RESULTS
3. RESULTS

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(Fig. 13)
3.1 CHARACTERIZATION OF NUCLEAR (NUCLEAR PORE COMPLEX) OXALATE BINDING PROTEIN

3.1.1 Nucleic acid contents of rat and human kidney nuclei

Nuclei were isolated and the purity was checked based on the DNA content. About 66 - 71% of DNA was recovered in the nuclear pellet of rat liver, kidney and human kidney. However nuclear RNA was present about only 5.7 - 8.7% of the total RNA in rat liver, kidney and human kidney respectively (Table 1).

3.1.2 FITC-anti-nuclear-antibody staining of isolated nuclei

Intactness of the isolated nuclei was checked by incubating with FITC-anti-nuclear-antibody. The nuclei showed rim staining showing the exclusion of the anti-nuclear-antibody from entering into the nucleoplasm through the nuclear pore complex. This confirms that the nuclei is intact (Plate 1).

3.1.3 Nuclear oxalate / oxalate bound histone uptake of rat and human kidney

i) Effect of pH on nuclear oxalate uptake

Nuclear oxalate uptake in rat and human kidney was pH-dependent showing two pH optima, one at pH 4.0 and the other at pH 7.4 (Fig.1).
# TABLE 1: NUCLEIC ACID CONTENTS OF RAT AND HUMAN KIDNEY NUCLEI

<table>
<thead>
<tr>
<th>S. No</th>
<th>Particulars</th>
<th>DNA Content</th>
<th>RNA Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homogenate (mg/g tissue)</td>
<td>Homogenate (mg/g tissue)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclear pellet (mg/g tissue)</td>
<td>Nuclear Pellet (mg/g tissue)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclear Pellet (%)</td>
<td>Nuclear Pellet (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Rat Liver</td>
<td>1.71 ± 0.15</td>
<td>2.81 ± 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.21 ± 0.15</td>
<td>0.174 ± 0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>71</td>
<td>6.2</td>
</tr>
<tr>
<td>2.</td>
<td>Rat Kidney</td>
<td>2.02 ± 0.2</td>
<td>1.45 ± 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.37 ± 0.41</td>
<td>0.126 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68</td>
<td>8.7</td>
</tr>
<tr>
<td>3.</td>
<td>Human Kidney</td>
<td>2.42 ± 0.21</td>
<td>1.16 ± 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6 ± 0.15</td>
<td>0.067 ± 0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Values are mean ± S.D for four determinations.

Nuclei were isolated and the nucleic acid contents were determined as described in Materials and Methods.
PLATE 1: FITC-ANTI-NUCLEAR-ANTIBODY STAINING OF ISOLATED NUCLEI

The Isolated Nuclei was incubated with FITC-ANA and stained as described in Materials and Methods.
Values are mean ± SD for 6 determinations.

100 μg of rat / human kidney nuclei were incubated at the indicated pH with 10,000 cpm of $[^{14}C]$ oxalate and the oxalate uptake was assayed as described in Materials and Methods.
ii)  **Effect of time on nuclear oxalate uptake**

Nuclear oxalate uptake of rat and human kidney reached a maximum at about 3 minutes and then attained an equilibrium (Fig.2).

iii)  **Effect of sodium chloride on nuclear oxalate uptake**

Even in the presence of high salt concentration - 150 mM NaCl, the uptake was not significantly inhibited (Fig.3).

iv)  **Effect of DIDS and Nucleotides on nuclear oxalate uptake**

DIDS - 4,4'-dithiocyanostilbene-2,2'-disulfonic acid, an anion transport inhibitor significantly (p< 0.01) inhibited by 50% of the nuclear oxalate uptake while the nucleotides, ATP and ADP significantly (p< 0.05) increased the uptake in both rat and human kidney (Fig.4).

v)  **Effect of DIDS and Nucleotides on nuclear oxalate bound histone uptake**

Nuclear uptake of oxalate bound histone was also significantly (p< 0.01) inhibited by 50% in presence of DIDS and the uptake was significantly increased (p< 0.05) in the presence of ATP and ADP (Fig.5).
Values are mean ± SD for 6 determinations.

100 μg of rat/human kidney nuclei were incubated with 10,000cpm of [14C] oxalate at pH 7.4 for various time and the oxalate uptake was assayed as described in Materials and Methods.
Values are mean ± SD for 6 determinations.

100 µg of rat / human kidney nuclei were incubated with 10,000 cpm of [14C] oxalate with or without 150 mM NaCl for 10 minutes and the oxalate uptake was assayed as described in Materials and Methods.
Values are mean ± SD for 6 determinations.

100 μg of rat / human kidney nuclei were incubated with 10,000cpm of [14C] oxalate-histone with or without DIDS / ATP / ADP (1 mM) for 10 minutes and the oxalate uptake was assayed as described in Materials and Methods. Values are statistically significant when compared to control. (p< 0.05*; p< 0.01**)
Values are mean ± SD for 6 determinations.

