RESULTS

Results obtained from the experiments are plotted as line and bar diagrams. Each data point represents the mean value ± standard deviation. In some cases, results are presented in a tabular form. Results obtained from two sets of data were statistically analyzed according to Student's t-test, with P< 0.05 taken as significant.

Glucocorticoid receptor binding studies

Concentration of [³H]dexamethasone and the time required to obtain maximum saturable binding.

Fig. 1 depicts the kinetic of [³H]dexamethasone binding by glucocorticoid receptors using increasing concentrations of [³H]dexamethasone from 2.5-120 nM. The specific binding increases with increasing hormone concentration up to about 60 nM but, thereafter there is no significant increase in the binding of [³H]dexamethasone by glucocorticoid receptors. This shows that maximum specific saturable binding was obtained at ~60 nM of [³H]dexamethasone.

The time kinetics for [³H]dexamethasone binding is shown in fig. 2. It is observed that the maximum saturable binding, using 60 nM [³H]dexamethasone was attained by about 4 h of reaction time. Each data point in the figures represent the mean value obtained from four separate assays.

Glucocorticoid receptor level at various postnatal ages

The glucocorticoid receptor levels, expressed as fmol/ mg protein were determined using high-affinity [³H]dexamethasone binding in the liver, kidney, cardiac muscle, skeletal muscle and the cerebral hemisphere at various postnatal ages (0-, 5-, 10-, 30-, 60- and 90-day) of male chicken. In the liver, the receptor level reaches a peak value by day 5 of postnatal age, which is significantly higher (+ 42%) than the value observed at day 0 of postnatal age. The receptor concentration values then show a gradual decline with age, i.e. − 25% at day 10 and − 18% at day 30 of postnatal age. The receptor levels at day 60 and 90 of postnatal age are similar to the values that of day 30 (Fig. 3). The slot blot analyses of receptor preparation (Fig. 4A), as well as the net intensity of the slot bands (Fig. 4B) confirmed the increased level of GR protein at day-5, as compared to day-0 of postnatal age. In the kidney, the receptor level is maximum in the early postnatal age (0-day). Thereafter, the receptor concentration declines by 18% at day 5 and remains unchanged up to day 30 of postnatal age. By day 60, the receptor level again decline by 16% and then remains unchanged up to day 90 of postnatal age.
Fig. 1. Concentration dependence of [³H]dexamethasone binding to glucocorticoid receptors in the liver of chicken. Aliquots of cytosol (100 µl) were incubated with increasing concentrations of [³H]dexamethasone ± 500-fold excess non-radioactive dexamethasone for 6 hr at 2°C. Unbound hormones were removed by dextran coated charcoal (DCC) treatment as described in the Materials and Method section. Specific binding was calculated as the difference between total binding and that in presence of non-radioactive hormone. Each point represents the mean value of 4 separate set of experiments, each performed in duplicate.

Fig. 2. Time kinetics of [³H]dexamethasone binding to hepatic glucocorticoid receptors. Cytosols were incubated with 60 nM [³H]dexamethasone ± 500-fold excess non-radioactive dexamethasone at 2°C. At the indicated intervals of time, specific binding was determined as mentioned in the Materials and Method section. Each data point is the mean value of 4 separate set of experiments.
Fig. 3. Specific saturable binding of [\(^3\)H]dexamethasone in liver of chicken at different postnatal ages. Tissue fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to each other groups.

Fig. 4. Slot blot analysis of hepatic glucocorticoid receptor from 0- and 5-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of liver cytosols containing GR from 0- and 5-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).
Fig. 5. Specific saturable binding of \(^3\text{H}\)dexamethasone in kidney of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to other each groups.

