CHAPTER II

COMPARATIVE STUDIES ON THE ABSORPTION SPECTRA OF HAEMOGLOBIN OF OX BLOOD AND ROOT NODULES

Sorby, in 1876, in his classical paper "On the evolution of haemoglobin", showed for the first time that the absorption spectra of haemoglobins of different origins are not the same. Subsequently, a wealth of literature has accumulated to demonstrate the diversities of haemoglobins from different sources in terms of absorption spectra, molecular weight, isolectric points, amino acid content, affinities for oxygen and carbon monoxide, crystalline structure, ease of crystallisation, solubility, auto-oxidizability of the iron, antigenic properties and ease of denaturation by alkali and other factors (Keilin and Hartree, 1951).

That the red pigment of root nodules of legumes is a haemoprotein with a strict analogy with blood haemoglobin in terms of reactions with oxygen and carbon monoxide is now firmly established. (Hartree, 1955). Virtanen (1955) showed that there was a good correlation between haemoglobin concentration in pea nodules and the amount of nitrogen fixed by them. Thus, one criterion for effectiveness in symbiosis is the capacity of the symbiotic system to synthesize haemoglobin.

It was, therefore, deemed desirable to study:

1. the synthesis and degradation of this pigment in root nodules,
2. the effects of environment in relation to nitrogen fixation and haemoglobin synthesis,
3. to define effectiveness in tropical species in terms of haemoglobin concentration in nodules,
4. the absorption spectra of haemoglobin of a few legume species,
5. and the influence of pathologically altered host metabolism such as occur in virus infection on haemoglobin formation.

For these reasons, comparative study of haemoglobin of ox blood and root nodules was carried out.

A. PREPARATION OF CRYSTALLINE HAEMIN FROM OX BLOOD.

Haemin was obtained in a purified state from ox blood following strictly the instructions of Hartree (1955) in all details (vide:Materials and Methods).

B. ESTIMATION OF TOTAL HAEMATIN:

Rationale of the Method: The standard for comparison was crystalline haemin (Ferriheme chloride, $C_{34}H_{32}O_4N_4FeCl$ with Cl attached to Fe) (Hertree, 1955).

In haematin the 5th and 6th co-ordination positions are, probably, occupied by an OH group and a water molecule. If this structure is accepted, the molecular weight of haematin is virtually equal to that of haemin (Hartree, 1955). In haemoproteins, the protein satisfies one Fe co-ordination valency and a prosthetic group as it occurs in such derivatives, will have a lower molecular weight corresponding to the loss of either the OH group or the water (Keilin and Hartree, 1951). Thus, according to Hartree (1955), the molecular weight of haematin prosthetic group will be about 3% less than that of haemin.

The method of expression of root nodule haemoglobin in terms of haematin of ox blood was chosen. Haemochromogen solutions prepared from haemin by addition of pyridine and sodium dithionite ($Na_2S_2O_4$) were kept in the dark and protected from air since their auto-oxidation leads to the destruction of the porhyrin nucleus. After addition of $Na_2S_2O_4$, the solutions were left standing for 30 minutes before readings were taken to facilitate adjustments in minor changes of density (details as in Table 4).

