liver is about 1 μM (9, 28, 32) and we have used a 700-fold dilution in these assays. We have also measured the cyclic AMP present in a separate liver extract made under the same conditions and found 1 to 1.5 nm after dilution to the concentration of the assay. This endogenous cyclic AMP would substantially dilute the lower concentrations of cyclic [3H]AMP used and reduce the radioactivity bound, resulting in the lower apparent

![Fig. 1](http://www.jbc.org/)

Source of extract: Liver, HTC; Supernatant fraction used: 27,000 x g, 105,000 x g; Protein kinase activity: - Cyclic AMP, + Cyclic AMP; Binding activity: gmoles/mg.
affinities observed and masking very high affinity binding. Dilution would be insignificant at the higher concentrations of cyclic [H]AMP; and thus, the intercept on the "bound" axis would not be changed by endogenous cyclic AMP, provided that complete exchange occurred between bound endogenous cyclic AMP and free cyclic [H]AMP. At pH 4 the "bound" intercept is fairly constant; but, at pH 0.5, it increases with dialysis (Fig. 1, A and C). This could be the result of less complete equilibration between endogenous and radioactive cyclic AMP resulting from the slower release rates observed at the latter pH (25).

Unlike liver, HTC extracts show high affinity binding at pH 4 without prior dialysis (Fig. 1B), and the shape of the curve is not altered by dialysis (data not shown). This is consistent with the presence of less cyclic AMP in the HTC extracts, either because of a lower endogenous level (6) or a greater rate of breakdown during sample preparation. Indeed, our measurements of the endogenous cyclic AMP level in HTC cells (0.04 to 0.2 pmole per mg of total protein) correspond to <0.2 nm at the dilution used in the binding assay. The binding observed in HTC extracts at pH 6.5 is also unaffected by dialysis for a day or 2 (Fig. 1D). However, this binding is of much lower affinity than that found in liver (Fig. 1C).

The apparent binding affinity in HTC extracts at pH 6.5 does eventually increase if very prolonged dialysis is used (Fig. 1D). The curve then becomes decidedly nonlinear and resembles somewhat more the HTC curve at pH 4 and the liver curves at pH 6.5 after dialysis. The increased curvature at pH 6.5 can be brought about more rapidly and more completely by exposing HTC extracts to pH 4 overnight (Fig. 2). The extremely long times required to obtain the effect by dialysis and its occurrence at pH 4 suggest that it does not involve the removal of cyclic AMP, but rather some other kind of alteration. A major contrast between liver and HTC cells seems to be the difference in apparent affinities at pH 6.5. Even before dialysis, liver has higher apparent affinity and its difference from HTC extracts increases markedly with dialysis for a few days. High affinity binding in HTC cells is revealed only by pH 4 treatment or by extremely long dialysis (or storage) at pH 7.6. Preparation of the HTC extract with 0.25 M sucrose-3.3 mM CaCl₂-6 mM mercaptoethanol and diisopropyl fluorophosphate treatment as described by Kumon et al. (33) does not affect the low affinity binding at pH 6.5 or its activation. Once the high affinity binding has been produced by pH 4 treatment of HTC extracts, it is not reversed by reincubating at pH 7.6 (Fig. 2).

The binding curves for liver and HTC cells at pH 4.0 also differ considerably in shape and are decidedly nonlinear (Fig. 1, A and B). In order to observe these nonlinear curves, it is essential to examine the binding at low concentrations of free cyclic AMP (<0.5 nm). The nonlinearity is not dependent upon our particular conditions for preparing extracts, since similar nonlinear results are obtained when the usual Triton X-100 homogenization buffer is modified by removal of dithiothreitol or by addition of sucrose, MgCl₂, or EDTA. Similar binding has been observed in whole homogenates, postmitochondrial and postmicrosomal supernatant fractions, and extracts from both frozen and fresh tissue. These results with crude extracts are also not qualitatively altered by diisopropyl fluorophosphate treatment as described above. There are several possible reasons for this nonlinear behavior, and these are discussed more fully in the accompanying article (25).