100 µg of rat/human kidney nuclei were incubated with 10,000 cpm of [14C] oxalate-histone with or without DIDS / ATP / ADP (1 mM) for 10 minutes and the oxalate uptake was assayed as described in Materials and Methods. Values are statistically significant when compared to control. (p < 0.05*; p < 0.01**)
3.1.4 Nuclear envelope oxalate binding activity

The oxalate binding activity was associated with nuclear membrane. In order to see whether the oxalate binding activity was associated with protein, further studies were undertaken with proteolysis.

i) Effect of trypsin

When the nuclear envelope was incubated with trypsin, a time-dependent decrease in oxalate binding activity was observed. About 50% of oxalate binding activity was lost after 30 minutes of trypsin treatment (Fig.6).

ii) Effect of pH

The nuclear envelope oxalate binding activity showed a pH optima at pH 7.4 for both rat and human kidney (Fig.7).

3.1.5 Distribution of oxalate binding activity in rat and human kidney nuclear fractions

Nuclei isolated by sucrose density gradient method showed enhanced oxalate binding activity of about 32.0 and 38.0 p moles/mg protein for rat and human kidney respectively. Among the subfractions of nucleus, the nuclear envelope showed a maximal binding activity of 75.0 and 36.0 p moles of oxalate bound/mg protein for rat and human kidney respectively. The nuclear pore complex showed a very high oxalate binding activity of 144.0 and 220.0 p moles/mg protein for rat and human kidney respectively. The nuclear lamina showed no oxalate binding activity for both rat and human kidney (Table 2).
Values are mean ± SD for 6 determinations.

100 μg of human kidney nuclei were incubated with 10,000cpm of [14C] oxalate with or without 0.05% trypsin solution and the reaction was arrested at varying time points and the specific activity was determined as described in Materials and Methods.
Values are mean ± SD for 6 determinations.

100 μg of nuclear envelope was incubated at the indicated pH with 10,000 cpm of [14C] oxalate and the oxalate uptake was assayed as described in Materials and Methods.
TABLE 2: DISTRIBUTION OF OXALATE BINDING ACTIVITY IN NUCLEAR FRACTIONS OF RAT AND HUMAN KIDNEY

| Subfractions of Nuclei       | Specific Activity
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p moles of oxalate bound /mg protein</td>
</tr>
<tr>
<td>Rat kidney</td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>32.0 ± 2.7</td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>75.0 ± 7.4</td>
</tr>
<tr>
<td>Nuclear Pore Complex</td>
<td>144.0 ± 17.2</td>
</tr>
<tr>
<td>Nuclear Lamin</td>
<td>No binding</td>
</tr>
<tr>
<td>Human Kidney</td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>38.0 ± 3.2</td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>36.0 ± 3.8</td>
</tr>
<tr>
<td>Nuclear Pore Complex</td>
<td>220.0 ± 25.0</td>
</tr>
<tr>
<td>Nuclear Lamin</td>
<td>No binding</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. for six determinations.

Nuclei were isolated by sucrose density gradient method. Nuclear envelope and Nuclear pore complex were then separated as described in Materials and Methods. Oxalate binding activity was checked by filter binding assay.
3.1.6 Extraction of oxalate bound nuclear pore complex protein

The $^{14}$C-oxalate bound nuclear pore complex protein was extracted from the nuclear envelope as described in Materials and Methods. Only 65% of oxalate binding protein was extracted even at 5% triton X-100. But when the nuclear pore complex was extracted along with high salt - 5% triton X-100 and 0.1 M KCl, about 75% of oxalate binding protein was extracted. However, when 0.3 M KCl was included with 5% triton X-100, complete extraction was possible (Table 3).

3.1.7 Purification of nuclear pore complex oxalate binding protein

The $^{14}$C-oxalate bound nuclear envelope had a specific activity of 135 and 150 p moles of oxalate bound/mg protein for rat and human kidney respectively. The triton-high salt extract of the rat and human kidney nuclear envelope showed a specific activity of 200 and 230 p moles of oxalate bound/mg protein with a fold purity of 1.48 and 1.53 with 44 and 61% yield respectively. When the same was loaded onto DEAE-Sephadex A-50 column, a protein peak with overlapping radioactive peak (F1, fraction nos. 2-4) was eluted with phosphate buffer and two other protein peaks were eluted with the same buffer containing 0.5 M (F2, fraction nos. 12-17) and 1 M NaCl (F3, fraction nos. 23-25) and these proteins showed no radioactivity. On elution with the buffer containing 3.0 M NaCl no protein was eluted (Fig. 8 & 9). The
TABLE 3: EFFECT OF VARYING CONCENTRATIONS OF TRITON X-100 WITH AND WITHOUT KCl ON THE EXTRACTION OF NUCLEAR PORE COMPLEX OXALATE BINDING PROTEIN

