CHAPTER FOUR

β-GLUCURONIDASE, ACID PHOSPHATASE, ESTERASE AND ALKALINE PHOSPHATASE IN THE SALIVA OF HUMAN BEINGS
INTRODUCTION:

After having established that the enzyme β-glucuronidase, acid phosphatase, esterase, and alkaline phosphatase are synthesized in the major salivary glands of the experimental animal, white rats, and that the synthesis of these enzymes in the salivary glands is regulated by the hormones viz. estrogen and androgen, we directed our efforts towards finding out, whether identical sex-related differences in the enzymes under investigation occur in the human beings and whether in human beings also, the synthesis and secretion of these enzymes are regulated by the sex-hormones. Our studies on human beings were limited to the study of saliva, since directly salivary glands could not be taken for study. The other limitation on our studies was naturally the impossibility of carrying out any ablative and subsequent hormone-administration experiments, naturally we selected to study the variations in the secretion of these enzymes in the human saliva under varying conditions of hormonal levels in the body during natural physiologic process such as menstrual cycle, pregnancy and growth.

The human saliva has been a subject matter of some such studies, though not directly on the enzymes selected in the present investigation, but on other constituents of saliva. The possibility of alterations in the acid and alkaline phosphatases, under varying hormonal levels in the body in the saliva secreted by women, was indicated, as elaborately described
in the introductory chapter, by the acid and alkaline phosphatase paper-strip techniques devised by Foster and Raymond (1967). During the ovulatory phases only the saliva of women exhibited an intense reactivity in these techniques. But it should be emphasized here that these enzymes were not studied quantitatively in the saliva of women during entire menstrual cycle or pregnancy. Moreover, the reaction observed on the paper strip was more or less that identical to the one seen in histochemical techniques, carried out under conditions which are not ideal from several points of view, the most important being the quantity of saliva applied to the paper. Since the enzymatic reaction rate depends upon the quantity of the enzyme that is present in the saliva, the reaction as seen from the development of colour on paper will largely depend upon the quantity of saliva applied. Hence, a detailed study employing well known and standard biochemical techniques was desired to confirm the validity of the paper-strip techniques. The literature that is available on the salivary glands with respect to these enzymes shows a distinct paucity of such work.

Human saliva has been a subject matter of studies on alterations in the electrolytes under varying hormonal conditions. In recent years, Puskulian (1972) investigated the variations in the salivary calcium content of women during menstrual cycle. Puskulian reported that the calcium content following menses in the early proliferative period is high and it decreases midway between the menstrual cycle at about the ovulatory phase.
following which in the luteal phase it again increases. Such alterations have been related to the varying levels of hormones such as parathorhone (Kraintz, 1960, 1966, 1967).

A study of the enzymes in human saliva under varying hormonal conditions such as those which occur during growth, menstrual cycle and pregnancy, has certain inherent deficiencies and has to face certain criticism. Some such deficiencies worth criticism are (1) Such a study does not give a clue as to which of the three major salivary glands is responsible for the secretion of varying concentrations of the enzymes, as found in total saliva under varying hormonal conditions. Since the total saliva contains secretions of all the three glands and in all probability those of the lingual salivary glands also, we cannot pinpointedly say which gland is responsible for the enzymatic variations. One has to rely on the results obtained on the experimental animals for the interpretations of the results on human saliva. (2) Since the total saliva, in addition to the secretions of the salivary glands, also contains a bacterial population and some degenerated mucosa cells, one has to make sure that the enzymatic alterations seen in the saliva are due to the enzymes secreted by the salivary glands and not due to these extraneous matters. (3) Though at a general level the women will exhibit a typical pattern of changes in the hormonal levels during menstrual cycle, pregnancy and growth, individual variations do occur which might interfere with the interpretation of the results. The objections to the last two points of criticism can be overcome by studying a large
number of human beings, so that the factors such as the presence of bacterial population and mucosal cells in the saliva become a common factor and since a large population is studied the individual variations also do not affect the results much.

The present work on the β-glucuronidase, acid phosphatase, esterase and alkaline phosphatase in the human saliva, thus, aims at:

1) Study of the activities of these enzymes in the saliva of sexually mature fertile men and women, with a view to find out differences, if any, in the activities of these enzymes which can be related to sex-differences. This part of the study, thus, aims at finding out, whether there exists any sexual dimorphism in the secretion of these enzymes in the human beings.

2) Study of variations in the activities of these enzymes in the saliva of sexually mature fertile women during their menstrual cycle, with a view to find out whether such variations in the enzyme activities bear any relationship with the known variations in the hormonal levels in the body occurring during menstrual cycle.

3) Study of variations in the activities of these enzymes in the saliva of women during pregnancy cycle and postpartum, with a view to find out whether such variations in the enzyme activities bear any relationship with the known variations in the hormonal levels in the body occurring during pregnancy and postpartum.
4) Study of variations in the activities of these enzymes in the saliva of human beings, both male and female, during their growth, attainment of maturity, adult reproductive period and subsequent senescence.

MATERIAL AND METHODS:

I. Collection of samples:

While selecting human subjects for the study of their saliva, following precautions were taken.

1) Human beings with any mouth and teeth diseases were avoided and those with clean mouth and clean saliva alone were accepted.

2) Human beings who habitually chewed tobacco or rubbed burned tobacco powder were avoided and in similar manner saliva of smokers was not taken.

3) While selecting subjects for the study of enzymatic variations in their saliva during menstrual cycle, women with a normal menstrual history, satisfactory body temperature and clean mouth were taken. Women taking any type of hormone-containing birth control pills were avoided.

4) While selecting subjects for the study of enzymatic variations in their saliva during pregnancy and postpartum, women who have at least one child and whose delivery history was normal were taken. The primies were generally avoided.
5) While selecting subjects for the study of enzymatic variations in the saliva during growth cycle, children of varying ages with clean mouth, clean saliva and normal body development were taken.

We shall fail in our duty, if we do not acknowledge the kind and considerate co-operation of the inmates of Ladies Hostel of Shivaji University, Tararani Girls' Highschool, Kolhapur, Private Highschool and Vivekanand Highschool for boys, Kolhapur, students in the departments of Botany, Zoology, Chemistry in the Shivaji University, who kindly consented to donate their saliva for the present study. A special mention must be made of lady doctors in Civil Hospital, Kolhapur and Savitribai Phule Maternity Home run by Kolhapur Municipal Corporation, for helping in getting subjects for the study of saliva of women during pregnancy and postpartum. But for the co-operation of all these, the present work would not have been completed.

II. Collection of saliva:

Each subject before taking saliva was requested to clean his or her mouth with tap water twice and with distilled water thrice. 15 minutes after the mouth washing, during which the subjects were requested to spit off their saliva, the saliva sample was collected in clean dry test-tubes. At a time a minimum of 10 ml saliva was collected. The test-tubes containing the saliva samples were immediately transferred to the ice-box and maintained at least at 4°C till they are taken for
further work. Occasionally samples were examined under microscope, to see if the bacterial population has increased beyond normal and if the mucosal cells also occur in abnormal number. Saliva containing thick sputum and traces of blood was also rejected. In such cases the samples were rejected. The saliva from the samples was directly used for enzyme assay, without being diluted.

III. Studies on sex-differences:

For studying the sex-related differences in the enzymes in the saliva, sexually mature fertile men and women within age group 28 to 30 years were selected. Saliva samples of about 120 men within this age group and about 200 women in this age group were studied. In the case of women, salivary samples were collected between 11 to 14 days of menstrual cycle which forms the late proliferative phase of menstrual cycle and on 1st and 2nd days of menses. In case of men saliva samples were taken randomly. A minimum of four samples were collected from each individual. The average values were calculated for 100 ml of saliva.

IV. Studies during menstrual cycle:

The variations in the activities of the enzymes under investigation in the saliva during menstrual cycle were studied, in the following age groups of women containing the number of subjects shown against the group for months as indicated:

1) Women within age group 21 - 25 years,
   40 subjects for 6 months continuously.
2) Women within age group 26 - 30 years, 
   40 subjects for 6 months continuously.
3) Women within age group 31 - 35 years, 
   40 subjects for 6 months continuously.
4) Women within age group 35 - 40 years, 
   40 subjects for 6 months continuously.

A detailed record showing menstrual history, body temperature charts etc. was maintained. As and when the body temperature was found to be abnormally high, the saliva samples of such individuals were rejected. Ovulation was confirmed with the help of body temperature which was nadir on the day of ovulation and also from the subject history. As and when needed, medical advise was taken from lady doctors in the Savitribai Phule Maternity Home, Kolhapur.

Saliva samples were collected every day for the period mentioned above, from each of the individuals belonging to different age groups in the morning between 8 a.m. to 10 a.m. This time was kept unchanged throughout the course of the work. All the four enzymes were assayed in the saliva samples by employing biochemical techniques described in chapter 2. The average enzyme values were calculated for each day and plotted as a function of the stages in the menstrual cycle.

V. Studies during pregnancy and postpartum:

The variations in the activities of the enzymes under investigation in the saliva during pregnancy and postpartum,
were studied in the following age groups of women containing the number of subjects shown against the group for months as indicated:

1) Women within age group 22 to 25: 25 subjects for 2 to 9 months of pregnancy and 5 days after parturition.

2) Women within age group 25 to 30: 40 subjects for 2 to 9 months of pregnancy and 5 days after parturition.

3) Women within age group 30 to 35: 40 subjects for 2 to 9 months of pregnancy and 5 days after parturition.

4) Women within age group 35 to 40: 22 subjects for 2 to 9 months of pregnancy and 5 days after parturition.

A detailed record showing progress of pregnancy, body weight etc. was maintained. The early pregnancy was confirmed by the gynecologists of Savitribai Phule Maternity Home. The saliva samples were taken on a day separately decided for each group per week, either by visiting the home of the subjects or in Maternity Home, for the complete period of pregnancy, after 45 days till parturition. Samples were generally collected in the morning and subjected to enzyme assay as described earlier. The average enzyme values were calculated for each month of pregnancy and for 5 days after parturition and plotted as a function of month of pregnancy and days after parturition.
VI. Studies during growth:

The variations in the activities of these enzymes during growth of human beings, attainment of maturity, active period of reproduction and subsequent senescence, were carried out in the saliva of human beings in the following age groups in the subjects shown against each group for as many trials as shown below:

1) Boys and girls of age 5 years: 20 subjects, 10 trials in a month.

2) Boys and girls of age 10 years: 20 subjects, 10 trials in a month.

3) For the age groups 20 to 40 years the values obtained from earlier studies on menstrual cycle of women were taken.

For men the following subjects were chosen:

a) Men of age 20 years: 50 subjects, 10 trials in a month.

b) Men of age 25 years: 80 subjects, 10 trials in a month.

c) Men of age 30 years: 80 subjects, 10 trials in a month.

d) Men of age 35 years: 40 subjects, 10 trials in a month.

e) Men of age 40 years: 40 subjects, 10 trials in a month.

4) Men and women of 45 years: 20 subjects, 10 trials in a month.

5) Men and women of age 55 years: 20 subjects, 10 trials in a month.
6) Men and women of age 60, 65 and 70 years: 20 subjects, 10 trials in a month.

In case of women above the age of 15 years, the enzyme activities were studied in the proliferative phase i.e. on 12th, 13th and 14th days of menstrual cycle. In case of men, the saliva was studied randomly. In case of women, pregnant women were avoided while studying this part of the investigation.