Fig. 6. Slot blot analysis of renal glucocorticoid receptor from 0- and 5-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of kidney cytosols containing GR from 0- and 5-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).
Postnatal age (Fig. 5). Slot blot analyses (Fig. 6A) and net intensity of the slot bands (Fig. 6B) corroborate the higher level of GR protein at day 0 in the kidney of chicken. The glucocorticoid receptor concentration in the cardiac muscle (heart) is constant in the early postnatal ages up to day 30. By day 60, the receptor level declines by 36% and the level at day 90 is similar to that at day 60 of postnatal age (Fig. 7). Slot blot analyses of the receptor preparation (Fig. 8A) and the intensity of the slot bands (Fig. 8B) confirm a decline in receptor protein level at day 60 compared to day 0 of postnatal age. In skeletal muscle, the glucocorticoid receptor level reaches the peak value by day 10 of postnatal age, which is significantly higher (+69%) than the value observed at day 5 of postnatal age. The receptor level then declines by 41% at day 60 of postnatal age. The receptor level at day 90 is similar to that at day 60 of postnatal age (Fig. 9). Slot blot analyses (Fig. 10A) and the intensity of the slot bands (Fig. 10B) show that the receptor protein level is higher at day 10 compared to day 0 of postnatal age. In the cerebral hemisphere, the receptor levels are quite low compared to the other tissues studied, however, the receptor level does not elicit any significant change in all the ages studied (Fig. 11). Slot blot analyses (Fig. 12A) and subsequent analyses of the intensity of the slot bands (Fig. 12B) corroborate the above findings. The glucocorticoid receptor concentrations in different tissues and at various postnatal ages are also summarized in Table I.

Scatchard analyses of glucocorticoid receptor
Scatchard analysis of the binding data was performed in the liver and kidney of chicken at the two age groups, where the changes in receptor concentration were maximum. Scatchard plot of the binding data in liver reveals no difference in the slopes of the linear-regressed plots obtained from 0- and 5-day old chicken (Fig. 13A). This shows that the dissociation constant (Kd), the value of which was found to be similar (2.84 nM for day 0 and 2.98 nM for day 5), indicating that affinity of hormone for the receptor remains unchanged at these two ages studied. However, the intercept of the plots on the x-axis showed that the value of specific binding sites in 5-day old chicken (435 fmol/mg protein) is significantly higher than that at day 0 (326 fmol/mg protein). In the kidney, Scatchard data from 0- and 5-day old chicken, similarly revealed no age-associated change in the dissociation constant values for both the age groups studied (Fig. 13B). However, the specific binding sites at day 0 (368 fmol/mg protein) is significantly higher than that at day 5 (313 fmol/mg protein). The data obtained from the Scatchard analyses in liver and kidney have also been outlined in Table II.

Studies on the activation of glucocorticoid receptor
Activation studies were performed in the liver and kidney of 0- and 30-day old chicken to monitor if there are any changes in the activation properties during these developmental
Fig. 7. Specific saturable binding of [\(^3\)H]dexamethasone in cardiac muscle of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to other each groups except day-90.

Fig. 8. Slot blot analysis of cardiac muscle glucocorticoid receptor from 0- and 60-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of cardiac muscle cytosols containing GR from 0- and 60-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).
Fig. 9. Specific saturable binding of [³H]dexamethasone in skeletal muscle of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to other each groups except day-30.

Fig. 10. Slot blot analysis of skeletal muscle glucocorticoid receptor from 0- and 10-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of skeletal muscle cytosols containing GR from 0- and 10-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).
Fig. 11. Specific saturable binding of \(^{3}H\)dexamethasone in cerebral hemisphere of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group.

![Graph](image)

Fig. 12. Slot blot analysis of cerebral hemisphere glucocorticoid receptor from 0- and 5-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of skeletal muscle cytosols containing GR from 0- and 5-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).
Table 1

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<th>Tissue</th>
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<th>10</th>
<th>30</th>
<th>60</th>
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<td>Liver</td>
<td></td>
<td>332±15</td>
<td>472±13*</td>
<td>355±7</td>
<td>290±10</td>
<td>284±10</td>
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<td>Kidney</td>
<td></td>
<td>374±12*</td>
<td>302±8</td>
<td>286±11</td>
<td>299±9</td>
<td>252±8</td>
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<td>178±18#</td>
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<td>383±18†</td>
<td>368±22</td>
<td>216±13</td>
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<td>Cerebral</td>
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<td>131±13</td>
<td>128±8</td>
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Glucocorticoid receptor levels are expressed as fmol/mg protein; values are mean ± standard deviation from 5-6 animals of each age group.