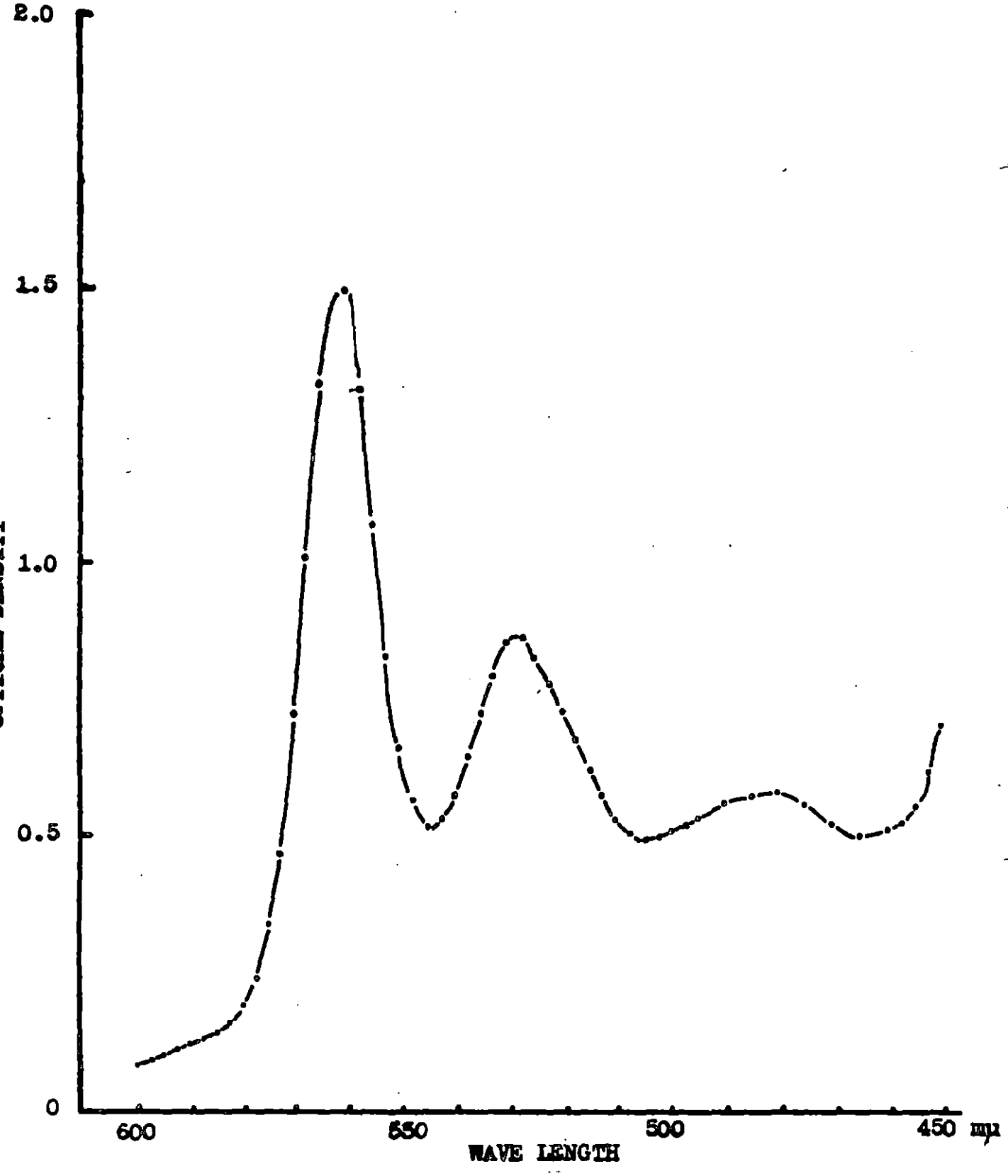

Fig. 2. SPECTRUM OF PYRIDINE HAEMOCHROMOGEN FROM OX BLOOD

C. THE SPECTRUM OF PYRIDINE HAEMOCHROMOGEN FROM OX BLOOD.

The spectrum presented in figure 2 shows a distinct maximum at 560 mμ and a weaker band at 527 mμ with a diffuse Soret band forming a plateau from 465 to 500 mμ.

D. THE SPECTRUM OF PYRIDINE HAEMOCHROMOGEN FROM THE ROOT NODULES OF ARACHIS HYPOGAEA. Vars. TMV 2 and TMV 3.

Nodules were freshly removed from field grown plants 45 days from the day of sowing. The plants were inoculated with a heavy suspension of an effective strain of Rhizobium (R_4) at the time of sowing in the field. The nodules collected from a total of ten plants were washed, dried and weighed. Five grams of nodules were than ground thoroughly in water and centrifuged at 3000 r.p.m. for ten minutes to yield a clear red supernatant.

A measured volume of the extract was then converted to the haemochromogen by addition of NaOH, followed by pyridine and $Na_2S_2O_4$. The absorption spectra of the solutions were then determined in a Uvispeck photo electric spectrophotometer (Hilger) using a suitable photocell selector (details of procedure are shown in Table 4) between 480-600 mμ.

