RESULTS

Results obtained from the experiments performed 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.

Studies on hormone binding to GR and its stability by modulators:

Hormone binding to GRs and stability of hormone-receptor (H-R) complexes, after attaining maximal saturable binding with 40 nM [³H]dexamethasone, [³H]dex, at 0°C in the absence (control) or presence of stabilizers such as DTT, ME and GSH were examined as a function of time (1-24 hr). Pilot experiments performed in the liver of 120-day old mice revealed that the maximal saturable binding of GR by [³H]dex reached a peak at about 4 hr without any change up to 8 hr and then sharply declined in control (Fig. 1A). However, in the presence of DTT (1mM) there was a significant higher (37%) specific binding of hormone to the receptor at 4 hr compared to control, and that was maintained for up to 24 hrs of incubation (Fig. 1A). This shows that DTT enhances the maximum specific binding of hormone to the receptor as compared to control. Interestingly, mercaptoethanol (Fig. 1B) and glutathione (Fig. 2) had no influence on the hormone binding to hepatic GR at 4 hr as compared to their respective controls.

In terms of stability of GR after attaining maximal saturable binding at 4 hr, there was a similar pattern of decline of H-R complexes up to 24 hr in both control and DTT incubated cytosols (Fig. 1A). This shows that DTT is ineffective in stabilizing the H-R complexes after maximal saturable binding. Also, both mercaptoethanol (Fig. 1B) and glutathione (Fig. 2) had no stabilizing effect on H-R complexes after maximal saturable binding as compared to control. Hence, results indicate that DTT, a potent reducing agent enhances the hormone binding to receptor, however, both mercaptoethanol and glutathione had no influencing role on this process. In contrast, none of these were able to stabilize the H-R complexes as a function of time.

Next, it was decided to employ these modulators to find out differences, if any, on steroid binding to GR and stabilization of hormone-bound GR from the liver and kidney of immature (15-) and mature (120-day old) mice to reveal age-specific difference, if any. Our data from control group show greater (33%) maximal specific binding of hormone to the hepatic receptor at 4 hr from immature mice as compared to mature and that was maintained up to 16 hrs of incubation (Fig. 3A), with similar observation in the kidneys (Fig. 3B). However, there was no observed difference in the stability of hepatic (Fig 3A) and renal (Fig 3B) GR complexes of control in immature and mature mice after maximal specific saturable binding is attained. In the
Figure 1. Effect of dithiothreitol (DTT) (A) and 2-mercaptoethanol (ME) (B) on the binding of \(^{3}H\)dexamethasone to hepatic glucocorticoid receptors and stabilization of hormone-receptor complexes. Aliquots of freshly prepared cytosol (100 µl) were incubated with 40 nM \(^{3}H\)dexamethasone ± 500-fold excess nonradioactive dexamethasone, and in the presence or absence (control) of 1 mM DTT or ME for 1-24 hr at 0°C. Unbound steroids were removed by dextran-coated charcoal (DCC) treatment as indicated in the materials and methods section. The values obtained from subtraction of non-specific binding from total binding represented the specific \(^{3}H\)dexamethasone bound to the receptor. Each data point represents mean ± standard deviation of 4 separate experiments performed in duplicate from 4-5 mice. *Statistically significant compared to control at 4 hr.
Figure 2. Effect of glutathione (GSH) on the binding of $[^3H]$dexamethasone to hepatic glucocorticoid receptors and stabilization of hormone-receptor complexes from mice. Cytosol preparation, hormone binding and further processing of the samples were performed as indicated in fig. 1. Results are mean ± standard deviation as given in fig. 1.
Figure 3. Effect of dithiothreitol (DTT) on the binding of [3H]dexamethasone to glucocorticoid receptors and stabilization of hormone-receptor complexes from the liver (A) and kidney (B) of 15- and 120-day old mice. Cytosol preparation, hormone binding and subsequent processing of the samples were performed as indicated in fig. 1. Each data point represents mean ± standard deviation of 4 experiments performed each time in duplicate with 4-5 mice of each age group. *Statistically significant (P<0.05) compared to 15-day control.
Figure 4. Effect of 2-mercaptoethanol (ME) on the binding of [\( ^3 \text{H} \)]dexamethasone to glucocorticoid receptors and stabilization of hormone-receptor complexes from the liver (A) and kidney (B) of 15- and 120-day old mice. Cytosol preparation, hormone binding and subsequent processing of the samples were performed as indicated in fig 1. Each data point represents mean ± standard deviation of 4 experiments performed each time in duplicate with 4-5 mice of each age group.
Figure 5. Effect of glutathione (GSH) on the binding of [³H]dexamethasone to glucocorticoid receptors and stabilization of hormone-receptor complexes from the liver (A) and kidney (B) of 15- and 120-day old mice. Cytosol preparation, hormone binding and subsequent processing of the samples were performed as indicated in fig. 1. Each data point represents mean ± standard deviation of 4 experiments performed each time in duplicate with 4-5 mice of each age group.
presence of DTT, there were no apparent differences in the maximal specific binding and stabilization of liver (Fig. 3A) and kidney (Fig. 3B) H-R complexes of immature and mature animals. Similarly, both mercaptoethanol (Fig. 4A&B) and glutathione (Fig. 5A&B) also showed no alteration in specific binding of hormone to the receptor and the stabilization of H-R complexes from the liver and kidney of immature mice and mature mice. These studies therefore indicate no age-specific differences in hormone binding to GR and the stability of H-R complexes from both the tissues of immature and mature animals by these modulators.

