AN ASSESSMENT OF THREE ENZYME ESTIMATIONS AS POSSIBLE
PLACENTAL FUNCTION TESTS

BY

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This thesis is presented for the Degree of
Doctor of Medicine.

University of Edinburgh
1972
I declare that this thesis has been composed by myself.

The work involved has been carried out by myself, with the exception of some assistance with scoring the slides in neutrophil alkaline phosphatase estimations, and with the initial experiment to establish an assay for glucose-6-phosphatase.

signed...
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DISCUSSION

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To gain a greater insight into the factors affecting fetal prognosis, the obstetrician has striven to find an ideal placental function test. As the search has taken place without a full understanding of placental physiology there is doubt as to the exact significance of the changes being measured.

What is meant by the term placental function test? It has been used loosely in recent years to mean the estimation in maternal urine or blood, of a hormone or enzyme, which has been metabolised partly or wholly by the placenta, or which has been liberated from the placenta. These values have been correlated with fetal outcome and perinatal mortality, thus forming the basis of a crude index of fetal prognosis. The placenta is a complex organ with many activities and so the main disadvantage of these estimations is that they record only one isolated aspect of its metabolism. Many of the functions of the placenta are as yet unexplored and much fundamental work needs to be done before deciding upon the most important and significant aspects of its physiology which affect fetal well-being.

A critical appraisal of tests, asking the specific questions to which the obstetrician is seeking the answer, has not always been carried out, thus raising false
hopes for individual tests. To be of value a placental function test should give the obstetrician certain information, such as a warning of imminent fetal anoxia, and of failure of fetal growth. Its assessment must be firstly in relation to fetal anoxia, namely with perinatal deaths attributed to anoxia, to fetal distress in labour, and to the Apgar score of the infant at birth.

Secondly, assessment must be in relation to the incidence of growth retarded babies, and thirdly to fetal survival in complications of pregnancy, such as hypertension.

Most important of all, the results must have a prognostic value. It is of limited value to show retrospectively a weak statistical association between placental function test levels, and, for example, fetal distress in labour, or the birth weight of the baby. Results of placental function tests are only of significant value if they form a substantial basis for clinical action. For the time being, estimations of placental hormones and enzymes will have to be critically assessed, and the knowledge thus gained added to the total sum of knowledge about the placenta. From this, a series of tests will probably evolve, enabling the obstetrician to assess placental function specifically and accurately, much as liver function is assessed at the present time.

In this study, three enzyme tests have been compared with total urinary oestrogen excretion. Despite the inaccuracies
of the latter it is still the most widely used and accepted assessment of the feto-placental unit in use today. Many of the two alkaline phosphatase estimations have been carried out concurrently, and these have been compared with each other.

It is against these clinical and laboratory criteria that the three enzyme estimations discussed in this thesis are evaluated as possible placental function tests.

The conclusions reached are that urinary oestrogen excretion is an acceptable but not very accurate guide to fetal prognosis. The three enzyme estimations, although yielding some information do not fulfil the criteria stated, and as such are not acceptable on their own, as placental function tests. Due to lack of knowledge about the function of enzymes in the placenta, the many variable factors influencing the enzyme levels, and the wide range found in normal pregnancy sera, enzyme placental function tests at the present time are unlikely to prove satisfactory.
I wish to acknowledge with grateful thanks, the help of many people who have made this work possible.

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SUMMARY

The value of urinary oestrogen estimations and their validity as a comparison with the three enzyme tests as an assessment of fetal prognosis is discussed.

The basis for using these enzyme estimations as a possible guide to fetal prognosis is reviewed.

The abnormal groups of patients studied were those with mild, and moderate to severe hypertension; growth retarded babies; fetal distress in labour; and those whose pregnancies ended in a perinatal death.

The problems of the method used for estimating urinary oestrogens, and the results obtained in 45 normal and 115 abnormal pregnancies are analysed and compared with the findings of other workers. The results of this study are similar to these findings, and so form a valid comparison with the three groups of enzyme estimations.

698 serial serum heat stable alkaline phosphatase (HSAP) estimations carried out during 72 normal and 124 abnormal pregnancies are analysed. The difficulties of drawing comparisons with the results of other workers who used less specific methods for estimating serum HSAP are stressed. The conclusions are that although the levels of this enzyme, specific to pregnancy, rise in a steady linear fashion during pregnancy, alterations in these levels do not give
an adequate guide to fetal prognosis in cases of hypertension
or fetal growth retardation.

High levels of serum HSAP found in the third trimester of
pregnancy are indicative of a poor fetal prognosis. The
reasons for this are obscure, but it is suggested that in
these pregnancies there may be excessive trophoblast
proliferation and subsequent placental infarction.

The reason for a reduction in serum HSAP values in severely
hypertensive patients during the last few weeks of pregnancy,
may be a diminished amount of syncytiotrophoblast,
containing the placental iso-enzyme of alkaline phosphatase.

The large overlap of values between normal and abnormal
pregnancies considerably reduces their prognostic significance.

731 serial histochemical estimations of neutrophil alkaline
phosphatase (NAP) were carried out during 103 normal and
123 abnormal pregnancies.

The values are found to be significantly lower than normal
in cases of mild, and moderate to severe hypertension, fetal
growth retardation, fetal distress and perinatal mortality.

There is a significant negative correlation between the NAP
values and the weeks of gestation in cases of moderate to
severe hypertension and perinatal mortality. The values,
in the hypertensive patients, fall several weeks prior to
the onset of hypertension.
Despite these reductions the prognostic value of the estimations are much reduced, due to the large overlap with the normal range.

Positive correlations are found in hypertensive pregnancies between NAP scores on the one hand, and total urinary oestrogen excretion and serum HSAP estimations on the other. There is no correlation between the estimations in normal pregnancy.

It is suggested that the reduction in placental production of oestrogens due to hypertensive damage may affect the NAP scores directly, or secondarily by reducing the circulating corticosteroid levels. Experiments showed that the alkaline phosphatase found in neutrophils is unrelated to the placental iso-enzyme and it is suggested that the correlation between these apparently unrelated estimations is coincidental.

A method using appropriate experimental conditions for estimating serum glucose-6-phosphatase (G-6-Pase) is described.

356 serial serum G-6-Pase estimations carried out during 78 normal and abnormal pregnancies are analysed. There is no trend of increasing values during the third trimester of normal pregnancy, and there is no significant difference between the mean values found during normal and abnormal pregnancies.

There is no correlation between serum G-6-Pase and serum
IISAP estimations, but there is a negative correlation between serum G-6-Pase levels and total urinary oestrogen excretion.

The levels in late pregnancy do not seem to differ significantly from those found in the non pregnant individual.

A possible explanation of these facts is that G-6-Pase is not liberated from damaged placental cells to the same extent as HSAP because of its intra-cellular situation. The negative correlation between serum G-6-Pase and total urinary oestrogen excretion can be explained by transient high levels of the former due to severe episodes of placental infarction at times when urinary oestrogen excretion is low.

Further elucidation of these problems is suggested.
INTRODUCTION

The aetiology of placental damage and subsequent fetal anoxia and fetal growth retardation is uncertain. In the latter case there is a chronic reduction in transfer of nutrients and oxygen across the placenta. Severe maternal hypertension may lead to a similar situation but usually there is a more acute failure of placental function, leading either to fetal distress in labour or to the intrauterine death of a normal weight for dates baby. Robertson et al. (1967) studied placental bed biopsies in cases of essential hypertension and pre-eclamptic toxaemia and found that in the former condition hyalinisation of small basal arteries and arterioles was rare but that hyperplastic arteriosclerosis of the placental bed arteries was common. Pre-eclamptic toxaemia was associated with fibrinoid necrosis and infiltration of the damaged vessel wall with foam and round cells. The extent of vascular damage correlated well with the severity of the hypertension. The lesion which they called acute atherosclerosis was comparable to the renal artery lesions found in rejected human kidney homotransplants and they suggested that there may be an immunological component in the vascular lesions of the placenta in pre-eclamptic toxaemia. The fact that intravascular coagulation is present in cases of pre-eclamptic toxaemia and growth retarded babies has been suggested by Bonnar et al. (1971) and Howie et al. (1971). This may lead to fetal growth retardation by diminishing the placental circulation, and its effects in the renal circulation may account for the
clinical signs of pre-eclamptic toxaemia. The improvement in fetal growth and in placental function tests brought about by the intravenous administration of heparin to patients with known fetal growth retardation strongly suggests that intravascular coagulation reduces the placental circulation (Bonnar, personal communication).