<table>
<thead>
<tr>
<th>% of Triton X-100 used</th>
<th>Nuclear pore complex oxalate binding protein extracted</th>
<th>% of nuclear pore complex oxalate binding protein extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>500μg of nuclear pore complex with 1000 cpm</td>
<td>50%</td>
</tr>
<tr>
<td>2%</td>
<td>550μg of nuclear pore complex with 1200 cpm</td>
<td>60%</td>
</tr>
<tr>
<td>5%</td>
<td>800μg of nuclear pore complex with 1300 cpm</td>
<td>65%</td>
</tr>
<tr>
<td>5% Triton X-100 + 0.1M KCl</td>
<td>1000μg of nuclear pore complex with 1500 cpm</td>
<td>75%</td>
</tr>
<tr>
<td>5% Triton X-100 + 0.3M KCl</td>
<td>1000μg of nuclear pore complex with 2000 cpm</td>
<td>100%</td>
</tr>
</tbody>
</table>

Values are mean ± 6 determinations

1.5 mg of nuclear envelope with 2000 cpm of bound ¹⁴C-oxalate was extracted with varying concentrations of triton X-100 with and without KCl and the % of extraction was determined in terms of radioactivity.
Triton-high salt rat kidney nuclear membrane extract containing [14C] oxalate bound protein (~2.5 mg protein with 4000 cpm) was loaded onto the column and was eluted with phosphate buffer containing 1% Triton X-100 with or with out NaCl. Protein was measured at 280nm and radioactivity was measured as described in Materials and Methods.
Triton-high salt human kidney nuclear membrane extract containing [14C] oxalate bound protein (~2.5 mg protein with 4000 cpm) was loaded onto the column and was eluted with phosphate buffer containing 1% Triton X-100 with or with out NaCl. Protein was measured at 280nm and radioactivity was measured as described in Materials and Methods.
F1 fraction showed a specific activity of 500 and 625 p moles/mg protein for rat and human kidney respectively. It also showed a fold purity of 3.7 and 4.16 with 11.0 and 17% yield for rat and human respectively (Table 4).

3.1.8 Determination of molecular weight of nuclear pore complex oxalate binding protein

The SDS-PAGE profile showed homogeneity and the molecular weight of the human kidney nuclear pore complex oxalate binding protein (F1) was found to be 205 kD. Western blotting of the gel also confirmed homogeneity of the protein (Plate 2 & 3).

3.1.9 Kinetic properties of human kidney nuclear pore complex oxalate binding protein

i) Effect of oxalate concentration on isolated human kidney 205 kD protein

The effect of different concentrations of oxalate (150-6000nM) on the human kidney 205 kD protein was studied. It was saturated at 2400nM oxalate concentration (Fig.10).

ii) Scatchard plot analysis

By Scatchard plot analysis, the dissociation constant (Kd) for the human kidney nuclear pore complex 205 kD protein was found to be 2.98 pM and Bmax was computed to be 197 p moles of oxalate bound/mg protein (Fig.11).
TABLE 4: PURIFICATION PROFILE OF NUCLEAR PORE COMPLEX OXALATE BINDING PROTEIN OF RAT AND HUMAN KIDNEY

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Total Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (pmoles of oxalate bound)</th>
<th>Specific Activity (pmoles of oxalate bound/mg protein)</th>
<th>Fold Purity</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RAT KIDNEY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>1.5</td>
<td>17</td>
<td>2295 ± 250</td>
<td>135 ± 9.8</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Triton extract</td>
<td>0.5</td>
<td>5</td>
<td>1000 ± 110</td>
<td>200 ± 22.0</td>
<td>1.48</td>
<td>44.0</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 (F1)</td>
<td>3.0</td>
<td>0.5</td>
<td>250 ± 21</td>
<td>500 ± 43.0</td>
<td>3.7</td>
<td>11.0</td>
</tr>
<tr>
<td><strong>HUMAN KIDNEY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>1.5</td>
<td>20</td>
<td>3000 ± 292</td>
<td>150 ± 21.0</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Triton extract</td>
<td>0.5</td>
<td>8</td>
<td>1840 ± 181</td>
<td>230 ± 26.0</td>
<td>1.53</td>
<td>61</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50 (F1)</td>
<td>3.0</td>
<td>0.8</td>
<td>500 ± 54</td>
<td>625 ± 59.0</td>
<td>4.16</td>
<td>17</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 determinations.