**Activation modulation of GR**

Activation of GR was studied using heat and salt in the liver and kidney of 15- and 120-day old mice. DNA-cellulose and purified nuclei were utilized as acceptors of activated GR binding. Also, the modulation of hepatic GR activation process was studied in 120-day old mice using various endogenous and exogenous agents.

DNA-cellulose binding assays reveal that heat (25°C) significantly enhanced the activation of hormone-receptor (H-R) complexes from the liver (2.5-3.5 fold) (Fig. 6A) and kidney (Fig. 6B) (2.5 fold) of mice. Salt (20 mM Ca²⁺) also enhanced the activation of H-R complexes at 0°C in the liver (Fig. 6A) (~3.3 fold) and kidney (Fig. 6B) (~3 fold). Since DNA-cellulose being a non-specific assay system, purified nuclei from both the tissues were utilized to mimic an in vivo assay system. Nuclear binding assay also exhibited increased activation of liver (Fig. 7A) and kidney (Fig. 7B) H-R complexes by heat and salt.

Experiments were also carried out in order to reveal any age-specific alteration in heat and salt activation of GR in the liver and kidney of immature (15-) and mature (120-day) mice. DNA-cellulose binding assay in liver (Fig. 6A) shows a higher magnitude (37%) of GR heat activation from mature animal as compared to immature ones, with no such differences in salt activation. Neither heat nor salt could reveal any age-related changes in GR activation in the kidney (Fig. 6B) of immature and mature mice. Nuclear binding assays also indicated a much higher (75%) degree of GR heat activation from mature mice’s liver (Fig. 7A) as compared to immature ones, without any apparent differences in salt activation. In the kidneys, no alterations in the magnitude of GR activation by heat as well as salt were observed in immature and mature animals (Fig. 7B).

Hence, both DNA-cellulose and nuclear binding assays revealed a significant increase in heat and salt activation of GR from the liver and kidney of mice albeit, with a higher magnitude of heat activation from mature animal’s liver compared to immature ones.

Cross-mixing experiments (i.e., binding of heat-activated H-R complexes from immature to nuclei of mature and vice-versa) were also carried out in the liver to reveal whether the observed increase in nuclear binding of heat-activated GR in mature mice’s liver is due to
Figure 6  Binding of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes to DNA-cellulose in 15- and 120- day old mice Cytosols from these tissues were prepared in buffer B (1) and the hormone-receptor complexes obtained by incubating with 40 nM [³H]dexamethasone for 4 hr at 0°C Activation conditions, DNA-cellulose binding and further processing of the pellets were performed as described in materials and methods The results are mean ± standard deviation of 4 experiments with 4-5 mice of each age group *Statistically significant (P<0.05) compared to 15- day old mice
Figure 7 Binding of hepatic (A) and kidney (B) [\(^3\)H]dexamethasone-receptor complexes to purified nuclei in 15- and 120- day old mice. Purified nuclei of these tissues were used instead of DNA-cellulose for activation studies. Other experimental procedures are same as in figure 6. The (a) and (b) barograms in (A) represent cross-mixing experiments in which heat-activated hepatic GR complexes from 15-day old mice were incubated with nuclei of 120-day old (a) and heat activated GR complexes from 120-day mice with the nuclei of 15-day (b). The results are mean ± standard deviation of 4 experiments performed with 4-5 mice of each age group. Statistically significant (P<0.05) compared to 15-day old mice.
alteration(s) in the nuclear property. Result shows an increased nuclear binding of mature GR to the nuclei of immature animal (Fig. 7A). Hence, result obtained demonstrates that the higher magnitude of heat activation of mature mice's hepatic GR is due to alteration(s) in receptor property at this phase of life span.

Next, the activation modulation of GRs by utilizing various exogenous and endogenous agents was studied to see their modulatory effects in the liver of 120-day old mice. To examine the effect of cadmium (Cd\(^{2+}\)) on receptor activation by heat, a concentration of 0-4 mM Cd\(^{2+}\) was used. Result exhibited a dose-dependent inhibition (maximally 60% at 2 mM) of heat activation of H-R complexes from the liver of mice as assessed by binding to DNA-cellulose (Fig. 8A) and purified nuclei (Fig. 8B). Selenite (SeO\(_3^2\)), a strong oxidant and a modifier of protein thiol groups, was used to reveal any modulatory effect on heat activation of hepatic GR. Results indicate that selenite when used at a concentration range from 0-8 mM, also inhibited the heat activation of hepatic GR, as assessed by binding to DNA-cellulose (Fig. 9A) and purified nuclei (Fig. 9B), to a magnitude of maximally 50% at 4 mM. Arsenite (AsO\(_2^-\)) was also utilized to see any modulatory effect on hepatic GR activation, as it was known that arsenite is a potent modifier of thiol groups in GR as well as in other proteins and enzymes. Arsenite (0-8 mM) caused a maximal inhibition (40%) of hepatic GR heat activation at 4 mM by DNA-cellulose (Fig. 10A) and nuclear binding assays (Fig. 10B).

Leupeptin, a bacterial peptide, is a potent inhibitor of ser/cys proteases and also a potent stabilizer of untransformed (non-DNA binding form) GR in cytosols. The effect of leupeptin on heat activation of GR was tested when used at a concentration range from 0-40 mM. Data revealed a significant inhibition of heat activation of hepatic GR by leupeptin, maximally (45-50%) at 20 mM by DNA-cellulose (Fig. 11A) and nuclear binding assays (Fig. 11B).