McLennan et al. (1972) have shown that the placental changes seen in pre-eclamptic toxaemia and fetal growth retardation are the same as those induced by hypoxia, which may be caused by a diminished placental circulation.

The above hypothesis illustrates the fact that the aetiology of placental damage in cases of fetal growth retardation and pre-eclamptic toxaemia is as yet obscure and that it is undoubtedly a complex process. Until placental physiology and pathology are more fully understood, placental function tests will remain largely empirical and consequently not very accurate in the information that they provide. Whatever the mechanism of placental damage it seems that there is a diminished maternal blood supply to the placental bed and it is the effects of this that are being hopefully assessed by current tests.

There are basically two groups of tests in use at the present time. One is the assessment of the feto-placental unit by means of hormone assays and the other is measurement in the maternal serum of enzymes released from the placenta.
Hormone assays that have been commonly used are urinary pregnanediol, the metabolic end product of progesterone, and urinary oestrogens. Blood levels of progesterone and oestrogens have also been used, but because of technical difficulties, their use to date has not been extensive.

The placenta can synthesise pregnenolone and progesterone from circulating precursors such as acetate and cholesterol, but it lacks the enzymes necessary to split off the side chain of C21 steroids such as progesterone, in order to convert them to androgenic C19 steroids such as dehydroepiandrosterone and androstenedione. The latter substance is an obligatory intermediate in the conversion of dehydroepiandrosterone to oestrogens (Fig. 1) and the necessary enzymes for this reaction are found only in the fetus. Thus, the placenta can synthesise progesterone but it can form oestrogens only in conjunction with the fetus.

**Urinary Oestrogens**

Dissatisfaction with urinary pregnanediol levels as a guide to fetal prognosis led obstetricians to examine urinary oestrogen excretion. After about the 32nd week of pregnancy urinary oestrogen excretion increases markedly, reflecting greatly increased secretion (Klopper & Billewicz, 1963), while pregnanediol excretion remains fairly constant. This rise is chiefly attributed to increased production and excretion of oestriol which forms about 90% of the total
Steroid metabolism in fetus and placenta.
oestrogens in pregnancy (Brown 1956). There are also high levels of oestriol found in maternal blood (Roy and Mackay, 1962), cord blood (Roy 1962) and amniotic fluid (Diczfalusy and Magnusson, 1958).

Urinary oestrogen estimations have turned out to be a better guide to fetal prognosis because, in contrast to progesterone, the fetus is involved in the metabolism of the end product measured and so the functional capacity of the feto-placental unit is being assessed and not just that of the placenta.

The placenta depends for its oestrogen synthesis on neutral C19 steroid precursors from the maternal and fetal circulations. These are converted to 16 desoxy and 16 oxygenated C19 neutral steroids.

Important fetal precursors of oestrogens in mid-pregnancy are dehydroepiandrosterone sulphate, and 16 α hydroxy dehydroepiandrosterone sulphate (Dell' Acqua. et al. 1967). Androstenedione is an obligatory intermediate in the conversion of dehydroepiandrosterone to oestrogens.

The essential enzyme systems required for oestrogen synthesis are distributed between the fetus and placenta in a characteristic manner. Only the placenta contains 3β hydroxy steroid dehydrogenases, whereas only the fetus contains 16 α hydroxylating enzyme systems acting on C18
and C19 steroids, and aryl sulphokinase activity.

The oestrogen pattern of all fetal tissues is dominated by the conjugated forms (Diczfalussy and Magnusson, 1958), a considerable proportion of which is oestriol-3-sulphate, and this may be trans conjugated in the fetus into oestriol glucosiduronates (Troen et al., 1961). Oestrogen sulphates reaching the placenta via the fetal circulation are hydrolysed by the placenta and rapidly transferred to the maternal compartment. Placental secretion of oestriol-3-sulphate to the mother is followed by metabolism to oestriol-3-glucosiduronate (Goebelsmann et al., 1965).

The metabolic pathways in the production of oestrogens are shown in Fig. 1.

Thus it can be seen that both fetus and placenta are inter-dependent for the synthesis of oestrogens and hence measurement of oestriol in maternal urine is a reasonable guide to the state of the feto-placental unit. The clinical evidence for this statement will now be given together with the situations in which the urinary oestrogen excretion pattern can be misleading.

Sources of Error

There is quite a large experimental error in all but the most sophisticated of methods. The more rapid the method, the more specificity and accuracy must be sacrificed. All
laboratories must establish their own normal values, as even within the limits of experimental error, two laboratories using the same method may not get the same results. The range of normal values is wide.

There is a large biological day to day variation in urinary oestrogen excretion, of up to 30%, and there are the difficulties associated with an accurate 24 hour urine collection. Therefore one value is unreliable, and it is the trend of serial assays that must be examined against the normal range. Low urinary oestriol excretion can be caused by non relevant conditions such as urinary tract infection (Taylor et al. 1963), anaemia (Beischer et al. 1968a) and by drugs such as ampicillin (Willman and Pulkkinen, 1971), mandelamine (Eraz and Hausknecht 1969) and corticosteroids (Brown et al. 1968). Anencephaly is associated with low urinary oestrogen excretion and this may be due to a diminution in oestriol precursors produced by the fetal adrenal glands (Frandsen and Stakemann, 1964).

**Urinary Oestrogen Estimations in normal and abnormal Pregnancies**

There is general agreement that urinary oestrogen excretion rises steadily in normal pregnancies to a peak at or about term, (Frandsen and Stakemann, 1963a; Coyle and Brown, 1963; Oakey et al. 1967). However, Jenkins et al. (1971) found that mean levels reached a plateau at about 38 weeks gestation. The normal range is wide and the coefficient of variation can be as high as 30%.
Fetal growth retardation is the clinical condition in which urinary oestrogen excretion is of most value in predicting fetal prognosis. Using serial assays it is possible to determine the optimum date of delivery for these fetuses. Many authors have shown that low urinary oestriol or total oestrogen excretion is associated with significant fetal growth retardation (Michie, 1967; Oakey et al. 1967; Elliott, 1970). However in about 10% to 20% of cases, fetal growth retardation can occur with normal oestriol output (Frandsen and Stakemann, 1963; Elliott, 1970).

Many authors have shown a correlation between fetal weight and oestriol excretion in normal pregnancy (Frandsen and Stakemann, 1960a; Coyle and Brown, 1963; Beling, 1967; Beischer et al. 1968b; Easterling and Talbert, 1970). In a mixture of normal and abnormal pregnancies, Curzen and Southcombe (1970) have shown a correlation between crude placental weight and urinary oestrogen excretion. No such correlation was shown by Frandsen and Stakemann (1960a); Coyle and Brown (1963), or Beling (1967) who were studying normal pregnancy. This difference of opinion may be due to differences in clinical material studied.

The reason for the reduction in urinary oestrogen excretion in cases of fetal growth retardation is unknown. Whether the primary fault lies in the fetus, placenta, or both, is open to conjecture. Possible causes are a lack of precursor steroids from the fetal adrenal glands, an enzyme deficiency, perhaps C16 hydroxylase, or placental sulphatase or a
reduction in trophoblast production of hydroxy steroid dehydrogenase caused by hypoxia (Sharp et al. 1972), which will reduce the synthesis of oestriol precursors.

Despite the various areas of controversy, there is no doubt that urinary oestrogen output is usually reduced when fetal growth is significantly retarded and the estimations are, in most cases, helpful in making or confirming a diagnosis, and in deciding the optimum time for induction of labour.

Prolonged severe pre-eclamptic toxaemia is often associated with fetal growth retardation and in this group of patients urinary oestrogen output is often low (Klopper, 1966; Michie, 1967). The value of these estimations is to warn the clinician when intrauterine death is imminent (Coyle et al. 1962) and so when induction of labour is essential. For this, critical limits for fetal survival must be set and these vary greatly from 1 - 3 mg. (Zondek and Pfeifer, 1959) to 12 mg. at term (McLeod et al. 1967). Therefore each laboratory must set its own critical value after a careful analysis of its results.

Severe pre-eclamptic toxaemia in the absence of fetal growth retardation is also associated with low urinary oestrogen excretion, but in these cases, both the onset of toxaemia and the reduction in urinary oestrogen levels are usually sudden. Unless these levels are being estimated frequently, and unless the results are available within the
same day, the test is of little value, and certainly no better than clinical events in helping to decide when to intervene, if necessary.

Mild pre-eclamptic toxæmia is associated with a minimal reduction in urinary oestriol excretion (Klopper, 1966). Hypertension per se may not cause a reduction in urinary oestriol excretion, but in cases of pre-eclampsia with impaired renal function, the latter may be the cause of a reduction in its excretion (Taylor et al. 1963).

