CHAPTER III

RESULTS

3.1 Purification of antigens.

a. Ceruloplasmin.

The eluate from 2.5 x 27.0 cm DEAE column and the modified glass tubing gave the same clectrophoretic pattern, but the time for fractions in the first column was longer than the other one. This is because the flow rate from glass tubing pluged with glass wool was faster. The modified glass tubing was selected.

The results of elution from CM-Sephadex chromatography are shown in Fig. 2, p 25. Immunoelectrophoresis of this peak when developed against anti human serum and anti ceruloplasmin shows that it contains almost pure ceruloplasmin except for a small contamination by β-globulin (Fig. 3, **p.26).**

The result of rechromatography of this purified ceruloplasmin on CM-Sephadex was shown in Fig. 4, p. 27. The fractions were tested by Ouchterlony Technic against anticeruloplasmin and anti normal human serum, it was demonstrated that it did not contain any contaminant (Fig. 5), p. 28. Immunoelectrophoresis of fraction 7 and 9 of eluate showed one band of purified ceruloplasmin against anti human serum (Fig. 6), p. 29.

Fig.2 Elution of ceruloplasmin from CM-Sephadex column.
at the first run, flow rate of 30 drops /min.

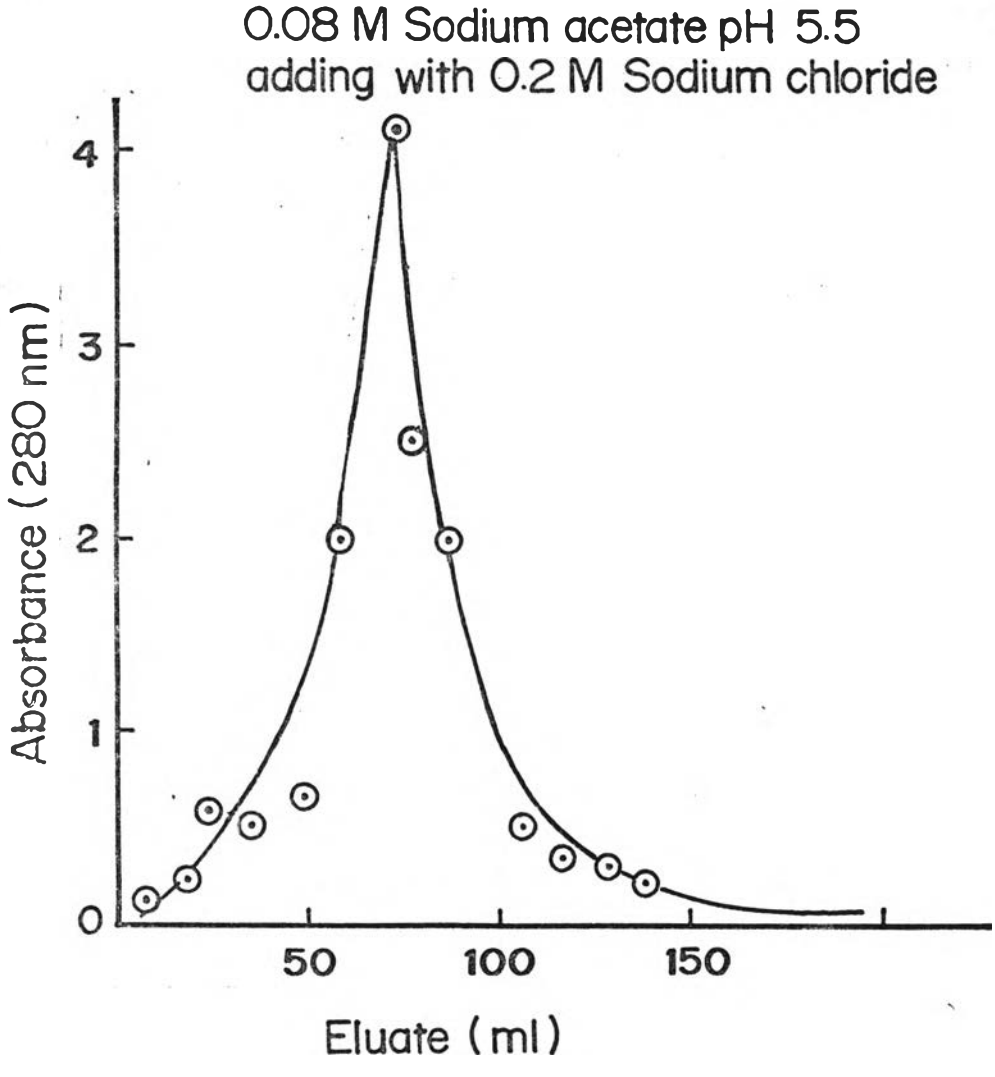
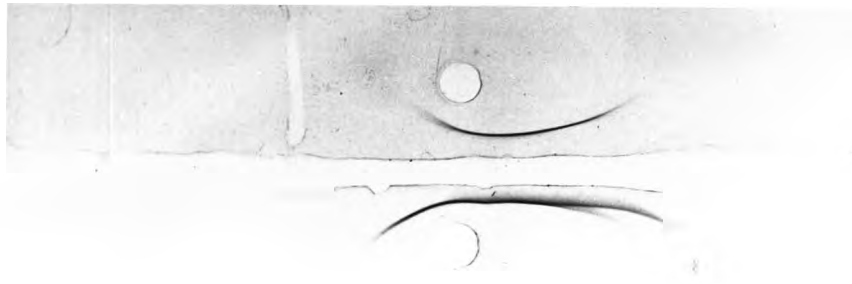


Fig. 3. Immunoelectrophoresis of protein peaks obtained from two CM-Sephadex chromatography.



