A STUDY OF SOME CONSTITUENTS

IN HUMAN FEMALE SALIVA

BY

ANWAR YACOUB YOUSIF B.Sc.

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Department of Pharmacy

University of Aston

Gosta Green

Birmingham B4 7ET

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SUMMARY

Changes in the concentration of some constituents in women's saliva during the menstrual cycle were studied. Saliva was used because it is easier to collect than other body fluids and is continuously available for analysis. Glucose, the enzyme N-Acetyl-β-D-glucosaminidase (NAG) and Calcium which are saliva constituents and belong to three different chemical groups were selected for the study.

Several analytical techniques were investigated. The fluorometric assay procedure was found to be the best because of its specificity and sensitivity for the estimation of these constituents. Besides the fluorometric method a spectrophotometric method was used in the NAG determination and an atomic absorption method in the calcium estimation.

Glucose was estimated by an enzymatic method. This is based on the reaction of glucose with the enzymes glucose oxidase and peroxidase to yield hydrogen peroxide, which in turn oxidises a non-fluorescent substrate, p-hydroxyphenylacetic acid, to a highly fluorescent product.

The saliva samples in this determination had to be centrifuged at high speed, heated in a boiling water bath, centrifuged again and then treated with a mixture of cation and anion resins to remove the substances that inhibited the enzyme system.

In the determination of the NAG activity the saliva samples were diluted with citric acid/phosphate buffer, and then centrifuged at high speed. The assay was based on the enzymic hydrolysis of the non-fluorescent substrate , 4-Methyl-umbelliferyl-β-D-glucosaminide to the highly fluorescent 4-Methyl-umbelliferone.

Calcium was estimated by a fluorometric procedure based upon the measurement of the fluorescence produced by the complex formed between calcein blue and calcium, at pH 9 - 13.

From the results obtained from the analysis of saliva samples of several women it was found that glucose showed a significant increase in its level around the expected time of ovulation. This was found in seven cycles out of ten. Similar results were found with the enzyme NAG. No significant change in the calcium levels was observed at any particular time of the cycle.

The levels of the glucose, the activity of the enzyme NAG and the concentration of the calcium were found to change daily, and to differ from one subject to another and in the same subject from cycle to cycle.

The increase observed in salivary glucose levels and the enzyme NAG activity could be monitored to predict the time of ovulation.

Key Words: Saliva, Menstrual Cycle, Glucose, N-Acetyl-β-D-glucosaminidase and Calcium.

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TO

MY WIFE AND PARENTS

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CHAPTER I

GENERAL INTRODUCTION

Biological fluids constitute 60 - 70% of the human body. Saliva is one of those complex biological fluids produced by specialized organs in the oral cavity.

It is the first digestive fluid that comes in contact with food and is secreted by four different types of salivary glands; the parotid, submandibular (or submaxillary)and sublingual glands, which are all bilaterally paired (1). In addition to the three major salivary gland pairs, saliva is also secreted by numerous minor mucous glands located in most areas of the oral mucosa and pharynx. (1).

Saliva is a slightly opalescent slimy liquid consisting mainly of water, but a variety of dissolved components, both inorganic and organic, are always present. Its specific gravity is 1.002 - 1.012 and its pH varies between 6.35 - 6.85, but in stimulated secretions it increases to more than 7.00. Hydrogen ion concentration of saliva has been found to vary directly with the carbon dioxide, CO_2, content of blood. That is when the CO_2 content of the blood is high, more CO_2 finds its way into the salivary secretion to lower its pH and vice versa (1).

Saliva helps to maintain the oral pH at about 7.0. Due to the buffering action, saliva is saturated with calcium and so the teeth do not lose calcium to the oral fluids. At more acid pH calcium loss is appreciable (2). The total amount of salivary fluid produced during 24 hours period by a healthy adult ranges between 1000 - 1500 ml, with a flow rate of about 0.5 - 1ml/minute at rest, but this increases with stimulation (3). About 80 -90% of the daily production of saliva is a result of stimulation, mainly gustatory and masticatory associated with eating. The unstimulated saliva consists of 25% v/v

parotid secretions, 70% v/v submaxillary secretions, 4% v/v of the sublingual secretions and 1 % of the minor gland secretions (4). A mixture of these secretions is termed whole or mixed saliva. Many studies have been made on whole saliva and on the individual secretions in an attempt to examine their constituents, flow rate and their relations to the physiological conditions in health and disease. Whole saliva varies in amount, rate of flow and composition between individuals and in the same individuals from time to time. This is of value in aiding the diagnosis and prognosis of systemic and salivary gland diseases. The results of these studies have concluded that flow rate and composition changes in saliva can reflect emotional stress, drug toxicity, irradiation, metabolic changes and local or systemic disease.

FUNCTIONS OF SALIVA (1, 3, 5, 6)

The main function of saliva is to maintain the integrity of the teeth, tongue and mucous membranes of the oral and the oral-pharyngeal areas. However, the following are the main specific functions:

a. Saliva helps to maintain the water balance of the body.

b. Due to the presence of the digestive enzyme ptyaline or salivary α- amylase saliva has a digestive function.

c. Salivary secretions have a considerable protective potential by the lubrication and protection of the mucous membranes due to the organic and inorganic components present in the secretions, for example the glycoproteins and mucoides produced form a protective coating and barrier to irritants.

d. Lubrication of tongue and lips to facilitate speech and aid swallowing of dry food.

e. Mechanical cleansing; the physical flow of saliva aids the removal of food, cellular and bacterial debris via the alimentary canal.

f. Buffering action; saliva has a considerable buffer capacity because of its content of bicarbonate, phosphate and amphoteric proteins.

g. Saliva acts as a solvent for molecules that facilitate the sense of taste.

h. Saliva functions in maintenance of tooth integrity by a number of ways:

   i. By providing minerals for post eruptive maturation.

   ii. By virtue of its calcium and phosphate entering plaque.

   iii. By forming a film of glycoprotein on teeth and reducing wear due to attrition and abrasion.

i. Saliva has antibacterial properties due to a number of components which can individually or in concert make a defence against

bacterial and viral invasion. The agents in this antimicrobial system are secretory IgA, lysozymes, lactoferrin, lactoperoxidase, hydrogen peroxide and thiocyanate ion (SCN^-) (7).

j. Saliva has been found to possess a potent inhibitory activity of the fibrinolytic process, by its inhibitory effect on the rate of the breakdown of radio-iodine-labeled, thrombin-coagulated euglobulin factors. This activity has been found to be most pronounced in saliva obtained from the parotid gland and the weakest in the cell-free supernatant of whole saliva. (8).

RATE OF FLOW

Normal Flow Rate

Saliva flow rate is variable and depends on the physiological state of the subject and on any exogenous or endogenous factors that are capable of stimulating or inhibiting the process of secretion. The continuous secretion of saliva in the absence of any known stimulating factors is called spontaneous or resting secretion (6). The flow rate is generally expressed as volume per unit time since the gland mass producing the fluid cannot readily be measured (8).

When resting conditions prevail, flow is about 0.5 - 1.0 ml/minute, but during the period of sleep flow is less than 0.5 ml/minute, and during the intense stimulation associated with eating, the flow is 1.5 - 2.3ml/minute (8). Also many substances can stimulate the production of saliva, such as the chewing of paraffin wax or rubber bands or non-sugared chewing gum, and it has been found that the resulting increase in flow rate causes salivary electrolyte composition to change. The flow rate generated by stimulation varies with the nature of the stimulus. Parasympathetic stimulation gives a watery secretion and sympathetic stimulation gives a viscous secretion containing up to 6% of saliva solids (6).

Secretion of saliva is brought about in two ways; either through the stimulation of the nerves of the mouth by the presence of food or other substances, or by stimulation of some organs of special sense other than that of taste. The former type of reflex is termed unconditioned or inherent and the latter conditioned or acquired (6). Resting mixed saliva shows a moderate increase in average flow rate between the ages of 8 and 29 years and thereafter there is a slight decline in flow rate. It was realised that, although an increase in salt content with increasing secretory rate is commonly observed in

salivary glands, not all the individual ion species contributing to the salivary salt content follow this relation. Among those that do are sodium, calcium and chloride, but not potassium and bicarbonate (3, 9).

Generally concentrations tend to increase with age and where there is a sex difference, the male saliva usually shows a slightly higher concentration of solute. Seasonal changes also have an effect on the flow rate of secretion. The lowest flow rate occurs in hot summer months and the maximum flow rate is found in the winter months (6).

Factors Affecting Flow Rate (6)

a. Emotional and psychic factors, for example anxiety can result in reduced flow. Depressed patients exhibit reduced flow rate (10).

b. Conditioned reflexes; sight or smell of food can produce a salivary flow rate. (11).

c. Unconditioned reflexes; since gustatory and masticatory reflexes are the major stimuli for salivation, interference with the ability to taste or chew has a profound effect (6).

d. Hormonal control; abnormalities in hormone function have been shown to affect salivary flow (6).

e. Light deprivation has been demonstrated to result in reduction of flow rate of about 50% (12).

f. Salivary gland disease; inadequate function of the glands as a result of infectious disease or irradiation will result in reduced salivary flow (6).

g. Pharmacological agents have a profound effect on salivary flow rate due to a variety of neural influences on the salivary glands. Increased salivation can also be a problem with those agents that stimulate the Central Nervous System (CNS) (6).

COLLECTION OF SALIVA

Since saliva is used to assess various physiological and pathological states, consideration of its role, and methods and conditions for its collection are important (9). Saliva must be collected under standardized conditions which should be always recorded. The main points to be noticed are: type of saliva needed, whether whole or individual secretion, stimulated or unstimulated, flow rate, nature and duration of stimulation, time of day, and kind of stimulation used.

The flow rate should be maintained constant in any experiment on salivary composition because changes in flow rates have pronounced effects on the composition (13). Subjects can be trained to produce constant flow rate by chewing gum or rubber bands in time to a metronome. Unstimulated saliva should be collected after an accomodation period of at least five minutes to reduce the influence of the collecting procedure as a stimulus. The collection should always be timed and the volume measured to 0.1 ml in order to calculate the rate as ml/minute/gland (9).

Stimulated saliva should be collected with sour candy or 2% w/v citric acid applied to the tongue. Throughout the study the same kind (in flavour and sourness) of candy should be used so that the degree of gustatory stimulation is constant (6).

Many salivary components are affected by the circadian rhythm (22), i.e. they exhibit high and low values at different times of the day due to hormonal influences. In comparative studies it is necessary to standardize the time of collection,

a. before breakfast and before brushing teeth (14).

b. two hours post prandial (breakfast or lunch).

The time of collection should always be recorded. In collecting all types of saliva it is better to discard the volume collected in the first five minutes to allow the concentration of the constituents to stabilise. If saliva is collected too frequently from the same subject then the actual collection influences the salivary composition. Thus saliva should not be collected more than once every one or two hours (15). The samples from whole saliva contain contributions from all the major and the minor salivary glands, shed mammalian and bacterial cells and remnants from food and liquid ingestion. Centrifugation to remove extraneous materials is essential (6). Soluble non salivary components, however, remain as contaminants. Many workers use resting secretions which are sampled in the absence of any exogenous stimulation (6).

In general the subject should be awake, seated comfortably, making the least possible movement, in a well lighted room and a warm environment, without talking or smoking at a time when the stomach is empty but before the sensations of hunger appear (16). If a sampling device is used it should not itself produce any stimulation.

METHODS OF COLLECTING SALIVA

Whole Saliva

Whole saliva can also be collected without exogenous stimulation to yield resting or unstimulated saliva. The subject at first should swallow all the saliva present in the mouth. Then the subject should allow the saliva to drain passively or by actively spitting accumulated fluid into a collecting funnel, or receptacle, or by use of an aspiration device such as a dentist's saliva ejector. The minimum collection time for a sufficient volume to be accurately estimated is probably about five minutes. Centrifugation can be applied to remove any debris and to spin down foam, making the estimation of volume more accurate.

Saliva can be stored in refrigerated conditions. Drug concentrations remain stable for one week at 4°C or for several weeks at -20°C (6).

Parotid Saliva (6)

For a long time parotid saliva has been readily collectable by the use of a simple cup-like suction device applied over the opening of the parotid duct. This device was first developed by Lashley in 1916 (17). Another modified collector of parotid saliva is called the ANOVEL (IO) Cup (18), which is a plastic intra-oral cup developed to facilitate collection.

Submaxillary Saliva (6)

Another collector developed by L. Schneyer in 1955 allows the collection of submaxillary and sublingual saliva separately (19).

The Block-Brotman device collects some sublingual saliva with the submaxillary, since the sublingual contribution is less than 5% of the submaxillary, this contribution can be ignored.

Mandle (1969) described a method of collecting submaxillary saliva using a cotton roll placed over the parotid gland ducts and the saliva which accumulates in the floor of the mouth is aspirated (20). Collection of minor mucous gland secretions and gingival fluid is most easily accomplished by using micropipettes.

COMPOSITION OF SALIVA IN A NORMAL INDIVIDUAL

In humans, mixed saliva varies enormously in amount and composition between individuals and the same individual from time to time.

The relative contributionsof various salivary glands vary in amount and composition by physical and psychic stimuli.

Saliva consists mainly of water (99.5%) and 0.5% of total solids which contain inorganic constituents commonly found in the plasma and certain organic constituents (1).

Tables, numbered, 1 (21), 2 (22), 3 and 4 (6) show detailed information about the physical properties and chemical composition of saliva and their individual secretions under different conditions.

ELECTROLYTES (5)

Salivary electrolyte content is different from that of plasma since it reflects an active transport system and is relatively independent of plasma concentrations. Nevertheless, the principal inorganic constituents of saliva (with the exception of bicarbonate), are derived directly from the blood (9).

The osmolality of saliva depends chiefly on its inorganic content, and, while organic components are present, they contribute little to the final osmolality.

In many respects saliva is similar to intracellular fluid.(6) In unstimulated saliva, values are often different from those in stimulated saliva. Some electrolyte levels are unaffected by change in saliva flow rate but others decrease or increase as the flow rate increases. For example, sodium and chloride are directly

Table 1. Physical Properties and Chemical Composition of Saliva.

Part I: MAN

With the exception of lines 86-91, values are for mixed secretions of the salivary glands. Values in parentheses are ranges, estimate "c" (cf. Introduction).

	Property or Constituent	No. of Observations	Saliva Stimulant	Value
	(A)	(B)	(C)	(D)
	Physical Properties and General Chemical Components			
1	Freezing point depression		None	(0.07-0.34) °C
2	pH	3405	None	6.75(5.6-7.6)
3		39	Paraffin	7.45(7.2-7.6)
4	Rate of flow	148	None	0.57(0.1-1.8) ml/min
5		148	Paraffin	1.9(0.4-4.8) ml/min
6	Specific gravity		None	(1.010-1.020)
7	Solids, total	69	Paraffin	581(386-860) mg/100 ml
	Electrolytes			
8	Bicarbonate	25	None	6.44(3.48-10.70) mEq/L
9		25	Paraffin	15.74(8.12-19.47) mEq/L
10	Calcium	650	None	5.8(5.2-9.7) mg/100 ml
11		39	Paraffin	5.5(3.5-9.2) mg/100 ml
12	Carbon dioxide		None	12(5-25) vol %
13			Paraffin	25(8-44) vol %
14	Chloride		None	15.5(8.4-17.7) mEq/L
15			Paraffin	11.8(8.7-17.7) mEq/L
16	Cobalt	37	Paraffin	2.44(0-12.53) µg/100 ml
17	Copper	20	None	6.3(2-22) µg/100 ml
18		48	Paraffin	25.9(10.0-47.5) µg/100 ml
19	Fluoride		None	(0-0.005) mEq/L
20	Iodine		None	(0-350) µg/100 ml
21	Magnesium		None	0.58(0.16-1.06) mEq/L
	Phosphorus			
22	Total P	50	None	20.4 mg/100 ml
23	Inorganic P	180	None	14.9(7.4-21.1) mg/100 ml
24	Organic P	50	None	5.5 mg/100 ml
25	Lipid P	207	None	0.119(0.02-0.24) mg/100 ml
26	Potassium	148	None	80.3(56-148) mg/100 ml
27		148	Paraffin	78.0(50-95) mg/100 ml
28	Sodium	147	None	23.2(8-56) mg/100 ml
29		148	Paraffin	57.3(19-133) mg/100 ml
	Nitrogenous Substances			
30	Protein, total	25	None	386(156-630) mg/100 ml
31		25	Paraffin	242(140-527) mg/100 ml
32	Mucin	30	None	250 mg/100 ml
33		30	Paraffin	270(80-600) mg/100 ml
	Amino acids			
34	Alanine	9	None	1.2(0.5-2.9) mg/100 ml
35	Arginine	18	Paraffin	(3.3-10.0) mg/100 ml
36	Aspartic acid	9	None	0.15(0.13-0.33) mg/100 ml
37	Cystine	18	Paraffin	(0.16-0.45) mg/100 ml
38	Glutamic acid	9	None	1.2(0.5-1.3) mg/100 ml
39			Paraffin	(3.0-12.6) mg/100 ml
40	Glycine	9	None	1.4(0.5-3.6) mg/100 ml
41		18	Paraffin	(1.9-15.5) mg/100 ml
42	Histidine	18	Paraffin	(0.35-2.00) mg/100 ml
43	Isoleucine	18	Paraffin	(0.2-0.9) mg/100 ml
44	Leucine	18	Paraffin	(0.025-0.300) mg/100 ml
45	Lysine	9	None	0.77(0.15-1.50) mg/100 ml
46		18	Paraffin	(0.4-1.5) mg/100 ml

Part I: MAN (Continued)

	Property or Constituent	No. of Observations	Saliva Stimulant	Value
	(A)	(B)	(C)	(D)
	Nitrogenous Substances (concluded)			
	Amino acids (concluded)			
47	Methionine	2	Paraffin	(0.005-0.010) mg/100 ml
48	Phenylalanine	18	Paraffin	(0.6-2.5) mg/100 ml
49	Proline	18	Paraffin	(0.35-1.50) mg/100 ml
50	Serine	9	None	0.66(0.33-1.20) mg/100 ml
51		18	Paraffin	(1.0-1.8) mg/100 ml
52	Threonine	18	Paraffin	(0.4-5.6) mg/100 ml
53	Tryptophan	18	Paraffin	(0.2-0.9) mg/100 ml
54	Tyrosine	18	Paraffin	(0.2-1.0) mg/100 ml
55	Valine	18	Paraffin	(0.7-2.2) mg/100 ml
56	Ammonia	81[1]	None	2.6 mM/L
57			Paraffin	3.5(0.8-7.1) mM/L
58	Creatinine	3	Paraffin	0.35(0.275-0.455) mg/100 ml
59	Urea	9	None	12.7(8.2-18.1) mg/100 ml
60		15	Paraffin	8.8(0-14.3) mg/100 ml
61	Uric acid		None	1.5(0.5-2.9) mg/100 ml
62		72	Paraffin	4.8(1.5-8.7) mg/100 ml
63	Thiocyanate	35	None	13.4(3.1-27.5) mg/100 ml
	Nitrogen			
64	Total N	20	Paraffin	90.0(36.1-125.3) mg/100 ml
65	Protein N	20	Paraffin	63.6(22.9-88.2) mg/100 ml
66	Non-protein N	20	Paraffin	36.4(8.2-62.4) mg/100 ml
67	Ammonia N	94[2]	None	3.8(0.5-9.9) mg/100 ml
	Lipids, Carbohydrates, Miscellaneous Organic Acids			
68	Cholesterol		None	7.5(3-15) mg/100 ml
69	Glucose	16	None	19.6(11.28-28.08) mg/100 ml
70		10	Paraffin	20.7(14.04-30.00) mg/100 ml
71	Citric acid	121	Paraffin	1.05(0.20-3.15) mg/100 ml
72	Lactic acid		None	0.17 mEq/L
	Vitamins			
73	B_1 (thiamine)	8	None	0.7 µg/100 ml
74		23	Paraffin	(0.2-1.4) µg/100 ml
75	B_2 (riboflavin)	8	None	5.0 µg/100 ml
76	B_6 (pyridoxine)	17	Paraffin	0.6(0.1-1.7) µg/100 ml
77	B_{12} (cyanocobalamine)	2	Paraffin	0.33(0.15-0.50) µg/100 ml
78	B_c (folic acid)	20	Paraffin	2.4(0.3-7.5) µg/100 ml
79	C (ascorbic acid)	110	Paraffin	0.07(0-0.372) mg/100 ml
80	H (biotin)	8	None	0.08 µg/100 ml
81	K	8	None	1.5 µg/100 ml
82	Choline	7	None	0.65(0.47-0.99) mg/100 ml
83		87	Paraffin	1.62(0.62-3.64) mg/100 ml
84	Niacin (nicotinic acid)	90	Paraffin	11.5(2.34-40.90) µg/100 ml
85	Pantothenic acid	41	Paraffin	8.8(1.2-19.0) µg/100 ml
	Enzymes[3]			
86	Cholinesterase		Paraffin	0.33(0.23-0.43) units/L[4]
87	Esterase, total		Paraffin	0.34(0.12-0.65) units/L[5]
88	β-Glucuronidase		Paraffin	(170-1750) units/L[6]
89	Lipase		Paraffin	1.42(0.25-2.58) units/L[7]
90	Lysozyme		Paraffin	670(250-1360) units/L
91	Phosphatase, acid		Paraffin	4.23(2.5-7.7) units/L[8]

/1/ 12-year-old children. /2/ 7-year-old children. /3/ Parotid gland secretion. /4/ β-Carbonaphthoxycholine iodide substrate. /5/ β-Naphthyl acetate substrate. /6/ Sodium-8-benzoyl amino-2-naphthyl glucuronide substrate. /7/ β-Naphthyl laurate substrate. /8/ Monosodium-β-naphthyl phosphate substrate.

Table 2. A Reference Table for Human Parotid Saliva (Chemical Composition and Physical Properties).

Parotid fluid variables	Number of subjects	Candy drop exogenous stimulant					
		peppermint		cherry		grape	
		mean	SD	mean	SD	mean	SD
Acid phosphatase							
Flow rate, ml/min	314	0.329	0.177	0.612	0.275	0.993	0.421
Acid phosphatase, units/100 ml		0.23	0.09	0.20	0.09	0.20	0.12
Ammonia							
Flow rate, /ml/min	72	0.275	0.153	0.477	0.234	0.929	0.458
Ammonia, mg/100 ml		0.21	0.14	0.11	0.10	0.06	0.03
Amylase							
Flow rate, ml/min	364	0.339	0.183	0.610	0.275	0.986	0.417
Amylase, units $\times 10^3$/100 ml		176.0	89.2	239.0	119.9	401.0	206.2
Bicarbonate							
Flow rate, ml/min	368	0.315	0.183	0.570	0.285	0.878	0.406
Bicarbonate, mEq/l		10.9	5.56	19.8	7.85	29.8	9.19
Calcium							
Flow rate, ml/min	100	0.343	0.130	0.556	0.173	0.830	0.235
Calcium, mg/100 ml		2.81	1.61	2.73	1.34	3.26	2.18
Chloride							
Flow rate, ml/min	292	0.325	0.119	0.565	0.170	0.920	0.303
Chloride, mEq/l		17.7	10.52	25.8	12.73	33.3	13.44
Creatinine							
Flow rate, ml/min	100	0.419	0.179	0.693	0.258	1.093	0.361
Creatinine, mg/100 ml		0.15	0.04	0.14	0.05	0.13	0.05
Fluoride							
Flow rate, ml/min	25	0.374	0.115	0.623	0.206	0.896	0.156
Fluoride, mg/l		0.020	0.005	0.019	0.005	0.020	0.005
Glucose							
Flow rate, ml/min	106	0.211	0.83	0.367	0.126	0.623	0.225
Glucose, mg/100 ml		0.29	0.12	0.19	0.08	0.15	0.06
Immunoglobulin-A							
Flow rate, ml/min	50	0.327	0.100	0.491	0.114	0.676	0.140
IgA, mg/100 ml		12.6	5.49	8.6	2.57	5.9	2.00
Iodine							
Flow rate, ml/min	87	0.303	0.168	0.503	0.248	0.861	0.388
Total iodine, μg/100 ml		20.5	14.0	11.8	6.1	9.2	3.7
Organic iodine, μg/100 ml		5.3	3.1	3.3	2.0	2.6	1.2
Magnesium							
Flow rate, ml/min	100	0.343	0.130	0.556	0.173	0.830	0.235
Magnesium, mg/100 ml		0.07	0.03	0.07	0.02	0.05	0.03
Nitrogen							
Flow rate, ml/min	100	0.419	0.179	0.693	0.258	1.093	0.361
Nonprotein nitrogen, mg/100 ml		15.6	4.1	13.0	4.0	11.5	3.4
Total nitrogen, mg/100 ml		45.8	18.7	50.9	18.8	69.6	29.7
Flow rate, ml/min	418	0.305	0.174	0.561	0.278	0.878	0.400
Urea nitrogen, mg/100 ml		9.0	2.45	7.7	1.84	7.5	1.83
Osmolality							
Flow rate, ml/min	314	0.333	0.189	0.619	0.298	1.019	0.458
Osmolality, mOsm/kg		57.1	18.44	8.72	28.25	127.2	34.95

Table 2 (continued)

Parotid fluid variables	Number of subjects	Candy drop exogenous stimulant					
		peppermint		cherry		grape	
		mean	SD	mean	SD	mean	SD
Protein, total							
Flow rate, ml/min	534	0.337	0.164	0.586	0.244	0.950	0.389
Total protein, mg/100 ml		146.0	55.5	205.0	78.5	319.0	122.8
Sialic acid							
Flow rate, ml/min	176	0.204	0.128	0.379	0.200	0.621	0.279
Sialic acid, mg/100 ml		0.74	0.50	0.73	0.43	1.05	0.53
Sodium							
Flow rate, ml/min	192	0.316	0.114	0.569	0.168	0.966	0.338
Sodium, mEq/l		15.4	9.96	33.2	18.23	54.9	16.91
Solids, total							
Flow rate, ml/min	318	0.363	0.176	0.634	0.284	0.943	0.402
Total solids, %		0.49	0.13	0.66	0.19	0.92	0.26
Specific gravity							
Flow rate, ml/min	384	0.323	0.171	0.577	0.256	0.943	0.402
Specific gravity		1.0024	0.0009	1.0036	0.0012	1.0061	0.0030
Steroids (free 17-OHCS)							
Flow rate, ml/min	50	0.333	0.168	0.633	0.291	0.962	0.389
Free 17-OHCS, μg/100 ml		2.48	1.08	2.20	0.81	2.06	0.74
Thiocyanate							
Flow rate, ml/min	79	0.153	0.047	0.253	0.069	0.428	0.119
Thiocyanate, mg/100 ml		5.47	3.23	3.81	1.83	3.20	1.77
Uric acid							
Flow rate, ml/min	314	0.329	0.177	0.612	0.275	0.993	0.421
Uric acid, mg/100 ml		3.82	1.09	3.15	0.91	2.85	0.80
Viscosity							
Flow rate, ml/min	384	0.323	0.170	0.577	0.267	0.943	0.402
Viscosity, cps		1.16	0.14	1.18	0.12	1.20	0.10
pH							
Flow rate, ml/min	368	0.350	0.196	0.631	0.302	1.015	0.457
pH		7.08	0.30	7.43	0.21	7.67	0.18
Phosphorus							
Flow rate, ml/min	100	0.343	0.130	0.556	0.173	0.830	0.235
Phosphorus, mg/100 ml		11.8	2.62	10.5	2.41	9.7	1.56
Potassium							
Flow rate, ml/min	192	0.316	0.114	0.569	0.168	0.966	0.338
Potassium, mEq/l		15.0	3.56	15.9	3.66	16.0	2.65

TABLE 3. Mean Value of Salivary Composition in Normal Adults (From Mandel, 1972).

Flow rate (ml/min per gland) mEq/l	0.7	0.6
	Parotid	Sub Mandibular	Plasma
Potassium	20	17	4
Sodium	23	21	140
Chloride	23	20	105
Bicarbonate	20	18	27
Calcium	2	3.6	5
Magnesium	0.2	0.3	2
Phosphate	6	4.5	2
Mg/100 ml			
Urea	15	7	25
Ammonia	0.3	0.2	-
Uric acid	3	2	4
Glucose	1	1	80
Total lipid	2.8	2	500
Cholesterol	1	-	160
Fatty acids	1	-	300
Amino acids	1.5	-	50
Proteins	250	150	6000
pH 6.8 - 7.2			

Table 4. Normal Values for Parotid Fluid Unstimulated (From Shannon, I.L. 1966).

Flow rate (ml/min per gland) mEq/l	0.04
Potassium	36.7
Sodium	2.6
Chloride	24.8
Bicarbonate	1.0
Calcium	3.0
Magnesium	0.3
Phosphate	15.3
Mg/100 ml	
Urea	19.6
Uric acid	9.5
Glucose	1

related to flow, potassium is independent of flow in the stimulated secretion, while calcium is flow dependent only at high flow rates. Hydrogen ions are present in saliva in greater concentration than in blood, except at high flow rates when the pH approximates that of blood. Bicarbonate concentration in saliva increases with salivary flow (5). At low rate of secretion its concentration may be from 5 - 10mEq/litre. With increased rate of secretion the bicarbonate concentration rises slightly but never to a value greater than that of the serum. With change in bicarbonate concentration there is a reciprocal change in the chloride concentration and the total anionic concentration is not markedly altered.

A linear relationship is found between the chloride concentration and flow rate (Fig No 1). However, at all flow rates the salivary chloride concentration is below that of the plasma and may vary between 5 - 70mEq/litre (5).

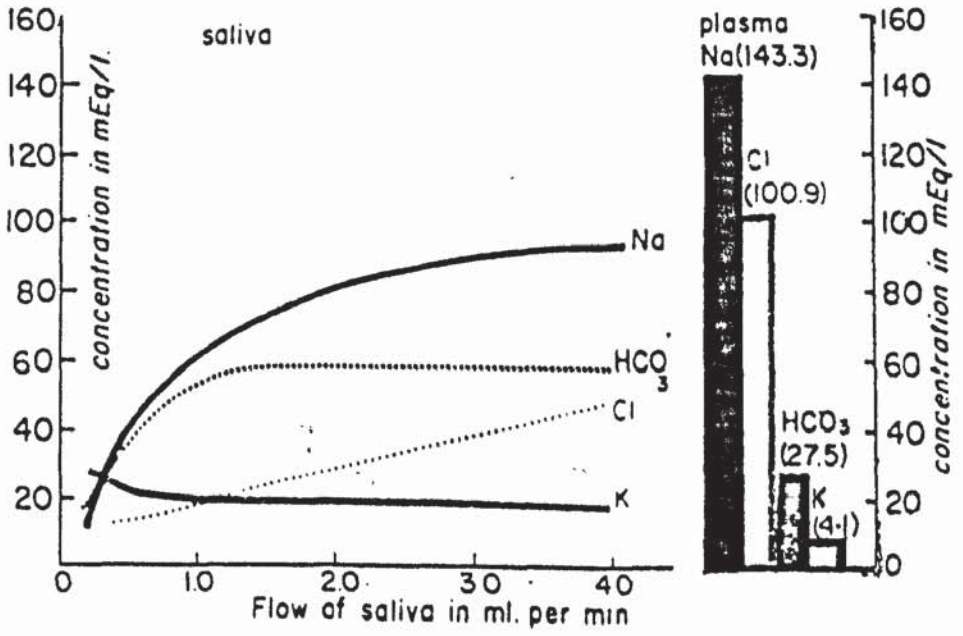


Fig. 1. Relationship between concentrations of sodium, potassium, chloride and bicarbonate in human parotid saliva and rate of salivary flow. Plasma concentration of the ions are also shown.

Salivary bromide is similar to that of chloride and the salivary/plasma ratio is slightly higher.

Salivary fluoride is between 0.08 and 0.25 parts per million and is unaffected by the level of fluoride in the drinking water unless the concentration exceeds 1.8 ppm.

Iodide in saliva is almost entirely inorganic in marked contrast to the plasma iodide (5).

Sodium concentration in saliva is highly dependent upon the rate of flow. In general the sodium content of saliva is low in the resting state and increases progressively as the secretory rate increases in response to cholinergic stimulation until the concentration approaches a maximum value (9). At low flow rates the concentration maybe less than 5mcg/litre, whereas at high flow rate it may exceed 100mcg/litre. This maximum is usually lower than the sodium concentration in both the primary saliva and in the plasma. Thus it is impossible to give a normal sodium concentration for saliva (5).

Potassium concentration of saliva is relatively high and exceeds that of the blood plasma. Ordinary mixed saliva usually contains 8 - 20mcg/litre of potassium which is 1.5 - 4 times the concentration in plasma (5).

Salivary calcium is present in the form of carbonate and phosphate and has an average concentration of 58mcg/ml and like sodium its concentration increases with increased flow rate(21). Not all calcium in saliva is ionized, about 20% of salivary calcium is protein-bound and the remainder (around 80%) is in the ionic form (9). But there is no necessary positive correlation between salivary calcium levels and plasma concentrations. Since salivary ionized calcium concentrations seem usually to be lower than those

of plasma, and since little is known of the primary fluid calcium content, it would be premature to speculate on the mechanism underlying the excretion of calcium in saliva (9). Submaxillary calcium concentration is about twice the parotid (6).

Other electrolytes are also present in saliva, such as ammonium, magnesium, cobalt, copper, cadmium, lead, iron and phosphate.

NON ELECTROLYTES (6)

The organic composition of saliva is quite variable and, as with the inorganic constituents, the amount and kind of each organic substance present depends on the gland as well as on the type and intensity of the stimulation.

Proteins

They comprise the bulk of the organic content of saliva. Mixed saliva contains about 0.3% protein which is responsible for its lubrication and digestive properties. This concentration increases with increasing flow rate.

1. Extrinsic proteins

These include albumin, orosomucoid, caeruloplasmin, β-lipoprotein, transferrin, β 2-macroglobulin and gamma globulins. Gamma globulins appear in saliva but the separable entities, such as IgG, IgA and IgM, aswell as others may have different points of origin (9).

The secretory immunoglobulin IgA (6)is the major

immunoglobulin found in saliva. The immunoglobulin IgG is present in saliva at an extremely low level, much lower than IgA. It seems that IgA is predominant in saliva and not IgG as in serum.

2. Intrinsic Proteins

Glycoproteins comprise the major group of salivary intrinsic proteins. A mixture of many glycoproteins form the mucin to which the viscous properties of whole saliva is attributed (6).

The submaxillary and sublingual glands produce blood group substances and the glycoproteins are responsible for the blood group type. From the secretion of the blood group substances (75% of the population) blood type ABO can be determined in a drop of saliva. The concentration of blood group in saliva is from 10 - 20mg/litre.

Enzymes

Whether carbohydrate containing or not, numerous enzymes are produced by the gland cells. The major salivary enzyme is amylase. It is an enzyme which catalyses the conversion of starch to maltose. Chloride and calcium ions are required for the activation of this enzyme (5). Copious quantities of amylase have been reported in the submandibular and parotid saliva, but the concentrations in the parotid saliva were very much higher than in the submandibular saliva (9).

Because of its high concentration in saliva, amylase values tend to parallel the total protein concentration of saliva.

Lysozyme

This is an enzyme which has the ability of lysing certain bacteria and is usually found in both parotid and submaxillary secretions (5).

Lactoperoxidase (6)

This is an enzyme which is part of the defence system present in exocrine secretions.

Lactoferrin

This red iron-binding protein is widely distributed in body fluids. It is an important factor in the antibacterial defence of saliva.

Other enzymes

A number of other enzymes have been identified in both parotid and submaxillary secretions, for example, alkaline phosphatases, non-specific esterases, lactate dehydrogenase, β-glucosaminidase, cholinesterase, phosphatase, lipase, kallikrein, renin, peptidase, peroxidase, ribonuclease, galactosidase, dehydrogenase and esterase (9).

Non Protein Compounds

Carbohydrates.

Earlier papers mentioned that it was doubtful whether glucose is present or not in saliva, but 110 - 300mg/litre of reducing substances were reported (2). Later on glucose was reported to be present but the quantity said to be present varies in different

papers according to the method used.

Smaller amounts of other hexoses, fucose, galactose, hexosamine and sialic acid are also present.

Free amino acids are found in saliva and their concentrations are uaually 10 -20% of the plasma level (5).

Vitamins.

Some vitamins have been reported but in very small concentrations, for example, B_1(thiamine), B_2(riboflavin), B6, B12, C, K, folic acid, biotin, choline, niacin and pantothenic acid (6). (Their names and concentrations are shown in table No 2). There are many small nitrogen containing compounds such as urea, uric acid, thiocyanate and purine which is present in a concentration of 9.5mg%. The prostaglandins pgA_2 or pgB_2 and pgF_2 have recently been estimated.

Recently hormones and their metabolites have been identified in saliva. Most of the steroid hormones have been identified, including cholesterol, cortisone, tetrahydrocortisol, testosterone, oestrogens and progesterone. The testosterone concentration in the saliva of males was 295ng/ml while that of females was 195ng/ml and in childrens saliva it was absent (9).

Factors Affecting Saliva Composition (6)

A number of factors can modify the saliva composition, these include:

1. Flow rate.
2. Nature and duration of stimulus.
3. Time of day.

4. Blood group and secretory status.

5. Dietary influence.

Rhythms In Saliva Flow Rate And Composition (13)

Rhythms in saliva composition and flow may have an effect on oral biology. In fact there appear to be many complex rhythms of varying frequency and amplitude. The circannual rhythm in salivary flow rate has already been considered but there are also underlying circadian and diurnal rhythms for sodium concentrations in unstimulated whole, parotid and submandibular saliva. Similar patterns can be shown for many other saliva constituents. Futhermore, the most important are the rhythms associated with the menstrual cycle in females which are probably related to the cycling in hormone levels.

Naturally, apart from their inherent importance, these rhythms should be considered in any experimental design or sampling procedure for saliva analysis.

SALIVA IN WOMEN

Recently sex hormone activity has been found to be related to the flow rate and concentration of electrolytes in human whole saliva. Therefore, the main conditions discussed are those in which sex hormones have a pronounced effect on salivary flow and composition during normal menstrual cycle, in pregnancy and during administration of oral contraceptives or other hormones.

1. Salivary Flow And Composition Changes During The Normal Menstrual Cycle (23)

The flow rate of stimulated whole saliva decreases prior to menstruation and increases during menstruation (24). Other workers found a decreased flow rate at time of ovulation of unstimulated submaxillary saliva.

Prout and Hopps (13) reported that there is no variation in unstimuulated whole salivary flow rate during the cycle. Hormonal changes may explain these flow rate changes.

Daily samples of parotid and submaxillary saliva collected from eight females during a complete menstrual cycle showed significant electrolyte changes in the submaxillary saliva.

Comparison of menstrual and midcycle concentration of calcium and sodium demonstrated a notable decrease in Ca^{++} and Na^{+} at midcycle, whereas K^{+} levels increased significantly. The changes were attributed to a probable hormonal effect on salivary composition. Tables, numbered, 5, 6, 7, and 8 demonstrate the submaxillary Ca^{++}, Na^{+} and K^{+} concentration with Na^{+}/K^{+} ratio during the menstrual cycle.

Table No. 5

SUBMAXILLARY SALIVA CONCENTRATION DURING THE MENSTRUAL CYCLE

Menstrual Cycle Period	Submaxillary Calcium (mEq/liter)
Entire cycle	3.9 ± 0.2
Menstrual	3.8 ± 0.2*
Preovulatory	4.2 ± 0.2†
Ovulatory	2.8 ± 0.2*†
Premenstrual	3.9 ± 0.3

Note: Eight woman; values are mean ± standard error.

* Significant difference between the respective means, $P < 0.001$.

† Significant difference between the respective means, $P < 0.001$.

Table No. 6

SUBMAXILLARY SALIVA SODIUM CONCENTRATION DURING THE MENSTRUAL CYCLE

Menstrual Cycle Period	Submaxillary Sodium (mEq/liter)
Entire cycle	31.5 ± 6.1
Menstrual	42.1 ± 4.7*
Preovulatory	33.7 ± 4.8
Ovulatory	30.8 ± 6.0*
Premenstrual	37.2 ± 5.2

Note: Eight women; values are mean ± standard error.

* Significant difference, $P < 0.05$.

Table No. 7

SUBMAXILLARY SALIVA POTASSIUM CONCENTRATION IN THE MENSTRUAL CYCLE

Menstrual Cycle Period	Submaxillary Potassium (mEq/liter)
Entire cycle	15.7 ± 1.1
Menstrual	15.3 ± 1.2*
Preovulatory	15.6 ± 1.2†
Ovulatory	17.9 ± 0.8*†
Premenstrual	15.6 ± 1.1

Note: Eight women; values are mean ± standard error.

* Significant difference between the respective means, $P < 0.05$.

† Significant difference between the respective means, $P < 0.05$.

Table No. 8

SUBMAXILLARY Na/K RATIO DURING THE MENSTRUAL CYCLE

Menstrual Cycle Period	Submaxillary Na/K Ratio
Menstrual	2.99 ± 0.4*
Preovulatory	2.26 ± 0.3
Ovulatory	1.78 ± 0.3*
Premenstrual	2.49 ± 0.4

* Significant difference, $P < 0.01$.

One of the findings was that of the decrease in calcium concentration in submaxillary saliva at ovulation day. This day was one or two days past the midcycle. The low submaxillary calcium at ovulation appears to be related to the oestrogen peak at that time.

Sodium concentration in submaxillary saliva reaches its highest level during menstruation and its lowest level at ovulation. A decreased flow rate was also observed at the time of ovulation. In parotid saliva, no significant changes occurred for any of these electrolytes.

Glucose appears to reach its maximum concentration in saliva at ovulation, and the concentration of glucose in an early morning sample of whole saliva could be related to the blueness of colour developed on an adapted dextrostix. At ovulation a maximum blue colour developed with no prior change and faded away in a few days (25).

Phosphate in unstimulated whole saliva reaches its peak concentration with ovulation at midcycle. Salivary hyaluronidase seemed to have a high level during the menstrual cycle, the maximum being reached towards the end of the cycle. Often the high level was maintained for only one day (26).

Salivary activity of N-acetyl-β-D-glucosaminidase (27)showed a characteristic change during the normal cycle with a distinct peak on days 13, 14 or 15 before the next cycle. This peak of enzyme activity occurred within one day of the nadir of basal body temperature and was absent in women with spontaneous or iatrogenic anovulatory cycles. These results indicate that the salivary determination of this activity could be a convenient method for determining the day of ovulation. A second characteristic increase of this enzyme is observed dt the end of the normal cycle about two

to four days before menstruation. It was noted that this peak was absent in all women who became pregnant. This absence, shortly before the time that menstruation is expected might be used as an indication of pregnancy. Since this absence occurs ten or twelve days after conception, it would be an earlier indicator than any other test available. Alkaline phosphatase presents a pattern similar to that shown by glucosaminindase, that is a characteristic midcycle peak in its activity (28).

2. Salivary Composition Changes During Pregnancy (29)

Since pregnancy represents a non pathological condition accompanied with many hormonal and metabolic changes a series of analyses have been done to observe what changes may occur in salivary composition during this period. The pH value was one of the significant changes noted in the salivary secretion. The pH value of saliva of non pregnant women is about 7.0, while that of pregnant women is 6.5 i.e. it becomes slightly acidic during pregnancy. The changes in salivary composition and secretions in the mouth during pregnancy can cause tooth decay. Marder, Wotman and Mandle (1972) found no change in calcium concentration in parotid secretion (30), whereas in the submaxillary secretions lower values were recorded for Ca^{++} during pregnancy than postpartum. In both parotid and submaxillary secretions, sodium concentration decreased and potassium concentration increased during pregnancy, that is the Na^{+}/K^{+} ratio described during pregnancy, returning to normal within six days of delivery.

The nitrogen content of the saliva of a non pregnant

woman has been estimated as 34.7mg/100ml, while that of the saliva of a pregnant woman was about 28.3mg/100ml of saliva (29). No obvious variations in Mg^{++} level during pregnancy were noted.

3. The Effect Of Oral Contraceptives(Hormones) On The Composition Of Human Saliva (30)

Sex hormones have a regulatory effect on the concentrations of electrolytes in human saliva. Although large individual variations in concentrations of various substances were found in response to the hormone administration, some of the parameters showed systematic and significant changes. In stimulated whole saliva, protein, sialic acid, hexosamine, fucose, hydrogen ion concentration and total electrolyte concentration decreased. The secretion rates for both parotid and submandibular secretions increased. The sodium and hydrogen ion concentrations increased in parotid secretion and sodium in submandibular secretion. The differences in concentration could not be explained by differences in secretion rate.

ADVANTAGES AND REASONS FOR UTILISING SALIVA FOR MONITORING (31)

1. Due to social and other personal reasons, saliva is the most easily collected of the body fluids and is continuously available for analysis. To collect a number of plasma samples, trained personnel and traumatic procedures are required, while saliva can be obtained by non invasive techniques and this is especially helpful when multiple serial samples are required daily.

2. Outpatients would avoid daily venipunctures for estimation of some substances in the blood if saliva were to be used, for example urea levels are easily measured in parotid saliva.

3. It could help in avoiding drug overdosage, and so it could be useful in the management of patients in chronic therapy, especially if the therapeutic concentration range of the drug is narrow.

4. Information on the concentration of some drugs or toxic chemicals in saliva would be more informative clinically than information on their concentraions in plasma. For example, values in saliva for several of the anticonvulsant drugs correspond to the free or pharmacologically active form of the drug in plasma. Excessive toxic levels of mercury and lead can be detected in saliva before they reach a dangerous level.

5. Saliva was suggested for monitoring of certain organic and inorganic environmental pollutants. The inorganic components include lead, mercury, silver, copper, zinc, fluorine and lithium.

In some instances saliva proved preferable to blood or urine. Such is the case for the salivary lead determination by atomic absorption (32). Mercury was measured in saliva and its levels compared to those in blood and urine (6, 33). The secretion of lithium was investigated in manic depressive patients. Lithium was found to be concentrated in saliva up to four times the plasma level (32). Besides lithium, strontium, iodine, fluorine, zinc and copper are found in saliva. The organic components measured in saliva include drugs, such as barbiturates, salicylic acid, antibiotics, theophylline and morphine (32). Tetrahydrocannabinol (the phenotoxic principal of hashish) was found to concentrate specifically in salivary glands, and can be monitored in saliva (32).

6. Salivary composition reflects certain physiological and pathological systemic changes, salivary composition and rate of flow are regulated by the hormonal balance of the body. For example, the sodium-potassium ratio reflects the rate of secretion of aldosterone and can be used in diagnosis of hypertension (32). In hyperthyroidism the rate of salivary secretion is increased. In diabetes, the protein composition of saliva changes. Salivary phosphate concentration may be used as an indicator of ovulation (32).

7. The concentration of most drugs in saliva corresponds to the free or unbound plasma drug concentrations and this is a more meaningful value for consideration of pharmacological activity than a value that reflects both bound and unbound drugs (6, 34). Electrolytes in saliva appear as a result of an active transport

system. As drug concentrations in saliva have been shown to be independent of flow rate, we would expect the concentration of other non electrolytes such as urea and uric acid which also passively diffuse into the saliva to be unaffected by flow rates. For saliva to be used in monitoring drug levels a correlation between plasma drug concentration and salivary drug concentration must exist. The excretion of drugs in the saliva occurs by simple diffusion. Also increasing the salivary flow rate simply dilutes the drug concentration, so that stimulated whole saliva will give similar results to unstimulated saliva (9). Most drugs, especially weakly acidic ones, are bound to some extent to plasma proteins; there being an equilibrium between the protein bound and free form. The free form reflects more accurately the biological activity of the drug. The differences between the total concentrations observed in saliva and the concentrations of free drug in plasma are attributable to the pH dependent transfer of these drugs.

SALIVA IN SYSTEMIC AND ORAL DISEASE (6)

1. Saliva And Oral Disease

Disturbances in the functions of the salivary secretions may cause an oral disease. Therefore the clinical importance of saliva in protection of the oral cavity becomes most apparent when malfunction of the salivary glands (due to irritation, disease or pharmacologic effects)results in a dry mouth. When teeth are present there is a heavy accumulation of plaque, material alba and debris. Caries progresses rapidly and extensively while gingivitis is exacerbated.

No correlation has been found to exist in whole stimulated saliva between the flow rate and extent of caries or between sodium, potassium, solids and ash contents with caries incidence.

The whole stimulated salivary citrate concentration appears to vary in some way with the incidence of caries. IgA concentration is low in patients with high caries incidences.

Measuring the oral glucose clearance time and capacity of the buffering system does give some indications of caries expectancy. Low salivary pH will tend to enhance the indication of caries. The mineral components of the supragingival calculus are derived almost entirely from the salivary fluids. Elevated levels of some of the components of either whole saliva, parotid or submaxillary secretions, for example calcium or phosphorus in adults or in children, are major determinants of calculus formation.

2. Saliva and Systemic Disease

Recently useful information has been established in diagnosis or prognosis and evaluating the effect of treatment of a disease has been obtained from studies of salivary fluid.

Salivary glands are affected by cystic fibrosis. The specific effects in the submaxillary and labial secretions may provide secondary diagnostic tests for this disease and may also provide clues as to the pathogenesis of the disease. Elevation in calcium is found in other secretions in cystic fibrosis patients.

Other diseases which affect salivary glands are cirrhosis, malnutrition and diabetes. Patients with cirrhosis have a secretion with a high flow rate. The diabetics show increased calcium in their saliva and a change in immunoglobulin composition.

Hypertensive disease: A correlation between changes in plasma renin, aldosterone, excretion and angiotensin with salivary electrolyte changes, if established, may provide a useful screen for patients with essential hypertension.

CONCLUSION

Blood, urine and saliva are the three main body fluids most readily available for analysis, diagnosis and prognosis. To establish saliva in the first line of analysis it must possess distinct advantages over the other two commonly used fluids. It is important in salivary studies to standardise and regulate the conditions if the results are to be valid. This places restrictions on the application of salivary studies.

Whole saliva also contains gingival fluid which contains substances such as certain immunoglobulins not found in the individual secretions. Thus care must be taken to exclude this fluid when studying these substances.

In many cases the bodily changes are mirrored in the individual secretions and not in the whole saliva. Thus a complication of collecting these secretions exists, limiting the convenience of the procedure. This is particularly the case in electrolyte studies. However, the use of saliva for analysis possesses many distinct advantages. Since saliva is collected at the point of its manufacture, it is therefore unaffected by collection or storage in the body as happens with other body fluids, this is very important for the determination of many organic and inorganic constituents specially those which are affected during storage.