**Stability of Binding Components**—Although the binding activity is very stable at pH 7.6, we have found that the stability is lower at the pH values used in the assay, especially at pH 4. The stability in the assay is directly related to the concentration of cyclic AMP present, as shown in Fig. 3. In all cases, the binding is stable at concentrations above 100 nm cyclic AMP. At pH 6.5, the loss of activity even at the lowest concentrations of cyclic AMP is fairly small (<5% for HTC cells and <18% for liver). However, at pH 4 there is a large loss (>50%) of binding activity in the liver preparation at the lowest cyclic AMP concentrations, but substantially less loss (<24%) in HTC extracts. In contrast, preincubation of HTC extracts at pH 4.0 results in an increase in pH 6.5 binding measured at 175 nm cyclic AMP (Fig. 2). This is probably related to the activation phenomenon. Preincubation at pH 4.0 without cyclic AMP for a second day does result in the loss of 16% of the binding observed after the first day (data not shown).

The effect of the binding losses on the Scatchard plots shown in Fig. 1 would be to cause the observed binding to be too low at low concentrations of cyclic AMP. The plots would have had an even stronger upward curvature if no binding losses had occurred. Thus, these binding losses cannot account for the upward curvature seen in most of our Scatchard plots. However, the binding instability could account for some of the complexity apparent in the curve for liver at pH 4 (Fig. 1A). The middle range of this curve shows a partial plateau in bound/free ratio as the amount bound decreases. This change is in the direction expected if a binding component were being increasingly lost as the...
cyclic AMP concentration decreased. The exact shape would depend upon the relative rates of loss and of binding, since cyclic AMP, once bound, appears to stabilize the binding sites. As the concentration of cyclic AMP is decreased below the middle range of the curve, the bound/free value again increases, suggesting that binding is occurring to some other sites having greater stability or a more rapid rate of binding.

**Chromatographic Fractionation of Binding Proteins and Protein Kinases**—Additional comparisons of HTC cells and liver were based upon the elution profiles of binding and kinase activities from DEAE-cellulose. Fig. 4, A and C, illustrates the chromatographic differences between undialyzed extracts from the two sources. The liver elution profile has two regions where protein kinase and cyclic AMP-binding activities coincide (Fractions 2 and 4 in Fig. 4A). As shown below, these kinase fractions are stimulated by cyclic AMP. They probably correspond to cyclic AMP-stimulated protein kinases B1 and B2 observed in liver by Kumon et al. (33) and to the two kinases resolved by other workers from liver (34, 35) and other tissues (16, 36-38). In addition, the liver profile contains kinase activity, but no significant amount of binding, in the void volume and in Fraction 1. There is reduced binding relative to protein kinase activity in the trailing portion of the liver profile (Fraction 5). Finally, there is a major region of binding activity which appears free of kinase (Fraction 8). This is probably the R protein of Kumon et al. (33). Many of these fractions appear chromatographically heterogeneous.

As shown in Fig. 4C, the results with HTC cells are different. There is almost no binding activity in the position corresponding to kinase B1 of liver (Fraction 2), there is no peak of binding which appears separate from kinase activity, and kinase Fraction 5 is much larger than in liver. The apparent differences between the liver and HTC profiles were substantiated by mixing experiments. When liver Fraction 2 and HTC Fraction 4 were combined and rechromatographed, they remained as two distinct peaks. Liver binding Fraction 3 was eluted ahead of the HTC binding peaks (Fractions 4 and 5) when chromatographed with an HTC extract.

In order to determine whether differences in endogenous cyclic AMP or salts were contributing to the differences in elution profiles, extracts were also chromatographed after dialysis at pH 7.6. This treatment did not affect the HTC profile except to eliminate the protein kinase peak in the void volume and increase somewhat the size of Fraction 1 (data not shown). The salt concentration in the undialyzed sample may have been high enough to prevent some of the kinase in Fraction 1 from binding to the resin. As shown in Fig. 4B, there were some additional changes in the elution profile for liver after dialysis. The free kinase peak in the void volume disappeared and that in Fraction 1 was greatly reduced. In addition, there was a relative increase in the first cyclic AMP-stimulated kinase (Fraction 2) and a decrease in the free binding peaks (Fraction 8). These results suggest the possible reassociation of some regulatory and catalytic subunits upon dialysis. Despite these changes, the liver profile is still very different from that obtained with HTC cells. Similar chromatographic differences between liver and HTC cells were also observed in extracts treated with diisopropyl fluorophosphate as described by Kumon et al. (33).