About 15-20mg of nuclear membrane was incubated with ~ 50,000 cpm of ¹³C-oxalate at 25°C for 30 minutes. Then centrifuged and washed the pellet twice to remove unbound oxalate and then extracted with 5% Triton X-100 + 0.3 M KCl and centrifuged. Triton X-100 extract was loaded onto DEAE - Sephadex A-50 column. Fractions 2-4 were pooled. Radioactivity was measured in the nuclear membrane, triton extract and DEAE-Sephadex eluate fractions as described in Materials and Methods.
PLATE 2: SDS - PAGE OF HUMAN KIDNEY OXALATE BINDING PROTEIN

Lane 1: Molecular weight markers
Lane 2: BSA alone
Lane 3: Isolated human kidney oxalate binding protein

50 μg of isolated human kidney nuclear pore complex oxalate binding protein (F1) was loaded onto 8% mini SDS gel and processed as described in Materials and Methods.
PLATE 3: WESTERN BLOT OF HUMAN KIDNEY OXALATE BINDING PROTEIN

Lane 1: Molecular weight markers
Lane 2: Isolated human kidney 205 kDa protein

The SDS gel was transbloted onto PVDF membranes and stained with Ponceau Red as described in Materials and Methods.
Fig. 10  EFFECT OF OXALATE CONCENTRATION ON HUMAN KIDNEY 205 kD PROTEIN

Values are average of 6 determinations.

100 µg of human kidney 205 kD protein was incubated with 600 - 4800 nM concentrations of [14C] oxalate and specific binding was assessed.
Values are mean ± SD for 6 determinations.

Oxalate binding activity was determined by Equilibrium dialysis as described in Materials and Methods.
3.1.10 Immunoreactivity of nuclear pore complex oxalate binding 205 kD protein

i) Effect of PBC serum containing autoantibodies against nuclear pore complex protein, gp210 on human kidney 205 kD protein

When different concentrations of the human kidney nuclear pore complex 205 kD protein was incubated with PBC serum containing autoantibody against gp210, a concentration dependent increase in the antigen - antibody complex was observed (Fig.12).

ii) Effect of gp210-autoantibody on oxalate binding activity of 205 kD protein

The human kidney 205 kD protein showed an oxalate binding activity with a specific activity of 368 p moles/mg protein. But when the same was pre-incubated with PBC serum containing autoantibody against gp210, its oxalate binding activity was found to be totally abolished. When the control serum and PBC serum were checked for oxalate binding activity, both showed about 150 p moles of oxalate bound/mg protein (Table 5).

iii) Effect of gp210-autoantibody on nuclear uptake of oxalate / oxalate bound histone

On pre-treatment with gp210 autoantibody, the human kidney nuclear uptake of oxalate and oxalate bound histone was significantly abolished (p< 0.001) (Fig.13).
FIG. 12 IMMUNOREACTIVITY OF HUMAN KIDNEY 205 kD PROTEIN WITH PBC SERUM CONTAINING gp210 AUTOANTIBODIES

Values are mean ± SD for 6 determinations.

Human kidney 205 kD oxalate binding protein was coated on ELISA plates and probed with PBC serum containing autoantibodies against gp210 as described in Materials and Methods.
TABLE 5: EFFECT OF gp210-AUTOANTIBODY ON OXALATE BINDING ACTIVITY OF HUMAN KIDNEY NUCLEAR PORE COMPLEX 205 kD PROTEIN

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Specific activity (p moles of oxalate bound/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>205 kD</td>
<td>368 ± 4.0*</td>
</tr>
<tr>
<td>205 kD + PBC serum</td>
<td>No binding</td>
</tr>
<tr>
<td>PBC serum</td>
<td>150 ± 12.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 determinations

100 μg of human 205 kD protein was incubated with 10,000 cpm of ^14^C-oxalate and assayed for oxalate binding activity. PBC serum and control sera were also assayed for the same. * Oxalate binding activity was checked by filter binding assay.
Fig. 13  EFFECT OF GP210-AUTOANTIBODY ON NUCLEAR UPTAKE OF OXALATE AND OXALATE BOUND HISTONE

Values are mean ± SD for 6 determinations.

500μg of human kidney nuclei were incubated with 10,000cpm of [14C]oxalate / histone- [14C]oxalate with or without PBC serum containing gp210-autoantibody (1:100 dilution) for 30 minutes and the oxalate uptake was assayed as described in Materials and Methods. Values are statistically significant when compared to control. (p< 0.001***)
3.2 FUNCTIONAL ASPECT OF NUCLEAR PORE COMPLEX OXALATE BINDING PROTEIN

3.2.1 Role of 205 kD protein on \textit{in vitro} calcium oxalate nucleation and aggregation \hfill (Fig. 14)

3.2.2 Role of 205 kD protein on nuclear transport of natural karyophilic protein (histone), artificial karyophilic protein (NLS-BSA) and non-karyophilic protein (BSA) \hfill (Plate 4)

\begin{itemize}
\item \textit{i)} Integrity of nuclear membrane
\item \textit{ii)} Localization of nuclear pore complex oxalate binding protein
\item \textit{iii)} Nuclear transport of proteins
\end{itemize}
3.2 FUNCTIONAL ASPECT OF NUCLEAR PORE COMPLEX
OXALATE BINDING PROTEIN