Polyunsaturated fatty acids (PUFAs) have also been used to study their modulatory role on in vitro receptor activation modulation. PUFAs such as oleic acid (C18:1), linoleic acid (C18:2) and arachidonic acid (C20:4) at a concentration range from 0-200 \(\mu\)M were used to study their modulatory effects on receptor activation in the liver of 120-day old mice. Interestingly, all the three PUFAs were found to be potential inhibitors of heat activation of hepatic GR. Oleic acid (C18:1) caused 38% maximal inhibition of heat activation at 40 \(\mu\)M as assessed by DNA-cellulose (Fig. 12A) and nuclear (Fig. 12B) binding assays. Linoleic acid (C18:2) also inhibited the heat activation of hepatic GR, albeit to a greater magnitude (~70% at 160 \(\mu\)M) compared to oleic acid (Fig. 13 A&B). Arachidonic acid (20:4) was also found to be a potential inhibitor of hepatic GR heat activation by both DNA-cellulose (Fig. 14A) and purified nuclei (Fig. 14B) binding assays, achieving a maximal inhibition of ~70% at 160 \(\mu\)M, similar to linoleic acid.
Figure 8. Effect of cadmium (Cd²⁺) on the heat (25°C) activation of hepatic [³H]dexamethasone-receptor complexes, as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [³H]dexamethasone for 4 hr at 0°C 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.
Figure 9. Effect of selenite ($\text{SeO}_3^{2-}$) on the heat (25°C) activation of hepatic $[^3\text{H}]\text{dexamethasone}$-receptor complexes, as assessed by binding to DNA-cellulose (A) and purified nuclei (B). Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM $[^3\text{H}]\text{dexamethasone}$ for 4 hr at 0°C to generate $[^3\text{H}]\text{dexamethasone}$-receptor complexes. Aliquots of cytosol containing $[^3\text{H}]\text{dexamethasone}$-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of selenite. DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Heat activation in the absence (control) of selenite is taken as 0% inhibition.
Figure 10. Effect of arsenite (AsO$_2^{-}$) on the heat activation of hepatic [³H]dexamethasone-receptor complexes, as assessed by binding to DNA-cellulose (A) and purified nuclei (B). Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [³H]dexamethasone for 4 hr at 0°C 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 arsenite. DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Heat activation in the absence (control) of arsenite is taken as 0% inhibition.
Figure 11. Effect of leupeptin on the heat activation of hepatic [³H]dexamethasone-receptor complexes, as assessed by binding to DNA-cellulose (A) and purified nuclei (B). Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [³H]dexamethasone for 4 hr at 0°C 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 leupeptin. DNA-cellulose, nuclear binding and further processing were performed as indicated in the materials and methods section. Heat activation in the absence (control) of leupeptin is taken as 0% inhibition.
Figure 12. Effect of oleic acid (C18:1) on the heat activation of hepatic [³H]dexamethasone-receptor complexes, as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosol from liver was prepared in buffer B (I) and incubated with 40 nM [³H]dexamethasone for 4 hr at 0°C 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 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.
Figure 13. Effect of linoleic acid (C18:2) on the heat activation of hepatic [³H]dexamethasone-receptor complexes, as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM [³H]dexamethasone for 4 hr at 0°C 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 linoleic 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 linoleic acid. Heat activation in the absence (control) of linoleic acid is taken as 0% inhibition.
Figure 14. Effect of arachidonic acid (C20:4) on the heat activation of hepatic $[^3H]$dexamethasone-receptor complexes, as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM $[^3H]$dexamethasone for 4 hr at 0°C 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 arachidonic 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 arachidonic acid. Heat activation in the absence (control) of arachidonic acid is taken as 0% inhibition.
Results indicate an increase in the magnitude of inhibition of hepatic GR activation concomitant to increase in chain length and unsaturation of PUFAs to a certain limit.

Pyrophosphates (PPI's) have been attributed to play important physiological and biochemical roles in animals. In this study, we have used tetrastodium pyrophosphate (Na₄P₂O₇) at a concentration range from 0-16 mM to see its effect on hepatic GR activation of 120-day old mice. Na₄P₂O₇ turned out to be an inducer of hepatic GR activation at 0°C, with maximum activation of ~65% at 8 mM, using both DNA-cellulose (Fig. 15A) and nuclear (Fig. 15B) binding assays.

All the above modulators of activation were also used to study the modulation of heat activation of liver and kidney GR from immature (15-) and mature (120- day) mice to see any tissue- and/or age-specific modulation. Cadmium was used at increasing concentration (0-4 mM) to see its inhibitory effect on GR heat activation in the liver and kidney of immature and mature mice. DNA-cellulose binding assay shows similar pattern of inhibition (~60% at 2 mM) of hepatic (Fig. 16A) and kidney (Fig. 16B) GR heat activation by cadmium in both the ages studied. Nuclear binding assay also revealed a similar pattern of inhibitory effect by cadmium in both the ages in the liver (Fig. 17A) and kidney (Fig. 17B) of mice. Selenite (0-8 mM), caused a maximal inhibition (~50% at 4 mM) of GR heat activation, but could not show any difference in % inhibition from the liver (Fig. 18A) and kidney (Fig. 18B) of immature and mature mice by DNA-cellulose binding assay, with similar result in the case of nuclear binding assay (Fig. 19A&B).

Arsenite (0-8 mM) also inhibited GR heat activation maximally 40% at 4 mM in both the tissues and ages albeit, to a similar extent as assessed by DNA-cellulose (Fig. 20 A&B) and nuclear (Fig. 21 A&B) binding assays. Leupeptin (0-40 mM) was also used to reveal any differences in GR heat activation inhibition in both the tissues and ages. However, both DNA-cellulose (Fig. 22 A&B) and nuclear (Fig. 23 A&B) binding assays exhibited similar extent of inhibition (~45-50% at 20 mM) in both the tissues and ages.