* statistically significant (p<0.05) as compared to other age groups
# statistically significant (p<0.05) as compared to other age groups except day-90
† statistically significant (p<0.05) as compared age groups except day-30
Fig. 13. Scatchard plot of [3H]dexamethasone binding in the liver (A) and kidney (B) of chicken of day 0 and 5. Cytosols from liver and kidney were incubated with 2.5-120 nM [3H]dexamethasone ± 500-fold excess non-radioactive dexamethasone for 4 hr at 2°C. Specific binding at each concentration point was calculated as the difference between total binding and that in presence of excess non-radioactive hormone. The data obtained was analysed according to the method of Scatchard. The slope of the curve provided the dissociation constant ($K_d$) and the intercept on the x-axis gave the maximum receptor concentration value. Each data point is the mean of 3 experiments performed each time with tissues from 5-6 chicken of each age group.
Table II

Concentration and affinity of $[^3]H$-dexamethasone receptors in the liver and kidney of chicken

<table>
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<td>0-day</td>
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<td>5-day</td>
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<tr>
<td>Number of specific binding sites (fmol/mg protein)</td>
<td>326±15</td>
<td>435±13*</td>
<td>368±8*</td>
<td>313±11</td>
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<tr>
<td>Dissociation constant, Kd (nM)</td>
<td>2.84±0.9</td>
<td>2.98±0.39</td>
<td>3.19±0.10</td>
<td>3.09±0.12</td>
</tr>
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</table>

Data were collected from three sets of experiments with pooled tissues from 5-6 chicken of each age group and analysed using Scatchard plot as given in the Materials and Methods section. Values are mean ± standard deviation. *statistically significant (p<0.05) as compared to other age group.
phases. These ages were selected, as the level of GR was similar, hence any change in activation binding was not attributed to the GR level, but rather to its activation properties. The optimum temperature for maximum activation of GR complexes was found to be 25°C (Fig. 14A) whereas, 25 mM CaCl₂ was the optimum concentration required for maximal binding of hormone-receptor complexes to the binding medium (Fig. 14B). The magnitude of thermal as well as salt (Ca²⁺) dependent activation processes were judged using DNA-cellulose and purified nuclear binding assays.

**DNA-cellulose binding assay**

DNA-cellulose binding assays in liver revealed that both the temperature and salt increase (2-2.5 fold) [³H]dexamethasone-receptor complexes binding to DNA-cellulose as compared to the binding of unactivated receptor complexes incubated at 0°C for 45 min. However, the degree of activation is similar for hepatic glucocorticoid receptors obtained from 0- and 30-day old chicken (Fig. 15A). The results indicate no postnatal difference in the *in vitro* activation of the hormone-receptor complexes under the conditions mentioned above.

In the kidney too, the thermal as well salt activation of [³H]dexamethasone-receptor complexes were similar to that in liver since there was no difference in the magnitude of *in vitro* activation of the hormone-receptor complexes in the two ages studied. However, the fold of activation of [³H]dexamethasone-receptor complexes both by temperature and salt is slightly lower (2-2.3 fold) compared to the liver (Fig. 15B).