It seems that pre-eclampsia needs to be severe before there is a significant reduction in urinary oestrogen excretion, but its estimation is useful in these cases for the prediction of a worsening fetal prognosis or of imminent intrauterine death.

The relationship between fetal distress in labour and oestriol values has been investigated by Jenkins et al. (1971). They found that there was a significantly higher incidence of fetal distress in labour (33%) when the urinary oestriol values were low (less than 14.5 mg. per 24 hours) compared with 13% when the levels were normal. They felt that falling or low values predicted acute anoxia causing fetal distress during labour in normal weight babies.

In cases ending in a perinatal death, there was a much higher incidence of low urinary oestriol values than expected. Reid et al. (1968) studied urinary oestriol
levels in cases of pregnancy complicated by pre-eclamptic toxaemia with proteinuria. The overall perinatal mortality was 15%. The mortality among those with normal urinary oestriol excretion was 3.4%, while among those with a low urinary oestriol excretion it was 31.7%. In cases of moderately severe pre-eclamptic toxaemia there were no perinatal deaths associated with normal urinary oestriol excretion, but when this was low, 24% of cases ended in a perinatal loss. Nelson (1969) and Easterling and Talbert (1970) recorded similar results and concluded that perinatal deaths were frequently associated with falling or low urinary oestrogen values.

In other high risk pregnancies, urinary oestrogen estimations have been of variable use. In pregnancies which have progressed beyond 42 weeks gestation, urinary oestrogen excretion that is not falling indicates that the fetus is in no immediate danger, whilst a falling level necessitates immediate delivery because of the increasing risks of fetal distress and perinatal mortality. (Lundwall and Stakemann, 1966). The problem with post mature pregnancies is that the urinary oestrogen excretion can fall very rapidly and so estimations must be carried out frequently if intrauterine death is to be avoided.

In diabetic pregnancies, Talbert et al. (1969) found that low urinary oestriol values (less than 10 mg. per 24 hours), were associated with a high incidence of small for dates
babies and a perinatal mortality rate of 4.3%, while Green et al. (1965) found that values over 12 mg. per 24 hours within 48 hours of delivery were not associated with any perinatal mortality.

However, in some diabetic pregnancies, intrauterine death may be immediately preceded by a sudden drop from normal levels, and so the value of urinary oestriol estimations in these patients is somewhat limited.

In pregnancies complicated by Rhesus iso-immunisation, Klopper and Stephenson (1966) found a wide scatter of values and no drop before intrauterine death, while Taylor et al. (1963) found high values. Fairweather et al. (1972) recorded low levels that were of no prognostic value. Schindler et al. (1967) found that neither urine nor plasma levels of oestriol reflected the condition of the baby in Rhesus iso-immunisation, but suggested that amniotic fluid oestriol levels were of prognostic value.

Despite the inadequacies of urinary oestrogen excretion as a placental function test in the conditions described, there is adequate evidence that the estimations fulfil the criteria necessary for such a test in many of the more common complications of pregnancy.

Although limitations such as a large day to day variation, experimental error, and the possibility of falsely low values must not be minimised, nevertheless serial estimations
are a good guide to the presence or absence of fetal growth retardation and chronic anoxia, to the probability of acute fetal anoxia in prolonged or hypertensive pregnancies, or as a warning of imminent fetal death. Serial normal or high values indicate a good fetal prognosis.

On the basis of this evidence, total urinary oestrogen excretion has been used as a comparison for the three enzyme estimations.
Serum alkaline phosphatase was found to be elevated in pregnancy by McMaster et al. (1964) and Curzen and Morris (1965). The former authors suggested that the rise was due almost entirely to an increase in the placental isoenzyme which was stable on heating to 56°C. This quality of the placental isoenzyme fostered hopes that its estimation might be of value as a placental function test. This hope was substantiated when Curzen and Morris (1966) found that in severe pre-eclamptic toxæmia, three quarters of the serum alkaline phosphatase values were outside the normal range after the sera had been pre-heated to 56°C for 30 minutes. This contrasted with their findings of the previous year when they found no difference in unheated serum alkaline phosphatase levels between normal and hypertensive pregnancies.

At this stage it seemed that serum heat stable alkaline phosphatase (H.SAP) estimations might be a useful index of placental function. However, Neale et al. (1965) and Hunter (1969) showed that a temperature of 56°C was inadequate for the complete inactivation of the non-placental iso-enzymes. Hunter (1969) felt that 65°C for 30 minutes was necessary, while Fishman et al. (1968b) and Stolbach et al. (1969) felt that 65°C for 5 minutes, or 55°C for 1 hour (Fishman et al. 1968a) was adequate.
Optimal conditions for the activity of the placental isoenzyme involving pH and substrate concentration have been described by Ghosh and Fishman (1968) and these are incorporated in the method used by Fishman et al. (1968b) and Stolbach et al. (1969).

Unfortunately these conditions, specific for the measurement of the placental iso-enzyme have not been used by many authors in estimating serum HSAP and so their conclusions as to the value of the estimations as a test of placental function are open to criticism. Comparison between various series is also difficult because of the different methods used. Despite this there is agreement that in normal pregnancy, serum HSAP levels show a progressive linear increase during the third trimester (Curzen and Morris, 1966; Hunter, 1969; Hunter et al. 1970; Watney et al. 1970; Quigley et al. 1970; Curzen and Varma, 1971; Pirani et al. 1972). There is however a wide range of normal values (Messer, 1967; Watney et al. 1970; Curzen and Varma, 1971; Pirani et al. 1972).

In cases of severe pre-eclamptic toxaemia there is again agreement among several authors that serum HSAP levels are significantly raised during the third trimester. (Curzen and Morris, 1966; Hunter et al. 1970; Quigley et al. 1970). Benster's (1970) results are in disagreement, in that he found that serum HSAP values before 32 weeks were high in pre-eclamptic toxaemia, and that thereafter they failed to show the progressive rise found in normal pregnancy. Thus by the last weeks of pregnancy the serum HSAP values in these patients were often low. He also noted that in three patients high serum HSAP levels preceded the onset of severe pre-eclamp-
tic toxaemia by several weeks. This suggested that the estimations might be of some value in the prediction of hypertension in pregnancy.

The ability of serum HSAP estimations to predict fetal growth retardation is doubtful. Messer (1967) found that low serum HSAP values were a bad prognostic sign, and that there was a correlation between these values, and urinary oestrogen excretion. A criticism of this finding is that only groups, and not actual values, were used, and no correlation coefficient was stated. Watney et al. (1970) found that 20% of patients who delivered themselves of growth retarded babies had significantly high serum HSAP levels, and that 14% had significantly low levels. Curzen and Southcombe (1970) did record a correlation between serum HSAP levels and urinary oestrogen excretion, and a positive correlation between the former levels and fetal and placental weight, suggesting that serum HSAP estimations might be of value in predicting the presence of a growth retarded fetus. Subsequent work (Curzen and Varma, 1971; Curzen and Hensel, 1972) refuted this and concluded that these estimations were of no value in the prediction of these babies. The difference in the results of these papers must be attributed to differences, albeit slight, in the method used, and to the interpretation of the results.

Despite the number of papers on serum HSAP levels in pregnancy, few authors have related these levels to the clinically important parameters of fetal distress in labour.
or the Apgar score of the infant. Curzen and Varma (1971) did carry out such a study, and they concluded that serum HSAP estimations were of no value in predicting fetal distress, nor were they related to the Apgar score of the infant one minute after birth, whereas urinary oestrogen estimations did relate quite well to these clinical conditions.

Hunter et al. (1970) felt that when serum levels of HSAP exceeded the upper limits of normal, and especially if the rise was rapid, then fetal prognosis was poor. Quigley et al. (1970) recorded one case of severe pre-eclamptic toxaemia at 29 weeks gestation in which the serum HSAP levels were four times the normal one week prior to the onset of the toxaemia. Benster (1970) found no relationship after 35 weeks gestation between serum HSAP estimations and perinatal death, although there was an association between high levels earlier in the third trimester of pregnancy and fetal loss. These findings suggest that high values of serum HSAP, especially early in the third trimester are indicative of a poor fetal prognosis.