PUFAs (oleic, linoleic and arachidonic acid, 0-200 μM) were also employed to show any differences in tissue- and age- specific inhibition of heat activation. Oleic acid caused similar extent of inhibition (~38% at 40 μM) of heat activation of hepatic and renal GR in both the ages as observed by DNA-cellulose binding assay (Fig. 24 A&B), with a similar extent using in nuclear (Fig. 25 A&B) binding assay. Linoleic acid exhibited ~70% inhibition at 160 μM of hepatic and renal GR heat activation, albeit to a similar degree in both the ages studied using DNA-cellulose (Fig. 26 A&B) and nuclear (Fig. 27 A&B) binding assays. Inhibition of heat activation of GR by arachidonic acid also showed a similar pattern (~70% at 160 μM) in both
Figure 15. Effect of tetrasodium pyrophosphate (Na$_4$P$_2$O$_7$) on the activation of hepatic $[^3H]$dexamethasone-receptor complexes of mice using DNA-cellulose (A) and nuclear (B) binding assays. Cytosol from liver was prepared and incubated with 40 nM $[^3H]$dexamethasone for 4 hr at 0°C. Hormone-receptor (H-R) complexes were then incubated with increasing concentrations of pyrophosphate [prepared as 0.1-1 M stock in buffer B (l)] for 45 min at 0°C, control tubes received buffer B (l) only. Additionally, aliquots of H-R complexes were also incubated at 0°C and 25°C separately for 45 min, to yield unactivated and heat activated receptor complexes, respectively. The magnitude of activation was then assessed by allowing the H-R complexes to bind to DNA-cellulose and purified nuclear pellets for 60 min at 0°C. Further processing of the pellets was done as described in materials and methods in the text. Results are mean ± standard deviation of 4 separate experiments with 4-5 mice. *Significant (P<0.01) heat activation compared to 0°C. #Significant activation (P<0.05) compared to control.
Figure 16. Effect of cadmium (Cd²⁺) on the heat activation of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes from 15- and 120-day old mice by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig.8.
Figure 17. Effect of cadmium (Cd$^{2+}$) on the heat activation of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes from 15- and 120-day old mice by nuclear binding assay. Experimental procedures and assay conditions are similar as given in fig. 8.
Figure 18. Effect of selenite on the heat activation of hepatic (A) and kidney (B) [3H]dexamethasone-receptor complexes from 15- and 120-day old mice by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 9.
Figure 19. Effect of selenite on the heat activation of hepatic (A) and kidney (B) [3H]dexamethasone-receptor complexes from 15- and 120-day old mice by nuclear binding assay. Details of experimental procedures and assay conditions are as given in fig. 9.
Figure 20  Effect of arsenite on the heat activation of hepatic (A) and kidney (B) $[^3]$H]dexamethasone-receptor complexes from 15- and 120-day old mice by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 10.
Figure 21. Effect of arsenite on the heat activation of hepatic (A) and kidney (B) [\(^3\)H]dexamethasone-receptor complexes from 15- and 120-day old mice by nuclear binding assay. Receptor preparation and activation procedures are same as described in fig. 10.
Figure 22. Effect of leupeptin on the heat activation of hepatic (A) and kidney (B) [3H]dexamethasone-receptor complexes from 15- and 120-day old mice by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 11.
Figure 23. Effect of leupeptin on the heat activation of hepatic (A) and kidney (B) $[^3H]dexamethasone$-receptor complexes from 15- and 120-day old mice by nuclear binding assay. Receptor preparation and activation conditions are similar as given in fig. 11.
Figure 24 Effect of oleic acid (C18:1) on the heat activation of hepatic (A) and kidney (B) [3H]dexamethasone-receptor complexes from 15- and 120-day old mice by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig.12.
Figure 25. Effect of oleic acid (C18:1) on the heat activation of hepatic (A) and kidney (B) [$^3$H]dexamethasone-receptor complexes from 15- and 120-day old mice by nuclear binding assay. The details of procedures and experimental assay conditions are similar to fig. 12.
Figure 26. Effect of linoleic acid (C18:2) on the heat activation of hepatic (A) and kidney (B) \(^{[3}H\)dexamethasone-receptor complexes from 15- and 120-day old mice by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig 13.
Figure 27. Effect of linoleic acid (C18:2) on the heat activation of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes from 15- and 120-day old mice by nuclear binding assay. Experimental procedures are same as detailed in fig. 13.
Figure 28. Effect of arachidonic acid (C20:4) on the heat activation of hepatic (A) and kidney (B) $[^3]$Hdexamethasone-receptor complexes from 15- and 120-day old mice by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 14.
Figure 29. Effect of arachidonic acid (C20:4) on the heat activation of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes from 15- and 120-day old mice by nuclear binding assay. Details of experimental procedures are as given in fig. 14.
Figure 30 Effect of tetrasodium pyrophosphate (Na₄P₂O₇) on the activation of hepatic (A) and kidney (B) 
[³H]dexamethasone-receptor complexes of 15- and 120-day old mice by using DNA-cellulose assay
Cytosol preparation, activation conditions, binding to DNA-cellulose pellets and further processing of 
pellets are similar to those indicated in fig 15 Results are mean ± standard deviation of 4 separate 
experiments with 4-5 mice of each age group *Significant (P<0.01) heat activation compared to 0°C
#Significant activation (P<0.05) compared to respective control
Figure 31. Effect of tetrasodium pyrophosphate (Na₄P₂O₇) on the activation of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes of 15- and 120-day old mice by using purified nuclei binding assay. Cytosol preparation, activation conditions, binding to nuclear pellets and further processing of pellets are similar to those indicated in fig. 15. Results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Significant (P<0.01) heat activation compared to 0°C. #@Significant activation (P<0.05) compared to respective control.
the ages and tissues of mice assessed by DNA-cellulose (Fig 28 A&B) and nuclear (Fig 29 A&B) binding assays