Upper well : second run (rechromatography)

Lower well : first run
(having traces of β-globulins as
contaminants)

Trough : anti normal human serum.

Fig. 4 Rechromatography of ceruloplasmin on CM – Sephadex column. (Flow rate 36 drops / min.)

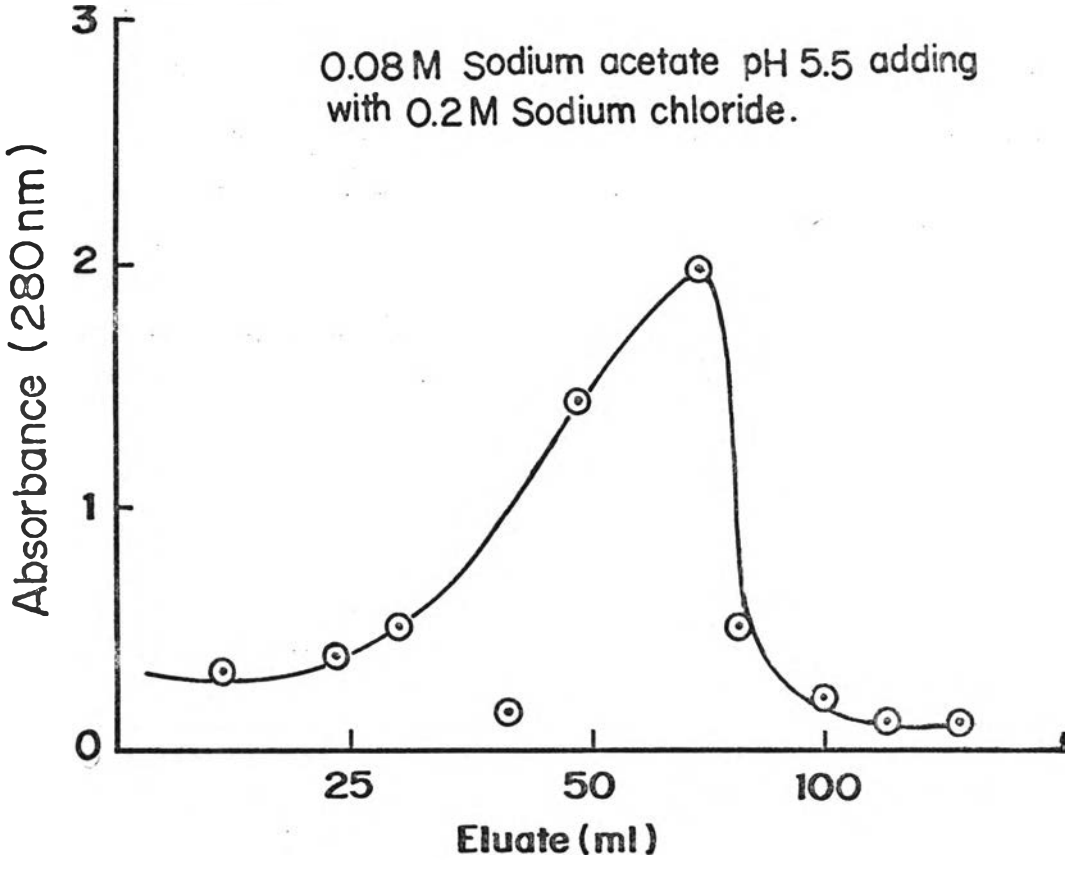
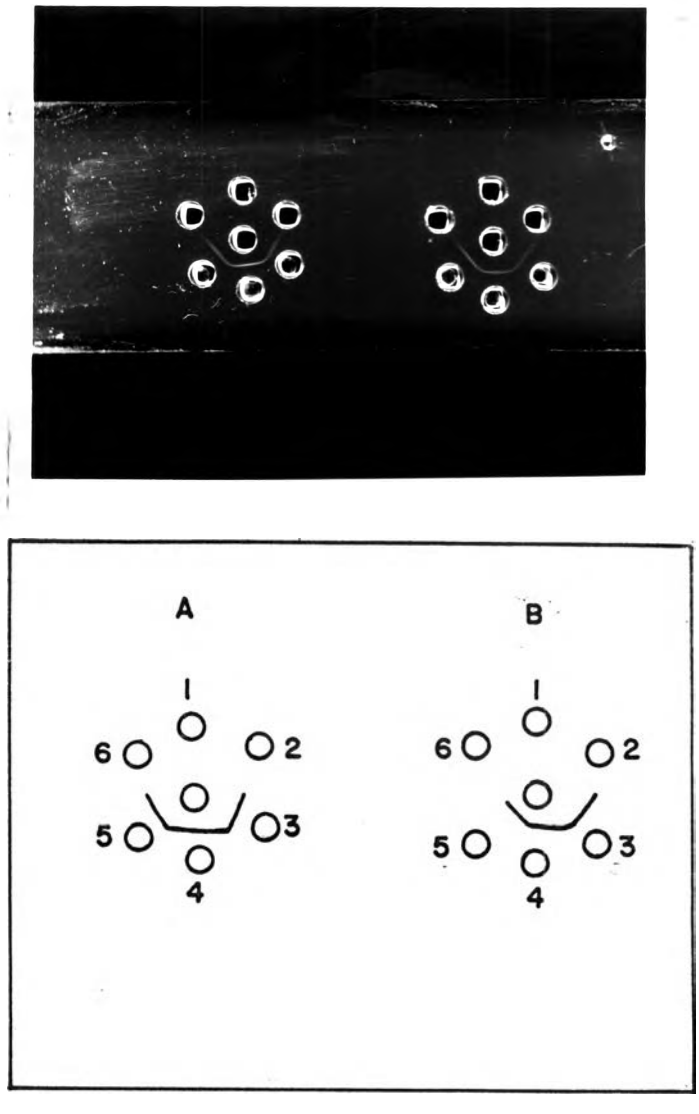
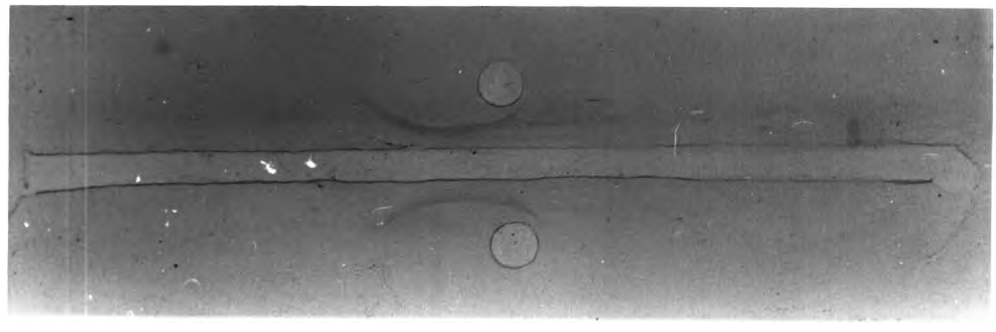


