EXPERIMENT (8)

8 Haemoglobin ,Anaemia ,HCT and ESR

8.1 Haemoglobin

8.1.1 Introduction

8.1.1.1 Haemoglobin Synthesis

The circulating blood of a normal adult man contains about 750 gm of **haemoglobin**, and of this about 1/12o or 7 – 8 or 7 – 8 gm are degraded daily. This amount has to be newly synthesized each day because:

- The globin part of haemoglobin can be reutilized only after catabolism into its constituent amino acids,
- the haem moiety is broken down into bile pigment, which is excreted,
- iron alone is reutilized in the synthesis of Haemoglobin.

The rates at which haemoglobin is synthesized and at which red cells are formed, are related to the oxygen content of the blood. And therefore depend not only upon the amount of oxygen reaching the blood but also upon the capacity of the blood to carry oxygen, which in turn depends on the amount of circulating haemoglobin. Therefore, haemoglobin synthesis is stimulated by anoxia, whether due to oxygen deficiency or due to anaemia. The erythrocytes are derived from primitive nucleated cells in the bone marrow by successive processes of mitosis and maturation.

A primitive stem cell divides to form two cells, one of which retains its behavior as a stem cell while the other successively divides to form two basophile normoblasts, four polychromatic normablasts and eight ortho-chromatic normoblasts, after which maturation through late normoblast and reticulocyte stages to the mature non-nucleated fully haemoglobionized erythrocyte involves

no further mitotic division. These processes must involve the biosynthesis not only of haemoglobin, but also of large quantities of purine bases, nucleic acids and protein. The ability of the haemopoietic tissues to manufacture erythrocytes depends on a variety of hormones, trace metals, enzymes, coenzymes and an adequate provision of essential amino acids, glycine, acetyl coenzyme A and iron. There is a strong evidence that the marrow response to the stimulus of hypoxia is dependent upon glycoprotein hormone, erythropoietin, which in response to hypoxia may act on differentiation of the stem cells rather than upon any particular step in haemoglobin synthesis. Erythropoietin is formed in the kidney from a prohormone (erythrogenin) by the action of a plasma factor synthesized in the liver.

8.1.1.2 HaemoglobinCatabolism

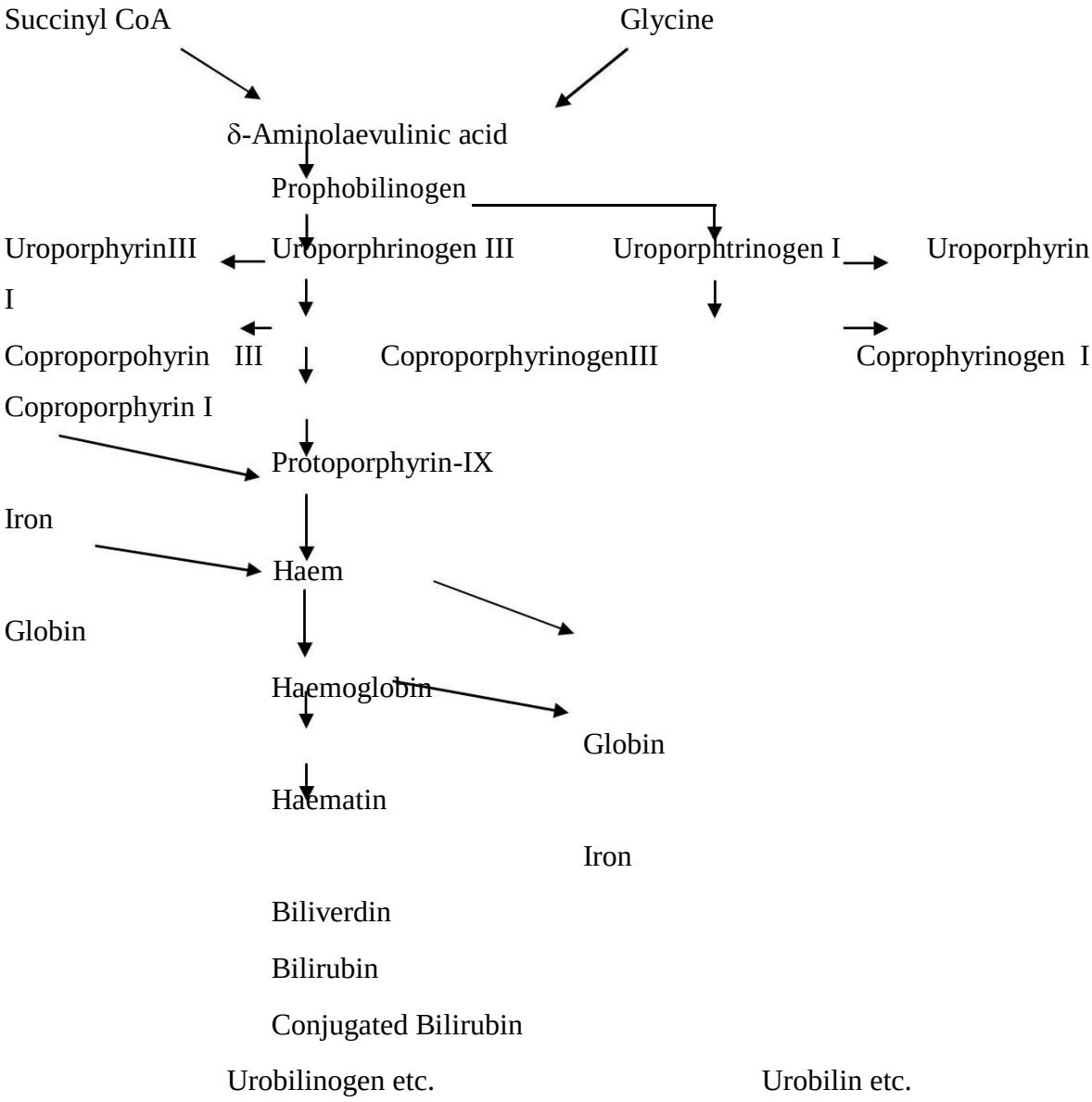
1- In the reticuloendothelial system, erythrocytes are destroyed and the haemoglobin is released.