Tetrasodium pyrophosphate was turned out to be an inducer of GR activation (65% at 8 mM) at 0°C from both the tissues and ages of mice, but without any apparent change in percent activation in terms of tissue- and age- specificity as assessed by binding to DNA-cellulose (Fig 30 A&B) and purified nuclei (Fig 31 A&B)

Taken together, results exhibit no significant change in percent modulation of heat activation by these modulators in a tissue- and age- specific manner. This indicates that probably the mechanism(s) of activation modulation does not get altered in both the tissues at these ages of mice

**Acceptor binding modulation of GR:**

DNA binding of activated receptor can be modulated by a number of endogenous and exogenous agents. In our study, acceptor (DNA) binding modulation of activated GR was carried out by using PLP, MMTS and ATA in the liver of 120-day old mice to observe their modulatory effect, if any. Pyridoxal 5-phosphate (PLP) is an active coenzyme form of vitamin B₆ and a potent modifier of lysine residues in proteins. It is known that the DNA binding domain (DBD) of GR contain many lysine residues that play an important role in DNA binding. Hence, it was decided to use PLP for its modulatory effect on DNA binding by activated GR. PLP when used at a concentration from 0-10 mM, maximally inhibited (98%) DNA binding of activated hepatic GR at 4 mM, showing an IC₅₀ of 0.75 mM, by both DNA-cellulose (Fig 32A) and nuclear (Fig 32B) binding assays

Methyl methanethiosulfonate (MMTS) is a synthetic, strong modifier of thiol groups in proteins. Hence, MMTS was used to study its modulatory effect on activated hepatic GR binding to acceptor at a concentration range from 0-8 mM. MMTS was also found to be a potent inhibitor of activated hepatic GR binding to DNA, causing highest inhibition (85%) of receptor binding as assessed by DNA-cellulose (Fig 33A) and nuclear (Fig 33B) binding assays at a concentration of 6 mM, with an IC₅₀ of 1.2 mM

Auntricarboxylic acid (ATA), a synthetic triphenylmethane dye, was also a very potent inhibitor of acceptor binding. ATA when used at 0-0.7 mM concentration caused a maximal inhibition (95%) of activated hepatic GR binding to both DNA-cellulose (Fig 34A) and nuclei (Fig 34B) at 0.2 mM, with an IC₅₀ of 0.05 mM
**Figure 32.** Effect of pyridoxal 5-phosphate (PLP) on the binding of heat (25°C)-activated hepatic [\(^3\text{H}\)]dexamethasone-receptor complexes to DNA-cellulose (A) and purified nuclei (B). Cytosol from the liver was prepared in buffer B (i) and incubated with 40 nM [\(^3\text{H}\)]dexamethasone for 4 hr at 0°C to generate [\(^3\text{H}\)]dexamethasone-receptor complexes. Aliquots of cytosol containing [\(^3\text{H}\)]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of PLP for 30 min at 0°C. Control and PLP incubated [\(^3\text{H}\)]dexamethasone-receptor complexes were then allowed to bind to DNA-cellulose and purified nuclei at 0°C for 60 min. Further processing was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition.
Figure 33. Effect of methyl methanethiosulfonate (MMTS) on the binding of heat (25°C)-activated hepatic [³H]dexamethasone-receptor complexes to DNA-cellulose (A) and purified nuclei (B). Cytosol from liver was prepared in buffer B (I) and incubated with 40 nM [³H]dexamethasone for 4 hr at 0°C 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. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of MMTS for 30 min at 0°C. Control and MMTS incubated [³H]dexamethasone-receptor complexes were then allowed to bind to DNA-cellulose and purified nuclei at 0°C for 60 min. Further processing of pellets was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition.
Figure 34. Effect of aurintricarboxylic acid (ATA) on the binding of heat (25°C)-activated hepatic $[^3H]$dexamethasone-receptor complexes to DNA-cellulose (A) and purified nuclei (B). Cytosol from liver was prepared in buffer B (i) and incubated with 40 nM $[^3H]$dexamethasone for 4 hr at 0°C 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. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of ATA for 30 min at 0°C. Control and ATA incubated $[^3H]$dexamethasone-receptor complexes were then allowed to bind to DNA-cellulose and purified nuclei at 0°C for 60 min. Further processing was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition.
Acceptor binding modulation study was also performed in the liver and kidney of immature (15-) and mature (120-day old) mice to reveal any tissue- and/or age- specific modulation. PLP was employed (0-10 mM) as an inhibitor of acceptor binding of heat-activated GR from both the tissues and ages. There was a significant inhibition (98% at 4 mM) of acceptor binding of GR in the liver and kidney in the presence of PLP, however, it could not exhibit any significant difference in acceptor binding inhibition by activated GR in terms of tissue- and age-specificity by DNA-cellulose (Fig. 35A&B) and nuclear (Fig. 36 A&B) binding assays. MMTS (0-8 mM) was also a significant inhibitor (85% at 6 mM) of acceptor binding by GR in both the tissues, but result indicates no apparent differences in per cent inhibition at either of the age and tissue by DNA-cellulose (Fig. 37 A&B) and nuclear (Fig. 38 A&B) binding assays. ATA (0-0.7 mM) also turned out to be a potent inhibitor (95% at 0.2 mM) of acceptor binding by GR in the liver and kidney. It also does not reveal any significant change in per cent inhibition at such ages and tissues studied, as assessed by binding to DNA-cellulose (Fig. 39 A&B) and purified nuclei (40 A&B).

