Chapter 3

RESULTS

3.1 Experiments to Find Out the Optimum Conditions for Electrophoresis

Although Van der Helm (1961) showed that there was a relationship between electrophoretic pattern of plasma proteins and that of LDH isoenzymes, wide variation in the conditions used in electrophoresis of LDH isoenzymes had been reported by several investigators (Cawley and Eberhardt, 1962; Ressler and Joseph, 1962; Van Der Helm et al, 1962; Wieme, 1966; Emery, 1967). In this part of the thesis, the experiments were performed in order to study the effect of the following parameters on the electrophoresis of plasma proteins.

a. concentration of Difco Special Agar-Noble

b. molarity of barbital buffer, pH 8.6

c. temperature

d. time

e. point of application of plasma samples

f. current applied

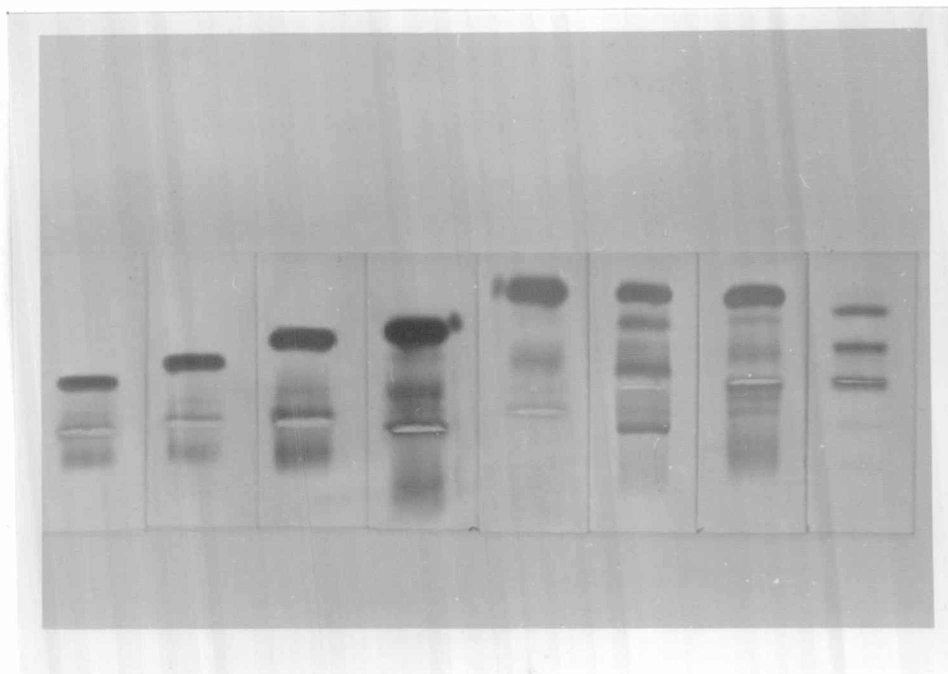
The various conditions used in the studies are given in Table 2 and the results obtained are shown in Figure 1. Ten microlitres of plasma were used throughout the experiments.

It can be seen that an increase in the percentage of the agar concentration from 0.4 - 0.7 % w/v to 0.8 - 1.5 % w/v and a change of the barbital buffer concentration from 0.025 - 0.035 M to 0.05 M

TABLE 2

Various conditions used in the electrophoresis of plasma proteins.

Number	Concentration of Agar (% w/v)	Concentration of Barbital buffer, pH 8.6 (M)	Temperature (°C)	Current mA./slide	Application point (cm from anode)	Distance of albumin from application point (cm.)	Time (hr)
1	0.4, 0.5, 0.7	0.025	0,5, 10, 15	7, 10, 15, 20	5	1 - 1.5	2
		0.035	0,5, 10, 15	7, 10, 15, 20	5	1 - 1.5	2
2	0.4, 0.5, 0.7	0.05	25, 37	7, 10, 15, 20	5	1.5 - 2	2
3	0.8,1.0,1.2,1.5	0.025	0,5, 10, 15	7	5	2 - 2.5	2
	0.8,1.0,1.2,1.5	0.035	0,5, 10, 15	7	5	2 - 2.5	2
4	0.8,1.0,1.2,1.5	0.05	0,5, 10, 15	7	5	2 - 3	2
5	0.8,1.0,1.2,1.5	0.025	25, 37	7	4.5	2.5 - 3	1½
6	0.8,1.0,1.2,1.5	0.05	25	7	4	2.5 - 3	1½
7	0.8,1.0,1,2,1.5	0.05	25	7	4	2.5	1
8	0.8,1.0,1.2,1.5	0.05	25	7	4	2.5	1



Number 1 2 3 4 5 6 7 8

Figure 1 The electrophoretograms of plasma proteins and LDH isoenzymes obtained from electrophoresis under different conditions. The conditions used are summarized in Table 2.

Number 1 - 4 are electrophoretograms of plasma proteins stained with bromphenol blue.

Number 5 - 7 are electrophoretograms of plasma proteins stained with Ponceau S.

Number 8 is an electrophoretogram of LDH isoenzymes stained with the solution 5.3.3 (page 21).

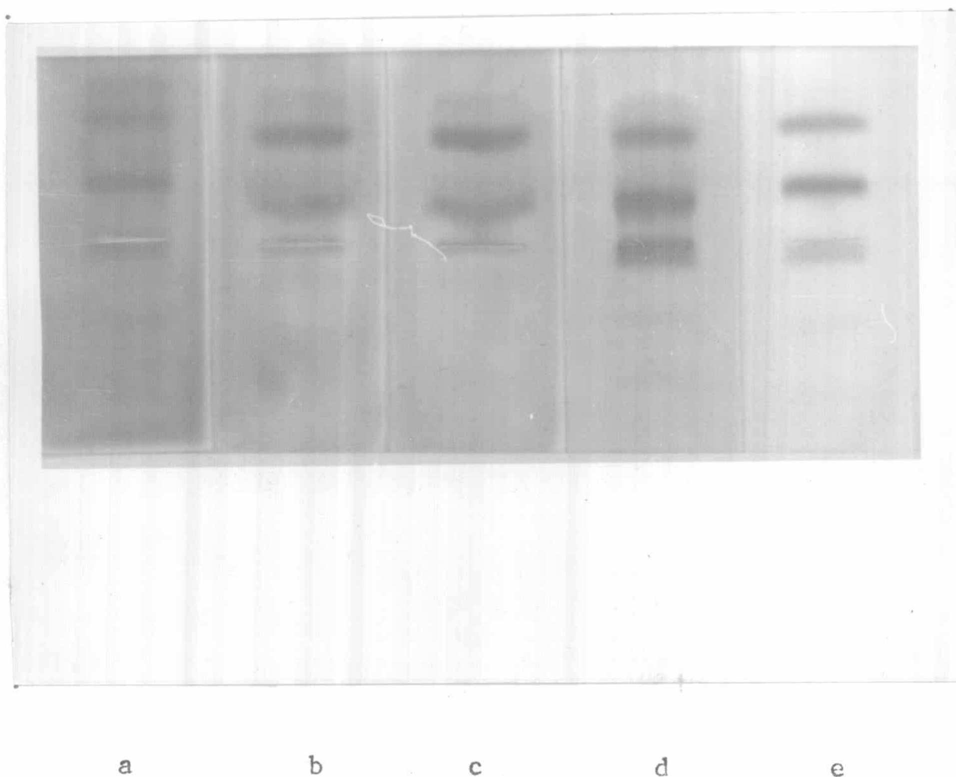
increased the mobility of the plasma proteins. The migration was also affected by a change in the temperature. An increase in the temperature from 0°- $15^{o}C$ to 25 ^{o}C decreased the electrophoresis time and also improved the separation of the plasma proteins.

It appeared from Figure 1 that the best separation of the plasma proteins and LDH isoenzymes was obtained when the experiment was carried out with 0.8 % w/v of Difco Special Agar-Noble, 3 ml of agar/slide, 0.05 M barbital buffer, pH 8.6 at $25^{o}C$ for 60 minutes with a current of 7 mA./slide. These conditions were used throughout the experiment.