One of the major advantages over any body fluid in monitoring drug levels, is that saliva reflects the current free plasma drug concentration which relates to the drug's biological activity (6). Saliva can be rapidly and conveniently collected in an "office type" environment and may be useful in dealing with outpatients. It can be obtained continuously in a restricted environment, by non-invasive

techniques avoiding the development of stress.

The salivary findings in hypertension and cystic fibrosis point to a relationship between salivary composition and hormonal and autonomic status. It has been suggested that changes in salivary electrolytes may be potentially useful in evaluating endocrine changes. It is also useful to know the begining of the fertile period, as then, intercourse will be most fruitful, or to avoid conception.

CHAPTER II

OBJECTIVES AND METHODS

1. Introduction

Since saliva reflects certain physiological and pathological systemic changes, any change in these processes will be indicated in changes in saliva (32).

Salivary composition is subject to many variables. A series of investigations into the composition of collected salivary secretions were carried out to determine what changes in electrolyte and non electrolyte composition occur in the normal menstrual cycle. Information gained from the changes observed could be of interest to salivary physiologists and endocrinologists and might also prove of value to the practising obstetricians (35).

Salivary composition changes have been found to be related to the hormonal changes and activity during the cycle. This change in composition and character could be used as a basis of a test for ovulation (23). The object of the research project was to develop methods for the determination of the level of some constituents of mixed unstimulated saliva of the human female during the normal menstrual cycle. In this way it might be possible to determine the beginning of the fertile period and the exact day of ovulation. Three components which belong to different chemical groups were chosen for this purpose. The three components were calcium, glucose and the enzyme N-acetyl-β-D-glucosaminidase and belongs to the electrolytes and the non electrolytes (carbohydrates and enzymes)groups respectively.

2. Glucose

Glucose was selected because in some early papers there appears to be doubt as to whether glucose was actually present in saliva. There also appeared to be a lack of a sensitive, specific and reliable method for the determination of very low concentrations of glucose in saliva. Suitable microanalytical techniques were then developed, enabling the glucose concentration to be quantified. Values reported depend on the method used (36). Early values, from an assay utilising the reducing properties of glucose, were overestimated as the method was non-specific for glucose (11.28 - 28.08 mg% for unstimulated and 14.04 - 30.00 mg% for stimulated whole saliva) (37). In whole saliva, a more recent study reports the level of glucose to be 3.65 mg% (38).

Another important reason for selecting glucose was that monitoring for salivary glucose levels has a number of applications, for example, in diabetes mellitus, glucose has been reported to be elevated in the parotid saliva. Several studies have reported success in employing parotid secretions as a means of performing the glucose tolerance test (39, 40). The glucose level is sensitive to an intravenous administration of glucose and parallels the serum concentration. It would thus appear that parotid secretions offer a convenient method of diagnosing sugar metabolising disorders and monitoring treatment. Another application is that it was shown that women could monitor their own fertility by regularly checking their saliva for the presence of glucose (41). It appears that two days before ovulation salivary glucose is usually at alevel which can be detected by a faint blue colour on a 'Dextrostix' test strip. The blue colour

then maximises at ovulation and fades away within two or three days after ovulation.

One of the aims of this research project was therefore to develop a very sensitive method for the determination of the glucose level of mixed unstimulated saliva of normal women during the menstrual cycle to confirm or disprove the above reports. This might also be useful in predicting the ovulation period during the menstrual cycle.

3. The Enzyme N-acetyl-β-D-glucosaminidase

In some earlier reports on the composition of saliva during the menstrual cycle, it was noted that the levels of the enzyme N-acetyl-β-D-glucosaminidase increased sometime during the menstrual period. Later on, some of the more recent reports have shown that the lysosomal enzymes activity change during the menstrual cycle was related to the time of ovulation. Of several lysosomal enzymes originally screened, only the activity of N-acetyl-β-D-glucosaminidase showed a characteristic pattern of changes during the normal menstrual cycle with a distinct peak on days 13, 14, or 15 before the next menstruation. This peak of enzyme activity occurred within one day of the nadir of basal body temperature and was absent in women with spontaneous or iatrogenic anovulatory cycles. These results are strongly suggestive that the salivary determination of this activity may be a convenient indicator for determining the day of ovulation (27).

It was decided to investigate whether the salivary enzyme activity was indeed a general and practical indicator of ovulation by studying the variation in the activity of this enzyme throughout the menstrual cycles of a number of women.

4. Calcium

In biological fluids (in serum and saliva) calcium is usually combined with protein. In saliva the binding of calcium to salivary phosphoproteins, protein A and protein C have been described (43). In diabetes mellitus an elevated level of calcium has been found in parotid and submaxillary saliva of a significant number of subjects (44). There are a few reports concerning the changes of the calcium level during the normal menstrual cycle. For example, in one study on electrolytes in saliva during the menstrual cycle, which was made by Puskulian in 1972, it was shown that in the submandibular saliva calcium and sodium levels were significantly lower at ovulation, whereas the potassium levels were significantly higher. In the parotid saliva no significant changes occurred for any of these electrolytes (23). Therefore, it was decided to investigate whether there were significant changes in the calcium levels of whole saliva during the menstrual cycle which might correlate with the time of ovulation. Calcium, therefore, could be a salivary electrolyte component, which perhaps could be used as a monitor of hormonal change.

CHAPTER III

CHOICE OF METHOD

1.Introduction

After surveying the different available methods for determining the selected constituents of saliva samples, glucose, calcium and the enzyme N-acetyl-β-D-glucosaminidase, it was decided to use fluorescence as the analytical technique. The decision was made on the basis of the known sensitivity and the frequently accompanying specificity of fluorescence assays.

i. Sensitivity

Fluorometric methods can detect concentrations of substances as low as one part in ten billion. This is a sensitivity of a 1000 times greater than that of most spectrophotometric methods. The reason for this increased sensitivity is that in fluorescence the emitted radiation is measured directly and can be increased or decreased by altering the intensity of the exciting radiant energy. An increase in signal over a zero background signal is measured in fluorometric methods. In spectrophotometric methods the analogous quantity, absorbed radiation, is measured indirectly as the difference between the incident and the transmitted intensities. This small decrease in the intensity of a very large signal is measured in spectrophotometry with a correspondingly large loss in sensitivity.(46).

ii. Specificity

The specificity of fluorescence is a result of two main factors (46):-

(a) There are fewer fluorescent compounds than absorbing ones because all fluorescent compounds must necessarily absorb radiation, but not all compounds that absorb radiation are fluorescent.

(b) Two wavelengths are used in fluorometry, but only one in spectrophotometry. Two compounds that absorb radiation at the same wavelength will probably emit at different wavelengths. The difference between the excitation and emission peaks ranges from 10 - 280 nm.

Another advantage (also a limitation) is that generally only certain aromatic molecules fluoresce (47). This excludes from fluorometry all acyclic and alicyclic molecules, aswell as those aromatic molecules that do not fluoresce. (This is of course an advantage when analysing a mixture containing a fluorescent aromatic molecule and several acyclic or alicyclic molecules.)

2 The Fluorescence Process

Every molecule possesses a series of closely spaced energy levels and can be excited from a lower energy level to a higher one by absorbing an integral quantum of light which is equal in energy to the difference between the two energy states (Fig. No. 2). Only certain molecules which are raised to this higher excited state are capable of exhibiting fluorescence or undergoing photochemical change. Between each main electronic state there are a number of vibrational levels, for the molecule. In Fig. No. 2 , which illustrates the various potential energy levels of a diatomic molecule, are indicated the various vibrational levels, represented as 0, 1, 2, 3, and 4, of each

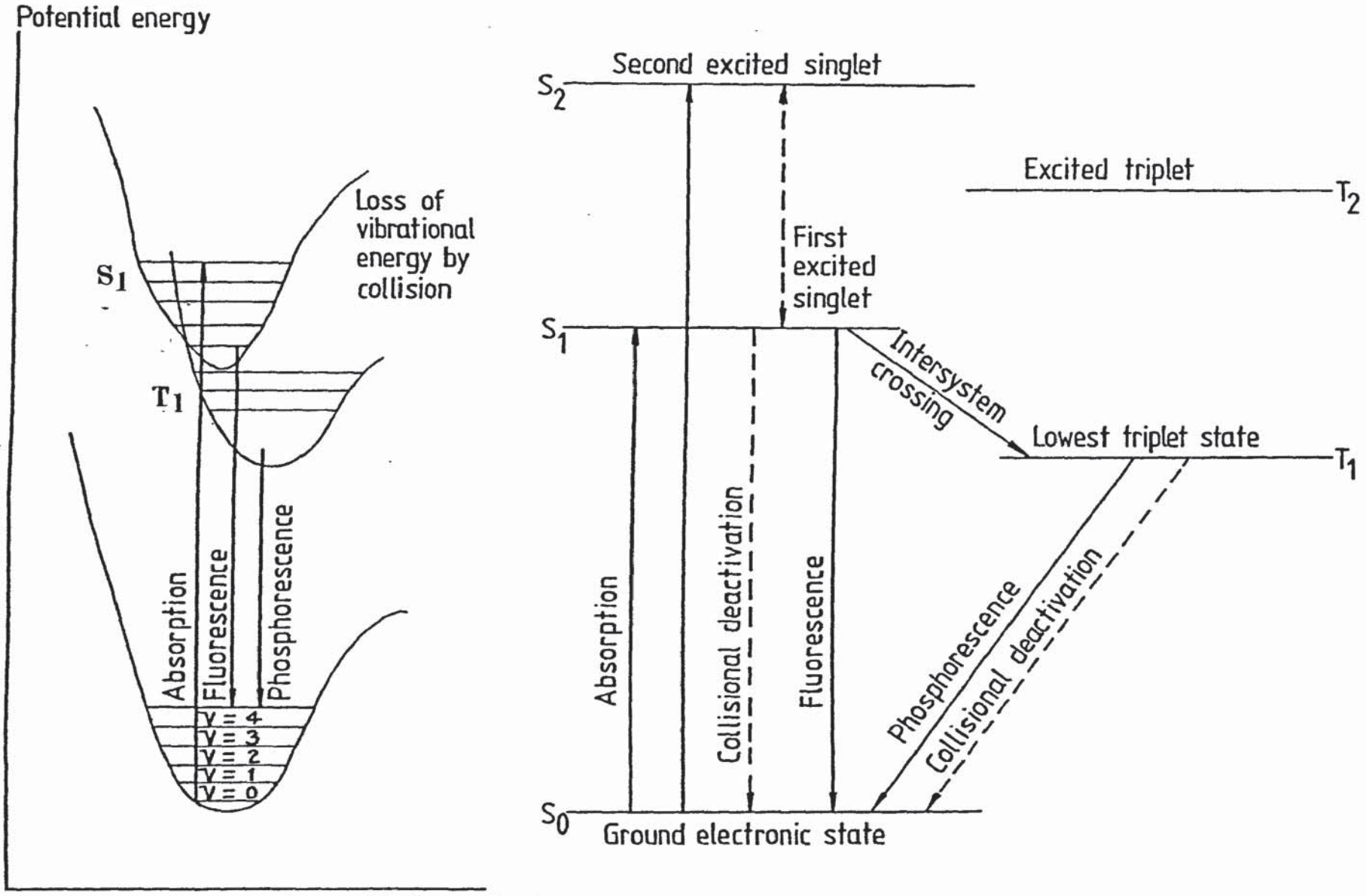


Fig 2 Schematic energy-level diagram for a diatomic molecule

curve. The ground state is indicated by G, the first excited singlet electronic state by S, and the first excited triplet electronic state by T. The differences between the singlet and triplet states resides in the different spins, S, of the electrons. All electrons have a spin equal to $\pm \frac{1}{2}$. The arrow designations, which are often used to denote spin (1↓), arise from the right-hand rule. If one curls the fingers of the right hand in the direction of the spin, the thumb points in the direction of the arrow, and vice versa.

A normal polyatomic molecule in the ground state, G, usually has an even number of electrons with paired spins. Multiplicity is a term used to express the orbital angular momentum of a given state and is related to the spin by the equation: $M = 2S + 1$. Thus, when all electrons are paired, $S = 0(+\frac{1}{2} - \frac{1}{2} = 0)$, and the multiplicity equals 1. This is called the singlet electronic state. When the spin of a single electron is reversed, the molecule finds itself with two unpaired electrons and $S = 1$, $(+\frac{1}{2} + \frac{1}{2} = 1)$, and the multiplicity is 3, $(2\ (1) + 1 = 3)$. This electronic state is called a triplet.

The relationship between wavelength, energy, and colour are shown in Table No. 9 . Photons in the ultraviolet and visible regions of the electromagnetic spectrum have energies of 145 - 35 Kcal/mole and promote electronic transitions. More energetic photons may cause photodecomposition. Less energetic photons have only enough energy to cause a vibrational or rotational transition. The less energetic vibrational transitions are often superimposed on the electronic transition and are observed as fine structure. When a quantum of light strikes a molecule, it is absorbed in about 10^{-15} sec, and a transition to a higher electronic state takes place (Fig. No. 2). This absorption of radiation is highly specific, and radiation of a particular energy is absorbed only by a characteristic structure. The electron is raised

Region	Wavelength (nm)	E (kcal/mole)	Color	Complement
Ultraviolet:				
Far	200	143.0	--	--
Near	250	114.5	--	--
	300	95.4	--	--
	350	82.0	--	--
Visible	380	75.5	Violet	Yellow-green
	400	71.5	Violet	Yellow-green
	450	63.8	Blue	Yellow
	500	57.3	Blue-green	Red
	550	52.1	Green	Purple
	600	47.7	Orange	Green-blue
	650	44.1	Red	Blue-green
	700	40.9	Red	Blue-green
	750	38.2	Red	Blue-green
Near infrared	780	36.7	--	--
	800	35.8	--	--

Table No. (9). Relationship of Wavelength to Energy and Color

to an upper excited singlet state, S1, S2, etc. These ground to singlet transitions are responsible for the visible and ultraviolet absorption spectra observed for molecules. The absorption transitions usually originate in the lowest vibrational level of the ground electronic state.

During the time the molecule can spend in the excited state, 10^{-8} sec, any energy in excess of the lowest vibrational energy level is rapidly dissipated. The lowest vibrational level (V = 0) of the excited singlet state, S, is attained. If the remaining excess energy is not further dissipated, say by collision with other molecules, the electron returns to the ground electronic state, with the emission of energy. This phenomenon is called fluorescence. Because some energy is lost in the brief period before emission can occur, the emitted energy (fluorescence) is of a longer wavelength than the energy that was absorbed. The phenomenon of phoshorescence involves an intersystem crossing, or transition, from the singlet to the triplet state. A triplet state results when the spin of one electron changes so that the spins are the same, or unpaired. The transition from the ground state to the triplet state is a forbidden transition (highly improbable).

Internal conversion from the singlet state to the triplet state (electron-spin reversal) is more probable since the energy of the lowest vibrational level of T is lower than that of S. Molecules in T can then return to the ground state, G, directly, since a return via S could result only by acquiring energy from the environment. (This sometimes occurs and is called delayed fluorescence). Transition times of 10^{-4} sec - 10 sec are observed in phosphorescence. Hence a characteristic feature of phosphorescence is an afterglow, that is, emission that continues after the exciting source is removed. Because of the relatively long lifetime of the triplet state, molecules in this state are much more susceptible to radiationless deactivation processes, and only substances dissolved in a rigid medium phosphoresce. This is

generally achieved by cooling the sample solution to liquid nitrogen temperatures.

3. Emission Processes accompanying Fluorescence.

The fluorescence normally observed in solutions is called Stokes fluorescence. This is the re-emission of less energetic photons, with a longer wavelength (lower frequency) than the absorbed photons.

If thermal energy is added to an excited state or a compound has many highly populated vibrational energy levels, emission at shorter wavelengths than those of absorption can occur. This is anti-Stokes fluorescence, often observed in dilute gases at high temperature. A common example is the green emission from copper activated cadmium sulfide excited by red light.

Resonance fluorescence is the re-emission of photons possessing the same energy as the absorbed photons. This type of fluorescence is never observed in solution because of solvent interactions, but it does occur in gases and crystals.

If a molecule is excited, by absorbing a photon, to a higher vibrational level with no electronic transition then energy is entirely conserved and a photon of the same energy is re-emitted within 10^{-15} seconds as the molecule returns to its original state. The emitted light has the same wavelength as the exciting light since the absorbed and emitted photons are of the same energy. The emitted light is referred to as Rayleigh scattering and occurs at all wavelengths. Its intensity, however, varies as the fourth power of the wavelengths, so its effect can be minimized by working at longer wavelengths. It is a problem when the intensity of fluorescence is low in comparison with the exciting radiation and when the absorption and fluorescence spectra of a substance are close together.

Another form of scattering emission related to Rayleigh scattering is the Raman effect. Raman scatter appears in fluorescence spectra at higher and lower wavelengths (the former being more common) than the Rayleigh scatter peak. These Raman bands are satellites of the Rayleigh scatter peak with a constant frequency difference from the exciting radiation. The bands result from vibrational energy being added to, or subtracted from, the excitation energy. The Raman bands are much weaker than the Rayleigh scatter peak but become significant when high intensity sources are used.

4. Instrumentation (46, 47)

The two types of fluorescence instruments that are used are the filter fluorimeter and the spectrofluorometer.

The four main components of any fluorescence instrument are the source of excitation, the sample cell, the detector, and the filter or gratings used to select the exciting and emitted radiation. Figure No. (3) shows the essential compounds of any filter fluorimeter or spectrofluorometer. The source is a mercury arc, or xenon arc lamp (the latter is usually used in spectrofluorometers). The exciting radiation passes through a grating or primary filter, allowing only a certain range of wavelength to strike the sample. Gratings are used in spectrofluorometers, filters in filter fluorimeters. The major advantages of the grating are that it selects any given wavelength from 200 - 600 nm and that it passes a constant bandwidth (such as 10 nm) of radiation, no matter what the wavelength. The spectrofluorometer with its more selective grating, may be adjusted so that only one substance is excited whereas a filter may allow two substances to be excited. The disadvantage of mercury source (plus filter) is that

there are a limited number of mercury lines available with which to excite a sample. Most of the exciting radiation passes through the sample cell without being absorbed. The radiation absorbed causes the sample to fluoresce in all directions, but only the emission that passes through the aperture or slit and through the secondary filter or fluorescence grating (which are located at 90° to the incident optical path) is measured by the phototube, or photomultplier. The output of the photodetector, a current that is proportional to the intensity of the fluorescent energy, is amplified to give a reading on a meter or plotted on a recorder. An x - y recorder is used to display the excitation and emission spectra.

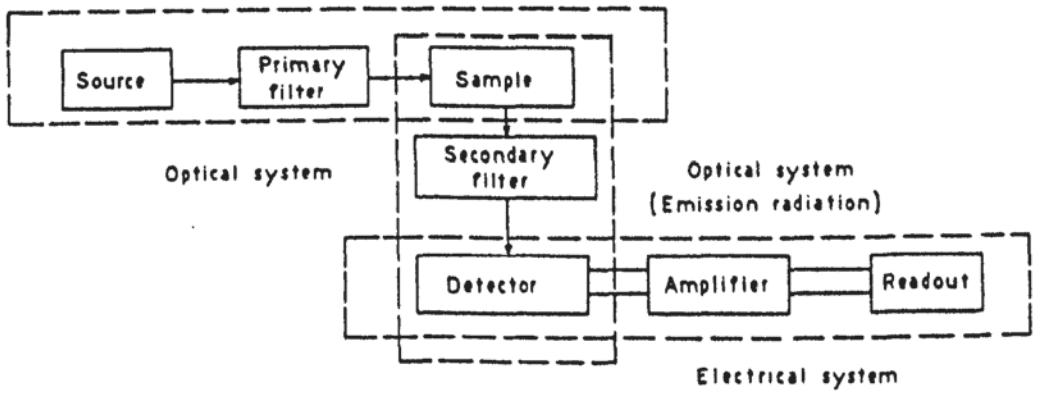


FIG. 3 Schematic diagram of the optical components of a typical filter fluorometer.

CHAPTER IV

PREPARATION OF SALIVA SAMPLES

Preparation of saliva for Calcium and N-acetyl-β-D-glucosaminidase Determination

About 5.0 ml of unstimulated whole saliva was collected daily by spitting into a polyethylene tube. The collection was always carried out before breakfast and after rinsing the mouth out with water, but without washing with toothpaste. The saliva samples were then diluted with the buffer and centrifuged at 10,000 r.p.m. for different periods of time, for example 5, 10, 20, and 30 minutes.

When the collected saliva samples were examined it was found that they were opaque or milky turbid solutions with both suspended and precipitated solid material. The amount of this solid material varied considerably between the individual donors. Centrifugation was adopted as a technique for the removal of this fraction to give clear supernatant saliva samples for analysis. These samples, however, were found to be relatively very viscous and volumes as large as 0.5 - 1 ml could not be measured out with an acceptable precision. To achieve satisfactory precision in handling the saliva it was found necessary to dilute the saliva by a factor of two with a buffer solution before centrifuging. When this dilution was done with distilled water, the clear supernatant, after storage in a freezer, still gave turbid solutions with precipitated solid material. Also different results were obtained for the same samples when estimations of selected constituents were made on successive days. Therefore, a buffer was chosen for dilution instead of distilled water. The buffer was a

citric acid/phosphate one of pH 5.00. This was selected because it was also to be the buffer in the enzyme N-acetyl-β-D-glucosaminidase reactions. After dilution with this buffer and centrifugation the clear, less viscous supernatant could be stored in a freezer and when thawed out still gave a relatively clear solution with very little solid material. This made the measurement of small volumes of it easier and more precise as was found from consecutive repeated determinations.

The procedure described was found to give satisfactory results in the determination of calcium and N-acetyl-β-D-glucosaminidase in saliva samples. When the same procedure was used for the preparation of samples for glucose analysis in saliva negative results were obtained. Clearly the technique was inadequate and further treatment of saliva was required in order to determine its glucose content.

Preparation of Saliva for Glucose Determination

Even when glucose solutions of different concentrations (10 - 40 mcg/ml) were mixed with the centrifuged saliva samples no reaction took place.

It was found that when glucose solutions on their own were used then the reaction started immediately with an accompanying increase in fluorescence. If saliva was added during this stage, the reaction stopped immediately. From these observations it was concluded that there were some constituents in the saliva that interfered with the glucose oxidase-peroxidase enzyme system and inhibit their action. It was suspected that the proteins, which are the main constituents in saliva, may have a strong inhibitory action on the enzymes. Therefore, several attempts were made to remove most of the proteins

from the saliva samples without affecting the glucose concentration. Saliva samples were treated with 10% w/v and 20% w/v solutions of $(NH_4)_2SO_4$. In each case about 3 ml of saliva was mixed with 3 ml of $(NH_4)_2SO_4$, and then centrifuged at 10,000 r.p.m. for 25 minutes. Samples collected before breakfast and after breakfast were used. No reaction occurred except with saliva samples to which glucose, of 10 mcg/ml, had been added. A very slow reaction started eight minutes after adding the sample.

Other saliva samples were treated with 5% w/v trichloroacetic acid, then centrifuged for 20 minutes at 10,000 r.p.m. The pH of this saliva sample had dropped down to 2.00. No reaction was observed at all, even when this treated saliva was mixed with tris buffer to bring the pH to 8.5. However, when glucose was added to this sample to give a final concentration of 50 mcg/ml, the reaction, and the change in fluorescence, started after 20 seconds.

Then perchloric acid (0.1N) was used as a protein precipitant. The conditions were similar to those of trichloroacetic acid. However, the saliva treated this way again gave no reaction with the enzymes to indicate the presence of glucose.

A molecular separator was next used for deproteinizing the saliva samples (48). In this technique the protein containing solution is drawn under reduced pressure through a membrane that acts as a molecular filter. Small molecules such as water, peptides, carbohydrates, and also salts dissolved in water pass through the membrane and are collected in the aqueous filtrate. Large molecules such as proteins and colloids cannot pass through and are retained by the membrane. When saliva samples were deproteinized by means of this separator they were found to be unreactive with the enzyme reagents. The molecular filter therefore was not able to remove the substances inhibiting the

enzyme system. This was clear from the observation that even with added glucose the saliva samples treated in this way still did not give positive results. In case this lack of reactivity was due to insufficient reagent levels the concentration of the enzymes, glucose oxidase and peroxidase, were increased but no better results were obtained. Then the tris buffer was changed for a phosphate buffer, of pH 8.4, in which the peroxidase enzyme and the substrate, p-hydroxyphenylacetic acid (49), was changed and tyramine was used instead (49). It was tested in the presence of different amounts of glucose oxidase, but once more no reaction was observed with the saliva even after waiting for over one hour. It was thought at this stage, that either the glucose in saliva was present in some unreactive form or that the enzyme inhibitors were non-protein in nature. It was decided to try and 'free' the glucose in the saliva by simply heating it. Saliva samples were therefore centrifuged and the clear supernatant was heated in a boiling water bath for fifteen minutes to precipitate the remaining proteins and then centrifuged again. Some of these saliva samples when mixed with a glucose solution, at 20 mcg/ml level, gave a positive reaction in two minutes after adding the sample, with a regular increase in fluorescence every fifteen seconds. It was only the saliva samples which had previously been diluted with distilled water and heated that gave positive results in mixing with glucose solutions. For on carrying out the procedure on an undiluted saliva sample which had been concentrated to half its original volume and then mixed with glucose no reaction was observed after several minutes. It appeared therefore that the effect of heat was not so much that of freeing the glucose, but of inactivating some enzyme inhibitors provided they were in relatively low concentration. But even with added glucose some of the saliva samples failed to give positive results.

Clearly there still remained some inhibitors and the saliva needed further treatment unless, of course, it contained no glucose in the first instance.

It is known that saliva contains a variety of cations, some of which could act as enzyme reaction inhibitors. An attempt was made to remove these by complexing with E.D.T.A. of different concentrations. For example, 0.05 M and 0.1 M,E.D.T.A. solution was used with centrifuged saliva, molecularly filtered, heated and centrifuged saliva. In each instance, however, there was no reaction without added glucose and even with added glucose the reaction was very slow. It was also observed that E.D.T.A. treated glucose solutions gave reduced fluorescence intensities of about 70% of that of untreated solutions. The E.D.T.A. did not therefore remove all the interfering ions and also acted as a fluorescence quencher. It appeared that a more efficient method was required to remove ionic inhibitors from saliva. It was thought that ion exchange resins would fulfill that requirement, and it was decided to remove both the cations and anions. Therefore a mixture of an anionic and cationic resin was used. The following ion exchange resins were tried initially:-

1. Amberlite IR 120 (Na) standard grade (50).
   This is a strongly acidic resin, used for water softening, deionization, and separation of rare earths, peptides, amino acids and inorganic substances.

2. Amberlite IRA 401 (HCL), standard grade (50).
   This is a strongly basic resin with high porosity for recovery or removal of large molecules, water conditioning, removal or recovery of high molecular weight anions, fractional separation of organic

acids, amino acids and metals as anionic complexes.

After they were regenerated, and made neutral by washing several times with 0.1 N HCL and 0.1 Na OH, 2 gms of each of these resins were mixed together and used for the treatment of saliva. The samples were diluted with the buffer, centrifuged, heated in a boiling water bath, for fifteen minutes, and centrifuged again. They were then shaken with the resins for thirty minutes and after centrifugation the supernatant was removed for the glucose estimation. After this treatment it was found that some samples gave a positive result immediately, although others still gave negative results in the absence of added glucose. On the addition of a 1.0 mcg/ml glucose solution to the resin treated saliva it was observed that the rate of the reaction was increased. From the above experiments it was concluded that treatment with the ion exchange resins was useful and could remove some inhibitors to give positive results. However, the results appeared to depend on the amount of glucose and inhibitors present in each sample. Therefore, further experiments were carried out to find the most suitable resins and conditions of treatment by which all the inhibitors could be removed. Thus analytical grade resins of both Amberlite IR 120 and IRA 401 were used instead of the standard grade. These resins were also washed, regenerated and made neutral.

Some standard glucose solutions were treated with the resins, for 45 minutes, and their fluorescence intensities were compared with the fluorescence intensities of resin untreated glucose solutions. It was found that there was a 30% loss for the treated 1 mcg/ml glucose solutions, 20% loss for the treated 2 mcg/ml glucose solution and 14% loss for the treated 4 mcg/ml treated solution. A comparison was also made between the regenerated and non-regenerated resins. The

Table No. 10 shows the different effect of the treatment of the resins on the glucose loss.

Table No.10

Glucose concentration in mcg/ml	Treated with regenerated resin	Treated with non-regenerated resin
1.0	30% loss	56% loss
2.0	60% loss	65% loss

Therefore, washing and regeneration of the resin is necessary in order to avoid a considerable loss of a glucose and also to obtain lower blank readings.

Another comparison was made between regenerated analytical grade resin and regenerated standard grade resin. The results are in the Table No.11 .

Table No.11

Glucose concentration in mcg/ml	Treated with regenerated analytical grade resin	Treated with regenerated standard grade resin
1.0	33% loss	45% loss
2.0	36% loss	50% loss
4.0	41% loss	50% loss

From Table No.11 it is seen that treatment with the regenerated analytical grade resin shows a smaller loss than with the regenerated standard grade resin. However, the loss of glucose onto these resins was thought to be unacceptably large. To try and reduce this loss it was decided to try and saturate the resins with some sugar, other than glucose, prior to their use for the deionization of saliva samples. It was hoped that in this way the saliva glucose might all be recovered without, presumably, much adsorption onto the resin. Sucrose was used for this purpose after verifying that it did not react with the enzyme reagents to give any appreciable fluorescence. Sucrose solutions in the range of 4 - 200 mcg/ml (4, 10, 20, 50, 100, 150 and 200 mcg/ml) were used and saturation was achieved by shaking the resins (2g. cation + 2g. anion) with the sucrose solution for fifteen minutes using a mechanical shaker. After centrifuging for 5 minutes the supernatant was separated and analysed for its glucose concentration. When the regenerated analytical grade resin was saturated with several sucrose solutions of different concentrations it was found that saturation with a solution which gives 20 mcg/ml sucrose in the final solution, gave the following results:-

Table No.12

Glucose concentration in mcg/ml	Percentage loss
1.0	0%
2.0	12.5%
4.0	17%

Saturation of the resins with sucrose solutions at concentrations greater than 20 mcg/ml, gave higher blank readings and higher fluorescence intensities than those of standard glucose solutions.

Following this the Amberlite IRA 401 resin was changed to Amberlite CG400 (Cl) type I, 100 - 200 mesh (50). This anion exchange resin is strongly basic and more specific for the recovery of high molecular weight anions, fractional separation of organic acids, separation of amino acids and of metals such as, Pb^{++}, Fe^{++}, Cu^{++}, and Ca^{++} as anionic complexes. This resin was washed and regenerated and used with an equal weight of the IR 120 resin after saturation with sucrose solution at a final concentration of 20 mcg/ml. It was found that this resin combination gave much improved results. The reaction of saliva samples with the enzyme reagents starts immediately with a linear increase in fluorescence intensity every fifteen seconds. For the saliva samples with added glucose the fluorescence intensity was equal to the sum of the fluorescence intensity of both the saliva sample and the standard glucose solution. However, when standard glucose solutions were treated with these resins, it was found that there was still a 5 - 10% loss of the glucose.

Further attempts were made to find the optimum procedure of saliva treatment with the resin. It was finally found that the best results were obtained when the regenerated cation and anion exchange resins were soaked in double their amount of sucrose solution, which gave a concentration of 20 mcg/ml in the final solution. The mixtures were stirred vigorously by a magnetic stirrer for fifteen minutes, then filtered off under reduced pressure. A mixture of 1.5 g of regenerated saturated anion resin CG400 and 1.5 g of regenerated cation resin IR120 was sufficient for the treatment of 5 ml of standard glucose solution or 5 ml of diluted, heated and centrifuged saliva sample. The blank,

standard or saliva sample were mixed with the resins and shaken rapidly on a mechanical shaker for 10 minutes. The mixture was then centrifuged, at 10,000 r.p.m. for 5 minutes, to give a clear supernatant. From the clear supernatant, 0.1 ml, 0.25 ml and 0.5 ml aliquots were taken and their glucose content was determined.

CHAPTER V

GLUCOSE ESTIMATION

The object of this study was the determination of the concentration of one of the non-electrolytes in saliva and its relation to the hormonal changes which occur during the menstrual cycle of the human female. Glucose was chosen because in some early papers doubt was expressed as to whether it was actually present in saliva. There also appeared to be a lack of a sensitive, specific and reliable method for the determination of very low concentrations of glucose in saliva. Most of the reported methods have been applied to biological tissues other than saliva.

Applications of monitoring glucose levels

Monitoring for salivary glucose levels has a number of applications, for example in diabetes mellitus, glucose has been reported to be elevated in the parotid saliva (42).

Several studies have reported success in employing parotid secretions as a means of performing the glucose tolerance test (39, 40). The glucose level is sensitive to an intravenous administration of glucose and parallelsthe serum concentration. It would thus appear that parotid secretions offer a convenient method of diagnosing sugar metabolising disorders and monitoring treatment.

Another application is that, at ovulation glucose has been reported to become maximally elevated in whole saliva and has been used to identify the beginning of the fertile period during the menstrual cycle (25). The aim of this project was therefore the

estimation of glucose levels in the saliva of normal women, to confirm or disprove the above report.

Methods for Glucose Determination (51)

Methods for the determination of glucose in biological fluids fall under two main categories; the chemical and the enzymatic methods. These can be further classified according to the type of measurement finally used, for example photometric, fluorometric, or colourimetric, and the enzyme system used. For example one qualitative determination is based on Benedict's copper reduction test (clinitest). Dextrostix, which depend upon enzymes, are used for semiquantitative estimation of glucose in biological fluids. Automated and manual procedures exist for the quantitative determination by both the enzymatic and chemical methods.

Choice of Method

Qualitative Tests for the Glucose Determination

A. Chemical Tests

i. With Fehling's Solution (52)

1 ml of centrifuged saliva was added to a mixture of 1 ml of solution A and 1 ml of solution B. The blue colour of the mixture changed slightly after shaking and boiling. When 2 ml of the centrifuged saliva were used, there was a change of the blue colour to brown and a brown precipitate was formed.

ii. With Schiff's Base (52)

When 1 ml of the centrifuged saliva was added to 1 ml of the colourless Schiff's reagent a faint pink colour was produced. While with 2 ml of the centrifuged saliva a deep pink colour was produced.

B. Thin Layer Chromatography (53)

Thin layer chromatographic technique has been devised for the detection of glucose in saliva. Visualization of the sugars was made with anilinephthalic acid reagent. Substances interfering with the application and migration of the sugars were removed by resin treatment of the samples. Identifications were made by comparing the mobility of the unknown with that of the standard on the same chromatogram.

Materials and Method

T.L.C. glass plates (20 x 20), precoated with 0.5 mm thick silica gel G No. 60, were used.

Chromatogram developing solvent: Butanol, pyridine and water were mixed in the proportions of 9:5:4.

Standard solutions: 100 mg/ml of each of L (+) Arabinose, D (-) Fructose, Galacturonic acid, D (-) Glucuronic acid, D (-) Glucose and L (+) Rhamnose were dissolved in distilled water.

Detection reagent: 1 ml aniline and 1.6 g phthalic acid were dissolved in a solution of 49 ml butanol, 49 ml ether and 2 ml water (54).

Resin: Mixed bed resin of amberlite IR 120, transformed to the free acid, and amberlite IRA 401 transformed to the acetate form. Equal amounts of the resin were mixed before use in each case.

Procedure

Some saliva samples were centrifuged at 10000 r.p.m. for 30 minutes and used immediately, while others were first heated in a boiling water bath for 10 minutes and then centrifuged at 10,000 r.p.m. for 10 minutes and then used. These samples were then treated with mixed ion exchange resins. The treatment consisted of mixing and shaking 2 mls of the centrifuged saliva with a mixture of 1 g of the cation plus 1 g of the anion resin. The clear supernatant from this was then used for the analysis.

Centrifuged saliva samples with added glucose and heated centrifuged samples with added glucose were also used. 10 mcl of each of the samples and the standards were applied with a stream of warm air to minimize diffusion of the samples.

Ascending chromatography was carried in a closed tank containing 5 - 10 mm of the developing solvent. The separation was allowed to continue until the solvent front reached a 15 cm height. After removal, the chromatograms were allowed to dry at 50°C for 10 minutes and were then sprayed with the aniline reagent and heated for 10 minutes. The sugars were located with the aid of long wave u.v. light. The following table gives the Rf values for the standards and the samples.

Table No. 13

	Sample.	Rf Value
a.	Centrifuged saliva.	0
a'.	Centrifuged saliva with added glucose.	0.645
b.	Centrifuged saliva treated with resin.	0
b'.	Centrifuged saliva treated with resin and then with added glucose.	0.677
c.	Heated centrifuged saliva.	0.71
c'.	Heated centrifuged saliva with added glucose.	0.71
d.	Heated centrifuged and treated saliva.	0
d'.	Heated centrifuged and treated saliva and then with added glucose.	0.71
1.	Arabinose.	0.71
2.	Fructose.	0.58
3.	Galacturonic acid.	0.87
4.	Glucoronic acid.	0.84
5.	Glucose.	0.71
6.	Rhamnose.	0.677

The concentration of the glucose solutions used was 170 mcg/ml. Although this T.L.C. method was useful for the determination of

moderate amounts of the sugars, it was not useful for the determination of very small quantities of sugars in heated and centrifuged and treated saliva samples. The search for simpler, more specific and sensitive methods continues.

C. Clinistix reagent Strips (55)

These are rapid, simplified, semiquantitative, enzymatic tests specific for the detection of glucose, based on glucose oxidase reaction. Clinistix may also give an indication of the amount present.

Chemical principles: Clinistix strips have a test area of cellulose impregnated with a buffered mixture of glucose oxidase, peroxidase and a chromogen system.

i. The glucose is oxidised by atmospheric oxygen in the presence of glucose oxidase to gluconic acid and hydrogen peroxide.

ii. The hydrogen peroxide in the presence of peroxidase oxidizes the chromogen system to a shade of purple.

Directions for use: The use of frosh samples is necessary. It was impossible to obtain positive results with frozen samples after they were thawed and brought back to room temperature. The test area of the strip is dipped in the sample briefly then removed immediately. After 10 seconds wetting, the colour of the test area is compared with the colour chart.

Procedure: Standard glucose solutions of serial concentrations were prepared (170, 340 and 680 mcg/ml). Saliva samples centrifuged at 10,000 r.p.m. for 30 minutes were prepared. Diluted (2x and 4x) centrifuged saliva samples and saliva samples with added glucose were also prepared. The following table shows the results obtained for the standards and the samples.

Table No.14

No.	Sample	Result
1.	Glucose solution containing 170 mcg/ml	+ ve Light
2.	Glucose solution containing 340 mcg/ml	++ ve Medium
3.	Glucose solution containing 680 mcg/ml	+++ ve Dark
4.	Centrifuged saliva sample only	- ve test
5.	50% v/v centrifuged saliva + 170 mcg glucose/ml	++ ve Medium
6.	25% v/v centrifuged saliva + 170 mcg glucose/ml	+ ve Light
7.	1 ml centrifuged saliva + 340 mcg glucose/ml	+ ve Light
8.	1 ml centrifuged saliva + 500 mcg glucose/ml	++ ve Medium
9.	50% v/v centrifuged saliva + 500 mcg glucose/ml	+++ ve Above Medium

From the above results, specially the glucose solution containing 340 mcg/ml and the centrifuged saliva with added glucose at 340 mcg/ml, it was concluded that these were unsuccessful. This was probably due to the lack of sensitivity of the tests or the presence of interfering substances or both.

Chemical Methods for Glucose Determination

1. Taurine Method

The method is based on the fluorometric determination of carbohydrates using taurine and borate buffer (56).

Taurine of any of its analogous amino acids was found to react with reducing carbohydrates by heating at neutral pH to give a solution with an intense fluorescence. The development of fluorescence was found to be greatly enhanced by the addition of borate buffer in the reaction mixture. This reaction is specific for a reducing sugar having a hydroxyl or a 2-amino group.

Materials and Methods

a. Reagents

All reagents were prepared using distilled water.

i. Taurine reagent: Taurine is 2-aminoethane sulphonic acid, $NH_2-CH_2-CH_2SO_3H$. This reagent was prepared by dissolving 67 m mol (8.4 g) of taurine in borate buffer pH 7.4. (The borate buffer was prepared by dissolving 7.6 g of KH_2PO_4 and 8.6 g of $Na_2B_4O_7$ in distilled water to make 1 litre.)

ii. D-Glucose stock solution of concentration 1 mg/ml: From this solution several solutions of concentration range from 1 mcg to 50 mcg/ml were prepared for the standard solutions.

iii. DL-phenylalanine: Aldrich Chemical Co. $C_6H_5-CH_2-\underset{NH_2}{\overset{H}{C}}-COOH$.

Stock solution of concentration 1 mg/ml was prepared.

iv. DL-Alanine: B.D.H. Stock solution of concentration 1 mg/ml was prepared.

v. L-Tyrosine: Aldrich Chemical Co. Stock solution of concentration 1 mg/ml was prepared.

vi. L-Tryptophan: B.D.H. Stock solution of concentration 1 mg/ml was prepared.

vii. Anthranilic Acid: Stock solution of concentration 1 mg/ml was prepared.

viii. β-Alanine crystalline: Sigma Chemical Co. Stock solution of concentration 1 mg/ml was prepared.

ix. KOH solution of different normalities.

x. Glycine Buffer (74)

Standard, blank and sample solutions were prepared for all the experiments.

b. Apparatus

The Aminco Bowman spectrophotofluorimeter was used.

Procedure

Initial experiments were carried out on aqueous glucose solutions to check the method. The taurine reagent and glucose concentrations of 10 mcg/ml to 50 mcg/ml after heating on a boiling water bath gave solutions with an intense fluorescence. However, there was no linear relation between the glucose concentrations and the fluorescence intensity. And when several solutions of taurine at different pH conditions (pH 5.45, 6.5, 7.5 and pH 8.) were prepared, maximum fluorescence intensity was found to be produced in solutions at pH 8. (Table 15) but again the relation between the glucose concentration and the fluorescence intensity was not linear, even after several different periods of heating.

Table 15 pH of Taurine Reagent against ΔF.

No.	pH of reagents.	Blank fluorescence.	Sample fluorescence	ΔF.
1.	5.4	15	16	1
2.	6.5	41	29	-12
3.	7.5	31	34	3
4.	8.0	4.5	24.5	20

The same procedure was repeated using phenylalanine as the reagent instead of taurine. The solutions were also made alkaline using N/1 KOH instead of borate buffer, but again the relation between the glucose concentration and the fluorescence intensity was irregular and unreliable.

When the phenylalanine was replaced by alanine and several solutions of different pH were prepared, maximum fluorescence intensity was obtained at pH 11.0 - 11.5. But none of these assays showed a linear relation between the glucose concentration and the fluorescence intensity, even after the reagents were heated in a boiling water bath for a prolonged period of time.

L-Tyrosine, tryptophan and anthranilic acid were used under the same conditions. Maximum fluorescence intensities were obtained at pH 11.00 with alanine and L-Tyrosine and at pH 9.5 with tryptophan (Table No.16).

Table No.16 Fluorescence Intensities of Alanine, L-Tyrosine and Tryptophan against pH

No.	pH	Alanine fluorescence (x.01)	L-Tyrosine fluorescence (x.01)	Tryptophan fluorescence (x1.0)
1.	4.0	6.5	5.5	26.5
2.	7.0	6.5	10.0	28.5
3.	9.5	8.0	20.0	58.0
4.	11.0	165.0	23.0	32.5
5.	12.0	84.0	21.0	17.0

But again no better results could be achieved with the above three reagents. A new amino acid, B-alanine was used instead of taurine. Solutions of β-alanine, 1.2 mcg/ml and 2.4 mcg/ml, were prepared at pH 7.4, pH 8.2, pH 8.4 and pH 9.00. A series of glucose solutions from 2 to 32 mcg/ml were also prepared. Then the reagents and glucose mixtures were heated in a boiling water bath for different periods of time. The fluorescence intensities were found to be non linearly related to the glucose concentration.

Then the β-alanine was purified by recrystalization in order to get rid of any impurities which might effect its reactions and the fluorescence intensities. Again it was found that the results obtained were not reproducible.

2. The 5-hydroxy-1-tetralone Method

The method is based on the report that 5-hydroxytetralone gives a sensitive fluorescent reaction selectively with a hexose when heated with sulphuric acid (57).

Materials and Methods

Reagents

i. 5-hydroxy-1-tetralone solution: 100 mg of 5-hydroxy-1-tetralone (0.1% w/v) (Sigma Chemical Company) was dissolved in 100 ml of analytical grade concentrated sulphuric acid, and stored in a refrigerator.

ii. Trichloroacetic acid solution: 5 g of analytical grade trichloroacetic acid was dissolved in 100 ml of distilled water.

iii. Standard glucose solution: Glucose solutions of 2 mcg/ml, 5mcg/ml, 10 mcg/ml and 20 mcg/ml were freshly prepared with distilled water.

Fluorescence measurement: This was read on the Aminco-Bowman spectrofluorimeter, set to read at the following conditions:-

Meter multiplier	= 0.03
Sensitivity	= 25
Slit for light source	= 1 mm
Slit for emitted light	= 3 mm
Photomultiplier slit	= 3 mm
Emission wavelength	= 535 nm
Excitation =	= 380 nm

Procedure

1 ml of the standard glucose solution or the blank was mixed with 1 ml of 5-hydroxy-1-tetralone reagent, and 3 ml of concentrated sulphuric acid was added with cooling in an ice bath and then heated in a boiling water bath for 40 minutes. Immediately after heating all solutions were cooled in an ice bath and diluted with 15 ml of distilled water. Then after the solutions had stood for 30 minutes at room temperature their fluorescence intensities were recorded. But linear relations were not obtained.

Other experiments were carried out using different concentrations of 5-hydroxy-1-tetralone in an attempt to obtain a linear fluorescence to concentration relation. However, the results were disappointing as no such relation could be obtained.

CONCLUSION

Therefore from the above series of experiments it was concluded that analytical procedures for the determination of glucose, which were dependent upon the chemical reactivity of glucose, were unsuitable for its assay in saliva. They could not be relied upon to give accurate and precise estimates of glucose concentrations in that fluid. It is not clear why this should be so, but the variations observed may be related to the day to day changes in the composition of human saliva.

It was therefore decided to try and apply an enzymatic analytical method for this purpose.

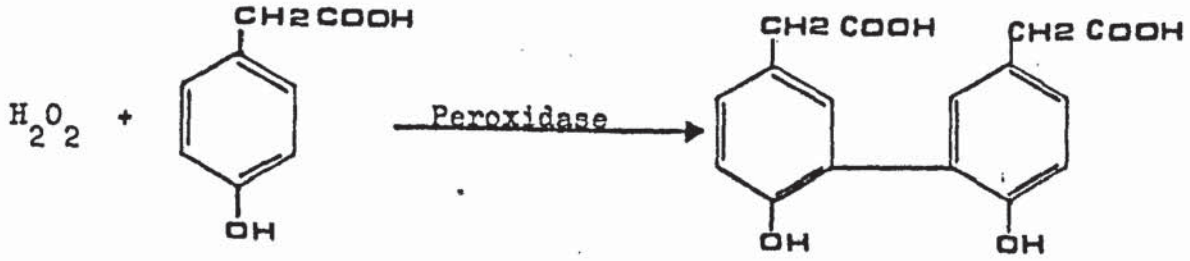
Development of Enzymatic Fluorometric Method of Estimating Glucose in Women's Saliva

Summary

A specific, sensitive and simple kinetic method has been developed to determine microgram quantities of salivary glucose.

Since glucose is present in very small quantities with other carbohydrates in saliva, the enzymatic fluorometric method was chosen because it offered a high sensitivity and specificity which permitted a quantitative determination of glucose to be made in the presence of other sugars. The method is based on the following highly selective reaction (58, 59).

β-D-Glucose + O_2 + H_2O $\xrightarrow{\text{Glucose Oxidase}}$ D-Glucuronic acid + H_2O_2



reduced chromogen (p-hydroxyphenyl-acetic acid)

oxidised chromogen Highly fluorescent

Free hydroxy-radicals are formed by the action of a peroxidase enzyme on the hydrogen peroxide which itself is produced by the action of glucose oxidase on β-D-Glucose. The hydroxy radicals attack the fluorogenic but non-fluorescent substrate, p-hydroxyphenyl acetic acid, and thus form a highly fluorescent compound in alkaline

medium . The fluorescence has a maximum at 405 nm and was used to determine glucose in the range of 0.5 - 4.0 mcg/ml.

Treatment of Saliva Samples

The saliva samples were first diluted by factor 2 with a citric acid/phosphate buffer of pH 5.0 and then centrifuged. The supernatant was now heated in a boiling water bath, centrifuged again, and treated with saturated regenerated mixed resins of IR-120 and CG-400. Heating the samples in a boiling water bath, centrifugation and treatment with mixed bed resin was found necessary for the removal from saliva of inhibitors of the glucose oxidase-peroxidase system.

Materials

1. Glucose Oxidase Enzyme from aspergillus niger.
   Activity: 20,000 units/gm solid (Sigma Chemical Company) 3 mg/ml was prepared in distilled water.

2. Peroxidase. From horseradish type VI.
   Activity: 250 purpurogallin units per mg solid. (Sigma Chemical Company) 1 mg/ml was prepared in distilled water.

3. Tris buffer. A 0.1 N tris (hydroxy methyl) aminomethane buffer, pH 8.5, was prepared by dissolving 12.1 g of pure compound (B.D.H. Chemical Company) in 1 litre of distilled water and adjusting the pH with concentrated HCl.

4. Substrate. p-hydroxyphenylacetic acid. (Sigma Chemical Company) This substrate is stable and not air oxidised in aqueous solutions, and has the advantages over others of low cost and a higher fluorescent coefficient (fluorescence/concentration in moles).

When mixed with peroxidase in tris-buffer at pH 8.5, the p-hydroxyphenylacetic acid is also stable to auto-oxidation. p-hydroxyphenylacetic acid was judged to be the best substrate for the peroxidase because it permits the determination of lower concentrations of sugars as it gives a zero blank reading (60).

To eliminate the need for three separate solutions, a mixed reagent solution was prepared by dissolving 2 mg peroxidase and 7.4 mg p-hydroxyphenylacetic acid in 50 ml tris buffer, pH 8.5. It has been reported that this reagent is stable for two weeks, if stored overnight under refrigeration (60).