The evidence for serum HSAP estimations being used as a placental function test is equivocal. The authors using the most accurate method (Benster, 1970; Curzen and Varma, 1971; Curzen and Hensel, 1972) as described by Fishman et al. (1968b) conclude that it's use, if any, is limited.
This study was set up to cast further light on the role of serum H3AP as a placental function test. A comparison with the best available test of placental function, namely urinary oestrogen estimations, was carried out, and a possible correlation with the activity of neutrophil alkaline phosphatase was also investigated in both normal and abnormal pregnancies.
Leucocytes contain alkaline phosphatase, this fact being first noted by Kay (1929). Roche (1931) demonstrated the presence of the enzyme in white blood cell preparations containing both granulocytes and lymphocytes. Gomori (1943) found marked evidence of the enzyme in the leucocytes of tissue sections. Wachstein (1946) found varying proportions of alkaline phosphatase present in neutrophils, and that the enzyme activity was present in large amounts in the bone marrow, suggesting that the phosphatase is present in the leucocytes before their release from the bone marrow.

Using biochemical methods, Haight and Rossiter (1950) found alkaline phosphatase to be confined chiefly to the polymorphonuclear leucocytes. The amount present varies from one pathological condition to another.

Leucocyte alkaline phosphatase activity has been found to be raised in polycythaemia vera (Valentine et al. 1952), acute infections associated with polymorphonuclear leucocytosis (Valentine and Beck, 1951; Kenny and Moloney, 1957) and trauma (Valentine et al. 1954). A common factor in the last two conditions was thought to be stress (Valentine et al. 1954), and this was confirmed by showing a rise in leucocyte alkaline phosphatase activity after the prolonged administration of ACTH gel. These authors felt that the increased activity caused by stress was dependent on adrenal and pituitary
function, and was only manifest after stress continuing for at least a day or two, or after the prolonged administration of ACTH. The enzyme activity in the leucocyte rises to a peak over a few days, presumably because the humoral effect is not exerted on the mature circulating leucocyte, but on the cell during its maturation.

The fact that increased adrenal and pituitary activity is associated with increased leucocyte alkaline phosphatase activity is also suggested by the fact that this is raised in pregnancy (Pritchard, 1957; Harer and Quigley, 1961; Zuckerman et al, 1969; Polishuk et al, 1970). The rise occurs as early as five weeks from the onset of the last menstrual period, rising from a normal score of 0 - 60, to a level of greater than 80 (Harer and Quigley, 1961). Scoring is carried out by assessing the intensity of histochemical staining at the sites of enzyme activity in 100 consecutive mature neutrophils, giving each cell an arbitrary score of 0 - 4. The levels rise steadily until about 28 weeks gestation after which time they remain relatively constant, with a reduction at and after term. Values have returned to the normal non-pregnant level within six weeks of delivery (Polishuk et al. 1970).

In pre-eclamptic toxaemia, Sadovsky et al. (1969) and Zuckerman et al. (1969) found that leucocyte alkaline phosphatase activity was reduced in proportion to the severity of the hypertension and that this reduction preceded the onset of symptoms by several weeks. It has been suggested
by the former authors that use can be made of this reduction in leucocyte alkaline phosphatase activity as a guide to placental function, on the basis that it is a reflection of placental hormonal changes. Polishuk et al. (1968) studied leucocyte alkaline phosphatase activity during the menstrual cycle and after the systematic administration of oestrogen, progesterone and chorionic gonadotrophin. During the menstrual cycle they found a sharp rise and fall immediately after ovulation, followed in some patients by a smaller rise a few days prior to menstruation. During anovulatory cycles they found no increase in leucocyte alkaline phosphatase activity.

The systematic administration of oestrogens to post-menopausal women produced a rise in leucocyte alkaline phosphatase activity to the levels found during the third trimester of pregnancy, which was followed by a slow fall, while the administration of a progestogen, an oestrogen-progestogen combination, or chorionic gonadotrophin, produced no rise at all. (Polishuk et al. 1968). These facts led them to suggest that oestrogens were responsible for the rise in leucocyte alkaline phosphatase activity, which was in agreement with Goldstein (1965). Polishuk et al. (1968) also concluded that progestogens can inhibit this rise. This is in disagreement with O'Kell and Axon (1965) who felt that progesterone levels did increase leucocyte alkaline phosphatase activity. The absence of an increase in activity after the administration of chorionic gonadotrophin suggests that this hormone is not responsible for the
rise found during pregnancy, although the amounts of it that were used were much smaller than those found during pregnancy. Polishuk et al. (1970) carried out the same investigations on pregnant women and obtained similar results. These results suggest that the enzyme activity may be a reflection of the relationship between placental oestrogen and progesterone production.

Valentine et al. (1954) showed that ACTH was responsible for a rise in leucocyte alkaline phosphatase activity and Pritchard (1957) felt that the rise found in pregnancy was a reflection of the increased circulating cortico-steroid levels. Polishuk et al. (1970) found a reduction in leucocyte alkaline phosphatase activity in pregnancy following the administration of hydrocortisone. This suggests that it is not so much the circulating steroid levels that are responsible for the increase in enzyme activity, but perhaps ACTH itself or a placental substance similar to ACTH (Assali and Hamermesz, 1954) which may be the regulating factor.

In conclusion, it can be stated that there is much controversy about the factors regulating the levels of alkaline phosphatase activity in leucocytes, but it seems possible that in pregnancy, oestrogens or indirectly some other placental steroid hormone may be responsible. The reduced activity found in hypertensive pregnancies may reflect a drop in the production of these placental steroids, and hence may crudely reflect the extent of placental damage. On this basis it is possible that neutrophil alkaline
phosphatase estimations might be useful as a placental function test. This study was set up to ascertain whether variations in neutrophil alkaline phosphatase activity were a guide to fetal prognosis, and whether these variations in any way correlated with the feto-placental production of oestrogens, or the levels of the placental iso enzyme of alkaline phosphatase in the maternal serum.
Glucose-6-phosphatase (G-6-Pase) is an important enzyme in carbohydrate metabolism and is found chiefly in the liver and kidney, and in small amounts in the intestine. The enzyme is responsible for splitting glucose-6-phosphate into glucose and inorganic phosphate. Glucose-6-phosphate is derived either from glycogenolysis or from gluconeogenesis. Glucose utilisation also proceeds through the glucose-6-phosphate stage, by the addition of a phosphate group from adenosine tri-phosphate, the enzyme responsible being hexokinase.

Villee (1953) incubated tissue slices of placenta and fetal liver in a Ringer phosphate solution containing C\textsuperscript{14} labelled glucose and pyruvate. He showed that the glycogen content of the placenta decreases and that of the fetal liver increases as gestation proceeds. Similarly he showed that the ability of the placenta to produce glucose decreases while that of the fetal liver increases with advancing gestation. It is concluded that the fetal liver is unable to store glycogen and regulate blood glucose adequately until about mid-pregnancy, presumably due to the delay in the production of G-6-Pase. The placenta, on the other hand, can store glycogen and secrete glucose in early pregnancy, thereby maintaining fetal blood glucose levels until the liver is able to assume this function. It is deduced from this work that the placenta contains G-6-Pase early in pregnancy and that its amount diminishes as pregnancy...
Wachstein et al. (1963) using histochemical techniques, showed evidence of phosphatase activity in the borders of syncytiotrophoblast cells, but were unable to show that it was specifically G-6-Pase. The technique that they used was that described by Wachstein and Meisel (1956) which is a modification of Chiquoine's method (1953) involving the precipitation of lead sulphide at the site of enzyme activity. G-6-Pase is thought to be destroyed by formalin (Chiquoine, 1953), and as a pre-fixation with formalin did not suppress the reaction, Wachstein et al. (1963) felt that the enzyme activity was not specifically G-6-Pase. Curzen (1964), using a similar histochemical technique, concluded that the enzyme was present in the syncytiotrophoblast, and that it's amount increased as pregnancy progressed.

Carter and Weber (1966) using similar histochemical techniques, and biochemical methods, found that the total placental glucose-6-phosphatase activity increased, but that in relation to fetal weight this activity decreased significantly as pregnancy advanced.

From the work described, it seems likely that G-6-Pase is present in the placenta in large amounts in early pregnancy but that this diminishes with advancing pregnancy. There seems however, to be some doubt about the ability in the histochemical techniques described, to record G-6-Pase activity specifically from that of other phosphatases.
As the enzyme is fundamental to the production of free glucose, it may have an important role in the maintenance of fetal blood glucose levels. The enzyme is contained in the endoplasmic reticulum of cells (Tice and Barrnett, 1962), and so if sufficient cell damage occurs, it may leak into the maternal serum. Therefore it is possible that the detection of elevated serum levels of the enzyme may indicate significant placental infarction, as is thought to be the case with the placental iso-enzyme of alkaline phosphatase. It is also possible that abnormal levels may be found in patients with a growth retarded baby. As serum levels of this enzyme have not been recorded in either normal or abnormal pregnancy, it was decided to investigate these with a view to assessing fetal prognosis by means of altered enzyme levels.