3.2 A Comparison of Different Methods for the Staining of LDH Isoenzymes

The formulae of the staining solution of LDH isoenzymes given by Van Der Helm (1962), Yakulis et al (1962), Mull and Starkweather (1965), Cawley (1969), and Fritz et al (1970) were tried. The results obtained are shown in Figure 2. The lithium lactate in the Fritz et al (1970)'s formula was replaced by sodium lactate because of the difficulty in getting the former compound.

Although it can be seen that the Fritz et al (1970)'s formula gave the best result, Figure 3 showed that a better stain could be obtained when a glycine buffer, pH 8.7, was used instead of glycine buffer, pH 10.0



a b c d e

Figure 2 Electrophoretograms comparing different methods used for the staining of LDH isoenzymes.

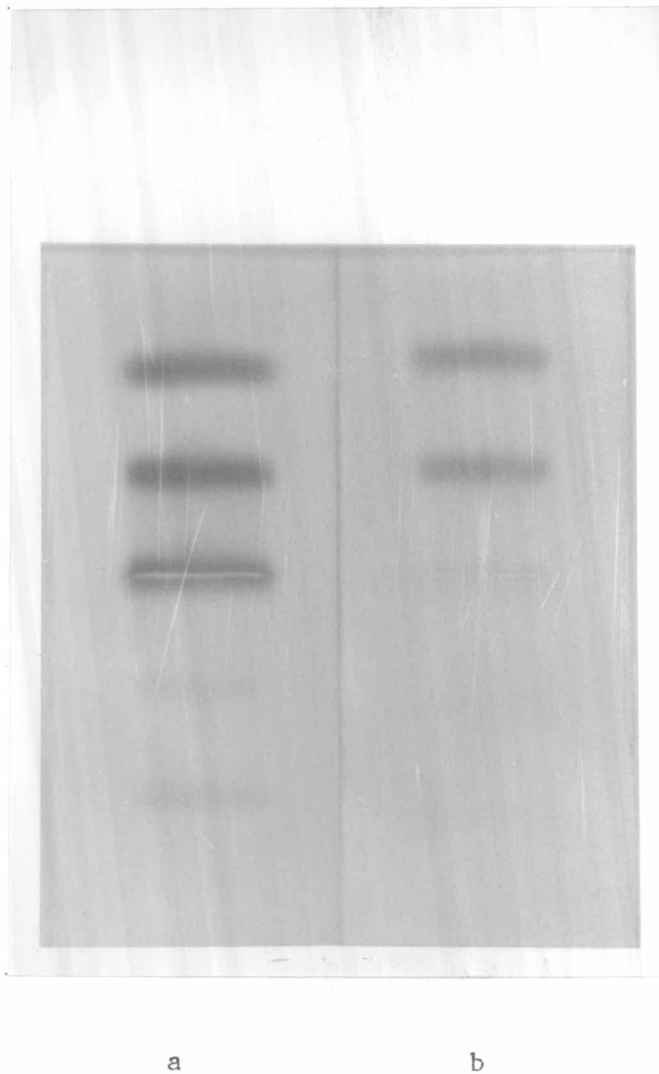
a. Yakulis et al (1962)

b. Van Der Helm (1962)

c. Mull and Starkweather (1965)

d. Cawley (1969)

e. Fritz et al (1970) slightly modified



a b

<u>Figure 3</u> Electrophoretograms comparing the staining solution of Fritz <u>et al</u> (1970) in glycine buffer

a. pH 8.7

b. pH 10.0

3.3 A Comparison of Different Methods for the Staining of Lipoprotein

In this experiment, different staining methods described by several investigators were compared. They were Cawley (1969), Papadopoulos and Kintzois (1969), and Dyerberg and Hjorne (1970). Figure 4 showed that the method of Dyerberg and Hjorne (1970) gave better stain than those of Cawley (1969) and Papadopoulos and Kintzois (1969).

3.4 Fixation of LDH Isoenzymes, Proteins, and Lipoproteins

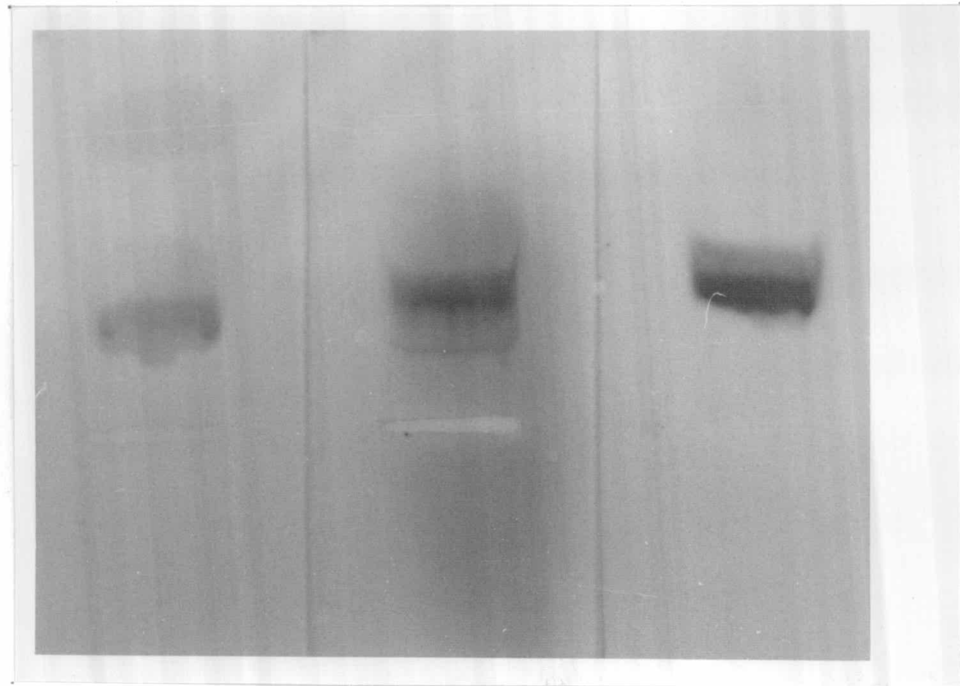
The following fixing solution were used:

1. 95% ethanol
2. 5% acetic acid in 75% ethanol
3. 10% acetic acid in methanol
4. Solution of methanol:water:glacial acetic acid=5:5:1 v/v.

The electrophoretograms of LDH isoenzymes were fixed after they had been stained and destained and it was found that a mixture of methanol:water:glacial acetic acid gave satisfactory results for the fixing of LDH isoenzymes.

The electrophoretograms of lipoproteins and proteins were fixed before the staining process and all the solutions were tried for the fixation of protein and lipoprotein electrophoretograms. Similar results were obtained but a solution of 10% acetic acid in methanol required the shortest time. Of interest was that the drier the gel, the easier in staining and destaining process of proteins and lipoproteins.

There was no difference when the fixed electrophoretograms were dried under filter paper either at room temperature or in an oven at 37°C.



a b c

Figure 4 Electrophoretograms comparing results obtained when lipoproteins were stained with

a. Oil Red O (Papadopoulos and Kintzois, 1969)

b. Sudan Black B (Cawley, 1969)

c. Sudan Black B in zinc acetate (Dyerberg and Hjorne, 1970)

3.5. Simultaneous Determination of LDH Isoenzymes and Plasma Proteins in Normal Subjects

The results obtained from 3.1, 3.2, 3.3 and 3.4 suggested that the conditions which should be used for the electrophoresis of the LDH isoenzymes in plasma samples were as those described on page 23.The selected methods for the staining of plasma LDH isoenzymes, proteins, and lipoproteins were described in the materials and methods section.

Figure 5 shows a typical electrophoretogram obtained by simultaneous separation of LDH isoenzymes and proteins in plasma of a normal subject showing the relative location of the plasma protein zones to those of LDH isoenzymes. The fastest migrating isoenzymes, LDH-1 lies between the albumin and the alpha$_1$-globulin. LDH-2 moves as far as the fastest part of alpha$_2$-globulin. LDH-3 lies between beta$_1$-and beta$_2$-globulin whereas LDH-4 is infront of gamma-globulin. The slowest migrating isoenzymes, LDH-5 is behind the gamma-globulin.