**Nuclear binding assay**

Since DNA-cellulose by virtue of it being a non-specific assay system, could not implicate any age-related differences, purified nuclei were used to provide a more physiological assay system. The results indicate that the thermal as well as salt activation of the [³H]dexamethasone-receptor complexes is similarly observed in both the age groups studied as seen using DNA-cellulose binding assays. However, in contrast to DNA-cellulose binding assay, nuclear binding of both thermal- and salt-activated glucocorticoid-receptor complexes is significantly higher in immature (0-day) compared to that of mature (30-day) chicken (Fig. 16A). Nuclear exchange (cross-mixing) assays, where in both the thermally and salt activated receptors of 30-day old were allowed to interact with the nuclei of 0-day and vice versa, showed significantly higher binding by the hepatic nuclei of 0-day old as compared to those from 30-day old chicken (Fig. 17A). These findings indicate higher binding capacity of immature nuclei to activated hormone-receptor complexes than those of mature ones.
Fig. 14. Effect of temperature (A) and of CaCl₂ (B) on activation of [³H]dexamethasone-receptor complexes. The hepatic hormone-receptor complexes were prepared and nuclear binding of the receptors activated by heat (A) and salt (B) were performed. Details of processing and determination of the bound complexes are as described in the Materials and Methods section. Each point represents the mean value of 4 separate set of experiments, each performed in duplicate.
Fig. 15. Binding of [\textsuperscript{3}H]dexamethasone-receptor complexes to DNA-cellulose in the liver (A) and kidney (B) of chicken of day 0 and 30. Cytosols from liver and kidney were prepared in buffer B with 60 nM [\textsuperscript{3}H]dexamethasone for 4 hr at 2°C. Activation conditions, binding to DNA-cellulose and further processing of the pellets was done as described in the Materials and Methods section. The results are mean ± standard deviation of three experiments, each set done in triplicate with pooled tissues from 5-6 chicken of same age group. * statistically significant as compared to other age.
Fig. 16. Binding of \[^3H\]dexamethasone-receptor complexes to purified nuclei in the liver (A) and kidney (B) of chicken of day 0 and 30. Cytosols from liver and kidney were prepared in buffer B with 60 nM \[^3H\]dexamethasone for 4 hr at 2°C. Activation conditions, binding to purified nuclei and further processing of the nuclear pellets was done as described in the Materials and Methods section. The results are mean ± standard deviation of three experiments, each set done in triplicate with pooled tissues from 5-6 chicken of same age group. * statistically significant as compared to other each age.
Fig. 17. Cross-mixing binding of [3H]dexamethasone-receptor complexes to purified nuclei in the liver (A) and kidney (B) of chicken of day 0 and 30. Cytosols from liver and kidney were prepared in buffer B with 60 nM [3H]dexamethasone for 4 hr at 2°C. Nuclear cross-mixing in which activated receptor complexes from 30-day old chicken were incubated with nuclei of 0-day old chicken (a) and vice versa (b). The results are mean ± standard deviation of three experiments, each set done in triplicate with pooled tissues from 5-6 chicken of same age group. * statistically significant as compared to other age.
In Kidney, the pattern of nuclear binding by heat and salt activated receptor complexes was similar to that observed in liver (Fig. 16B). Nuclear exchange assay also revealed the same pattern of activation as seen in liver (Fig. 17B).

DNase I digestion studies

Nuclear exchange studies in the liver and kidney revealed a higher degree of binding of the thermally activated [3H]dexamethasone-receptor complexes by the nuclei of 0-day as compared to 30 day nuclei. These findings indicated a change in nuclear chromatin property that may have contributed to the higher binding of hormone-receptor complexes in immature chicken. Therefore, DNase I digestion of the liver and kidney nuclei, from both the age groups were done to determine the extent of chromatin digested that will be estimated by the amount of bound hormone-receptor complexes from the nuclei. This will reflect the change in chromatin organization that might have been responsible for the observed changes in the nuclear binding at the two ages. DNase I digestion of the nuclear chromatin was able to extract a significantly higher number of nuclear bound, thermally activated [3H]dexamethasone-receptor complexes from both the liver and kidney of 0-day with respect to 30-day old chicken. The degree of extraction of the nuclear bound receptors in the liver is 70% (0-day) and 44% (30-day) as compared to their respective controls taken as 100% (Fig. 18A). In the Kidney too, the percentage of extraction was observed to be 60% for 0-day and 46% for 30-day chicken (Fig. 18B).