Simultaneous total urinary oestrogen excretion and serum HSAP estimations were carried out to see what relationship, if any, existed between these parameters on the one hand, and serum G-6-Pase levels on the other, in both normal and abnormal pregnancies.

However, there is controversy as to whether G-6-Pase can be measured in serum distinctly from non-specific acid and alkaline phosphatases. Ashmore et al. (1954), Koide and Oda (1959) and DiBella et al. (1963) felt that it was possible, while others felt that this was not the case (Eggermont and Hers, 1960; Zuppinger, 1961; Nordlie and Arion, 1964; Foz, 1967).
Not only is the attempted estimation complicated by the presence of non-specific phosphatases, but G-6-Pase itself seems to be far from specific in its actions (Barman, 1969). In view of this controversy, it was decided to investigate this aspect more fully, before proceeding to the comparative studies in normal and abnormal pregnancies.
Normal pregnancy, is defined as one in which there were no antenatal complications and which terminated in the delivery of a single normal weight for dates baby at between 37 and 42 weeks gestation, with no fetal distress.

Mild hypertension, is defined as a blood pressure reading of between 140/90mm.Hg and 150/100mm.Hg at any stage of pregnancy.

Moderate to severe hypertension, is defined as a blood pressure reading above 150/100mm.Hg at any stage of pregnancy. Many of the patients had unclassifiable hypertension and no attempt has been made to differentiate these from cases of essential hypertension or pre-eclamptic toxaemia.

Growth retarded babies, are defined as those babies whose birth weight fell below the 10th percentile employing the values illustrated in Figure 2. These smooth curves (Elder et al, 1970) are based on data from the Second Report of the 1958 British Perinatal Mortality Survey of the National Birthday Trust Fund (Butler and Alberman, 1969) supplemented by data from Gruenwald (1966) at the lower gestational ages.

Fetal distress, is defined as a fetal heart rate of less than 100 beats per minute on more than one occasion, or the presence of meconium in the amniotic fluid, during labour. The number of patients involved, and the number of estimations carried out, are recorded in the relevant chapters of the Experimental Section.
Fig. 2

Distribution of birth weights according to maturity. Mean birth weight, and the 10th and 90th percentiles are shown.
The variation of estimations from their mean value is expressed by one standard deviation. This is the square root of the sample variance which is \( \frac{(x - \bar{x})^2}{n - 1} \), where \( x \) is an individual estimation, and \( \bar{x} \) and \( n \) are the mean and number of estimations respectively, in the sample.

In the serum G-6-Pase study the standard error of the mean value was calculated from the formula \( \text{S.E.M.} = \frac{V}{\sqrt{n}} \), where \( v \) is the variance of the sample and \( n \) the number of estimations.

Comparison of the number of patients from one group with those of another group is carried out using the Chi squared distribution.

The correlation of the two variables \( x \) and \( y \) is obtained from the sample correlation coefficient.

\[
    r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}
\]

The regression line, the equation of which is \( y = bx + a \), is obtained by the method of least squares. The degree of significance of the correlation is obtained from the 't' distribution of \( b \), which is the slope of the line, divided by its standard error.
The value of $b$ is calculated from the formula

$$b = \frac{\xi(x - \bar{x})(y - \bar{y})}{\xi(x - \bar{x})^2}$$

and its standard error from the formula

$$\text{S.E.} = \sqrt{\frac{\xi(y - \bar{y})^2}{n - 2} \left(1 - r^2\right)}$$

Comparison of mean values of one group of patients with another for a particular week of gestation is carried out by Students 't' test. The value of 't' is obtained from the formula

$$t = \frac{\overline{x}_1 - \overline{x}_2}{\sqrt{\frac{v_1}{n_1} + \frac{v_2}{n_2}}}$$

where $\overline{x}$, $v$ and $n$ are the mean, variance and number of estimations in each sample.

In the NAP study comparison of the scores obtained from one group of patients, with those of another, is carried out by the Mann-Whitney U test.
REAGENTS USED FOR TOTAL URINARY OESTROGEN ESTIMATIONS

All reagents used were 'AnalaR' and supplied by British Drug Houses Ltd., except for oestriol standard and bauxite chips.

1) Concentrated hydrochloric acid 11N.
2) Sulphuric acid 10N - diluted with water to 6.5N.
3) Diethyl ether.
4) Oestriol standards 100 µg and 5 µg/ml in ethanol (Grade 1, Sigma Ltd., U.S.A.).
5) Sodium carbonate solution pH 10.5. 1 l. of N sodium bicarbonate solution was mixed with 150 ml. of 5N sodium hydroxide solution, and the pH adjusted to 10.5.
6) Quinol 2% (w/v) in ether.
7) Quinol 2% (w/v) in 6.5N sulphuric acid.
8) Bauxite chips.
9) Sodium sulphate, anhydrous.

Stability of reagents

Oestriol standards stored at 4°C were stable for some months.
Sodium carbonate solution, pH 10.5, was stable at 4°C for at least 2 weeks but in practice the pH of the reagent was checked on each working day.
Quinol 2% (w/v) in sulphuric acid was prepared freshly each day while quinol 2% (w/v) in ether was stored in a dark bottle at 4°C and prepared freshly each week.
Ether was stored at 4°C.
METHOD USED FOR TOTAL URINARY OESTROGEN ESTIMATIONS

The method used was that described by Oakey et al. (1967) which is a modification of the method described by Brown (1955).

Principle of the Method

The principle of the method involves acid hydrolysis of the conjugated oestrogens in urine, followed by their ether extraction from the aqueous solution. Shaking with sodium carbonate removes the acid fraction from the ether extract, without appreciable loss of oestriol.

Quinol in ether was then added prior to evaporation to dryness, thereby improving the Kober colour reaction (Bauld, 1954).

After evaporation, heating with quinol in sulphuric acid brings about the red Kober colour reaction. Reducing agents with oxidation-reduction potentials of the type produced by quinol-quinhydrone couple are required for the subsequent maximum production and stability of the red colour (Brown, 1952).

Critical conditions, such as the concentration of sulphuric acid and the duration of heating, have also been studied by Brown (1952). The reaction is completed by dilution
with water and reading the extinction at three wavelengths to exclude interfering chromagens.

**Method**

The volume of a 24 hour urine collection was measured, and diluted to 2 l. with distilled water. Two ml. of diluted urine were transferred to each of 3 Kober tubes, two being duplicate test specimens, and the third the internal standard. To each tube was added 0.3 ml. concentrated hydrochloric acid. When the urine volume was greater than 2 l. it was diluted to the nearest litre and one thousandth of this volume used, together with a proportionately increased volume of hydrochloric acid. The tubes were stoppered and placed in a boiling water bath for 1 hour. After cooling 0.2 ml. of \( \text{\AE} \) oestriol standard (100 \( \mu \)g/ml.) was added to the third Kober tube - the internal standard - to monitor loss of oestrogens during the assay and subsequent colour loss. Ten ml. of ether were then added to each tube and the contents shaken vigorously for half a minute. After separation the lower aqueous layer was removed. Sodium carbonate 0.5 ml. was added to neutralise acid in the ether layer and again each tube shaken vigorously for half a minute. Addition of anhydrous sodium sulphate 1 - 2 g. removed any residual water in the ether extract. A better recovery was obtained when the sodium carbonate layer was specifically removed prior to the addition of anhydrous sodium sulphate and this small modification was incorporated into the method. Three ml. of
ether from tubes 1 and 2 and duplicate aliquots of 3 ml. from the internal standard tube were transferred to clean Kober tubes. An external standard tube containing 1 ml. of oestriol standard \((5 \mu g/ml.)\) and 3 ml. of ether was set up in a 5th tube. 0.2 ml. of 2% quinol in ether was added to these 5 tubes and to a 6th for a reagent blank. After the addition of some bauxite chips, the tubes were evaporated to dryness, under suction, in a water bath at \(55^\circ C\). Two ml. of 2\% (w/v) quinol in sulphuric acid were added to each tube, which was then stoppered and placed in a boiling water bath for 40 minutes. After cooling in running water for 10 minutes, 1.7 ml. of distilled water were added and the extinction of each tube measured after 15 minutes at 472, 514 and 556 nm, against the reagent blank. The extinction at 514 nm was corrected to exclude interfering chromogens, by using the following formula:-

\[
\text{Corrected } D_{514} = 2x R_{514} - (R_{472} + R_{556}),
\]

where \(R\) is the actual reading of the extinction at these wavelengths.