2- Some haem is released in the narrow during erythroblast maturation or from the dead cells of ineffective erythropoiesis.

3- Globin is separated from haem, and haematin is formed in which the iron of the harm is oxidized to [ferric] iron (III).

4- The porphyrin ring is then opened and the iron is removed with the formation of the straight chain compound biliverdin, which is converted to bilirubin by reduction. A minor pathway first opens the ring to form choleglobin and then removes the iron and globin to produce biliverdin globin and then biliverdin.

5- The iron and the amino acids of the globin, are retained, the pyrrole rings are eventually excreted as bilirubin.



The principal steps in the synthesis and degradation of Haemoglobin

8.1.1.3.Role of Vitamins, Trace Metals and Cofactors Deficiencies in Producing HumanDiseases

1- Biotin, pantothenic acid, pyridoxal phosphate and coenzyme A; are essential coenzymes required for the synthesis of haem. The deficiency of pyridix I phosphate plays a role in human disease while the deficiency of folic acid can cause megaloblastic anaemia.

2- Of the trace metals, only copper and cobalt are known to play a role. Copper is playing a role in the absorption of ion, while Cobalt is an essential constituent of vitamin B_{12}.

3- Deficiency of intrinsic factor can cause vitamin B_{12} deficiency, with abnormal maturation of red cells leading to a megaloblastic stage and consequent failure to liberate sufficient red cells to maintain a normal amount of circulating haemoglobin.

I) Iron-Deficiency Anaemia

Deficiency of iron is essentially due to blood loss with failure to replace the iron stores because of dietary deficiency, increased requirement or defective absorption; plasma iron is low, the iron-binding capacity normal but percentage saturation is low. Microcytes containing a subnormal quantity of haemoglobin may be released into the circulation, and be ineffective in raising the haemoglobin level to normal. Accompanying changes include brittleness of the nail and atropy of mucous membranes.

II) Megaloblastic Anaemia

This may be due to deficiency of folic acid or cobalamin (vitamin B_{12}) both of which acts as coenzymes and exist in various forms within the body. In megaloblastic erythropoiesis, there is general disturbance of metabolism which leads not only to the characteristic megaloblastic marrow but also to lesions of the

oral, gastrointestinal and vaginal epithelium. Deficiency of both cobalamins and folate has aroused much interest with regard to CNS function, which is affected in deficiencies of two other members of the vitamin B group, nicotinamide and thiamine. Thus psychoses, confusion and depression as well as sub acute combined degeneratin occur in cobalamin deficiency while there is an association between anticonvulsant drug therapy for epilepsy and later development of the folate deficiency with megaloblastosis. The effectiveness of anticonvulsant therapy is interfered with by folate leading to an increase in fit frequency.