Standard Solutions

These were prepared at 0.5, 1.0, 2.0 and 4.0 mcg/ml concentrations. These and all other solutions and samples were delivered with adjustable Finn pipettes.

Fluorescence Measurement

All fluorescence emission spectra were measured on an Aminco-Bowman spectrophotofluorometer as described previously.

The 1.0 cm cuvette was maintained at 30°C by means of circulating water in the cuvette holder.

Influence of Temperature

When the experimental work for the glucose estimation was carried under the same conditions but at different temperatures for the reaction medium, for example 21°C, 30°C and 37°C, it was found that the highest enzyme activity and fluorescence intensity were obtained at 30°C (Fig. No. 4).

Procedure

All reagents were kept in a circulating water bath at 30°C. To 2.2 ml of the mixed reagent in the cuvette, 0.1 ml of glucose oxidase solution was added. At zero time 0.1 ml of the blank, the standard solution or the sample to be assayed, were ejected from the pipette to allow mixing of the reagents. Immediately after adding the reagents the cuvette was stoppered, shaken, inserted into the cuvette compartment and the clock started. The reaction and the fluorescence intensity started at zero time with a regular increase every fifteen seconds. This regular increase in fluorescence intensity was recorded for up to three minutes. For every standard, sample or blank the assay was performed twice.

The blank was set up and the increase in fluorescence was similarly determined. Thus from the results the mean increase in fluorescence was calculated and the initial rate of reaction (ΔF/minute) was recorded. From the calibration plots of ΔF/minute versus the glucose concentration in mcg/ml in the final solution, the amount of glucose present in the unknown samples could be calculated (Fig. No. 5).

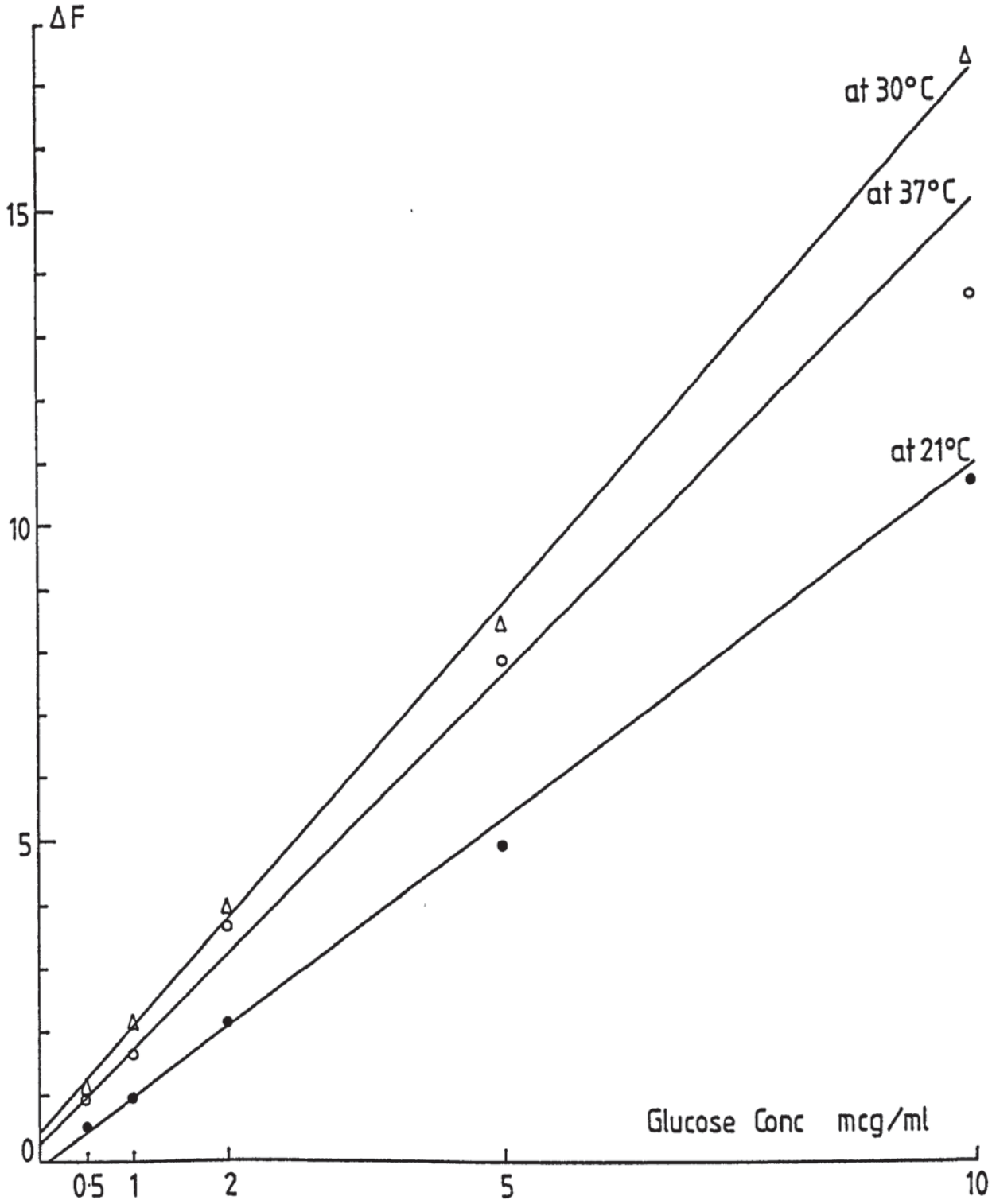


Fig 4 Shows the effect of temperature on enzyme activity and fluorescence intensity

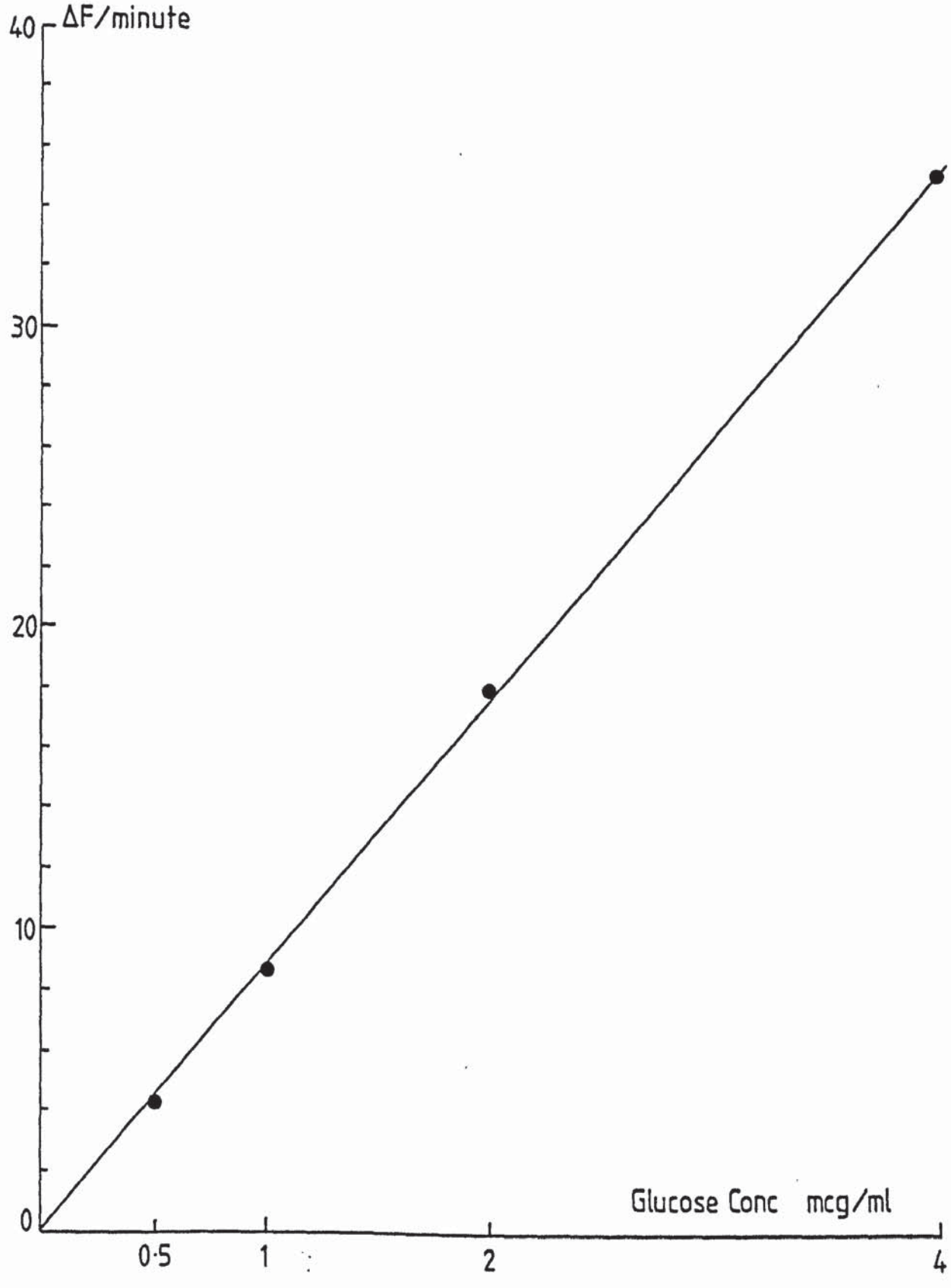


Fig 5 Shows the relation between standard glucose concentration and ΔF per minute

CHAPTER VI

ESTIMATION OF N-ACETYL-β-D-GLUCOSAMINIDASE ACTIVITY

Summary

An accurate and specific fluorimetric method for the estimation of salivary N-Acetyl-β-D-Glucosaminidase (NAG) has been developed. A high sensitivity has been achieved which permits the determination of 0.00054 units/ml. of NAG in a 0.1 ml. sample of centrifuged saliva.

The method consists of the enzymic hydrolysis of the substrate, 4-Methylumbelliferyl-β-D-Glucosaminide, at an acid pH, followed by a fluorimetric determination of the liberated methyl-umbelliferone at a predetermined alkaline pH (61).

Introduction

Before the study of the cyclic changes in enzymatic salivary activity was begun, the conditions of the fluorimetric enzymatic assays were carefully established in order to ensure linear kinetics under all conditions of the test. For comparison purposes absorption spectrophotometric method was also applied. The fluorimetric method for the assay of NAG was modified for the determination of this enzyme in saliva.

The sensitivity of the fluorimetric technique is such that saliva can be diluted prior to the assay. This dilution eliminates the effect of NAG inhibitors present in saliva and the differences between two successive determinations due to the high viscosity of the saliva.

Materials and Methods

Reagents

1. The fluorescent substrate, 4-Methylumbelliferyl-N-Acetyl-β-D-Glucosaminide (0.02 mg/ml), was dissolved in citric acid/phosphate buffer, pH 4.8, (citric acid 0.1 M plus sufficient Na_2HPO_4, 0.2 M to give a pH of 4.8). This substrate solution was freshly prepared. (Sigma Chemical Company.)

2. Enzyme N-Acetyl-β-D-Glucosaminidase suspension in 2.5 M $(NH_4)_2SO_4$. (Sigma Chemical Company.)

3. Glycine/Sodium hydroxide buffer of pH 10.8.

4. 2.5 M Ammonium Sulphate solution.

5. $HgCl_2$ solution of 0.1 M.

6. $AgNO_3$ solution of 0.1 M.

7. E.D.T.A. solution of 0.01 M.

Apparatus

Fluorescence spectra were determined with an Aminco-Bowman spectrofluorimeter. The instrument was standardized to read the emitted fluorescence at 445 nm while the excitation was at 365 nm. The meter multiplier was at 0.3, and the slit for the excitation was 1 mm and for the emission was 3 mm. The photomultiplier slit was 2 mm

and sensitivity was at 30.

Samples

Saliva samples from healthy subjects were diluted with a citrate buffer prior to freeze storage. Centrifuged, non-centrifuged and filtered samples were used. A portion of 0.5 ml of saliva sample was diluted to 2.5 ml and from this 0.5 ml was taken for analysis.

Procedure

N-Acetyl-β-D-Glucosaminidase (NAG) activity was determined using 0.02 mg/ml of 4-Methyl-umbelliferyl-β-D-N-Acetyl-Glucosaminide as substrate dissolved in a citric acid/phosphate buffer of pH 4.8. An aliquot of 0.5 ml of the standard enzyme suspension, or the diluted sample, was added to 1 ml of a 0.02 mg/ml substrate solution. The mixture was incubated for 30 minutes at 37°C after which 4.5 ml of glycine/NaOH buffer, pH 10.8, were added and the fluorescence was measured. The following dilutions of NAG in distilled water were used as standards, 0.00054 unit/ml, 0.00108 unit/ml, 0.00216 unit/ml, 0.00324 unit/ml, 0.00432 unit/ml and 0.0054 unit/ml.

The standard graph was drawn of the increase in fluorescence (ΔF) against the enzyme concentration. From this the enzyme activity of an unknown was determined. One unit of the enzyme has been defined as the amount of enzyme which hydrolyses 1 mc. mole of 4-Methyl-umbelliferyl-β-D-Glucosaminide to 4-Methyl-umbelliferone and D-N-acetyl-glucosamine per minute at pH 5.0 at 25°C (62).

When p-nitrophenyl-acetyl-β-D-glucosaminide was used as the substrate in the colourimetric determination, the assay principle was the same as that used for 4-Methyl-umbelliferyl-N-Acetyl-β-D-Glucosaminide.

The specific activity of the enzyme was expressed as units per mg of protein (63).

The Stability of 4-Methyl-umbelliferyl-N-Acetyl-β-D-Glucosaminide.

Solutions of this substrate slowly liberated methyl-umbelliferone on storage at 0 - 5°C. All substrate solutions were therefore prepared freshly before use (64). Small amounts of methyl-umbelliferone were liberated on incubation under the conditions of the enzyme assay.

This non enzyme hydrolysis was linear with time and substrate concentration, but did not vary greatly over the pH range studied (4.0 - 6.0).

The Effect of Temperature of Incubation

With an incubation period of 30 minutes and the conditions standardized, different temperatures of incubation were tried, for example 21°C (room temperature), 30°C, 37°C and 45°C. Optimal fluorescence intensity and the best linear relation between the standard enzyme suspension and ΔF were obtained at an incubation temperature of 37°C (Figure No. 6).

Time of Incubation

The effect of time of incubation on the enzymic hydrolysis of 4-Methyl-umbelliferyl-glucosaminide was studied. This substrate was incubated with the enzyme suspension for different periods of time, as for example for 10, 20, 30, 60 and 90 minutes. It was found that at the pH 4.0 - 5.0 the ΔF was linear with time for at least 90 minutes. Therefore a 30 minute incubation period was chosen as a reasonable time of incubation.

Effect of Enzyme Concentration

With substrate concentration of 0.02 mg/ml and the standard conditions such as incubation at 37°C for 30 minutes, and 4.5 ml of the glycine/NaOH stopping buffer, the enzyme concentrationswhich gave a linear relation with ΔF range from 0.00054 unit/ml up to 0.0054 unit/ml. Above this concentration quenching of the fluorescence was observed (Figure No. 7).

The Substrate Concentration

Experiments on the effect of various substrate concentrations on the activity of the enzyme were carried out. Concentrations of 0.1

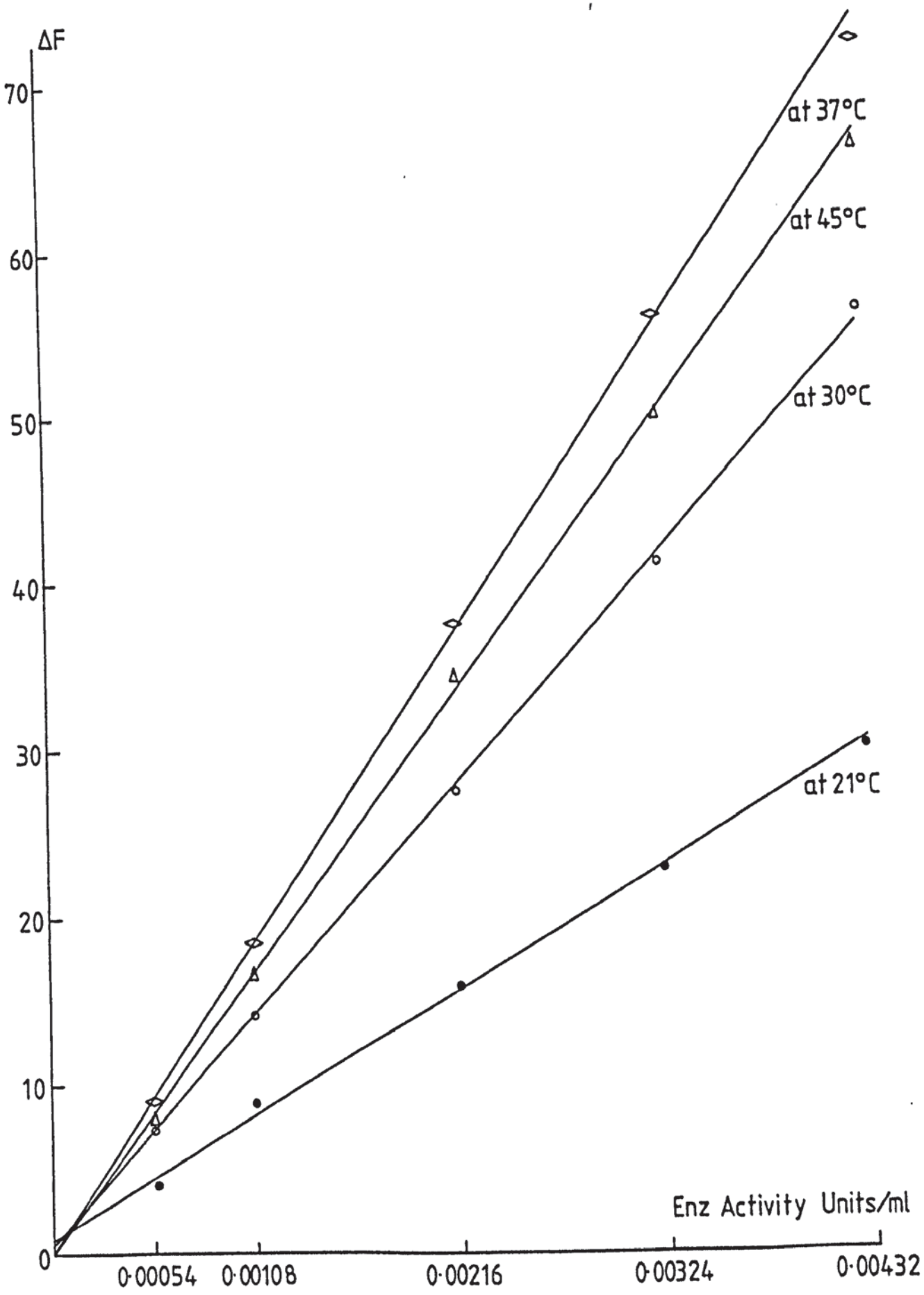


Fig 6

Effect of temperature of incubation on the fluorescence intensity and the enzyme (NAG) activity

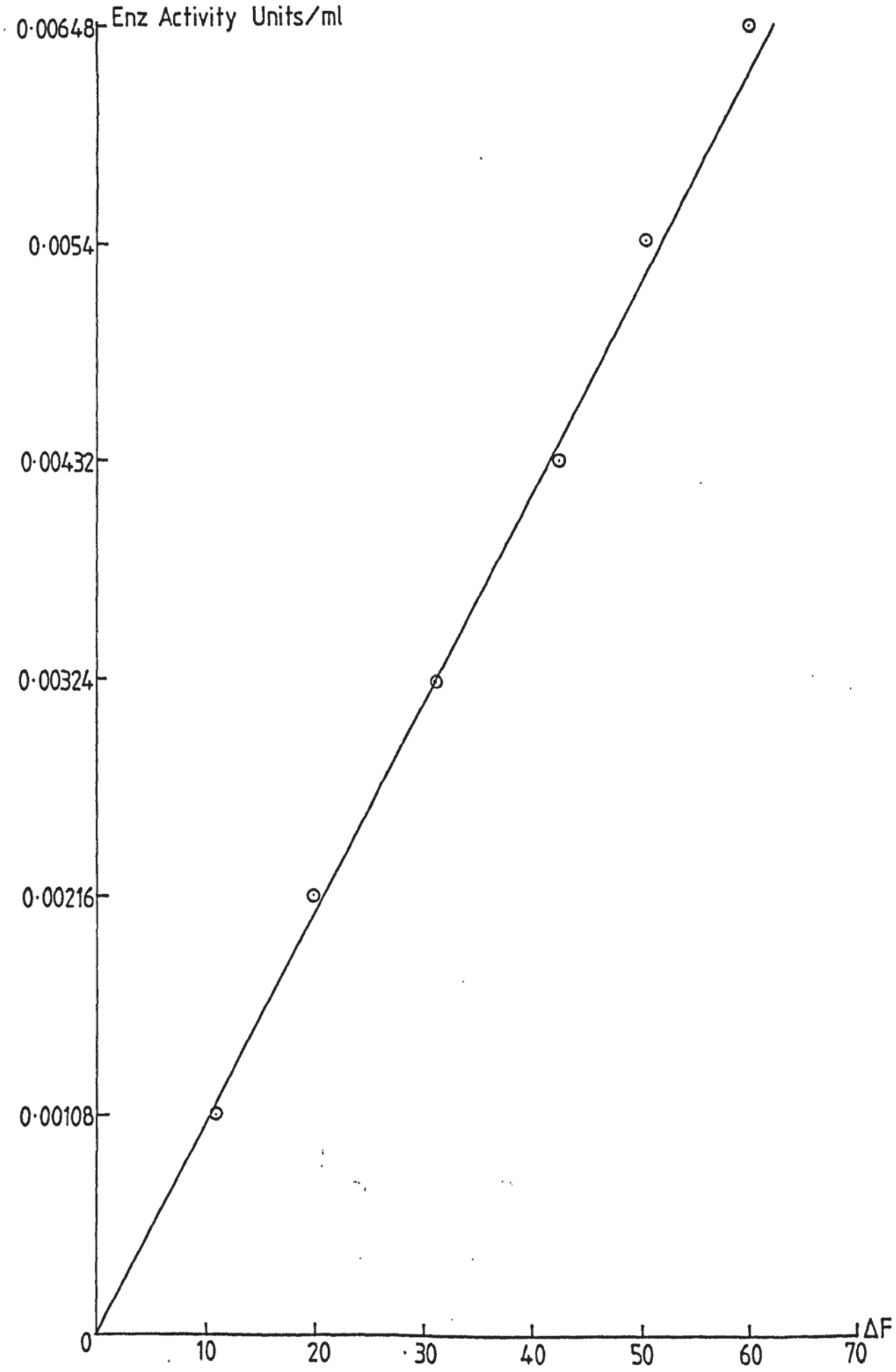


Fig 7 Effect of the enzyme concentration on the fluorescence intensity

mg/ml, 0.05 mg/ml, 0.04 mg/ml, 0.02 mg/ml, and 0.01 mg/ml of substrate dissolved in citric acid/phosphate buffer were used. The best linear relation was obtained with a concentration of 0.02 mg/ml under the above mentioned conditions and the range of enzyme concentration. (Figure No. 8).

The Effect of the pH of the Citric Acid/Phosphate Buffer

With 4-Methyl-umbelliferyl-N-Acetyl-β-D-Glucosaminide as substrate, the activity of the enzyme varied with the pH. The optimum activity of the enzyme was obtained with 0.02 mg/ml substrate concentration in citric acid/phosphate buffer of pH 4.6 - 5.0 (Figure No. 9).

The Effect of pH and Volume of Glycine/NaOH Stopping Buffer

Saliva samples were used in addition to the standard enzyme suspension and the assays were carried out using buffers of pH 9.6, 10.4, 10.8 and 11.5. The highest ΔF values, that is the optimum enzyme activity, and best linear relation was obtained with a buffer of pH 10.8 (Figure No. 10). When these solutions were left overnight at room temperature, 70 - 100% loss was found with the samples at pH 9.6, 5 - 20% loss for solutions with a buffer of pH 10.8, and 25 - 32% loss for solutions with a buffer of pH 11.5.

The volume of the glycine/NaOH buffer was found to be important, because 3 ml of the pH 10.8 buffer gave a final solution of pH 7.1, which had a lower and irregular fluorescence intensity. While with 4.5 ml of the buffer the pH of the final solution becomes 8.5, and an optimum fluorescence and linear relation with the concentration is obtained. With more than 4.5 ml buffer no better results could be obtained (figure No. 11).

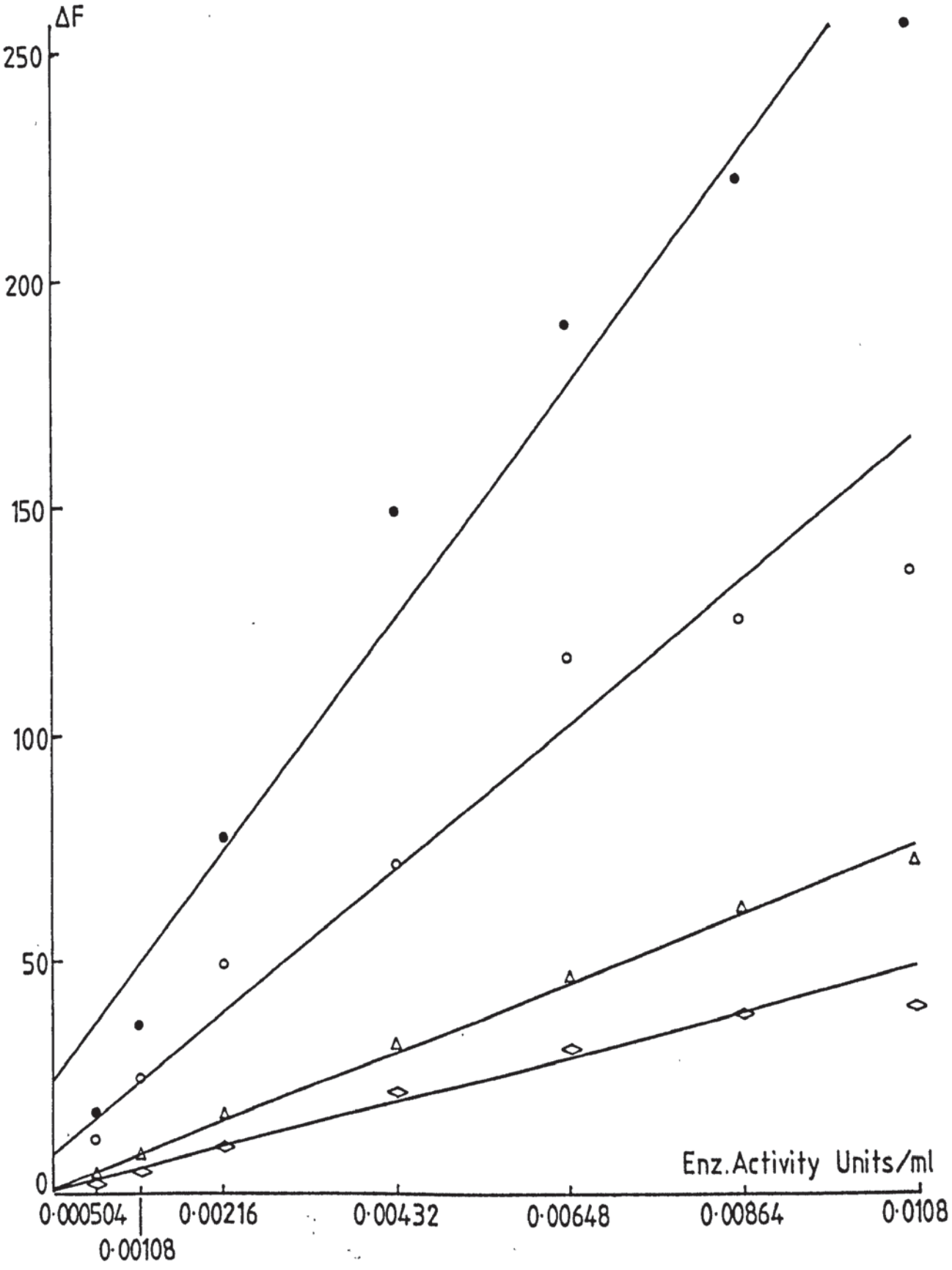


Fig 8 Effect of the concentration of the substrate N-Acetyl-β-D-glucosaminide on the linearity of the standard curve

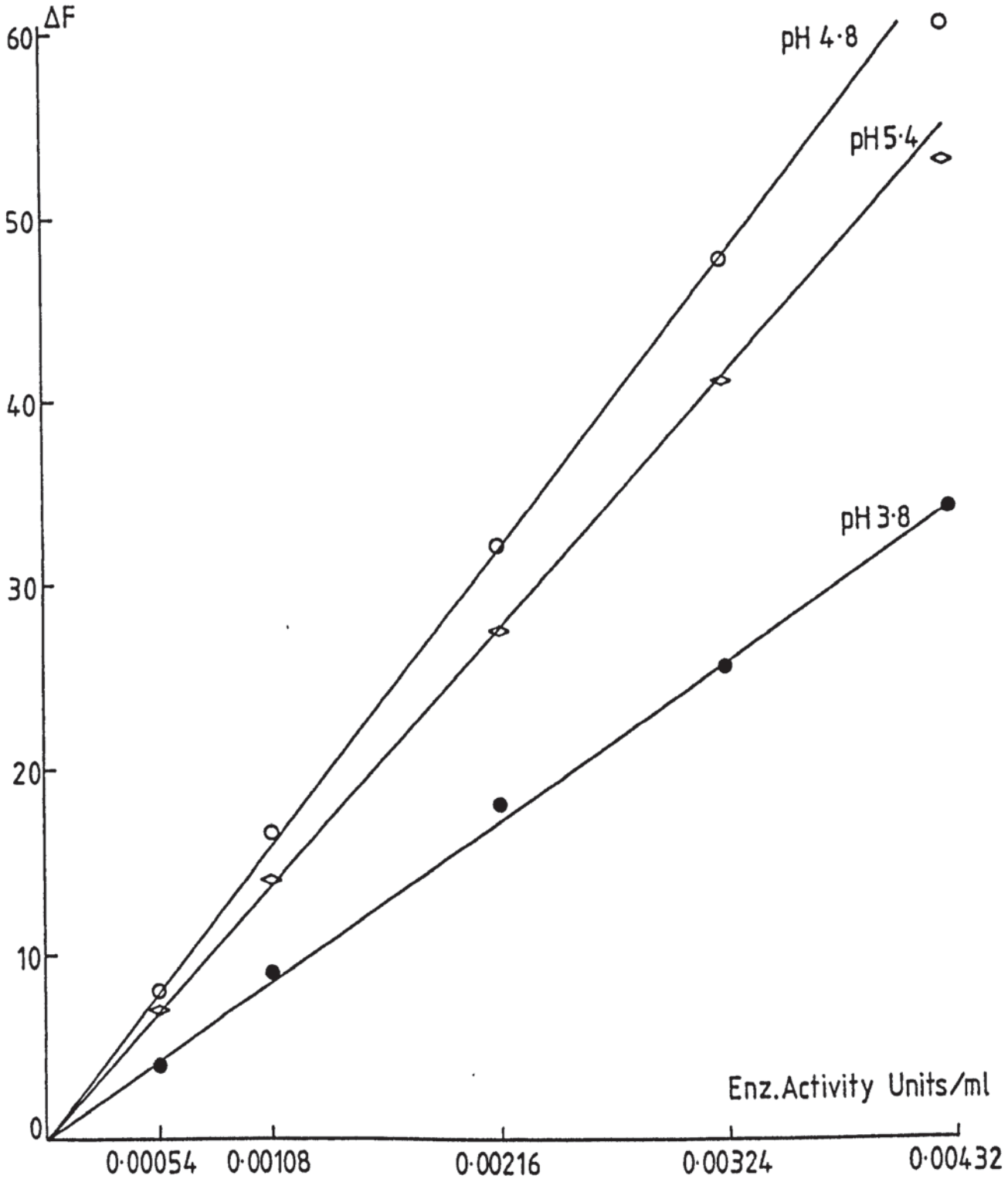


Fig 9 The effect of the pH of citric acid/phosphate buffer on the activity of the enzyme N-Acetyl-β-D-glucosaminide

Concentration of the substrate 4-Methylumbelliferyl-N-Acetyl-β-D-glucosaminide = 0·02 mg/ml

pH of the stopping enzyme glycine/NaOH = 10·8

Enzyme suspension diluted with distilled water

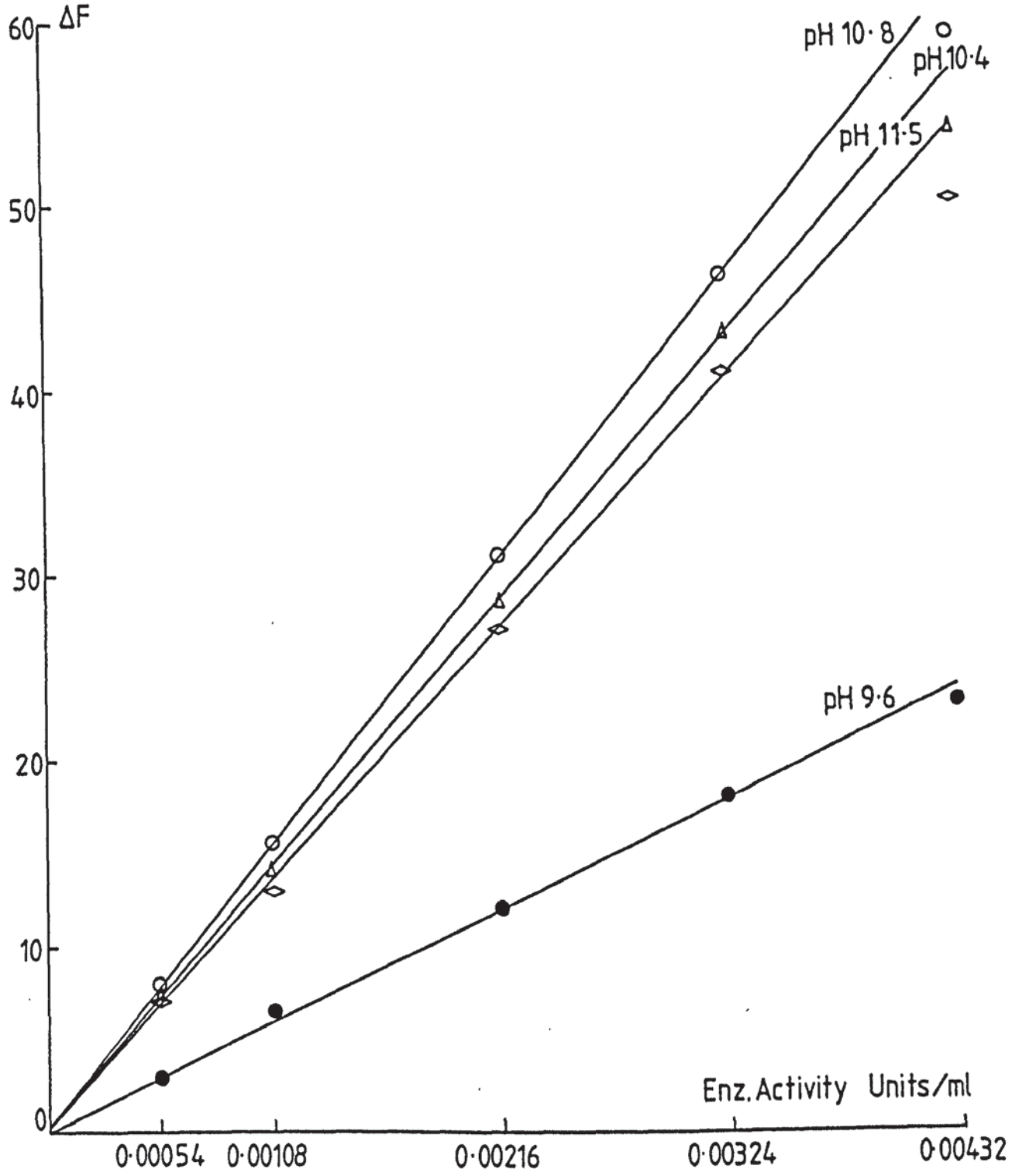


Fig 10 The effect of the pH of the stopping buffer Glycine/Na OH on the enzyme activity

Substrate 4-Methylumbelliferyl-N-Acetyl-β-D-glucosaminide of concentration 0·02mg/ml in citric acid/phosphate buffer pH 4·8

Enzyme suspension diluted with distilled water

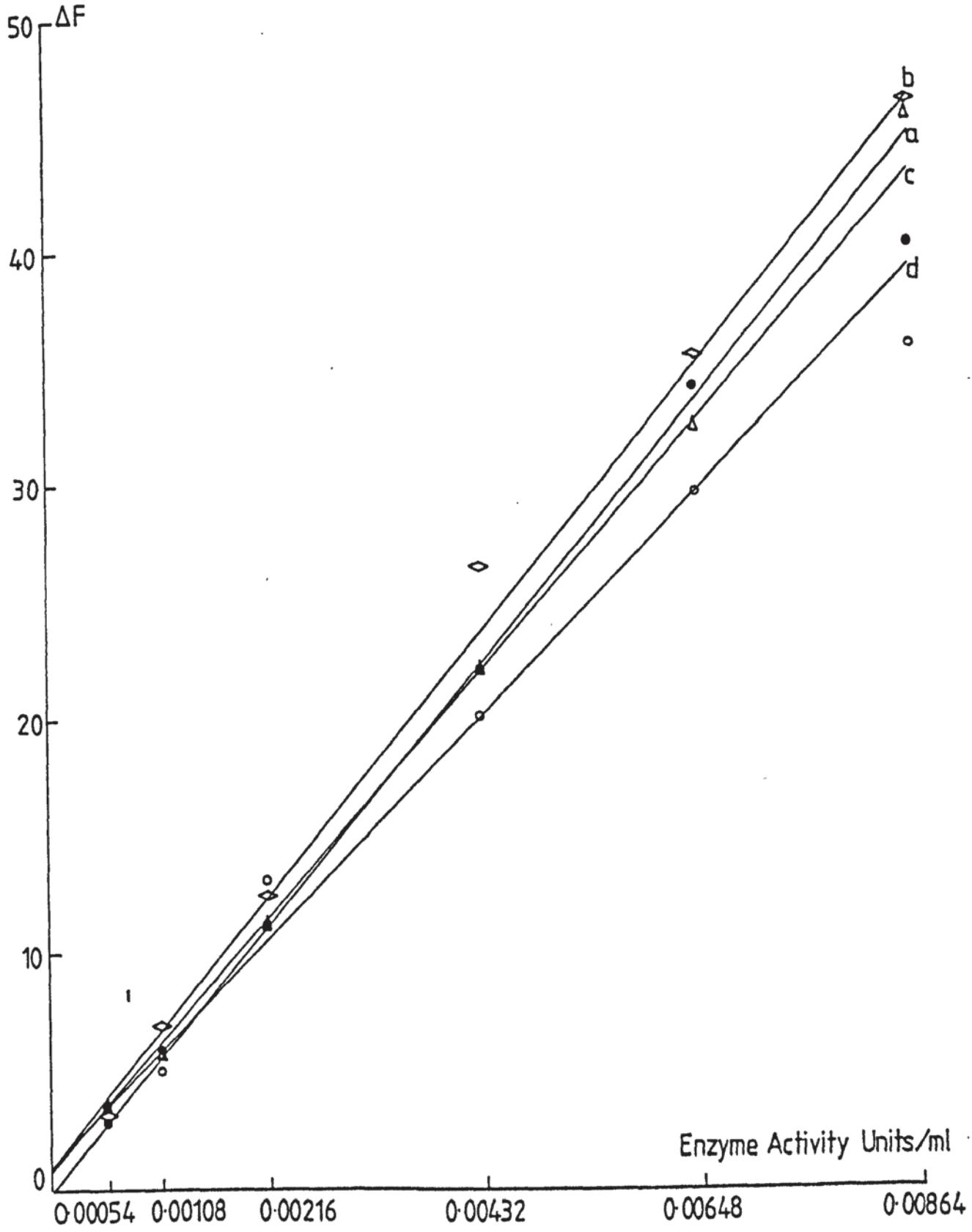


Fig 11 Effect of the volume of the stopping buffer Glycine/NaOH
a. With 2·5ml glycine buffer (•)
b. With 4·5ml glycine buffer (◇)
c. With 6·5ml glycine buffer (△)
d. With 8·5ml glycine buffer (○)

Substrate 4-Methylumbelliferyl N-Acetyl-β-D-glucosaminide used of concentration 0·02mg/ml in citric acid/phosphate buffer pH 4·8

The Effect of Dilutents of the Enzyme Suspension

The enzyme used was supplied as a suspension in 2.5 M $(NH_4)_2SO_4$ solution of pH 7.0. In the preparation of different enzyme concentrations for the standards dilutions were made with 2.5 M $(NH_4)_2SO_4$ solution at pH 7.0. Then another set of enzyme solutions were prepared by dilution with distilled water. Saliva samples were also used and some were diluted with $(NH_4)_2SO_4$ solution and the others with distilled water. Higher fluorescence, or enzyme activity, was obtained from the standards and samples diluted with distilled water (about double the activity).

A Citric acid/phosphate buffer at pH 4.8 was also used for dilution of the enzyme suspension and the standards and a comparison was made with those solutions diluted with distilled water. It was found that the standards and the enzyme suspension diluted with distilled water showed a higher enzyme activity and a better linear relation between ΔF and enzyme activity (Figure No. 12).

The Effect of Inhibitors

The enzyme suspension was treated with some substances that might inhibit its activity, such as 0.1mM $HgCl_2$ and 0.1mM $AgNO_3$. It was found that $HgCl_2$ did not have any inhibitory effect, while $AgNO_3$ showed a remarkable inhibitory effect which reduced the activity to 68 - 81% of the original value. The effect of ethanol was also examined. Ethanol 25% (v/v) was mixed with the enzyme suspension, and centrifuged and non-centrifuged saliva. This had the effect of quenching the fluorescence and the enzyme activity decreased by about 30 - 40%.

Treatment with E.D.T.A. and Ion Exchange Resin

The standard enzyme suspension, and centrifuged and non-

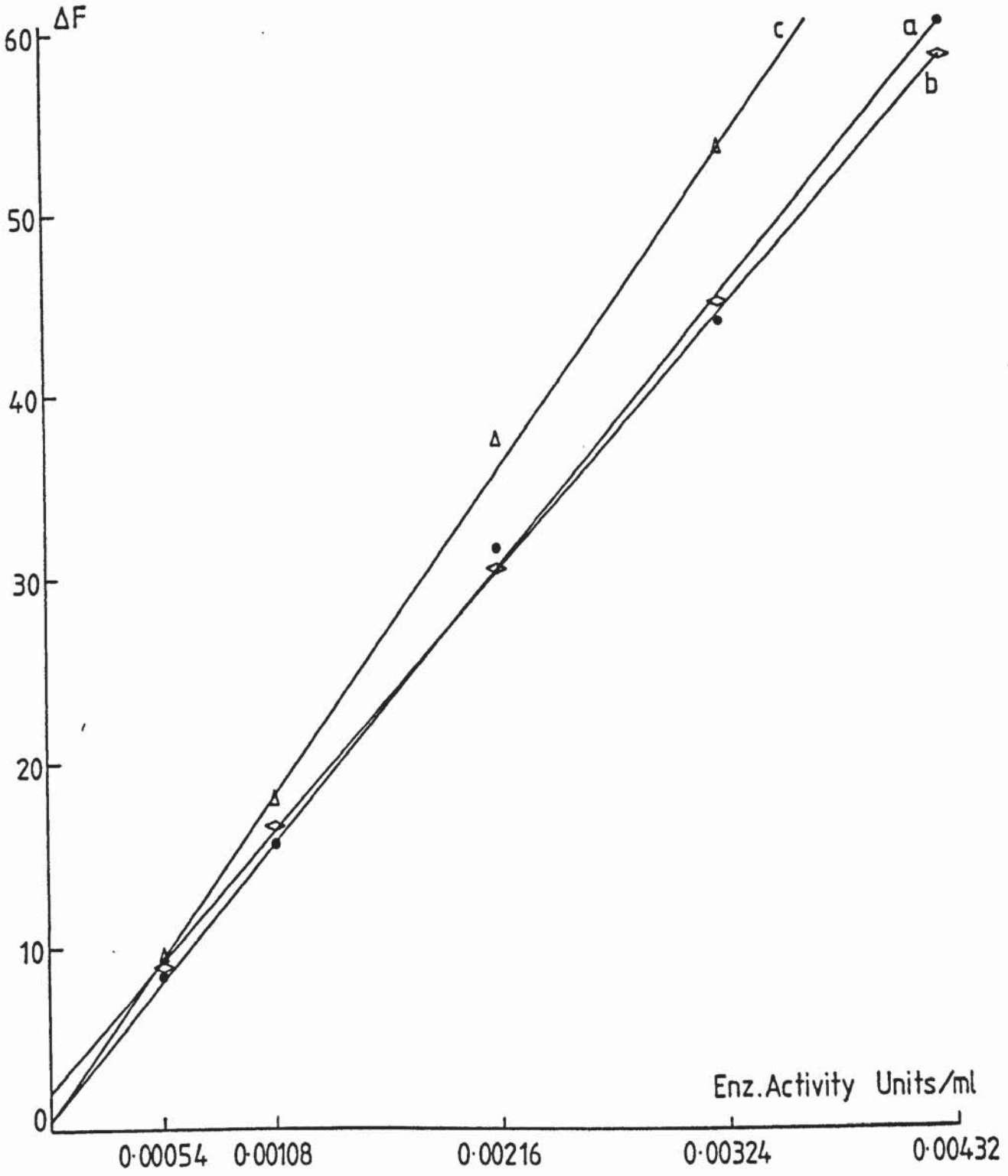


Fig 12 Comparison between enzyme suspensions diluted with

a. $(NH_4)_2 SO_4$ (2·5M) —(•)

b. citric acid/phosphate buffer pH 4·8 —(◇)

c. distilled water —(△)

Substrate 4-Methylumbelliferyl-N-Acetyl-β-D-glucosaminide of concentration 0·02mg/ml in citric acid/phosphate buffer pH 4·8

centrifuged saliva were treated with E.D.T.A. in order to find out whether this reagent could remove some of the enzyme inhibitors and what effect it would have on the enzyme activity. It was found to quench the fluorescence intensity of both the standards and the samples. Similarly, when the same standard enzyme suspension and the saliva samples, both centrifuged and non-centrifuged, were treated with mixed anion and cation resins, it was found that the saliva samples had lost 50%, and the enzyme suspension a 100% of its activity.

Samples Used

Filtered, centrifuged and non-centrifuged saliva samples were used. It was found that the filtered saliva samples showed the lowest enzyme activity. This was about 20 - 30% less than centrifuged samples and about 40 - 50% less than non-centrifuged samples. But due to the presence of protein particles and turbidity in the non-centrifuged samples, it was the centrifuged saliva that was selected for the enzyme activity determination.

The Spectrophotometric Estimation of N-Acetyl-β-D-Glucosaminidase

This method is based on the same principle as the fluorimetric determination of the enzyme activity, except that the substrate used was p-nitrophenyl-N-acetyl-β-D-glucosaminide. This substrate is a colourless phenolic derivative of N-acetyl-β-glucosamine which reacts in the presence of glucosaminidase, at an acid pH, to form a phenol and this has a distinct colour at an alkaline pH (65).

A Beckmann spectrophotometer (Acta V), with tungsten lamp, was used. The wavelength was set at 400 nm and a slit of 0.65 mm was used. A 1 ml portion of substrate solution, at 0.4 mg/ml in citric acid/phosphate buffer pH 4.8, was added to 0.5 ml of the enzyme

suspension. The enzyme suspension was used over a range of concentrations from 0.00054 unit/ml to 0.00432 unit/ml. The reaction was terminated by the glycine/NaOH stopping buffer at pH 10.8 and the absorption was then read.

Under the above mentioned conditions a linear relation with optimum enzyme activity could be obtained. (Figure No. 13).

Discussion

The fluorimetric method for the determination of salivary NAG described in this chapter has been modified and the sensitivity increased to the point where it was possible to determine the enzyme activity in 0.1 ml of centrifuged saliva and down to 0.00054 unit/ml in a standard enzyme suspension. The small volume of the sample used and its dilution, and that of the standard enzyme suspension, appeared to reduce the effect of some of the inhibitiors and interfering substances that affected the enzyme activity.

The excellent linear correlation between the results obtained (ΔF vs. enzyme activity in unit/ml), and the agreement between two successive measurements of the saliva samples confirm the choice of conditions. This high sensitivity of the fluorimetric method was achieved by:-

a. Low substrate concentration and optimal pH (0.02 mg/ml in citric acid/phosphate buffer of pH 4.8).

b. Small volumes of the standard and samples used.