Inhibition of activation studies

The inhibition of activation of GR by utilizing various exogenous and endogenous agents was studied to see their modulatory effects in the liver of 30-day old chicken. To examine the effect of molybdate (MO\(^{2-}\)) on receptor activation by heat, a concentration of 0-100 mM (MO\(^{2-}\)) was used. Result exhibited a dose-dependent inhibition (maximally 80 % at 80 mM) of heat activation of H-R complexes from the liver of chicken as assessed by binding to DNA-cellulose (Fig. 19A) and purified nuclei (Fig. 19B). Tungstate (WO\(^{4+}\)) was also utilized to see any modulatory effect on hepatic GR activation. Results indicated that tungstate (0-20 mM) appears to be a more effective inhibitory agent (80% at 15 mM) compared to molybdate, as assessed by binding to DNA-cellulose (Fig. 20A) and purified nuclei (Fig. 20B). Cadmium (0-6 mM) caused a maximal inhibition (60%) of hepatic GR heat activation at 2 mM by DNA-cellulose (Fig. 21A) and nuclear binding assays (Fig. 21B). Oleic acid, a polyunsaturated fatty acid (PUFA) has also been used to study its inhibitory role on in vitro receptor activation. Oleic acid (C 18:1) caused 45% maximal inhibition of heat activation at 40 µM as assessed by DNA-cellulose (Fig. 22A) and nuclear binding assays (Fig. 22B).
Fig. 18. Extraction of the nuclear bound \[^3H\]dexamethasone-receptor complexes by DNase I. Thermally activated, nuclear bound hormone-receptor complexes were extracted using DNase I (150 µg/100 µg DNA) in the liver (A) and kidney (B), for 45 min at 2°C. Details of the procedure and determination of the bound radioactivity are given in the text. The values are mean ± standard deviation of three experiments. * statistically significant with respect to other age groups.
Fig. 19. Effect of molybdate (MoO$_4^{2-}$) on the heat activation of hepatic [H]$\Delta$examethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM [H]$\Delta$examethasone for 4 hr to generate [H]$\Delta$examethasone-receptor complexes. Aliquots of cytosol containing [H]$\Delta$examethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of molybdate. DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Heat activation in the absence (control) of molybdate is taken as 0 % inhibition.
Fig. 20. Effect of tungstate (WO₄²⁻) on the heat activation of hepatic [³H]dexamethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM [³H]dexamethasone for 4 hr to generate [³H]dexamethasone-receptor complexes. Aliquots of cytosol containing [³H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of tungstate. DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Heat activation in the absence (control) of tungstate is taken as 0 % inhibition.
Fig. 21. Effect of cadmium (Cd²⁺) on the heat activation of hepatic [³H]dexamethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM [³H]dexamethasone for 4 hr to generate [³H]dexamethasone-receptor complexes. Aliquots of cytosol containing [³H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of cadmium. DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Heat activation in the absence (control) of cadmium is taken as 0 % inhibition.
Fig. 22. Effect of oleic acid (C 18:1) on the heat activation of hepatic \([{^3}H]\)dexamethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM \([{^3}H]\)dexamethasone for 4 hr to generate \([{^3}H]\)dexamethasone-receptor complexes. Aliquots of cytosol containing \([{^3}H]\)dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of oleic acid (prepared as stock in dimethyl sulfoxide, DMSO). DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Control tubes received appropriate volume of DMSO instead of oleic acid. Heat activation in the absence (control) of oleic acid is taken as 0 % inhibition.
All the above inhibitors of activation were also used to study the modulation of heat and salt activation of liver and kidney GR from 0- and 30-day old chicken to see any tissue- and/or age-specific modulation. Molybdate was used at increasing concentration (0-100 mM) to see its inhibitory effect on GR heat and salt activation in the liver and kidney of 0- and 30-day old chicken. DNA-cellulose binding assay shows similar pattern of inhibition (~80% at 60 mM) of hepatic (Fig. 23A) and kidney (Fig. 23B) GR heat activation by molybdate in both the ages studied. Nuclear binding assay also revealed a similar pattern of inhibitory effect of molybdate in both the ages in the liver (Fig. 24A) and kidney (Fig. 24B) of chicken. DNA-cellulose binding assay shows inhibition (75-80% at 60mM) of hepatic (Fig. 25A) and kidney (Fig. 25B) GR salt activation by molybdate in both the ages studied. Nuclear binding assay also revealed a similar pattern of inhibitory effect of molybdate in both the ages in the liver (Fig. 26A) and kidney (Fig. 26B) of chicken. Tungstate (0-20 mM) also inhibited GR heat activation maximally 80-85% at 15 mM in both the tissues and ages although, to a similar extent as assessed by DNA-cellulose (Fig. 27A&B) and nuclear (Fig. 28A&B) binding assays. Tungstate also inhibited GR salt activation in the same pattern as above in both tissues and ages as assessed by DNA-cellulose (Fig. 29A&B) and nuclear (Fig. 30A&B) binding assays. Cadmium (0-6 mM), caused a maximal inhibition (60% at 2 mM) of GR heat activation, but could not show any difference in % inhibition from the liver (Fig. 31A) and kidney (Fig. 31B) of 0- and 30-day chicken by DNA-cellulose binding assay, with a similar result in the case of nuclear binding assay (Fig. 32A&B). Similar observations were found where cadmium caused maximal inhibition (60% at 2 mM) of GR salt activation, but as above could not show any difference in % inhibition from the liver (Fig. 33A) and kidney (Fig. 33B) of 0- and 30-day chicken by DNA-cellulose binding assay, with a similar result in the case of nuclear binding assay (Fig. 34A&B). Oleic acid which is an endogenous PUFA was also employed to show if any difference in tissue- and age- specific inhibition of both heat and salt activation of GR. Oleic acid caused similar extent of inhibition (~42-47% at 40 µM) of heat activation of hepatic and renal GR in both the ages as observed by DNA-cellulose binding assay (Fig. 35A&B), with a similar extent in nuclear (Fig. 36A&B) binding assay. Oleic acid also caused a similar extent of inhibition of salt activation of hepatic and renal GR in both the ages as observed by DNA-cellulose binding assay (Fig. 37A&B), with a similar extent in nuclear binding assay (Fig. 38A&B).