III) Membrane Defects

In these conditions there is a defect of the erythrocyte membrane and an abnormally in the sodium pumps: the fundamental causes have not been elucidated. The best-known disorders are hereditary spherocytosis and hereditary elliptocytosis.

8.1.1.4 Glucose-6-phosphate dehydrogenate (G6PD) deficiency [non-spherocytic]

This is relatively common, especially in negroes, south Chinese, and Mediterranean people, and may protect against malaria. G6PD is the enzyme responsible for the initial deviation of glucose into pentose phosphate pathway to form 6-phosphogluconate. This pathway provides $NADPH_2$ in the erythrocyte for the conversion of oxidized reduced glutathione and for other reactions such as reduction of methaemoglobin.

Deficiency of G6PD

- The enzyme deficiency may cause haemolysis, but chiefly occurs after sensitization of the erythrocyte by a wide variety of agents e.g. primaquine, broad beans (favism) or in infections.

- The cells accumulate methaemoglobin and are deficient in reduced glutathione which is necessary for cell integrity. Haemolysis, dark urine and jaundice are present.
- In homozygote, enzyme activity is reduced to less than 15% of normal.
- The deficiency of G6PD may also produce neonatal jaundice.

Normal Range

Men	14 – 18 g/dl
Women	12 – 16 g/dl

8.1.2 Principle

The Fe(II) atom in each of the haem in the red haemoglobin is oxidized by ferricyanide to Fe(III)-methaemoglobin. A cyanide group is then attached to the iron atom (which is positively charged) by reaction with KCN to give the brown cyanmethaemoglobin. Qyanmethaemoglobin can be estimated quantitatively.

Haemoglobin + Cyanide + Ferricyanide →

Cyanmethaemogloibn

8.1.3 Material

- Potassium hexacyanoferrate (III) solution

Potassium hexacyanoferrate (III)0.6 mmol/l

Potassium phosphate buffer 0.5 mmol/l, pH 7.20

-Potassium cyanide solution

Potassium cyanide 0.75 mmol/l

-Potassium phosphate buffer2.50 mmol/l, pH 7.20

-Working Solution

Mix equal volumes of both reagents 1 and 2.

-Sample Preparation

Use whole blood immediately. Heparinized blood and EDTA blood can be stored up to 4 days at +4 to 25^{o}C.

-Caution

All solutions are poisonous. Use safety pipettes.

-Requirements

Wavelength	546 nm
Cuvette	1 cm light path
Incubation temp.	20-25$_{o}$C
Measure against	redist. Water

8.1.4 Procedure

Pipette into two dry clean test tubes

	Blank	**Test**
Working Solution	---	5.00 ml
Blood Sample	---	0.02 ml
Redist. Water	5.00 ml	---

Flush pipette thoroughly with the working solution.

Mix solution well, and incubate at 20-25^{o}C for 5 min.

Measure the absorbance of the sample (A_{HGB}).

8.1.5 Calculation

C_{HGB} = 36.77 x A_{HGB} [g/dl]

= 22.82 x A_{HGB} [mmol/l]

- **Note:**

  You can obtain the concentration of HGB in your sample from the attached table of values.

Table of values for measurement at Hg 546 nm

Absorbance A	Haemoglobin			Absorbance A	Haemoglobin		
	g/100 ml	Hb/4 mmol/l	%		g/100 ml	Hb/4 mmol/l	%
0.100	3.7	2.3	23.1	0.400	14.7	9.1	91.8
105	3.9	2.4	24.1	405	14.9	9.2	93.2
110	4.0	2.5	25.2	410	15.1	9.4	94.3
115	4.2	2.6	26.4	415	15.3	9.5	95.4
120	4.4	2.7	27.6	420	15.5	9.6	96.6
125	4.6	2.9	28.7	425	15.6	9.7	97.7
130	4.8	3.0	29.9	430	15.8	9.8	98.7
135	5.0	3.1	31.1	435	16.0	9.9	99.9
140	5.1	3.2	31.7	440	16.2	10.0	101.1
145	5.3	3.3	33.2	445	16.4	10.2	102.3
150	5.5	3.4	34.5	450	16.6	10.3	103.4
155	5.7	3.5	35.6	455	16.7	10.4	104.3
160	5.9	3.7	36.7	460	16.9	10.5	105.6
165	6.1	3.8	37.9	465	17.1	10.6	107.8
170	6.3	3.9	38.9	470	17.3	10.7	108.0
175	6.4	4.0	40.1	475	17.5	10.8	109.1
180	6.6	4.1	41.1	480	17.7	11.0	110.4
185	6.8	4.2	42.5	485	17.8	11.1	111.5
190	7.0	4.3	43.6	490	18.0	11.2	112.5
195	7.2	4.5	44.8	495	18.2	11.3	113.7
0.200	7.4	4.6	45.9	0.500	18.4	11.4	114.8
205	7.5	4.7	47.1	505	18.6	11.5	116.0
210	7.7	4.8	48.2	510	18.8	11.6	117.1