We wondered whether any of the cyclic AMP-binding peaks in these profiles might correspond to cyclic AMP phosphodiesterases. When phosphodiesterase was measured in the chromatographic fractions, the major peak of activity in both HTC cells and liver was found in Fraction 5. However, the tubes of maximum activity were offset from the nearest cyclic AMP binding peaks. In liver, there was an additional minor peak of phosphodiesterase activity which overlapped parts of Fractions 3 and 4.

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**Fig. 4.** Chromatographic comparison of cyclic AMP-binding proteins and protein kinases from liver and HTC cells. Postmicrosomal supernatant fractions were chromatographed on DEAE-cellulose as described under "Experimental Procedures." The KCl gradient was begun at Fraction 21 in each case. Binding activity (O-O) was measured at pH 4 with 100-μl aliquots of effluent. Protein kinase activity (●-●) was determined in 50-μl aliquots with a 15-min assay containing cyclic AMP and 2.5 μM [γ-32P]ATP. Blank values have not been subtracted. The following samples were chromatographed: A, a fresh undialyzed extract from 1.8 g (wet weight) of liver; B, an extract identical with that in (A) but which had been dialyzed for 3 days against several changes of Tris-dithiothreitol buffer, pH 7.6; C, a fresh undialyzed extract from 1.6 × 10⁶ HTC cells. The solid bars indicate the grouping of fractions for further analysis.
Properties of Separated Protein Kinase Fractions—The protein kinases resolved by DEAE-cellulose chromatography were tested for their ability to be stimulated by cyclic AMP with histones as substrates (Table II). As expected, those kinase fractions which contain no binding activity (liver Fraction 1, HTC Fractions 1 and 2) are not affected by cyclic AMP. The data in Table II also show that Fraction 5 from both liver and HTC cells is largely unresponsive to cyclic AMP. Although this fraction is not completely separated from binding activity, its ratio of kinase to binding activity is very high. This protein kinase fraction is especially prominent in HTC cells, and its position of elution is similar to the “casein kinase” referred to by Kumon et al. (33). The protein kinase fractions which correspond to major peaks of binding activity (liver Fractions 2 and 4, HTC Fraction 4) are stimulated by cyclic AMP (Table II). The greatest effect is shown by liver Fraction 2, while HTC Fraction 4 is less responsive than the analogous liver fraction. The somewhat limited stimulation (<2-fold) given by the HTC fraction was not enhanced by increasing the cyclic AMP concentration used in the assay.

Properties of Separated Cyclic AMP Binding Fractions—The binding characteristics of the fractions resolved by chromatography were compared with each other and with the properties of the original extract. Since salt has a strong influence on the binding at pH 4 for both liver and HTC cells (25), the fractions were dialyzed before analysis. The Scatchard plots for liver Fraction 2 at pH 4 and at pH 6.5 are shown in Fig. 5. At pH 4, the curve passes through a maximum and then declines sharply as the concentration of bound cyclic AMP decreases. The decline is probably due to the extreme lability of this fraction at low concentrations of cyclic AMP, as demonstrated by the broken curve in Fig. 5A. At pH 6.5, binding Fraction 2 is more stable at all concentrations of cyclic AMP; and the Scatchard plot is more nearly linear, although there is still some downward curvature at very low cyclic AMP concentrations (Fig. 5B). The apparent KD for the linear part of the curve is 1.2 nM. Liver Fraction 3 resembles Fraction 2 in its binding properties, although the lability at pH 4 and the curvature of the Scatchard plot are less severe (data not shown). The lability of liver Fractions 2 and 3 at pH 4 is reminiscent of that of part of the unfraccionated liver binding activity. In contrast, the HTC chromatographic profile contains no binding components that are this unstable at pH 4, in agreement with the greater stability of the binding in the original HTC extract (Fig. 3).