3.6 Patterns of LDH Isoenzymes Found in Normal Subjects

Although five bands of the LDH isoenzymes as shown in Figure 5 were normally detected both in electrophoretograms of plasma samples from normal subjects and patients, two other patterns with four and three bands of the isoenzymes were also found in electrophoretograms obtained from plasma samples of the former cases. The relative activity of the isoenzymes was determined by scanning the stained electrophoretograms in a densitometer. The patterns obtained are shown in Figure 6. The activity of each isoenzyme was calculated from the area under each

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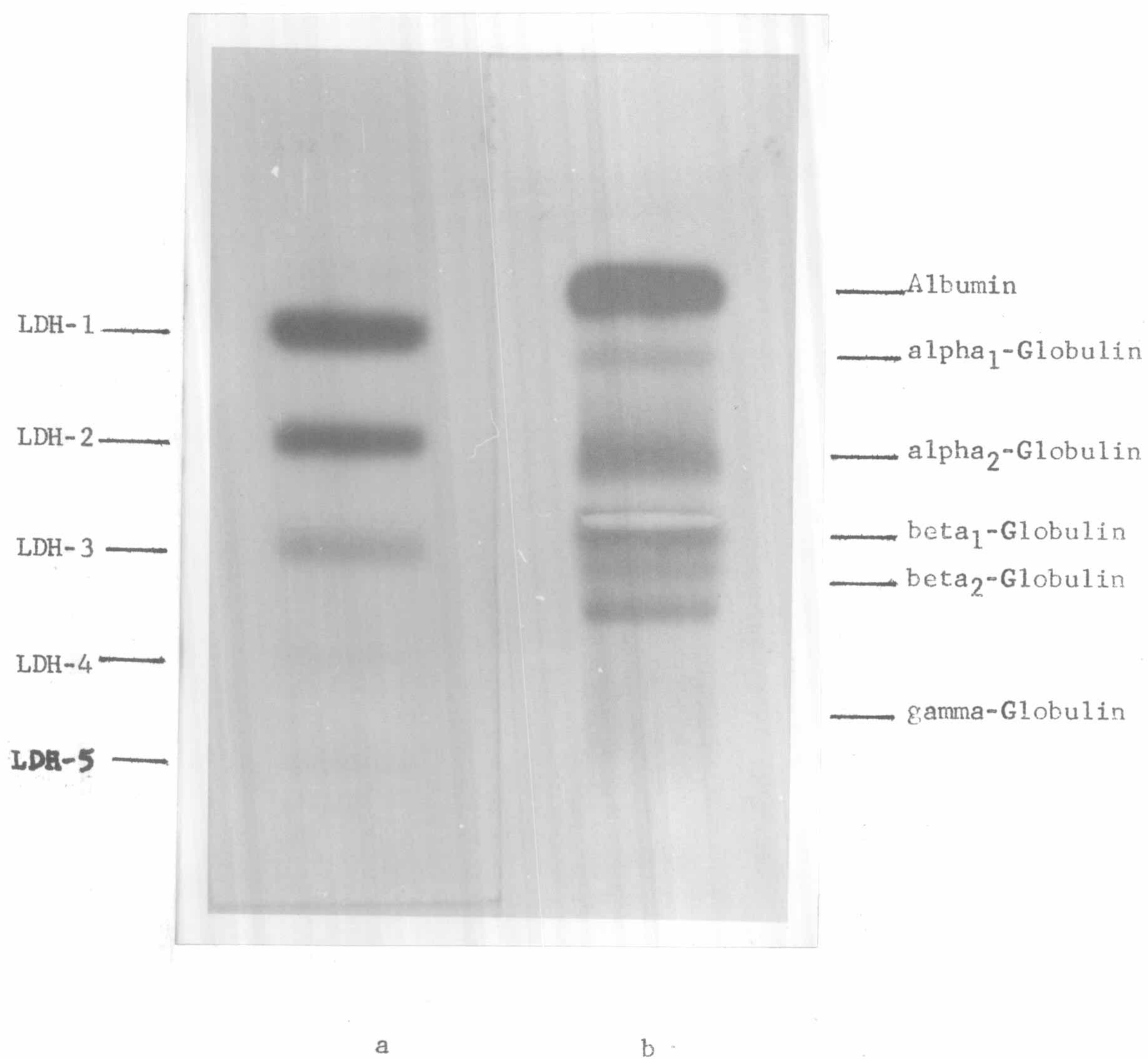


Figure 5 The relative locations of plasma LDH isoenzymes (a) to that of proteins (b). The details of the experimental procedures were described on page 23.

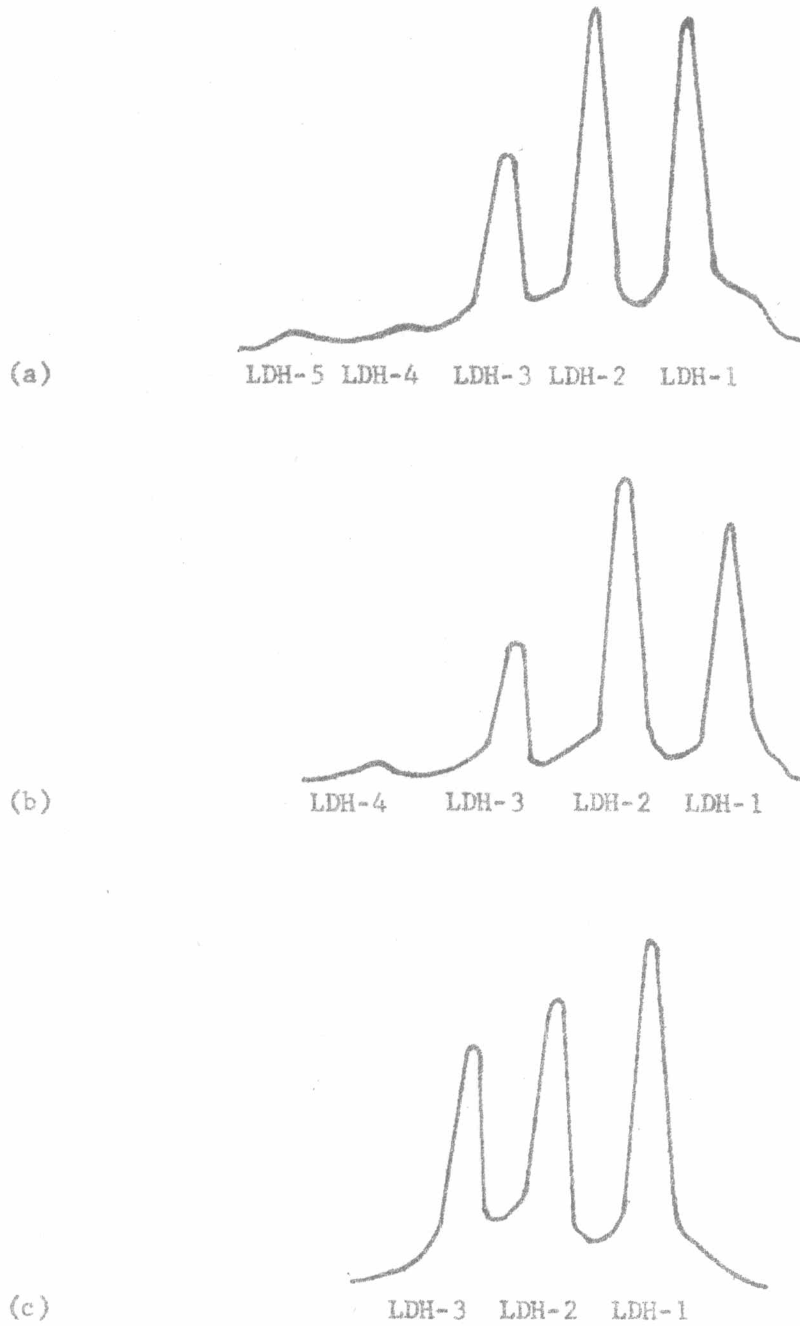


Figure 6 Different electrophoretic patterns of LDH isoenzymes peaks found in normal subjects

a. 5 peaks, the most common pattern

b. 4 peaks

c. 3 peaks

peak as a percentage of the total area under peaks. The total activity was determined spectrophotometrically as described below.