215	7.9	4.9	49.4	515	18.9	11.8	118.3
220	8.1	5.0	50.6	520	19.1	11.9	119.5
225	8.3	5.1	51.7	525	19.3	12.0	120.6
230	8.5	5.2	52.8	530	19.5	12.1	121.9
235	8.6	5.4	54.0	535	19.7	12.2	123.0
240	8.8	5.5	55.1	540	19.9	12.3	124.0
245	9.0	5.6	56.2	545	20.0	12.4	125.3
250	9.2	5.7	57.4	550	20.2	12.6	126.4
255	9.4	5.8	58.6	555	20.4	12.7	127.6
260	9.6	5.9	59.8	560	20.6	12.8	128.7
265	9.7	6.0	60.9	565	20.8	12.9	129.7
270	9.9	6.2	62.0	570	21.0	13.0	131.0
275	10.1	6.3	63.2	575	21.1	13.1	132.1
280	10.3	6.4	64.3	580	21.3	13.2	133.3
285	10.5	6.5	65.5	585	21.5	13.4	134.5
290	10.7	6.6	66.6	590	21.7	13.5	135.6
295	10.9	6.7	67.8	595	21.9	13.6	136.6
0.300	11.0	6.8	68.9	0.600	22.1	13.7	138.3
305	11.2	7.0	70.1	605	22.2	13.8	139.0
310	11.4	7.1	71.3	610	22.4	13.9	140.3
315	11.6	7.2	72.3	615	22.6	14.0	141.4
320	11.8	7.3	73.5	620	22.8	14.2	142.5
325	12.0	7.4	74.7	625	23.0	14.3	143.6
330	12.1	7.5	75.8	630	23.2	14.4	144.6
335	12.3	7.6	77.0	635	23.3	14.5	145.8
340	12.5	7.8	78.2	640	23.5	14.6	147.8
345	12.7	7.9	79.2	645	23.7	14.7	148.2
350	12.9	8.0	80.5	650	23.9	14.8	149.3
355	13.1	8.1	81.6	655	24.1	15.0	150.5
360	13.2	8.2	82.7	660	24.3	15.1	151.5
365	13.4	8.3	83.9	665	24.5	15.2	142.8
370	13.6	8.4	85.0	670	24.6	15.3	154.0
375	13.8	8.6	86.2	675	24.8	15.4	155.3

380	14.0	8.7	87.4	0.680	25.0	15.5	156.3
385	14.2	8.8	88.4				
390	14.3	8.9	89.6				
0.395	14.5	9.0	90.8				

8.2 Determination of Hematocrit (HCT)

8.2.1 Introduction

Hematocrit, or packed cell volume (PCV), determination is part of the daily routine in hematological laboratories. It is used as a simple screening test for anemia and is used in conjunction with the mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC). Blood is collected in heparinised capillary tube, which is then sealed, centrifuged and the red cell volume expressed as a percentage of the whole blood.

8.2.2 Procedure

1- The blood of a colleague may be taken in a heparinised capillary tube or a blood sample in such a tube may be used which has been taken not more than 6 hours before and stored at 4^{o}C.

2- Seal the dry end of the tube, and centrifuge for 5 min.

3- The column of red cells will be seen, topped by the grayish-red layer of leukocytes and above this a thin creamy layer of platelets, the “Buffy coat”.

4- Measure the length of the column of red blood cells (A), and the total length of blood components (B).

$$\text{Haematocrit} = \frac{A}{B}$$

Normal Range Men 0.40 – 0.54

Women 0.37 – 0.47

8.3 C. Determination of Erythrocyte Sedimentation Rate [ESR]

8.3.1 Principle

- ESR is the rate at which erythrocytes settle out of anticoagulated blood in 1 hour. This test is based on the fact that inflammatory and necrotic processes cause an alteration in blood proteins, resulting in an aggregation of red cells, which make them heavier and more likely to fall rapidly when placed in a special vertical tube.
- ESR is dependent on the plasma concentration of asymmetric macromolecules such as globulins, fibrinogen, besides the concentration of red cells.
- ESR is used clinically as a non-specific screening test to detect the presence of infection in the body in general. It is used as well as a means of monitoring the status of chronic inflammatory disease such as rheumatoid arthritis.
- ESR is not diagnostic of any particular disease, but rather is an indication that a disease process is ongoing and must be investigated.