The means of the duplicate tests and internal standard were used in the subsequent calculation.

The quantity of Kober chromogen was calculated from the following formula:-

\[
\text{Kober chromogen} = 20 \times \frac{D_{514} T}{D_{514} IS - D_{514} T} \text{ mg./24 Hr.}
\]

Where \(D_{514} T\) is the mean corrected extinction at 514 nm of the test, and \(D_{514} IS\) is the same for the internal standard.
RESULTS

Percentage Recovery

The percentage recovery was estimated by the addition of 10, 30 and 50 mg. oestriol standard, dissolved in a minimal amount of ethanol and made up to 2 l. with male urine. The urinary oestriol was estimated five times at each concentration, and the results are shown below in Table I.

<table>
<thead>
<tr>
<th>Mean of 5 estimations</th>
<th>10 mg.</th>
<th>30 mg.</th>
<th>50 mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SD in mg.</td>
<td>6.74 ± 1.2</td>
<td>22.7 ± 2.8</td>
<td>38.5 ± 4.6</td>
</tr>
</tbody>
</table>

Mean % recovery

| 67.4 | 75.7 | 77.0 |

The percentage recovery in individual estimations was calculated from the following formula:

\[
\% \text{ Recovery} = 0.84 \times \frac{D_{514}^{IS} - D_{514}^{T}}{D_{514}^{ES}}
\]

Where \( D_{514}^{ES} \) is the corrected extinction at 514 nm of the external standard.
Reproducibility of Results

The preceding results show that the coefficients of variation of the method are 17.9%, 12.3% and 11.9% depending on whether the amount of oestriol being estimated was 10, 30 or 50 mg. per 2 litres of urine.

The means of 4 estimations carried out on two pregnancy urines, selected to contain a large and small amount of oestrogen were 48.1 ± 5.9 mg. and 5.1 ± 0.7 mg. giving coefficients of method variation of 12.3% and 13.7% respectively.

TOTAL URINARY OESTROGEN EXCRETION IN NORMAL PREGNANCY

The results of 174 total urinary oestrogen estimations carried out between 30 and 42 weeks gestation in 45 normal pregnancies are shown in Table II. These results show that there is a rise up to 37 weeks gestation after which time the urinary oestrogen excretion falls gradually to term and then more sharply to 42 weeks gestation. The normal range is taken as 2 standard deviations from the mean.

TOTAL URINARY OESTROGEN EXCRETION IN PREGNANCIES COMPLICATED BY HYPERTENSION

Mild Hypertension
The results of 148 total urinary oestrogen estimations carried out between 30 and 42 weeks gestation in 32 pregnancies complicated by mild hypertension are shown in Table III.

These results do not differ significantly from the normal until 34 weeks gestation at which time they become significantly reduced. There were inadequate estimations at and after term for valid comparison.

Moderate to Severe Hypertension

The results of 179 total urinary oestrogen estimations carried out between 30 and 42 weeks gestation in 39 pregnancies complicated by moderate to severe hypertension are shown in Table IV. These values are significantly lower than normal.

When pregnancies resulting in the delivery of a growth-retarded baby are excluded from this group, the results of the remaining 107 estimations are shown in Table V. These show a reduction from the normal only between 34 and 37 weeks gestation, there being inadequate numbers for comparison thereafter. There is no significant difference between these values and those shown in Table IV.
TOTAL URINARY OESTROGEN EXCRETION IN
NON-HYPERTENSIVE PREGNANCIES COMPLICATED
BY THE DELIVERY OF A GROWTH RETARDED BABY

The results of 147 total urinary oestrogen estimations carried out between 30 and 42 weeks gestation in 29 pregnancies complicated by the delivery of a growth retarded baby, are shown in Table VI. These results are significantly lower than those found in normal pregnancy. Seven patients had estimations entirely within the normal limits.

TOTAL URINARY OESTROGEN EXCRETION IN
PREGNANCIES COMPLICATED BY FETAL DISTRESS

Between 30 and 40 weeks gestation, 50 estimations of total urinary oestrogen excretion were carried out in 15 pregnancies complicated by fetal distress. This number of estimations is too small for a weekly comparison with the values found during normal pregnancy.

As the conditions leading to fetal distress can be acute or chronic, only the last value before the onset of labour, and always within one week of labour, has been used in the calculations. Of the 15 patients, 2 had a final oestrogen excretion that was normal, 8 had a value that was more than 1, but less than 2 standard deviations from the normal mean. Thus one third of patients had a final value outside the normal range.
<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean mg./24 hrs.</th>
<th>± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>4</td>
<td>17.5</td>
<td>3.1</td>
</tr>
<tr>
<td>31</td>
<td>3</td>
<td>17.6</td>
<td>3.2</td>
</tr>
<tr>
<td>32</td>
<td>9</td>
<td>19.3</td>
<td>7.4</td>
</tr>
<tr>
<td>33</td>
<td>12</td>
<td>20.0</td>
<td>6.6</td>
</tr>
<tr>
<td>34</td>
<td>15</td>
<td>21.6</td>
<td>7.6</td>
</tr>
<tr>
<td>35</td>
<td>14</td>
<td>27.6</td>
<td>9.7</td>
</tr>
<tr>
<td>36</td>
<td>23</td>
<td>28.3</td>
<td>9.7</td>
</tr>
<tr>
<td>37</td>
<td>20</td>
<td>28.5</td>
<td>8.1</td>
</tr>
<tr>
<td>38</td>
<td>21</td>
<td>25.8</td>
<td>8.5</td>
</tr>
<tr>
<td>39</td>
<td>15</td>
<td>26.1</td>
<td>8.2</td>
</tr>
<tr>
<td>40</td>
<td>12</td>
<td>25.2</td>
<td>9.6</td>
</tr>
<tr>
<td>41</td>
<td>13</td>
<td>20.2</td>
<td>6.8</td>
</tr>
<tr>
<td>42</td>
<td>8</td>
<td>15.4</td>
<td>5.9</td>
</tr>
</tbody>
</table>

174
### TABLE III

**Total urinary oestrogen excretion in pregnancies complicated by mild hypertension**

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>Number of estimations</th>
<th>Mean mg./24 hrs.</th>
<th>± S.D.</th>
<th>Significance of difference from normal mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2</td>
<td>10.9</td>
<td>1.3</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>31</td>
<td>2</td>
<td>14.6</td>
<td>0.8</td>
<td>NS</td>
</tr>
<tr>
<td>32</td>
<td>6</td>
<td>16.1</td>
<td>7.3</td>
<td>NS</td>
</tr>
<tr>
<td>33</td>
<td>8</td>
<td>18.1</td>
<td>6.1</td>
<td>NS</td>
</tr>
<tr>
<td>34</td>
<td>11</td>
<td>20.5</td>
<td>7.0</td>
<td>NS</td>
</tr>
<tr>
<td>35</td>
<td>19</td>
<td>20.7</td>
<td>6.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>36</td>
<td>28</td>
<td>21.2</td>
<td>9.4</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>37</td>
<td>31</td>
<td>19.4</td>
<td>8.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>38</td>
<td>24</td>
<td>19.4</td>
<td>9.5</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>39</td>
<td>9</td>
<td>15.0</td>
<td>4.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>40</td>
<td>4</td>
<td>20.0</td>
<td>5.9</td>
<td>NS</td>
</tr>
<tr>
<td>41</td>
<td>3</td>
<td>14.9</td>
<td>8.8</td>
<td>NS</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
<td>16.1</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**NS** = No significance
TABLE IV

Total urinary oestrogen excretion in pregnancies complicated by moderate to severe hypertension: all weights of babies.