While taking the saliva samples of boys and girls below age of 15 years, firstly they were given chewing gum and then the first three lots of saliva were discarded. They were asked to clean their mouth as described before and then first lot of saliva was discarded and the second lot was taken for study. In case of grown up men and women, saliva was taken as described before. The average enzyme values were calculated for each age group and plotted as a function of age.

METHODS:

Since no histochemical techniques could be applied for such saliva study, only the biochemical techniques of the enzyme assay were employed. The results were calculated for enzyme units per 100 ml of total saliva. Studies on pH optima and effects of various activators and inhibitors on random saliva samples obtained for the growth study were carried out.
OBSERVATIONS:

A) Enzyme differences in relation to sex:

The enzyme values in the saliva of men within the age group 28 to 30 years of age and of women in late proliferative phase of menstrual cycle (11 to 14 days of menstrual cycle) of the same age group. At a general level, the activities of all the four enzymes in the saliva of men were more than those in the saliva of women, but the differences in the enzyme activities were not significant. At no time in the entire study, the enzyme values in the saliva of men were found to be less than those in the women. If the enzyme values in the saliva of men are considered in comparison with those of the women during first and second days of menstruation, a distinct and very remarkable difference could be observed.

1) β-glucuronidase:

The saliva of men within age group 28 to 30 years exhibited β-glucuronidase activity equivalent to 4010 ± 210 F.U., while in the saliva of women in this age group of late proliferative phase, this activity was equivalent to 3520 ± 180 F.U., thus, showing a difference of about 500 F.U. between the saliva of men and women in this phase of menstrual cycle. Curiously enough, the women in first and second days of menses exhibited the enzyme activity equivalent to 455 ± 47 F.U., thus, indicating a very significant difference of more than 3500 F.U.
2) **Acid Phosphatase** :

The average value of acid phosphatase in the men within age group 28 to 30 years, was equivalent to $2873 \pm 121$ units, whereas the average values of this enzyme in the saliva of women in this age group but in the late proliferative phase of menstrual cycle was equivalent to $2770 \pm 102$ units, thus, showing only a difference of about 100 units between the saliva of men and women. The women in the 1st and 2nd days of menses, on the other hand, exhibited very low enzyme value which was equivalent to $376 \pm 39$ units. Thus, the saliva of men exhibited a difference of about 2200 units from the enzyme values of women during the first two days of menses.

3) **Esterase** :

The esterase activity of men within age group 28 to 30 years was equivalent to $55 \pm 13$ units, while in the saliva of women in this age group but in late proliferative phase this activity was equivalent to $40.5 \pm 10$ units, thus, showing a very minor difference of about 12.5 p-nitrophenol units between the saliva of men and women in this phase of menstrual cycle. But very significant difference of 36 units was observed between the esterase activity of saliva of men and women, when the enzyme activity of women saliva during first two days of menses, was compared with men saliva. In the saliva of women during this stage of menstrual cycle, the activity was equivalent to $16 \pm 11$ units, whereas in men it was $55 \pm 13$ units.
4) **Alkaline Phosphatase**:  

Average value of alkaline phosphatase activity in the saliva of men within age group of 28 to 30 years, was 2972 ± 208 units, whereas in the saliva of women of the same age group in the late proliferative phase of menstrual cycle alkaline phosphatase value was 2960 ± 107 units. Thus, the saliva of men and women in this phase of menstrual cycle contained practically the same quantity of this enzyme. But when the enzyme values of saliva of women in first two days of menses was compared with the enzyme values of men saliva, there was a significant difference of 2200 units. At this stage the average alkaline phosphatase activity of women saliva was about 750 ± 97 units.

B) **Alterations in enzyme activities during menstrual cycle**:  

The human menstrual cycle was timed from the day on which menses begins, this phase lasts for 4 days, which is then followed by proliferative phase which lasts from 5th to 13th days, at the end of which ovulation occurs, this phase is followed by luteal phase which continues until the 28th day which is the last day of menstrual cycle.

At a general level, it was found that the enzyme activities in the saliva during menses marked a minimum and remained in this condition for the first 3 days of menses. From the 4th day the
enzyme activities exhibited a progressive increase, reaching a maximum during the late proliferative phase on 13th to 14th days of the menstrual cycle. Following ovulation when the luteal phase was initiated, the enzyme activities exhibited a significant decrease which continued till 21st day. On 23rd day the enzyme activities showed a slight increase and again from 24th day to the last day of menstrual cycle, they exhibited a sharp and significant decrease. Thus, during menses the enzyme activities are minimum, they increase during proliferative phase reaching a maximum at the time of ovulation and then again decrease in the luteal phase showing an insignificant increase in mid-luteal phase. Identical alterations in the enzyme values are obtained in the menstrual cycle of women belonging to the different age groups with some very minor differences. The exact values of the variations in the enzyme activities during the complete menstrual cycle of women belonging to different age groups are shown in Table 17.

For further consideration of the alterations in the individual enzymes during menstrual cycle, average values of all the age groups are taken for description.

1) β-glucuronidase:

The alterations in β-glucuronidase during menstrual cycle are shown in Table 17 and these alterations are graphically represented in graph No.37. All the values for β-glucuronidase described hereafter are calculated for 100 ml of the saliva.
During menses proper on the first 3 days, the enzyme activity varied between 455 ± 35 F.U. and 606 ± 51 F.U. On the last day of menses the activity showed a little increase, it being 740 ± 62 F.U. Following menses in the early proliferative period, the activity showed a sharp increase. On 5th day the activity was equivalent to 823 ± 60 F.U., whereas on 6th day it increased to 1372 ± 65 F.U. During 9th to 13th days the activity increased from 2348 ± 72 F.U. to 3312 ± 157 F.U., on 14th day the activity was practically identical to that observed on 13th day. Following ovulation in the early luteal phase the activity started decreasing. On 15th day the activity dropped down to 3002 ± 120 F.U., on 17th day it was 2132 ± 72 F.U. 19th day witnessed a further fall in the enzyme activity, it being 1773 ± 74 F.U. On 21st day it again underwent a further decrease, it being 1051 ± 75 F.U. On 22nd and 23rd days a little increase in these activities was observed, on 23rd day the activity being 1876 ± 65 F.U. From 25th to 28th days further decrease was observed, on 25th day it was 1510 ± 161 F.U., on 27th day 754 ± 52 F.U. and on 28th day it was 377 ± 56 F.U.

Thus during menses the β-glucuronidase activity marks a minimum, during proliferative phase it gradually increases reaching a peak on 13th and 14th days of menstrual cycle which correspond to the ovulatory period, the increase being more than 8-fold and in the luteal phase the activity again decreased, except on 22nd and 23rd days on which some increase is observed, reaching a minimum very close to the menses values on the last day of menstrual cycle.
2) **Acid Phosphatase** :

The variations in the acid phosphatase during menstrual cycle are shown in Table 17 and they are graphically represented in graph No. 37. The activity of acid phosphatase in the human saliva is calculated on the basis of p-nitrophenol units per 100 ml of saliva. At the time of menses during the first 3 days, acid phosphatase activity varied between 376 ± 36 to 527 ± 42 units. On 4th day the activity showed slight increase, it being 610 ± 21 units. During the early proliferative phase, the activity showed a significant increase. On 5th day the enzyme activity was about 805 ± 129 units, whereas on 7th day the enzyme activity increased to 1351 ± 135 units. In the late proliferative phase during 9th to 13th days the activity showed an abrupt increase from 1955 ± 28 units to 3006 ± 132 units. On 14th day the enzyme activity was practically equivalent to that observed on the 13th day. After the ovulation in the early luteal phase, the acid phosphatase activity of saliva started decreasing, on 15th day the activity decreased to 2852 ± 126 units. On 17th day it exhibited a further decrease to 2304 ± 129 units. This fall in the enzyme activity was continued up to 21st day, on 19th day it was 2056 ± 36 units and on 21st 2053 ± 39 units. During 22nd and 23rd days, there was a slight increase in the enzyme activity, on 23rd day the acid phosphatase activity was equivalent to 2376 ± 142 units. In the late luteal phase i.e. from 25th to 28th days again there was a considerable decrease in the enzyme activity, on the 25th day it was 1801 ± 36 units and on 27th the enzyme
activity was 1100 ± 137 units. At the end of the cycle i.e. the 28th day, the activity decreased to 868 ± 130 units.

Thus, during the menses the acid phosphatase activity is minimum and during the oвуляtory phase the activity gradually increases up to 14th to 15th day of menstrual cycle which corresponds to the ovulatory period, the increase being more than 9-fold and in the luteal phase the activity again decreases, except on 22nd and 23rd days some increase is observed, reaching a minimum level just before the beginning of next menses.

3) Esterase:

The changes in the enzyme activity during menstrual cycle are described in Table 17, these alterations are graphically represented in graph No.38. As in the case of other enzymes, the esterase activity is expressed on the basis of p-nitrophenol units per 100 ml of the saliva. During the menses proper on the first two days the activity was about 16 ± 7 units. On the third day of menses it was about 17 ± 5 units. From 4th day onwards, the enzyme activity exhibited progressive increase, which was 20 ± 5 units on 5th day of menstrual cycle. On 7th day it increased to 25 ± 6 units. During 9th to 13th days the enzyme activity increased from 31 ± 9 to 47 ± 13 units. On 14th day it was observed that the enzyme activity was more or less equivalent to that on the 13th day. Following ovulation in the early luteal phase, the enzyme activity started decreasing, on 15th day it was 43 ± 7 units and 33 ± 15 units on 17th day. 19th day witnessed a
further decrease in the enzyme activity to 27 ± 11 units. The decrease in the enzyme activity still continued, on 21st day it was about 25 ± 7 units. But on 23rd, 24th and 25th days, a little rise in the enzyme activity was observed, on 23rd day activity being 26 ± 5 units and on 25th day it was 25 ± 9 units. Following this in the remaining period of menstrual cycle, the enzyme activity exhibited a progressive decrease till the end of menstrual cycle. On 27th day enzyme activity was 19 ± 5 and 15 ± 3 units on 28th day.

In this way, during menses period esterase activity is minimum. During the proliferative phase it gradually increases reaching a peak at the time of ovulation. The increase in the enzyme activity on 13th day being about 3-fold. The enzyme activity again decreases in the luteal phase, but there is a little increase in the activity on 23rd and 25th days. Then the enzyme activity again decreases till the end of the menstrual cycle.

4) Alkaline Phosphatase:

The changes in the alkaline phosphatase activity during the menstrual cycle are shown in Table 17 and these alterations are graphically represented in graph No.38. All the values of the enzyme activities described henceforth are calculated in p-nitrophenol units for 100 ml of saliva. During the menses proper, alkaline phosphatase activity on the first two days varied between 750 ± 162 and 829 ± 153 units. On the 3rd and 4th days of menses
there was a distinct increase in this activity, on the 3rd day the activity was $1182 \pm 153$ units and on 4th day it was $1450 \pm 69$ units. Following menses in the early proliferative phase, the activity showed a sharp increase. On the 5th day, the activity was equivalent to $1824 \pm 171$ units and on the 7th day it increased to $1923 \pm 170$ units. During 9th to 13th days the alkaline phosphatase activity increased from $2181 \pm 69$ units to $3375 \pm 70$ units. On the 15th day, saliva showed highest alkaline phosphatase activity which was about $3402 \pm 163$ units, but after 15th day the enzyme activity gradually decreased up to 21st day, on 17th day the saliva showed about $3103 \pm 179$ units, on 19th day it was $2603 \pm 150$ units and on 21st it was about $2250 \pm 152$ units. The enzyme activity again showed some increase on 23rd to 25th days, on 23rd day it was $2856 \pm 159$ units and 25th day it increased to $3254 \pm 145$ units. But after this period of menstrual cycle, the enzyme activity dropped to $2002 \pm 163$ units, on 27th day. On the last day of the menstrual cycle the activity decreased to $1626 \pm 51$ units.