Fig. 5. Ouchterlony double diffusion showing precipitation of purified ceruloplasmin.



Well 1 to 5 contain purified ceruloplasmin fractions 7 to 9 which showed one band against anti human serum (A) and against commercial anti ceruloplasmin (B).

Fig. 6. Immunoelectrophoresis of two fractions obtained from rechromatography on CM Sephadex column.



Upper well : fraction number 7

Lower well : fraction number 9

Trough : Anti normal human serum

The prepared ceruloplasmin was used to immunize rabbits and the antiserum obtained was tested by immunoelectrophoresis showing antibodies to ceruloplasmin, to IgG and to other serum proteins. The anticeruloplasmin was found monospecific to ceruloplasmin after absorption with purified IgG, and then with eluate from DEAE-Sephadex chromatography containing most of other serum proteins except ceruloplasmin. The monospecificity to ceruloplasmin was demonstrated by IEP (Fig. 7, p. 32).

The final dilution of anticeruloplasmin in Mancini agar plate was found to be optimum at 1:7. At this dilution, clear and sharp immunodiffusion precipitation was observed with undiluted human sera. Lack of double concentric rings within the immunodiffusion reconfirm its monospecificity (Fig. 8, p. 33).

The levels of ceruloplasmin from 150 specimens were shown in (Table I, p. 34). The levels among those with liver diseases and those with hemoglobinopathies were highly increased when compared to that of normal ones.

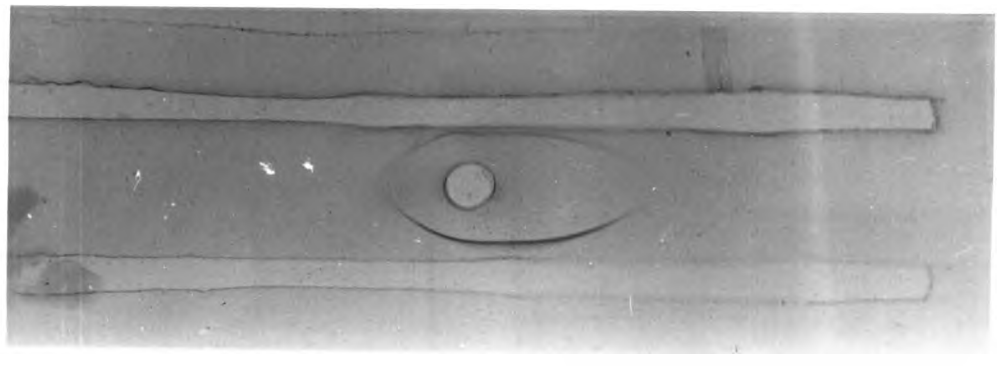
b. Haptoglobins.