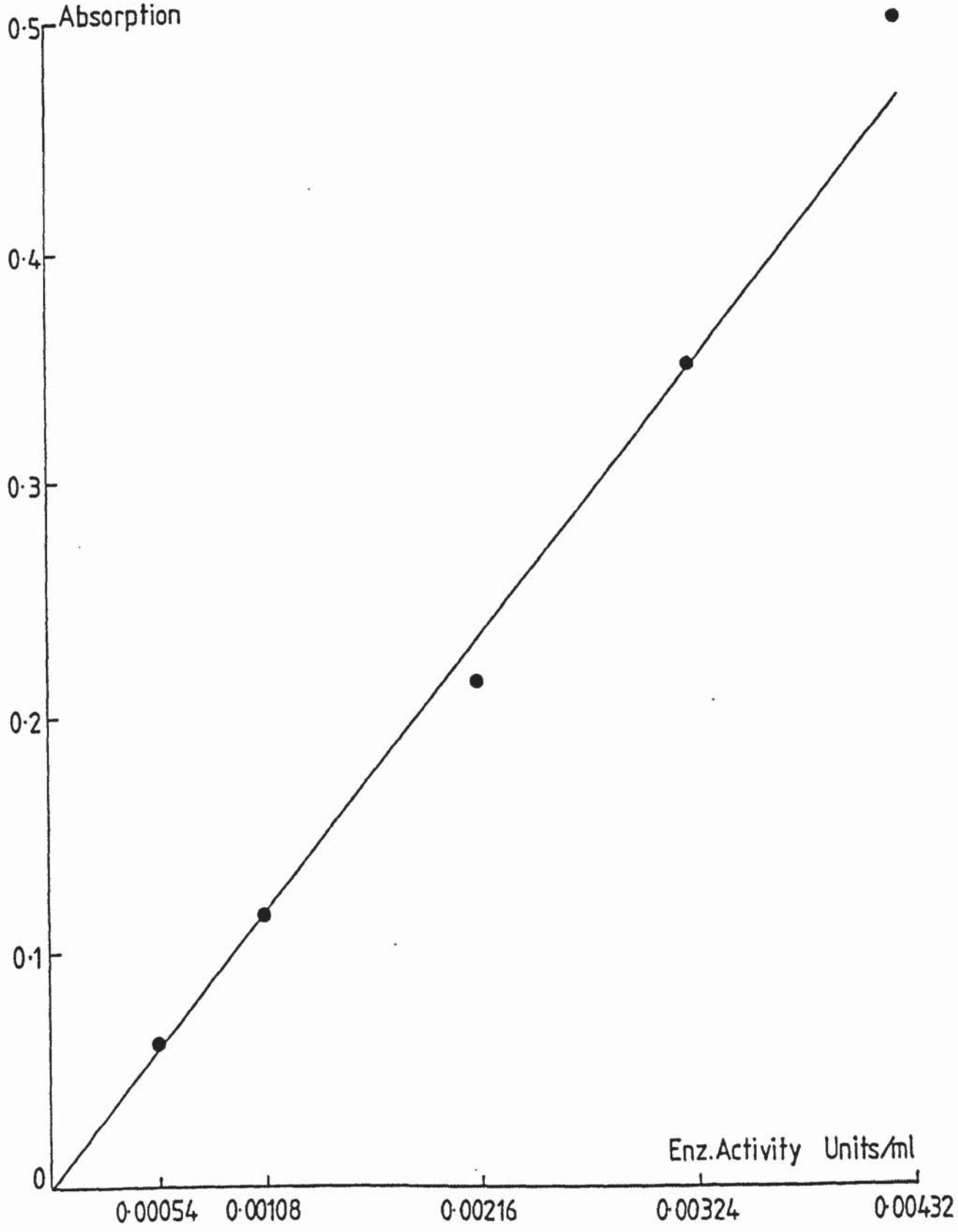


Fig 13 Spectrophotometric determination of the enzyme N-Acetyl-β-D-glucosaminidase
Using 0·4 mg/ml substrate p-Nitrophenyl-N-Acetyl-β-D-glucosaminide in citric acid/phosphate buffer pH 4·8
Stopping buffer (Glycine/NaOH) pH 10·8
Final solution pH 9·7

c. Incubation at 37°C for 30 minutes.

d. Small volumes of the strong alkalinizing buffer to terminate the reaction (4.5 ml).

Measurement of the pH of the final mixture demonstrated in all cases that 4.5 ml of the stopping buffer of pH 10.8 was sufficient to provide a pH of 8.0 - 8.5 which is the optimal pH for the fluorescence of 4-Methyl-umbelliferone. A high fluorescence blank was occasionally observed which could have been due to the fluorescence of impurities in the substrate itself, or the presence of free Methyl-umbelliferone in the preparation or due to the non-enzyme release of Methyl-umbelliferone from the substrate (64). To decrease this high fluorescence blank substrate solutions were always freshly prepared.

The liberated aglycone was found stable for a considerable period of time, and this offers another advantage for the accuracy of the method.

The high sensitivity of this method permits a reliable estimate to be made of the low levels of NAG concentrations found in normal saliva.

CHAPTER VII

CALCIUM DETERMINATION

Introduction

In general, the formation of a highly fluorescent chelate by the combination of the calcium ion with an organic ligand has proved to be one of the most sensitive and highly specific methods for the determination of the element (46). The following fluorogenic substances known to complex with calcium were obtained and their fluorescence emission spectra in acid, neutral and alkaline solution with and without calcium were determined. Tetracycline has been reported to be a highly sensitive fluorescent indicator for the complexometric titration of calcium in ammoniacal buffer at pH 10 (66). Fluorochromic indicators such as calcein have also been used (67). The calcium salt retains a strong fluorescence with calcein at pH values above 12. Several determination methods are based on this principle. For example, the automated fluorometric procedure for serum calcium depends upon the measurement of the fluorescence produced by calcein with calcium in a serum dialysate (68). Also a derivative of calcein, 4-methylumbelliferone-methylene-iminodiacetic acid, known as Calcein Blue, has been found to give a brilliant blue fluorescence (415 nm) at pH 11 in the presence of calcium. It is used for the chelatometric determination of metal ions which form highly coloured complexes with chelatometric reagents.

FLUOROMETRIC DETERMINATION OF SALIVARY CALCIUM

1. Summary:

A fluorometric procedure for the determination of salivary calcium levels has been developed. The method is based upon measurement of the fluorescence produced by Calcein Blue with calcium with which it forms a highly fluorescent compound at pH from 9 - 13. The maximum fluorescence occurs between pH 12.5 - 12.8 (Fig. 14).

2. Choice of Method:

Experiments with tetracyclines and with calcein indicated that they were not suitable of use in salivary calcium estimations. A method was selected based on the use of tetracyclines as a fluorescent indicator for complexometric titration of calcium in ammoniacal buffer at pH 10 (66). Tetracycline and chlortetracycline hydrochloride were used. The fluorescence intensity was measured with the excitation at 350 nm and an emission at 420 nm but no linear relation was obtained between fluorescence intensity and calcium concentration. Another method was used based on the solvent extraction of fluorescent tetracycline complexes with calcium and barbiturates (69). The solutions fluoresced but no linear relation between their fluorescence and their calcium concentration could be established. The same experiment was repeated but glycine buffer was used to dilute the tetracycline solutions and adjust their pH to 11.00. No linear relationship between calcium concentration and fluorescence was obtained. Then dimethylchlortetracycline was used following the same procedure.

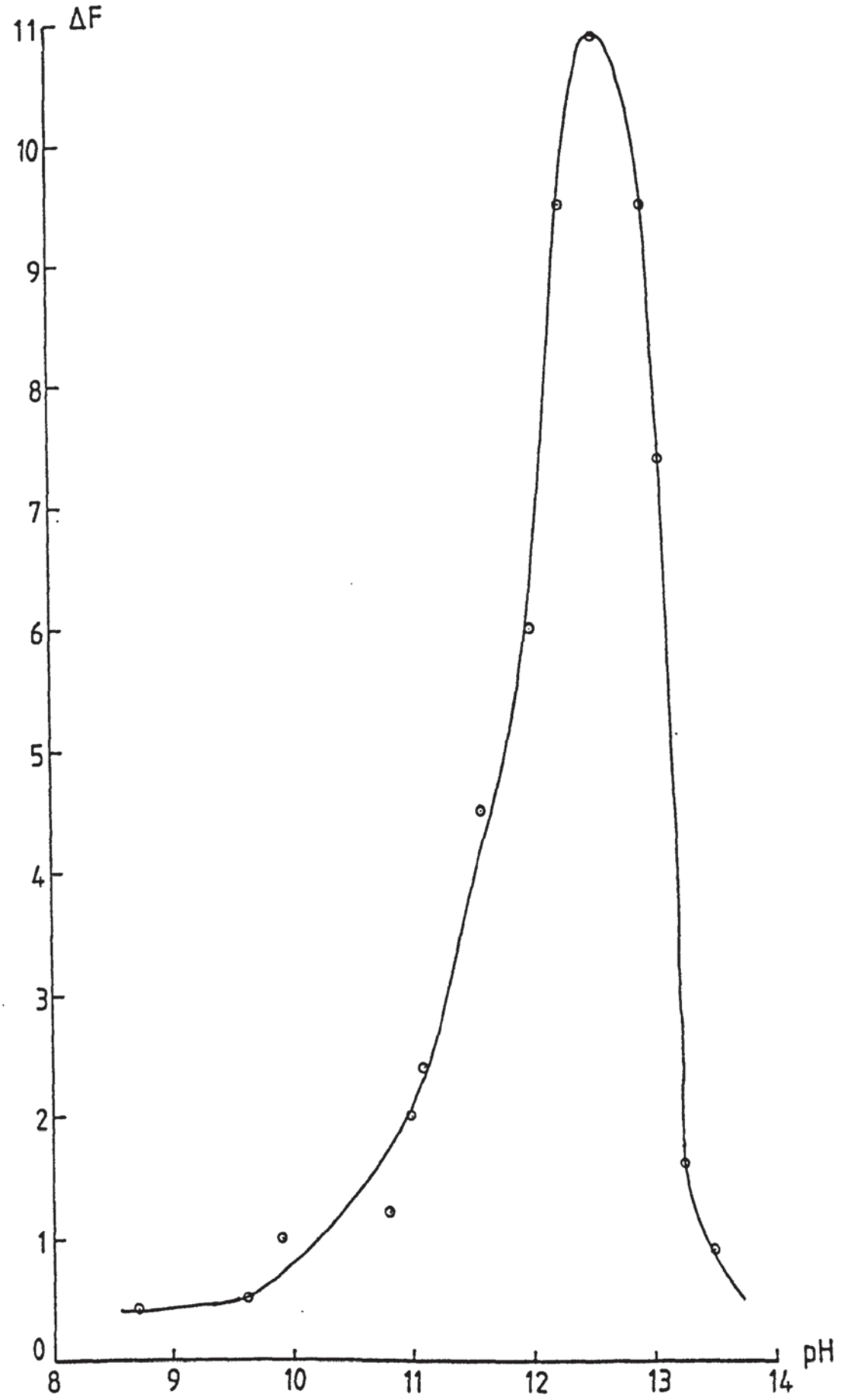
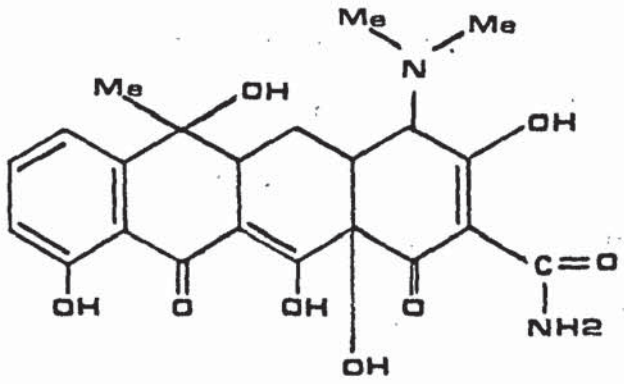


Fig 14

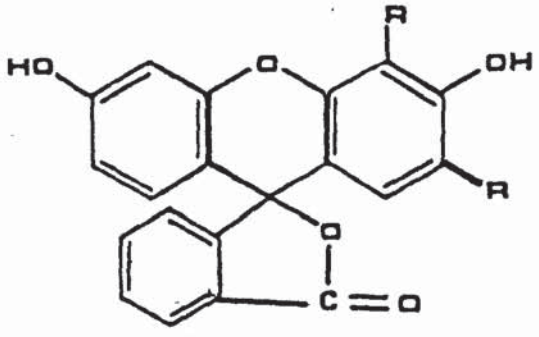
Relation between Calcein Blue with calcium against pH
Calcein Blue Concentration 12 mcg/ml
Calcium Concentration 1 mcg/ml
ΔF = F.Calcein Blue with Ca^{++} - F. Calcein Blue

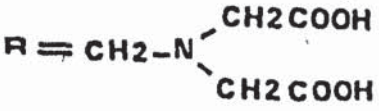
No linear relation between fluorescence intensity and calcium concentration was obtained over a wide range of calcium levels. The fluorescence produced was found to be unstable. The intensity changed with time. With chlortetracycline, it was found that the fluorescence intensity decreased as the calcium concentration increased, and with tetracycline hydrochloride the fluorescence increased with increased calcium concentration but without a linear relation.



Tetracycline

Following this the use of calcein was investigated. Calcein which is a derivative of fluorescein forms a yellow green complex with calcium ion (70).



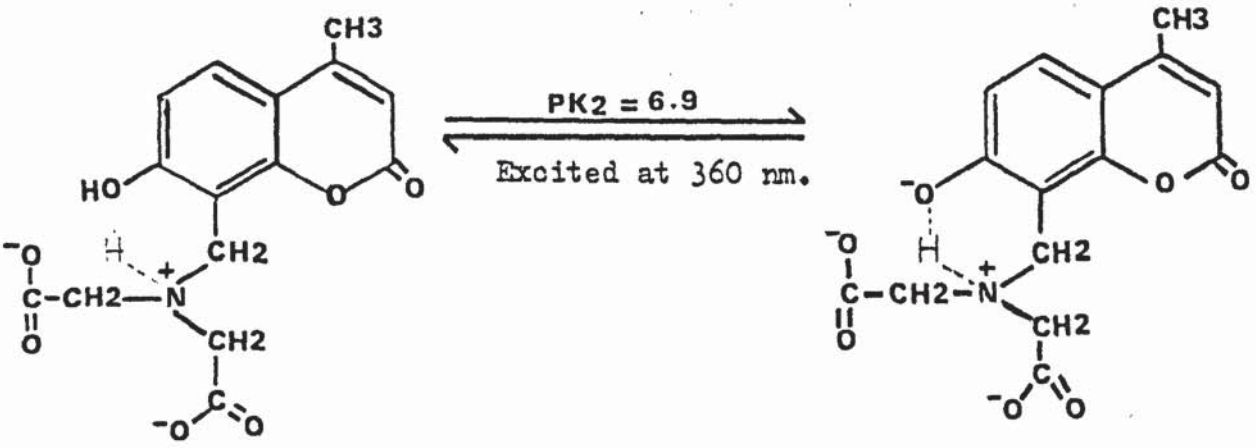


Calcein Structure

A method using calcein solutions made alkaline with 1N.KOH was applied. Fluorescence of the solutions was measured and an irregular relation was obtained between fluorescence intensity and calcium concentration. Another method described by Hercules in 1963 using calcein indicator was tested and the results obtained were also irregular (71).

Then the calcein derivative called calcein blue, used for the chelatometric determination of metal ions, was tried out. Wilkins reported that the material is useful as an indicator in the EDTA titration of calcium and certain transition elements (67). Several papers describe the use of this compound in the determination of various metals (46, 67, 72, 73).

The free calcein blue is a zwitter-ion. It fluoresces in both acidic and basic solutions when excited at a suitable wavelength.



Non-Fluorescent fluorescent

Calcein Blue

The fluorescence of doubly charged anion formed on the neutralization of the phenol group, when excited at 360 nm, reaches a maximum at pH range 8.6 - 10 (Fig. No.15) and decreases to zero with the neutralization of the ammonium ion at pH 13.8. In the presence of calcium the flourescence is maintained from pH 9 - 13 with maximum fluorescence at pH 12.5 - 12.8.

The two protons displaced by calcium in the formation of the calcium compound must come from the phenolic and ammonium groups. Because the calcium compound is highly fluorescent and the fluorescence is about the same intensity as that of calcein blue the structure corresponding to the conditions of maximum fluorescence at pH 9.

The phenolate oxygen atom, ammonium nitrogen atom, and probably both carboxylate oxygen atoms are bound to the calcium ion. Everything is tightly packed, rotation is restricted and the possibility of collisional deactivation reduced.

3. Experimental:

Reagents -

All reagents were prepared using deionised water.

i. Calcein Blue stock solution of concentration 60 mcg/ml. (Sigma Chemical Company) Mol. wt. = 321.3.

ii. Calcium chloride stock solution of concentration 25 mcg Ca^{++}/ml. $CaCl_2.2H_2O$ (Analar). (Hopkin & Williams Ltd.)

iii. Potassium hydroxide solutions of different normalities.

iv. Glycine buffer of different pH (12.5 - 12.8) (74).

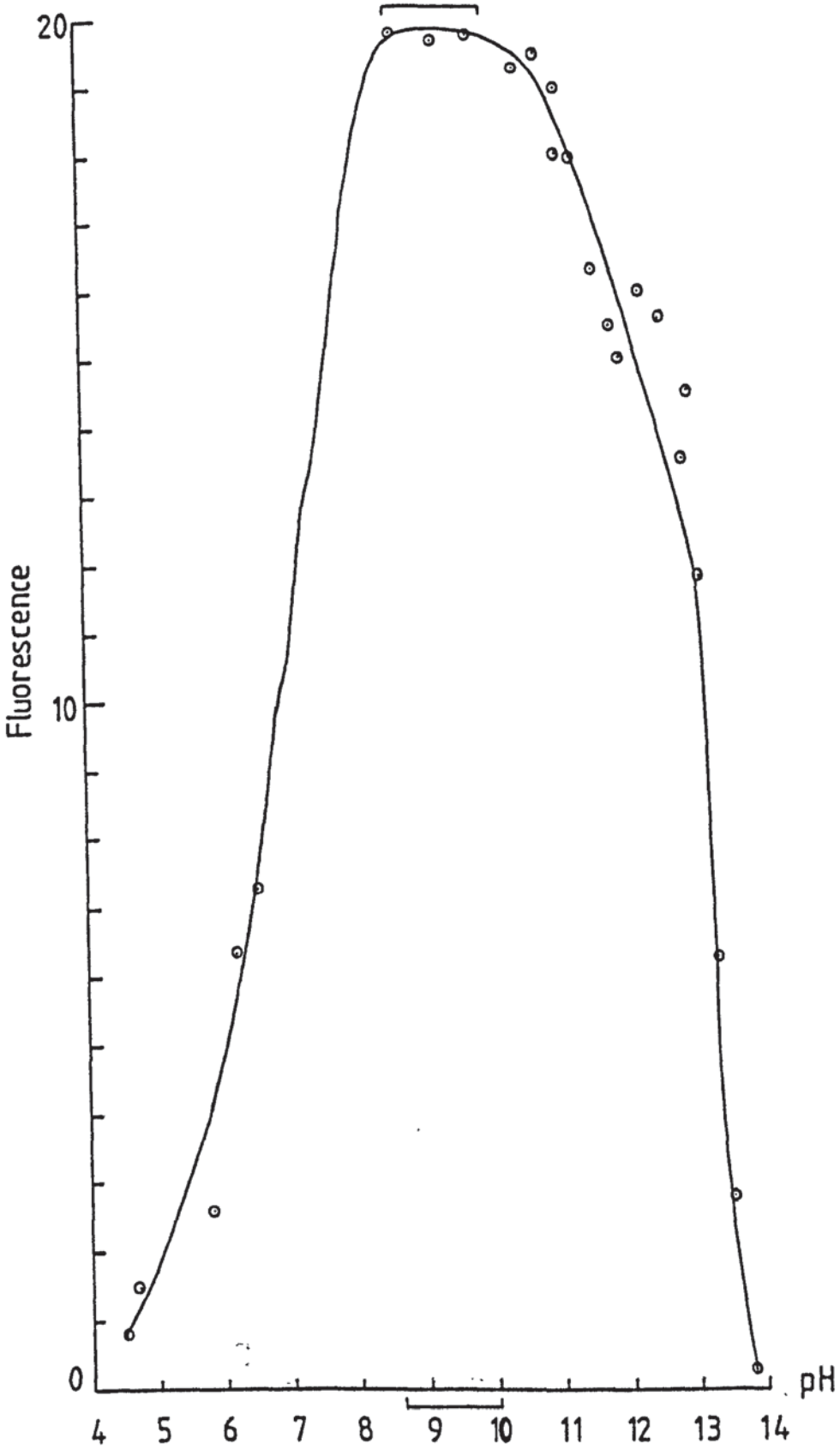


Fig 15

Fluorescence intensity of Calcein Blue against pH
Calcein Blue Concentration 12 mcg/ml in aqueous solution

Apparatus -

Fluorescence measurments were made with an Aminco Bowman spectrophotofluorimeter with an EMI 9871B photomultilplier tube, and a 250-W Xenon-arc excitation source.

All measurements were made under the same conditions:-

Monochromator excitation slit = 1 mm

Monochromator emission slit = 3 mm

Photomultiplier shutter slit = 0.5 mm

Sensitivity at 25 and meter multiplier at 1 and 0.3

A 1 cm silica cuvette was used.

In all the experiments standard, blank and sample solutions were prepared.

4. Procedure:

Initial experiments showed that the maximum fluorescence of calcein blue (of a concentration of 12 mcg/ml) occurred in the pH range of 8.6 - 10 (Fig. No.15). Further work showed the pH at which the maximum fluorescence of calcein blue occurred in the presence of calcium. Solutions of different pH were prepared containing calcein blue, 12 mcg/ml, and Ca^{++} at a concentration of 1 mcg/ml.

From Fig. no. (14) it can be seen that the maximum fluorescence is obtained at pH 12.7. When the calcium concentration was reduced to 0.5 mcg/ml and calcein blue at 12 mcg/ml, the maximum fluorescence was still at pH 12.7, but its intensity was about 20% lower than in the previous case with calcium at 1 mcg/ml.

(Fig. No.14, compared with Fig. No.15). Using a non-polar solvent, 50% methanol in water, for solutions of calcein blue at 12 mcg/ml with calcium at 0.5 mcg/ml (ratio 1:24) and another solution containing only calcein blue at 12 mcg/ml the ΔF (fluorescence of calcein blue with calcium - fluorescence of calcein blue) was plotted against pH. It was found that the maximum ΔF was at pH 12 but the fluorescence was 57% lower than that in water. (Fig. No.16).

The following table illustrates the above results (Table No.17).

Table17. Comparison of ΔF in polar and non-polar solvent against pH

No.	pH	ΔF (for polar solvent) (water)	ΔF (for non-polar solvent) (50% methanol)
1.	10.8	0.9	1.9
2.	11.6	3.0	
3.	12.0	5.4	3.8
4.	12.3	6.7	
5.	12.6	9.0	1.4
6.	12.9	9.0	0.9
7.	13.1	6.7	-1.0

ΔF = Fluorescence of calcein blue with calcium - fluorescence of calcein blue

Ca^{++} concentration = 0.5 mcg/ml

Calcein blue concentration = 12 mcg/ml

Different solution conditions were tested in order to determine an optimum assay procedure which would give a linear relation between fluorescence intensity and concentration with

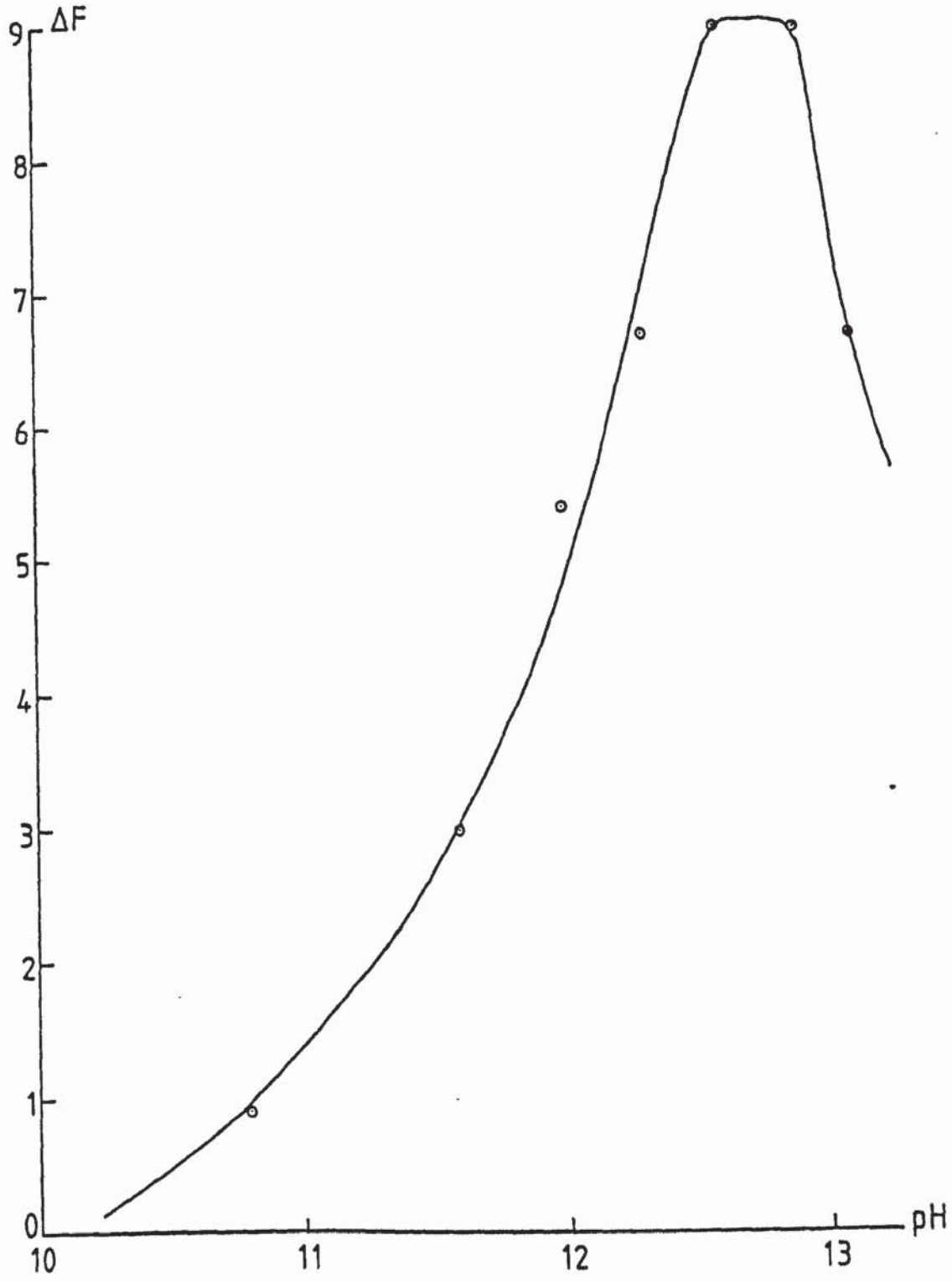


Fig 16

Using non-polar solvent, 50% methanol in water
Solutions of Calcein Blue 12 mcg/ml
With Calcium Concentration 0·5 mcg/ml
} Against pH

ΔF = F. Calcein Blue with Ca^{++} - F. Calcein Blue

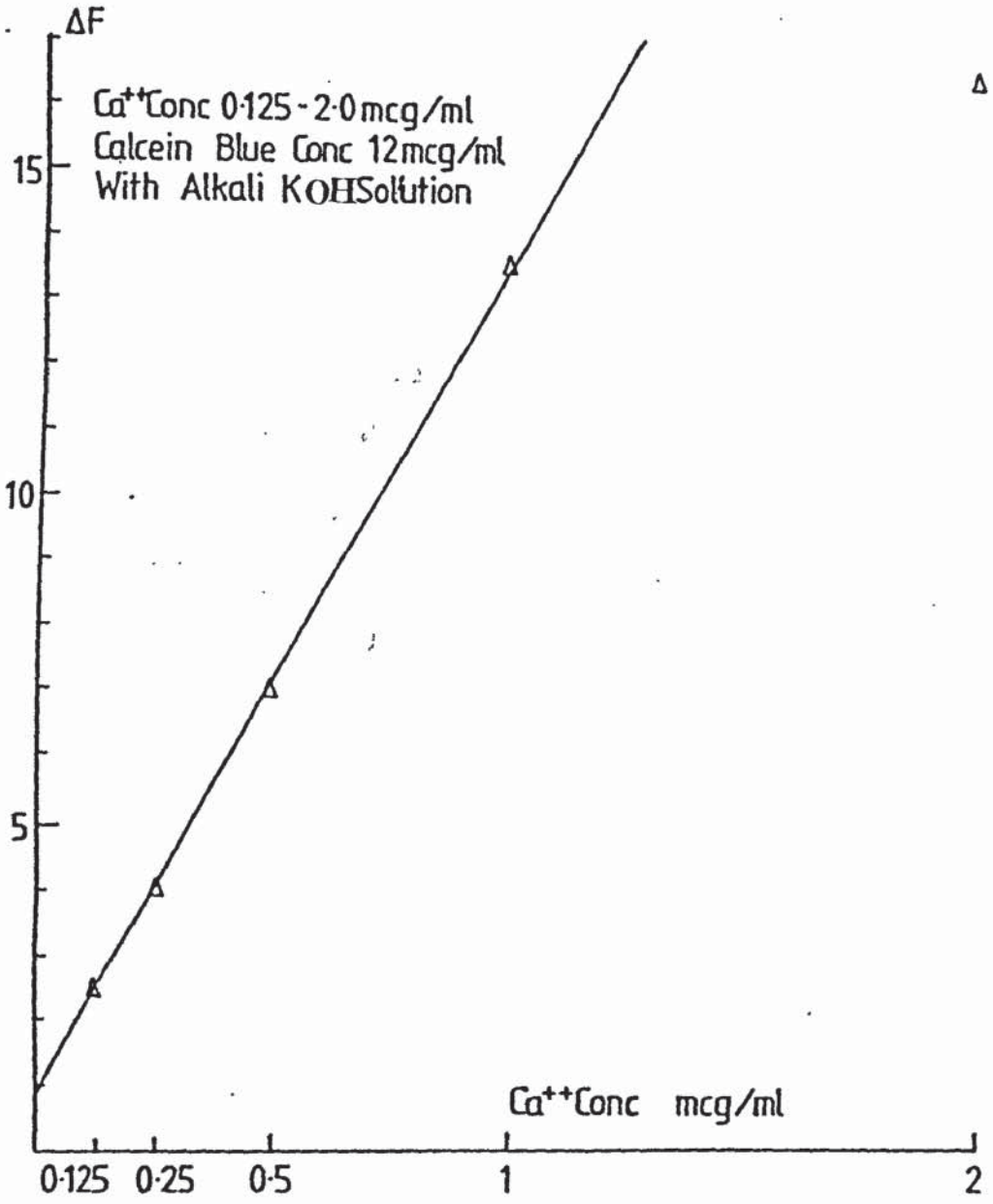
maximum precision and ease of manipulation. Thus different ranges of concentration of calcium and calcein blue were evaluated under different pH conditions (Fig. No.17).

For example some solutions were made up in a glycine buffer whereas others were made up using KOH solutions only. The effect of varying amounts of KCl on the fluorescence intensity as well as the ratio of calcium to calcein blue was investigated. Some of the results are shown in (Fig. 18,19, and20).

Different volumes of saliva samples were tested (0.05 ml, 0.1 ml, 0.2 ml, 0.5 ml and 1.0 ml) in order to find the appropriate volume that can be used in this analysis so that its calcium content was within the range of the calcium concentration of the standards. (Tables No.18 and 19).

Table 18. Standard solutions of calcium with Calcein Blue against ΔF.

No.	Ca^{++} Conc. mcg/ml	Fluorescence	ΔF
B.	0	33.0	-
1.	0.1	38.5	5.5
2.	0.2	44.5	11.5
3.	0.4	54.5	21.5
4.	0.6	63.0	30.0
5.	0.8	75.0	42.0
6.	1.0	82.0	49.0



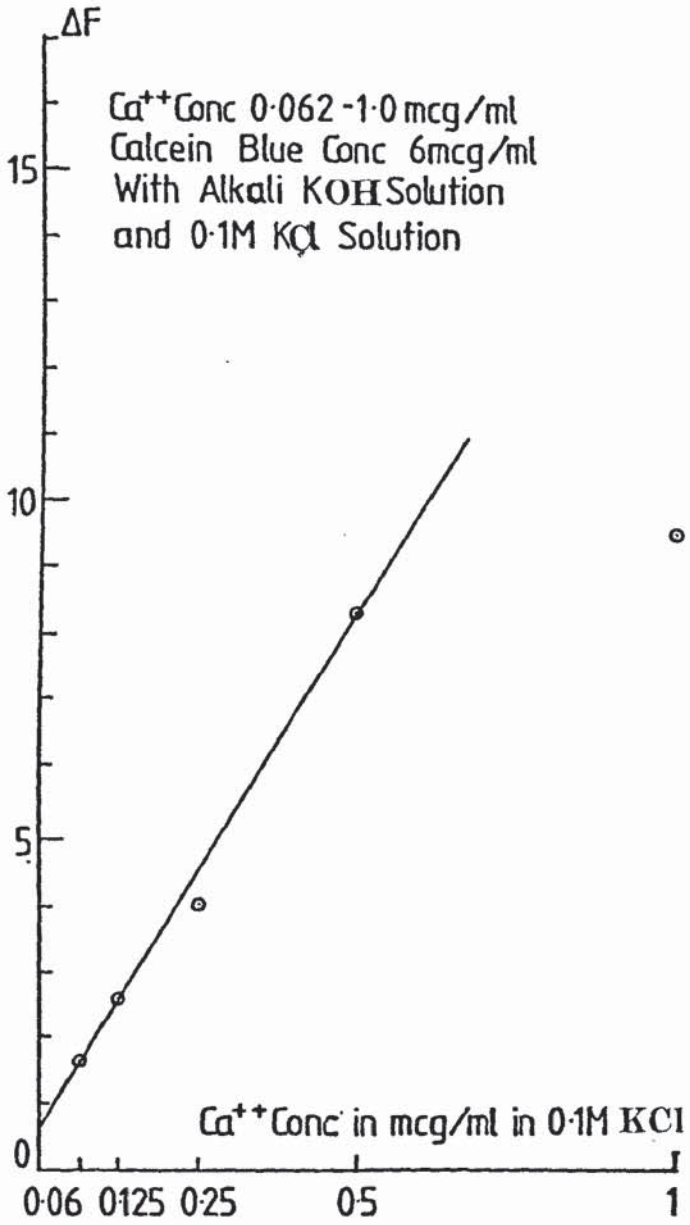


Fig 17 Using different ranges of concentration of Calcium and Calcein Blue under different pH conditions

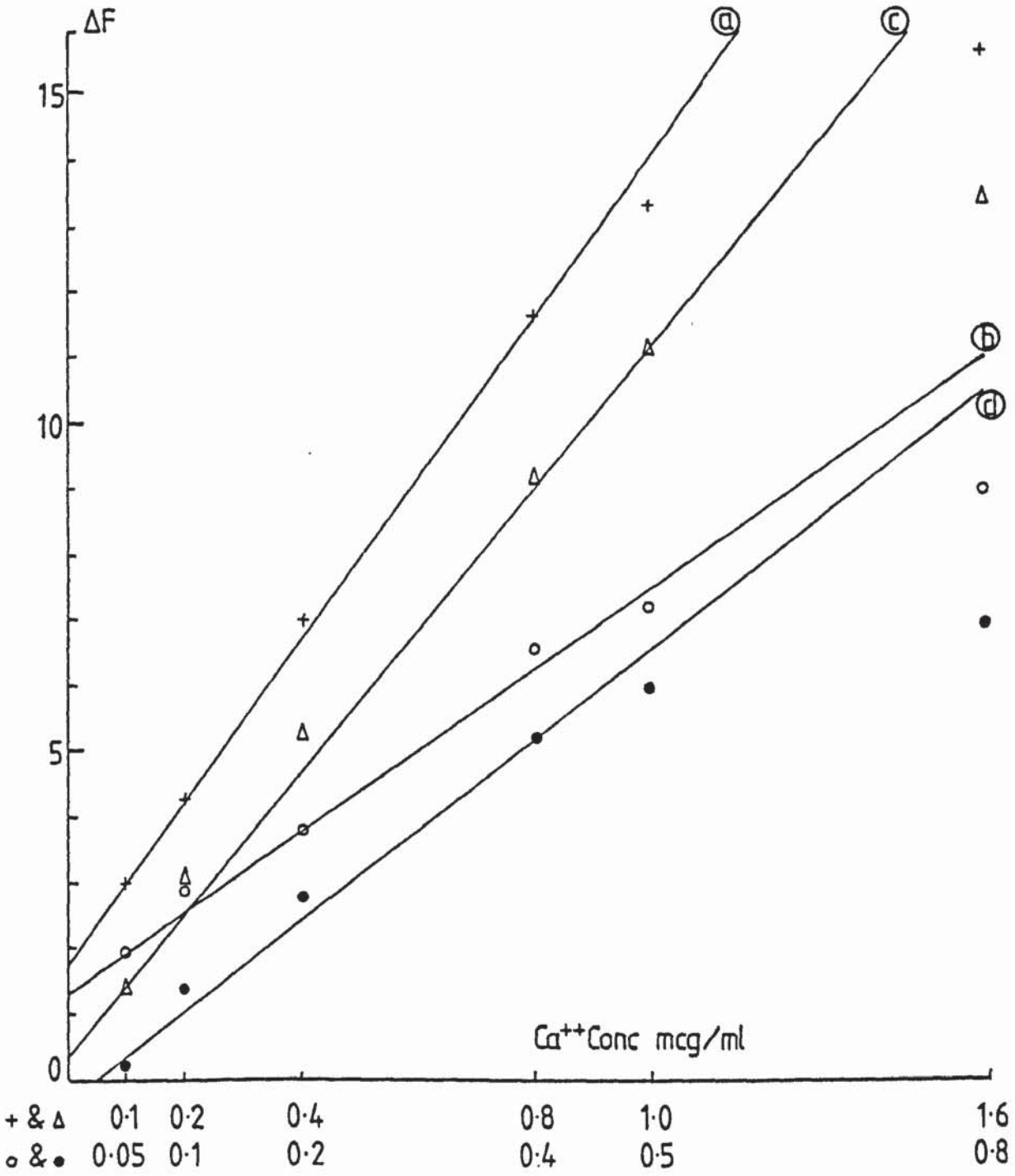


Fig 18 Comparison between different solutions prepared with

1. Glycine Buffer pH 12·77
2. Distilled Water + KOH Solution
3. 0·1M KCl

a. Diluted Glycine Buffer pH 12·77 (+)
b. Diluted Glycine Buffer pH 12·77 (o)
c. Diluted with Distilled Water (Δ)
d. Diluted with 10ml 0·1MKCl (•)

Calcein Blue Conc = 12 mcg/ml

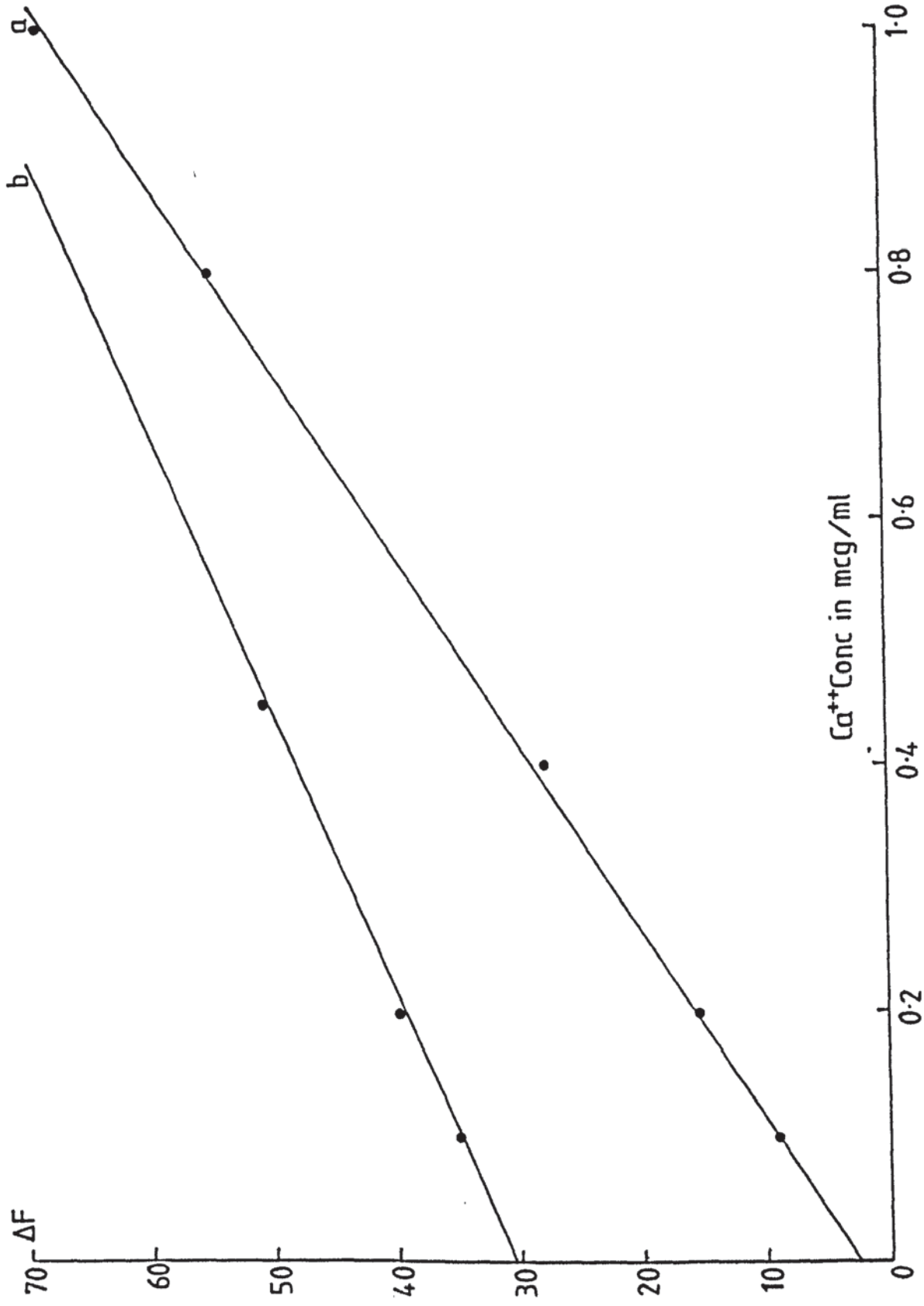


Fig 19 The effect of pH on the fluorescence of the blank

a. pH 12·55
b. pH 12·80
With 12 mcg calcein blue/ml and Ca^{++} Conc 0·1 - 1·0 mcg/ml

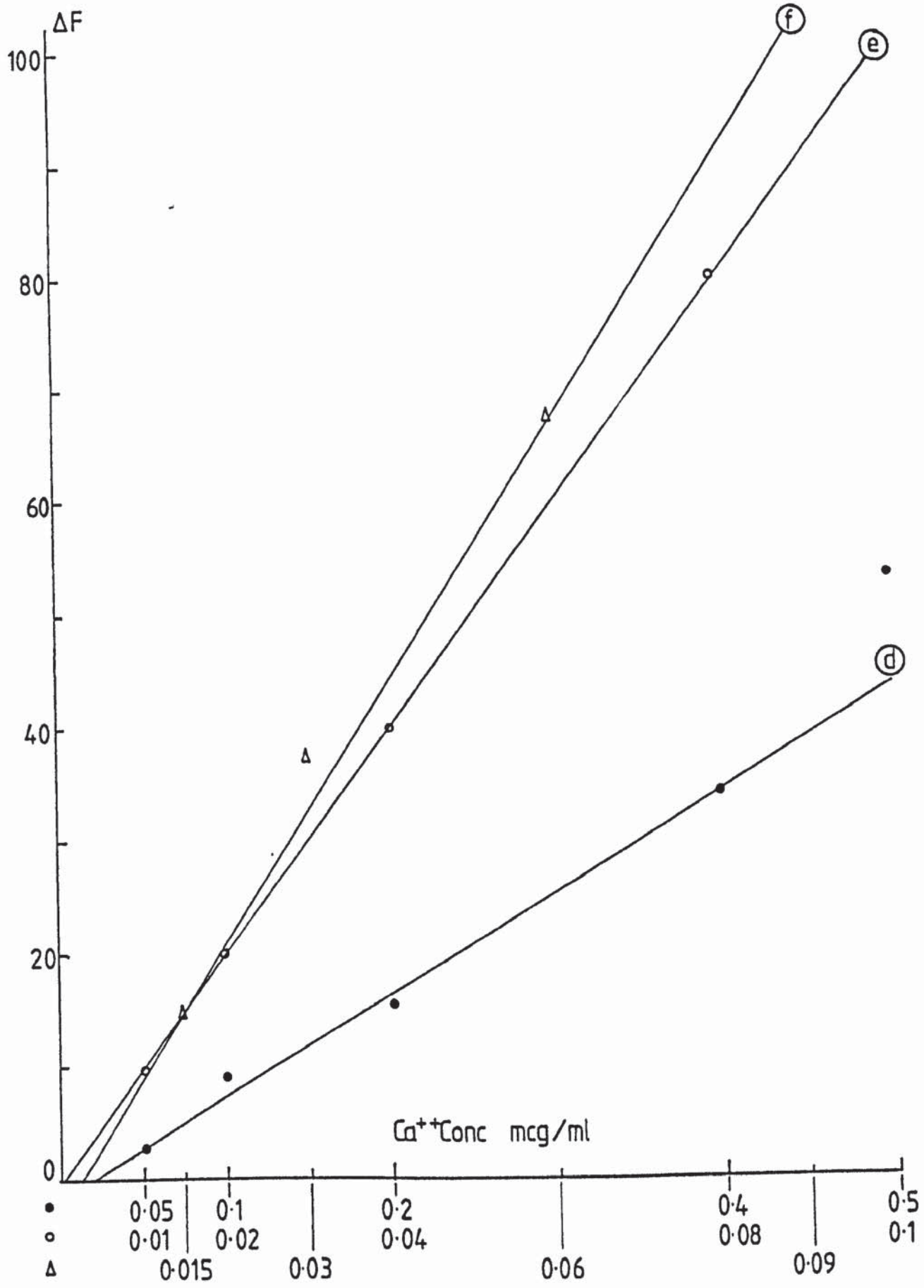


Fig 20 1ml N/1 KQHused - pH 12·55

d = ·6 mcg. calcein blue/ml
e = 1·2 mcg. calcein blue /ml
f = 1·8 mcg. calcein blue /ml

Table No.19 . Fluorescence of different volumes of saliva samples

No.	Saliva sample in ml	Fluorescence	ΔF
1.	0.05	38.0	5.0
2.	0.1	45.5	12.5
3.	0.2	59.5	26.5
4.	0.5	93.0	60.0
5.	1.0	95.5	62.5

These tables show that the 0.1 ml and 0.2 ml of saliva samples give fluorescence intensities within the range of the standard solution.

Since some salivary calcium is believed to be bound to protein, and in order to get rid of the protein and other constituents which might effect the fluorescence intensity, saliva samples were centrifuged, and the calcium levels of the centrifuged samples were compared with those of non centrifuged samples. Calcium at 0.2 mcg/ml was added to each of the centrifuged and non centrifuged samples in order to be sure that the calcium levels obtained were correct.

Table No.20 . Standard solution

No.	Ca^{++} conc. in mcg/ml	Average Fluor.	ΔF
0.	0	26.5	-
1.	0.1	31.5	5.0
2.	0.2	37.0	10.5
3.	0.4	47.0	20.5
4.	0.6	53.5	27.0
5.	0.8	63.0	36.5
6.	1.0	71.5	45.0

Table No.21 . With non centrifuged saliva sample

No.	Saliva volume	Average Fluor.	ΔF
1.	0.1 ml	36.0	9.5
2.	0.2 ml	48.3	21.8

Table No.22 . With non centrifuged saliva sample + 0.2 mcg/ml Ca^{++}

No.	Saliva volume	Average Fluor.	ΔF (0.3 mm)
1.	0.1 ml	46.0	19.5
2.	0.2 ml	60.5	34.0

Table No.23 . With centrifuged saliva sample

No.	Saliva volume	Average Fluor.	ΔF
1.	0.1 ml	34.5	8
2.	0.2 ml	43.5	17

Table No.24 . With centrifuged saliva samples + 0.2 mcg Ca^{++}

No.	Saliva volume	Average Fluor.	ΔF
1.	0.1 ml	45.5	19
2.	0.2 ml	53.5	27

From these tables it is seen that saliva samples with the added 0.2 mcg Ca^{++} have fluorescence intensities which are equal to the sum of the fluorescence intensity of the 0.2 mcg Ca^{++}/ml for the standard solutions plus the fluorescence intensity of the saliva sample only. This was found for both centrifuged and non centrifuged saliva samples.

When the saliva sample was diluted with water by factor of 2, the calcium concentration was found to be reduced also by a factor of 2.

The saliva samples were then treated with protein precipitants, trichloroacetic acid (15% w/v) and ammonium sulphate (25% w/v and 15% w/v). The samples were centrifuged and their calcium levels were determined and compared with the centrifuged samples without protein precipitants. It was found that they showed a lower fluorescence intensity and no linear relation could be obtained with their

standard solutions. This was probably because their pH had changed from 12.3 to 11.9. The following table shows the results obtained.

Table 25. Standard without Trichloroacetic acid

No.	Ca^{++} Conc. in mcg/ml	Average Fluor.	ΔF
0.	B-	27.0	-
1.	0.1	32.5	5.5
2.	0.2	38.0	11.0
3.	0.4	48.0	21.0
4.	0.6	55.5	28.5
5.	0.8	68.0	41.0
6.	1.0	78.0	51.0

Table 26. Standard with 15% w/v Trichloroacetic acid.
Calcium concentration diluted by factor of 2
(3 ml calcium solution + 3 ml TCA)

No.	Vol. of calcium solutions in ml	Ca^{++} conc. in mcg/ml	Average Fluor.	ΔF
0.	B	0	27.0	-
1.	0.1	0.05	28.0	1.0
2.	0.2	0.1	29.5	2.5
3.	0.4	0.2	35.3	8.3
4.	0.6	0.3	40.5	12.5
5.	0.8	0.4	44.5	16.5
6.	1.6	0.8	66.5	38.5

Table 27. Centrifuged saliva sample without TCA.

No.	Vol. of saliva sample in ml	Average Fluor.	ΔF
1.	0.1	38.3	11.3
2.	0.2	50.0	23.0
3.	0.3	62.3	35.3
4.	0.4	74.3	47.3

Table 28 . Centrifuged saliva samples with 15% w/v TCA.

No.	Vol. of saliva sample in ml	Average Fluor.	ΔF
1.	0.1	36.5	9.5
2.	0.2	46.5	19.5
3.	0.3	49.0	22.0
4.	0.4	61.5	34.5

Similar results were obtained when 25% w/v and 15% w/v of ammonium sulphate solution was used instead of TCA. In addition to the above results it was found that centrifuged saliva samples showed slightly lower fluorescence intensities than the non centrifuged saliva samples (Fig. No. 21).