Thus, during menses activity of the alkaline phosphatase is comparatively very low, which increases during proliferative phase and reaches a highest value at the time of ovulation. Following ovulation, activity gradually decreases till the end of menstrual cycle, showing some increase during 23rd and 25th days of cycle.
GRAPH NO. 37

ACID PHOSPHATASE

B-GLUCURONIDASE

DAYS MENSTRUAL CYCLE

ENZYME ACTIVITY

1 5 9 13 17 21 23 25 29
GRAPH NO 38

ALKALINE PHOSPHATASE

ESTERASE

ENZYME ACTIVITY

DAYS MENSTRUAL CYCLE
C) Alterations in enzyme activities during pregnancy and postpartum:

In the women pregnancy period is of about 270 to 279 days, which can be easily divided into three phases: first trimester, second trimester and third trimester. The third trimester ends with parturition, the period following parturition is called as postpartum.

At a general level, it was found that the enzyme activities in the saliva during the first trimester of the pregnancy were moderate, which gradually increased in the second trimester. The enzyme activities exhibited a sharp increase in the third trimester, reaching peak values at the end of pregnancy just prior to delivery. Following parturition the enzyme activities exhibited a sharp and significant decrease in the first 5 to 6 days. Identical alterations in the enzyme values were obtained in the pregnancy of women belonging to different age groups, with some very minor differences. For further considerations of the alterations in the individual enzymes during pregnancy, average values of all the age groups are taken for description.

1) θ-glucuronidase:

The alteration in the θ-glucuronidase activity during pregnancy are tabulated in Table 18 and these changes are graphically presented in graph No.39. All the activities of θ-glucuronidase described henceforth are calculated for 100 ml of saliva.
During the first trimester of the pregnancy, the enzyme activity was comparatively low. On the 45th day of pregnancy it was 2002 ± 50 F.U. On 60th day it increased to 2248 ± 52 F.U. and on 90th day it was about 2999 ± 62 F.U. During the second trimester, the enzyme activity increased gradually, on the 120th day of pregnancy the enzyme activity was about 3749 ± 70 F.U., on 150th day of pregnancy it was about 4020 ± 72 F.U. and on 180th day it reached a value of 4897 ± 73 F.U. In the third trimester the enzyme activity increased very sharply, on 210th day of pregnancy the activity was about 5361 ± 74 F.U., it increased to 6755 ± 69 F.U. on 240th day of pregnancy. During the last phase of trimester i.e. 9th month of pregnancy, the β-glucuronidase activity reached a peak which was about 8010 ± 50 F.U.

In the postpartum period, the β-glucuronidase activity decreased sharply which was about 6301 ± 120 F.U. on the first day of postpartum. On the 2nd day it was near about 3000 ± 50 F.U. and on 3rd day the activity was about 2998 ± 50 F.U. The β-glucuronidase activity was very low on the 4th and 5th days of postpartum. On the 4th day it was 1603 ± 50 F.U. and on the 5th day it was about 1009 ± 70 F.U.

2) Acid Phosphatase:

The alterations in the acid phosphatase activity during pregnancy are shown in Table 18 and they are graphically represented in graph No.39. The activity of acid phosphatase in the human
saliva was calculated in the p-nitrophenol units per 100 ml of the saliva.

During the first trimester on the 45th day of pregnancy, the acid phosphatase activity was 960 ± 30 units, which increased to 1067 ± 70 units on the 60th day of pregnancy. The average values during the 3rd month were about 1263 ± 129 units. In the second trimester the activity was slightly high, on the 120th day of pregnancy the enzyme activity was 1379 ± 151 units and on 150th day it increased to 1764 ± 120 units. On 180th day the activity was slightly high as compared to that in the previous month, which was 2253 ± 135 units. During third trimester activities were considerably higher as compared to those in the first and second trimesters on 210th day of pregnancy it was 2508 ± 145 units, which increased to 3252 ± 132 units on 240th day of pregnancy. On 270th day of pregnancy the activity was maximum on the saliva of women, which was 3709 ± 251 units.

In the postpartum period the acid phosphatase activity decreased considerably. On first day of postpartum it was about 2728 ± 36 units. The enzyme activity on the 2nd day of postpartum was 2118 ± 26 units and 2001 ± 52 units on the 3rd day. On the 4th and 5th days the enzyme activities were 1256 ± 29 and 938 ± 27 units respectively.

3) Esterase:

The changes in the esterase activity during the pregnancy are described in Table 18, and these alterations are graphically
represented in graph No. 40. All the esterase activities hereafter described are calculated on the basis of p-nitrophenol units per 100 ml of saliva.

During the first trimester on 45th day of pregnancy, the enzyme activity was 70 ± 7 units, which increased slightly on 60th day of pregnancy when it was about 75 ± 11 units. At the end of the first trimester, the enzyme activity was considerably high as compared to those observed in the first two months of pregnancy, on the 90th day it was about 105 ± 9 units. In the second trimester, the enzyme activity was still higher than those observed in the first trimester. It was about 125 ± 8 units on the 120th day of pregnancy. The enzyme activity increased to 168 ± 17 units and 208 ± 11 units on 150th and 180th days respectively. During the 3rd trimester on 210th day of pregnancy, the esterase activity was about 240 ± 9 units and on 240th day it reached to 256 ± 13 units. At the end of the third trimester enzyme activity was maximum, on the 270th day of pregnancy the esterase activity was 261 ± 72 units.

During the postpartum period the esterase activity showed a gradual decrease. On the 1st day after parturition it was about 226 ± 25 units, on the 2nd day the activity was 205 ± 20 units and on 3rd day it was 148 ± 18 units. On the 4th and 5th days of postpartum the activities were too low, they were 58 ± 2 units and 45 ± 7 units respectively.
4) **Alkaline Phosphatase:**

Alterations in the alkaline phosphatase activity during pregnancy are shown in Table 18 and they are graphically illustrated in graph No. 40. The activity of alkaline phosphatase in the women saliva was calculated on the basis of p-nitrophenol units per 100 ml of saliva.

During the first trimester on 45th day of pregnancy, the alkaline phosphatase activity was about 1560 ± 60 units, which increased slightly on 60th day when it was 1874 ± 70 units. At the end of the first trimester, alkaline phosphatase activity increased 2355 ± 70 units. During the second trimester there was gradual but sizeable increase in the enzyme activity, on 120th day, the enzyme activity was about 2504 ± 50 units and on 150th day it increased upto 2774 ± 50 units. At the end of the second trimester the enzyme activity reached a value of 3760 ± 120 units. In the third trimester alkaline phosphatase activity was highest, on 210th day of pregnancy it was 4507 ± 50 units and on 240th day it was about 4809 ± 72 units. At the end of the pregnancy the alkaline phosphatase activity was maximum, which was 6503 ± 71 units just prior to delivery.

In the postpartum period, alkaline phosphatase activity gradually decreased, on 5th day of postpartum it was about 1/7th of that observed during late pregnancy. On the first day of the postpartum, enzyme activity was 5280 ± 56 units, which decreased to 3748 ± 29 units on the 2nd day, and it was about 3008 ± 71 units.
GRAPH NO. 40

ALKALINE PHOSPHATASE

ESTERASE

DAY OF PREGNANCY  DAYS POST PARTUM

[Graph showing enzyme activity over days of pregnancy and days post partum for alkaline phosphatase and esterase.]
D) Alterations in enzyme activity during growth, attainment of reproductive maturity and senesence:

Very distinct and marked alterations in the activities of β-glucuronidase, acid phosphatase, esterase and alkaline phosphatase, were observed during the period of growth, attainment of reproductive maturity and senesence of human beings. At a general level, the average enzyme activities were very low in the children belonging to both the sexes within the age group of 5 to 10 years. Following this age, in the next 5 years i.e. within the age group of 10 to 15 years, a distinct difference could be observed in the alterations in the enzyme activities in the two sexes, the girls showed a very distinct and sizeable increase in the activities of all the enzymes by the time they reached an age of about 13 years, whereas in the case of boys very little and insignificant increase in the enzyme activities could be observed till they reached the age of 15 years. In the subsequent growth phase of 5 years i.e. within age group 15 to 20 years, the male sex exhibited a very sharp increase in the enzyme activities, the females also exhibited a sizeable increase in the enzyme activities, but in comparison with the male sex it was comparatively less sharp. No significant alterations could be observed during the functional reproductive period in the activities of
various enzymes in the male and female adults. In case of women the activities of all the enzymes remained practically unaltered within the age group 20 to 45 years. The four enzymes under investigation showed a decrease in their activity at different ages following the age of 45 years in women. In the case of men, on the other hand, the enzyme activities reached a maximum at the age of 20 to 35 years, following which the enzyme activities remained practically constant in the functional reproductive period. The activities of the enzymes did not undergo any change till the age of 60 years, following which the activities of all the enzymes exhibited decrease which was not as sharp as that observed in the case of women belonging to this age group.

The details of the alteration in the four enzymes during growth, attainment of reproductive maturity and senescence are described hereafter:

1) β-glucuronidase:

The alteration in the β-glucuronidase activities during growth, attainment of reproductive maturity and senescence both in men and women are tabulated in Table 19, these alterations are graphically represented in graph No.41 in which the alterations in β-glucuronidase activity are plotted as a function of age.
The saliva of the 5 year old boys exhibited β-glucuronidase activity equivalent to 332 ± 69 F.U. During the growth from 5 to 10 years, not very significant increase in the enzyme activity could be observed, the β-glucuronidase activity in the saliva of the 10 years old boys was equivalent to 501 ± 63 F.U. From 10 to 15 years period of growth, the enzyme activity exhibited a very gradual but insignificant increase. At the age of 12 years, the average activity was 562 ± 108 F.U., whereas at the age of 15 years, it was 660 ± 98 F.U. Thus, during the growth from 5 years to 15 years the enzyme activity doubled.

During the growth from 15 years to 25 years, very distinct, sharp and marked alterations in the β-glucuronidase activity were observed. In the growth period from 15 to 20 years, the enzyme activity showed a 3½ fold increase. At the age of 15, the average enzyme activity was 660 ± 98 F.U., whereas at the age of 20 years, it was 2332 ± 121 F.U. In comparison with the enzyme activity found in the 5 years old boys, this activity is more than 7-fold. The growth from 20 to 25 years also witnessed further sharp increase in the enzyme activity. At the age of 25, the average activity was 4011 ± 137 F.U., which in comparison with the activity observed at the age of 5, was about 13-fold. During the functional reproductive period from 25 years to about 60 years, no significant alterations in the β-glucuronidase activity could be noted, the average activity was about that described for the age of 25 years.
Following the age of 60 years, some decrease, not very significant, could be observed in the β-glucuronidase activity. In the old men of the age of 65 years, the average enzyme activity was $3834 \pm 149$ F.U., whereas in the 70 years old men, it was $2678 \pm 137$ F.U. How further aging affects the β-glucuronidase secretion in the saliva of very old men beyond the age of 70 years, could not be studied for want of enough number of subjects in this advanced age group.