**Physicochemical characterization of glucocorticoid receptor**

To study some of the physicochemical characteristics of the unactivated as well as activated glucocorticoid receptor from immature (0-day) and mature (30-day) chicken, gel filtration analyses and anion-exchange chromatography were performed only on the hepatic glucocorticoid receptors of chicken.
Fig. 23. Effect of molybdate (MO$_4^{2-}$) on the heat activation of hepatic (A) and kidney (B) $[3H]$dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.
Fig. 24. Effect of molybdate (MO\textsuperscript{2+}) on the heat activation of hepatic (A) and kidney (B) \textsuperscript{[3H]} dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.
Fig. 25. Effect of molybdate (MO_4^{2-}) on the salt activation of hepatic (A) and kidney (B) [^3H] dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.
Fig. 26. Effect of molybdate (MoO$_4^{2-}$) on the salt activation of hepatic (A) and kidney (B) $[^3H]$dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.
Fig. 27. Effect of tungstate (WO$_4^{2-}$) on the heat activation of hepatic (A) and kidney (B) $[^3H]$dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.
Fig. 28. Effect of tungstate (WO₄²⁻) on the heat activation of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.
Fig. 29. Effect of tungstate ($WO_4^{2-}$) on the salt activation of hepatic (A) and kidney (B) $[^{3}H]$dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.
Fig. 30. Effect of tungstate (WO$_4^{2-}$) on the salt activation of hepatic (A) and kidney (B) [$^3$H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.
Fig. 31. Effect of cadmium (Cd\textsuperscript{2+}) on the heat activation of hepatic (A) and kidney (B) \textsuperscript{[3H]}dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.
Fig. 32. Effect of cadmium (Cd\(^{2+}\)) on the heat activation of hepatic (A) and kidney (B) \([^{3}H]\)dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.
Fig. 33. Effect of cadmium (Cd^{2+}) on the salt activation of hepatic (A) and kidney (B) [^3H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.
Fig. 34. Effect of cadmium (Cd$^{2+}$) on the salt activation of hepatic (A) and kidney (B) [$^3$H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.
Fig. 35. Effect of oleic acid (C 18:1) on the heat activation of hepatic (A) and kidney (B) [\(^{3}H\)]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 22.
Fig. 36. Effect of oleic acid (C 18:1) on the heat activation of hepatic (A) and kidney (B) $[^{3}H]$dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 22.
Fig. 37. Effect of oleic acid (C 18:1) on the salt activation of hepatic (A) and kidney (B) \[^{3}H\]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 22.
Fig. 38. Effect of oleic acid (C 18:1) on the salt activation of hepatic (A) and kidney (B) 
$[^3]H$-dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear 
binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and 
further processing of pellets are same as described in fig. 22.
Gel filtration analyses

Fig. 39 depicts the elution profile of the unactivated \(^{3}H\)dexamethasone-receptor complexes analysed on a sephadex G-200 column. The receptor from both the ages (0-and 30-day) eluted as a single peak at the same elution volume between the standard molecular weight markers, ferritin and \(\beta\)-amylase. The data generated from the above studies were plotted and linear regressed curves obtained, from which molecular weight and the stokes radii \((R_s)\) were calculated. The plot of log M vs. \(V/V_0\) gave molecular weight of 255 kDa and 256 kDa for 0- and 30-day receptor, respectively (Fig. 40). The stokes radii were calculated from a plot of \(-\log K_a\) vs. \(R_s\) and calculated to be 5.65 and 5.68 nm for the receptors of 0- and 30-day, respectively (Fig. 41).