3.7 Determination of the Activity of LDH Isoenzymes

The activity of TLDH, ULDH, and HLDH were determined spectrophotometrically using lactate as a substrate. The activity was obtained by measuring the changes in the optical density of the nicotinamide adenine dinucleotide coenzyme at 340 nm.

Figure 7 shows the results obtained from the determinations of LDH isoenzymes activity in plasma samples of a normal subject and a subject with myocardial infarction.

Plasma TLDH activity in the normal subjects was found to be 121 ± 35 units in men (19 cases) and 88 ± 12 units in women (13 cases). The TLDH activity was suppressed when the plasma were treated with 2 M urea, and at 60°C for 60 minutes (see Table 3).

3.8 Studies on Changes of LDH Isoenzymes Activities in Patients with Myocardial Infarction

The changes of LDH isoenzymes activities were studied in 22 patients, using plasma samples taken on day 1, 2, 3, 5, 10, and 15 after the onset of the attack of myocardial infarction. The results are given in Table 3 and they are shown graphically in Figures 9, 10 and 11.

Figure 8 shows the electrophoretograms from plasma of a patient compared with the plasma proteins and LDH isoenzymes of a normal subject. The electrophoretograms showed that the changes were apparent within 24 hours after the onset of attack and this elevation persisted

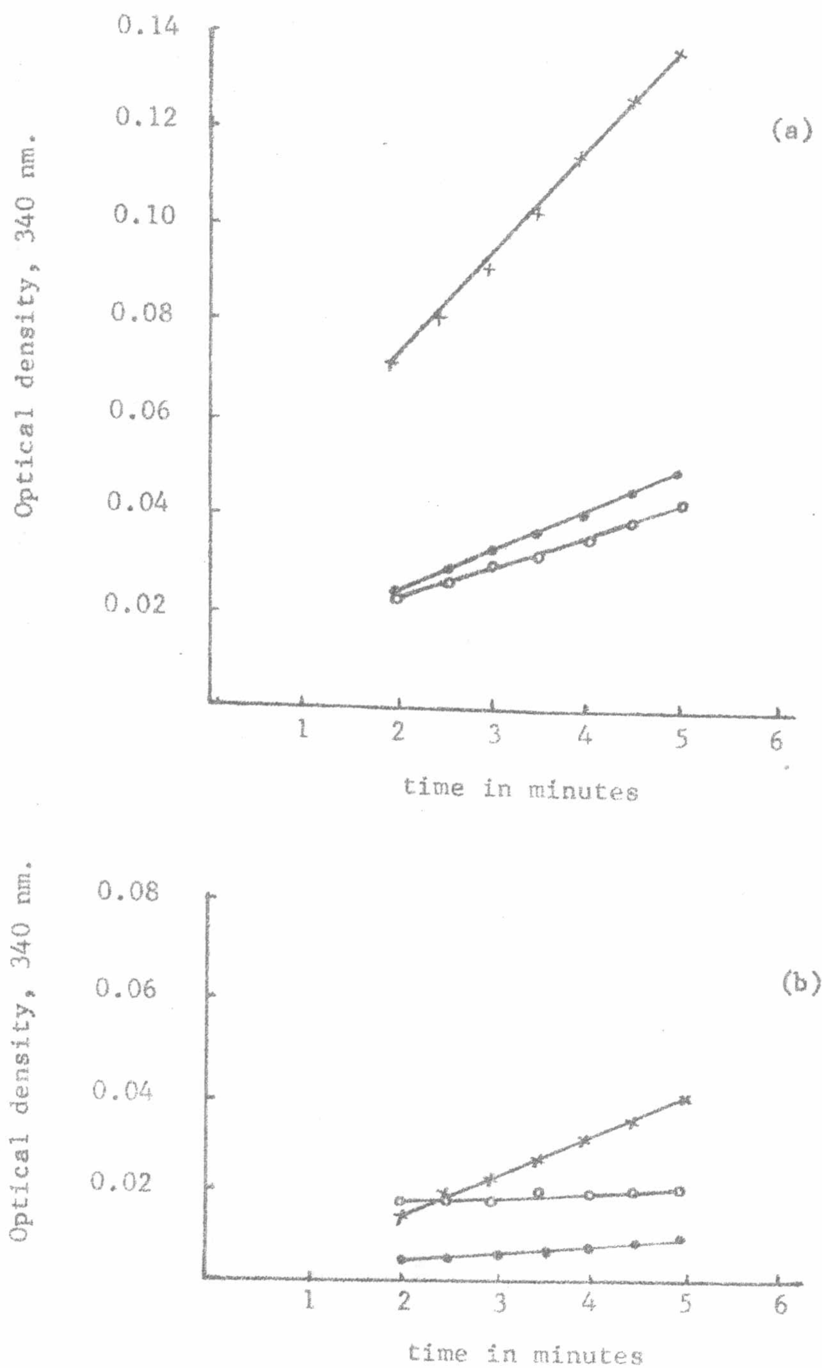


Figure 7 Rate of reaction of LDH isoenzymes from plasma of a patient with myocardial infarction (a) and of a normal subject (b)

x———x total activity

●———● urea-stable activity

o———o heat-stable activity

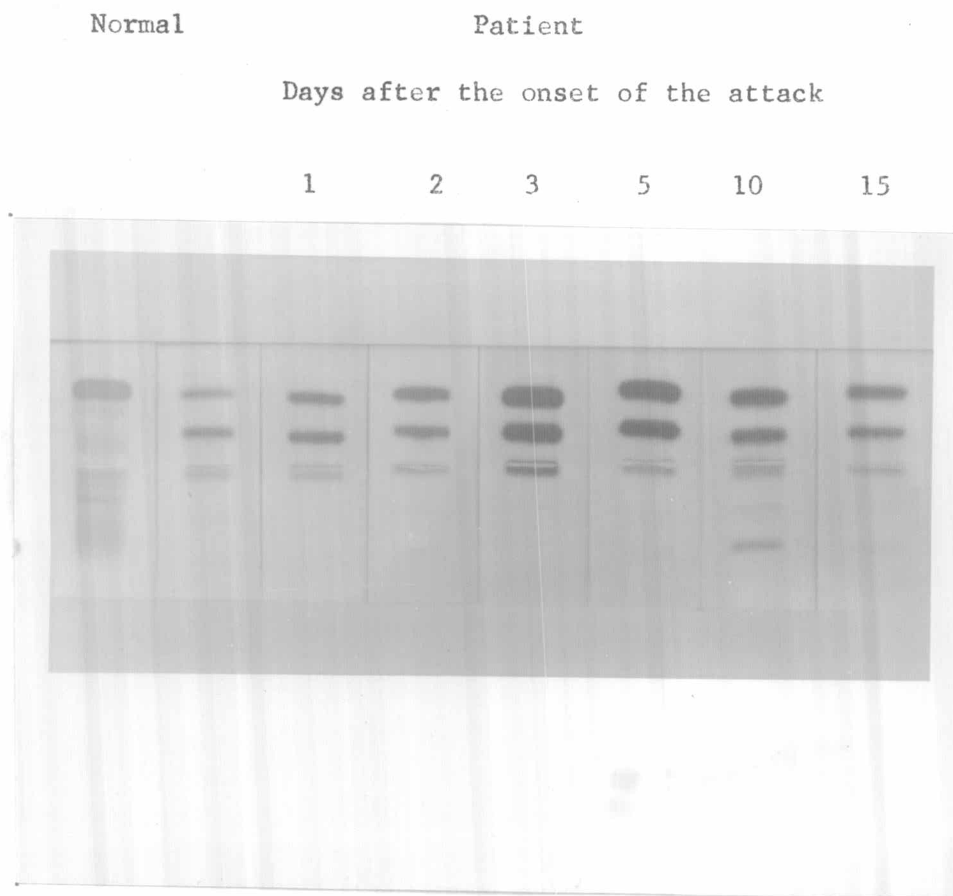
TABLE 3

Activity of lactic acid dehydrogenase isoenzymes in plasma of normal subjects and patients with myocardial infarction. (Mean ± S.D.)