Different experiments were done in order to determine whether the salivary calcium level is affected during storage for a long period. Samples of centrifuged and non centrifuged saliva were stored in a frozen state and the tests on these samples were done

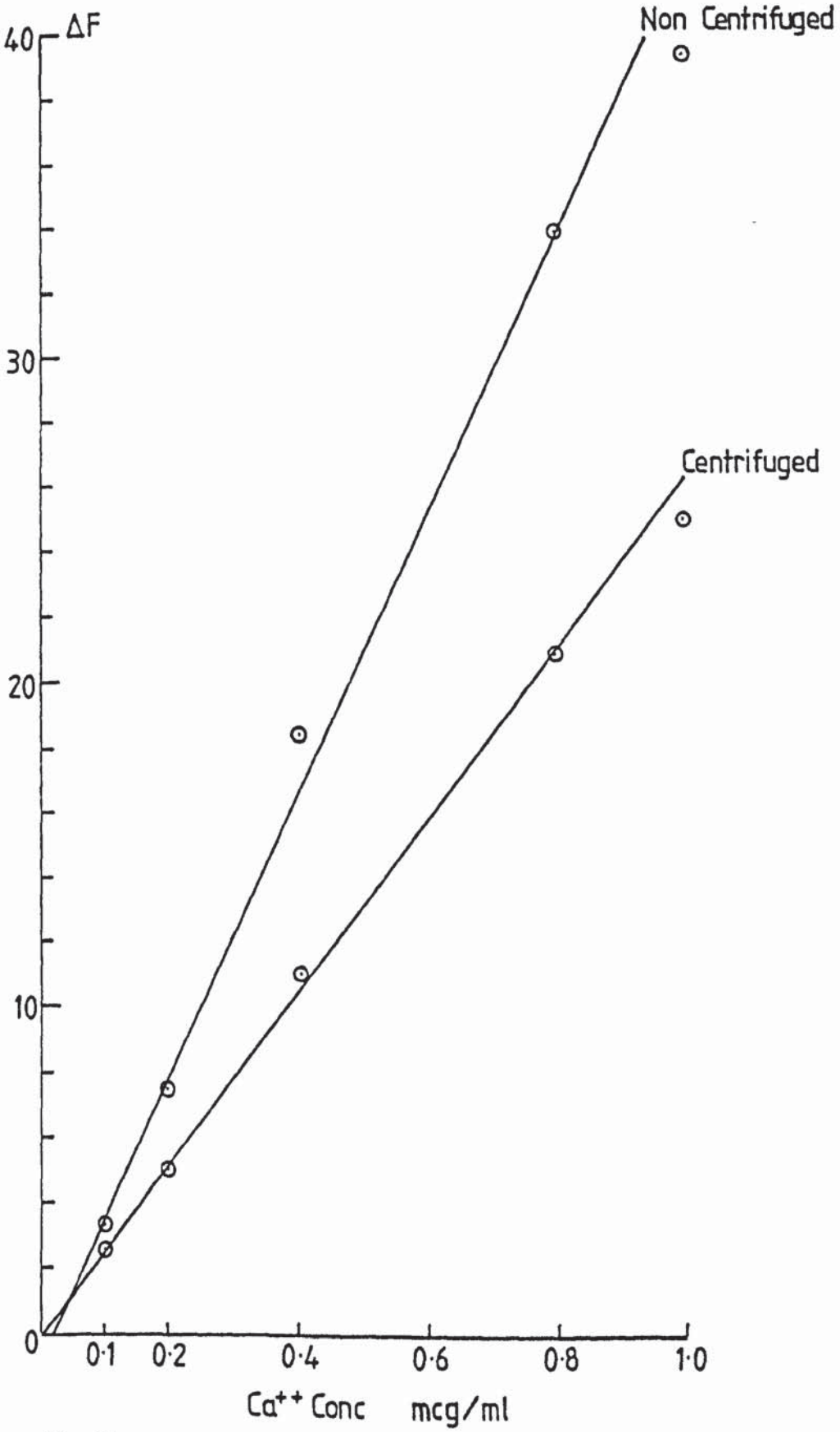


Fig 21

Calcium concentration in centrifuged and non centrifuged saliva

every two or three days for a twenty day period. At the same time the calcium levels of fresh samples were determined, for comparison. The following Tables (No.29 , 30) show the results obtained. It was found that the calcium concentrations of centrifuged saliva samples decreases by about 65% during twenty days, while in the non-centrifuged saliva samples the decrease is only 7.5%.

The calcium levels of saliva samples collected at 8.00 o'clock in the morning before breakfast were compared with the calcium levels of the samples collected at 12.00 noon, about three hours after breakfast. Centrifuged and non-centrifuged samples were used, and a comparison obtained. From the following Table (No.31) it can be seen that there is no difference in calcium levels of these two samples.

An experiment was also done on Calcein Blue solution in order to test its solubility and stability over a period of time. From Table No.(32) it is found that the solubility of Calcein Blue increases with increasing pH. It was found that the aqueous solution of Calcein Blue at the concentration required in the experiments (6 mg/100 ml) had a pH of 3.8, and the fluorescence intensity of this solution decreased by about 38.5% after one week. While a solution at neutral pH in a glycine buffer and kept in the dark in a refrigerator showed a decrease in fluorescence of only 4.5% after one week.

Table No.29 . Date of collection of the saliva sample 30-6-78

Date	Centrifuged	
Sample of	0.1 ml Sample mcg Ca^{++}/ml	0.2 ml Sample mcg Ca^{++}/ml
30/6	62.50	73.75
3/7	25.0	37.5
5/7	27.5	30.0
7/7	12.5	22.5
11/7	12.5	21.25
17/7	20.0	21.87
18/7	22.5	32.5

Date	Non Centrifuged	
Sample of	0.1 ml Sample mcg Ca^{++}/ml	0.2 ml Sample mcg Ca^{++}/ml
30/6	66.25	82.5
3/7	60.0	63.75
5/7	62.5	68.75
7/7	60.0	61.25
11/7	57.5	68.75
17/7	80.0	77.5
18/7	77.5	70.0

Table No.30

Date	Centrifuged saliva mcg Ca^{++}/ml	
Sample of	0.1 ml Sample	0.2 ml Sample
30/6	62.5	73.75
3/7	49.0	50.05
17/7	72.5	71.25
18/7	72.5	65.0

Date	Non Centrifuged saliva mcg Ca^{++}/ml	
Sample of	0.1 ml Sample	0.2 ml Sample
30/6	66.25	82.5
3/7	56.5	47.5
17/7	77.5	77.5
18/7	75.0	75.0

Table No. 31

Date	Sample collected at 8.00 o'clock			
	Centrifuged mcg Ca^{++}/ml		Non Centrifuged mcg Ca^{++}/ml	
	0.1 ml Sample	0.2 ml Sample	0.1 ml Sample	0.2 ml Sample
17/7	72.5	71.25	77.5	77.5
18/7	72.5	65.0	75.0	75.0

Date	Sample collected at 12.00 o'clock			
	Centrifuged mcg Ca^{++}/ml		Non Centrifuged mcg Ca^{++}/ml	
	0.1 ml Sample	0.2 ml Sample	0.1 ml Sample	0.2 ml Sample
17/7	65.0	71.25	72.5	81.25
18/8	77.5	73.75	80.0	75.0

Table No.32 . Solubility of Calcein Blue at different pH (67)

pH	Solubility in mg/100 ml
2.36	1.20
2.80	1.71
3.39	3.77
4.45	32.37
4.65	48.19
4.74	59.44

ATOMIC ABSORPTION METHOD FOR CALCIUM DETERMINATION:

1. Summary:

This method was used in order to check the accuracy of the salivary calcium concentration as determined by fluorimetric methods.

2. Experimental:

(a) Reagents:

All reagents were prepared in deionised water.

i. Stock calcium solution 100 mg/l

Analar $CaCl_2.2H_2O$ was used.

ii. Stock lanthanum chloride solution 5% La^{+3}.

iii. Standard calcium solutions: These solutions contained 2, 5, 10, 15 and 20 mcg Ca^{++}/ml in 1% La^{+3} solution. A blank was also prepared.

iv. Saliva sample.

The saliva sample was diluted so that its calcium concentration lay between 2 and 15 mcg/ml and a sufficient quantity of the stock lanthanum chloride solution was added to give a final concentration of 1% La^{+3}.

(b) Apparatus:

A Unicam SP90 atomic absorption spectrophotometer was used and adjusted under the following conditions:

Wave length	422.7 m
Slit width	0.08 mm
Filter	2
Lamp current	.12 mA
Burner	10 cm acetylene
Burner height	10 mm

Fuel-acetylene pressure 0.7 Kg/cm^2 (10 p.s.i.) and flow rate of 1500 cc/min.

Air pressure 2.1 Kg/cm^2 (30 p.s.i.) and a flow rate of 5l/min.

3. Procedure:

Experiments were done by aspiration of the blank, the standard and the saliva sample solutions (both centrifuged and non centrifuged saliva were always used).

Standard curves were plotted for calcium concentration in mcg/ml against optical density (atomic absorption). From these graphs it was found that over a concentration of 20 mcg of Ca^{++}/ml the graph is slightly curved, Fig. No. 22.

All the determinations made by the fluorometric method were checked by atomic absorption method. The following table (No. 33) shows the results obtained in Fig. 21 and 22.

Table No. 33

BY FLUORESCENCE		
Vol. used	Non centrifuged saliva	Centrifuged saliva
0.1 ml	52.5 mcg Ca^{++}/ml	47.5 mcg Ca^{++}/ml
0.2 ml	57.4 mcg Ca++/ml	53.75 mcg Ca++/ml

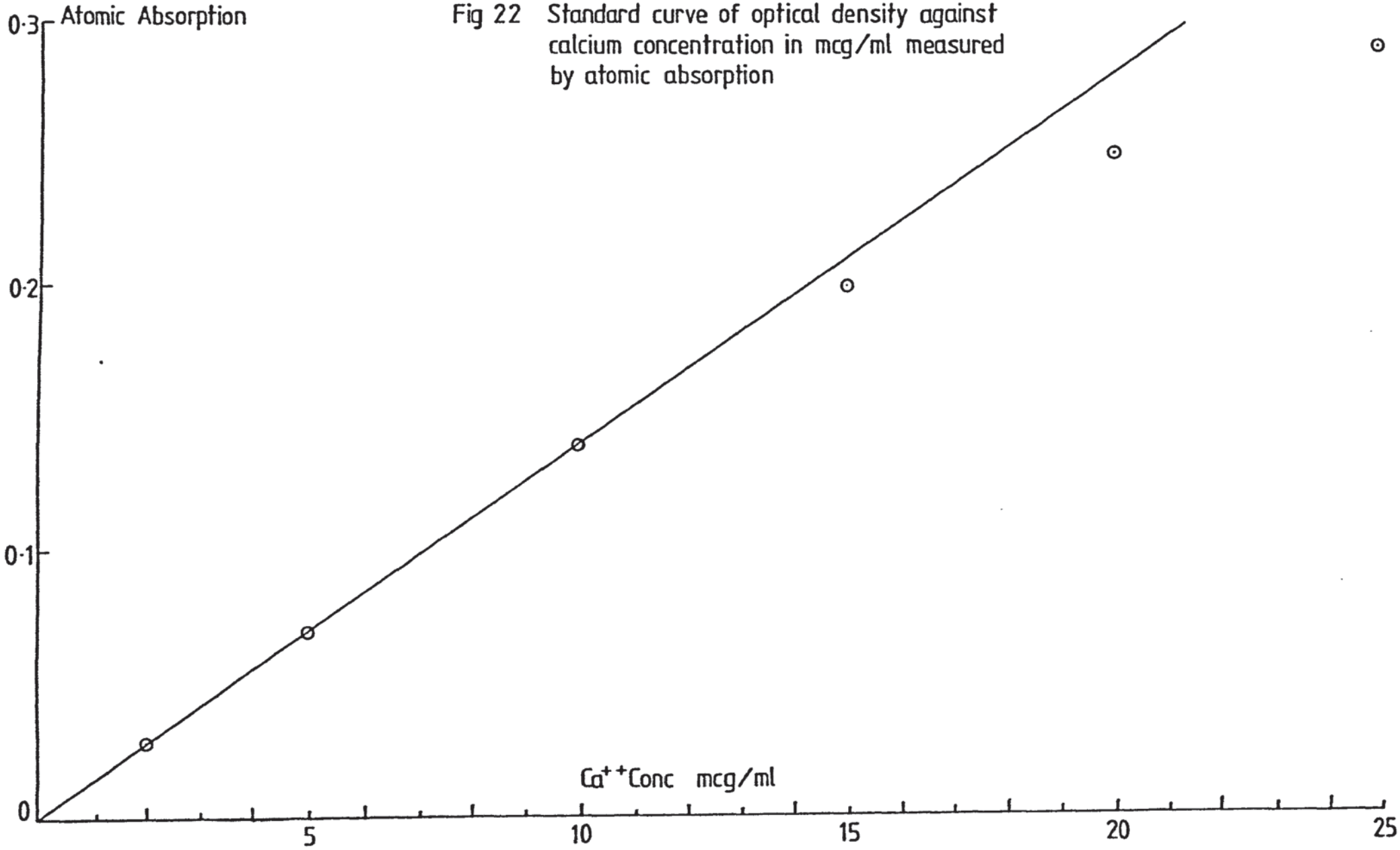


Fig 22 Standard curve of optical density against calcium concentration in mcg/ml measured by atomic absorption

Table No. 33. Continued.

	BY ATOMIC ABSORPTION	
Vol. used	Non centrifuged saliva	Centrifuged saliva
0.2 ml	56.25 mcg Ca^{++}/ml	55 mcg Ca^{++}/ml
0.4 ml	48.125 mcg Ca^{++}/ml	51.25 mcg Ca^{++}/ml

This table illustrates the results obtained by using different volumes of saliva samples.

Table No.34 . The date of determination was 29-6-78

Ca^{++}Concentration in mcg/ml. saliva.

BY FLUORESCENCE				
	Centrifuged saliva sample		Non centrifuged saliva sample	
Date of collection	0.1 ml	0.2 ml	0.1 ml	0.2 ml
29-6-78	87.5 mcg	92.5 mcg	102.5 mcg	92.5 mcg
28-6-78	52.5 mcg	52.5 mcg	72.5 mcg	71.5 mcg
27-6-78	-	33.75 mcg	-	50.0 mcg

BY ATOMIC ABSORPTION				
	Centrifuged saliva sample		Non centrifuged saliva sample	
Date of collection	0.5 ml	1.0 ml	0.5 ml	1.0 ml
29-6-78	88 mcg	82 mcg	88 mcg	83 mcg
28-6-78	58 mcg	-	72 mcg	63 mcg
27-6-78	-	39 mcg	-	46 mcg

DISCUSSION:

The best method for the estimation of salivary calcium levels should meet the following criteria:-

i. The calcium level in whole unstimulated saliva, as reported by some workers, lies between 2.3 - 5.5 mg% and between 5.2 - 9.7 mg% as reported in Table No.(1).

ii. The method should be simple and precise in order to be acceptable for the routine estimation of calcium levels in human saliva daily.

iii. The range of the concentrations of calcium standards to be determined must be around the range of saliva calcium-concentration.

iv. The standard solution prepared should remain stable for a considerable length of time in order to avoid fluctuations to obtain a reliable standard curve.

In conclusion, it was found that the most intense fluorescence was obtained at a pH of 12.5 - 12.8 in a glycine buffer with calcium concentrations between 0.1 and 1.0 mcg/ml. If the Calcein Blue was at 12 mcg/ml, then there was a linear relationship between the calcium concentrations and the fluorescence intensities. This range of calcium concentrations was such that it covered the average calcium concentrations in saliva if 0.1 ml - 0.2 ml of saliva sample was used. The Fig. (No.22a) shows that the final results obtained by using the

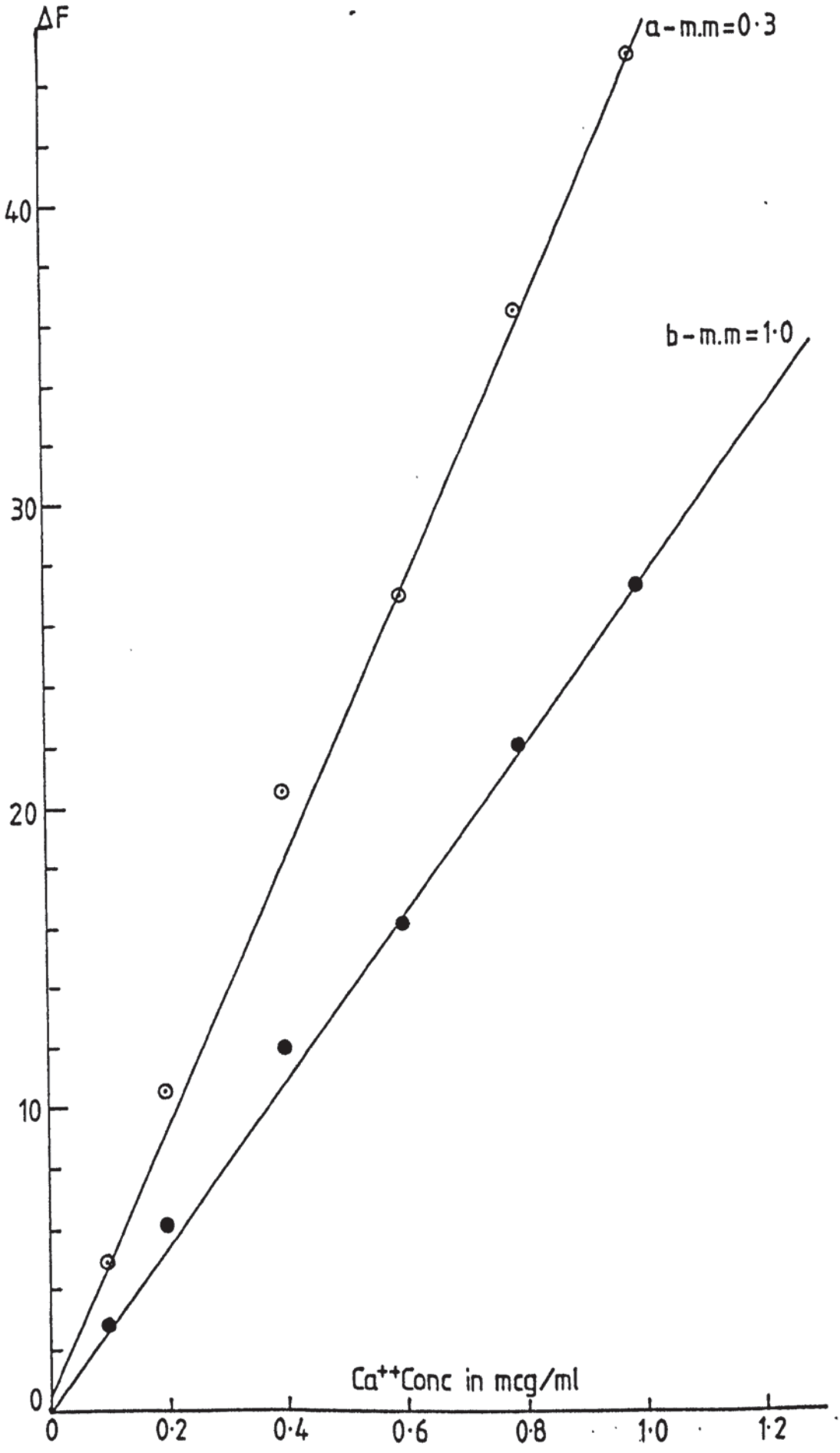


Fig Nº 22a With Glycine Buffer pH 12·66
5ml Calcein Blue used Conc 12mcg/ml
Standard Ca^{++}Solution Conc 25mcg/ml
Conc used 0·1mcg/ml to 1·0 mcg/ml
m.m = Meter multiplier

above procedures.

It was found that due to the viscosity of saliva, some of it remained at the tip of the pipette after ejection of the sample. These errors were corrected by diluting the saliva samples with water using 1 : 1 ratio.

When 0.2 mcg Ca^{++}/ml was added to centrifuged and non-centrifuged saliva samples, the results obtained indicated that only calcium was being determined.

Protein precipitants such as 15% w/v trichloroacetic acid and 25% w/v or 15% w/v of ammonium sulphate solutions were added to the saliva samples, in order to precipitate the protein bound to calcium, and to determine calcium only without interferance of any other constituents. It was found that the samples treated with protein precipitants showed a lower and irregular fluorescence intensities, i.e. lower calcium concentrations than those samples without protein precipitants.

When saliva samples were centrifuged in order to get rid of the protein bound to calcium and other suspended impurities, it was found that centrifuged samples showed lower fluorescence intensities than the non-centrifuged saliva samples. The higher fluorescence intensity observed using saliva without centrifugation or addition of any protein precipitants was probably due to the presence of protein and the other impurities suspended in the sample.

The calcium concentrations of the centrifuged saliva samples decreased by a considerable amount during long periods of storage. the decrease was about 65% during twenty days. Only a small decrease in calcium concentrations of the non-centrifuged samples was observed. The decrease was about 7.5% for the same period of storage.

No difference was observed with the calcium concentration of

the saliva sample collected at 8.00 o'clock in the morning and the calcium concentration of the saliva sample collected at 12.00 noon. This indicated that there are no diurnal variations in the calcium concentrations.

The results obtained for centrifuged and non-centrifuged saliva samples, fresh and old saliva samples using different volumes were checked by the atomic absorption method. It was found that similar results were obtained as those by fluorimetric methods. These results confirm that the real amount of calcium in saliva was determined by the fluorimetric method.

Tables No. (33) and (34) show the results obtained from the Fig. No.21 and 22.

It is recommended that the calcein blue stock solution should be made neutral with glycine buffer and kept in the refrigerator in the dark to avoid the considerable decrease of its fluorescence intensity produced during storage. This solution can then be stored for more than two weeks without any apparent change.

After measuring the fluorescence intensities of the standard solutions and the sample, it was found that their fluorescence intensities decrease over a period of one hour. To overcome this difficulty, each solution was prepared fresh and its fluorescence intensity was measured within five minutes of its preparation. Also a fresh blank was prepared and its fluorescence measured just before measuring the fluorescence of the sample. This was necessary to reduce instrumental errors.

By following the above procedure, it was found that a linear calibration graph which passed through the origin could be drawn and from which precise and reliable calcium concentrations in saliva samples could be obtained.

CHAPTER VIII

RESULTS AND DISCUSSION

RESULTS

1. Glucose

Data are presented for one complete menstrual cycle of seven normal women, between the ages of 16 and 38 years, who did not use oral contaceptives or become pregnant during this time.

All of the data are expressed with respect to the date of the onset of the next menstruation. Ovulation appears to be more closely related to the next menstrual period than to the last. This method of identifying the days of the cycle is therefore more reasonable than the alternative of relating to the end of the previous period of menstruation (27).

The saliva samples before use were diluted with citric acid/ phosphate buffer, pH 4.8 (1:1), and centrifuged at 10,000 r.p.m. for 10 minutes. They were then heated in a boiling water bath for 15 minutes, centrifuged, treated with a mixture of equal amounts of cation and anion exchange resins by shaking for 10 minutes, centrifuged again and then the clear supernatant was analysed for glucose. The concentration of glucose is expressed in microgram per millilitre.

The results of duplicate determinations indicate a high degree of precision for the enzymatic fluorimetric assay. Daily changes in the level of salivary glucose in ten menstrual cycles are shown in figures 23,24 , 25,26 ,27 , 28, 29,30 ,31 , and 32.

In all cycles, apart from the cycles of subjects D1,E1and G where the rise was not so obvious, a marked increase in glucose

concentration was found during the menstrual period on days 12, 13, 14 and 15 before the onset of the next menstrual cycle which is believed to be close to the expected time of ovulation. It can be seen therefore that a marked increase in the glucose level occured in the menstrual period of seven of the ten cycles studied. In general the high level persists for only one day and this change is larger than the maximum daily variation during the other parts of the cycle. There was a marked variation in the range of the level of glucose between the individual subjects. In subject C1 for example, the level of glucose was about six times higher than those found in subjects A and G, about four times higher than those found in subjects E1, B2 and D2, and about three times higher than those found in subjects D1 and F, and about two times higher than those found in subjects B1 and C2. For several of the subjects a second characteristic peak in glucose was observed around two to four days before the onset of the next menstruation. The mean glucose levels in saliva samples in the cycles of seven women are shown in figures 23, 24, 25, 26, 27, 28, 29, 30, 31, and 32. In subject A, Fig.23, the glucose level on days 2, 3, 4, 5, 10, 11, 12, 14, 15, 16, 17,18 and 19 were zero. A marked increase in glucose level occured on day 8 of the 22 days cycle, i.e. 14 days before the beginning of the next period. Another peak of elevated glucose levels was found on day 22 of the cycle. Subject B1 Fig.24 , showed a maximum glucose level of 88 mcg/ml on day 16 of a 31 days cycle, i.e. 15 days before the onset of the next period. Subject C1, Fig.25 , showed an increase from about 20 mcg/ml to about 160 mcg/ml on day 10 in a 23 days cycle, i.e. 13 days before the next menstruation. Another peak was found three days before the beginning of the next cycle.

Subject D1, Fig.26 , showed the highest glucose level of more than 50 mcg/ml on day 18, i.e. ten days before the onset of the next cycle. Another two peaks of glucose levels of about 48 - 49 mcg/ml were found on days 5, 6, 7 and 13 and 14 of the cycle. Another increase in glucose level of 39 mcg/ml was found on day 26, i.e. two days before the onset of the next cycle.

Subject E1, Fig.27 , showed an increase in the glucose level to 40 mcg/ml on day 14, i.e. 15 days before the next menstruation in a 29 day cycle. Another peak of 44 mcg/ml was also found two days before the next menstruation.

Subject F, Fig.28 , showed the first peak of 58 mcg/ml on day 2. A second characteristic peak of a glucose level of 50 mcg/ml occured on day 13, i.e. 16 days before the next menstruation, and again a third peak was observed two days before the next menstruation.

Subject G, Fig.29 , had a forty days cycle but supplied only 34 samples. Therefore there is some doubt about the significance of the results obtained in this case.

The 33 days cycle of subject B2, Fig.30 , showed several peaks for the glucose level. However, the one on day 19, that is 14 days before the beginning of the next cycle shows the highest glucose level of 40 mcg/ml. The other peaks were of 36 mcg/ml on day 1, 35.5 mcg/ml on day 6, 35 mcg/ml on day 11, 27 mcg/ml on day 29. On the last day of the cycle, i.e. one day before the next menstruation, the glucose level was 24.5 mcg/ml.

Subject C2, Fig.31 , had a 32 dayscycle. The highest glucose level of 76 mcg/ml was found to occur 13 days before the onset of the next menstruation. A second peak of 52 mcg/ml glucose appeared 5 days before the next menstruation, and the third peak of 40 mcg/ml appeared one day before the next menstruation.

Subject D2, Fig. 32, with a 30 day cycle had the first peak of glucose at a level of 37 mcg/ml on day 2. The second peak with the highest glucose level at 45 mcg/ml came 13 days before the onset of the next menstruation. The last peak, which was on the last day of the cycle, i.e. one day before the next menstruation, had a glucose level of 34 mcg/ml.

Table No. 35, summarises the results obtained in the seven subjects during one of their menstrual cycles.

Table (35). Glucose Levels in the Saliva of 7 Females during one Menstrual Cycle

S	A	D.C.D.	L.L. mcg/ml	D.L.L. B.N.M.C.	H.L. mcg/ml	D.H.L. B.N.M.C.	L.E. O.D.	E.O.D. B.N.M.C.
A	20	22	0	21,20,19, 18,12,11, 10,9,8,7, 6,5,4	40	1	24	15
B1	26	31	36	2	88	16	88	16
B2	26	33	10	31,17,2	40	15	40	15
C1	23	23	13	23,18,12	160	14	160	14
C2	23	32	10	33,26,20	76	13	76	13
D1	38	28	24	9	51	11	51	11
D2	38	30	9	24,18	46	13	46	13
E1	17	29	19	26	45	2	40	16
F	23	29	12	8	58	28	50	17
G	15½	40	5.5	39,9	41	27	23	13,12

S = Subject

A = Age

D.C.D. = Duration of the Cycle in Days

L.L. = Lowest Level

D.L.L. = Days of Lowest Level

H.L. = Highest Level

D.H.L. = Days of Highest Level

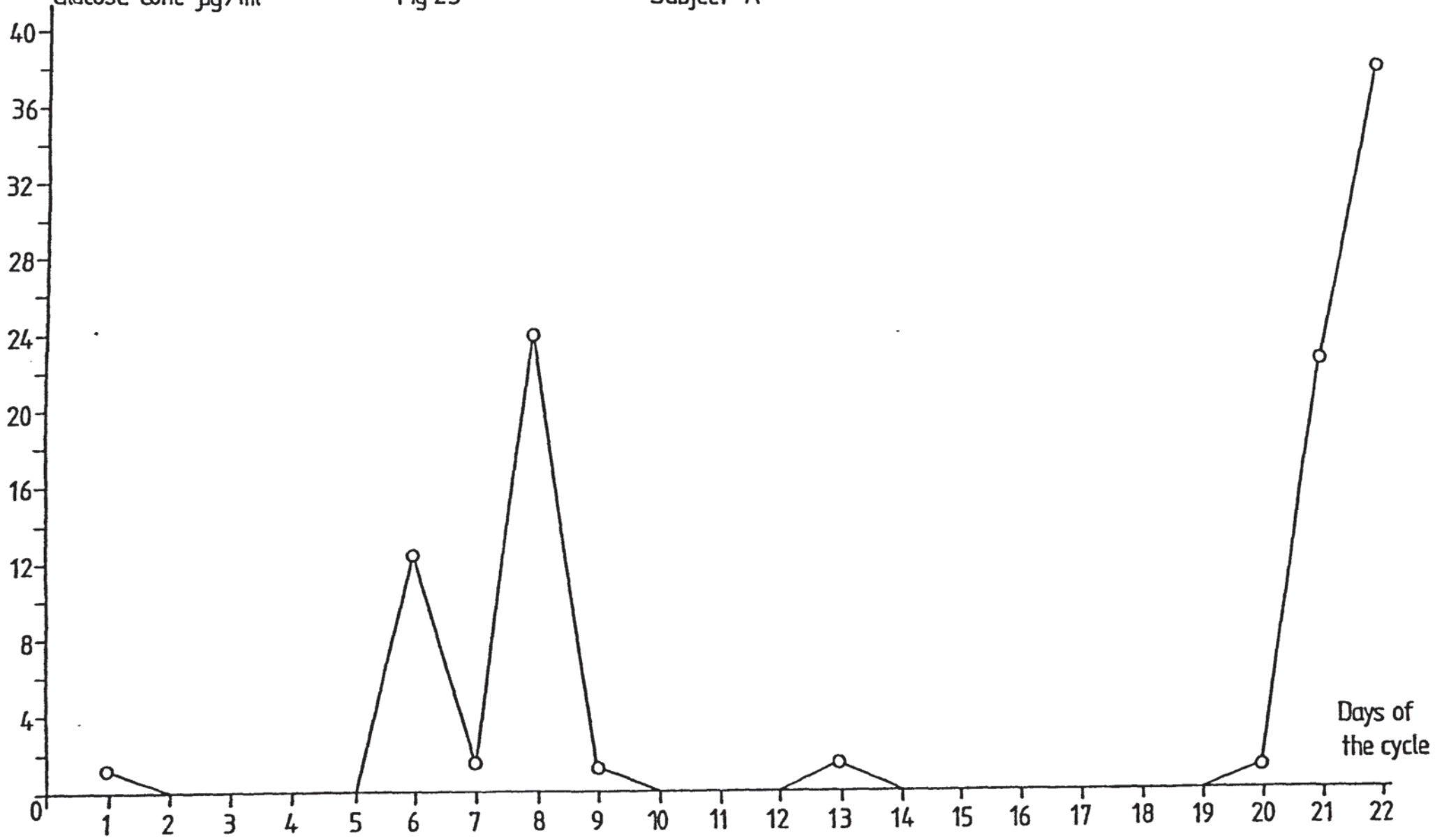
L.E.O.D. = Level on Expected Ovulation Day

E.O.D. = Expected Ovulation Day

B.N.M.C. = Before Next Menstrual Cycle

Subjects 1, 2, refer to a 1st and 2nd cycle of the same subject

Glucose Conc µg/ml Fig 23 Subject A



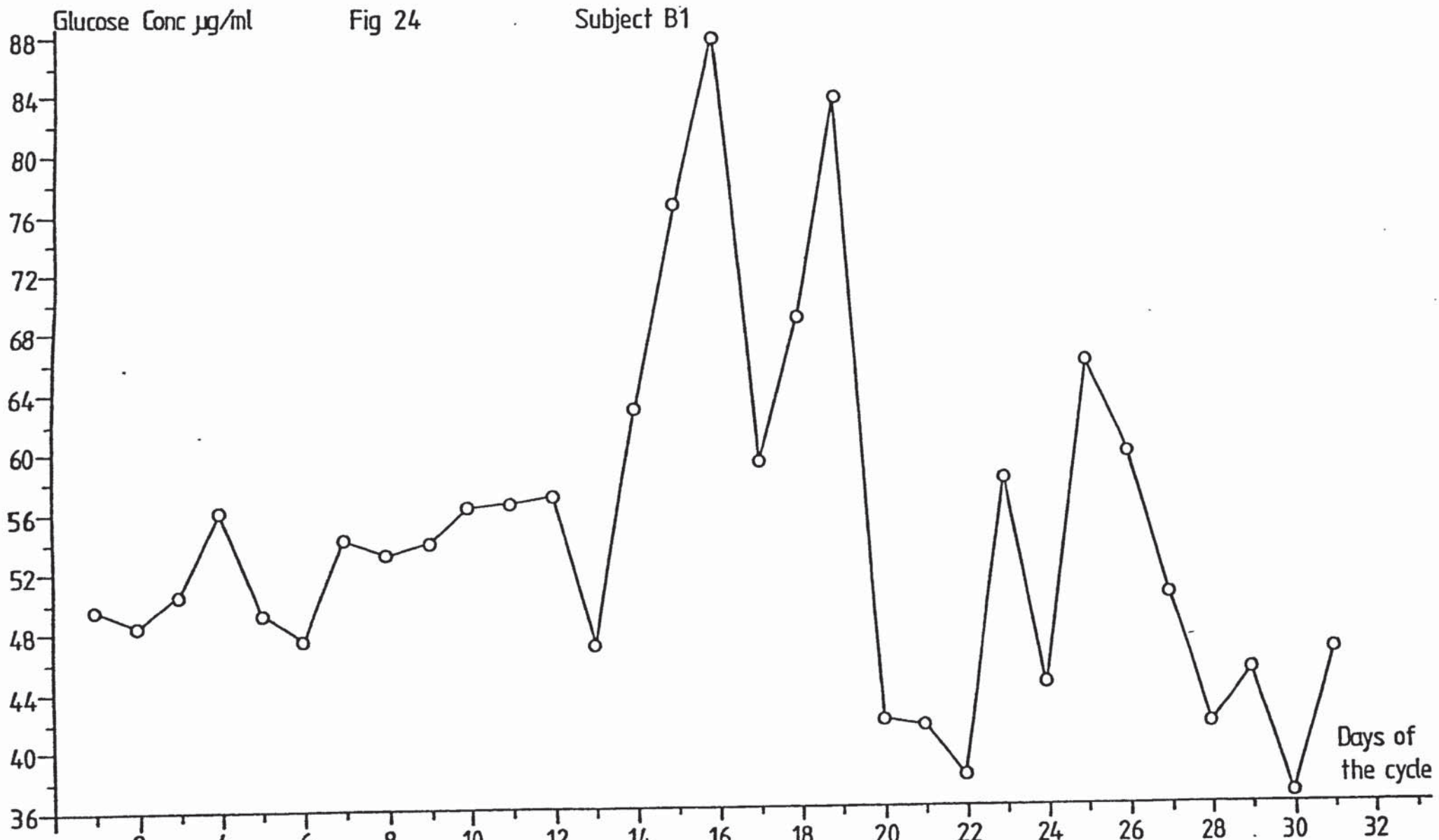
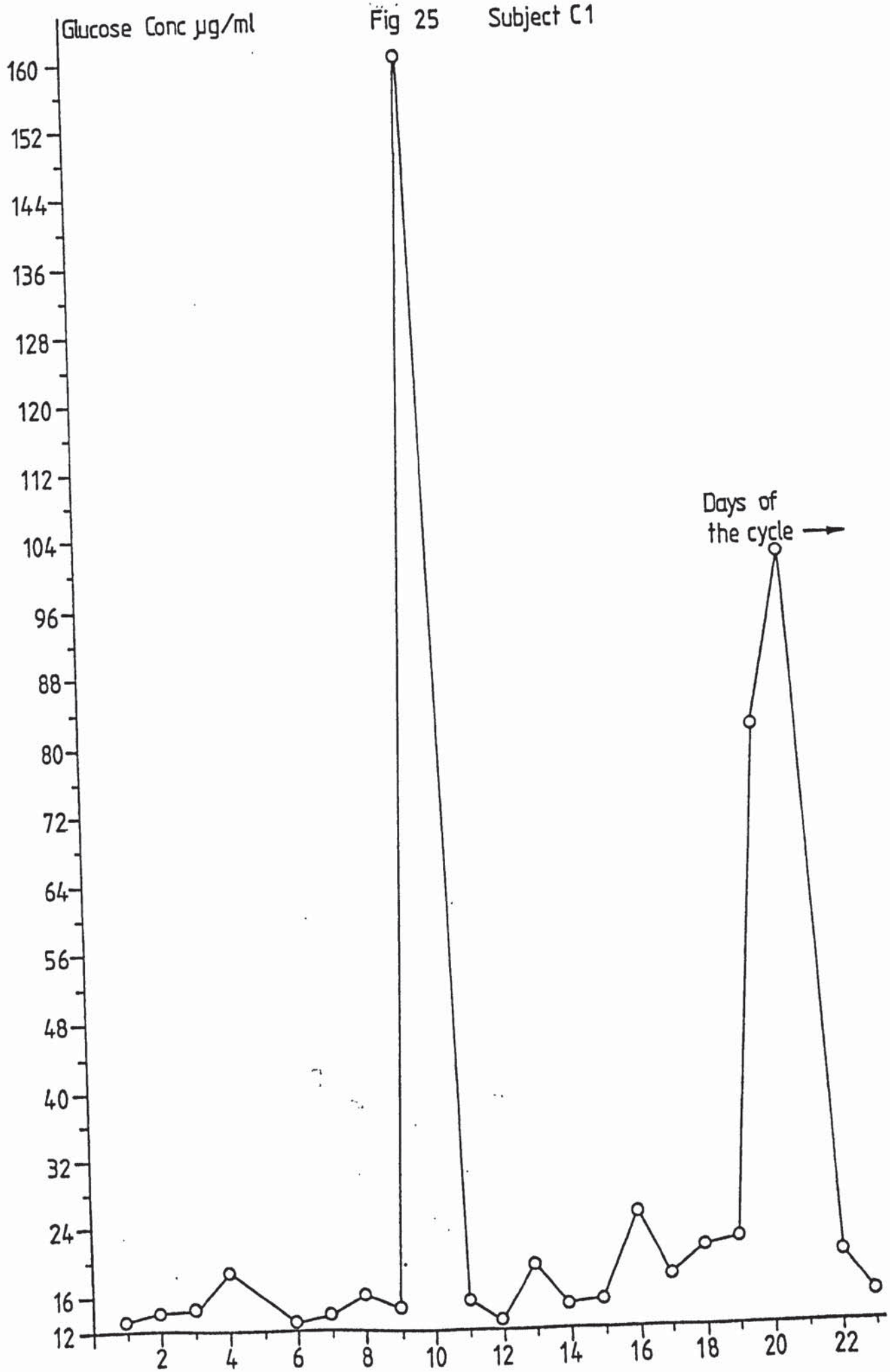
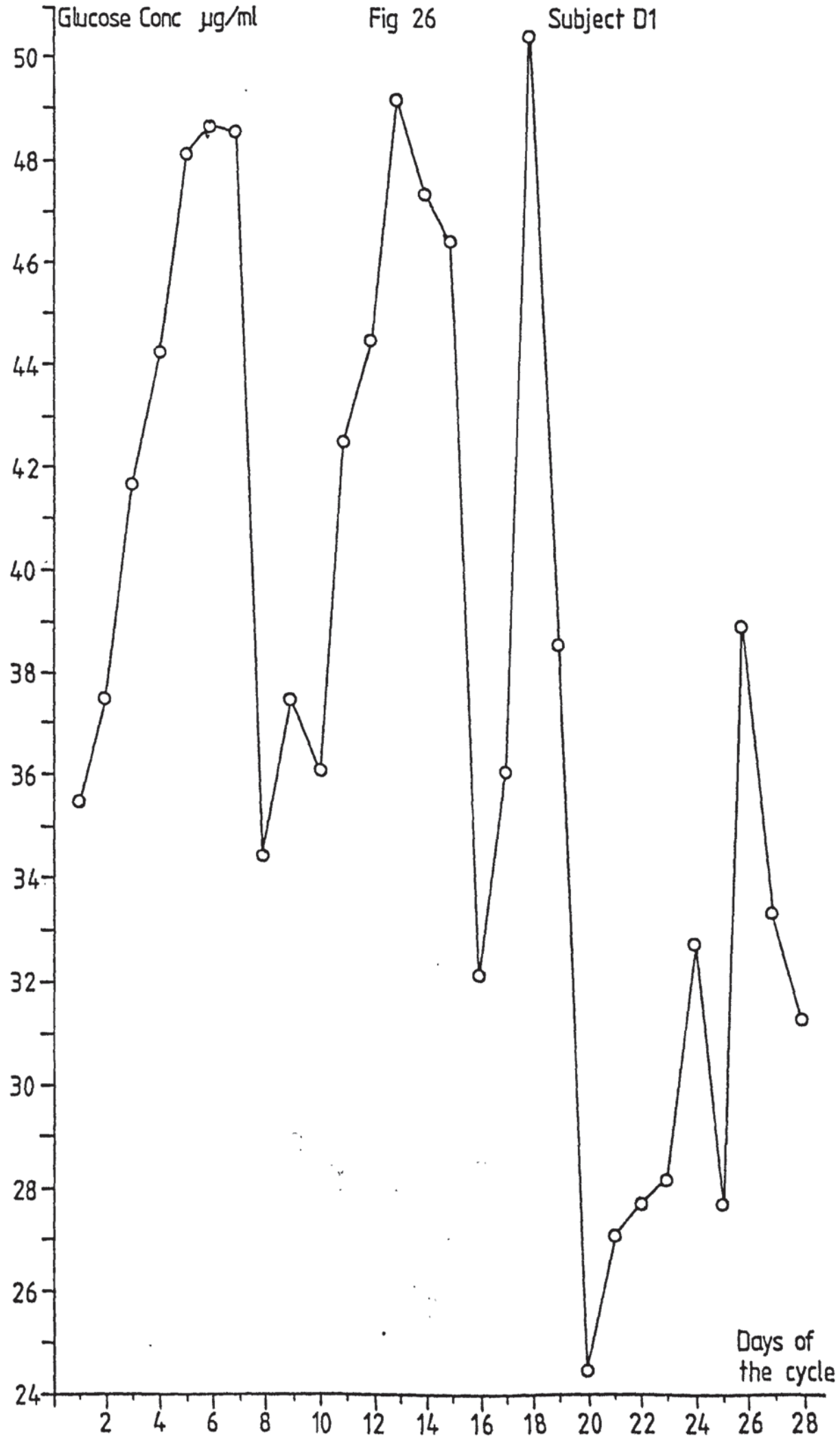
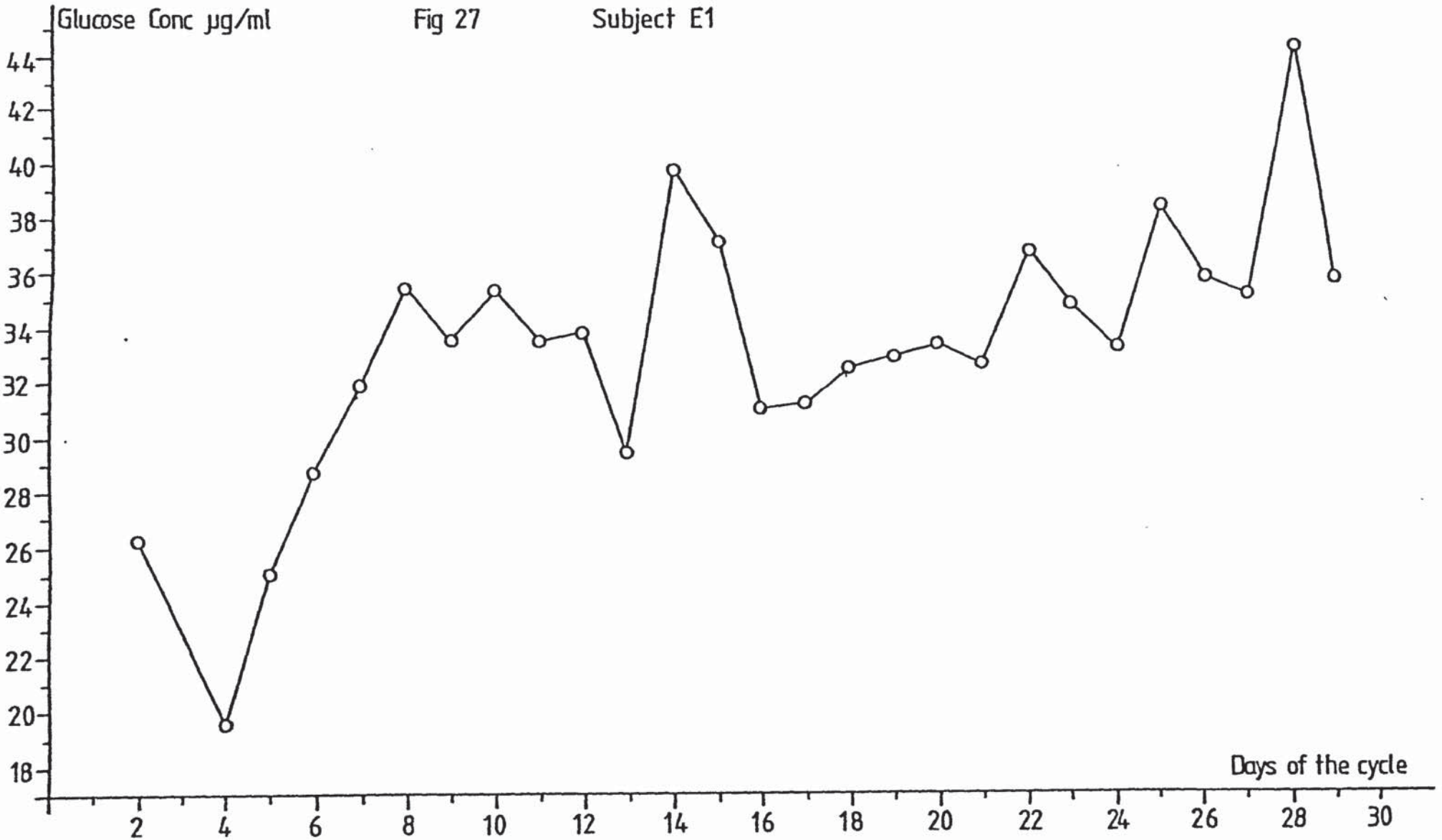
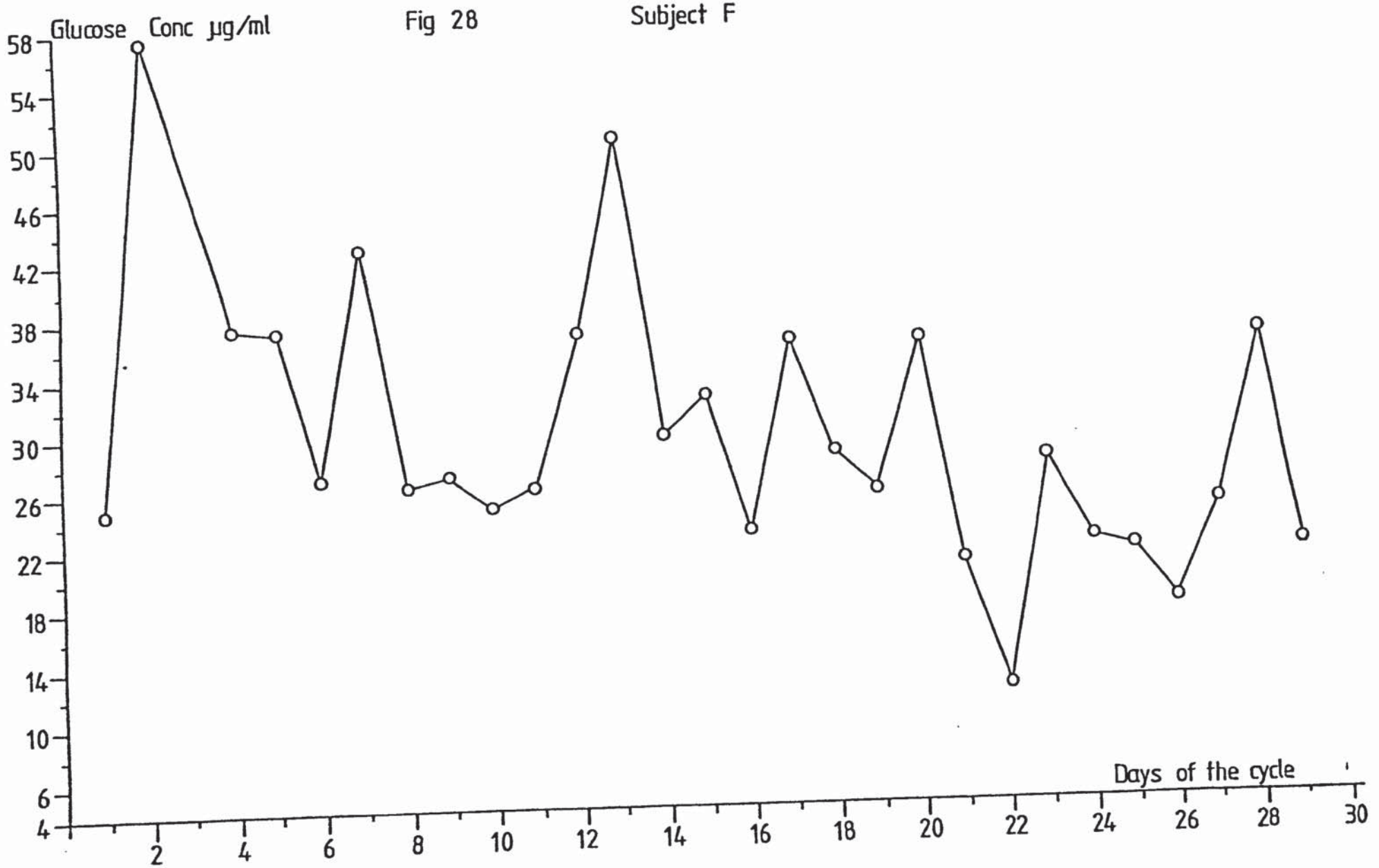

Glucose Conc µg/ml
Fig 24
Subject B1
88
84
80
76
72
68
64
60
56
52
48
44
40
36
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Days of the cycle