II. Variations in the β-glucuronidase in saliva of women:

The saliva of the 5 year old girls exhibited β-glucuronidase activity which was practically identical to that found in the 5 year old boys, and equalled to $332 \pm 130$ F.U. During the growth from 5 to 10 years, as in the case of boys, an increase in β-glucuronidase activity could be observed, which was a little more than that observed in case of boys. The 10 year old boys showed the enzyme activity which was equivalent to $501 \pm 63$ F.U., but in the case of the girls belonging to this age group the enzyme activity was $663 \pm 141$ F.U. As against the gradual and insignificant increase found in the growth period from 10 to 15 years in the case of boys, the girls during identical growth period showed a very sharp and remarkable increase in their saliva β-glucuronidase content. From the average enzyme activity of $663 \pm 141$ F.U. found in the saliva of 10 year old girls, the girls who had attained the age of 15 years showed enzyme activity which was equivalent to
2008 ± 154 F.U., thus exhibiting an increase of more than 3-fold within a span of 5 year growth during which the girls attained reproductive maturity. In comparison with the enzyme activity exhibited at the age of 5 years, this activity at the age of 15 years was more than 6-fold.

During the growth from 15 years to 25 years a very distinct, sharp and remarkable increase, as in the case of boys belonging to this age group, could be noted in case of girls also. At the age of 20 years, the girls exhibited the enzyme activity which was equivalent to 3007 ± 148 F.U. and at the age of 25 years this activity reached the value of 3510 ± 198 F.U., the activity from 15 to 25 years being determined in the late proliferative phase of menstrual cycle. During the functional reproductive period ranging from 20 years to 45 years, the activity as determined in the late proliferative phase of menstrual cycle did not show any significant alterations and fluctuated within very narrow and statistically insignificant ranges averaging at 3510 ± 96 F.U. It should be mentioned here that the females within the age group 15 to 45 years did exhibit the typical alterations in their saliva β-glucuronidase activity during the menstrual cycle as described before.

Following the age of 45 years, some change in the saliva β-glucuronidase activity could be noted. The enzyme activity exhibited a gradual decrease from the age of 45 to 70 years. At the age of 50 years, the average enzyme activity was 3160 ± 152 F.U., whereas at the age of 55 years it was 2834 ± 45 F.U., which
further decreased in the saliva of women at the age of 60, when the enzyme activity was $2116 \pm 151$ F.U. At the age of 65 years the activity was $1958 \pm 157$ F.U., whereas at the age of 70 years the activity further decreased to $1868 \pm 152$ F.U. How further aging affected the $\beta$-glucuronidase secretion in the saliva of very old women beyond the age of 70 years, could not be determined for want of enough number of subjects belonging to this advanced age group.

The enzymatic alterations after the age of 45 years in men and women show a distinct but at the same time interesting difference. In the case of men, decrease in the enzyme activity was observed after the age of 60 and this decrease was also not very significant, whereas in case of women the enzyme activity started decreasing at the age of 45 years and decrease with the advancing age from 45 to 70 years was remarkable in comparison with that observed in case of men.

2) Acid Phosphatase:

The enzyme activity for the acid phosphatase has been calculated on the basis of $\mu$-nitrophenol units per 100 ml of the saliva and hence all the figures of enzyme values described hereafter are for 100 ml of the saliva. The variations in the acid phosphatase during growth, attainment of reproductive maturity and senescence in both the sexes are illustrated in Table 19 and these variations in the activity are graphically
represented in graph No. 41, in which activities are plotted as a function of age.

I. Variations in the acid phosphatase in saliva of men:

The saliva of 5 year old boys contained the acid phosphatase activity equivalent to 249 ± 32 units. There was no remarkable increase during the growth from 5 to 10 years, 10 years old boys saliva showed the acid phosphatase activity equivalent to 510 ± 69 units. From 10 to 15 year period of growth, the enzyme activity exhibited very gradual and insignificant increase, at the age of 15 years it was 1565 ± 132 units, thus, during the growth from 5 to 15 years, the enzyme activity increases by more than 5 fold.

Very distinct alterations in the acid phosphatase activity were observed during the growth from 15 to 25 years. In the period from 15 to 20 years, the enzyme activity showed 1½ fold increase. At the age of 15, the average enzyme activity was 1565 ± 132 units, and at the age of 20 it was 2508 ± 149 units. When this activity was compared with the activity of 5 years old boys it could be observed that the increase in the activity is more than 10 fold. The growth from 20 to 25 years witnessed further increase in the enzyme activity which was insignificant. At the age of 25 the average activity was 2809 ± 162 units. From 25 to 30 years, no further increase in the enzyme activity could be discerned. Some increase was observed from the age 30 to 40 years, when the enzyme activity increased by about 125 units.
At the age of 30 years, the average enzyme activity was 2814 ± 139 units, whereas at the age of 40 years it was 2910 ± 152 units. From 40 to 55 years, the average activity varied between 2949 to 2978 units.

After the age of functional reproductive period, some insignificant decrease could be observed in the acid phosphatase activity. In the men of the age of 65 years the average enzyme activity was 2046 ± 109 units, whereas in the 70 year old men it was 2000 ± 130 units. How the enzyme activity is affected in further aging beyond the age of 70 years could not be studied for want of enough number of subjects for such a study.

II. Variation in the acid phosphatase in saliva of women:

The enzyme activity in the saliva of 5 year old girls was practically identical to that of the saliva of 5 years old boys and equalled to 251 ± 43 units. During growth from 5 to 10 years, as in the case of boys, there was an increase in the enzyme activity, which was little more than that observed in the case of boys. The enzyme activity at the age of 10 years in case of boys was 510 ± 39 units, but in case of 10 year old girls this activity was 666 ± 152 units. As against the gradual and insignificant increase found in the growth period from 10 to 15 years in the case of boys, the girls during identical growth period showed a very sharp and remarkable increase in their saliva acid phosphatase. The enzyme activity in the saliva of 10 year old girls was 666 ± 52 units, whereas the girls who had attained the
age of 15 years the average enzyme activity was equivalent to 2008 ± 143 units, thus exhibiting an increase of more than $3\frac{1}{2}$ fold within a span of 5 years growth during which the girls attained reproductive maturity. In comparison with the enzyme activity exhibited at the age of 5 years, this activity at the age of 15 years was more than $6\frac{1}{2}$ fold.

Very distinct, sharp and remarkable increase in the enzyme activity was observed in the saliva of girls during their growth from 15 to 25 years. At the age of 20 years, the enzyme in the saliva of girls exhibited activity equal to 2166 ± 147 units, whereas at the age of 25 years this activity increased to 2445 ± 151 units. At the age of 30 years, the enzyme activity was 2634 ± 142 units and at the age of 35 years, it increased to 2736 ± 142 units, the activity from 15 to 35 years being determined in the late proliferative phase of menstrual cycle. During the 35 to 55 years, the activity determined in the late proliferative phase of menstrual cycle, did not show any significant alterations and fluctuated within very narrow and insignificant ranges averaging in between 2708 ± 169 to 2683 ± 153 units. It should be mentioned here that the females within age group 15 to 45 years did exhibit the typical alterations in their saliva acid phosphatase activity during the menstrual cycle as described before.

Following the age 55 years, some changes in the saliva acid phosphatase activity could be noted. The enzyme activity
showed decrease from the age of 55 to 70 years. At the age of 60 years, the average enzyme activity was 2289 ± 131 units, whereas at the age of 65 years the average enzyme activity was about 2134 ± 123 units and at the age of 70 years the activity further decreased to 2066 ± 38 units. In comparison with \( \beta \)-glucuronidase, the decrease in the acid phosphatase activity in saliva of women in the age group 55 to 70 years is not very sharp. How further senescence affected the acid phosphatase secretion in the saliva of very old women beyond the age of 70 years, could not be determined as it was difficult to get enough number of subjects of this advanced age group.

The sharp difference in the decrease in the activity of \( \beta \)-glucuronidase in the men and women in the advanced age noted earlier, could not be found in the case of acid phosphatase activity. But there was a distinct difference in the age of men and women, when such a decrease in the enzyme activity occurred. In the case of men, decrease sets in at the age of 60 to 65 years but in the case of women, this sets in at the age of 50 years.

3) Esterase:

The variations in the esterase activity during growth, attainment of reproductive maturity and senescence both in men and women are recorded in Table 19, these variations in esterase activity are plotted as a function of age in graph No.42. The
enzyme activity was calculated on the basis of p-nitrophenol units for 100 ml of saliva.

I. Variations in the esterase in saliva of men:

The saliva of 5 year old boys exhibited esterase activity equivalent to 7 ± 2 units. During the growth from 5 to 10 years, there was no significant increase in the esterase activity. The enzyme activity of 10 years old boys was about 17 ± 5 units. During 10 to 15 years period of growth, the enzyme activity exhibited very gradual but insignificant increase. At the age of 12 years, the average enzyme activity was 23 ± 6 units, whereas at the age of 15 years it was 27 ± 5 units. It could be seen that during the growth from 5 to 15 years, the enzyme activity increased by about 4-fold.

During the growth from 15 to 25 years, increase in the enzyme activity was observed. In the growth period from 15 to 20 years, the enzyme activity exhibited an increase by about 10 units. At the age of 15 years the average enzyme activity was 27 ± 5 units, whereas at the age of 20 years it was 37 ± 5 units. When the enzyme activity of 5 year old boys was compared with that of the 20 year old boys, the enzyme activity exhibited an increase by about 5-fold. Growth from 20 years to 25 years also witnessed further sharp increase in the enzyme activity. At the age of 25, the average enzyme activity was 47 ± 5 units, which in comparison with the activity observed at the age of 5, was about 7 fold. During the age of 30 to 60 years, no signifi-
cant alterations in the esterase activity could be noted, the average activity varying about 55 units.

Following the age of 60, some decrease but not very significant, could be observed in the esterase activity. At the age of 65 years the average esterase activity was 43 ± 7 units. In the old men at the age of 70 years the enzyme activity was 42 ± 2 units. Alterations in the esterase secretion in the saliva of very old men beyond the age of 70 years could not be studied, as it was difficult to get enough number of the subjects in this advanced age group.

II. Variation in the esterase in saliva of women:

The saliva of 5 year old girls showed esterase activity, which was practically identical to that found in 5 year old boys and was equivalent to 7 ± 5 units. During the growth from 5 to 10 years, as in case of boys, an increase in the esterase activity could be observed. In the saliva of 10 year old boys the esterase activity was 17 ± 5.2 units, but in the girl belonging to the same age group the enzyme activity was 25 ± 5 units. As against the gradual insignificant increase found in the growth period from 10 to 15 years in the case of boys, the girls during identical growth period exhibited remarkable increase in the esterase activity. The average enzyme activity in 10 year old girls was about 25 ± 5 units, while girl who had attained the age of 15 years showed enzyme activity which was equivalent to 37 ± 8 units.
In comparison with the activity exhibited at the age of 5 years, this activity at the age of 15 years was more than 5 fold.

During the growth from 15 to 25 years, gradual increase in the esterase activity was observed. In the saliva of girls at the age of 20 the enzyme activity was equivalent to 40 ± 5 units, whereas at the age of 25 years it increased to 42 ± 3 units, the activity from 15 to 25 years being determined in the late proliferative phase of menstrual cycle. During the 25 to 50 year growth period, the enzyme activity as determined in the late proliferative period of menstrual cycle, did not show any significant alterations and fluctuated within very narrow and statistically insignificant ranges, averaging at 47 units. It should be mentioned here, that the women within the age group 15 to 45 years did exhibit the typical alterations in their saliva esterase activity during the menstrual cycle as described earlier.