	Normal subject		Patient (22)					
			Days after the onset of attack					
	Men (19)	Women (13)	1	2	3	5	10	15
TLDH	121 ± 35	88 ± 12	171 ± 59	276 ± 174	310 ± 200	299 ± 189	199 ± 40	168 ± 85
ULDH	21 ± 11	12 ± 3	58 ± 48	120 ± 105	101 ± 78	102 ± 95	71 ± 66	42 ± 17
HLDH	12 ± 2	11 ± 2	44 ± 35	64 ± 60	72 ± 65	39 ± 32	19 ± 14	20 ± 11
LDH-1	36 ± 9	26 ± 5	53 ± 17	89 ± 64	90 ± 51	88 ± 67	61 ± 30	45 ± 20
LDH-2	37 ± 11	27 ± 5	54 ± 26	77 ± 47	87 ± 55	90 ± 59	58 ± 28	50 ± 27
LDH-3	28 ± 10	20 ± 6	40 ± 18	59 ± 37	64 ± 32	64 ± 43	47 ± 24	43 ± 33
LDH-4	10 ± 3	7 ± 2	14 ± 7	23 ± 16	24 ± 13	20 ± 10	18 ± 10	16 ± 11
LDH-5	12 ± 6	7 ± 2	13 ± 7	19 ± 8	23 ± 12	25 ± 13	22 ± 15	15 ± 16

Values are expressed in unit activity. One unit of LDH activity equals to an increase in absorbance of 0.001 per minute per milliliter of plasma at 25°C

Figure in parenthesis = number of cases.



<u>Figure 8</u> The changes of activities of 5 isoenzymes bands in plasma samples of a patient with myocardial infarction comparing with those of plasma proteins and LDH isoenzymes of a normal subject.

for 15 days. The activity of the isoenzymes reached a maximum level between the third and the fifth days and the values was about 2 to 4 folds of the activity found in plasma of normal subjects.

Figure 9, 10 and 11 show the means and standard deviations of the activities as TLDH, ULDH, and HLDH respectively.

Figure 12 shows the mean values of the activity of TLDH compared to those of the ULDH and HLDH.

Figure 13 shows the mean activities of five LDH isoenzymes in plasma samples from 22 patients with myocardial infarction.

It can be seen (Figure 12) that the changes of the TLDH activity at different times after the onset of the attack was similar to those of the ULDH and HLDH. An increase in the activity was observed in all the patients who were attacked with myocardial infarction and the activity was higher than the upper normal level of the isoenzymes. There was,however, a wide variation in the isoenzyme concentrations among individuals. The increase was still apparent at 15 days after the onset of the attack.

3.9 Determination of Lipids and Lipoproteins

Myocardial infarction is a disease that is very closely related to artherosclerosis, thrombosis, and embolism which associated with lipoid materials. Fredrickson et al (1967) found that lipids are transported in plasma as lipoproteins. It is interesting, therefore, to study the changes of the lipids and lipoproteins in the patient with myocardial infarction.

Cholesterol, triglycerides and lipoproteins were investigated

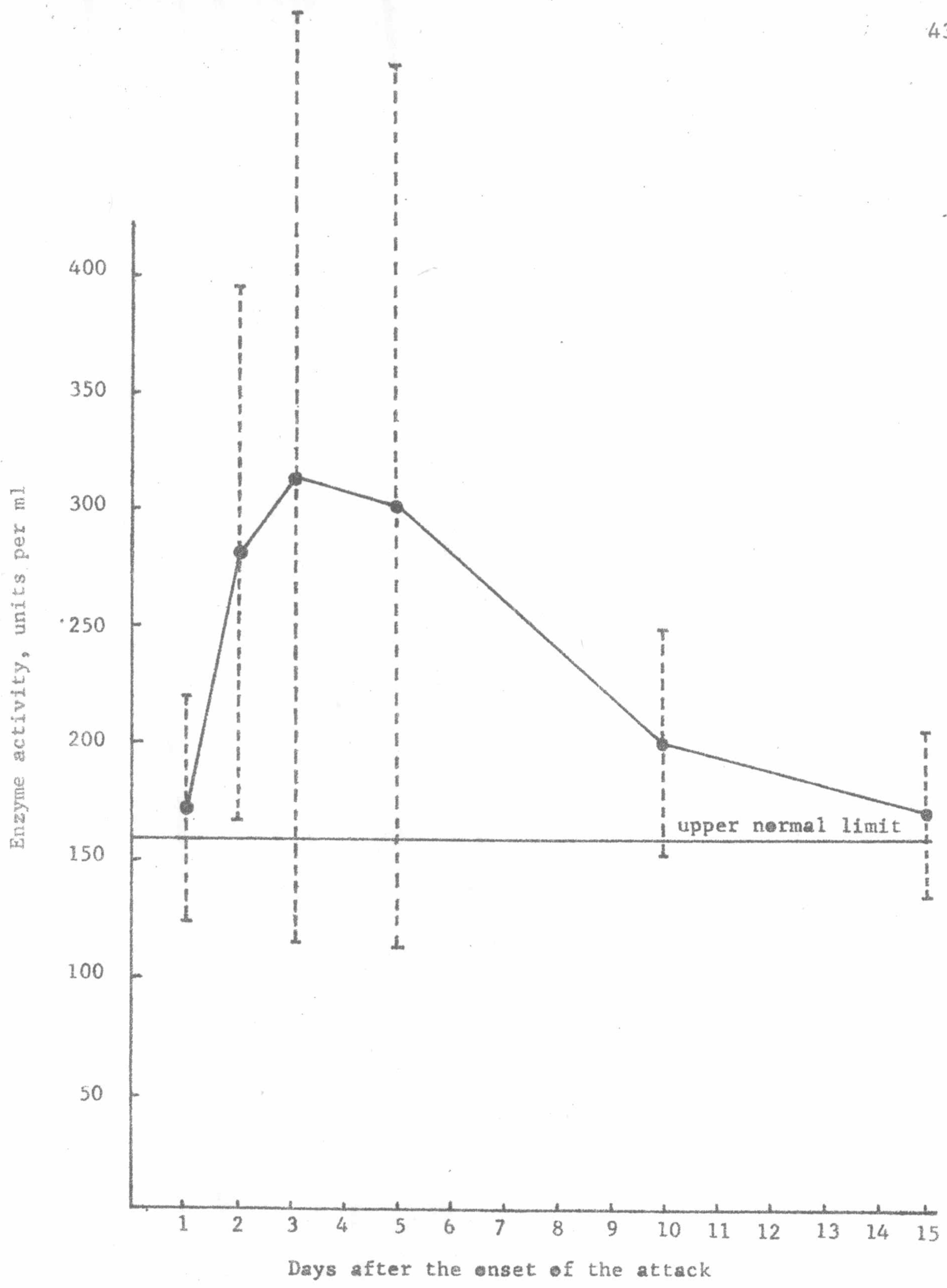


Figure 9 Total activities of lactic acid dehydrogenase isoenzymes (Mean±S.D.) in plasma samples from 22 patients with myocardial infarction