Hb was found to be readily bound to CNBr activated Sepharose complex, and thoroughly washed to be free from contaminating plasma protein, the bound Hp was eluted from Hb:-Sepharose by the use of 3.5 M Urea pH 5 (Fig. 9, p. 35). Test for its purity was made by immunodiffusion against commercial

antihaptoglobin and anti human serum. No precipitation lines of other contaminant serum proteins were observed in both immuno-electrophoretic and immunodiffusion methods(Fig. 10, p. 36, Fig. 11, p. 37). However after immunization into rabbits with this Hp, antibody produced showed reaction to IgG; this indicated minute contamination of IgG in the prepared Hp. This reactivity to IgG was readily eliminated by absorption with IgG. The monospecificity of prepared Hp was then demonstrated in Fig. 12, p. 38, Fig. 13, p. 39, Fig. 14, p. 40 .

Then antihaptoglobin was used to determine Hp level in normal human sera and in those with confirmed hemoglobinopathies patient sera (Fig. 15, p. 41). The results were shown in Table II, p.42. The level of Hp in patients' sera were lower than normal ones (Table II, p. 42). Only occasionally that the levels among hemoglobinopathies reached the normal level.

Fig. 7. Immunoelectrophoretic comparison between commercial and prepared anticeruloplasmin.

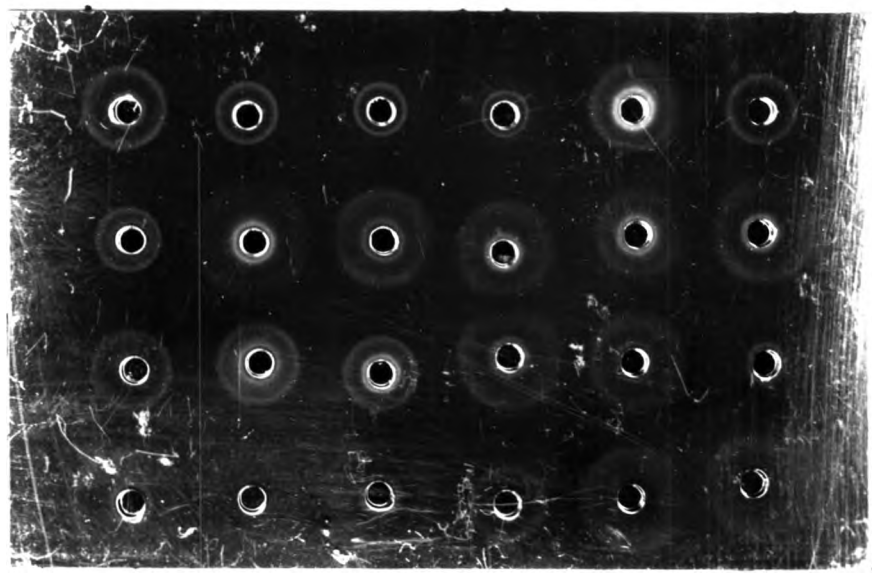


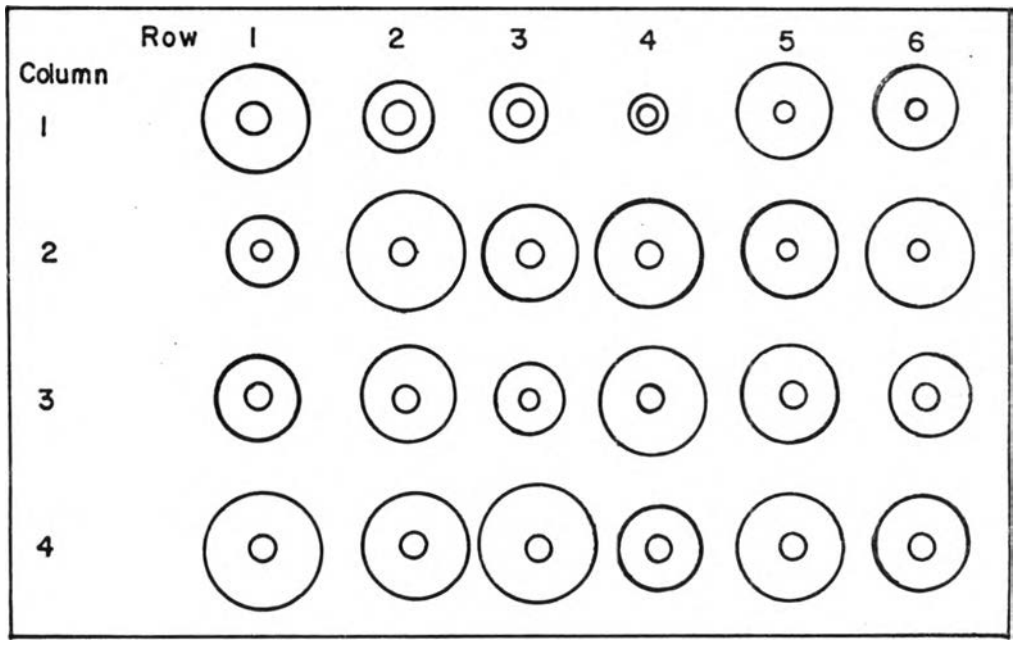
Upper trough : prepared anticeruloplasmin