It seems, none of these agents showed any age- and tissue- specific response in modulating the acceptor binding of heat-activated GR complexes, thereby indicating that the mechanism(s) of inhibition modulation of activated receptor binding to DNA do(es) not get altered during these phases of animal's life span.

**Diabetes and GR modulation:**

Diabetes was experimentally induced in mice (15- and 120- day old) by injecting intraperitoneally a single dose (20 mg/100 g body weight) of streptozotocin (STZ). Blood glucose level was determined at regular intervals, and on day 7 of STZ treatment, animals were sacrificed. Thereafter, STZ-induced diabetic effects were analyzed for GR level, affinity, and for *in vitro* activation (by heat and salt) in the liver and kidney of immature and mature animals.

In this study, blood glucose levels (mg/dl) of STZ-treated animals were elevated by approximately 3.5 fold to that of controls and hence, ensured that animals had responded to STZ and were diabetic (Fig. 41). The diabetogenic effect of STZ in inducing blood glucose level was similar at immature and mature ages of mice.

Scatchard plot analyses of the data obtained indicates a reduced (22-33%) level of GR in the liver (Fig. 42A) and kidney (Fig. 42B) of mature (120-day) mice as compared to immature (15-day) in control groups (Table I). However, slopes of the plots exhibit no alteration in the affinity (Kd) of GR for its cognate hormone at these two ages. STZ-induced diabetes (Fig. 43A&B) had no effects on the level and affinity of GR in either of the tissues and ages of mice studied (Table I). Studies on the activation process of the GR complexes at two ages in the liver and kidney of control and diabetic mice using DNA-cellulose (Fig. 44A&B) and nuclear (Fig.
Figure 3.5 Effect of PLP on the binding of heat-activated $[^{3}H]dexamethasone$-receptor complexes from liver (A) and kidney (B) of 15- and 120-day old mice to DNA-cellulose. Cytosol from liver and kidney of respective ages were prepared in buffer B (i) and incubated with 40 nM $[^{3}H]dexamethasone$ for 4 hr at 0°C 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. Activated complexes were then incubated in the presence or absence (control) of varying concentrations of PLP for 30 min at 0°C. Control and PLP incubated $[^{3}H]dexamethasone$-receptor complexes were then allowed to bind to DNA-cellulose at 0°C for 60 min. Further processing was done as described in the materials and methods section. Results are expressed as per cent (%) inhibition for each age group.
Figure 36. Effect of PLP on the binding of heat-activated [3H]dexamethasone-receptor complexes from liver (A) and kidney (B) of 15- and 120-day old mice to purified nuclei. Cytosol preparation, heat activation, treatment with PLP, binding to purified nuclei and further processing were performed as described in fig. 32. Results are expressed as per cent (%) inhibition.
Figure 37. Effect of methyl methanethiosulfonate (MMTS) on the binding of heat-activated \[^{3}H\]dexamethasone-receptor complexes from liver (A) and kidney (B) of 15- and 120-day old mice to DNA-cellulose. Cytosol preparation, heat activation, treatment with MMTS, binding to DNA-cellulose pellet and further processing were performed as described in fig. 33. Results are expressed as per cent (%) inhibition for each age group.
Figure 38. Effect of methyl methanethiosulfonate (MMTS) on the binding of heat-activated [³H]dexamethasone-receptor complexes from liver (A) and kidney (B) of 15- and 120-day old mice to purified nuclei. Cytosol preparation, heat activation, treatment with MMTS, binding to purified nuclear pellet and further processing were performed as described in fig 33. Results are expressed as per cent (%) inhibition for each age group.
Figure 39. Effect of aurintricarboxylic acid (ATA) on the binding of heat-activated [³H]dexamethasone-receptor complexes from liver (A) and kidney (B) of 15- and 120-day old mice to DNA-cellulose. Cytosol preparation, heat activation, treatment with ATA, binding to DNA-cellulose pellet and further processing were performed as described in fig. 34. Results are expressed as per cent (%) inhibition for each age group.
Figure 40. Effect of aurintricarboxylic acid (ATA) on the binding of heat-activated \(^{3}H\)dexamethasone-receptor complexes from liver (A) and kidney (B) of 15- and 120- day old mice to purified nuclei. Cytosol preparation, heat activation, treatment with ATA, binding to nuclear pellet and further processing were performed as described in fig. 34. Results are expressed as per cent (%) inhibition for each age group.
Figure 41. Blood glucose level in streptozotocin-treated diabetic and control mice of 15- and 120-day old. Values are mean of 4-5 mice of each age group. Bars represent standard deviation. *Statistically significant (P<0.001) as compared to control.
Figure 42 Scatchard plot of the $[^3]$H[dexamethasone binding studies from the liver (A) and kidney (B) of 15- and 120- day old control mice. Cytosols from these tissues were incubated with 1-120 nM $[^3]$H[dexamethasone ± 500-fold excess of nonradioactive dexamethasone for 4 hr at 0°C. The specific binding at each concentration was obtained by subtracting nonspecific binding from total binding and the data obtained was analyzed according to the method of Scatchard. The slope of the curve gave the dissociation constant ($K_d$), while the intercept on the X-axis gave the maximal number of specific binding sites. Each data point represents the mean of 4 separate experiments with 4-5 mice of each age group.
Figure 43. Scatchard plot of the $[^3]H$[dexamethasone binding studies from the liver (A) and kidney (B) of 15- and 120- day old diabetic mice. Experimental procedure, Scatchard analysis of the data were carried out as indicated for fig. 42. Each data point represents the mean of 4 separate experiments with 4-5 mice of each age group.
Table I.