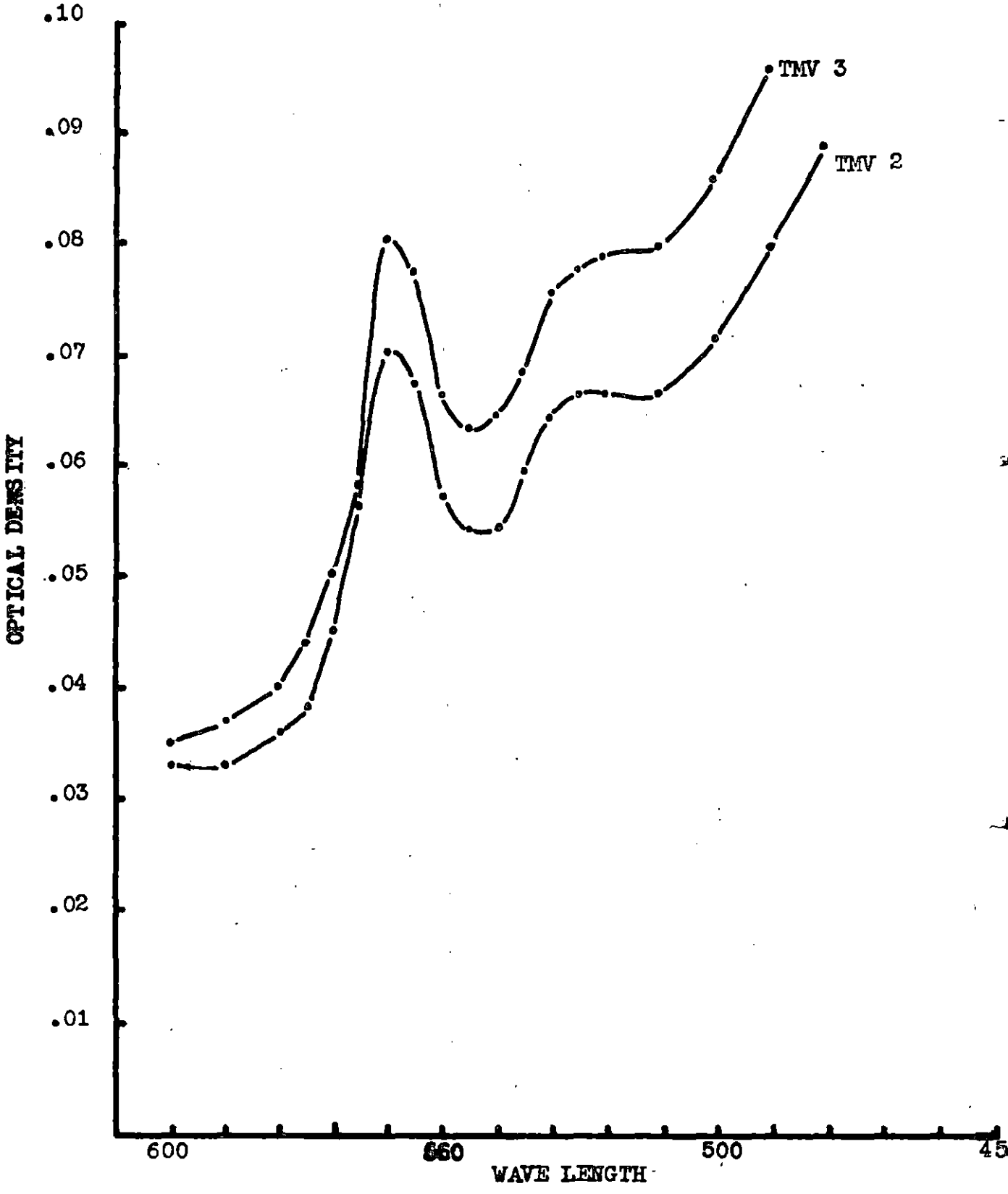

Fig. 3. SPECTRA OF PYRIDINE HAEMOCHROMOGENS FROM THE ROOT NODULES OF THE BUNCH (TMV 2) AND SPREADING (TMV 3) VARIETIES OF ARACHIS HYPOGAEA.

The curves in fig.3 show the presence of a clear maximum at 560 mμ. and a diffuse band spread over at 525 mμ. while the Soret band was absent.

The absorption curves of the pyridine haemochromogens prepared from both haemoglobins were essentially identical.

E. CALIBRATION CURVE FOR HAEMOGLOBIN:

Ox blood haemoglobin was used as the standard since the absorption curves of the pyridine haemochromogens of nodule and blood haemoglobins were very similar. The light absorption of various concentrations of haeamin, after conversion to pyridine haemochromogen were measured at 560 mu. as well as at 527 mμ. Values plotted from the α-band maximum (560 mμ) were used in all subsequent studies as the standard for comparison. Quantitative determinations of nodule haemoglobin were made as pyridine haemochromogen per gram of nodule tissue from the calibration graph. The light absorption by solutions of pyridine haemochromogen at the α-band maximum (560 mμ.) and the β-band maximum (527 mμ.) are represented in Table 4, and the calibration curves in fig.4.