Lower trough : commercial anticeruloplasmin

Well : normal human serum

Fig. 8. The radial immunodiffusion plate demonstrating various concentration of ceruloplasmin.





Column 1, Row 1-4 are standard antigen showing a concentric precipitin reaction in various sizes.

Table I

Serum Ceruloplasmin in normal human and patients with liver diseases expressed in percentage of a standard preparation.

	No.	Mean	Standard Deviation	range %	p*
Normal	50	33	20	13-53*	
Liver diseases	150	141	30	111-171	<0.05
Hemoglobinopathies	30	137	42	95-179	<0.05

* p - Compare to normal

(p = probability)

Fig. 9 Elution of haptoglobin with 3.6 M Urea from affinity chromatography.

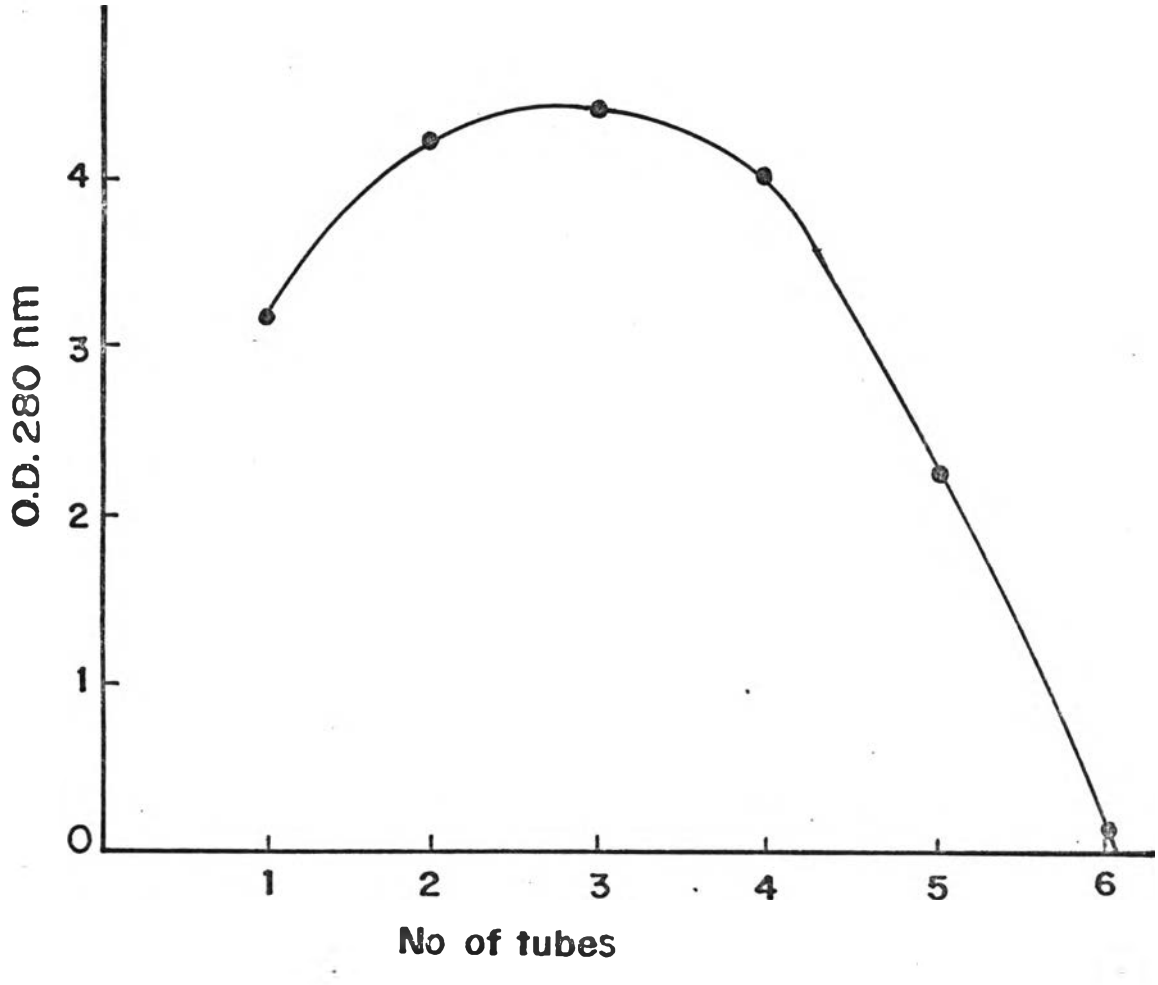
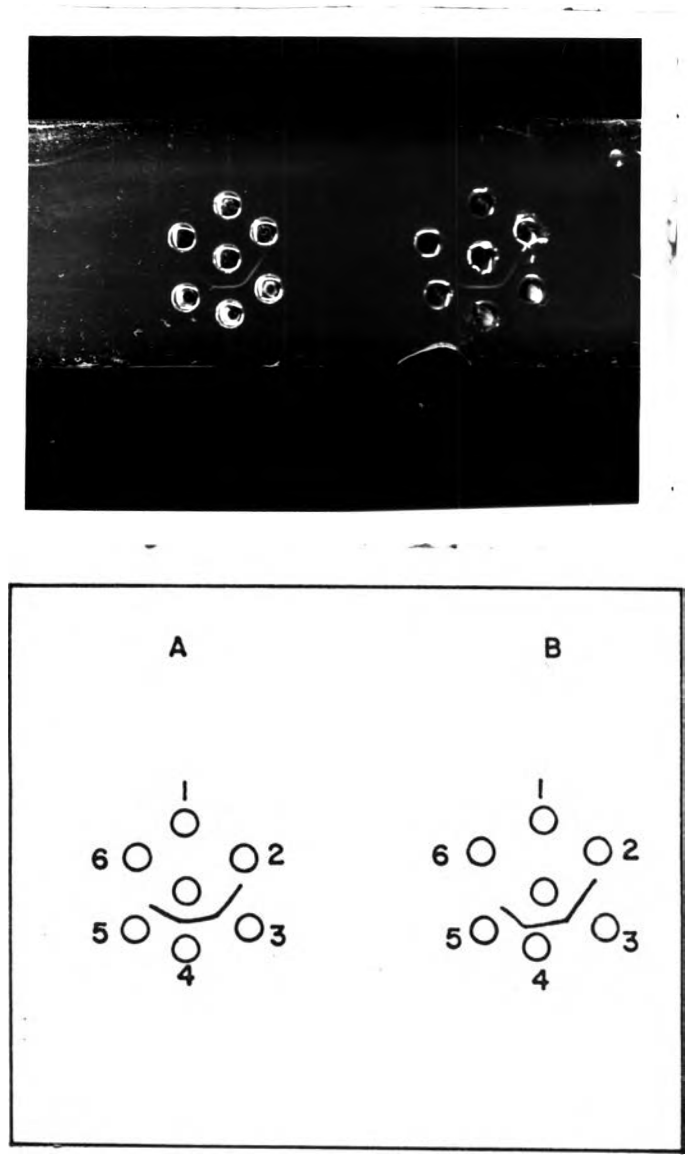