Concentration and affinity of [3H]dexamethasone-receptors in the liver and kidney of immature (15-) and mature (120-day) control and diabetic mice *

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Tissues</th>
<th>Age (Days)</th>
<th>B_max (fmol/mg protein)</th>
<th>K_d (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Liver</td>
<td>15</td>
<td>192.00±7.10</td>
<td>3.10±0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>148.80±6.63</td>
<td>2.78±0.30</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>15</td>
<td>144.00±5.93</td>
<td>3.80±0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>97.00±4.89</td>
<td>3.70±0.33</td>
</tr>
<tr>
<td>Diabetic NS</td>
<td>Liver</td>
<td>15</td>
<td>189.70±6.79</td>
<td>3.20±0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>147.30±5.69</td>
<td>2.78±0.30</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>15</td>
<td>141.00±6.82</td>
<td>3.70±0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>95.10±5.08</td>
<td>3.80±0.35</td>
</tr>
</tbody>
</table>

* The data were collected from 4-5 mice of each age group and analyzed using Scatchard plot as given under materials and methods. The results are mean ± standard deviation of four separate experiments for each age group.

Statistically significant (P<0.05) with respect to day 15; NS, not significant as compared to control.
Figure 44. Binding of hepatic (A) and kidney (B) [3H]dexamethasone-receptor complexes to DNA-cellulose in 15- and 120-day old control and diabetic mice. Cytosols from these tissues were prepared in buffer B (i) and the hormone-receptor complexes obtained by incubating with 40 nM [3H]dexamethasone for 4 hr at 0°C. Activation procedure, DNA-cellulose binding and further processing of the pellets were performed as described in materials and methods section in the text. The results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant (P<0.05) compared to control. #Significantly (P<0.05) higher heat activation as compared to 15-day control.
Figure 45. Binding of hepatic (A) and kidney (B) [³H]dexamethasone-receptor complexes to purified nuclear pellets in 15- and 120-day old control and diabetic mice. Purified nuclei of these tissues were used instead of DNA-cellulose for activation studies. Other experimental procedures are same as for fig.44. The (a) and (b) barograms in (A), represent cross-mixing experiments in which heat-activated GR complexes from 120-day old diabetic mice were incubated with the nuclei of 120-day old control (a) and heat-activated GR complexes from 120-day old control mice with the nuclei of 120-day diabetic mice (b). The results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant (P< 0.05) compared to control. #Significantly (P< 0.05) higher heat activation as compared to 15-day control. $Statistically significant (P< 0.05) compared to (b).
binding assays indicate that both temperature and salt significantly increase the DNA-cellulose binding of the H-R complexes in both the ages and tissues in diabetic and control animals. The GR from both the tissues underwent heat activation, albeit the extent of activation was more pronounced in mature liver compared to immature (Fig. 44A), with no such difference of activation in the kidney (Fig. 44B). In diabetic mice, the heat activation of hepatic GR exhibits reduced (~20-23%) DNA-cellulose binding (Fig. 44A) compared to control. In contrast, thermal activation of kidney GR does not show marked difference in diabetic mice at either of the ages studied (Fig. 44B). Salt-dependent activation of GR complexes does not exhibit any change in either of tissues and ages of mice (Fig. 44A&B). Nuclear binding of heat-activated hepatic GR complexes is also significantly reduced (24-39%) in diabetic mice compared to control. However, the nuclear binding of heat-activated H-R complexes from kidney (fig. 45B) does not show marked difference in diabetic mice at either of the ages. Data from cross-mixing experiments (heat-activated GR of diabetic and nuclei of control and vice-versa) performed only on mature mice's liver showed significantly decreased (25%) nuclear binding (Fig. 45A) by diabetic GR, thus indicating receptor specificity.

**Aging and GR modulation:**

The regulation of GRs in young (4-) and old (120-week) mice was studied to investigate possible changes in GR level, affinity, activation (by heat), activation modulation by PUFAs and nuclear-bound GR extraction by DNase I in the liver and kidney.

Scatchard plot (Fig. 46A&B) analyses of the data indicates a decreased level of GR in the liver (25%) and kidney (33%) of old mice compared to young ones. However, slopes of the plots exhibit no alteration in the affinity ($K_d$) of GR for its ligand at these two different ages (Table II).