Some changes were observed in the enzyme activity after the age of 50 years. The enzyme activity exhibited a gradual decrease from the age of 50 years to 70 years. The enzyme activity was about 41 ± 17 units at the age of 55 years; which further decreased at the age of 60 when it was equivalent to 40 ± 11 units. At the age of 65 years the enzyme activity was 38 ± 5 units, whereas at the age of 70 years it further decreased to 34 ± 3 units. How further ageing affected the esterase secretion in the saliva of very old women beyond the age of 70 years, could not be determined for want of enough number of subjects belonging to this advanced age group.
As in the case of β-glucuronidase and acid phosphatase, certain differences in the ages when the salivary esterase activity starts decreasing could be noted in the two sexes. In the case of men, decrease in the enzyme activity was observed after the age of 60 years and this decrease was not very significant, whereas in case of women the enzyme activity started decreasing at the age of 50 years and continued through 70 years. The decrease in the women is more sharper than in men.

4) **Alkaline Phosphatase**

The alkaline phosphatase activity was calculated on the basis of β-nitrophenol units for 100 ml of saliva. The variations in the enzyme activities during growth, attainment of reproductive maturity and senescence both in men and women are recorded in Table 19, these alterations in the alkaline phosphatase activity are illustrated graphically in graph No.42 in which variations in the alkaline phosphatase activity are plotted as a function of age.

I. **Variations in the alkaline phosphatase in saliva of men**

Saliva of 5 year old boys exhibited alkaline phosphatase activity which was equivalent to 466 ± 146 units. During the growth from 5 to 10 years, no significant increase in the enzyme activity could be noticed, the enzyme activity in the saliva of 10 year old boys being about 832 ± 151 units. During 10 to 15 year the enzyme activity showed a very gradual but not significant
increase. At the age of 12 years, the average activity was 987 ± 149 units, whereas at the age of 15 years, it was 1166 ± 163 units. Thus, during the growth from 5 to 15 years, the enzyme activity increased by about $2\frac{1}{2}$ fold.

During the growth from 15 to 25 years, very sharp increase in the alkaline phosphatase activity was observed. During the period of growth from 15 to 20 years, the enzyme activity showed an increase of about 350 units. At the age of 15 the enzyme activity was 1166 ± 51 units, whereas at the age of 20 years it was 950 ± 148 units. The growth from 20 to 25 years also witnessed further increase in the enzyme activity. At the age of 25, the activity was about 2008 ± 43 units, which in comparison with the activity observed at the age of 5, was about $4\frac{1}{2}$ fold. During the functional reproductive period from 25 to about 60 years, no significant alterations in the alkaline phosphatase activity could be noted, the enzyme activity varying between 2005 to 2317 units.

Following the age of 60 years, some insignificant decrease was observed in the alkaline phosphatase activity. In the old men of the age of 65 years the average enzyme activity was 1849 ± 144 units, whereas in the 70 year old men it was 1517 ± 146 units. How further aging affects the alkaline phosphatase activity in the saliva of very old men beyond the age of 70 years, could not be studied for want of enough number of the subjects in this advanced age group.
II. Variations in the alkaline phosphatase in saliva of women:

The enzyme activity in the saliva of 5 year old girls was practically identical to that found in the 5 year old boys, which was about 468 ±151 units. During 5 to 10 years of growth phase, as in the case of boys, an increase in alkaline phosphatase activity was observed, but this increase was sharper in girls than in the boys. The 10 year old boys saliva showed the alkaline phosphatase activity equivalent to 832 ± 152 units, but in case of girls belonging to the same age group the saliva enzyme activity was 915 ± 59 units. As against the gradual and insignificant increase found in the growth period from 10 to 15 years in the case of boys, the girls during identical growth period showed a very sharp increase in their enzyme activity. The average enzyme activity in 10 year old girls was 915 ± 59 units, whereas the girls who had reached the age of 15 years showed enzyme activity which was about 1501 ± 53 units, thus, exhibiting an increase of more than 2-fold within a span of 5 year growth, during which girls attained reproductive maturity. In comparison with the enzyme activity exhibited at the age of 5 years, this activity at the age of 15 years was more than 3 fold.

During 15 to 20 years a very sharp and remarkable increase, as in the case of boys belonging to this age group, could be noted in the case of girls also. At the age of 20 years, the girls exhibited the enzyme activity which was equivalent to 2166 ± 158 units. In further growth no significant change in the esterase
activity seemed to occur. At the age of 25 years, this activity reached the value of $2385 \pm 152$ units. The activity from 15 to 25 years was determined in the late proliferative phase of menstrual cycle. During the 25 years to 55 years, the activity did not show any significant alterations and fluctuated within very narrow and statistically insignificant ranges averaging at 2395 units. It should be mentioned here that the females within age group 15 to 45 years did exhibit the typical alterations in their saliva alkaline phosphatase activity during the menstrual cycle as described before.

Following the age of 55 years, some change in the saliva alkaline phosphatase activity could be noted. The enzyme activity exhibited a gradual decrease from the age of 55 to 70 years. At the age of 60 years the enzyme activity was $2009 \pm 137$ units. At the age of 70 years the activity further decreased to $1666 \pm 41$ units. What happens with the alkaline phosphatase secretion in the saliva in very old age beyond 70 years could not be investigated, for want of enough number of subjects in this advanced age group.

The enzymatic alterations after the age of 55 years in men and women did not show distinct difference as found in case of other enzymes. In the case of men the decrease in the enzyme activity was observed after the age of 60, whereas in case of women the enzyme activity started decreasing at the age of 55 years. In both the cases the decrease in the enzyme activity was gradual and identical.
GRAPH NO. 42

ALKALINE PHOSPHATASE

○ MAN
● WOMAN

ESTERASE

○ MAN
● WOMAN

ENZYMES:

ENZYME ACTIVITY

ENZYME ACTIVITY

0 10 20 30 40 50 60 70

AGE IN YEARS
ENZYME CHARACTERISATION:

The characterisation of the enzymes was performed in the saliva of men and women, in case of women saliva was randomly collected irrespective of their menstrual stage. The characterisation was carried out under three heads:

1) Effects of pH on the enzyme activities.
2) Effects of activators and inhibitors on the enzyme activities.
3) Effects of temperature on enzyme activities.

Experimental details of these studies have already been described under Material and Methods (chapter 2).

1) Effects of pH on the enzyme activities:

A) β-glucuronidase:

Graph No. 43 shows effects of pH on the β-glucuronidase activity of saliva of men and women in acetate buffer (0.01 M) in terms of optical density read at 540 μ. In acetate buffer (0.01 M) β-glucuronidase showed single pH optima in the saliva of both men and women at pH 4.4. At a general level, it was observed that the pH tolerance of saliva of human being was moderate. The β-glucuronidase activity increased sharply from pH 3.6 to 4.4 following which it decreased very sharply at pH 4.6 and then from pH 4.6 to 5.6 there was further sharp decrease.
B) Acid Phosphatase:

Graph No. 43 shows effects of pH on the acid phosphatase activity in the saliva of men and women in citrate buffer (0.05 M) in terms of optical density read at 400 μm. In the citrate buffer (0.05 M) the acid phosphatase in the saliva of human beings showed single pH optimum pH 4.6. The enzyme activity increased gradually from pH 3 to 4.6 and after pH 4.6 it decreased gradually up to pH 5 and then sharply up to pH 5.8.

C) Esterase:

The effects of different pH on the esterase activity in the saliva of men and women in phosphatase buffer (0.66 M) are described in graph No. 44. The optical densities of enzymatic reactions are plotted as a function of pH. The optical densities were read at 400 μm. In phosphate buffer (0.66 M) rather restricted pH optima was exhibited by esterase in the women saliva, but in the saliva of men a rather wide optimum pH range was observed. In case of women the enzyme activity gradually increased from pH 5.7 reaching a maximum at pH 7.1 following which it decreased gradually till pH 8.5, whereas in case of men it increased gradually up to 7.1 and remained unchanged till 7.3 and then it decreased gradually till pH 8.5.

D) Alkaline Phosphatase:

Graph No. 44 describes the pH optima of alkaline phosphatase activity of the saliva of men and women in glycine NaOH buffer
Graph No. 43

**Acid Phosphatase**

- **Man:**
- **Woman:**

**B. Glucuronidase**

- **Man:**
- **Woman:**
The enzyme activities in terms of optical density are plotted as a function of various pH levels. pH optima of saliva of both men and women alkaline phosphatase in glycine-NaOH buffer was found to lie at 10.6, though the enzyme showed appreciable activity at pH 10, 10.3, 10.9 and 11.2. The enzyme showed a sharp rise in the activity from pH 9.1 to pH 10.6, but a decrease from 10.6 to 12.1 which was very gradual.

II. Effects of activators and inhibitors on the enzyme activities:

The values of the enzymes are expressed as percentage of controls, where the control activity is treated as 100%.

1) β-glucuronidase:

The albumen was found to increase the enzyme activity, whereas mucic acid, glucuronic acid, mercuric chloride and saccharo-1-4-lactone were found to inhibit the β-glucuronidase activity, the degree of inhibition being different. The glucuronic acid was found to be less potent inhibitor, whereas mucic acid and saccharo-4-4-lactone were effective inhibitors of this enzyme. Heavy metal ion such as Hg²⁺ was most effective inhibitor.

Albumin at a concentration of 0.01% in the substrate medium showed 23% activation of the enzyme activity. The glucuronic acid in a concentration of 0.001 M showed 11%
inhibition. The mucic acid (boiled) at a final concentration of the substrate medium 0.001 M showed 79 % inhibition and saccharo-
1-4-lactone (0.01 M) showed 96 % inhibition, whereas HgCl\textsubscript{2} at a concentration of 0.0005 M showed 100 % inhibition. Not much difference could be noted in the salivary $\beta$-glucuronidase of men and women in response to various activators and inhibitors.

2) Acid Phosphatase:

Effects of ammonium molybdate, sodium fluoride, calcium chloride, pyridoxine and nicotinic acid on acid phosphatase of saliva of human beings showed that, ammonium molybdate inhibited more than 95 % acid phosphatase activity. The pyridoxine and sodium fluoride were also found to be strong acid phosphatase inhibitors, whereas calcium chloride and nicotinic acid were moderate inhibitors of this enzyme. MgCl\textsubscript{2} did not inhibit the acid phosphatase activity.

The ammonium molybdate at the concentration of 0.001 M showed 99 % inhibition of acid phosphatase activity. The pyridoxine (0.01 M) and sodium fluoride (0.01 M) were found to inhibit 84 % acid phosphatase activity, whereas 25 % enzyme activity was inhibited by calcium chloride and nicotinic acid. Not much difference could be noted between the acid phosphatase in the saliva of men and women in its response to various inhibitors.
3) **Esterase** :

The mercuric chloride seems to be the most strong inhibitor for esterase, whereas sodium fluoride and eserine sulphate are moderate inhibitors. Mercuric chloride at a concentration of 0.01 M in the substrate medium showed 100% inhibition, whereas sodium fluoride (mg/ml) and eserine sulphate (0.0001 M) showed 62% and 57% inhibition respectively. Practically identical values of inhibition were obtained in the salivary esterase of men and women.

4) **Alkaline Phosphatase** :

The magnesium ions enhanced the alkaline phosphatase activity, whereas zinc and beryllium sulphate inhibited the enzyme activity. The enzyme activity is also inhibited by anions such as phosphate and arsenate. At a concentration of 0.01 M in the substrate mixture, MgSO₄ enhanced the alkaline phosphatase activity by 24%. Zinc and beryllium sulphate at 0.0001 M concentration effected 80% inhibition of the activity, whereas disodium phosphate (0.01 M) and sodium arsenate (0.001 M) inhibited the enzyme activity by 47%.