3.2.1 Role of 205 kD protein on *in vitro* calcium oxalate nucleation and aggregation

*In vitro* calcium oxalate crystallization studies showed that the nuclear pore complex oxalate binding protein, gp210 was an inhibitor. Control assay showed the maximum nucleation at about 7 minutes with 0.14 OD unit. As the protein isolated contained 1% triton X-100, control assay was repeated with 1% triton X-100, which showed the maximum nucleation at about 0.12 OD unit. Increasing concentrations of gp210 gradually reduced the crystal nucleation and aggregation. Lower concentrations (1, 2.5 and 5 µg) of gp210 showed the maximum nucleation at about 0.1, 0.8 and 0.5 respectively but did not postpone the nucleation. Higher concentration of gp210 (10µg) very much reduced the nucleation giving an OD unit of 0.05. It also postponed the nucleation time to 30 minutes. The extent of inhibition of calcium oxalate crystallization by 1, 2.5, 5 and 10 µg of gp210 was 42, 45, 67 and 87 % respectively (Fig.14).

3.2.2 Role of 205 kD protein on nuclear transport of natural karyophilic protein (histone), artificial karyophilic protein (NLS-BSA) and non-karyophilic protein (BSA)

VERO monkey kidney cell line was the system used for the study (Plate 4).
Fig. 14 EFFECT OF INCREASING CONCENTRATION OF 205 kD PROTEIN ON CALCIUM OXALATE CRYSTAL GROWTH

Values are average of 6 determinations.

Varying concentrations of gp210 was added to the assay medium and the calcium oxalate crystal nucleation and aggregation was determined by auto rate assay method.
i) **Integrity of nuclear membrane**

Integrity of the nuclear membrane was checked by FITC-anti-nuclear-antibody staining. The cells treated with 40 µg/ml digitonin for 5 minutes showed cytosolic staining of FITC-ANA suggesting incomplete permeabilization (Plate 4A). However, cells treated with 40 µg/ml digitonin for 15 minutes showed nuclear rim staining only (Plate 4B) suggesting that the nuclear membrane is intact. Hence, further studies were carried out with cells treated with 40µg/ml digitonin for 15 minutes.

ii) **Localization of nuclear pore complex oxalate binding 205 kD protein**

The localization of nuclear pore complex oxalate binding protein in the nuclei was also confirmed by immunofluorescence assay. The permeabilized cells showed punctate pattern of staining at the nuclear rim (Plate 4C) indicating the presence of 205 kD protein in the nuclear pores.

iii) **Nuclear transport of proteins**

Nuclear transport of natural karyophilic protein (histone), an artificial transport protein (NLS-BSA), and a non-karyophilic protein (BSA) was studied in our system. Histone, a natural karyophilic protein with an inbuilt nuclear localization signal sequence was transported into the nuclei in the presence of ATP and cytosolic protein factors (Plate 4D). Individually in
the absence of ATP, it showed rim staining with much reduced uptake of histone and in the absence of cytosolic protein factors the nuclear uptake was not much reduced (Plate 4E & F) stating that the nuclear transport is ATP-dependent and only partially on cytosolic protein factors. Like histone, NLS-BSA, an artificial nuclear protein was also transported into the nuclei in the presence of ATP and cytosolic protein factors (Plate 4H) and was ATP-dependent but not with cytosolic protein factors (Plate 4I & J). When BSA, a non-nuclear protein with out any conjugated signal sequence was studied, it was not transported at all (Plate 4L).

The effect of gp210 antibody on nuclear transport of histone and NLS-BSA and BSA was also studied. The nuclei were pre-incubated with PBC serum containing gp210 autoantibody and further proceeded for transport studies. Plate 3.2 G, K and M showed no uptake of histone, NLS-BSA and BSA respectively. The nuclei showed rim staining only suggesting that gp210 is involved in nuclear protein transport.
Plate 4:

A. VERO kidney cells on permeabilization with digitonin (40μg/ml) for 5 minutes at 4°C and treatment with FITC-ANA for 30 minutes showing cytosolic staining stating incomplete permeabilization.

B. Cells on treatment with digitonin (40μg/ml) for 15 minutes showing nuclear rim staining of FITC-ANA stating complete permeabilization.

C. Permeabilized cells on incubation with PBC serum (1:100) containing autoantibodies against nuclear pore complex protein gp210, punctate pattern of staining at the nuclear rim is seen.

D. Nuclear transport of FITC-Histone showing complete uptake into the nuclei.

E. Nuclear transport of FITC-Histone in the absence of ATP showing minimum uptake into the nuclei with deposition in the nucleoli.

F. Nuclear transport of FITC-Histone in the absence of cytoplasmic protein factors showing uptake in almost all the cells but with less intensity.

G. Nuclear transport of FITC-Histone on pre-incubation with PBC serum containing autoantibodies against gp210 showing nuclear rim staining with no uptake.

H. Nuclear transport of FITC-NLS-BSA showing complete uptake into the nuclei.

I. Nuclear transport of FITC-NLS-BSA in the absence of ATP showing minimum uptake into the nuclei with deposition in the nucleoli.