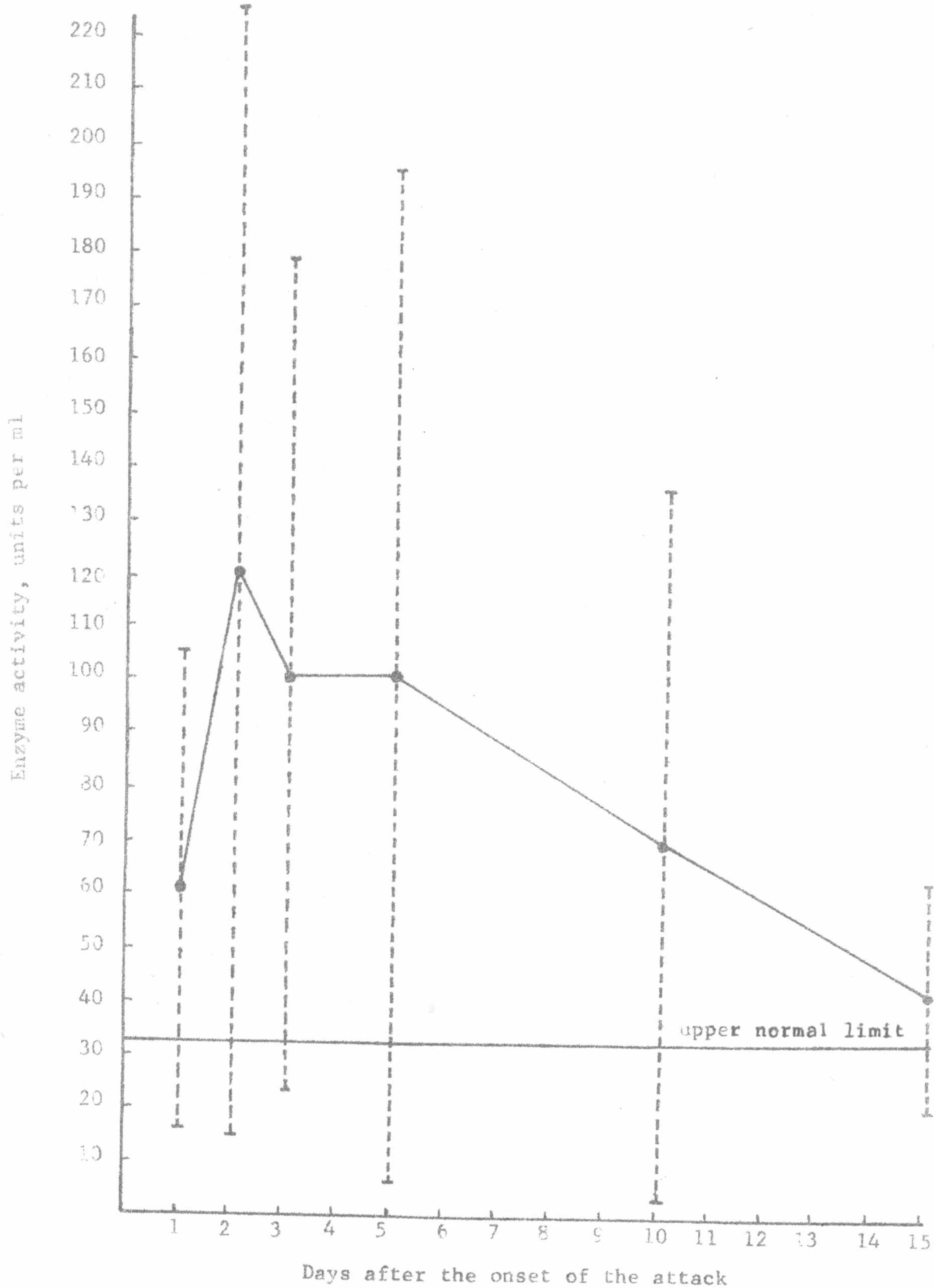


Figure 10 Urea-stable activities of lactic acid dehydrogenase isoenzymes (Mean±S.D.) in plasma samples from 22 patients with myocardial infarction

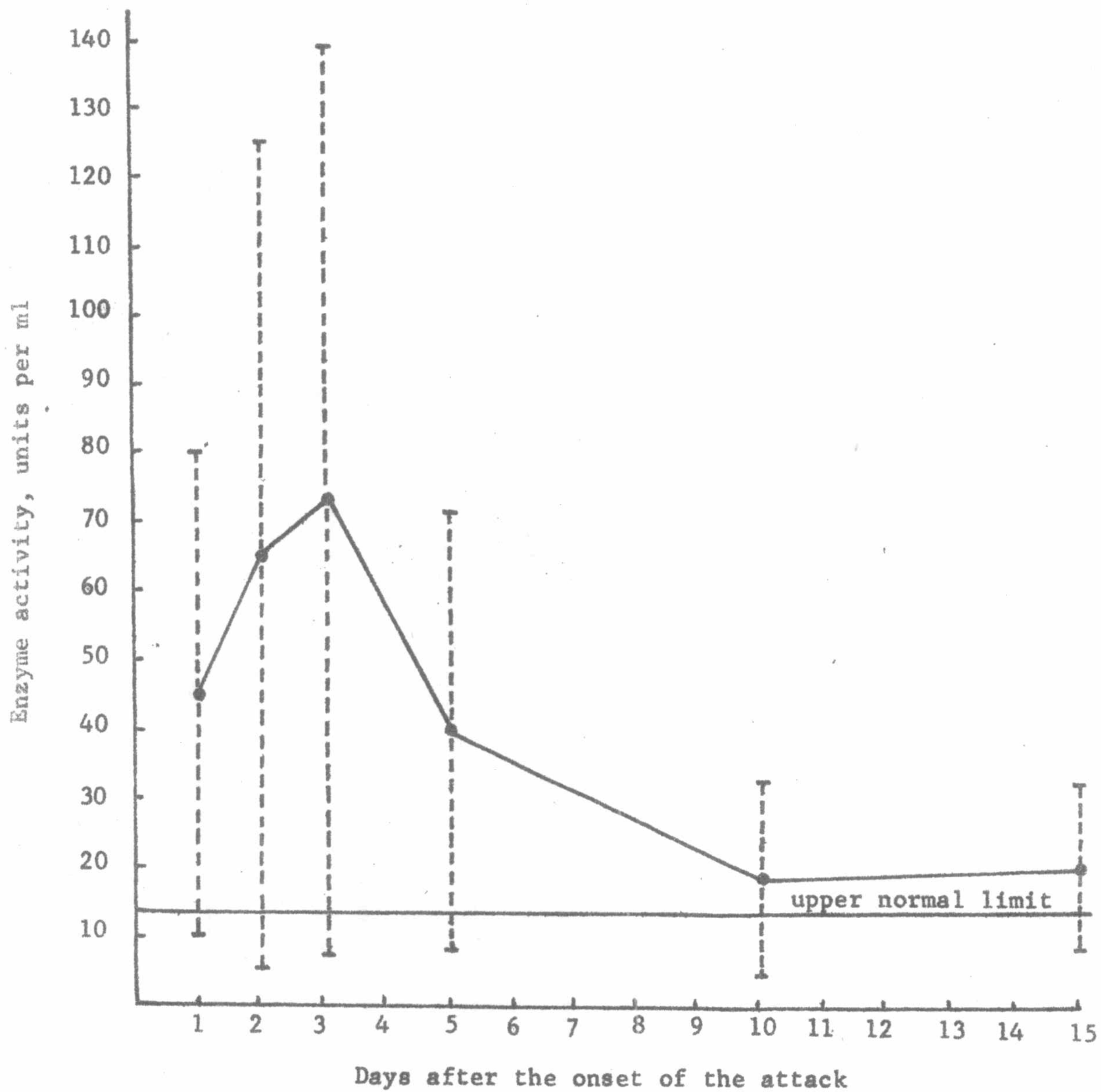


Figure 11 Heat-stable activities of lactic acid dehydrogenase isoenzymes (Mean±S.D.) in plasma samples from 22 patients with myocardial infarction