**TABLE II**

**Effect of cyclic AMP on protein kinase fractions obtained from liver and HTC cells**

Protein kinase activity was determined by a 5-min reaction containing 2.5 μM [γ-32P]ATP either with or without cyclic AMP. Stimulation is defined as the percentage of increase in activity caused by cyclic AMP. Each reaction contained enough kinase activity to transfer at least 0.1 pmole of phosphate per min. The samples were from tubes near the centers of the chromatographic fractions designated in Fig. 4 and were dialyzed for several days before analysis.

<table>
<thead>
<tr>
<th>Chromatographic fraction</th>
<th>Stimulation by cyclic AMP</th>
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<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>1</td>
<td>%</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>710</td>
</tr>
<tr>
<td>4</td>
<td>225</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

The samples were from tubes near the centers of the chromatographic fractions designated in Fig. 4 and were dialyzed for several days before analysis.

**FIG. 5.** Scatchard binding plots for liver chromatographic Fraction 2. Binding (●—●) was determined at either pH 4.0 (A) or pH 6.5 (B) on samples which had been dialyzed for several days against Tris-dithiothreitol buffer, pH 7.6. The activity remaining (O—O) at the end of the incubation was determined with a duplicate set of samples by adding 175 nM cyclic [3H]AMP for the final 2 hours of the reaction time.

**FIG. 6.** Comparison of Scatchard binding plots at pH 6.5 for liver and HTC chromatographic Fraction 4. Binding of cyclic AMP was measured either immediately after chromatography (●—●) or following dialysis against Tris-dithiothreitol buffer at pH 7.6 for 7 days (O—O). The activity remaining at the end of the binding assay (O—O) was determined with the dialyzed samples as described in the legend to Fig. 5. A, liver; B, HTC cells.

For Fraction 4 from both liver and HTC cells, the Scatchard plots obtained after dialysis are similar to each other. At both pH 6.5 (Fig. 6) and pH 4 (data not shown) the plots demonstrate upward curvature and apparent KD values less than 1 nM at the lowest cyclic AMP concentrations. The binding activity is relatively stable at pH 6.5 (Fig. 6). At pH 4, Fraction 4 shows about 45% loss of binding activity at the lowest cyclic AMP concentrations, but this is still considerably less than observed with liver Fractions 2 (Fig. 5A) or 5. The binding properties of Fraction 5 were similar to those of Fraction 4 (data not shown). Little evidence was found for changes in the shape of the Scatchard plot with the position of elution within Fractions 4 and 5. Whatever factors contribute to the nonlinearity of these plots are apparently not resolved by the chromatographic system.
Since the Scatchard plot obtained with the HTC chromatographic Fraction 4 shows a relatively high affinity at pH 6.5 (Fig. 6D), a conversion has occurred from the low affinity form seen in crude extracts (Fig. 1D). To see whether this activation was brought about by the chromatography or by the subsequent dialysis to remove salt, the binding was also analyzed within 1 day after elution from the column. This can be done without dialysis since binding at pH 6.5 is minimally affected by salt, in contrast to the marked effect at pH 4 (25). Also, the dilutions used in the binding assay were sufficient to lower the salt concentrations below 50 mM. This experiment showed (lower curve in Fig. 6B) that activation was not brought about by chromatography itself, but required dialysis for several days after elution. The activation also occurred during storage without dialysis and seemed to take place somewhat more readily in the column fractions than in the crude extract. Unlike the HTC binding protein, liver Fraction 4 did not alter its Scatchard binding profile with time after chromatography (Fig. 6A). It exhibited the same nonlinear, high affinity type of plot even when analyzed soon after elution. This is in agreement with the differences between liver and HTC cells seen in the analyses of unfractionated extracts at pH 6.5 (Fig. 1, C and D).