J. Nuclear transport of FITC-NLS-BSA in the absence of cytoplasmic protein factors showing uptake in almost all the cells but with less intensity.

K. Nuclear transport of FITC-NLS-BSA on pre-incubation with PBC serum containing autoantibodies against gp210 showing nuclear rim staining with no uptake.

L. Nuclear transport of FITC-BSA showing nuclear rim staining with no uptake.

M. Nuclear transport of FITC-BSA on pre-incubation with PBC serum containing autoantibodies against gp210 showing nuclear rim staining with no uptake.

Original Magnification: X 400
PLATE 4: EFFECT OF GP210-AUTOANTIBODY ON NUCLEAR TRANSPORT OF NATURAL KARYOPHILIC (HISTONE), ARTIFICIAL KARYOPHILIC (NLS-BSA) AND NON-KARYOPHILIC PROTEIN (BSA)
3.3 EXPRESSION OF NUCLEAR PORE COMPLEX OXALATE BINDING PROTEIN, gp210 IN OXALATE STRESS CONDITION

3.3.1 Effect of oxalate on cell density (Table 6)

3.3.2 Effect of oxalate on expression of gp210 (Fig. 15)

3.3.3 Oxalate induced and cell cycle-dependent expression of gp210

   i) By ELISA (Fig. 16)

   ii) By SDS-PAGE (Plate 5)

3.3.4 Effect of substrate analogues on expression of gp210 (Fig. 17)
3.3 EXPRESSION OF NUCLEAR PORE COMPLEX OXALATE BINDING PROTEIN

3.3.1 Effect of oxalate on cell density

Exposure of VERO cells to oxalate in the culture medium produced changes in cell density. 24 hours exposure to varying concentrations (0.1mM, 0.3mM and 1.0mM total oxalate) of oxalate produced biphasic effects on cell numbers. Cells exposed to 0.1mM total oxalate showed 40% increase in cell density, while cells exposed to 0.3mM total oxalate showed 25% increase in cell density and that exposed to 1.0mM total oxalate showed a slight decline of 10% in cell density when compared to that of the control cells unexposed to oxalate (Table 6).

3.3.2 Effect of oxalate on the expression of gp210

When the triton extract of cells grown in different concentrations of oxalate were quantitated for gp210 concentration by ELISA, there was a significant increase in its concentration when exposed to 0.1mM (p< 0.05), 0.3 mM (p< 0.01) and 1.0 mM (p< 0.001) total oxalate concentration (Fig.15). The residual pellet showed no presence of gp210.

3.3.3 Oxalate induced and cell cycle-dependent expression of gp210

i) By ELISA: The concentration of gp210 in synchronized cells grown in the medium containing different concentrations of oxalate showed an increase when compared to that of the control cells unexposed to oxalate (Fig.16).
TABLE 6: EFFECT OF OXALATE ON CELL DENSITY

<table>
<thead>
<tr>
<th>Particulars</th>
<th>% of cell density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM Oxalate</td>
<td>+ 25</td>
</tr>
<tr>
<td>0.3 mM Oxalate</td>
<td>+ 40</td>
</tr>
<tr>
<td>1.0 mM Oxalate</td>
<td>- 10</td>
</tr>
</tbody>
</table>

+ Increase - Decrease

Values are average of 3 experiments.

1x10^6 cells were incubated with growth medium containing varying concentrations of oxalate. After 24 hours, 10μl of cells from each category were mixed with 0.05% trypan blue and the cell density was assessed as described in Materials and Methods.
Values are mean ± SD for 3 experiments with 2 replicates for each experiment.

30μg of triton extract of each category was coated onto ELISA plates and probed with PBC serum containing gp210-autoantibodies (1:500) and the gp210 concentration was assessed based on the data on Fig.12. Values are statistically significant when compared to control. (p< 0.05*; p< 0.01**; p< 0.001***)

Fig.15 EFFECT OF OXALATE ON EXPRESSION OF gp210

<table>
<thead>
<tr>
<th>Concentration of oxalate (mM)</th>
<th>µg of gp210/1x10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50</td>
</tr>
<tr>
<td>0.1mM</td>
<td>100</td>
</tr>
<tr>
<td>0.3mM</td>
<td>150</td>
</tr>
<tr>
<td>1.0mM</td>
<td>200</td>
</tr>
</tbody>
</table>
Values are mean ± SD for 3 experiments with 2 replicates for each experiment.