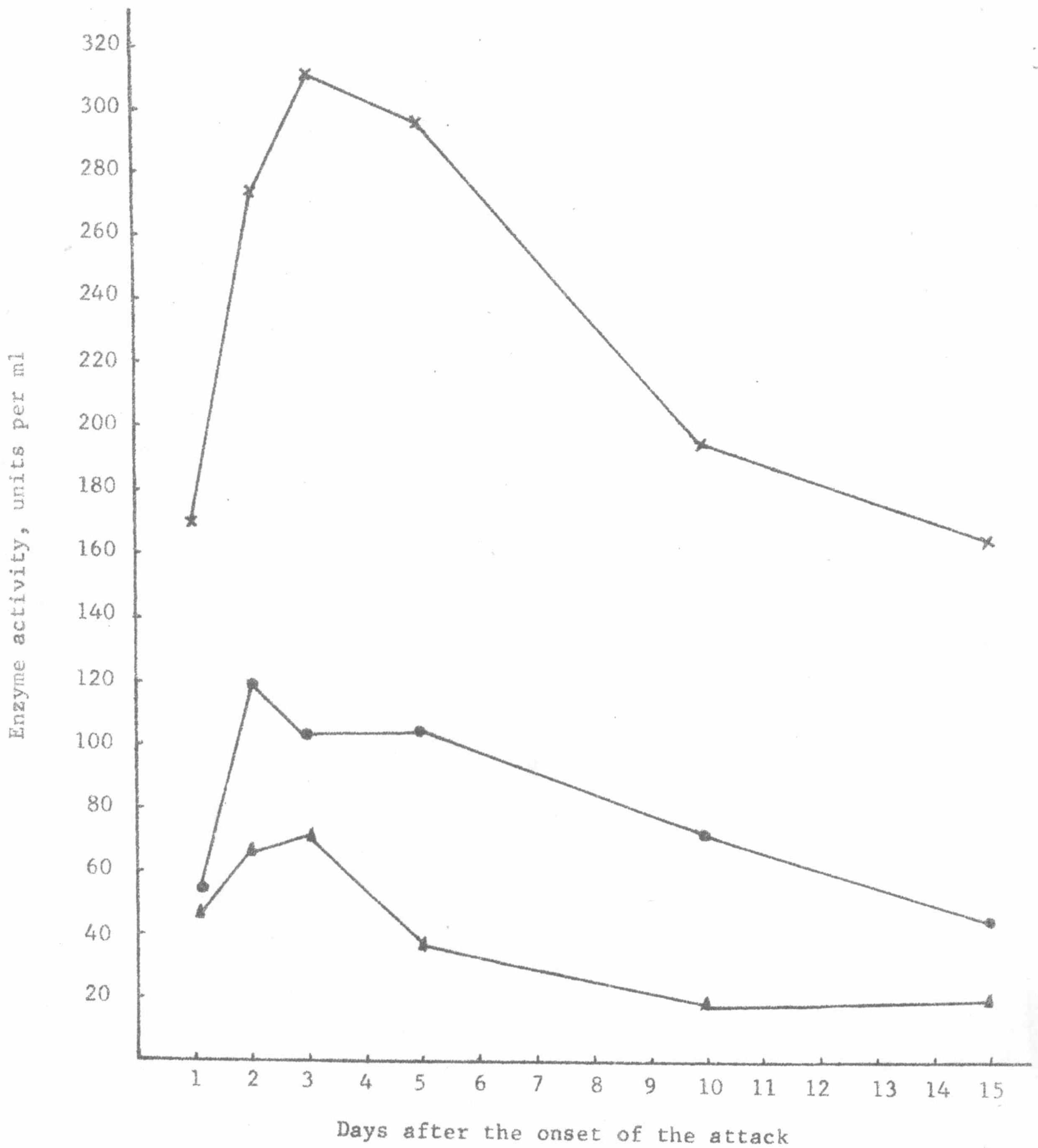


Figure 12 Mean activities of lactic acid dehydrogenase isoenzymes in plasma samples from 22 patients with myocardial infarction

x———x TLDH

●———● ULDH

▲———▲ HLDH

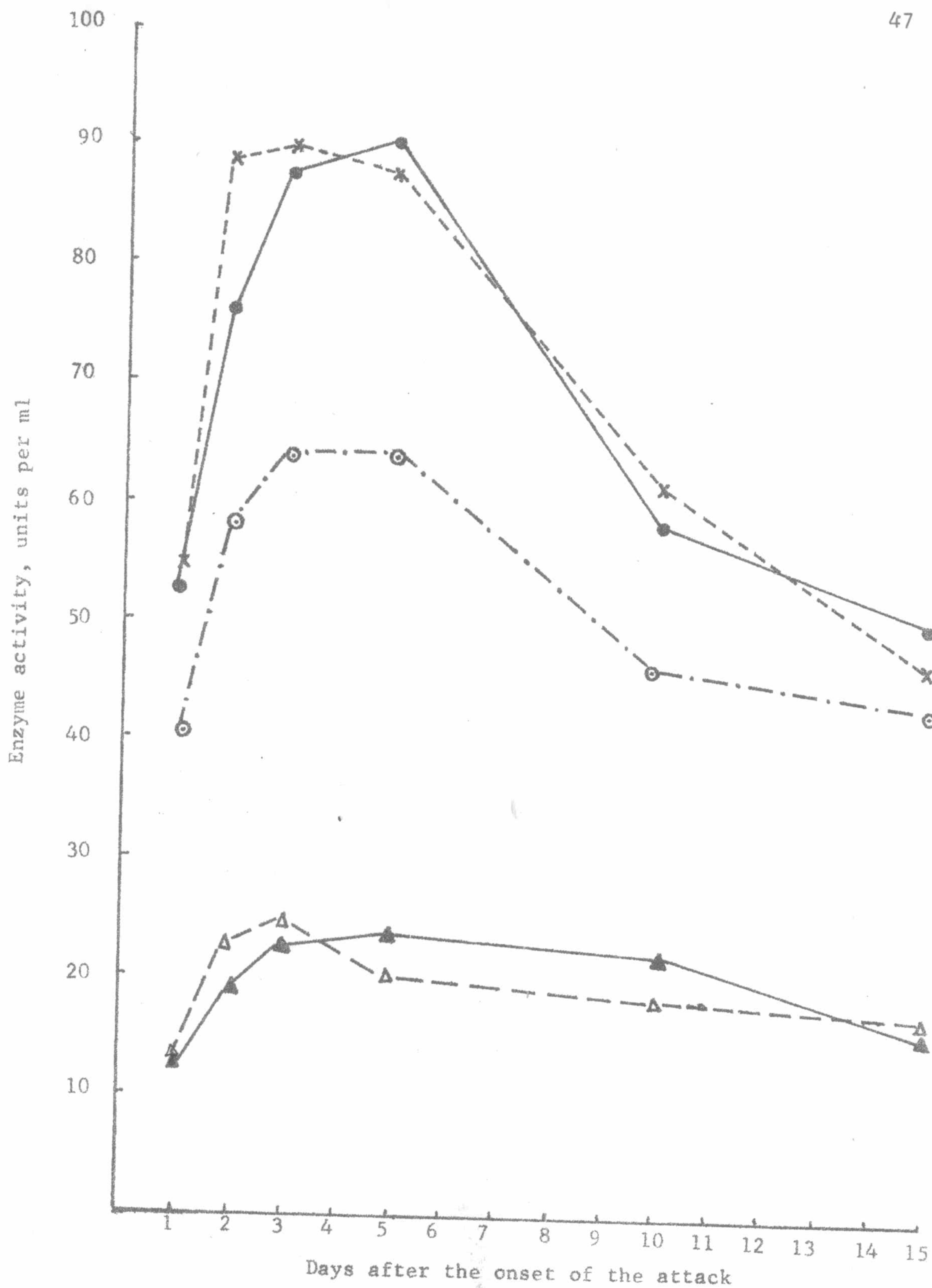


Figure 13 Mean activities of five LDH isoenzymes in plasma samples from 22 patients with myocardial infarction.

LDH-1, ●———●; LDH-2, x———x; LDH-3, o———o; LDH-4, ▲———▲

LDH-5, △———△

in the same series of plasma samples of LDH isoenzymes.

Figure 14 shows a calibration curve of cholesterol determined by the method of Parekh and Jung (1970) and Jung and Parekh (1971). A linear relationship was obtained over a wide range of optical density and concentration of the standard cholesterol. Similar results were also obtained in the determination of triglycerides by the method of Fletcher (1968). A calibration curve using glycerol trioleate as a standard is shown in Figure 15.

The concentrations of plasma cholesterol and triglycerides of normal subjects and patients with myocardial infarction determined by the above mentioned methods were shown in Table 4. There was no significant difference in the lipid levels either between sex or between normal subjects and the patients.