Pilot experiments performed reveal that heat (25°C for 45 min) significantly enhanced the activation of H-R complexes from liver (2-2.5 fold) (Fig. 47A & 48A) and kidney (1.5-2.5 fold) (47B & 48B) in both the ages, albeit the magnitude of activation was higher (24-29%) in young hepatic and renal GR with respect to old. Our earlier experiments reveal that PUFAs (linoleic and arachidonic acid) inhibit the heat activation of H-R complexes in a dose-dependent manner. Both were most effective at a concentration of 160 μM, exhibiting 40-75% maximal inhibition of receptor activation. Linoleic acid caused significant magnitude of inhibition in the liver (64%) (Fig. 47A) and kidney (68%) (Fig. 47B) of young mice as compared to old (41% and 43%, respectively). Arachidonic acid also showed a similar extent of activation inhibition of hepatic GR (Fig. 47A) in young (74%) compared to old (50%) animals. In kidney (Fig. 47B), however, arachidonic acid inhibited the heat activation of GR, albeit to a similar magnitude (~57%) in both the ages. Thus, arachidonic acid showed its age-specific inhibitory effect only in
Figure 46. Scatchard plot of the $[^3H]$dexamethasone binding studies from the liver (A) and kidney (B) of young (4-) and old (120-weeks) mice. Cytosols from these tissues were incubated with 1-120 nM $[^3H]$dexamethasone ± 500-fold excess of nonradioactive dexamethasone for 4 hr at 0°C. The specific binding at each concentration was obtained by subtracting nonspecific binding from total binding and the data obtained was analyzed according to the method of Scatchard. The slope of the curve gave the dissociation constant ($K_d$), while the intercept on the X-axis gave the maximal number of specific binding sites. Each data point represents the mean of 4 separate experiments with 4-5 mice of each age group.
Table II.
Specific binding sites (B_{max}) and dissociation constant (K_d) of glucocorticoid receptors in the liver and kidney of young (4-) and old (120-week) mice \(^a\)

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Age (Weeks)</th>
<th>B_{max} (fmol/mg protein)</th>
<th>K_d (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4</td>
<td>195±20.00</td>
<td>3.40±0.27</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>146±13.70 (^1)</td>
<td>3.68±0.24</td>
</tr>
<tr>
<td>Kidney</td>
<td>4</td>
<td>143±15.10</td>
<td>3.17±0.39</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>96±8.73 (^1)</td>
<td>3.38±0.33</td>
</tr>
</tbody>
</table>

\(^a\) Data were obtained from 4-5 mice of each age group and analyzed using Scatchard plot as given in materials and methods section. The results are mean ± standard deviation of 4 separate experiments for each age group. \(^1\)Statistically significant (P< 0.001) with respect to 4-week (young) mice.
Figure 47. Binding of hepatic (A) and kidney (B) [3H]dexamethasone-receptor complexes to DNA-cellulose in young (4-) and old (120-weeks) mice. Cytosols from these tissues were prepared in buffer B (i) and the hormone-receptor complexes obtained by incubating with 40 nM [3H]dexamethasone for 4 hr at 0°C. The hormone-receptor complexes were then subjected to heat (25°C) activation for 45 min alone or in presence of dimethyl sulfoxide (DMSO) as control, linoleic (C18:2) and arachidonic (C20:4) acids, which were added to a final concentration of 160 μM. DNA-cellulose binding and further processing of the pellets were performed as described in materials and methods. The results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant (P<0.01) as compared to young mice. #, $Statistically significant (P<0.01) to control for their respective ages. **Significantly higher binding compared to young mice.
Figure 48. Binding of hepatic (A) and kidney (B) [\(^{3}H\)]dexamethasone-receptor complexes to purified nuclei in young (4-) and old (120-weeks) mice. Purified nuclei from respective tissues were utilized instead of DNA-cellulose for activation-inhibition studies. Other experimental procedures are same as mentioned for fig. 47. Results are mean ± standard deviation of 4 separate experiments with 4-5 mice of each age group. *Statistically significant (P<0.01) compared to young mice. #, $ Statistically significant (P<0.01) to control for their respective ages. @Significantly (P< 0.05) higher binding as compared to young mice.
Figure 49. DNase I digestion-extraction of hepatic (A) and kidney (B) nuclear-bound [\(^{3}H\)]dexamethasone-receptor complexes of young (4-) and old (120-weeks) mice. Heat-activated, nuclear-bound hormone-receptor complexes from both the tissues were extracted using DNase I (prepared in buffer C) at a concentration of 100U/100 µg DNA for 45 min at 2-4°C. Experimental protocols are explained under materials and methods. The results are mean ± standard deviation of 4 separate experiments performed with 4-5 mice of each age group. *Statistically significant (P<0.001) compared to old mice.
the liver of mice. Since DNA-cellulose being a non-specific assay system, could not unequivocally implicate differences in the inhibitory effects of PUFAs on acceptor binding by activated H-R complexes. Hence, the purified nuclei from both the tissues of respective ages were used to provide a more relevant physiological assay system. Nuclear binding assay results also show linoleic acid as being equally effective in causing inhibition of GR heat activation, with greater magnitude of inhibition in young liver (66%) (Fig. 48A) and kidney (65%) (Fig. 48B) as compared to old age tissues (42% and 40%, respectively). Again, arachidonic acid showed tissue specificity in causing greater inhibition of hepatic GR activation (Fig. 48A) in young (68%) as compared to old (45%). The age-specific difference in arachidonic acid-mediated inhibition of activation was not significant in case of kidney (Fig. 48B), wherein the inhibition was ~57% at both the ages of mice. Hence, both DNA-cellulose and nuclear binding assays revealed a similar pattern of inhibition of heat activation of GR in the liver and kidney by these two PUFAs.

DNase I digestion (Fig. 49) of hepatic and renal nuclei bound with activated GR complexes from young and old mice revealed significant higher extraction of nuclear-bound, heat-activated [3H]dexamethasone-receptor complexes from young liver and kidney with respect to old age tissues. The degree of extraction of the nuclear-bound receptors in the young mice’s liver (Fig. 49A) was higher (59%) as compared to old (33%) ones, when compared to their respective controls, taken as 100%. In kidney too (Fig. 49B), the per cent extraction was higher in young mice (57%) as compared to aged (33%) ones.