30μg of triton extract of each category was coated onto ELISA plates and probed with PBC serum containing gp210-autoantibodies (1:500) and the gp210 concentration was assessed based on the data on Fig.12. Values are statistically significant when compared to control. (p< 0.05*; p< 0.01**; p< 0.001***
In the control cells, the gp210 concentration was increased maximum in the telophase of the mitotic phase than that of the 'S' phase. When the cells were exposed to 0.1mM total oxalate, the concentration of gp210 was significantly increased (p< 0.01) in all the three phases when compared to that of the control. On exposure of the cells to 0.3 mM total oxalate a significant (p< 0.001) increase in gp210 concentration was observed in all the three phases when compared to that of the control. In the mitotic phase, the telophase showed a maximum concentration of gp210 when compared to metaphase and 'S' phase. When the cells were exposed to higher concentration of oxalate (1.0 mM total oxalate), a significant (p<0.001) increase in the gp210 concentration in all the three phases was observed. The maximum increase was observed in the telophase. Among the different concentrations of oxalate, the cells exposed to 1.0mM oxalate showed a maximum concentration of gp210 in the telophase.

ii) SDS-PAGE: The triton extract of synchronized cells grown in different concentrations of oxalate were electrophoresed on 8% SDS-PAGE along with molecular weight markers. Relatively thicker bands were observed with increase in oxalate concentration (Plate 5).

3.3.4 Effect of substrate analogues on the expression of gp210

The triton extract of cells grown with 0.1mM concentration of oxalate and its structural analogues - oxamate, malate, succinate and citrate were checked for gp210 concentration. When compared with control, only cells grown with oxalate showed the significant (p< 0.01) increase of gp210, while the others did not show any significant increase in gp210 concentration (Fig.17).
Plate 5: SDS-PAGE PROFILE OF OXALATE INDUCED AND CELL CYCLE-DEPENDENT EXPRESSION OF GP210

Lane 1: 'S' Phase cells exposed to 0.1mM oxalate
Lane 2: 'S' Phase cells exposed to 0.3mM oxalate
Lane 3: 'S' Phase cells exposed to 1.0mM oxalate
Lane 4: Metaphase cells exposed to 0.1mM oxalate
Lane 5: Metaphase cells exposed to 0.3mM oxalate
Lane 6: Metaphase cells exposed to 1.0mM oxalate
Lane 7: Telophase cells exposed to 0.1mM oxalate
Lane 8: Telophase cells exposed to 0.3mM oxalate
Lane 9: Telophase cells exposed to 1.0mM oxalate
Lane 10: Molecular weight markers

50µg of triton extract of cells were electrophoresed on 8% mini-gel along with molecular weight markers and silver stained as described in Materials and Methods.
Values are mean ± SD for 3 experiments with 2 replicates for each experiment.

30μg of triton extract of each category was coated onto ELISA plates and probed with PBC serum containing gp210-autoantibodies (1:500) and the gp210 concentration was assessed based on the data on Fig.12. Values are stastically significant when compared to control. (p< 0.01**)
3.4 STATUS OF NUCLEAR PORE COMPLEX OXALATE BINDING PROTEIN, gp210 IN KIDNEY DISEASES

3.4.1 In hyperoxaluria

i)  Gp210 and its autoantibody in serum  (Fig. 18)

ii) Gp210 in renal tissue  (Fig. 19)

iii) Urinary excretion of gp210  (Table 7)

iv) Immunofluorescence staining of gp210 in cryo-sections  (Plate 6)

3.4.2 In other kidney diseases

i)  Gp210 and its autoantibody in serum  (Fig. 20)

ii) Gp210 in renal tissue  (Fig. 21)

iii) Immunofluorescence staining of gp210 in cryo-sections  (Plate 6)
3.4 STATUS OF NUCLEAR PORE COMPLEX OXALATE BINDING PROTEIN, gp210 IN KIDNEY DISEASES

3.4.1 In hyperoxaluria

i) *Gp210 and its autoantibody in serum*

Control subjects showed \(/\) ml of serum, while in hyperoxaluric condition, a significant increase \((p< 0.001\) about \(0.25\mu g\) of gp210) in concentration of \(2.1\mu g\) of gp210/ml serum was observed. The hyperoxaluric condition showed \(0.8\mu g\) of gp210-autoantibody/ml serum \((p< 0.001\) while control subjects showed no autoantibody against gp210 (Fig.18).

ii) *Gp210 in renal tissue*

Human cadaver kidney served as the control. Biopsy tissue homogenate of hyperoxaluric condition was probed for the presence of gp210 which showed an significantly increased concentration of \(225\mu g/g\) tissue when compared with that of the control which showed \(120\mu g/g\) tissue (Fig.19).

iii) *Urinary excretion of gp210*

24 hours urine sample of healthy individuals with no history of kidney disease served as the control. The dialyzed and concentrated urine of control and hyperoxaluric patients was probed for the presence of gp210. About \(380\mu g\) of gp210 was found to be excreted in hyperoxaluria when compared to \(200\mu g\) in control urine. The total oxalate excreted was found to
Values are mean ± SD for 6 determinations.

Gp210 and its autoantibody concentration was assessed in the control and hyperoxaluric sera based on the data on Fig.12. Values are statistically significant when compared to control. (p< 0.001***)
Values are mean ± SD for 6 determinations.