Fig. 10. Double immunodiffusion showing precipitation lines of reaction of prepared haptoglobin.

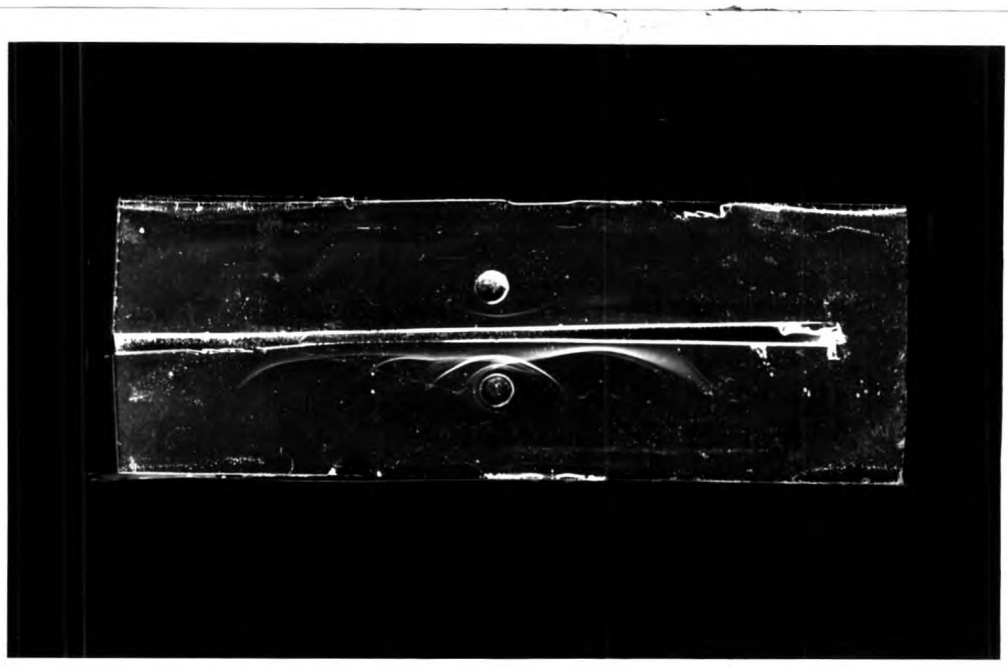


Well 1 to 5 contain prepared haptoglobin

A central well contain anti human serum

B central well contain anti commercial haptoglobin

Fig. 11. Immunoelectrophoresis of prepared haptoglobin.