8.3.2 Procedure

In this technique, cells are allowed to sediment under the effect of gravity, using a Westergren‟s tube. 106 cc of blood is drawn out from the vein of a subject by a syringe. Transfer it into an EDTA tube, and thend raw the blood up into a Westergren‟s tube exactly to the zero mark. The tube is placed upright in the rack and left undisturbed. The length of the column of clear plasma at the top is noted at the end of 1 hour and again at he end of 2 hours.

Normal Range

	After 1 hour	**After 2 hours**
Men	0 – 5 mm	7 – 15 mm
Women	0 – 10 mm	10 – 20 mm

8.3.3 References Ranges

	Male	**Male/Female**	**Female**	**Units**
RBC	4.2 – 5.5		3.7 – 5.0	10^{12}/l
HCT	38 – 48		36 – 46	%
MCV		80 – 100		Fl
RDW		11.5 – 15		%
HGB	135 – 165		115 – 140	g/l
MCH		28– 35		Pg
MCHC		330– 360		g/l
PLT		150– 380		10^{9}/l
MPV		7.5 – 10.5		Fl
WBC		4.0– 9.0		10^{9}/l
LYM		1.2– 3.5		10^{9}/l
MID		0.1– 0.6		10^{9}/l
GRA		1.4– 7.0		10^{9}/l
LYM		20– 48		%
MID		2 – 10		%
GRA		42– 80		%
Bands		0– 5		%
Neutrophils		55– 65		%

Eosinophils	1 – 5	%
Basophils	0 – 1	%
Lymphocytes	22 – 35	%
Monocytes	3 – 8	%
Reticulocytes	0.2 – 2.0	%

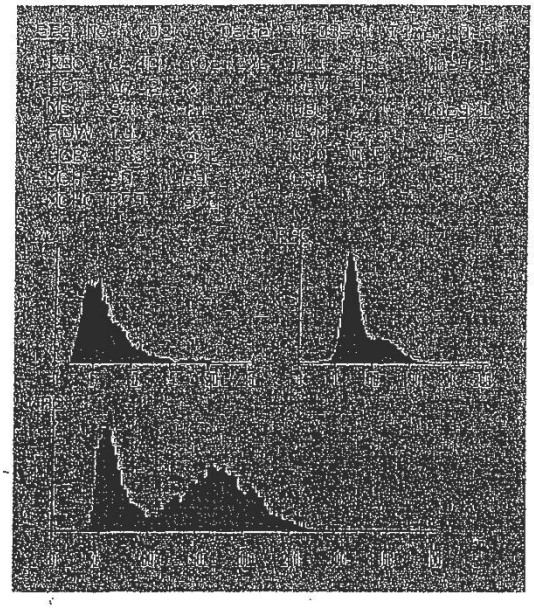
Abbreviations

RBC	Red Blood Cells
HCT	Haematocrit
MCV	Mean Cell Volume
RDW	Red Cell Distribution Width
HGB	Haemoglobin
MCH	Mean Cell Haemoglobin Concentration
MCHC	Mean Cell Haemoglobin Concentration
PLT	Platelets
MPV	Mean Platelet Volume
WBC	White Blood Cells
LYM	Lymphocytes
MID	Midcells
GRA	Granulocytes

fl	femtolitre	10^{-15} litre
pg	picogram	10^{-12} gram

8.3.4 Normal Sample

Haematologically healthy individual. The blood sample was taken with K_3EDTA as the anti-coagulant and analysed 30 min after venepuncture. It is important to allow the blood sample to adapt to the EDTA-environment. The sample cannot be kept too long before analysis otherwise the leukocyte differential count will be erroneous. The analysis should take place 15 min – 8 hours after drawing. If the particle concentration alone is requested then analysis can be performed up to 24 hours after drawing. It is essential that the sample is not older than 8 hours if a leukocyte differential count is requested.



The cytogram presents 16 parameters as numbers and 3 histograms.