The thermally activated \(^{3}H\)dexamethasone-receptor complexes were analysed on a sephadex G-100 gel column. The elution profile of the activated receptors showed that the receptors from both the ages eluted between the molecular markers, alcohol dehydrogenase and bovine serum albumin as a single peak and at the same elution volume (Fig. 42). A small peak of radioactivity, which eluted in the void volume, is probably due to the fraction of receptors remaining in the unactivated state. From the plot of log M vs. \(V/V_0\) the molecular weight was calculated to be 86 kDa (0-day) and 87 kDa (30-day) (Fig. 43). The stokes radii, calculated from the plot of \(-\log K_a\) vs. \(R_s\) gave the values as 3.28 nm (0-day) and 3.31 nm (30-day) (Fig. 44).

Ion-exchange analyses

To study the charge content of the unactivated as well as activated \(^{3}H\)dexamethasone-receptor complexes from liver of 0- and 30-day chicken, ion-exchange analyses were performed. The elution profile of the unactivated receptors (Fig. 45) does not reveal any age-related difference in the concentration of salt required to elute the receptors of the two ages. The hormone bound receptors from both the age groups eluted as a single peak at \(-248\) mM of KCl. Upon thermal activation, the elution of receptors from DE-52, reveals two radioactivity associated peaks, one eluting at \(-100\) mM KCl and the other at \(-247\) mM of KCl (Fig. 46). The peak which eluted at \(-100\) mM KCl represents the fraction of glucocorticoid receptors that have undergone thermal activation, while the peak at higher salt concentration is contributed by the fraction of receptors that remained unactivated. The data obtained from the above studies are also presented in a tabular form in Table III. However, these data did not reveal any differences in the charge content of the GR from the the two ages of chicken.
Fig. 39. Gel permeation column chromatography of hepatic unactivated glucocorticoid receptors. A column (1.8 x 90 cm) of sephadex G-200, equilibrated with buffer C (i) at 2-4°C was used. Cytosol prepared in buffer A was incubated with 60 mM [³H]dexamethasone for 4 h at 0°C. After DCC treatment, 2 ml of the cytosol was loaded onto the column and eluted with buffer C (i). Fractions of 2 ml each were collected and 100 µl from each fraction was counted for bound radioactivity. Standard protein markers used were F, ferritin (443 kDa); A, β-amylase (200 kDa); Al, alcohol dehydrogenase (150 kDa); B, bovine serum albumin (66 kDa) and C, cytochrome C (12.4 kDa). V₀ and Vᵢ represent the elution volume of blue dextran and [³H]dexamethasone, respectively. Each point in the elution volume represents the mean value of 4 experiments.
Fig. 40. Plot of log M vs. $V_d/V_o$ for the determination of molecular weight of unactivated glucocorticoid receptors. The data from sephadex G-200 gel chromatography were plotted to obtain a linear-regressed curve. The standard protein markers used were ferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (156 kDa), BSA (66 kDa) and cytochrome C (12.4 kDa). GR<sub>0</sub> and GR<sub>30</sub> represent the positions of unactivated hepatic glucocorticoid receptors from 0- and 30-day old chicken, respectively. The data is the mean of 4 separate experiments.
Fig. 41. Plot of \((-\log K_{av})^{1/2}\) vs. Rs for the determination of stokes radii of unactivated liver glucocorticoid receptors. The plot was generated using data from G-200 gel chromatography. The protein markers of known stokes radius were ferritin (7.0 nm), alcohol dehydrogenase (4.6 nm), bovine serum albumin (3.55 nm) and cytochrome c (1.84 nm). The positions of the receptor from the two age groups are indicated as GR_0 and GR_30, respectively. The values are mean of 4 experiments.