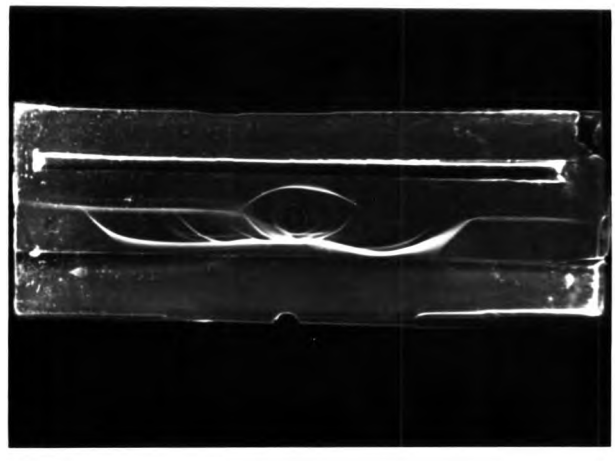


Upper well : prepared haptoglobin

Lower well : normal human serum

Trough : anti normal human serum

Fig. 12. Immunoelectrophoresis precipitation demonstrating the precipitin line of prepared anti-haptoglobin.



Upper trough : prepared anti-haptoglobin

Lower trough : anti normal human serum

Well : normal human serum

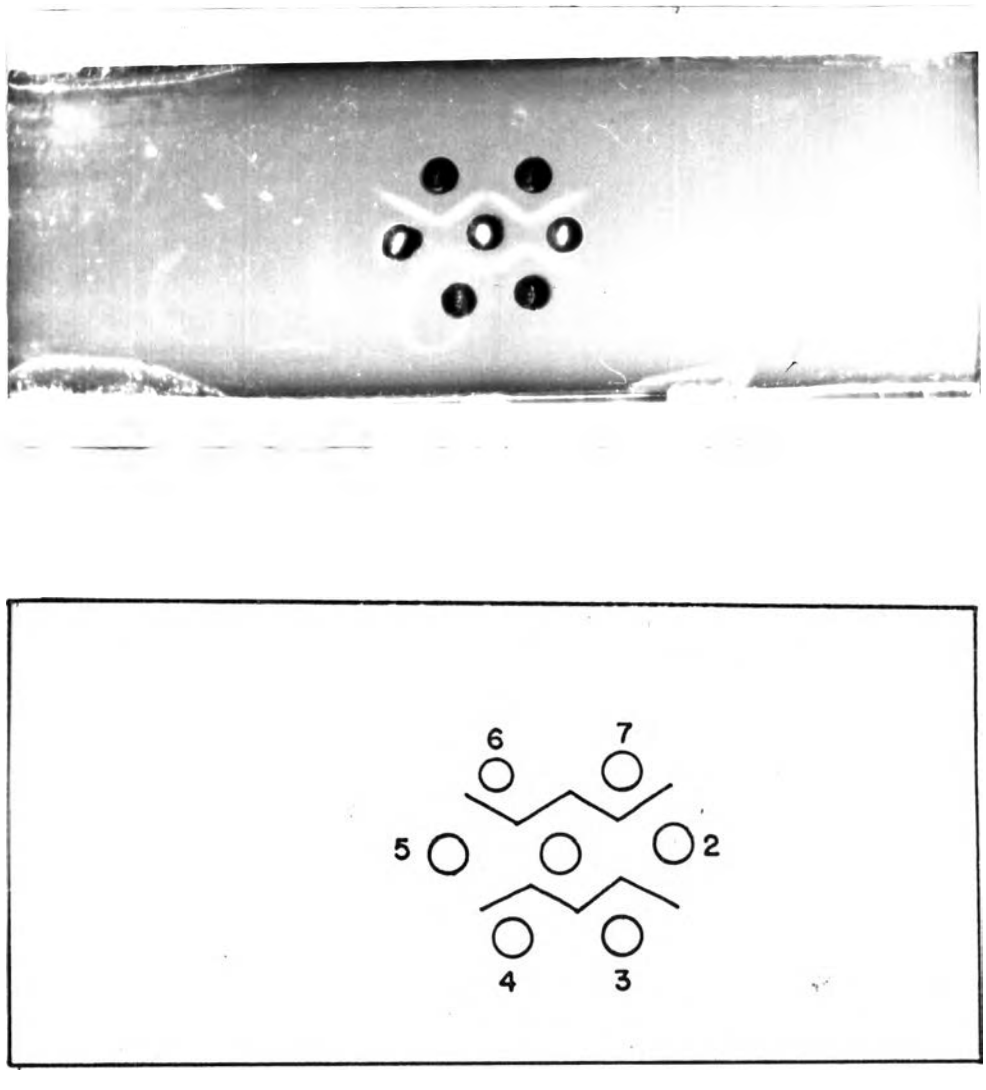
Fig. 13. Immunoelectrophoretic comparison between commercial and prepared antihaptoglobin.