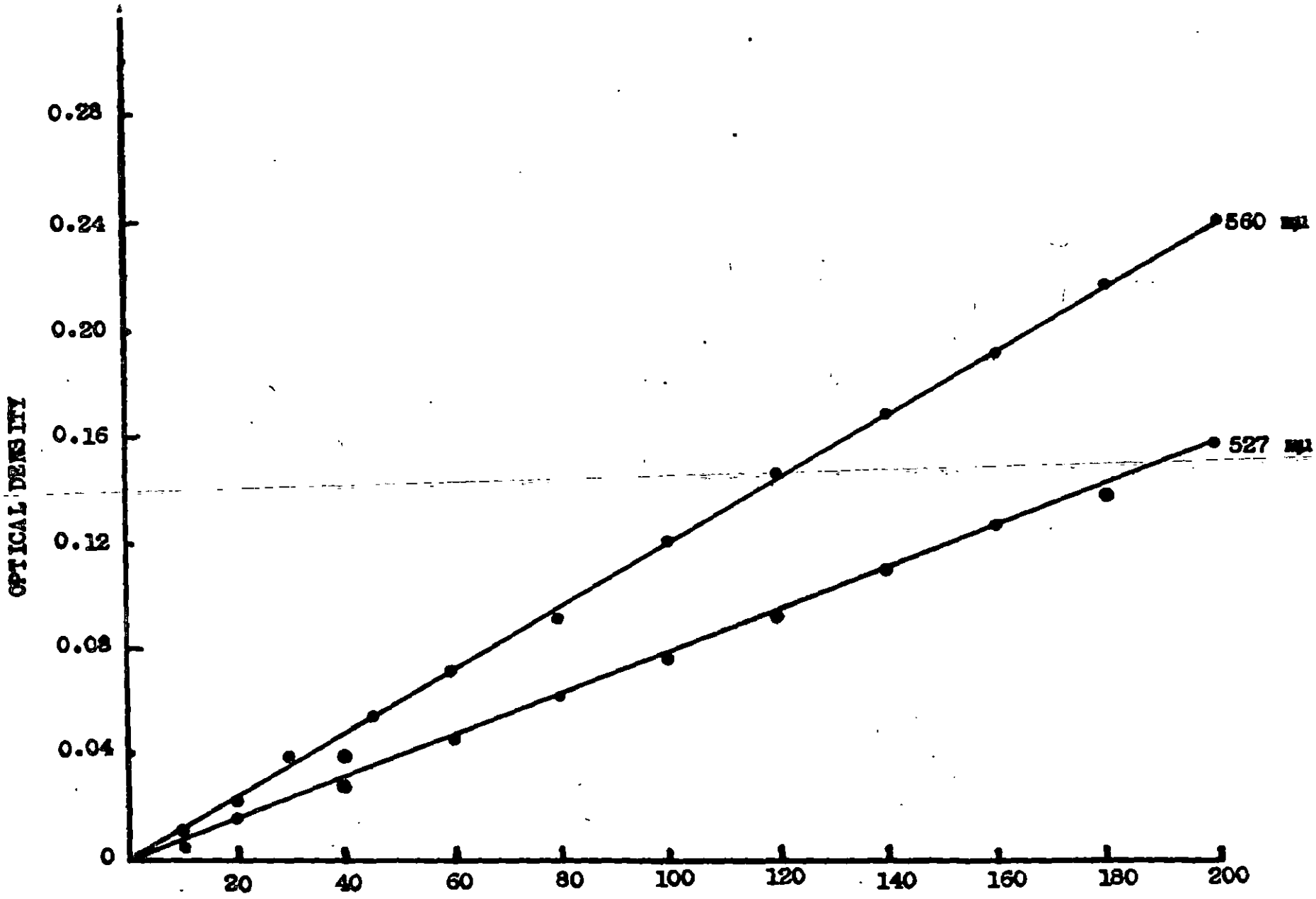

Fig.4. CALIBRATION CURVE OF PYRIDINE HAEMOCHROMOGEN FROM CRYSTALLINE HAEMIN OF OX BLOOD(μg / 25 ml.)

TABLE 4.

LIGHT ABSORPTION IN THE GREEN REGION OF THE SPECTRUM BY SOLUTIONS OF PYRIDINE HAEMOCHROMOGEN.

Haematin solutions: 25.0 mg. haemin was dissolved in 2.5 ml. N.NaOH and diluted with distilled water to 25 ml.

1 ml. of haematin solution was placed in each of two 25 ml. volumetric flasks treated with pyridine, reduced with 0.25 g. sodium dithionite ($Na_2S_2O_4$) and diluted with distilled water to 25 ml. The optical density was measured against blanks containing all but the haematin in a Hilger "Uvispek" photoelectric Spectrophotometer (1 Cm. cells, path length 5 mm.) at the α-band (560 mμ) and the β-band (527 mμ) maxima.

Haemin (μg/25 ml).	Optical density at α-band maximum.	Optical density β-band maximum.
10	0.011	0.006
20	0.022	0.014
30	0.039	0.031
40	0.040	0.028
60	0.071	0.046
80	0.092	0.062
100	0.120	0.077
120	0.146	0.093
140	0.168	0.110
160	0.190	0.125
180	0.214	0.136
200	0.269	0.180