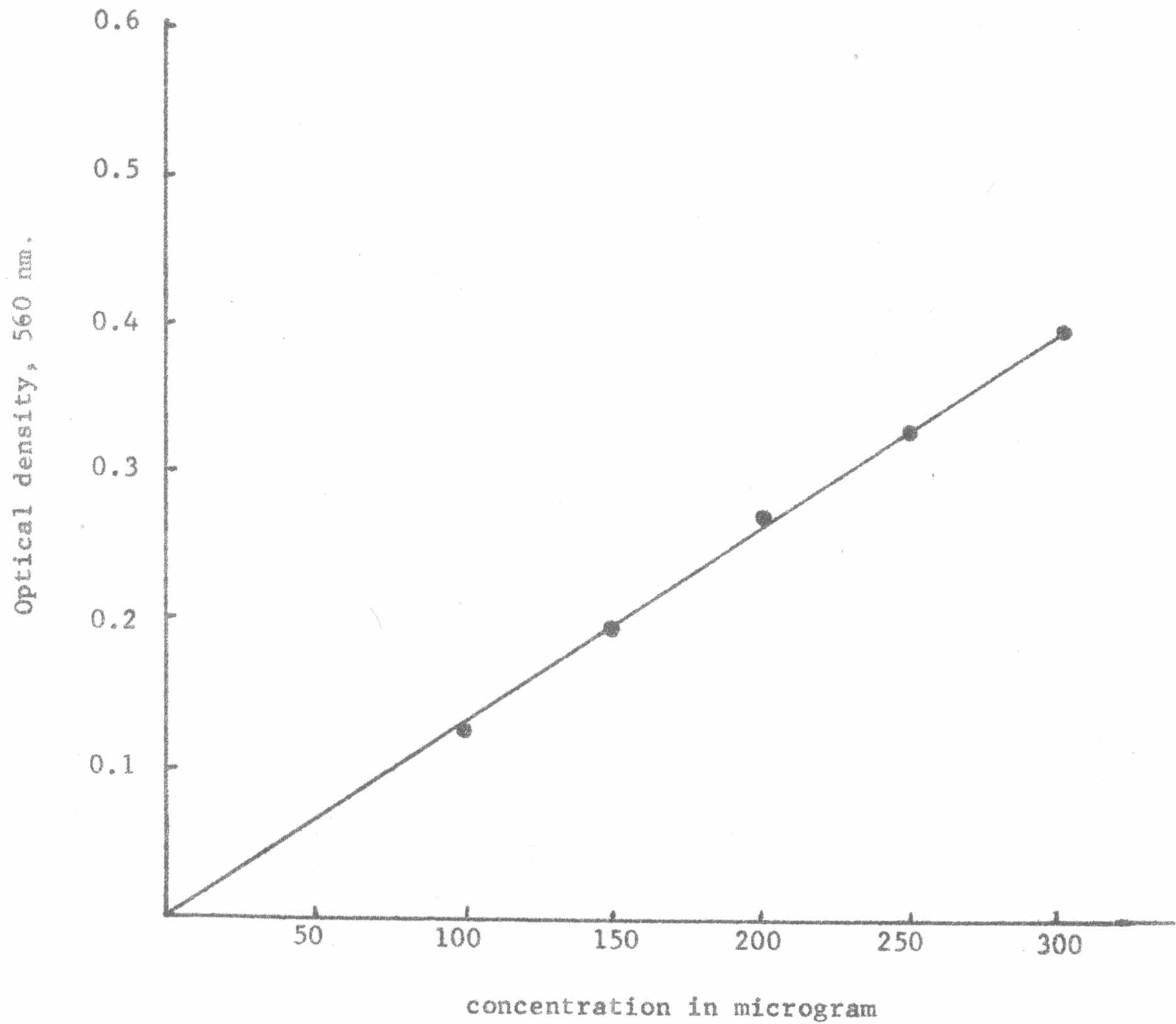


Figure 14 A calibration curve of cholesterol using cholesterin as a standard

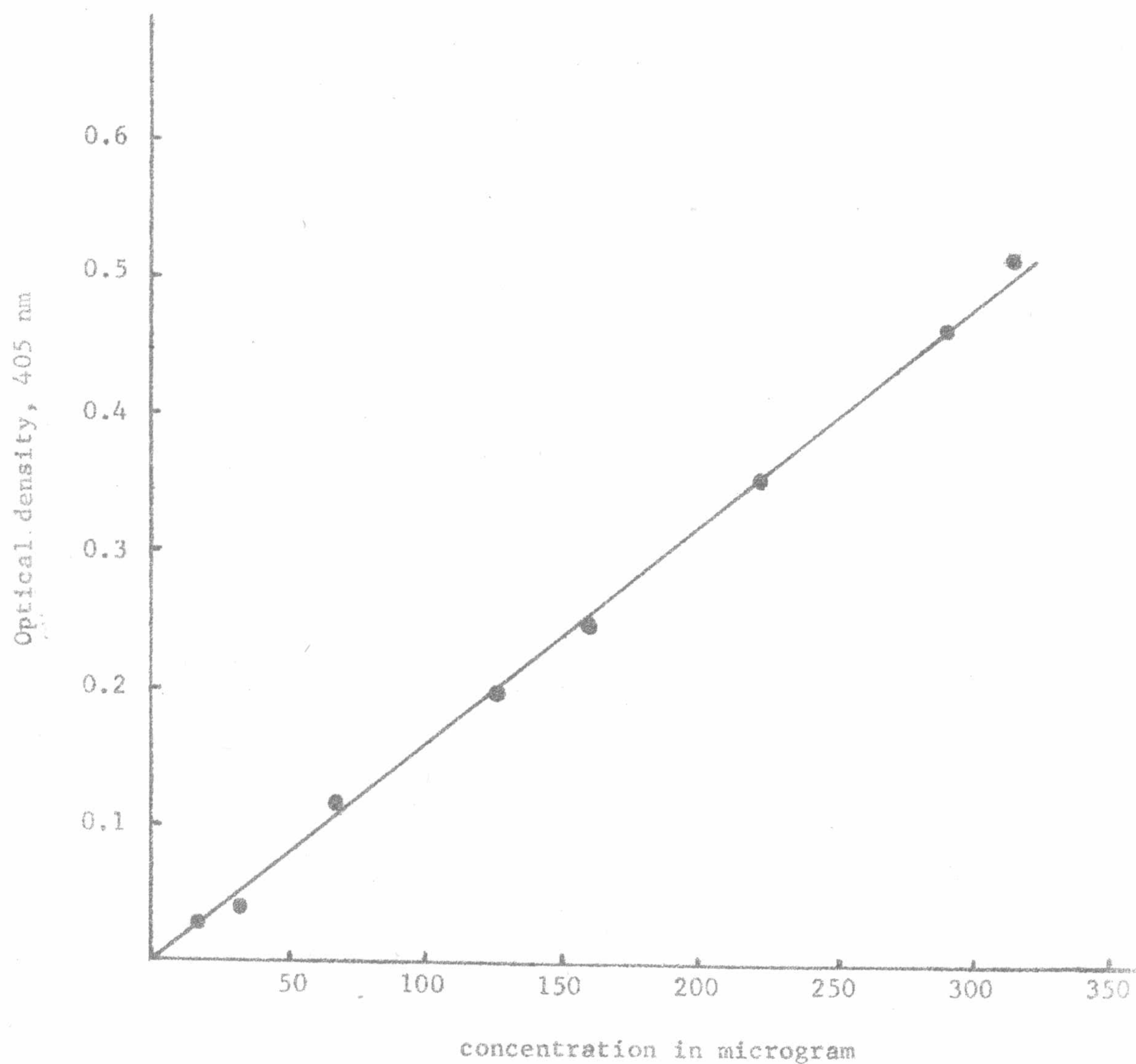


Figure 15 A calibration curve of triglycerides using glycerol trioleate as a standard

TABLE 4

Plasma lipids concentrations of normal subjects and patients with myocardial infarction

Concentration (mg /100ml)	Patient (22) Days after the onset of the attack with myocardial infarction					
	1	2	3	5	10	15
Cholesterol	277 ± 72	282 ± 71	295 ± 72	287 ± 67	259 ± 73	284 ± 66
Triglycerides	171 ± 76	180 ± 87	161 ± 59	166 ± 63	136 ± 22	172 ± 63

Concentration (mg /100 ml)	Normal Subjects	
	Men (19)	Women (13)
Cholesterol	271 ± 34	264 ± 40
Triglycerides	132 ± 42	136 ± 50

Figure in parenthesis = the number of cases.