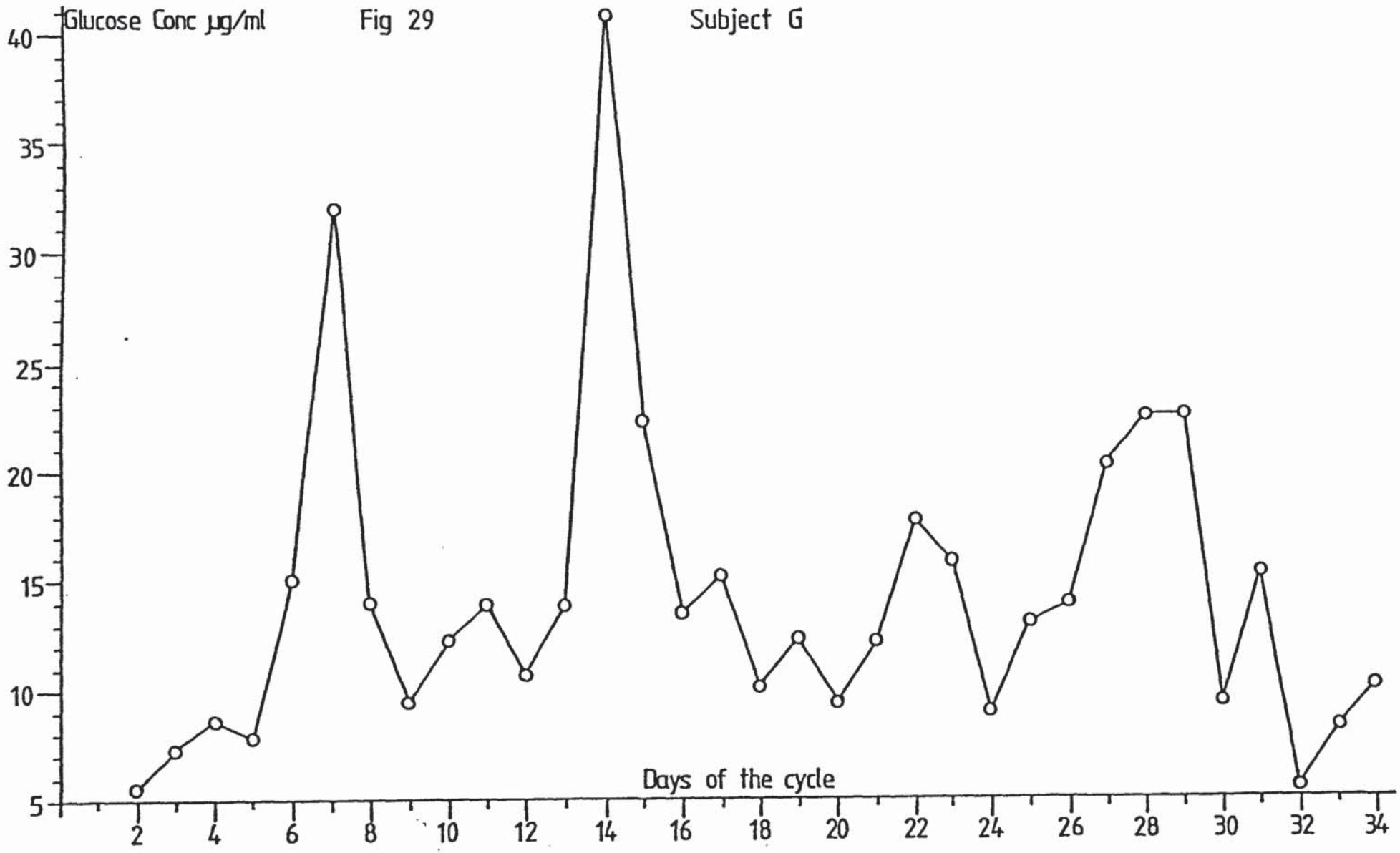
Fig 25 Subject C1




Glucose Conc µg/ml
Fig 26
Subject D1
50
48
46
44
42
40
38
36
34
32
30
28
26
24
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Days of the cycle


Glucose Conc µg/ml
Fig 27
Subject E1
44
42
40
38
36
34
32
30
28
26
24
22
20
18
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days of the cycle


Glucose Conc µg/ml
Fig 28
Subject F
58
54
50
46
42
38
34
30
26
22
18
14
10
6
4
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days of the cycle


Glucose Conc µg/ml
Fig 29
Subject G
40
35
30
25
20
15
10
5
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

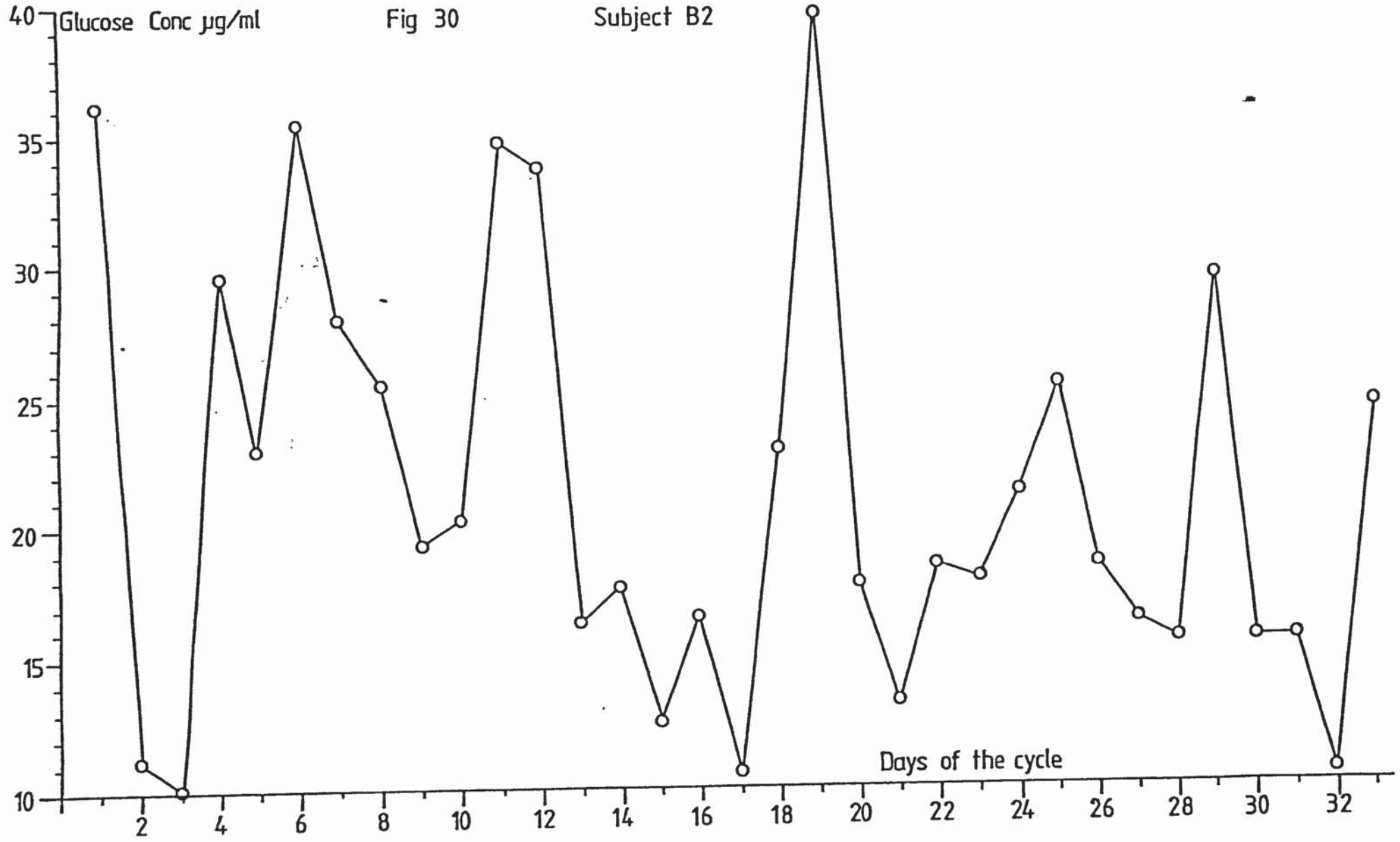
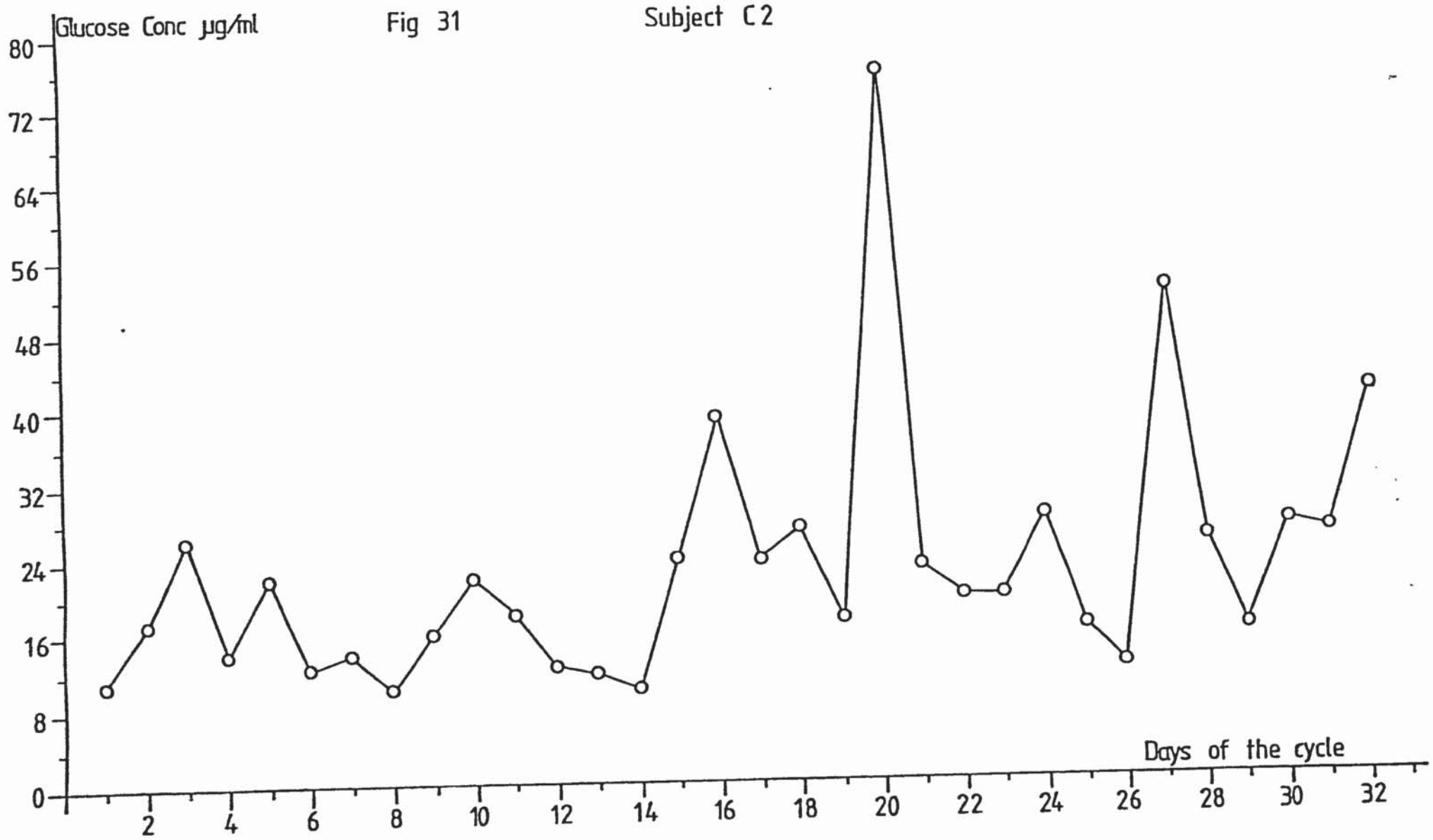
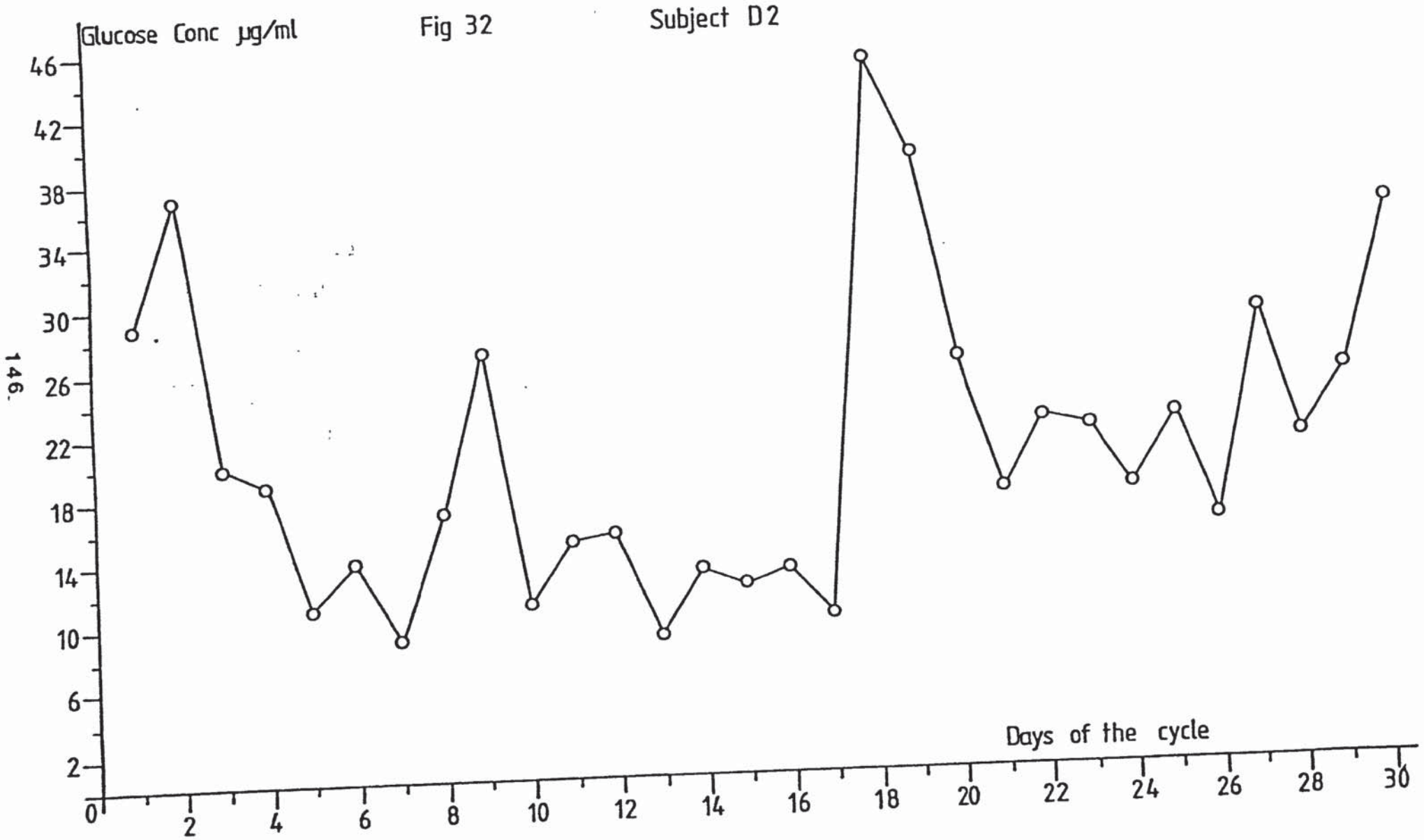

Glucose Conc µg/ml
Fig 30
Subject B2
40
35
30
25
20
15
10
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

Fig 31 Subject C 2




Glucose Conc µg/ml
Fig 32
Subject D2
46
42
38
34
30
26
22
18
14
10
6
2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days of the cycle

2. Results for N-Acetyl-β-D-glucosaminidase Enzyme

The daily changes in the levels of activity of salivary N-acetyl-β-D-glucosaminidase are presented for one (in some instances two) complete menstrual cycle of ten healthy women between the ages of 16 and 38 years. The subjects did not take any medicine, except for subjects B1 and D1 who used cold and headache preparations for 3 days of their cycle. None of the volunteers became pregnant during this time. Because the ovulation time is usually related to the date of the beginning of the next cycle all of the data are presented in relation to that date (27).

The levels of activity of the enzyme are presented in units/ml. The results of duplicate determinations made on two successive days are indicated by the two dashes which represent the upper and lower limits of the enzyme activity. The small difference between these two determinations indicate the precision of the fluorimetric method, and the handling of the saliva samples used for the analysis.

The daily changes in the levels of activity of salivary enzyme in the fifteen menstrual cycles are shown in Figs. 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, and 47. The mean value for each was used.

In nine cycles out of the fifteen cycles shown, a significant increase in the level of enzyme activity was found on days 12, 13, 14 and 15 before the beginning of the next menstrual cycle, close to the expected time of ovulation.

Subjects D1, E1, G, C2, D2, H and J2 (Figs. 36, 37, 39, 41, 42, 43 and 47, respectively) show a marked increase in the enzyme activity at the beginning and also at the end of the menstrual cycle two to four days before the next menstruation. The range of values of the enzyme activity varies markedly between individual subjects. The

highest enzyme activity of 0.375 units/ml was found in the saliva of subject B2, Fig. 40; while the lowest enzyme activity of 0.075 units/ml was found in the saliva of the subject D1, Fig. 36. In order to clarify presentation mean values of all determinations for each day of each cycle have been shown.

Figures 46 and 47 show the daily changes in the levels of the enzyme activity in two successive cycles of the same subject. In those two cycles there was no obvious peak of enzyme activity. This may have been because she had been taking contraceptive pills for some time before she had started supplying the saliva samples.

The high level of enzyme activity was found to persist for one day except for subjects A, D1, E1, C2 and D2. For example, subject A, Fig. 33, shows a peak 16 and 17 days before the onset of the next cycle. While subject D1, Fig. 36, shows a peak at 10, 9 and 8 days before the beginning of the next cycle. Subject E1, Fig. 37, shows a peak at 19, 18, 17, 16 and 15 days before the beginning of the next cycle.

Subjects C2, Fig. 41, had the highest enzyme activity at 15 and 13 days before the next menstruation. Table No.36 presents the results obtained in this trial.

Table (36). N-Acetyl-β-D-glucosaminidase Activity in the Saliva of 10 Females during one Menstrual Cycle.

S	A	D.C.D.	L.L. units/ ml	D.L.L. B.N.M.C.	H.L. units/ ml	D.H.L. B.N.M.C.	E.A. E.D.O.	E.O.D. B.N.M.C.
A	20	22	0.06	6	0.24	16	0.24	16,17
B1	26	31	0.065	27	0.185	16	0.185	16
B2	26	33	0.05	19,33	0.375	14	0.375	14
C1	23	23	0.065	23	0.135	17	0.12	13
C2	23	32	0.05	8	0.28	27	0.18	13,15
D1	38	28	0.035	15	0.075	8	0.06	8,9,10
D2	38	30	0.03	9	0.10	27	0.06 0.064	12
E1	17	29	0.026	7	0.095	22,23	0.088- 0.085	15,16,17, 18,19
E2	17	28	0.07	6	0.35	26	0.12	17
F	23	29	0.05	2,24	0.18	5,25	0.115	11
G	15½	40	0.06	30	0.20	20	0.20	20
H	23	26	0.01	15,16	0.10	1	0.022	8
I	30	34	0.02	20	0.12	16	0.12	16
J1	26	30	0.02	17	0.18	29	0.09	20
J2	26	33	0.052	30	0.10	5	0.082	17,18

S = Subject

A = Age

D.C.D. = Duration of the Cycle in Days

L.L. = Lowest Level

D.L.L. = Days of Lowest Level

H.L. = Highest Level

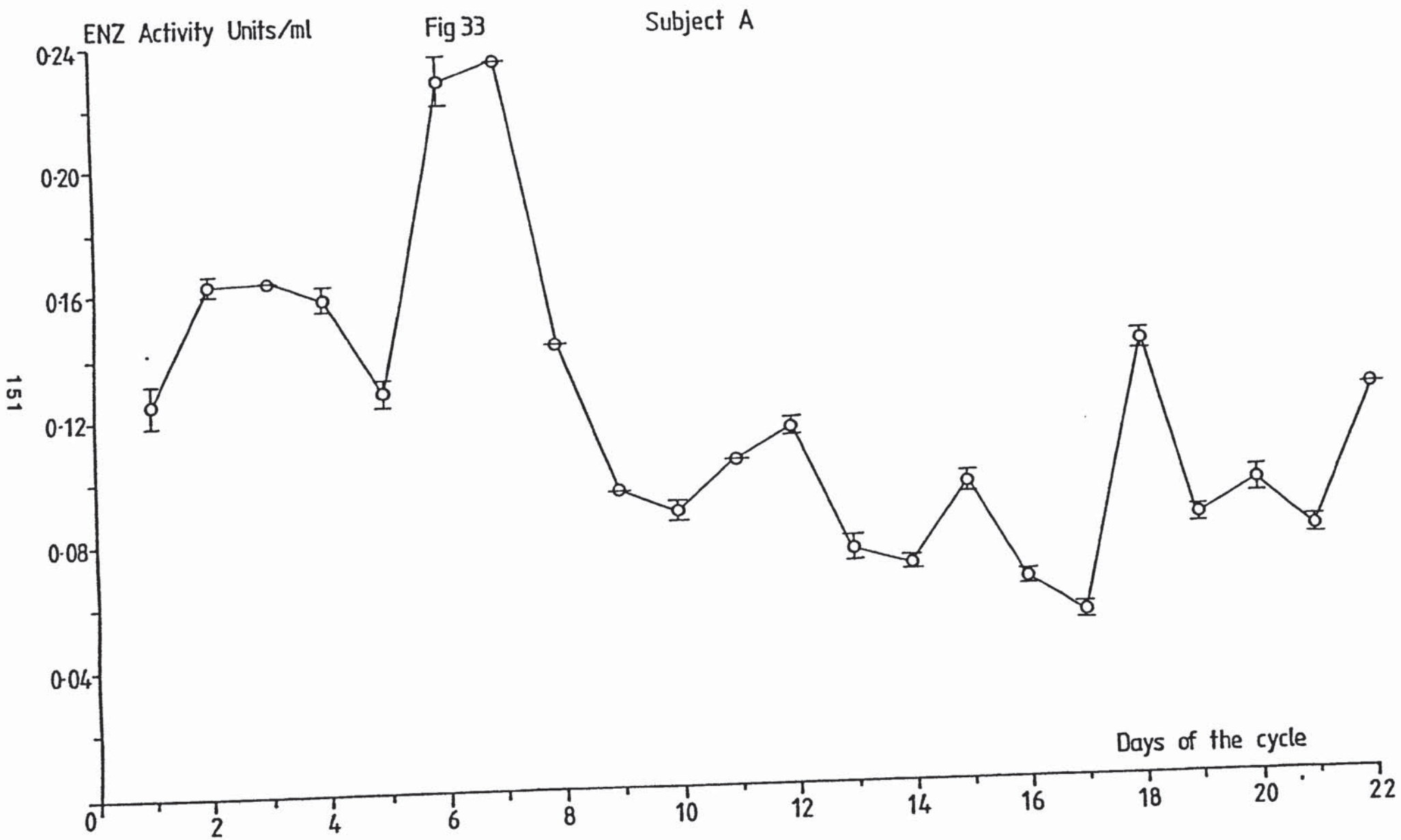
D.H.L. = Days of Highest Level

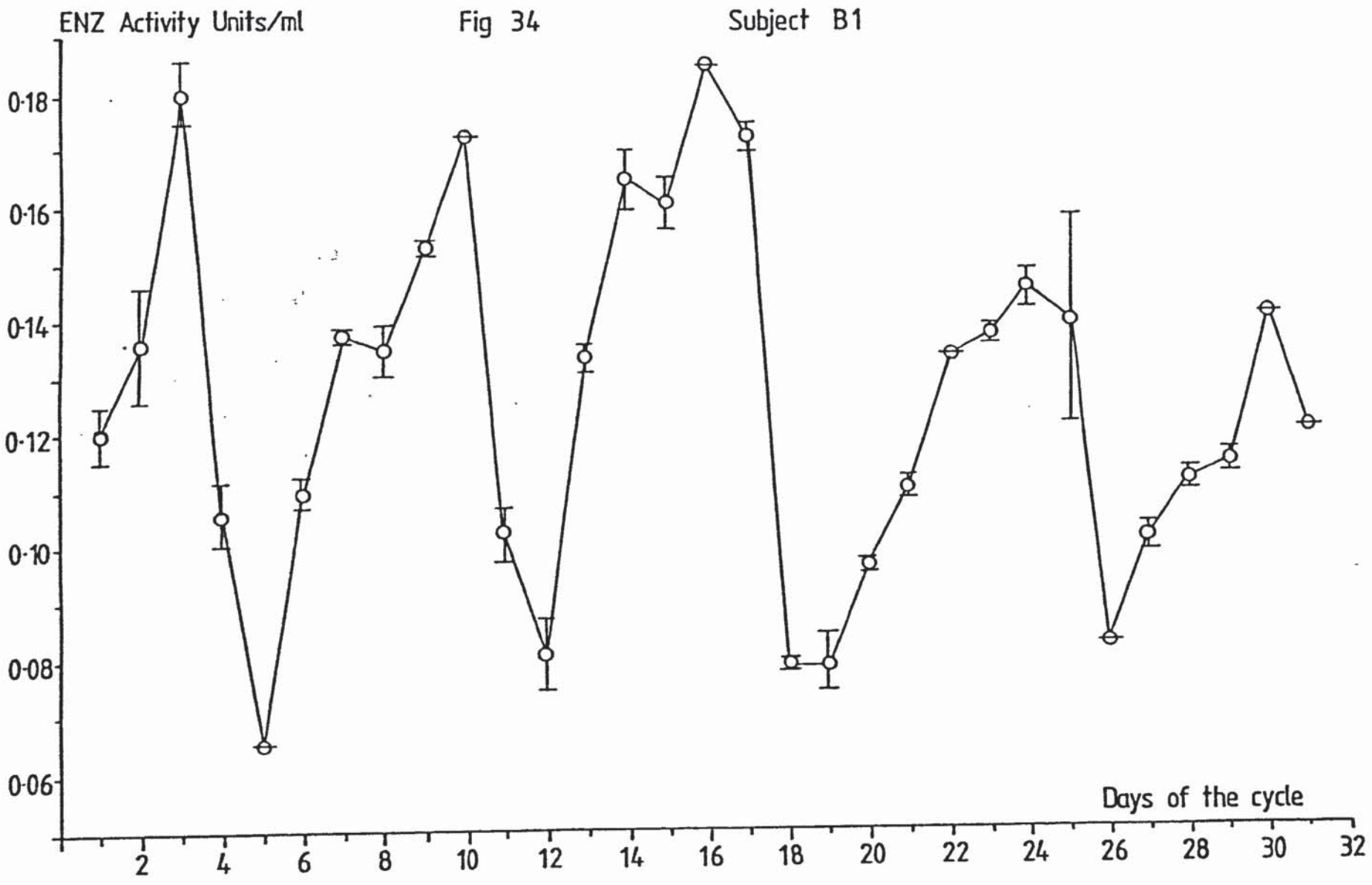
E.A.E.D.O. = Enzyme Activity on Expected Day of Ovulation

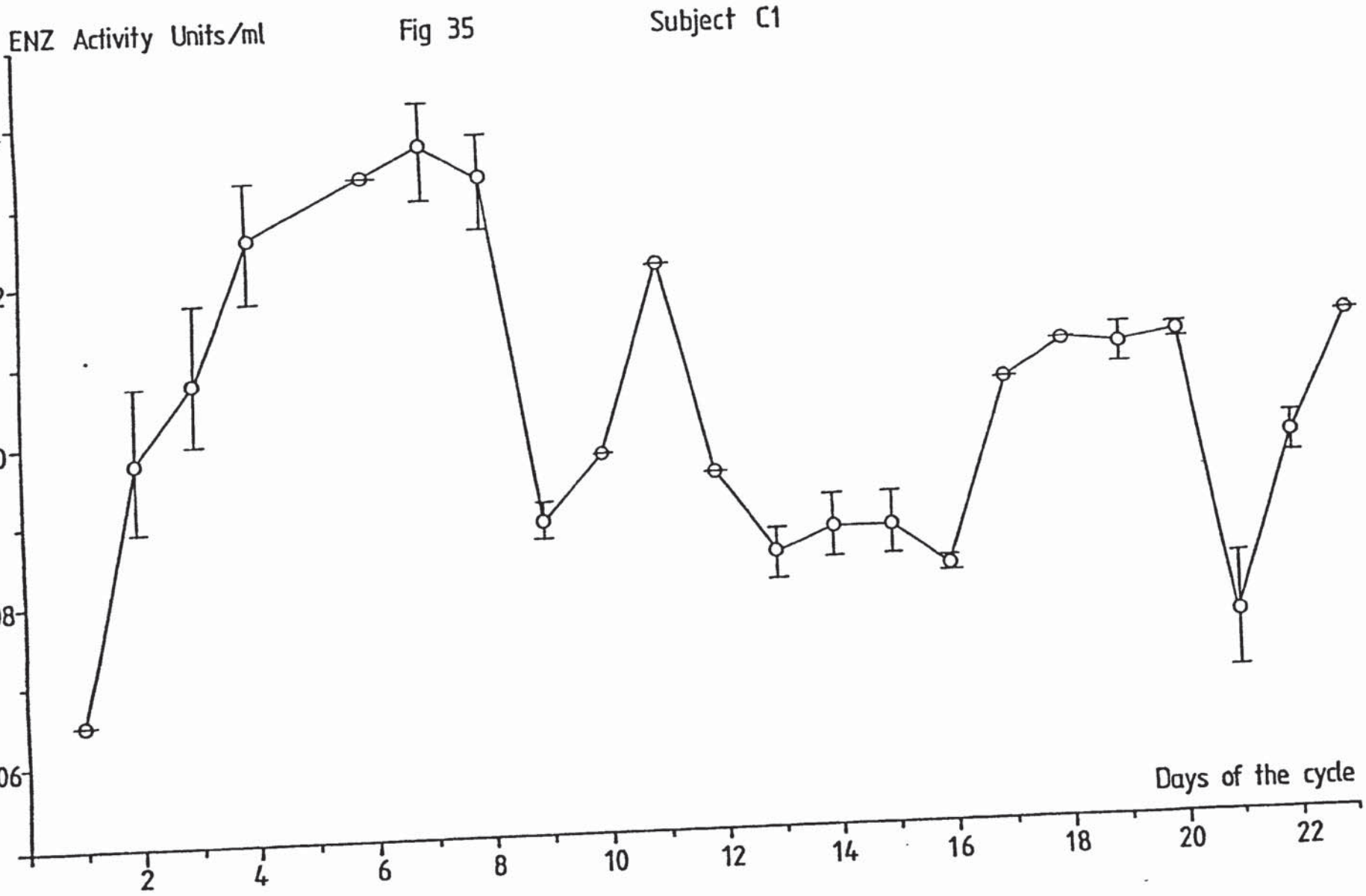
E.O.D. = Expected Ovulation Day

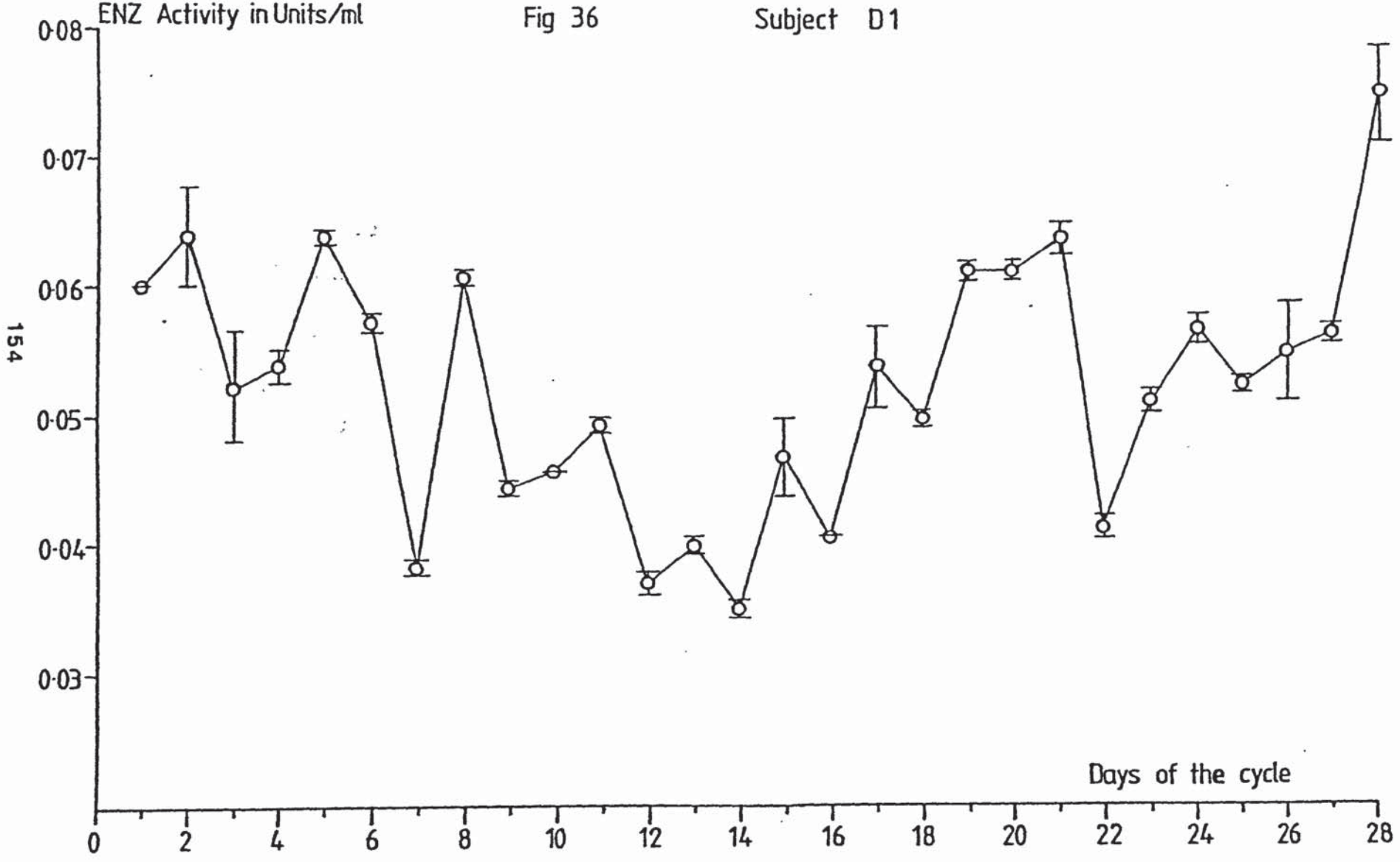
B.N.M.C. = Before Next Menstrual Cycle

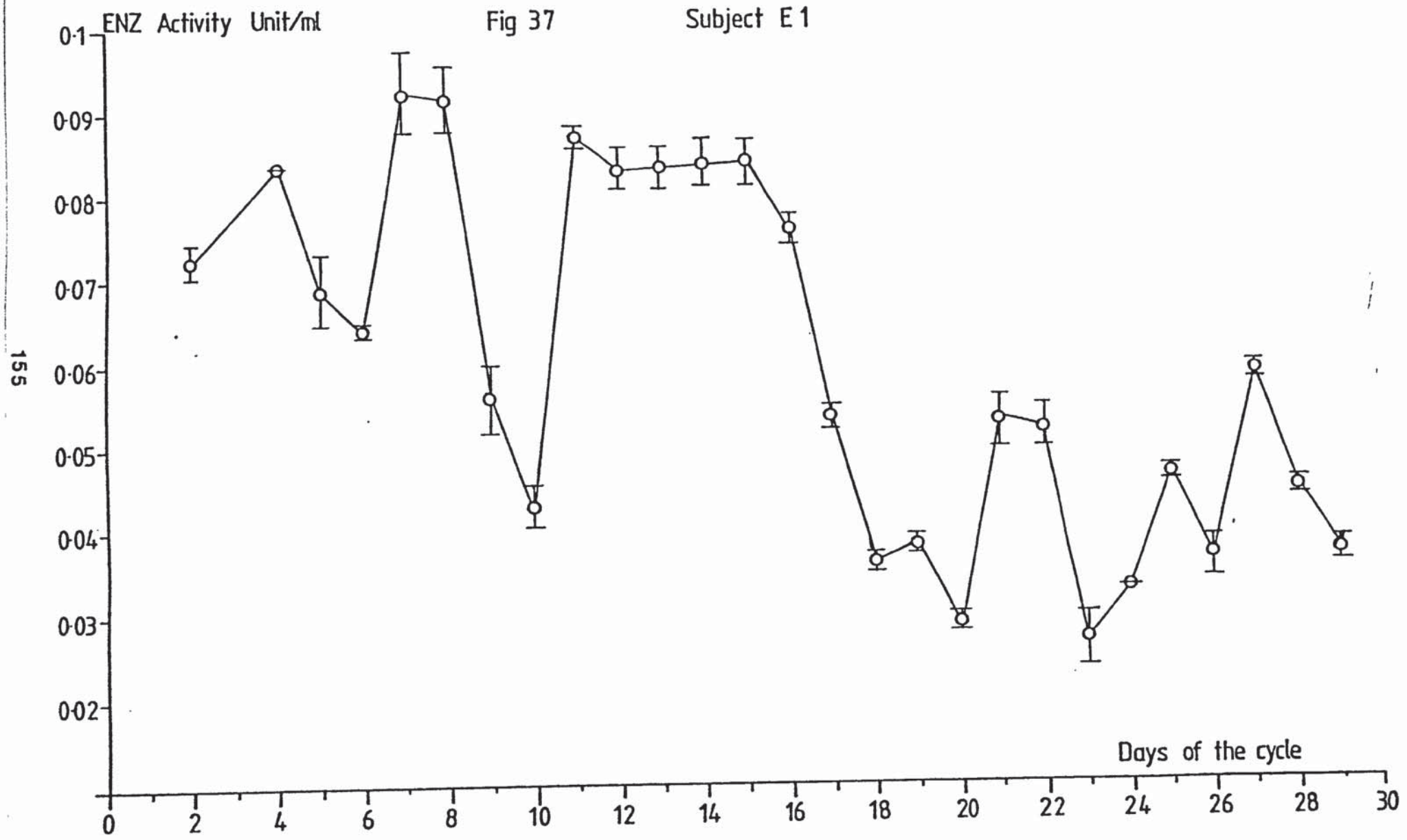
Subjects 1,2 refer to a 1st and 2nd cycle of the same subject

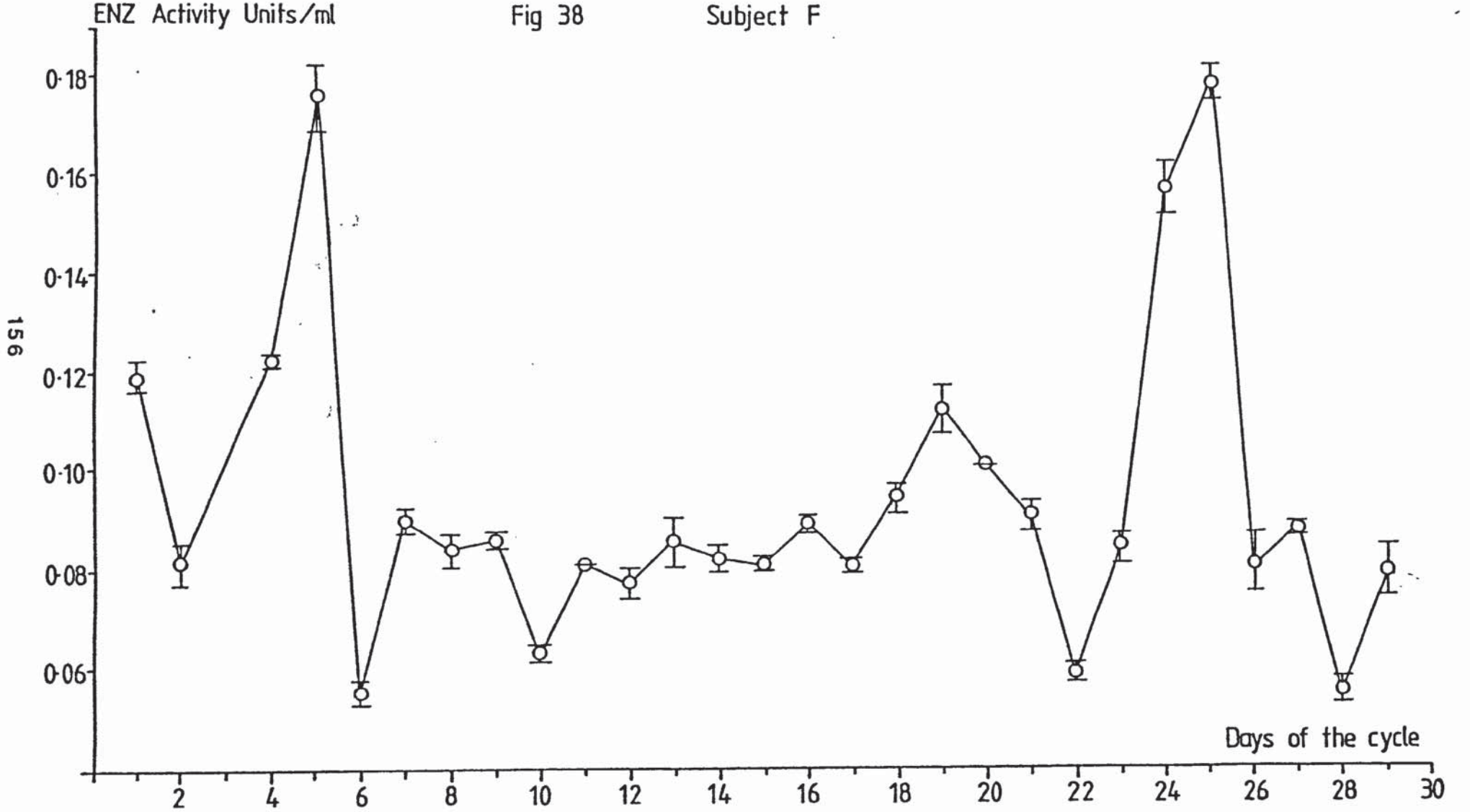

ENZ Activity Units/ml
Fig 33
Subject A
0·24
0·20
0·16
0·12
0·08
0·04
0
2
4
6
8
10
12
14
16
18
20
22
Days of the cycle

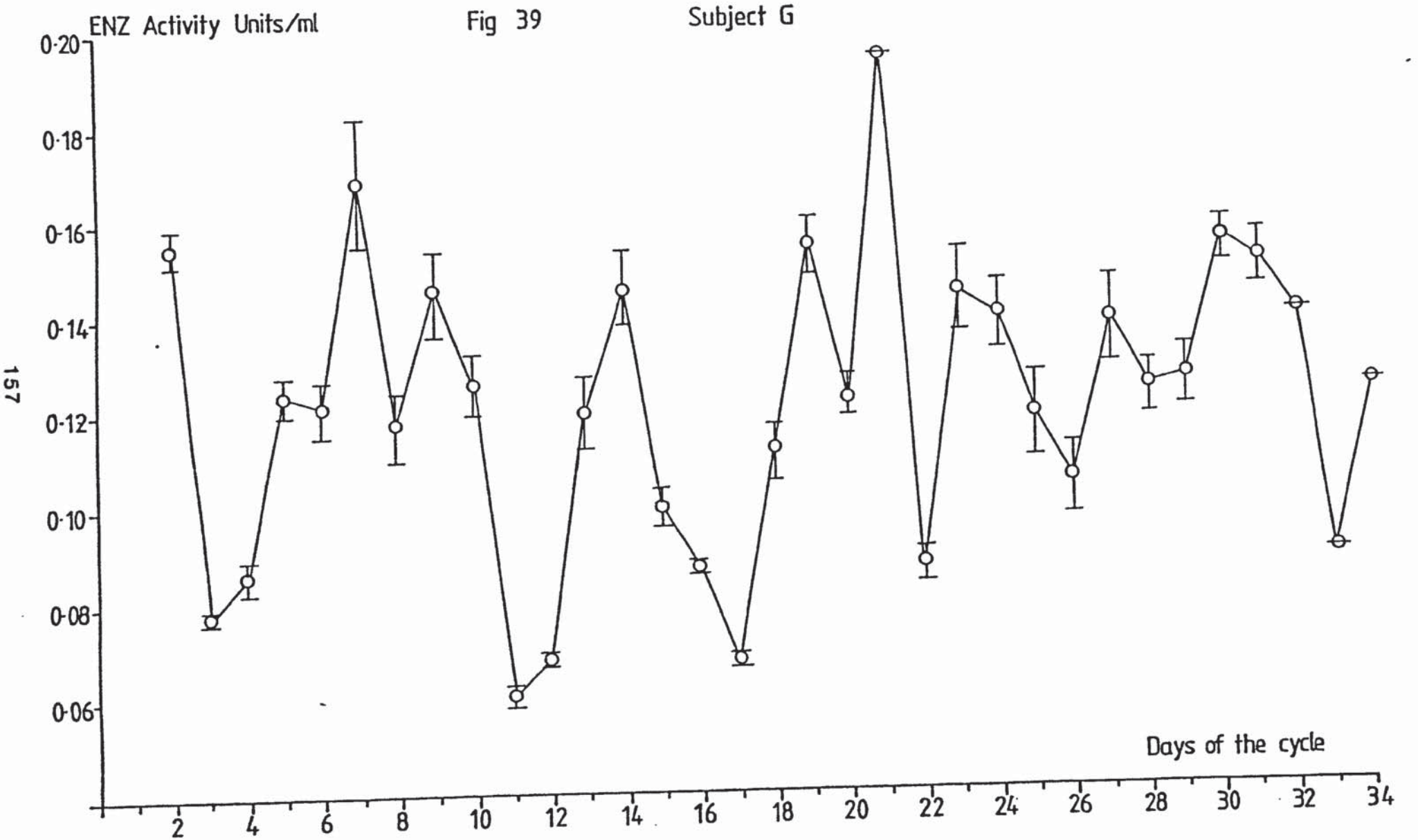

ENZ Activity Units/ml
Fig 34
Subject B1
0·18
0·16
0·14
0·12
0·10
0·08
0·06
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

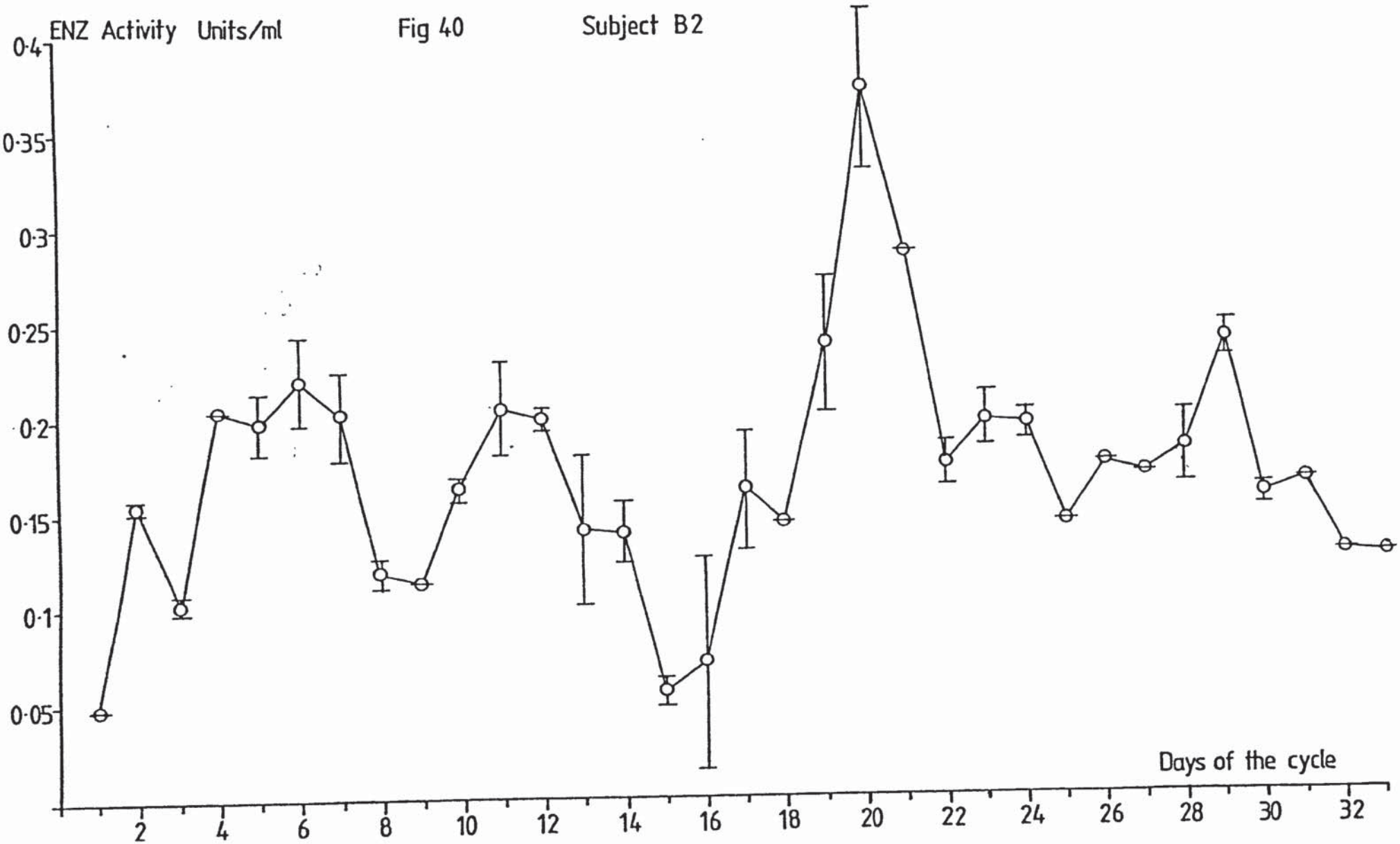

ENZ Activity Units/ml
Fig 35
Subject C1
0·14
0·12
0·10
0·08
0·06
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22