**Thermal stability** :

1) **β-glucuronidase** :

The effects of heat treatment on the β-glucuronidase of human saliva both of women and men are shown in graph No.45.
The graph shows a single thermal decay of the enzyme activity. As in the case of β-glucuronidase of salivary glands of rat, the β-glucuronidase of human saliva also exhibited high heat stability in comparison with acid phosphatase and esterase. It lost 62% of the total activity after 45 min treatment at 65.5°C.

2) Acid Phosphatase:

The effects of heat treatment on acid phosphatase activity in the saliva of men and women are compiled in graph No. 45. The enzyme acid phosphatase showed a single decay as that of β-glucuronidase. But it was heat-labile as compared to β-glucuronidase. It showed 94% loss with the treatment of 45 min at 65.5°C.

3) Esterase:

Graph No. 46 shows effects of heat treatment on the activity of esterase in human saliva of both the sexes. The esterase activity showed 85.7% loss after 45 min treatment at 65.5°C, thus, indicating its heat-labile nature.

4) Alkaline Phosphatase:

Effects of heat treatment on the alkaline phosphatase activity of men and women saliva are reported in graph No. 46. As compared to other three lysosomal enzymes, the alkaline phosphatase was more heat-labile, where it showed 58% loss after the treatment for 45 min at 65.5°C.
ALKALINE PHOSPHATASE
© MAN
© WOMAN

ESTERASE
© MAN
© WOMAN

Graph No. 46

Log % Remaining Enzyme Activity

Minutes at 65.5°C

Log % Remaining Enzyme Activity
DISCUSSION:

I. Sexual dimorphism:

From the biochemical point of view, the situation in the human beings is practically the same as that observed in case of white rats, with the difference that in the human beings the enzyme were assayed in the whole saliva secreted by the three major salivary glands, whereas in the rats the enzymes were assayed in the homogenates of the glands. This is an interesting point, which shows that the enzymes under investigation which are elaborated in the salivary glands are actually secreted out into the saliva. Especially from the point of view of the lysosomal enzymes, which function within the living cells (De Duve and Wattiaux, 1966; De Duve, 1969), this is a partinent thought and will be discussed elaborately in a later part of the present chapter.

While searching for sexual dimorphism in these enzymes in the human beings, a random choice of sexually mature and potent men is acceptable, but such a random choice of sexually mature and fertile women is not acceptable, since in the case of women the activities of the enzymes vary with the phases of the menstrual cycle. If women in proliferative phase of menstrual cycle and sexually mature men are taken for such a study, practically no difference in the average enzyme activities could be seen, thus, indicating absence of any quantitative sexual dimorphism in these enzymes in the saliva of men and women.
But if women in their menses stage are taken, a distinct sex-related quantitative difference in the enzyme activities could be observed. The saliva of men contained these enzymes in concentrations which are about 8 to 7 times in case of β-glucuronidase and acid phosphatase, and 3 to 4 times in case of esterase and alkaline phosphatase that in the women in the menses period of menstrual cycle.

Leaving aside such a quantitative sex-related difference in the activities of these enzymes, we have no other criteria to describe the sexual dimorphism in these enzymes in the salivary secretion of men and women. Especially which of the three major salivary glands are responsible for the secretion of these enzymes and which glands exhibit variations in their secretions of these enzymes in women in menstrual cycle and pregnancy, cannot be said from the present observations, since these enzymes were assayed in the whole saliva.

There are some stray reports which describe the localization of these enzymes in the human salivary glands. Glocker et al. (1938) were the first to describe the presence of acid phosphatase activity in human saliva. Chouncy et al. (1954) also examined mixed saliva of human beings and reported that the acid phosphatase activity varied between 0.5 to 13.025 % μmols in their assay technique. They also reported that this enzyme was also present in the parotid saliva and in its kinetics it resembled the acid phosphatase of blood and prostate. With respect to β-glucuronidase, this enzyme was studied in the human
mixed saliva by Chouncy et al. (1954), Harvey and Panse (1960) and Fishman et al. (1962). No histochemical studies to illustrate the distribution and localisation of this enzyme in the human salivary glands have yet been made. Esterase activity has been reported in the sublingual gland of human beings by Burstone (1956). Though the presence of alkaline phosphatase in the mixed saliva has not been rejected, its localisation in the myoepithelial cells in the human submandibular gland is not universally accepted (Gomori, 1941; Kawakatsu and Mori, 1962; Archer and Koa, 1968; Garrett and Harrison, 1970).

The present report, hence, is first of its kind and illustrates not only the presence of acid phosphatase, β-glucuronidase, esterase and alkaline phosphatase in the mixed saliva of human beings but also a sex-related quantitative difference in the activities of these enzymes.

II. Enzymatic alterations in menstrual cycle and their possible relation to hormonal variations:

The alterations in the activities of the enzymes in the mixed saliva of women during menstrual cycle, form an interesting parallel to the alterations seen in these enzymes in the submaxillary of rat during estrus cycle. At a general level, the activities of all these enzymes are lowest in menses, they increase gradually during early proliferative phase but in late proliferative phase they increase sharply attaining a peak on 13th or 14th days of the cycle, following which in early luteal
phase they exhibit some decrease but in late luteal phase on 23rd or 24th days they exhibit some increase and again decrease from 25th to 28th days of the cycle. The enzymes exhibit such cyclic variations in their activities during consecutive menstrual cycles, unless and otherwise pregnancy occurs. Since this is a set pattern of the enzymatic alterations in the menstrual cycle, they can very well be interpreted in the light of the hormonal variations in such cycles. The present efforts are, thus, directed at building a relationship between the circadian rhythm in the enzyme activities and the rhythm in the hormonal milieu of the body during menstrual cycle, as has been done in case of rat submaxillary enzymes and hormonal variations in estrus cycle.

During the menstrual cycle in women, not one but several different hormones exhibit a typical rhythm. The alterations in such hormones have been reported by several investigators. Various investigators such as Loéine and Bell (1968), Midgeley and Jaffe (1968), Orr and Elstein (1969), Yussman et al. (1970), Johansson et al. (1971) and Watson (1972) have studied the alterations in the LH levels in the women during menstrual cycle. Classically the LH levels have been shown to be low during proliferative phase as well as luteal phase but showing a sharp increase at the time of ovulation i.e. on 13th to 15th days. The aforementioned authors have studied the LH levels in blood plasma either by bioassay techniques or by radioimmunological technique and have shown that the LH levels attain a peak
on 14th to 16th days. Yokota et al. (1965) showed that the serum level of LH during menses is about 35 μg/100 ml of serum, during 5th to 9th days of early proliferative phase it increases to 50 μg/100 ml of serum, on 10th day it reaches to 80, during 12th to 14th days it varied between 110 to 130 μg/100 ml of serum, following which in luteal phase it again decreased and varied between 35 to 40 μg/100 ml of serum. In a similar manner Ross et al. (1967) showed that the plasma levels of LH average at 27 ± 3 μg/100 ml from 1st to 13th day, whereas on 14th day i.e. the day of ovulation they reach a value of 100 μg/100 ml and during 16th to 27th days they average at 15 ± 2 units.

If the aforedescribed pattern of rhythm of LH secretion is compared with the pattern of variations in the activities of the enzymes under study, the only correlation is that the enzyme activities are maximum when LH secretion is maximum. In the proliferative phase though some increase in LH secretion is observed as in the study of Yokota et al. (1965), it does not form a parallel to the increase observed in the enzyme activities. Moreover, in the menstrual cycle the LH levels drop down very sharply in post-ovulatory period, but the decrease in the enzyme activities in this period is not equally sharp, but actually on 23rd day the enzyme activities show a second peak when the LH levels are actually very low. Thus, excepting the parallelism that is seen in the peak enzyme activities and peak LH levels on the day of ovulation, no other parallel behaviour between the two can be seen.
Another hormone which exhibits typical rhythm during menstrual cycle in the women is the progesterone. Classically it is described that the progesterone levels in the luteal phase are high. In recent years, various workers have attempted to estimate the alterations in the progesterone levels during menstrual cycle by employing modern techniques. Dominguez et al. (1962) showed that the progesterone levels during follicular phase of normal women are 2.3 to 5.4 μg/kg body weight, whereas in the luteal phase it increases to 22-43 μg/kg body weight. In the ovariectomised women, Little et al. (1962) showed that progesterone level was equivalent to 1.3 μg/kg body weight. Mikhail et al. (1963) showed that the levels of progesterone in ovarian vein blood was 110 μg/100 ml on 18th and 19th days of cycle, whereas on 24th day it reduces to 48 μg and on 25th day to 29 μg/100 ml. Short and Levett (1961) measured the progesterone levels in the plasma of blood taken from peripheral blood vessels and showed that, in the proliferative phase of the cycle the progesterone levels varied between 1.0 to 1.4 μg/100 ml, on the day before ovulation it was 0.9 μg/100 ml, on 17th day it increased to 1.5 μg, on 21st day to 3.3 μg and on 25th day it decreased to 2.9 μg/100 ml.

If the alterations in the enzyme activities are viewed in comparison with the above described alterations in progesterone secretion, no parallelism can be built up between the two. The activities of the enzymes increase sharply and attain a peak during proliferative phase and the day of ovulation respectively,
when progesterone levels are very low. In a similar manner in the post-ovulatory period, excepting the increase observed on 23rd day, the enzyme activities decrease gradually when the progesterone levels are high. Naturally progesterone cannot be the hormone responsible for inducing the enzymatic alterations observed during the menstrual cycle.

A better parallelism can be built up between the alterations in the enzyme activities and the estrogen levels during the menstrual cycle. It is classically said that estrogen levels are low during the first 6 days of the menstrual cycle, which increase gradually up to 13th day following which they decrease and again increase on 21st day and then decrease gradually till menses. Brown (1955) and Brown et al. (1958) reported that the levels of estrogen are lowest in the first week of menstrual cycle, they rise in the late proliferative period and attain an ovulatory peak at approximately day 13, secretion rates fall and then rise again to a second peak, somewhat lower than the ovulatory peak, termed as luteal maximum. As the cycle draws to an end, estrogen secretion again falls heralding the onset of menses. Goering and Herrman (1963) reported that the production rate of 17-β estradiol during the premenstrual phase is 20 μg/24 hr, which increases to 150 to 300 μg/24 hr at the time of ovulation.

Thus, the activities of the enzymes under investigation are lowest during menses when the estrogen levels are also lowest,
in the first week of the menstrual cycle the estrogen levels are low when the enzyme activities also exhibit very gradual increase, but when the estrogen levels increase sharply in the second week of the menstrual cycle reaching a peak on 13th or 14th day i.e. the day of ovulation, the enzyme activities also exhibit a parallel sharp increase reaching a peak on 13th and 14th days. Thus, the ovulatory peaks of estrogen and enzyme activities coincide with each other. As the estrogen secretion is lowered down in post-ovulatory period, the enzyme activities also exhibit slight decrease. The second peak in the enzyme activities attained on 23rd day very well coincides with the luteal peak of estrogen activity, following which both the estrogen and the enzyme activities fall down sharply. Thus, when the estrogen levels are low the enzyme levels are also low and when the estrogen levels increase the enzyme levels also increase.