Fig. 42. Gel permeation column chromatography of hepatic activated glucocorticoid receptors. A column (3 x 40 cm) of sephadex G-100, equilibrated with buffer C (ii) at 2-4°C was used. After DCC treatment, the hormone-receptor complexes were activated at 25°C for 45 min and 2 ml of it loaded onto the column and eluted with buffer C (ii). Fractions of 2 ml each were collected and 100 µL from each counted for bound radioactivity. Standard protein markers used were A, alcohol dehydrogenase (150 kDa); B, bovine serum albumin (66 kDa); O, ovalbumin (45 kDa); Ca, Carbonic anhydrase (29 kDa) and C, cytochrome C (12.4 kDa). $V_0$ and $V_1$ represent the elution volume of blue dextran and [H]$\text{H}$examethasone, respectively. Each point in the elution volume represents the mean value of 4 experiments.
Fig. 43. Plot of $\log M$ vs. $V_d/V_o$ for the determination of molecular weight of activated glucocorticoid receptors. The data from sephadex G-100 gel chromatography were plotted to obtain a linear-regressed curve. The standard protein markers used were alcohol dehydrogenase (150 kDa), BSA (66 kDa), ovalbumin (45 kDa) and cytochrome C (12.4 kDa). GR₀ and GR₃₀ represent the positions of the activated glucocorticoid receptors from 0- and 30-day old chicken, respectively. The data presented is the mean of 4 separate experiments.
Fig. 44. Plot of $-\left(\log K_{av}\right)^{1/2}$ vs. $R_s$ for the determination of stokes radii of activated liver glucocorticoid receptors. The plot was generated using the data from sephadex G-100 gel chromatography. The molecular markers used were alcohol dehydrogenase (4.6 nm), bovine serum albumin (3.55 nm), ovalbumin (3.05 nm) and cytochrome C (16.4 nm). The positions of the receptor from the two ages are indicated by GR$_0$ and GR$_{30}$, respectively. The values are mean of 4 experiments.
Fig. 45. Elution profile of the unactivated glucocorticoid receptors from anion-exchanger DE-52. A 5 ml glass syringe containing 3 cm high gel-bed layered over a 2 mm thick dextran coated charcoal layer was used. The gel was equilibrated at 2-4°C with buffer D. Unactivated [3H]dexamethasone receptor complexes from the liver were prepared as given in the Materials and Methods section and 2 ml loaded onto the column. After washing off the unbound proteins, the bound proteins were eluted with a linear salt gradient (0-400 mM KCl in the above buffer). One ml fractions were collected and 100 µl from each fraction counted for radioactivity. Each point in the profile represents the mean of 4 experiments.
Fig. 46. Elution profile of the activated glucocorticoid receptors from anion-exchanger DE-52. A 5 ml glass syringe containing 3 cm high gel-bed layered over a 2 mm thick dextran coated charcoal layer was used. The gel was equilibrated at 2-4°C with buffer D. Heat activated [3H]dexamethasone receptor complexes from the liver were prepared as given in the Materials and Methods section and 2 ml loaded onto the column. After washing off the unbound proteins, the bound proteins were eluted with a linear salt gradient (0-400 mM KCl in the above buffer). One ml fractions were collected and 100 µl from each fraction counted for radioactivity. Each point in the profile represents the mean of 4 experiments.
### Table III

<table>
<thead>
<tr>
<th>Parameters</th>
<th>0-Day</th>
<th>30-Day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unactivated receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>255.50 ± 2.50</td>
<td>256.20 ± 2.80</td>
</tr>
<tr>
<td>Stokes radius (nm)</td>
<td>5.65 ± 0.06</td>
<td>5.68 ± 0.14</td>
</tr>
<tr>
<td>Elution from DE 52</td>
<td>248 ± 4.00</td>
<td>241 ± 5.00</td>
</tr>
<tr>
<td><strong>Activated receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>86.60 ± 4.00</td>
<td>87.30 ± 2.00</td>
</tr>
<tr>
<td>Stoke radius (nm)</td>
<td>3.28 ± 0.20</td>
<td>3.31 ± 0.07</td>
</tr>
<tr>
<td>Elution from DE 52 by KCl (mM)</td>
<td>100 ± 2.00</td>
<td>101 ± 3.00</td>
</tr>
</tbody>
</table>