**DISCUSSION**

This study has revealed several differences between HTC cells and liver. Analysis of the binding of cyclic AMP by the two cellular extracts has produced Scatchard plots which differ in shape. The added complexity of the liver binding curve at pH 4 correlates with the presence of binding component(s) which are quite labile at this pH when not complexed with cyclic AMP (25). The liver binding fraction which is especially unstable at pH 4 corresponds to the first of two cyclic AMP-stimulated protein kinases eluted from DEAE-cellulose (both of which may be heterogeneous). This chromatographic fraction is missing from HTC cells, which also have less labile binding in extracts examined at pH 4.

Extracts from liver and HTC cells have fairly similar levels of binding activity relative to the total amounts of soluble protein they contain (about 30% greater for liver). However, at pH 6.5, the apparent affinity of binding in HTC extracts is much lower than that in liver extracts. This difference becomes quite substantial after dialysis of the liver extracts for a few days, apparently due to removal of endogenous cyclic AMP. Such short times of dialysis do not increase the binding affinity observed in preparations from HTC cells, which contain very little endogenous cyclic AMP. However, the higher affinity binding at pH 6.5 can be caused to appear in HTC extracts by aging or treatment at pH 4. Even the chromatographic fraction which is common to both HTC cells and liver (Fraction 4) appears to have lower affinity in the HTC case before activation. The need for this activation process, which seems to be irreversible, suggests that HTC cells may contain lower affinity binding than liver in vivo.

The specific activity of protein kinase in HTC extracts is higher than that in liver preparations (by greater than 2-fold). Upon elution from DEAE-cellulose, HTC samples contain all the protein kinase peaks observed in liver, although the proportions differ. There is a large increase in HTC extracts of a protein kinase which appears late in the elution profile and is not stimulated by cyclic AMP. There are also two early eluting protein kinase fractions which do not respond to cyclic AMP. The first of these corresponds to an unstimulated protein kinase peak in the liver profile, but the second HTC fraction differs from the second liver fraction in that it lacks binding activity and is not stimulated by cyclic AMP. This is the binding fraction which appears to be missing from HTC cells. We do not know whether the free protein kinase in the first position in the HTC profile is analogous to the catalytic subunit of the first cyclic AMP-stimulated kinase in liver. However, the hepatic catalytic subunit found by Kumon et al. (33) does elute in this approximate position from DEAE-Sephadex.

A decrease compared to liver in a cyclic AMP-stimulated protein kinase fraction has recently been observed in two rapidly growing hepatomas by Criss and Morris (39). Measurements of cyclic AMP-binding and protein kinase activities in HTC extracts have also been made by Granner (40). Our studies agree with his observation that the ratio of binding protein to protein kinase activity is lower in HTC cells than in liver. However, his report differs quantitatively from ours in detecting only about 10% as much binding activity in HTC cells as in liver. One possible reason for this difference is the lower affinity we have observed for the HTC binding at pH 6.5. Granner’s assays were done at pH 5, where we also observe the binding affinity to be lower for HTC extracts than for liver.3 His assay procedure, which involved a correction to eliminate nonspecific binding, may have underestimated the HTC binding because of its lower affinity. However, despite its lower affinity, we believe the HTC binding to be analogous to some of that observed in liver because it can be activated to higher affinity, resembles some liver components chromato graphically, and has a high degree of specificity for cyclic AMP (25).

At this point, we can only speculate on the significance of the differences in binding properties between liver and HTC cells. Since derivatives of cyclic AMP can still induce tyrosine aminotransferase in HTC cells (7), components able to mediate this event must be present. Thus, the binding protein which is missing from HTC cells would appear to be excluded from a compulsory role in the induction process. Also the fact that the magnitude of the induction in HTC cells is rather low (7) could correlate with the less dramatic stimulation by cyclic AMP of their protein kinase fraction compared to liver. The lower affinity shown by HTC binding protein at pH 6.5, if also true in vivo, could be the reason HTC cells respond only to very high doses of cyclic nucleotides (7). Thus, many of the characteristics of protein kinases and binding proteins from HTC cells do seem reasonably consistent with the physiological responses of the cells.

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