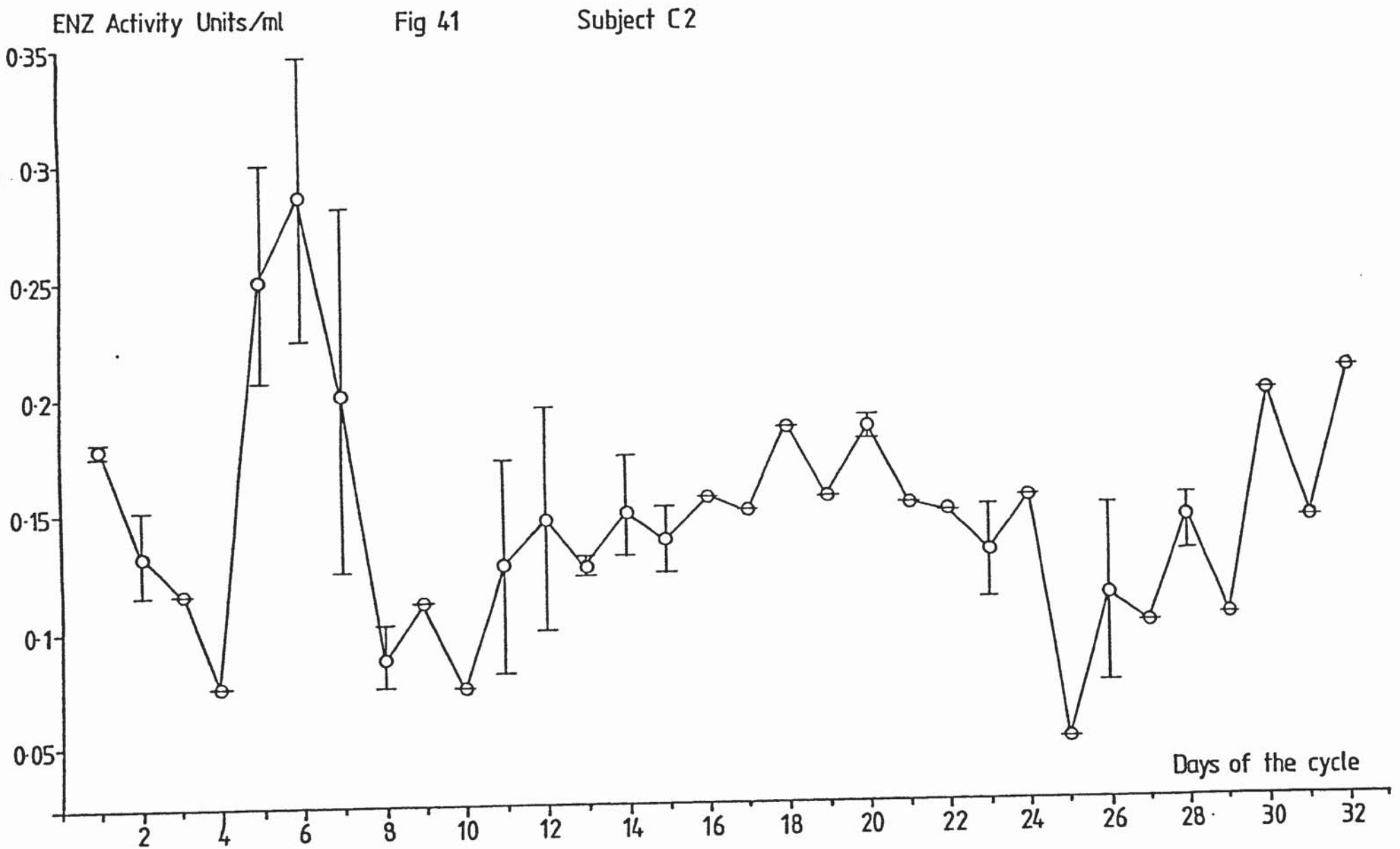

ENZ Activity in Units/ml
Fig 36
Subject D1
0·08
0·07
0·06
0·05
0·04
0·03
Days of the cycle
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28

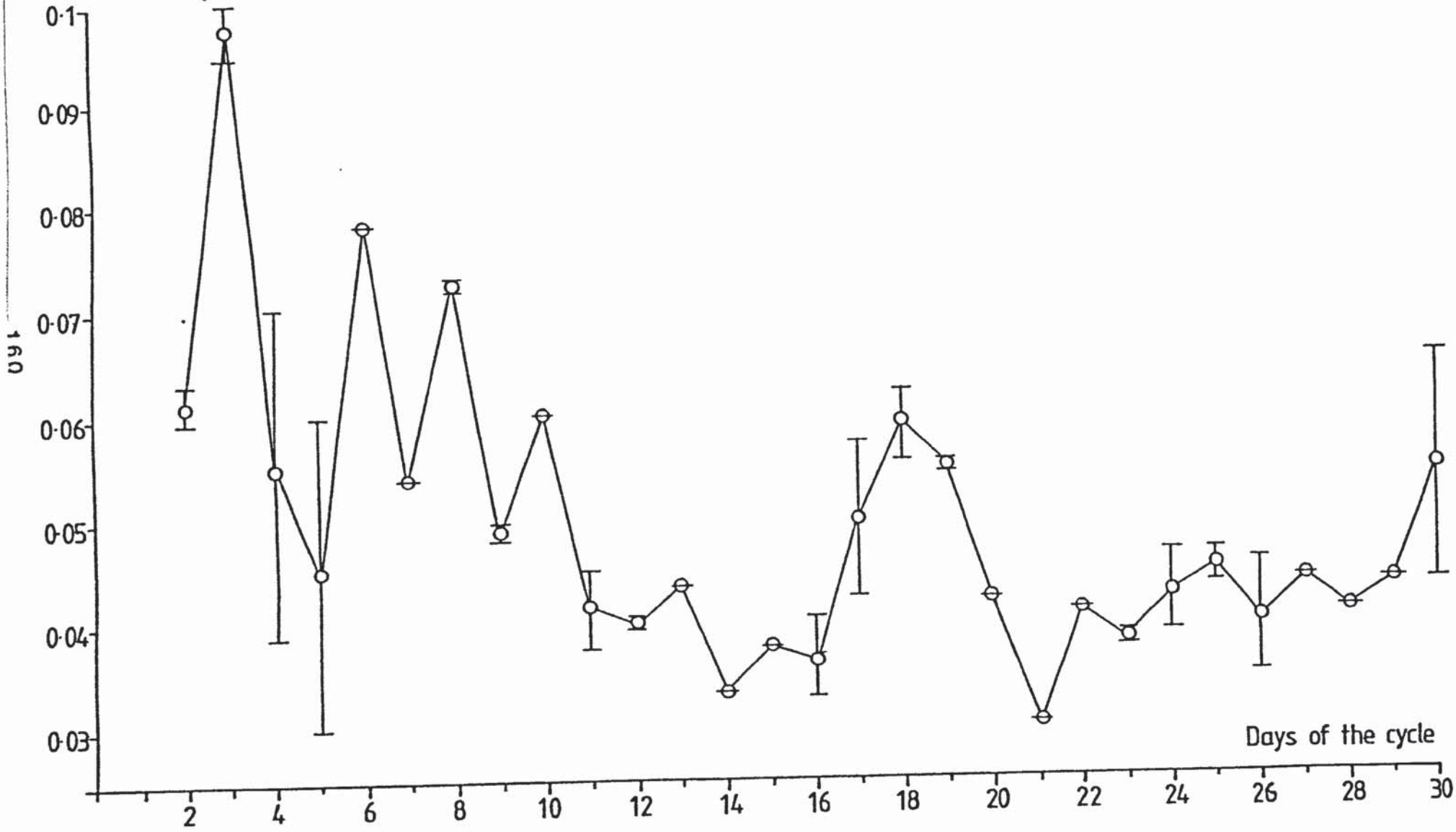

ENZ Activity Unit/ml
Fig 37
Subject E 1
0·1
0·09
0·08
0·07
0·06
0·05
0·04
0·03
0·02
Days of the cycle
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

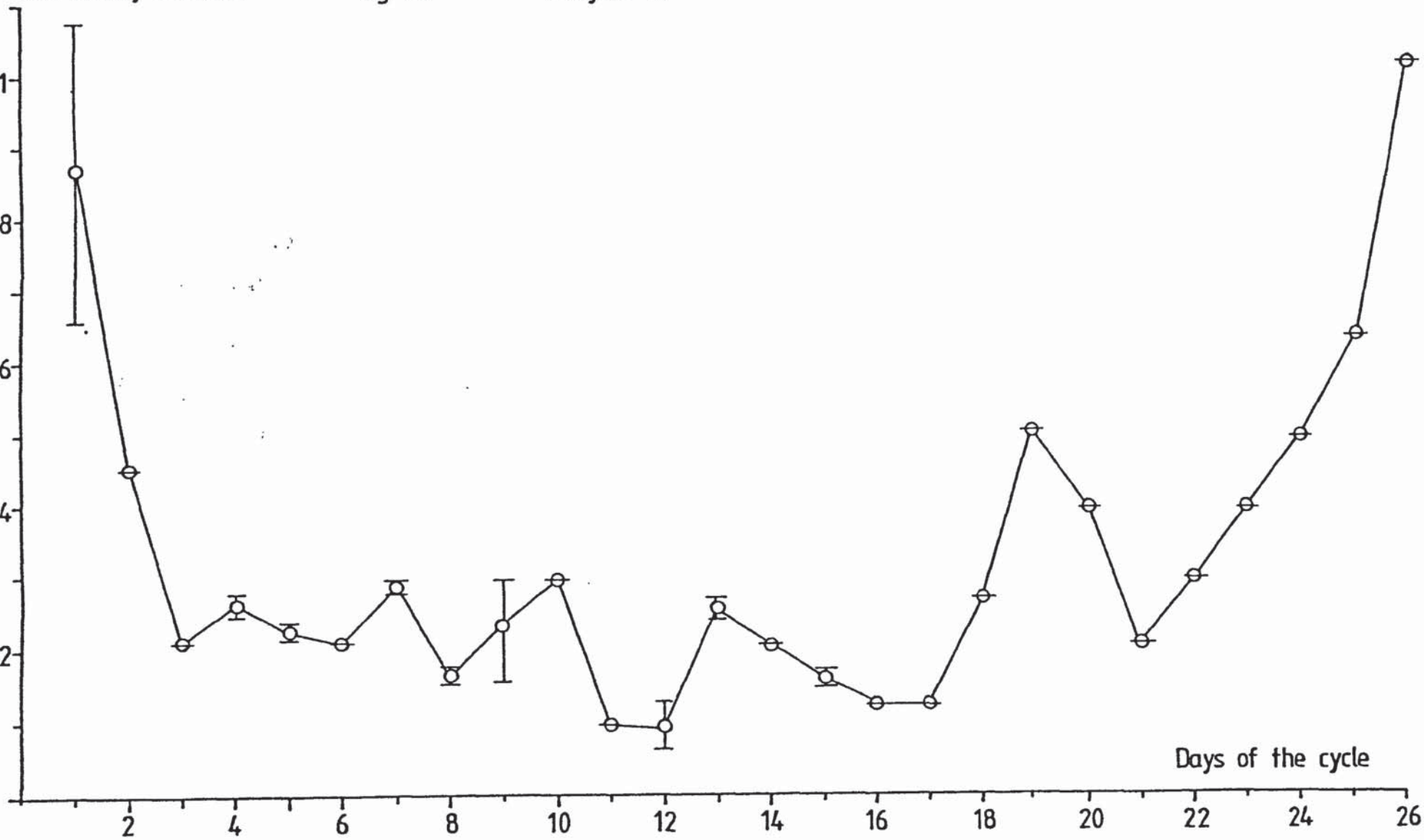

ENZ Activity Units/ml
Fig 38
Subject F
0·18
0·16
0·14
0·12
0·10
0·08
0·06
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30


ENZ Activity Units/ml
Fig 39
Subject G
0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34


ENZ Activity Units/ml
Fig 40
Subject B2
0·4
0·35
0·3
0·25
0·2
0·15
0·1
0·05
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32


ENZ Activity Units/ml
Fig 41
Subject C2
0·35
0·3
0·25
0·2
0·15
0·1
0·05
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32


ENZ Activity Units/ml
Fig 42
Subject D2
0·1
0·09
0·08
0·07
0·06
0·05
0·04
0·03
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30


ENZ Activity Units/ml
Fig 43
Subject H
0·1
0·08
0·06
0·04
0·02
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26

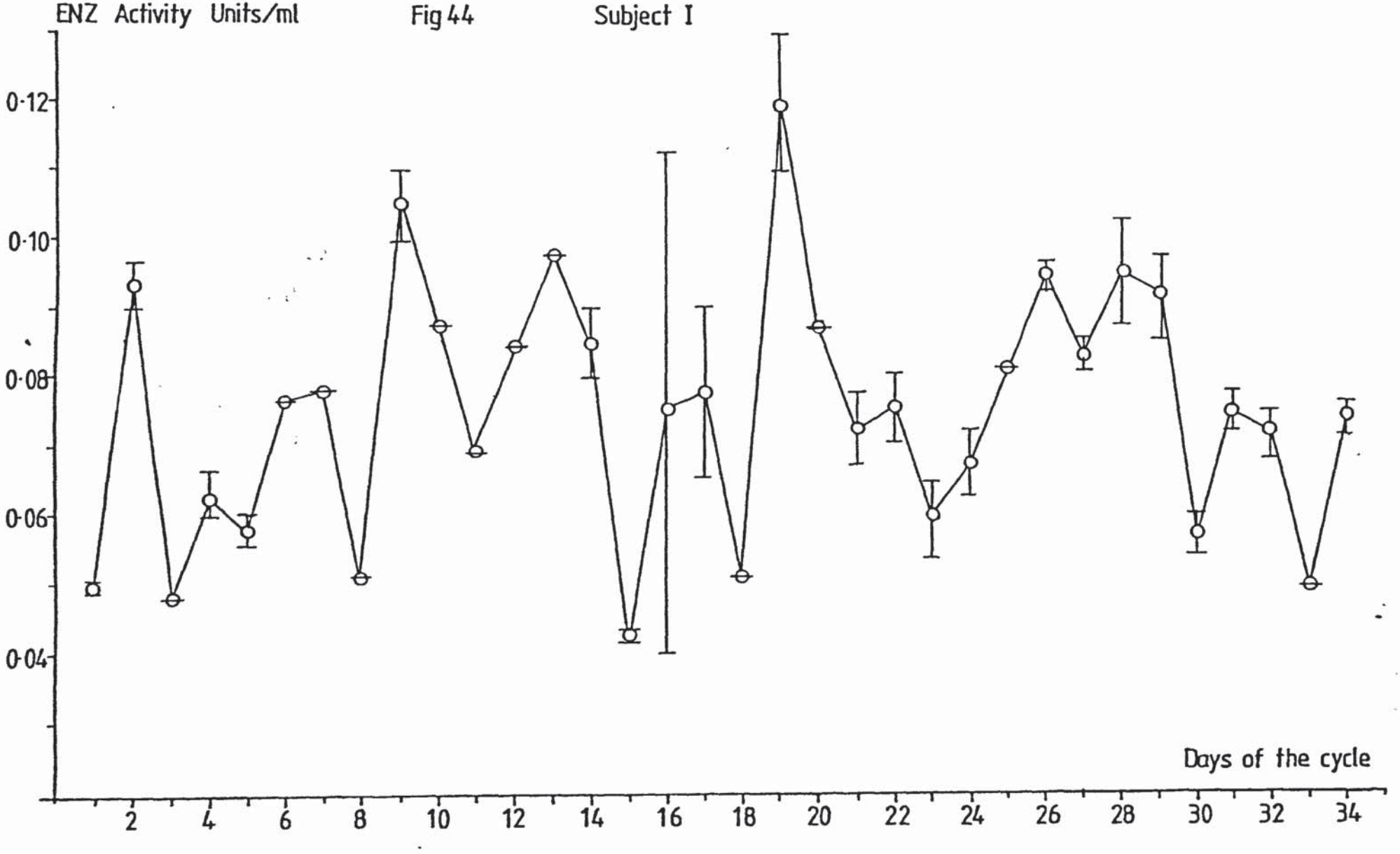

ENZ Activity Units/ml
Fig 44
Subject I
0·12
0·10
0·08
0·06
0·04
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

Fig 45 Subject E2

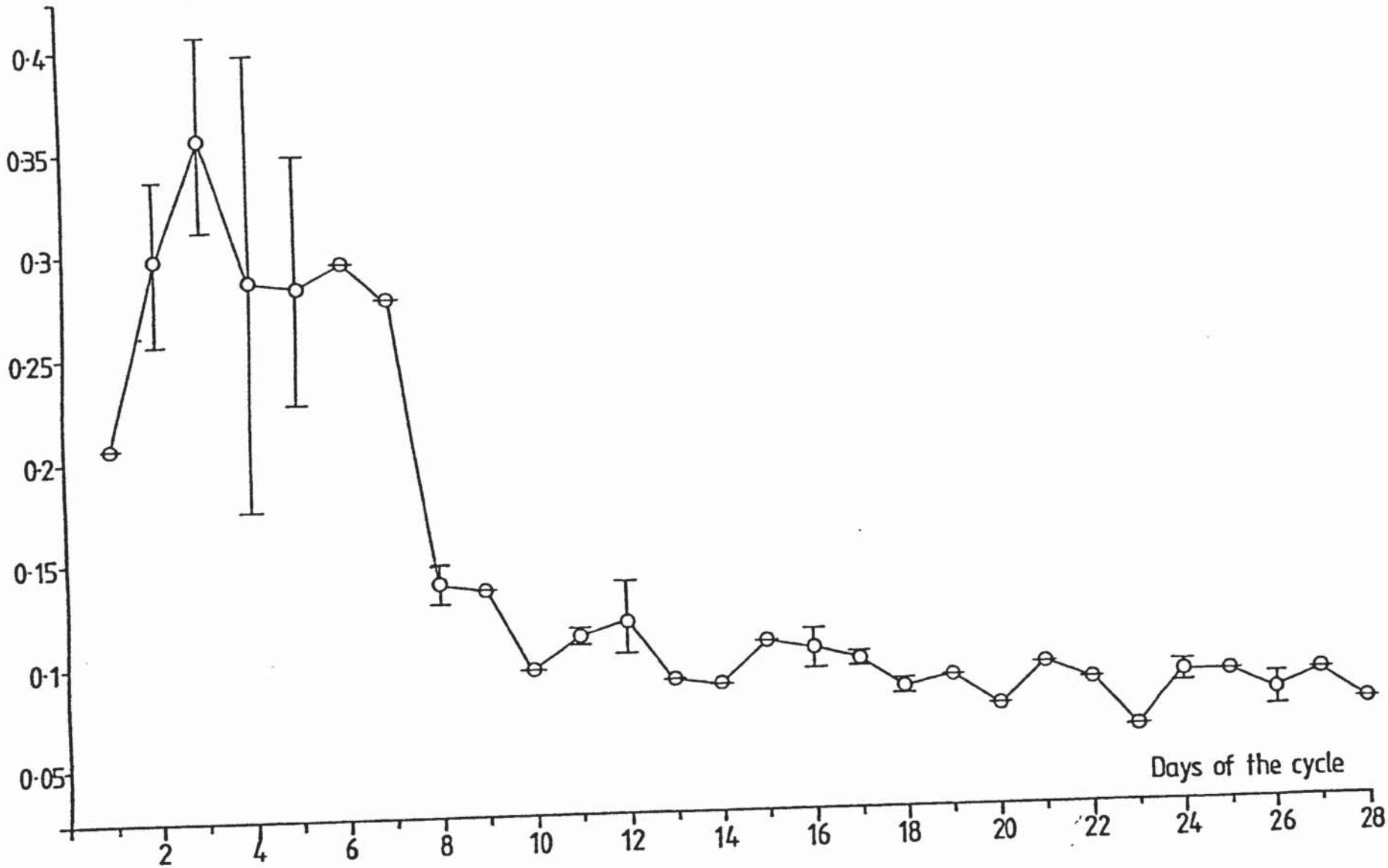
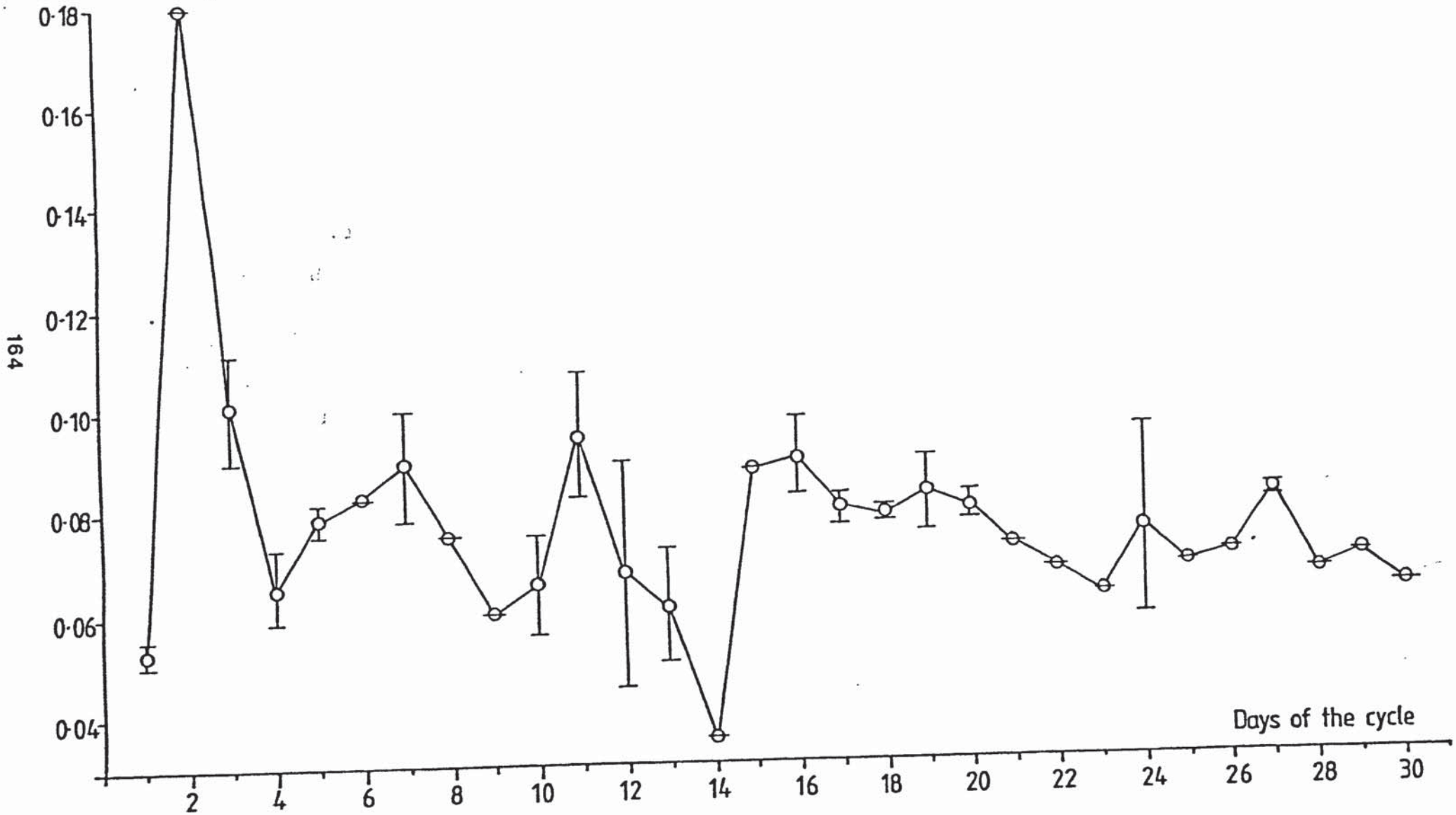
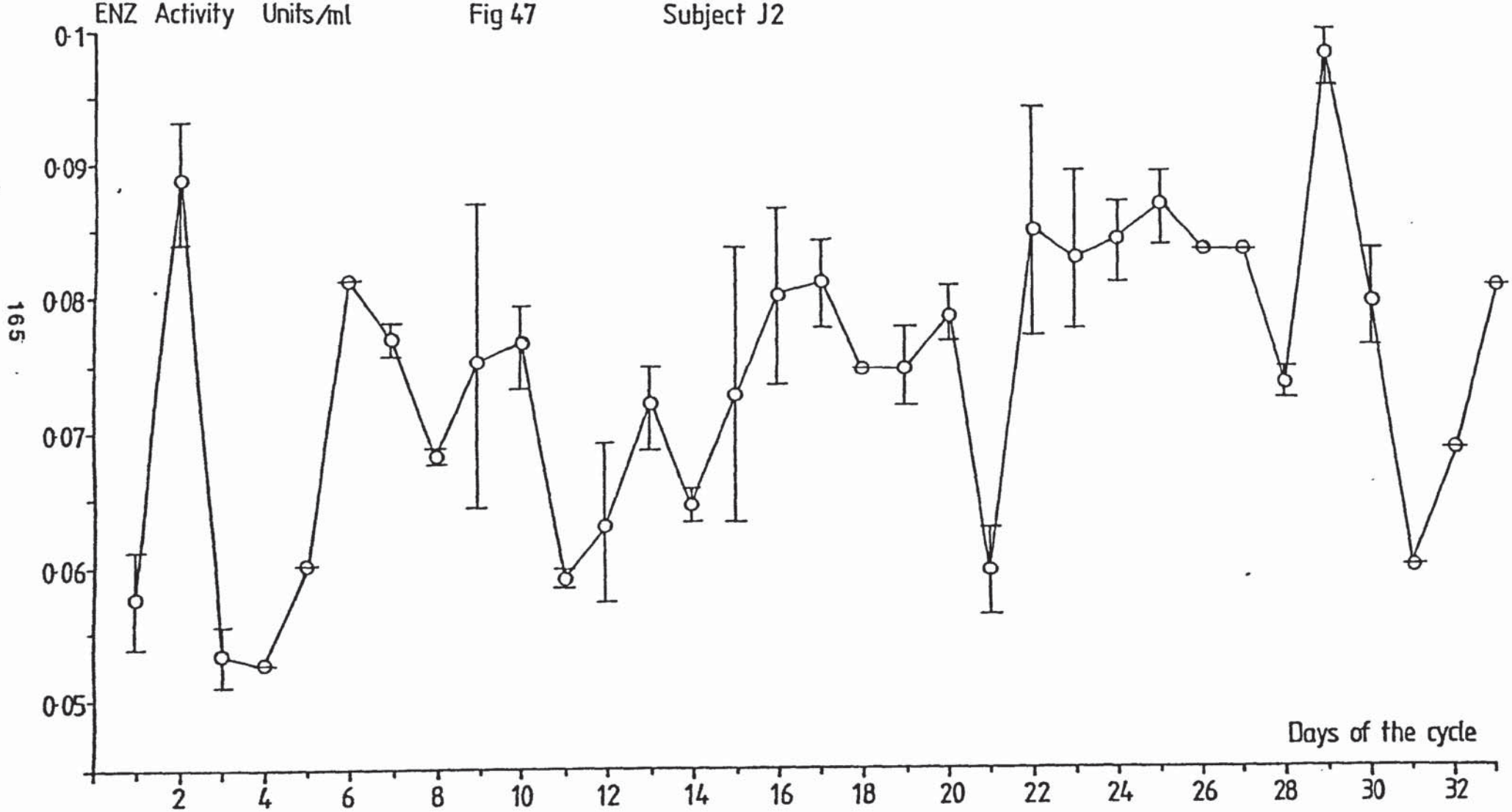


Fig 46 Subject J1




ENZ Activity Units/ml
Fig 47
Subject J2
0·1
0·09
0·08
0·07
0·06
0·05
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

3. Calcium

Calcium levels were determined in the same saliva samples as used for the estimation of the enzyme activity of N-acetyl-β-D-glucosaminidase and the glucose concentration.

All the data available for the calcium levels, in mcg/ml, during the normal menstrual cycles of 10 healthy women are presented in Figs. 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, and 62.

The results of the determinations of salivary calcium on two successive days for each sample in every day of the cycle are represented by two dashes, and the circles in between those two dashes represent the mean of these two successive determinations. As can be seen no regular significant changes, either an increase or a decrease can be detected during any particular time of the menstrual cycles. The daily changes in the calcium levels are irregular and their range varies between the individual subjects, and also in the same subject from one cycle to another. For example, in subject C1, calcium concentrations during one cycle ranged between 77 mcg/ml to 260 mcg/ml (Fig. 50), while in another cycle, C2, Fig. 56, the level ranged between 41.5 mcg/ml to 83 mcg/ml. The calcium level was between 26 mcg/ml and 60 mcg/ml for subject H, Fig. 58, and between 77 mcg/ml and 260 mcg/ml in Subject C1, Fig. 50. Table No. 37 presents the results of all determinations in fifteen normal cycles.

Table (37). Calcium Levels in the Saliva of 10 Females during one Menstrual Cycle

S	A	D.C.D.	L.L. mcg/ml	D.L.L. B.N.M.C.	H.L. mcg/ml	D.H.L. B.N.M.C.
A	20	22	56.5	6	102.5	18
B1	26	31	68	10	108	15
B2	26	33	50	32	96	18
C1	23	23	77	15,16	260	11
C2	23	32	41	31	90	1
D1	38	28	57	17	105	1
D2	38	30	46	22	70	1
E1	17	29	42	7	130	22
E2	17	28	62	22	108	18
F	23	29	50	7	105	5
G	15½	40	50	33,25	117	20
H	23	26	26	22	60	26
I	30	34	21	2	94	12
J1	26	30	22	23	69	18
J2	26	33	29	33	65	15

S = Subject

A = Age

D.C.D. = Duration of the Cycle

L.L. = Lowest Level

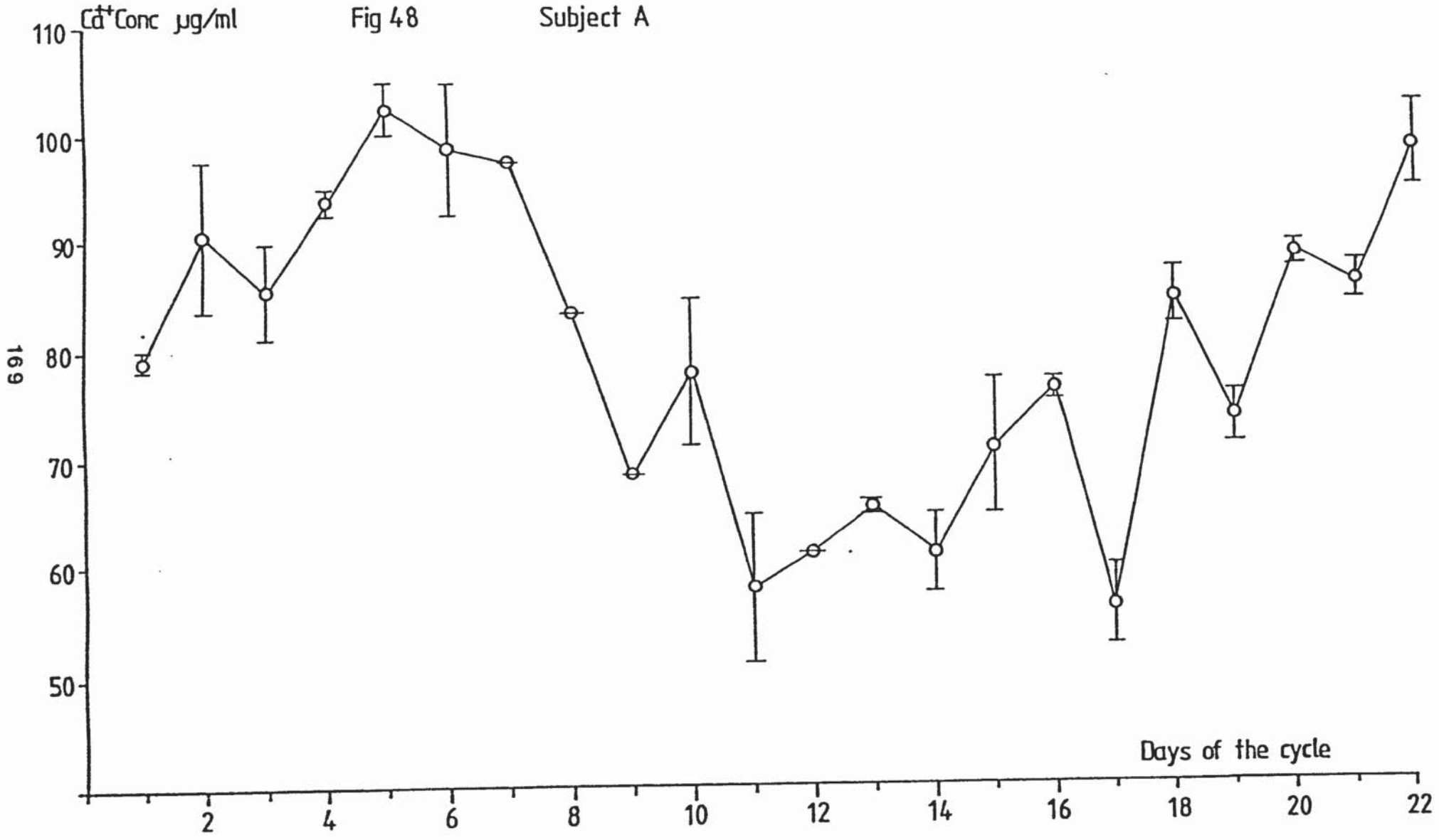
D.L.L. = Days of Lowest Level

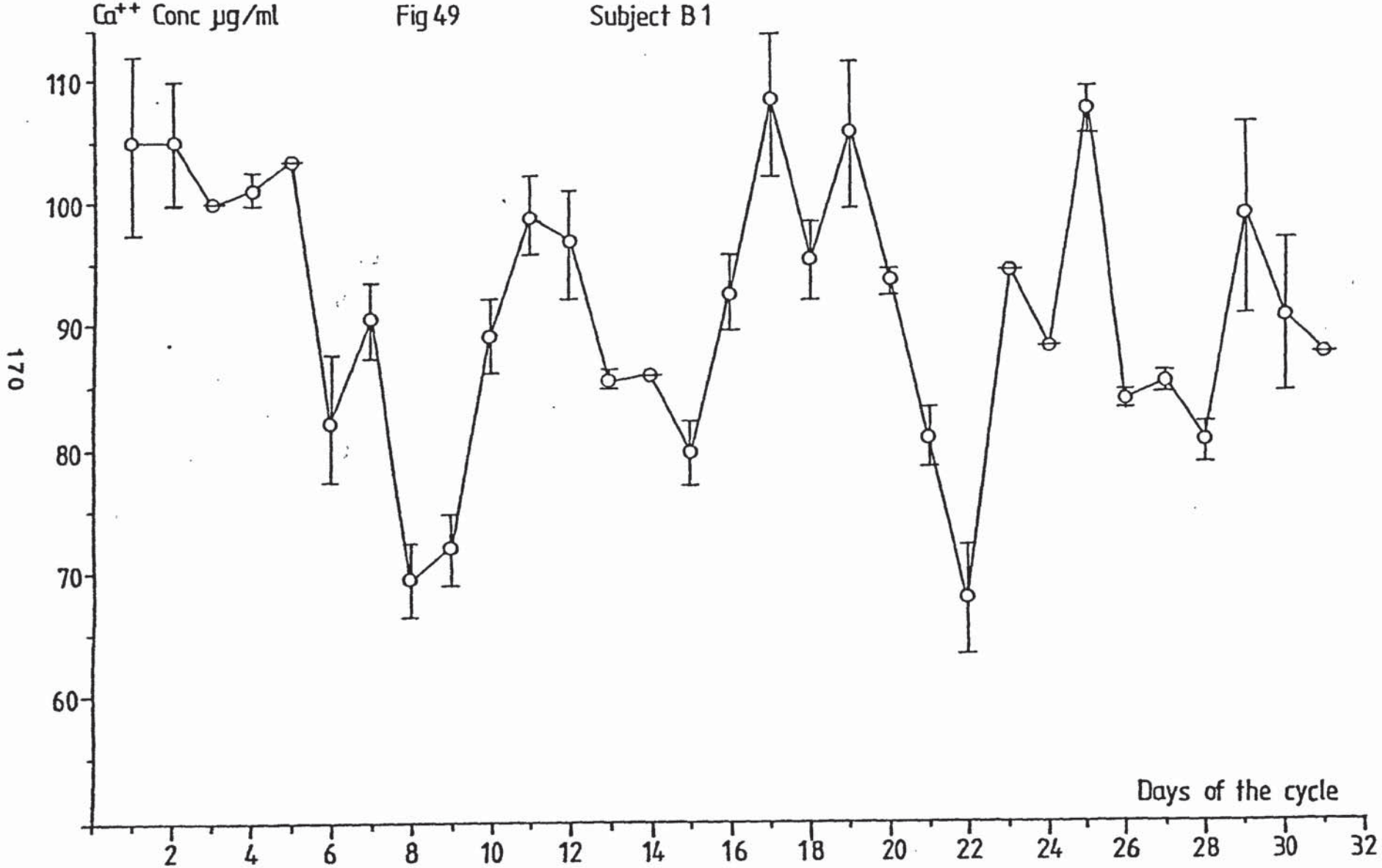
H.L. = Highest Level

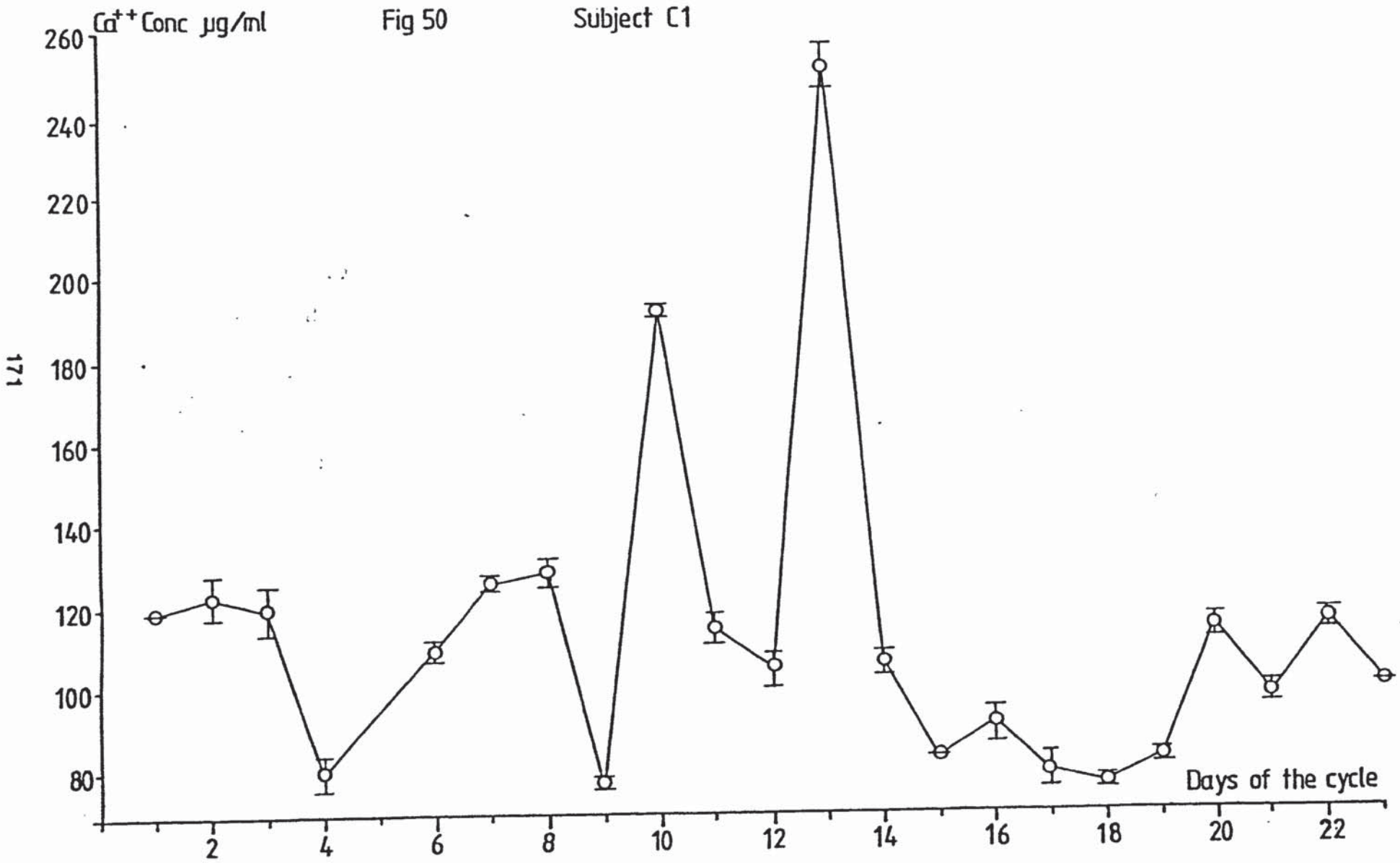
D.H.L. = Days of Highest Level

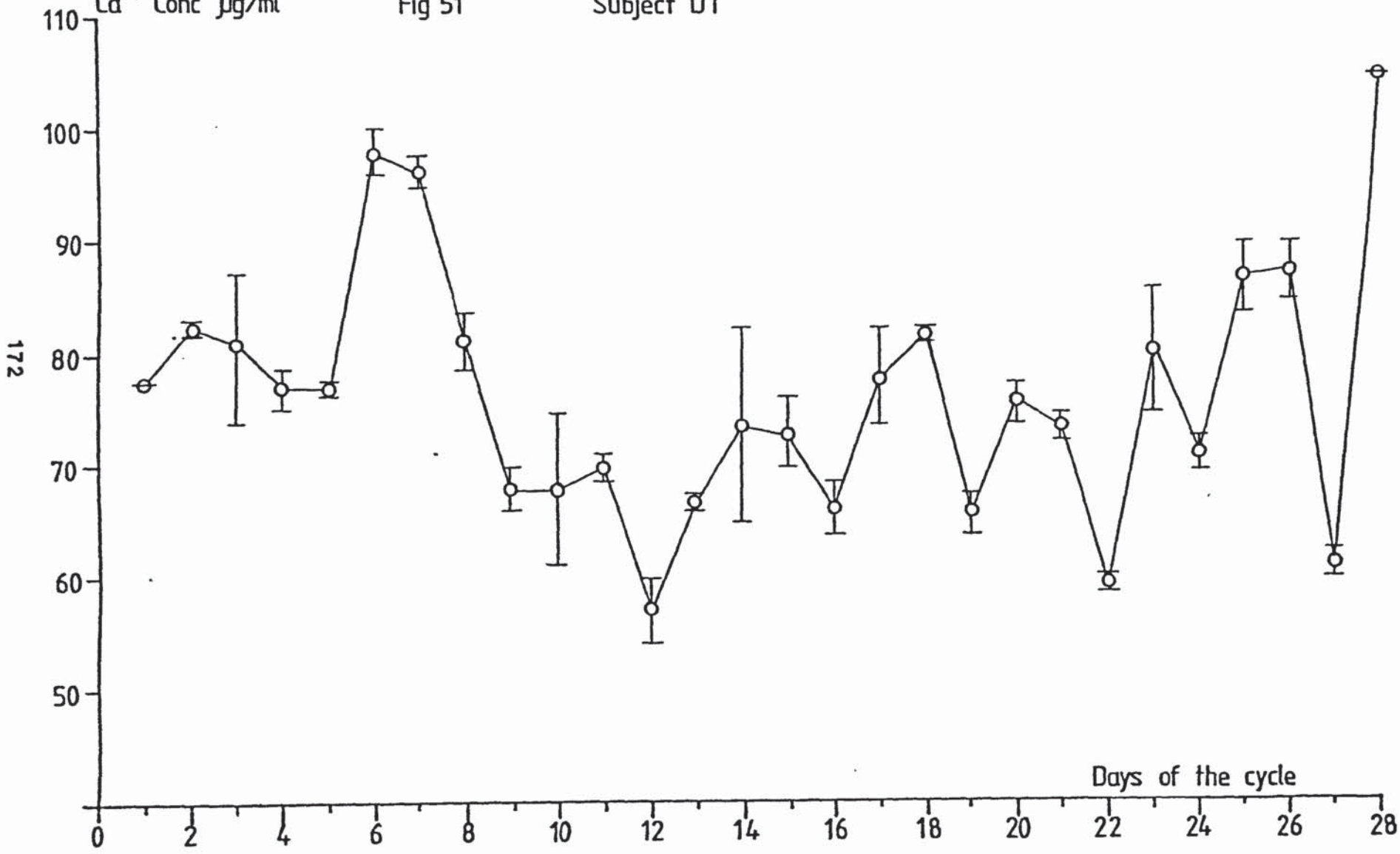
B.N.M.C. = Before Next Menstrual Cycle

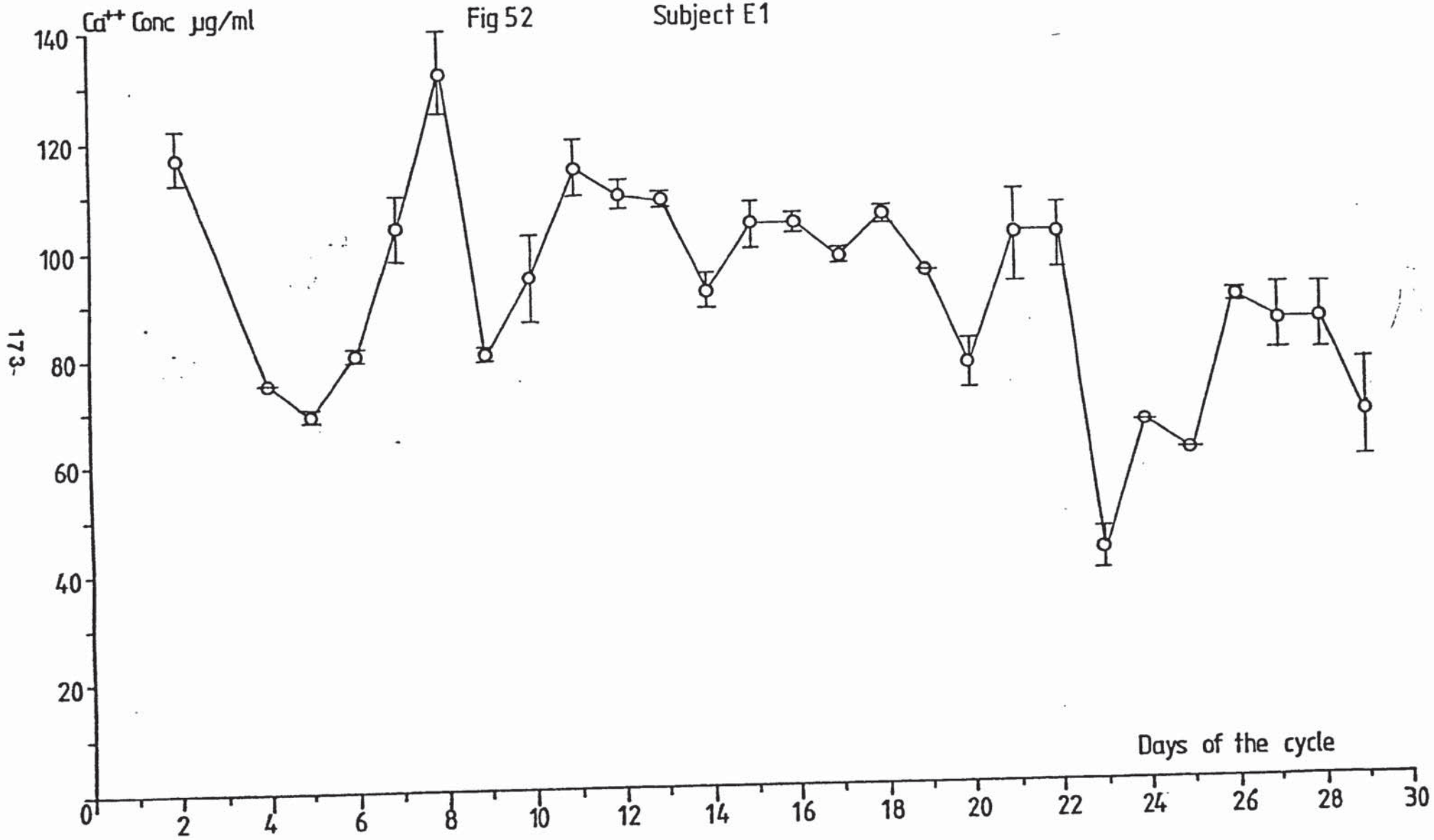
Subjects 1, 2 refer to a 1st and 2nd cycle of the same subject

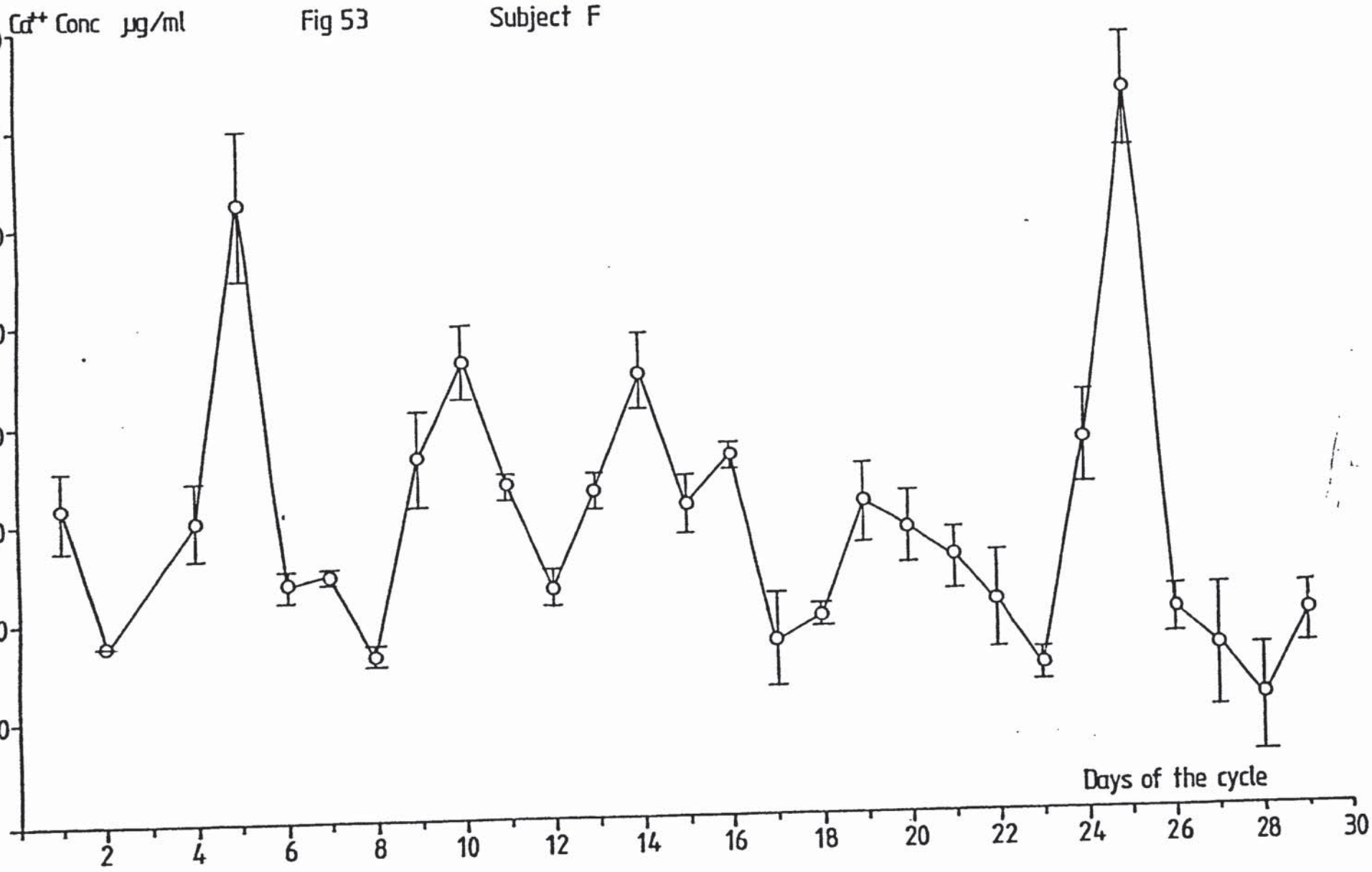

Ca++Conc µg/ml
Fig 48
Subject A
110
100
90
80
70
60
50
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22