RBC	Red Blood Cells
HCT	Haematocrit
MCV	Mean Cell Volume
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HGB	Haemoglobin
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Concentration
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WBC	White Blood Cells
LYM	Lymphocytes
MID	Midcells (monocytes)
GRA	Granulocytes

Red Blood Cell Histogram shows the distribution of erythrocytes between 35 and 250 fl. The low peak to the right shows erythrocytes which have passed the measuring point at the same time, usually two or three together (doublets and triplets respectively) These large "cells" are not included in the calculation of mean cell volume but are nevertheless presented in the histogram. *Further information is to be found in the instruction manual.*

Platelet Histogram shows the size distribution of the platelets. In normal cases this lies between 3 and 20 fl and has a positive skew shape. *Further information is to be found in the instruction manual.*

White Blood Cell Histogram shows three different populations within the 35 – 420 fl area.

LYM	35 – 85 fl	lymphocytes	Nucleated red blood cells, clumped platelets, macrocytic platelets, atypical lymphocytes, blasts)
MID	85 – 115 fl	monocytes	(atypical lymphocytes, blasts, immature granulocytes, plasma cells, eosinophils, basophils, precursor cells, agranular neutrophils, hyposegmented granulocytes)
GRA	115 – 420 fl	granulocytes	(eosinophils, bands, immature granulocytes, hypersegmented granulocytes)

Index calculation

HCT	=	RBC x MCV	%
MCH	=	HGB / RBC	pg
MCHC	=	HGB / HCT	g/l
RDW	=	SD / MCV	%

	Male	Male/Female	Female	Units
RBC	4.2 – 5.5		3.7 – 5.0	10^{12}/l
HCT	38 – 48		36 – 46	%
MCV		80 – 100		Fl
RDW		11.5 – 15		%
HGB	135 – 165		115 – 140	g/l
MCH		28– 35		Pg
MCHC		330– 360		g/l
PLT		150– 380		10^{9}/l
MPV		7.5 – 10.5		Fl
WBC		4.0– 9.0		10^{9}/l
LYM		1.2– 3.5		10^{9}/l
MID		0.1– 0.6		10^{9}/l
GRA		1.4– 7.0		10^{9}/l
LYM		20– 48		%
MID		2 – 10		%
GRA		42– 80		%
Bands		0– 5		%
Neutrophils		55– 65		%
Eosinophils		1– 5		%
Basophils		0– 1		%
Lymphocytes		22– 35		%
Monocytes		3– 8		%
Reticulocytes		0.2– 2.0		%

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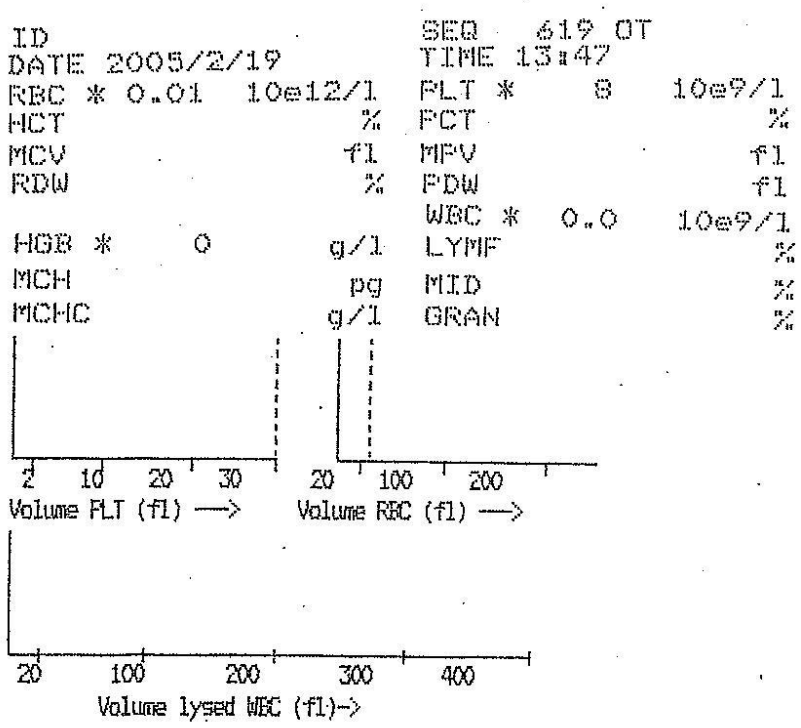

8.4 References

- Dacie .J.V and Lewis.S.M. Practical Haematology, Ninth Edition, 2001. Churchill-Livingston
- Russell. N J; Powell. G. M; Jones J .G., Winterburn P J and Basford J M. 1982. Blood Chemistry. Croom Helm, London and Canberra.