Upper trough : prepared antihaptoglobin

Lower trough : commercial antihaptoglobin

Well : normal human serum

Fig. 14. Double diffusion showing lines of identity in precipitin reaction.



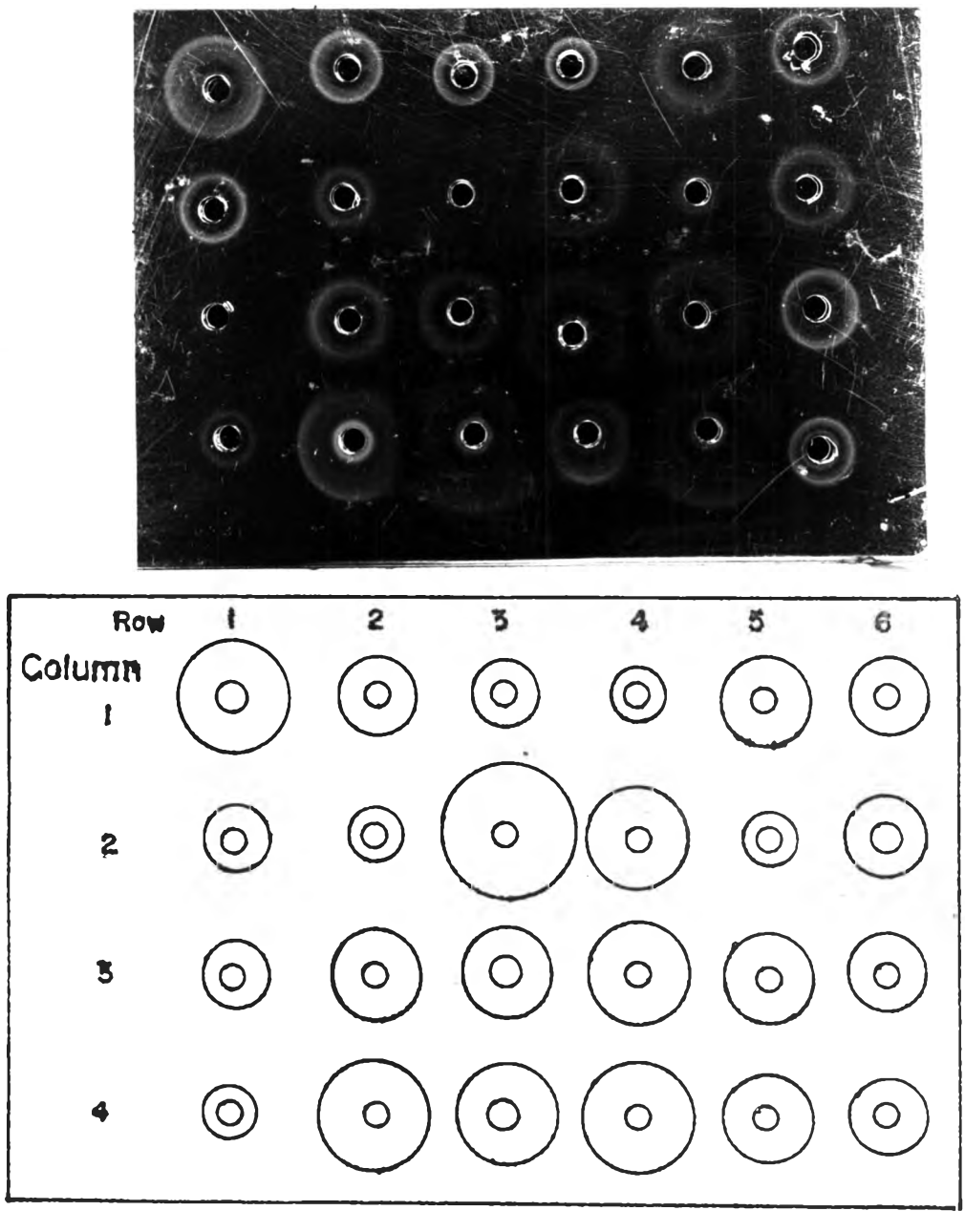
Well 1, 4 - Commercial anti haptoglobin

Well 3, 6 - prepared anti haptoglobin

Well 2, 5 - prepared haptoglobin antigen

Well 7 - normal human serum

Fig. 15. The quantitative determination of haptoglobin by Radial Immunodiffusion.



Column 1, Row 1 to 4: various dilutions of the standard antigens

Table II

Serum haptoglobin in normal human serum and patients with hemoglobinopathies expressed in percentage of a standard preparation.

	No.	Mean	Standard Deviation	range %	p*
Normal	50	198	90	108-288*	
Hemoglobinopathies	100	34	18	16-52	<0.05

*p Compare to normal

(p = probability)