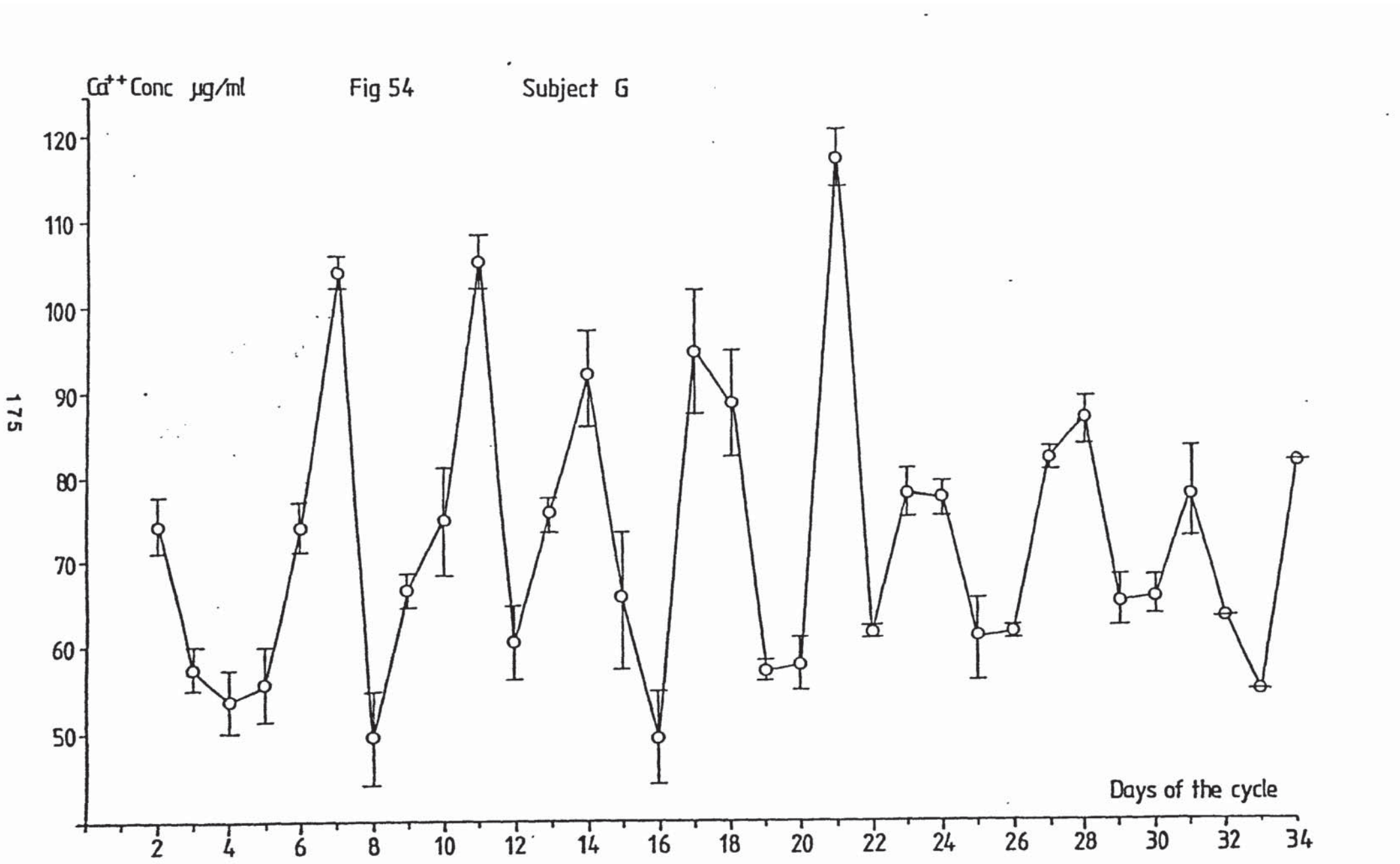

Ca++ Conc µg/ml
Fig 49
Subject B 1
110
100
90
80
70
60
170
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

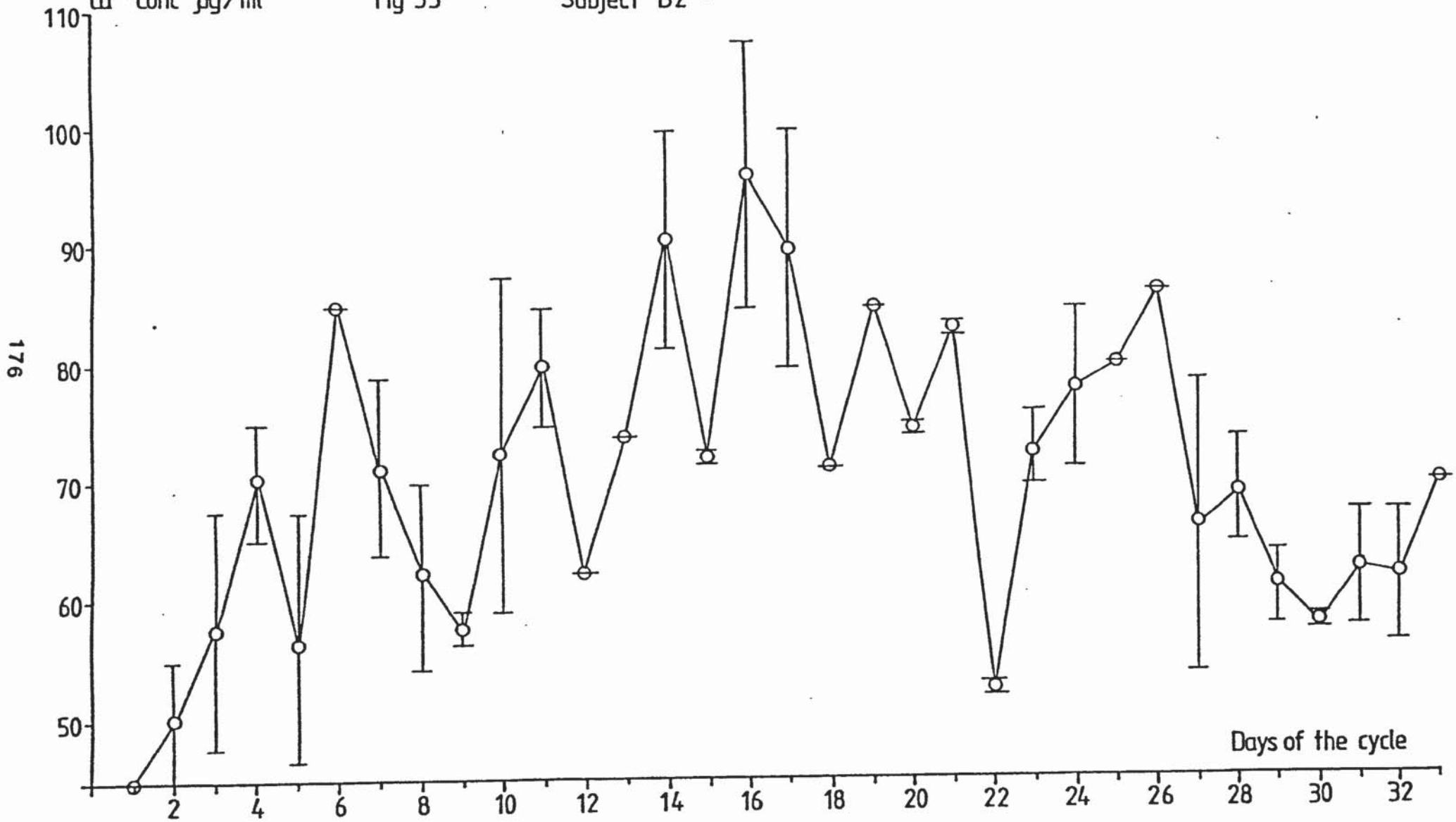

Ca++ Conc µg/ml
Fig 50
Subject C1
260
240
220
200
180
160
140
120
100
80
2
4
6
8
10
12
14
16
18
20
22
Days of the cycle

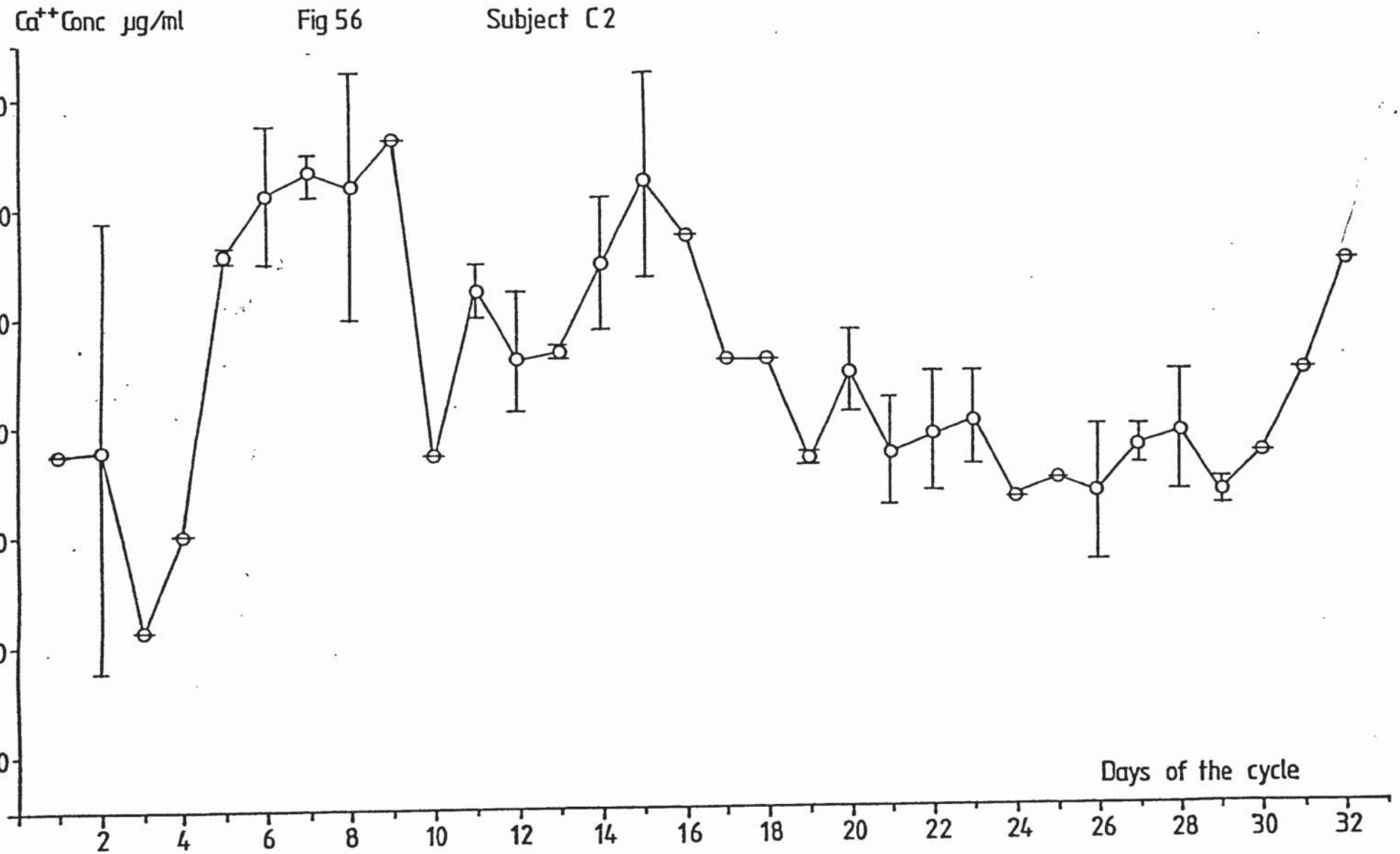

Ca++ Conc µg/ml
Fig 51
Subject D1
110
100
90
80
70
60
50
Days of the cycle
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28

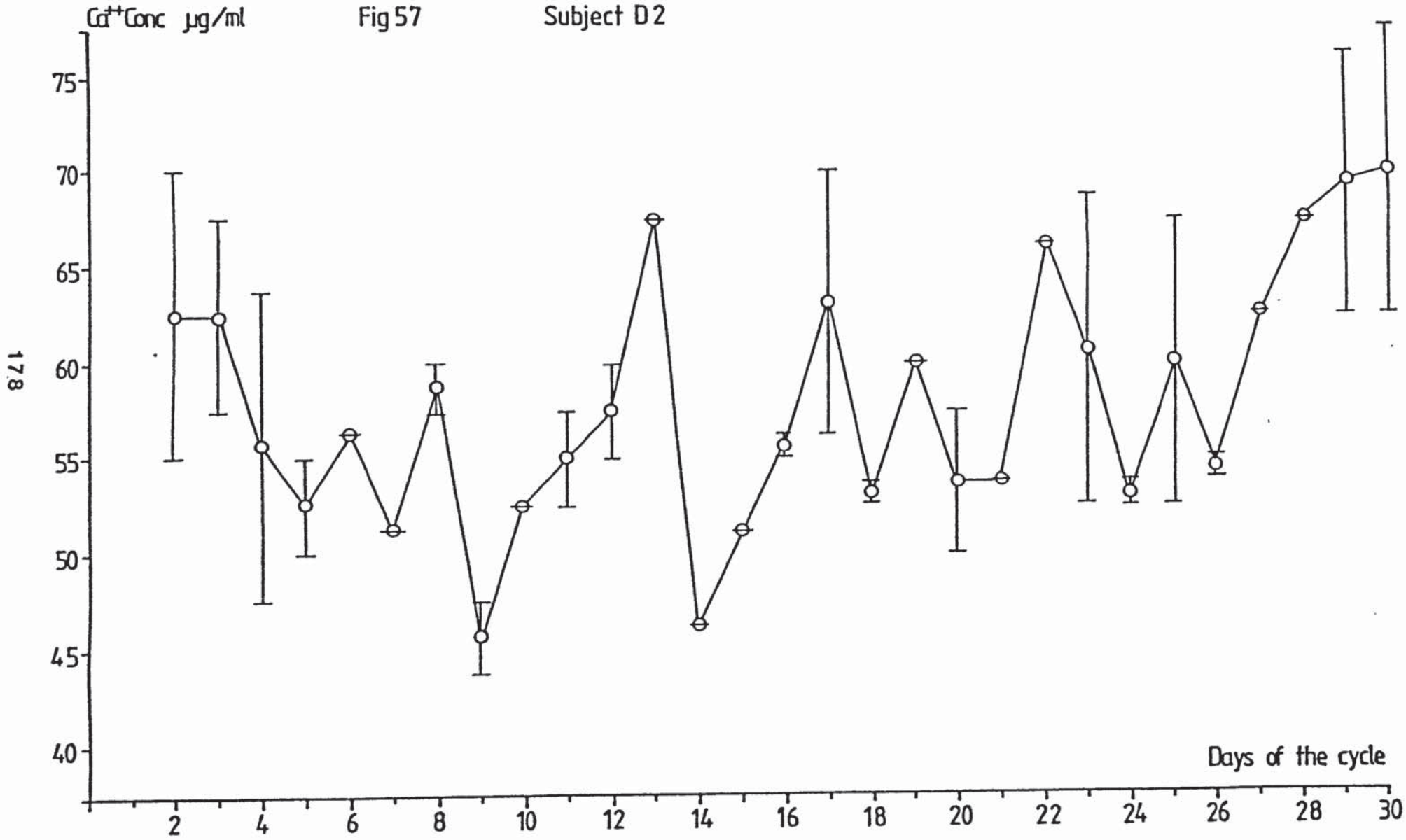

Ca++ Conc µg/ml
Fig 52
Subject E1
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days of the cycle

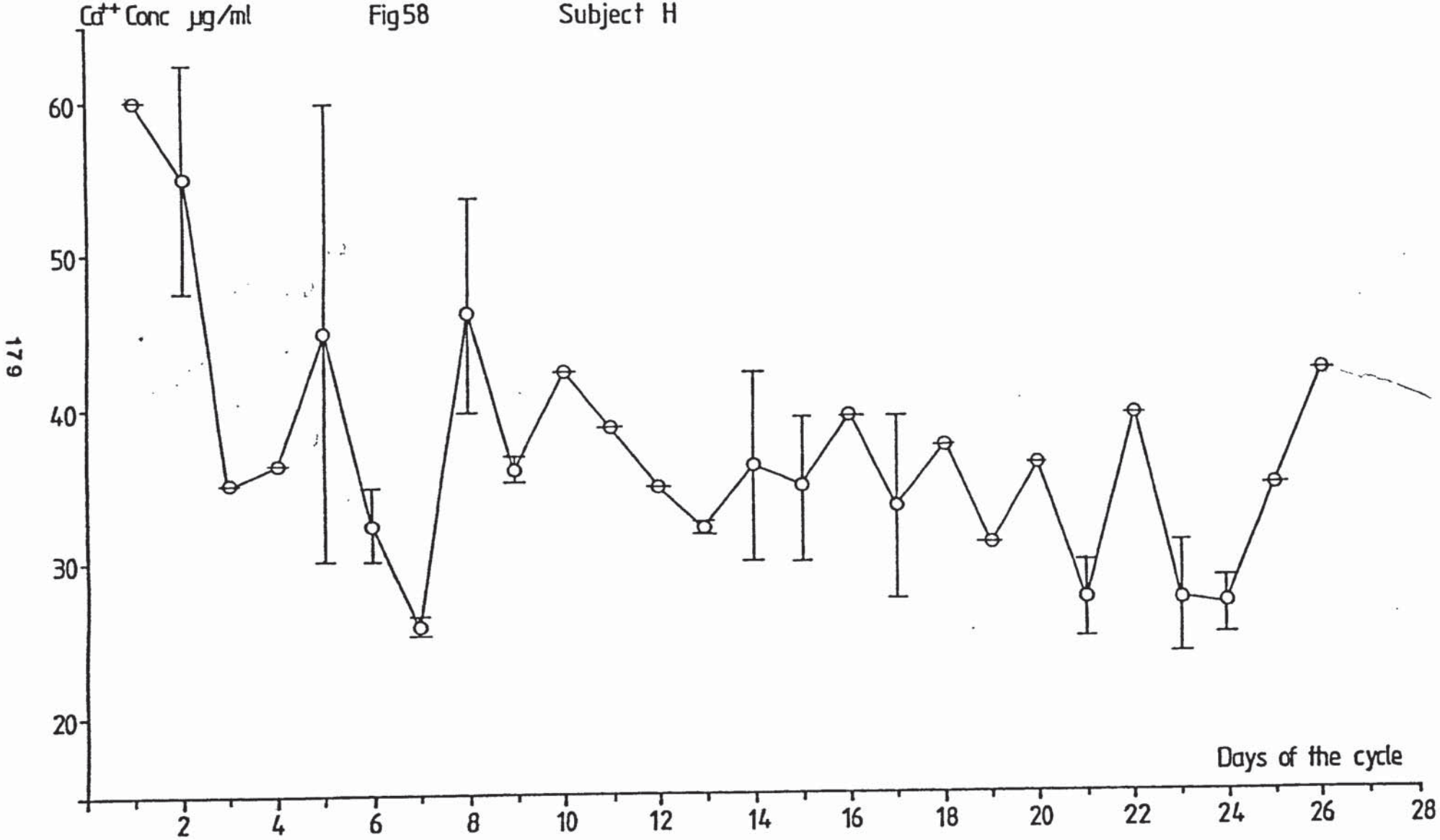

Ca++ Conc µg/ml
Fig 53
Subject F
120
110
100
90
80
70
60
50
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

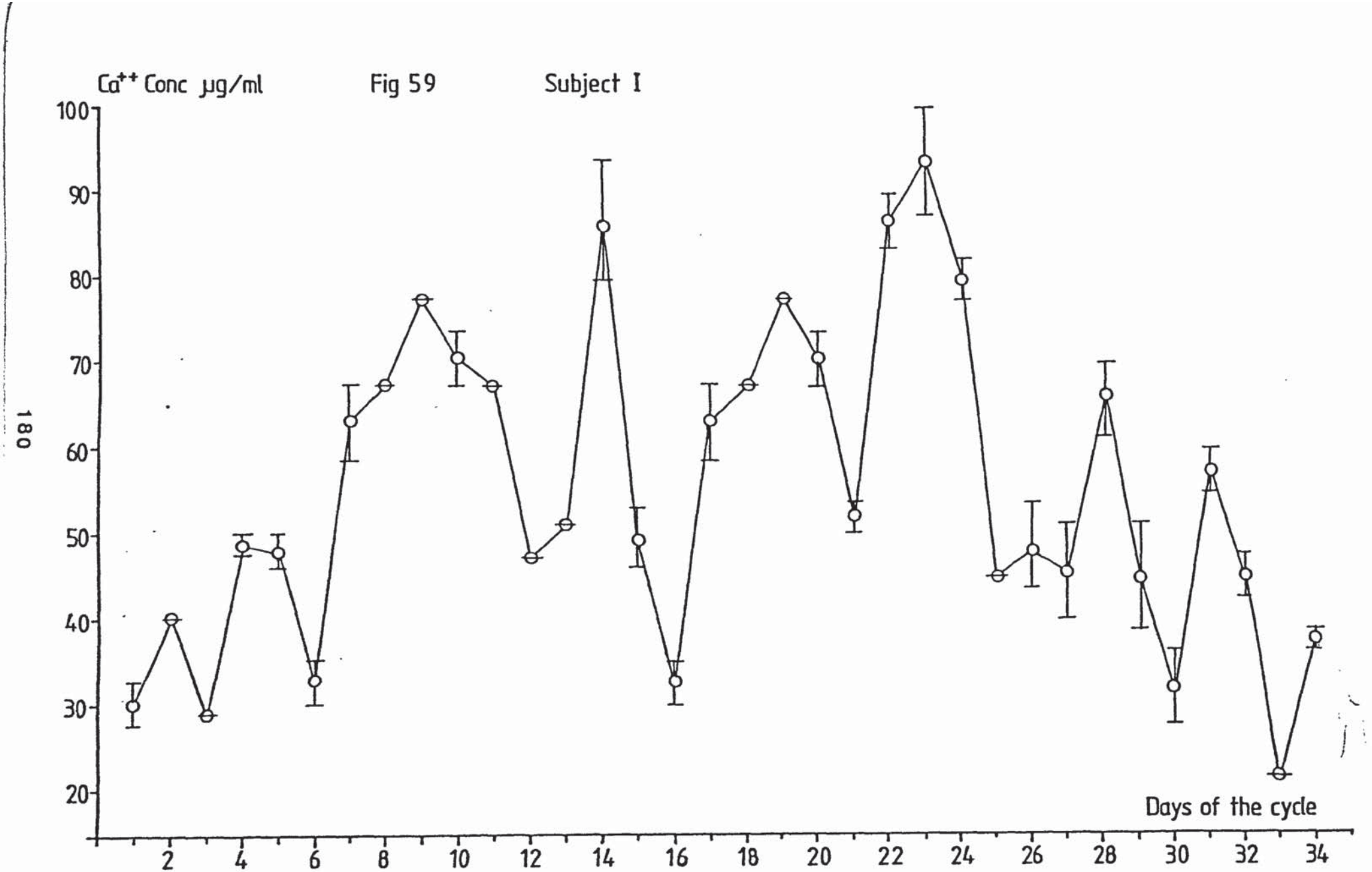

Ca++ Conc µg/ml
Fig 54
Subject G
120
110
100
90
80
70
60
50
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

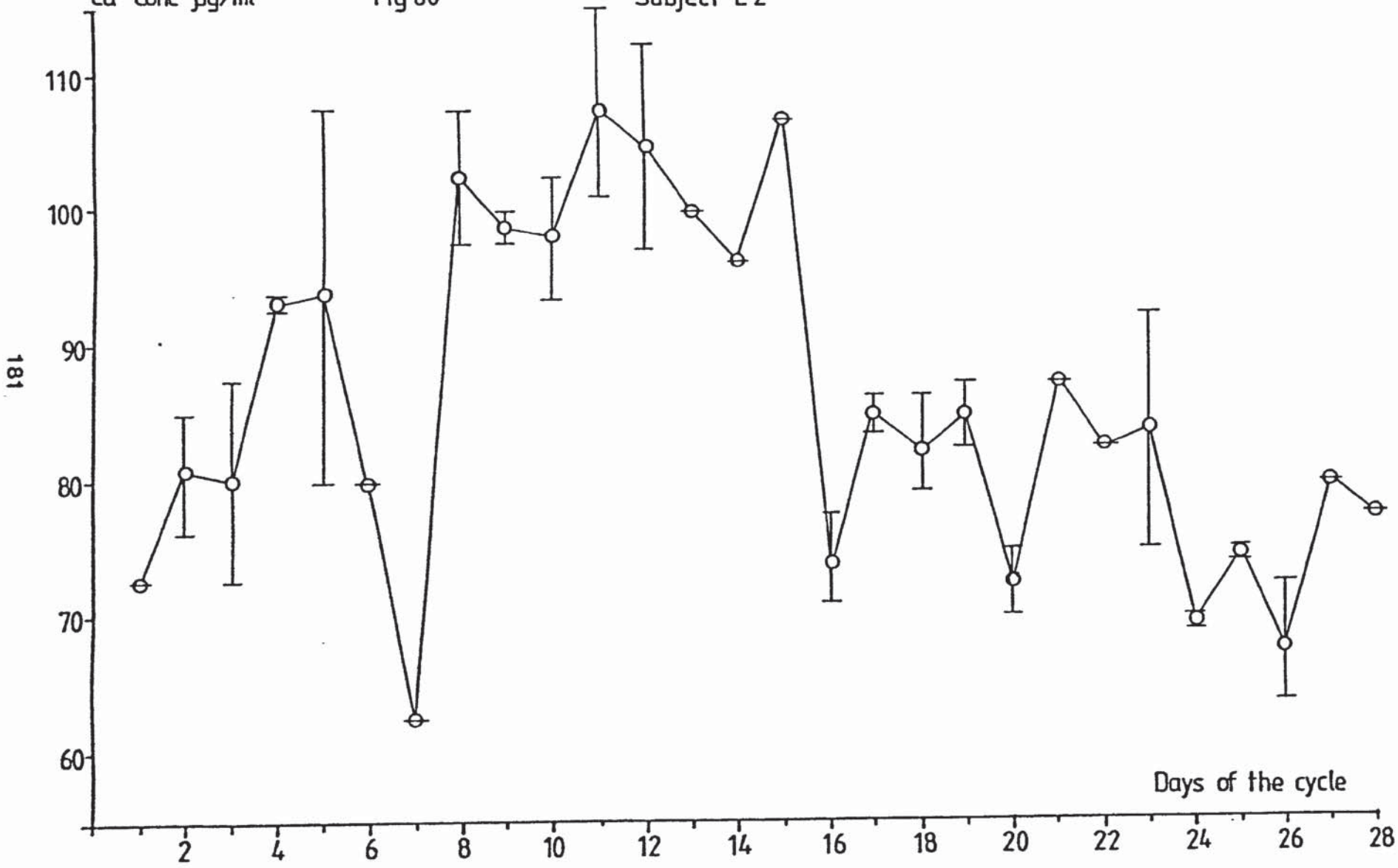

Ca++ Conc µg/ml
Fig 55
Subject B2
110
100
90
80
70
60
50
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

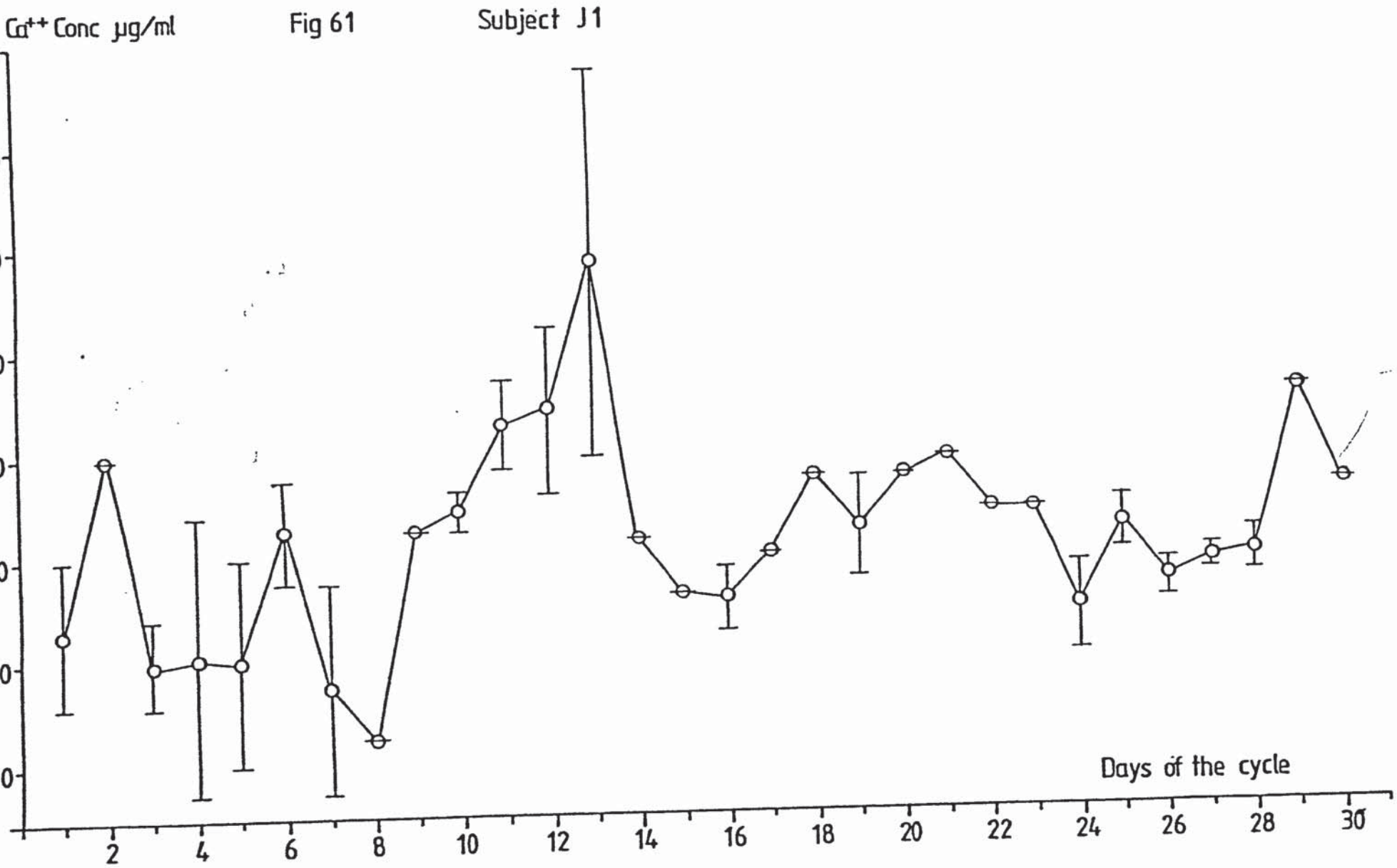

Ca++ Conc µg/ml
Fig 56
Subject C2
90
80
70
60
50
40
30
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

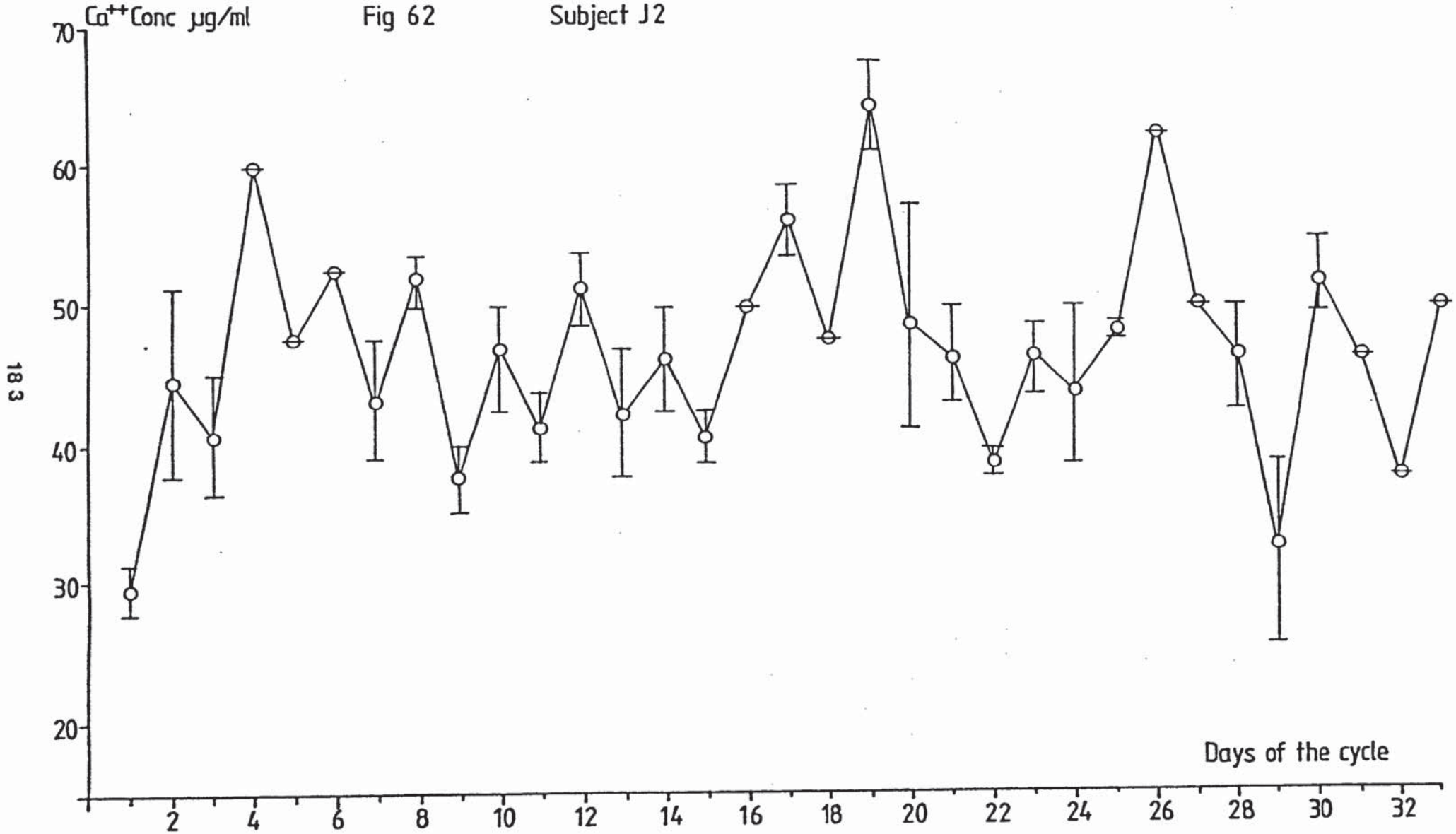

Ca++Conc µg/ml
Fig 57
Subject D2
75
70
65
60
55
50
45
40
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30


Ca++ Conc µg/ml
Fig 58
Subject H
60
50
40
30
20
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28


Ca++ Conc µg/ml
Fig 59
Subject I
100
90
80
70
60
50
40
30
20
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34


Ca++Conc µg/ml
Fig 60
Subject E 2
110
100
90
80
70
60
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28


Ca++ Conc µg/ml
Fig 61
Subject J1
90
80
70
60
50
40
30
20
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30


Ca++Conc µg/ml
Fig 62
Subject J2
70
60
50
40
30
20
Days of the cycle
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32

GENERAL DISCUSSION

Human physiological changes are often accompanied by changes in the compositions of body fluids. An investigation has been carried out to discover whether the time of ovulation in the human female can be determined by monitoring the composition of saliva. A number of female subjects were required to produce samples of their saliva daily for one complete menstrual cycle. The saliva samples were used to determine the levels of glucose, N-Acetyl-β-D-glucosaminidase and calcium contained in it. The subjects selected were not receiving any medical treatment involving the taking of any medicine during the test cycle. If they had to take something like an aspirin or cold tablets then they were to mark the saliva sample, following the taking of the aspirin, to indicate the event.

The first saliva sample was collected on the first day of the beginning of the menstrual flow. The last sample was collected on the day before the beginning of the next menstrual flow. The saliva was collected each morning before breakfast, after a brief mouth rinse with water only. About 5.0 ml of saliva was collected each day directly into a tube marked for the appropriate day, beginning with 1 for day 1 of the menstrual flow. Each tube had a line marking the 5.0 ml volume. The tubes were then handed in later on the same day. At weekends the samples were refrigerated and then handed in on the following Monday.

Discussion for Glucose

The high sensitivity and specifity of a reported fluorimetric enzymatic method (58) for glucose determination madepossible the development of a procedure for the determination of very small amounts of glucose in saliva.

The original method (58) was used for the quantitative determination of low concentrations of glucose in biological fluids. The glucose concentration was estimated by enzymatic colourimetric method which is based on the oxidation of the chromogenic substrate, O-dianisidine, by the peroxide formed by the action of glucose oxidase on the β-D-glucose.

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{D-Gluconic acid} + H_2O_2$$

Another enzymatic fluorimetric method has been described for the determination of glucose concentrations in the urine. This method is an adaptation of a spectrophotometric procedure using hexokinase (HK) and glucose-6-phosphate dehydrogenase (64). However, when all of the data and researches related to the present project were reviewed none of the methods surveyed could be considered as immediately applicable for the accurate determination of the expected very low glucose levels in female saliva. Attempts at using non enzymatic methods did not yield reproducible results. The first attempts with an enzymatic method also gave negative results. In both instances this failure may be ascribed to the effect of inhibiting materials present in human saliva.

The enzymatic method using glucose oxidase was chosen because it appeared to offer a selectivity not shown by most non enzymatic

methods. This is necessary since there are other carbohydrates present besides glucose in the female saliva. It also appeared that a fluorimetric technique would have the sensitivity to detect the low level of glucose present in the saliva. Another advantage could be that less material would be required in the fluorimetric assay.

In order to eliminate the effect of the many inhibitors present in the saliva, the samples were centrifuged at high speed to precipitate most of the larger proteins, some of which might act as enzyme inhibitors. Then the sample was heated in a boiling water bath and centrifuged to coagulate and get rid of the remaining smaller protein particles. De-proteinization was found to improve the results. Then to deal with the inorganic inhibitors in the saliva such as Mn^{+2}, Pb^{+2}, Fe^{+2}, Cu^{+2} and Ca^{+2}, the samples were then treated successively with E.D.T.A. solution, charcoal and a combination of cation and anion resins of different types and grades such as IR.120, IRA.401 and CG.400. Of the three techniques applied it was found that the treatment with resins gave positive results. From the different types of resins used it was found that a mixture of equal amounts of analytical grade of IR.120 and CG.400 gave the best results. The problem of the adsorption of glucose in saliva by the resins was overcome by saturating the mixture of resins by a sucrose solution containing 20 mcg/ml of sucrose.

In order to obtain accurate and precise results, the optimum conditions had to be established. The mixed reagent of 7.4 mg of p-hydroxyphenyl acetic acid, 2 mg of peroxidase in 50 ml tris buffer was used instead of preparing and using each reagent separately. In this way the errors in the measurement and handling of the reagents

were minimised. Since temperature has a considerable effect on enzyme activity,the mixed reagent, the glucose oxidase solution, the samples and the cuvette compartment in the fluorimeter were all kept constant at 37°C by a circulating water bath. Each sample was analysed twice, and each assay lasted 3 minutes. By following this procedure, glucose concentration down to 1 mcg/ml, in the final solution, could be estimated. The rate of change in fluorescence was recorded every fifteen seconds. A linear relationship between the glucose concentration of 1 mcg/ml to 8 mcg/ml, in the final solution, against ΔF could be achieved.

The volume of diluted and treated saliva samples used was 0.25 - 0.5 ml, because this volume of saliva contained a glucose level that fell within the range of the glucose standard solutions.

The results obtained from the analysis of the saliva samples of seven healthy women, during a complete cycle, show an increase in the glucose level, on about the 12 - 15 day before the beginning of the next menstrual flow, i.e. around the reported time of ovulation. Another increase in the glucose level was found 2 - 4 days before the onset of the next menstruation. It is thought that these variations in the salivary glucose level are related to the hormonal changes that occur in the body during the menstrual cycle. These results confirm the previous report by Davis R. H. in 1973 (25) who used an adapted "dextrostix" to detect the time of ovulation.

The results of the study presented here demonstrate clearly that there is in many women a very significant increase in salivary glucose at about the ovulation time. To fully establish this method as a monitor of ovulation time more subjects should be studied over several menstrual cycles in each case. Such a study should also be accomplished by the determination of serum hormones, such as

progesterone, which are accepted as indicators of the ovulation time.

Although this method is sensitive and specific it is not a practical or easy method to apply by a woman at home. It probably cannot be applied easily even in a simple laboratory. It would need to be simplified for use daily at home. It might be possible to develop a stick (or a paper strip) like the dextrostix stick. If this were made sensitive enough to measure the glucose level in the female saliva in the presence of all the other substances, and the change in the intensity of the colour of the stick could be matched with a colour chart then a woman would be able to use it to see that ovulation was about to occur or had just occured. The lowest peak glucose concentration in the saliva samples, encountered in the present study, was about 24 mcg/ml. Therefore, the sensitivity of the stick or any other means that could be developed would have to be such that it could measure down to 10 mcg/ml of glucose in the saliva. Sticks of different sensitivity might be required for use with saliva samples of different glucose concentration ranges.

Discussion for N-Acetyl-β-D-Glucosaminidase (NAG)

Beta-N-acetyl-glucosaminidase is widely distributed in animal tissue and serum, including that of humans. It is particularly abundant in organs in which high rates of mucoid turn over might be expected, for example, epididymis, kidney, liver, testes, spleen and salivary glands. It is generally assumed that the function of this enzyme is to contribute to the hydrolytic degradation of glycoproteins, mucopolysaccharides, or glycolipids (66).

A number of reports have appeared which affirm a correlation between salivary composition and hormonal state, and some of the more recent ones have shown characteristic patterns of behaviour of salivary components such as glucose, sodium and sialic acid and of certain enzyme activities in relation to the time of ovulation (27). Some of these changes have been correlated with the endocrine status of the women (27). Of several lysosomal enzymes originally screened only the activity of NAG showed an increase at about the time of ovulation (27).

The method is based on the enzymatic hydrolysis of methyl-umbelliferyl-β-glucosaminide, by the enzyme followed by fluorimetric estimation of the liberated methyl-umbelliferone (64).

The stability of the liberated aglycone, the methyl-umbelliferone, offers an advantage in the fluorimetric measurements of the standard and sample solutions.

The high sensitivity of the present fluorimetric method was achieved by the use of very dilute enzyme and sample preparations. The use of very dilute samples tended to eliminate the effect of the interfering substances present in saliva. The standard enzyme suspension used had an activity of between 0.0005 unit/ml

and 0.004 unit/ml, and the volume of the sample used was 0.05 ml and 0.1 ml made up to 1 ml with distilled water. The optimal pH was established to allow the use of low substrate concentration with the consequent advantage of conserving substrate. The high sensitivity of the fluorimetric estimation of methyl-umbelliferone had been demonstrated by Mead etal (65), and the present work was undertaken to assess methyl-umbelliferyl-N-acetyl-β-glucosaminide as a substrate for the enzyme as present in saliva. Two disadvantages of this fluorimetric technique were found to be the low solubility of the methyl-umbelliferyl glycoside substrate and its instability in solution. The difficulty of solubility was overcome by using it in very low concentrations. While the effect of the instability was reduced by ensuring that all the substrate solutions were freshly prepared before use. A small amount of methyl-umbelliferone was found to be liberated by non-enzymatic hydrolysis. Its production was linearly related to time and substrate concentration, and a correction for its effect could be incorporated within the blank reading.

The standard enzyme suspension and the samples were diluted with distilled water. When dilution was carried out with 2.5 M $(NH_4)_2SO_4$ solution or citric acid/phosphate buffer of pH 4.8, a lower enzyme activity and consequently a lower fluorescence intensity was obtained.

There was no other special treatment required for the samples or the standard enzyme suspension. When the samples were treated with E.D.T.A. or a mixture of ion excahnge resins, to remove any inhibitors which might affect the enzyme activity only a lower enzyme activity and fluorescence intensity were obtained. In the present technique 4.5 ml of glycine/NaOH buffer of pH 10.8 was used to stop

the enzyme reaction. This volume of buffer was sufficient to provide a pH of 8.0 - 8.5 in the final solution and gave fluorescence intensities linearly related to concentrations. The fluorescence intensity of 4-methyl-umbelliferone was found to remain constant when left overnight in the medium.

All the determinations were carried out in duplicate, including some made with half the amount of saliva.

The activity of NAG as found in the saliva is expressed in units/ml saliva.

The colourimetric assay of NAG was carried out using p-nitro-phenyl-glycoside as the substrate. The enzyme activity was estimated under similar conditions used for the fluorimetric method.

From the study carried out it appears that serial salivary NAG assays could be employed to determine the changes in the level of the enzyme during the menstrual cycle. The information gained confirms the earlier suggestions by Adolfo Rosado etal. (27), that the enzyme activity is increased sometime around the time of ovulation and thus could be used for predicting the time of ovualation.

It can be seen from the results shown in figures 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, and 47, that the enzyme activity varies daily in one menstrual cycle and in the same subject from cycle to cycle. For example, in subject C1, Fig. 35, the enzyme activity ranges from 0.065 unit/ml to 0.135 unit/ml while the same subject, C2, Fig. 41, in another cycle shows an enzyme activity of 0.05 unit/ml to 0.28 unit/ml. The highest enzyme activity was found with subject B2, Fig. 40. It was 0.375 unit/ml, twelve days before the next menstruation. Subject D1, Fig. 36, showed the lowest peak enzyme activity at 0.075 unit/ml.

Due to the fluctuations in daily salivary NAG levels observed in this study, the location of a single peak is not as definite as found in a previous study (27), or as found for glucose in this study. Clearly further studies using a greater number of subjects and menstrual cycles per subject are required, and as suggested for the application of salivary glucose levels, an ovulation confirmatory test should be done in conjunction with the NAG level determinations.

Finally, to make use of salivary NAG levels,applicable by a woman at home, for establishing ovulation time a stick or paper strip with a colour response would need to be developed. Considerations similar to the ones mentioned for a glucose salivary stick would have to be taken into account.

Discussion for Calcium

The results obtained suggest that the fluorimetric method used is suitable for salivary calcium determination. However, much more work would be required with a larger number of subjects to verify if any relation exists between changes in whole saliva calcium levels and hormonal changes in the menstrual cycle.

From the results obtained and shown in Figs. 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, and 62, it is clear that at no time within a menstrual cycle is the salivary calcium level characteristically low or high. This is in contrast to the findings of Puskulian, who reported that there was a significant decrease in the calcium concentration in submaxillary saliva at about ovulation time, although there was no significant change in the parotid salivary calcium concentration at this point in the cycle. From the results obtained in this study of calcium in whole saliva it is clear that the daily changes in calcium levels in female saliva are irregular within a menstrual cycle. This could indicate that salivary calcium concentration is not related to, or affected, directly by the hormonal changes that occur during the menstrual cycle. It may also be that as reported (23), submaxillary saliva calcium levels do vary within a cycle but the variation is not seen in the total of whole saliva as used in this study.

From a comparison of the results obtained in this study for salivary glucose and N-acetyl-β-D-glucosaminidase with those obtained for calcium it can be concluded that salivary calcium levels cannot be used for monitoring hormonal changes within a menstrual cycle. Its concentration is therefore not a suitable indicator of ovulation time.

CONCLUSION

The enzymatic fluorimetric method for glucose determination as described in this work, can be considered to be a sensitive, specific and reliable method for the assay of glucose in saliva. Down to 0.5 mcg/ml of glucose can be determined.

When the assay was used to measure salivary glucose levels in seven normal women, who were not receiving any drugs, a distinct peak of the glucose concentration was observed twelve to fourteen days before the beginning of the next menstrual cycle.

Similar, but not as clearly conclusive, observations were found in the determination of salivary N-Acetyl-β-D-glucosaminidase. A distinct peak in the activity of the enzyme was found during the midcycle of some of the ten normal women studied. While with the calcium determination it is difficult to say that there is a characteristic change in its concentration during the menstrual cycle.

From the above observations it can be concluded that the changes in the concentrations of glucose and the enzyme N-Acetyl-β-D-glucosaminidase in women's saliva can be considered as a possible criteria for the determination of ovulation time. Those findings demonstrate the potential usefulness of the analysis of the salivary secretions in diagnosis and prognosis.

With the ever increasing data being compiled on saliva and its relationship to physiological and pathological conditions, any clinical laboratory of the future must surely include salivary analysis as part of its service to physicians and dentists. Another hope for the future of utilising saliva is that a special, very

sensitive and specific stick will be developed for the determination of ovulation during the menstrual cycle.

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