Such a close parallelism between the cyclic secretion of estrogen and the salivary enzyme activities, thus, indicate that, in all probability, the cyclic secretion of the three lysosomal enzymes and alkaline phosphatase in the saliva of women in menstrual cycle are regulated by the levels of estrogen circulating in the body fluid. Such circumstantial conclusion is well supported by our experimental work on rat, wherein it has been conclusively proved that the elaboration of β-glucuronidase, acid phosphatase, esterase and alkaline phosphatase is under the control of estrogen and it is a process of hormone induced enzyme protein synthesis.
The results of the observations on the behaviour of acid phosphatase and allied lysosomal enzymes and of alkaline phosphatase, are of great significance from the point of view of the acid phosphatase and alkaline phosphatase test strip techniques developed by Fester and Raymond in 1966 and 1967. Fester and Raymond (1966, 1967) developed a peculiar test-strip method for alkaline phosphatase study in the saliva of women. This test strip consisted of bibulous material (paper) which was impregnated with a buffer to maintain the pH at 10 to 10.3 and an indicator indoxyl phosphate or 5-bromo indoxyl phosphate. In the presence of the enzyme alkaline phosphatase, there is a cleavage of ester bond of the indoxyl compound which release the indoxyl radicals, in the presence of air two indoxyl radicals combine to form indigo, a dark blue dye. The women to be tested were asked to apply their saliva to this paper strip. According to Fester and Raymond (1966, 1967) the reaction takes place within 0.5 to 2 minutes and the degree of intensity of blue colour formation is related to the enzyme present in the saliva. Phosphate ions in the saliva and those formed by the cleavage of the indicator may inhibit the enzyme reaction, but this difficulty was overcome by including MgSO₄ and MgCl₂ in the test-strip which react with the phosphate ions forming phosphate salts. In the test the female touches the test paper to her tongue to wet it with saliva and waits for a few minutes to see if the colour changes develop. Fester and Raymond showed that such tests indicated that there was an increase in the alkaline phosphatase during ovulatory phase of women.
In a similar manner Faster and Raymond (1967) also developed a test-strip technique for acid phosphatase by changing the pH of the buffer impregnated on the paper strip to 4 to 4.5 i.e. the pH at which the acid phosphatase acts maxillary. The mechanism involved in this technique was very much the same as that involved in alkaline phosphatase technique and in a similar manner an intense blue dye was formed in the presence of acid phosphatase. In this case also, Faster and Raymond (1967) showed that the intensity of colour development depends upon the acid phosphatase content of the saliva. Through this test also, Faster and Raymond (1967) showed that the acid phosphatase content of the saliva is maximum at the ovulatory phase of the women. In a similar manner, Faster and Raymond (1967) also developed a test-strip technique for esterase.

A critical revaluation of these techniques shows that these techniques are more or less parallel to the enzyme histochemical techniques generally employed for tissue sections, in which also the quantitative enzyme contents could not be determined in exact mathematical units and one has to depend upon a visual estimation of the colour developed. Increase or decrease in the enzyme contents depended to a large extent on the intensity of the colour produced on the test-paper strip. The validity of such techniques depends, naturally on the fundamental observations on the variations in these enzymes in exact enzyme units derived from the use of the bioassay techniques, not only in the saliva of women at the ovulatory phase but throughout the entire
menstrual cycle. Such techniques will further need confirmation from experimental work in which salivary contents of these enzymes need be shown to be related to the hormones of either gonadal origin or of pituitary origin which play a role in menstrual cycle and ovulation.

Though these test-strip techniques were evolved in 1966-67, no such confirmatory evidences were available till the time the present work was undertaken. The observations in the present work on one side confirm the validity of the paper-strip techniques and, on the other, raise some interesting points which throw some doubt on such validity.

Our observations show that in relation to peak estrogen surge at the time of ovulation as on 13th or 14th days of the cycle, the enzymes such as alkaline phosphatase, acid phosphatase and esterase and also β-glucuronidase, which was not studied by Fester and Raymond by this technique, also attain a peak, which we have called as the ovulatory peak. Naturally at this time, per unit of the saliva secreted by the women will have maximum contents of these enzymes and hence they will give the maximum intensity of indigo on the paper strip. Thus, Fester and Raymond believed that the intensity of colouration is maximum at the ovulatory period and our observations also show that the contents of these enzymes in the saliva, as assayed by recent and well-accepted biochemical techniques, are maximum at this period. But our observations further show that the ovulatory peak is not the only peak these enzymes attain during the menstrual cycle, since
in the luteal phase on 23rd day they attain a second peak. In the paper strip method, at this time also intense indigo development may occur, which might be misinterpreted as the ovulatory phase. Indigo development on the 23rd day of the luteal phase, thus, may be mistaken for the day of ovulation. Our observations show that difference in the enzyme activities in these two peaks is not vast, the luteal phase maxima activities being somewhat lower than the ovulatory phase maxima. To get such a difference between these two maxima, one has to assay the enzyme activities under the standard conditions of assay such as buffer, pH, temperature, and moreover, one has to carry out estimations in the saliva of number of women at these phases, since individual variations in the activities of these enzymes occur very frequently. Considering this it will be indeed difficult to capture a difference in the intensity of the colour development on the paper strip by the saliva of the women in these two phases of menstrual cycle. Hence there is every probability that the 23rd day of the cycle may be misunderstood as the ovulation day and vice-versa, this being especially true if the menstrual cycles of the subjects to be tested are irregular.

Though the above discussion throws some doubt on the validity of the paper-strip technique, the fundamental concept that the enzyme activities attain a maximum at the ovulatory phase cannot be disputed. Moreover, the present experimental work on female rats has confirmed that the elaboration of these enzymes in the salivary glands is regulated by the estrogen
circulating in the body fluid. In the present study on human saliva also, there is a further evidence which proves this fact. Our studies on the salivary enzyme activities in the growth, attainment of reproductive maturity and senescence have shown, that the cyclic alterations in the salivary enzyme activities come to a halt after the menopause, when the hormonal interplay also recedes.

III. Enzymatic alterations in pregnancy and postpartum
and their possible relation to hormonal variations:

During pregnancy the enzyme activities undergo interesting alterations. During the first trimester, though the enzyme activities can be seen, they are low from 2nd to 3rd months of pregnancy. In the second trimester with the progress in pregnancy, the activities of all these enzymes increase progressively, though the increase is gradual. In the third trimester, the activities increase sharply and attain a peak on the day before the parturition. All the activities decrease very sharply following the delivery in the postpartum.

The hormones that are most directly involved in pregnancy and lactation originate from the pituitary gland, ovary, placenta, and probably from the uterine endometrium. Like many other physiologic processes, these events involve a whole train of balanced forces and cannot be accounted for on the basis of hormones functioning in isolation. This may be true from the point of view of initiation and progress of the pregnancy, but
from the point of view of the secretion of the enzymes in the saliva, all these hormones may not be responsible for such typical alterations in these enzymes.

Of the several hormones, progesterone is one which shows very significant change in the pregnancy, since it is needed for the maintenance of pregnancy. Classically it is said that progesterone levels are low up to the 20th week of pregnancy and they increase in later phases of pregnancy (Guyton, 1964). According to Guyton (1964) progesterone is equivalent to 5 to 7 rat units up to 20th week of pregnancy and it increases up to 35 units in 40 weeks. Amongst various workers, Pearlman (1957), Solomon et al. (1962), Domingêx et al. (1962) and Zander and Munstermann (1966) have shown that the rate of progesterone secretion is very low in early pregnancy till about 15 to 20 weeks i.e. about the first trimester of the pregnancy, which then increases several folds in the second and the third trimesters and these values show a significant drop at the time of parturition. Thus, the behaviour of the salivary enzymes and the progesterone levels during the pregnancy form a very good parallel, thus, indicating that progesterone may be responsible for inducing the enhanced enzyme elaboration in the salivary glands, especially during the second and the third trimesters.

Similar condition exists when the estrogen changes in the pregnancy are viewed in comparison with the salivary enzymatic alterations. Classically it is said that the estrogen levels are low up to the 16th week of pregnancy, which increase sharply
in the late pregnancy up to parturition and following parturition they decrease considerably. The estrogen levels are equivalent to 7000 to 8000 rat units in the first 16 weeks of pregnancy, which then increase sharply to 50,000 units up to the 40th week of pregnancy (Guyton, 1964). Such observations have repeatedly been confirmed by various workers. Pearlman et al. (1954) showed that in the late pregnancy estrone secretion is equivalent to 20 mg/day, whereas estriol is equivalent to 85 mg/day and estradiol 17-β is 5 mg/day. Gurpide et al. (1962) studied in detail the rates of biosynthesis of estrone, estradiol 17-β and estriol. In 4, 5 and 9 month pregnancies the estrone secretion is respectively 7, 9.5 and 31 mg/kg body weight. In case of the estradiol 17-β these values are 7, 8.5 and 26 mg/kg body weight and in case of estriol they are 83, 86 and 230 mg/kg body weight respectively. Similar observations have also been made for the 6 month pregnant women by Fishman et al. (1962). Slaunwhite (1964) also showed that the total estrogen secretion is 7 to 10 mg/100 ml plasma in the 3rd month, whereas it is 37 to 54 mg/100 ml plasma in the 8th month of pregnancy. Steffl and Rose (1969) studied the urinary excretion of estrogen and observed similar increase during the pregnancy.

Thus, if one views the behaviour of estrogen in comparison with that of the salivary enzymes during pregnancy, one finds that when the estrogen secretion is low as in first trimester the enzyme activities are also low, and when the estrogen secretion increases and attains a peak, the enzyme activities also increase
and attain a peak. Both these show a significant fall following parturition. Thus, the enzyme activities closely follow the estrogen levels in the circulatory body fluid.

Thus, both the progesterone and estrogen qualify as hormonal candidates which might be responsible for provoking the enhanced enzyme elaboration and secretion in the saliva. LH need not be considered here, since the LH, which is a human chorionic gonadotropin, can be detected as early as the 24th day and rises to a peak at about 50th day and remains at this concentration for about 14 days and then falls down and remains at this low concentration through the remainder of pregnancy (Rowlands, 1964). Thus, the variations in LH levels do not form a parallel to those observed in the enzyme activities. Of the two candidates viz, progesterone and estrogen, which one is responsible for exerting influence over the elaboration and secretion of the enzymes, cannot be decided from the observations at hand. But in the case of rat, it has been conclusively proved that progesterone has no capacity to regulate the salivary enzyme activities and it is the estrogen which functions as the regulatory hormone of these enzymes. Moreover, in the studies on the alterations in the activities of these enzymes in the menstrual cycle of women, no parallelism could be observed between the behaviour of progesterone and enzyme activities. In the light of these evidences, though circumstantial and in an animal unrelated to human beings, we can exclude progesterone as the regulatory hormone and accept the estrogen as the regulatory hormone affecting alterations in the salivary enzymes during pregnancy.
The above discussion on the alterations in the activities of the enzymes under investigation and their relation to estrogen, thus, shows that as in the case of rats, there also exists a gonad-salivary gland hormone-enzyme axis in the human beings and since the estrogen is regulated by the pituitary, this idea can be stretched further to pituitary-gonad-salivary gland hormone-enzyme axis.