30μg of tissue homogenate of each kidney disease was coated onto ELISA plates and probed with PBC serum containing gp210 - auto antibodies (1:500) and the gp210 concentration was assessed based on the data on Fig.12. Values are statistically significant when compared to control. (p< 0.001***)
be 35 and 58mg/24hour for control and hyperoxaluric patients respectively. The total protein excreted was also higher in hyperoxaluric patients -500mg/24hours when compared to that of the control (Table 7).

**iv) Immunofluorescence staining of gp210 in cryo-sections**

By indirect immunofluorescence staining, biopsy kidney tissue sections of control (donor for kidney transplantation) and that of hyperoxaluric patients were stained for gp210 using the PBC serum containing gp210 autoantibodies. In control tissue, tubules and glomeruli were weakly positive (Plate 6A & B) while the hyperoxaluric kidney section showed a much higher level of staining for gp210 (Plate 6C).

### 3.4.2 In other kidney diseases

**i) Gp210 and its autoantibody in serum**

Control subjects showed about 0.25µg of gp210/ml serum while ARF, CRF, NS and SLE with NS showed 1.65, 1.42, 1.33 and 1.85µg of gp210/ml serum. SLE with NS cases showed 0.9µg of gp210-autoantibody/ml serum while it was absent in control subjects (Fig.20).

**ii) Gp210 in renal tissue**

Human cadaver kidney served as the control which showed 120µg/g tissue while ARF, NS and SLE associated with NS showed 168, 142 and 190µg of gp210/g tissue (Fig.19).
TABLE 7: EXCRETION PROFILE OF OXALATE, PROTEIN AND gp210 IN 24 HOURS HYPEROXALURIC URINE

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Total oxalate excreted mg / 24 hours</th>
<th>Total protein excreted mg / 24 hours</th>
<th>Total gp210 Excreted μg / 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35 ± 3.0</td>
<td>120 ± 10</td>
<td>200 ± 22.0</td>
</tr>
<tr>
<td>Hyperoxaluria</td>
<td>58 ± 6.2</td>
<td>500 ± 43</td>
<td>380 ± 41.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 determinations.

30μg of the dialyzed and concentrated urine was coated onto ELISA plates and probed with serum containing gp210-autoantibody (1:500) and the gp210 concentration in the urine was determined as described in Materials and Methods. Oxalate and protein excreted were also determined as described in Materials and Methods.
Plate 6:
A: Control tubular section showing immunofluorescence staining for gp210
B: Control glomeruli section showing immunofluorescence staining for gp210
C: Hyperoxaluric section showing immunofluorescence staining for gp210
D: Glomerulonephritic section showing immunofluorescence staining for gp210
E: Membranous nephropathic section showing immunofluorescence staining for gp210
F: SLE associated with NS section showing immunofluorescence staining for gp210
G: IgA nephropathic section showing immunofluorescence staining for gp210
H: Tubular necrotic section showing immunofluorescence staining for gp210
I: Diabetic nephropathic section showing immunofluorescence staining for gp210

Original magnification X 400

Cryo-sections of different kidney disease biopsy tissues were incubated with 1:100 diluted PBC serum for 1 hour, washed with buffer and incubated with 1:40 diluted FITC-anti-human-IgG for 1 hour, washed with buffer and viewed under fluorescent microscope and photographed as described in Materials and Methods.
PLATE 6: IMMUNOFLUORESCENCE STAINING OF 205 kD PROTEIN IN CRYO-SECTIONS OF BIOPSY TISSUES OF KIDNEY DISEASES
Values are mean ± SD for 6 determinations.

30μg of control and other kidney disease sera were coated onto ELISA plates and probed with PBC sera containing gp210 - auto antibodies (1:500) and the gp210 concentration was assessed based on the data on Fig.12. Values are stastically significant when compared to control. (p< 0.05*; p< 0.01**; p< 0.001***)
iii) **Immunofluorescence staining of gp210 in cryo-sections**

By indirect immunofluorescence staining, biopsy kidney tissue sections of control (donor for kidney transplantation) and other kidney disease patients were stained for gp210 using the PBC serum containing its autoantibodies. In control tissue, tubules and glomeruli were weakly positive (Plate 6A & B) while diseased kidney sections showed a significant increase in the intensity of immunoreactive gp210 in the same region. Glomerulonephritic and membranous nephropathic tissue with the characteristic club-shaped glomerular lobules and tubular atrophy also showed much higher level of fluorescence for gp210 (Plate 6D & E). SLE with NS and IgA nephropathic tissues with their characteristic fibrinoid immune complex material deposition and deformed glomeruli showed strong positive signals for gp210 (Plate 6F & G). Acute tubular necrotic and diabetic nephropathic tissues with their characteristic tubular atrophy and fibrinoid deposition on glomerular capillaries showed much strong staining (Plate 6H & I) when compared with control tubules and glomeruli.