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean mg./24 hrs.</th>
<th>± S.D.</th>
<th>Significance of difference from normal mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>7</td>
<td>9.1</td>
<td>5.5</td>
<td>(&lt;0.02)</td>
</tr>
<tr>
<td>31</td>
<td>11</td>
<td>11.4</td>
<td>7.9</td>
<td>(&lt;0.05)</td>
</tr>
<tr>
<td>32</td>
<td>17</td>
<td>15.3</td>
<td>8.9</td>
<td>NS</td>
</tr>
<tr>
<td>33</td>
<td>22</td>
<td>13.7</td>
<td>6.5</td>
<td>(&lt;0.02)</td>
</tr>
<tr>
<td>34</td>
<td>23</td>
<td>12.8</td>
<td>6.4</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>35</td>
<td>27</td>
<td>13.7</td>
<td>8.5</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>36</td>
<td>28</td>
<td>14.7</td>
<td>8.0</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>37</td>
<td>22</td>
<td>14.2</td>
<td>6.5</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>38</td>
<td>14</td>
<td>16.9</td>
<td>9.3</td>
<td>(&lt;0.005)</td>
</tr>
<tr>
<td>39</td>
<td>4</td>
<td>14.8</td>
<td>8.2</td>
<td>(&lt;0.025)</td>
</tr>
<tr>
<td>40</td>
<td>3</td>
<td>10.1</td>
<td>1.0</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>41</td>
<td>1</td>
<td>24.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[179]
TABLE V

Total urinary oestrogen excretion in pregnancies complicated by moderate to severe hypertension:

normal weight babies only

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean mg./24 hrs.</th>
<th>± S.D.</th>
<th>Significance of difference from mean of normal pregnancy</th>
<th>Significance of difference from mean values in Table IV</th>
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<td>9.6</td>
<td>&lt;0.005</td>
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<td>&lt;0.01</td>
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<td>&lt;0.001</td>
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<td>1.4</td>
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### TABLE VI

Total urinary oestrogen excretion in non-hypertensive pregnancies complicated by delivery of a growth retarded baby

<table>
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<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean mg./24 hrs.</th>
<th>± S.D.</th>
<th>Significance of difference from normal mean</th>
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</thead>
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<td>&lt; 0.01</td>
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<td>6.1</td>
<td>&lt; 0.005</td>
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<tr>
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<td>11.6</td>
<td>7.4</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>35</td>
<td>17</td>
<td>11.6</td>
<td>6.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>36</td>
<td>18</td>
<td>10.5</td>
<td>6.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>37</td>
<td>21</td>
<td>11.3</td>
<td>7.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>38</td>
<td>15</td>
<td>11.3</td>
<td>6.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>39</td>
<td>7</td>
<td>11.7</td>
<td>2.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>40</td>
<td>9</td>
<td>11.4</td>
<td>4.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>41</td>
<td>5</td>
<td>9.1</td>
<td>3.5</td>
<td>&lt; 0.001</td>
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<td>42</td>
<td>2, 147</td>
<td>8.3</td>
<td>2.7</td>
<td>&lt; 0.05</td>
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</table>
Twenty seven estimations of total urinary oestrogen excretion were carried out between 30 and 42 weeks gestation in 12 pregnancies ending in a perinatal death. Of these 12 patients, 6 had a final oestrogen excretion value, within one week of labour, that was normal, while 6 had a value that was outside the normal range, i.e. more than 2 standard deviations from the normal mean.

Of the 6 perinatal deaths with a final urinary oestrogen excretion within the normal range, two were due to respiratory distress syndrome in premature but normal weight for dates babies, one was due to a congenital heart abnormality and another due to shoulder dystocia.

In the remaining 2 cases, the urinary oestrogen excretion was misleading in that in one patient the values were normal immediately prior to intrauterine death, and in the second, the values were normal in a growth retarded baby dying of respiratory distress syndrome.

Thus, from 8 cases of perinatal death in which the total urinary oestrogen excretion would be expected to be of value, the final result was outside the normal range in 6 (75%) but within the normal range in 2 (25%).
RELATIONSHIP OF TOTAL URINARY OESTROGEN EXCRETION TO Fetal AND CRUDE placental Weights

Most of the patients studied had weekly serial estimations carried out, and it was felt that a single value of urinary oestrogen excretion carried out immediately prior to the onset of labour was not necessarily representative of the preceding values and hence not necessarily related to the fetal or placental weight. Consequently, when there were three or more estimations, these were examined to determine their trend, the equation of the line best fitting the results was calculated, and the last value before delivery adjusted to take into account the preceding results. This was the figure used in both correlations. In cases where there were less than three estimations, the last value, always within one week of the onset of labour, was used.

Fetal Weight

The results of urinary oestrogen output related to fetal weight in 37 pregnancies are shown in Fig. 3.

There is a correlation between these two parameters. The correlation coefficient is 0.533, the regression coefficient is 7.224, and as this is 3.205 times its standard error of 1.95, this is statistically significant at the p < 0.001 level. The regression equation is \( y = 7.224x - 4.065 \), where \( x \) is the fetal weight and \( y \) the urinary oestrogen output.
Relationship of total urinary oestrogen excretion to fetal weight.
Crude Placental Weight. This is defined as the weight of the placenta, membranes, and umbilical cord trimmed to a constant length. These results, again in 37 pregnancies, are shown in Fig. 4.

There is a correlation between these two parameters. The correlation coefficient is 0.402, the regression coefficient is 27.94, and as this is 2.597 times its standard error of 10.76, this is statistically significant at the p < 0.02 level. The regression equation is \( y = 27.94x + 1.36 \), where \( x \) is the crude placental weight and \( y \) the urinary oestrogen output.
Fig. 4.

Relationship of total urinary oestrogen excretion to crude placental weight.
DISCUSSION

The large increase in urinary oestrogen excretion during pregnancy reflects greatly increased secretion (Klopper and Billewicz, 1963). The placenta plays a major part in the biosynthesis of oestrogens, while the fetus is the site of intermediary metabolism. Brown (1956) found that of the oestrogens excreted in pregnancy, oestriol constituted about 90% of the total. Despite low levels in such non-relevant conditions as anencephaly (Frandsen and Stakemann, 1964), urinary tract infection (Taylor et al. 1963), and anaemia (Beischer et al. 1968a), urinary oestrogen excretion is of value as a guide to the functional state of the feto-placental unit, and so it is of prognostic value to the clinician. This value is most marked with impending intrauterine death, for in this situation many authors, among them Klopper et al. (1961) and Cartlidge et al. (1961), have found markedly reduced oestriol excretion.

Kober (1931), noted that when the fluorescent solution produced by the reaction of concentrated sulphuric acid on ether extracted urine, was diluted with water and warmed, a clear red colour with a greenish fluorescence appeared. This reaction was almost specific for natural oestrogens. Phenol added to the reaction mixture, quenched the final fluorescence making the red colour more intense and suitable for colorimetry. This reaction is known as the Kober reaction. The conditions necessary for maximum colour production have been described by Brown (1952). Assays of
specific oestrogens such as oestriol, oestrone and oestradiol, involving this reaction, have been described by Brown (1955) and Brown et al. (1957), but separation of the individual oestrogens is relatively complex and time consuming. Shorter methods have been described by Klopper and Wilson (1962), Brown and Coyle (1963) and Palmer (1964), but these too are time consuming.

In pregnancy, where the amount of oestrogen excreted in the maternal urine is high, with oestriol forming as much as 90% of it, shorter but less specific methods are acceptable. These methods shorten the extraction process, and measure oestrogens by the Kober reaction without separation into its individual components. These have been described by, among others, Montague (1964) and Oakey et al. (1967). The method of Oakey et al. (1967) has been used in this study.

The recovery rate of approximately 75% of the added oestriol is acceptable and compares closely with the figures obtained by Montague (1964) and Oakey et al. (1967). The oestriol is lost during hydrolysis and the subsequent handling. In individual pregnancy urines, the percentage recovery as calculated from the formula given on page 46, occasionally rose to over 100%. This is due to failure of the correction factor described by Allen (1950) to allow for contaminating chromagens. As this occurred only when the urinary oestrogen excretion was high, it was of little clinical significance.

The coefficient of method variation ranged between 12% and
18%, being highest when small amounts of oestrogens were being assayed. These figures are comparable with those recorded by Oakey et al. (1967) but nevertheless, they are still quite high, and when this and the large day to day variation in urinary oestrogen excretion are taken into account, it is obvious that no reliance can be placed on a single value. Clinical action can only be taken on the evidence of an established trend in results.

The results in this study, carried out during normal pregnancy (Table II) compare closely with those of Oakey et al. (1967).

The results in pregnancies complicated by mild hypertension show a significant reduction when compared to normal pregnancy. (Table III), but only after 34 weeks gestation.

The results in pregnancies complicated by moderate to severe hypertension are significantly lower than normal, (Table IV), the difference occurring earlier in the pregnancy and being more marked, than in cases of mild hypertension. When pregnancies complicated by moderate to severe hypertension, and resulting in the delivery of a normal weight baby are examined, the results (Table V), although a bit higher, show no significant difference from those found in all cases of moderate to severe hypertension. (Table IV).