The present studies afford evidences, though circumstantial, to build up a relation between the secretion of acid phosphatase, \( \beta \)-glucuronidase, esterase and alkaline phosphatase and estrogen in the females, but they are at a loss when one tries to theorise on the specific gland and the tissue and cellular sites which respond to varying levels of estrogen. As already emphasised, in the present study mixed saliva has been taken for enzyme studies and such saliva will contain secretions from all the three major salivary glands. Hence which particular gland and its secretion is responsible for the enzymatic alterations in the mixed saliva cannot be deduced from the present observations. Moreover, all the three salivary glands viz., submaxillary, sublingual and parotid have been shown to elaborate the lysosomal enzymes (Chowdry 1954). The literature on sex-related differences in the human salivary glands is poor and hence is of little use in arriving at certain conclusions with respect to the above lacuna in the present study. Harvey and Panse (1961) observed an enhancement in the salivary \( \beta \)-glucuronidase in patients having salivary carcinomas. Similar observation has also been made by Kim and
Plaut (1965). Lamberts (1967) studied amylase activity in the mixed saliva and also parotid saliva, but did not pay any attention to the sex-related differences, if any. Mugner (1964) showed that the submaxillary gland of man is seromucous, serous cell number being predominant, whereas in the women mucous cell number is more. Puskulian (1972) reported that in the women saliva Ca\(^{++}\) content decreases midway between the menstrual cycle, whereas Na\(^+\) and K\(^+\) contents increase. Such variations in electrolytes have been related to ovarian cycle.

In the absence of any decisive data in the available literature, we have to depend upon work on rat and other mammals. Excepting very few cases, in most of the mammals it is the submaxillary gland which has been shown to exhibit sexual dimorphism. In rat it has already been proved that in the female submaxillary gland is sensitive to estrogen and such sensitivity lies at the level of acinar cells and striated ducts. We can assume a similar situation in women and project a hypothetical viewpoint that the enzyme elaboration in the submaxillary of women, especially in the acinar cells and striated ducts, is regulated by estrogen. As in the case of rat, these tissue sites might be the sites of hormone-induced elaboration of acid phosphatase, \(\beta\)-glucuronidase and esterase. If evidences to prove otherwise are put forward, these viewpoints can be taken as directive principles in the gonad-salivary gland relationship in the women.
III. Enzymatic alterations in growth, attainment of reproductive maturity and senescence:

In the studies on growth, attainment of puberty and senescence, some distinct differences could be noted in males and females. In the latter, up to the age of 5 years, the enzyme activities are very low. During 5 to 10 years of growth, there is a gradual increase in the enzyme activities, and from 10 to 15 years of growth, there is a sharp increase in the activities of the enzymes. Girls in India mature at an age of 12 to 18 years. Following such maturation, they start exhibiting the typical enzymatic alterations in the menstrual cycle. If the enzyme activities in the proliferative phase are taken as an index, from 15 to 20 years of growth phase, there is a gradual increase in the enzyme activities. Activities remain more or less constant from 20 to about 45 years. Following menopause, the enzyme activities decrease gradually till 70 years.

These alterations can be interpreted on the basis of manifestation of growth processes and effects of sex-hormones. The gradual increase in the enzyme activities in very early childhood, i.e., till the age of 8 to 9 years, can be taken as an expression of growth. In the first day of tetus, the estrogen activity in the blood plasma is low as assayed by Schwers (1962) which declined to one tenth of the initial value on 3rd and 4th day. At the age of 12 years in Continental countries, the estrogen level in the girls is about 8000 to 10,000 rat units which increase to 15,000 to 20,000 rat units during the attainment of the sexual maturity.
and which they decreased to 10,000 rat units in menopause (Guyton, 1964). According to Berr et al. (1961) the intermediary metabolism of 17-β estradiol in infants and children differs from that of adults. The metabolism of 17-β estradiol assumes the adult pattern afterwards. Thus, in infants and young children estriol apparently does not represent a final stage in estrogen metabolism but constitutes an intermediate stage. Interesting observation here concerns with the enzyme activities after menopause. After menopause the estrogen secretion is lowered down considerably, but actually there is no parallel decrease in the enzyme values. For example acid phosphatase values of 40 year women in the proliferative phase are 2708 ± 69 units, which become equivalent to 2683 ± 53 at the 45 years, in 50 year old women this value is 2066 ± 38, in 60 year old 2033 ± 38 units and in 65 year old women 2010 ± 49 units. Thus, the decrease from 45 years to 65 years is only from 2683 ± 53 units to 2010 ± 49 units i.e. a loss of about 500 units in the old age. In a similar manner, in case of β-glucuronidase the enzyme values at the age of 40, 45, 50, 55, 60 and 65 years are 3510 ± 98, 3166 ± 52, 2923 ± 62, 2634 ± 45, 2516 ± 51 and 2166 ± 54 units. Thus, there is only a loss of about 1350 units in a span of about 15 years during old age. Similar observations can also be made if the values of esterase are studied. Thus, actually a very significant decrease in the enzyme values was expected in the old age, when sex-hormone secretion decreases considerably, but the actually observed depletion in the enzyme activities is insignificant. This fallacy cannot be interpreted from the
available literature and present observations. But one possibility can be suggested. Ellison (1967) studied acid phosphatase, the lysosomal marker enzyme, in the submaxillary of human beings. Bogart (1970) showed that in old age the acid phosphatase activity does not decrease, which he related to the formation of lipofuscin granules as an effect of senescence. Garret (1963) observed large number of lipofuscin granules in the submaxillary of aged human beings. Thus, the lysosomes and their acid hydrolysing enzymes in the salivary glands seem to be released from their hormonal control once menopause and old age set in, but they are caught in the process of senescence. The former process will lead to depletion in these enzymes but the latter, especially the lipofuscin formation, an increase. As a result the lysosomal enzymes do not exhibit very marked decrease after menopause in advanced old age.

In the growth, attainment of puberty and senescence of males also a similar situation is witnessed. In the growth till 5 years the enzyme activities are very low, there is slight increase in the growth from 5 to 10 years and gradual increase in the growth from 10 to 15 years. From 15 to 25 years there is very sharp increase in the enzyme activities. From 25 to 60 years the enzyme activities remain more or less constant. From 60 to 70 years, there is gradual decrease in the enzyme activities.

In the case of male sex, at a general level, the secretion of testosterone is low prior to the attainment of puberty and during the phase of reproductive maturity it increases considerably.
Guyton (1964) described the alterations in the secretion of testosterone in various age groups of men. At the age of 5 years, it is at a concentration of 1 to 3 mg/100 ml of plasma, it increases to 23 mg at the age of 20 to 35 years and during 35 to 40 years it is at the level of 25 mg, during old age 45 to 60 years it is in between 23 to 17 mg/100 ml of the plasma. Thus in case of men also, with the approach of puberty secretion of androgen increases and concomitantly there is an increase in the salivary enzyme values. During the period of active reproduction both the androgen and enzyme activities attain a maximum. In the old age with the depletion in the androgen secretion, there is depletion in the enzyme activities. As in the case of old women, in old men also the decrease in the enzyme activities is not very drastic. For example in case of acid phosphatase the activity decreases from 2410 ± 52 to 2000 ± 130 units in the old age from 60 to 70 years. In case of β-glucuronidase such decrease is from 2166 ± 51 to 1868 ± 152 units. Thus in case of old men also, once the salivary glands escape from the hormonal influence, they come under the influence of senescence which sets in at this stage during which lipofuscin granules are synthesised, in which lysosomes play an important role. Hence the depletion in the enzyme activities is not as sharp as expected.

IV. Problem of secretion of the lysosomal enzymes:

Since in the present study, the lysosomal enzymes such as acid phosphatase, esterase and β-glucuronidase have not only
been detected in the saliva of human beings, but their contents have also been shown to vary according to the ovarian function in the females and testicular function in the males, both being finally under pituitary control, a problem of some academic interest to the cell-physiologists and enzymologists arises. These enzymes have been reported to be localised in the endoplasmic reticulum (microsomes) and lysosomes, and their functional significance is closely related to the functional significance of the lysosomes in the life of the cells. Functions such as autophagic cell-nutrition, heterophagic cell-nutrition, pinocytosis and phagocytosis, cellular autolysis, cellular rejuvenation and cellular scavenging have been attributed to the intracellular vacuolar system of which the lysosomes form an important part (de Duve and Wattiaux, 1966; de Duve, 1969). Conditionally all these functions take place within the living cells and in the literature there are very few references to "secretion" of lysosomal enzymes outside the cells and their extracellular role. The problem of extracellular secretion of the lysosomal enzymes has recently been excellently reviewed by Dingle (1969). Acid hydrolyses are present in almost all tissue fluids but there is no unequivocal evidence of the lysosomal origin of these enzymes in the normal animals. For example it may well be that the acid phosphatase, DNase, β-glucuronidase and the various glucosidases of seminal plasma originate at least in part from the lysosomal complex of undifferentiated spermatozoa (Dott and Dingle, 1968), whilst considerable lysosomal enzyme activity of the prostatic cells
may contribute to that of the prostatic secretion. There are evidences of the secretion of the lysosomal enzymes into plasma (Weissmann et al., 1964) and synovial fluid (Hammerman and Bartland, 1966), but they rest almost entirely on pathological studies. De Duve and Wattiaux (1966) also cite secretion of lysosomal enzymes into bile from the peribiliary liver lysosomes. Most of the evidence for the secretion of the lysosomal enzymes outside the cells, is derived from organ culture studies, such as cartilage in organ-culture (Fell and Dingle, 1963; Dingle et al., 1966; Sledge and Dingle, 1965; Dingle et al., 1968; Dingle et al., 1969) and bone in organ-culture (Dingle, 1963; Vae, 1965) and other tissues in culture (Lasnitzki et al., 1965; Benassi, 1968; Munro et al., 1964). The stimulation of the lysosomal enzyme secretion by retinal, other vitamins and hormones such as parathyroid hormone have also been described (Dingle, 1961; Fell and Dingle, 1963; Vae, 1966; Raynolds and Dingle, 1968). The temporal relationship of enzyme synthesis to secretion was studied by Dingle et al. (1968). Dingle (1969) believes that the extracellular secretion of the lysosomal enzymes is an integral part of the lysosomal activity of the normal cells, often acting synergistically with the endocytotic mechanism, the secretion of the hydrolases playing an important role in the relationship of the cell to its environment.

The observations in the present study add another interesting parameter to the available information on the secretion of the lysosomal hydrolases. The facts that these enzymes are present in
significantly high concentration in the saliva of men and women, that their concentrations undergo cyclic rhythmic alterations in response to ovarian activity in the menstrual cycle and pregnancy and that their activities get enhanced in growth and puberty in relation to enhanced biosynthesis of the sex-hormones, indicate that these enzymes are not only secreted into the saliva, but also that their elaboration in the salivary glands, in all probability in the submaxillary, and secretion are hormonally controlled.

In the present studies, a pituitary-gonad-submaxillary axis of hormone-enzyme relationship is proposed in women from circumstantial evidences available from the behaviour of these enzymes in the menstrual cycle and pregnancy. In all probability a similar axis also exists in males in which the androgen regulates the enzyme activities. Some evidence in support of this can be seen in the studies on growth and attainment of puberty in men. For want of physiological processes in human beings in which androgen undergo cyclic changes, no further evidence can be cited. But our studies on rat have already confirmed presence of such a pituitary-testis-submaxillary hormone-enzyme axis in the male sex. Hence it can be assumed that such axis also functions in the man.

The present observations are of some significance from clinical aspects of human welfare which has been discussed in the last chapter on general discussions. During the course of
the present work on human saliva, certain abnormal results in women having abnormal menstrual cycles and in women who had the misfortune of undergoing abortion, have been obtained.

They are also discussed in the last chapter on general discussions.
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