When these results (Table V) are compared with the normal, there is a significant reduction between 35 and 37 weeks of
gestation only, this being similar to the findings in cases of mild hypertension. Again there are an inadequate number of values from 38 weeks gestation onwards for proper assessment. This indicates that the more pronounced reduction in total urinary oestrogen excretion found in cases of moderate to severe hypertension, is associated with a growth retarded baby. The reduction, associated with the hypertension alone is slight. It is not significantly different between mild and severe cases, and is not apparent until about 35 weeks gestation.

The results in pregnancies terminating in the delivery of a growth retarded baby (Table VI) are markedly reduced from normal from 30 weeks onwards.

The results of serial urinary oestrogen levels adjusted to take into account their trend, give a good correlation with fetal weight (Fig. 3), this being in agreement with Frandsen and Stakemann (1960a); Coyle and Brown (1963); Beling (1967); Beischer et al. (1968b); Curzen and Southcombe (1970); Curzen and Hensel, 1972). There is also a correlation with placental weight, though this is not very strong \( p < 0.02 \). This finding agrees with Curzen and Southcombe (1970) but other authors have been unable to demonstrate this relationship (Klopper and Billiewicz, 1963; Coyle and Brown, 1963; Beling, 1967; Curzen and Hensel, 1972).

This is surprising because there is a good correlation between fetal and placental weights, and as the placenta is
as significant as the fetus in the metabolism of oestrogens, one would expect urinary oestrogen excretion and placental weight to be in some way related. The inaccuracies caused by using crude placental weight, and other variables, probably obscure a weak correlation.

It would seem from this study that total urinary oestrogen values below 10 mg./24 hrs after 32 weeks gestation are suggestive of a growth retarded baby, and there is agreement with Elliott's (1970) findings that these estimations are valuable in assessing fetal prognosis in these cases.

However, 7 of 29 patients (24%) delivering themselves of growth retarded babies had total urinary oestrogen estimations that were within normal limits. This compares with corresponding figures of approximately 10% (Klopper, 1969) and 18% (Elliott, 1970). The differences found in various series may be due to lack of a standard definition for a growth retarded baby. It may be that a birth weight below the 10th percentile is not a sufficiently strict criterion, thus accounting for some of the cases of normal urinary oestrogen excretion, in association with growth retardation, that was minimal.

One third of cases of fetal distress had values outside the normal range, and 87% had values between 1 and 2 standard deviations from the normal mean. As the normal range is wide the prognostic value in these cases is limited. The position in cases of perinatal death is more clear cut, for three quarters of the anoxic deaths had estimations outside
the normal range. Two out of eight cases of perinatal death (one due to growth retardation and one to sudden intrauterine death in a toxaemic patient) had normal urinary oestrogen excretion. The explanation in the latter case was probably the suddenness of the intrauterine death, due to an accidental haemorrhage, while the former case is difficult to explain. Frandsen and Stakemann (1960b) and Oakey et al. (1969) have recorded similar cases of intrauterine deaths preceded by normal urinary oestrogen excretions. Irrespective of the cause of perinatal death, one third of cases had normal urinary oestrogen excretion in late pregnancy. This is in agreement with the findings of Frandsen and Stakemann (1963), and Nelson (1969) that low urinary oestrogen excretion is associated with a high perinatal mortality. Persistently low values do give a good guide to growth retarded babies, and subsequent fetal prognosis, some guide to fetal distress in labour, and although the normal range is wide, the test does to a large extent fulfil the criteria that are necessary for a placental function test.
REAGENTS USED FOR SERUM HEAT STABLE

ALKALINE PHOSPHATASE ESTIMATIONS

All reagents used were 'Analar', and supplied by British Drug Houses Ltd.

Disodium phenyl phosphate 72 m.mol/l.
Sodium carbonate - bicarbonate buffer, pH 10.7
4 Amino phenazone
Potassium ferricyanide
Phenol standard 5mg./100ml. distilled water

"Phosphatase reagent": A 72 m.mol/l. solution of disodium phenyl phosphate was made in 250ml. sodium carbonate - bicarbonate buffer; 0.5g. 4 amino phenazone was added and the pH adjusted to 10.7.

Potassium ferricyanide, 12g. was dissolved in 250ml. of the same buffer.

Stability of reagents

The phosphatase reagent was stored at 4°C and was prepared weekly.

The potassium ferricyanide was stored in a dark bottle.

The phenol standard was stored at 4°C and was stable for months.
METHOD USED FOR ESTIMATING SERUM HEAT

STABLE ALKALINE PHOSPHATASE

The method is based on a simplification of Kind and King's method (1954), described by Hansen (1966). The further modification of heating the sera to 65°C for 5 minutes to destroy non-placental iso-enzymes was described by Fishman et al. (1968b) and Stolbach et al. (1969). The critical pH (10.7) of the enzyme reaction was described by Ghosh and Fishman (1968).

Venous blood was taken carefully to avoid haemolysis, and at a constant time. After 10 minutes the clot was gently separated from the side of the tube, which was then centrifuged to separate the serum. All the enzyme estimations were carried out at once, making storage of the sera unnecessary.

The serum, 0.5ml., was diluted with 0.5ml. of distilled water, and then heated in a water bath at 65°C for 5 minutes. The dilution was necessary to avoid coagulation of the sera.

For each test serum, two tubes were placed in a water bath at 37°C and into each were pipetted 3ml. of phosphatase reagent. To one tube - the blank - 1ml. of potassium ferricyanide was added, preventing any reaction from taking place; 0.1ml. of serum was then pipetted into both tubes and the reaction in the 'test' tube stopped after 15 minutes by the addition of 1ml. of potassium ferricyanide.
For each batch of enzyme assays a standard and a standard blank estimation were carried out.

The standard tube contained 3 ml. of phosphatase reagent, and 0.1 ml of phenol standard, to which 1 ml. of potassium ferricyanide was added after 15 minutes. The standard blank tube contained 3 ml. of phosphatase reagent, 1 ml. of potassium ferricyanide and 0.1 ml. of distilled water.

The strength of the colour reaction is proportional to the amount of phenol released by the alkaline phosphatase from the substrate. The phenol combines with the 4 amino phenazone, and in an alkaline medium, in the presence of potassium ferricyanide, oxidation takes place to quinone.

The chemical reaction is illustrated below:

\[
\text{Ph} \quad \text{Ph} \\
\text{N} \quad \text{N} \\
\text{Me—N} \quad \text{C} = \text{O} + \text{Phenol} \quad \text{OH} \quad \text{K}_3\text{Fe(CN)}_6 \quad \text{Me—N} \quad \text{C} = \text{O} \\
\text{Me—C} = \text{C—NH}_2 \quad \text{Phenol} \quad \text{Alkali} \quad \text{Me—C} = \text{C—N} \quad \text{Red Quinone}
\]

After allowing 5 minutes for the full development of the colour reaction, the extinction of each tube was read on a Unicam SP 600 spectrophotometer (Pye Ltd.) at 520 nm against distilled water as a blank.
One placental iso-enzyme unit (P.I.U.) is defined as the amount of enzyme which will liberate 1.0 mg. phenol from 72 m.mol.L^{-1} phenyl phosphate in 15 minutes at 37°C and at pH 10.7.

The units can be calculated from the following formula:

\[
\frac{\text{Test reading} - \text{Blank reading}}{\text{Standard reading} - \text{Standard blank reading}} \times 10 \quad \text{P.I.U.}
\]
Reproducibility of Serum HSAP results

Ten estimations were carried out on the same sample of serum. The mean value was $8.2 \pm 0.3$ P.I.U. The coefficient of method variation was 3.7%.

SERUM HSAP LEVELS IN NORMAL AND ABNORMAL PREGNANCY

The results of serum HSAP estimations carried out during 72 normal and 113 abnormal pregnancies are shown in Tables VII to XI, and illustrated in Fig. 5. The normal range is taken as 2 standard deviations from the mean.

SERUM HSAP LEVELS IN PREGNANCY COMPLICATED BY PERINATAL DEATH

Twenty four serum HSAP estimations were carried out on 10 cases of perinatal death, this data being inadequate for comparison on a weekly basis with the normal group. Five patients had estimations outside the normal range, and this number differs significantly from the normal group ($p<0.001$).

In the complicated pregnancies described, there were a total of 24 serum HSAP estimations outside the normal range. Eighteen of these were higher than normal and occurred at 36 weeks gestation or earlier, while 6 were lower than normal and occurred at 36 weeks gestation or later.
TABLE VII

Serum HSAP estimations in 72 normal pregnancies.

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean P.I.U.</th>
<th>± S.D.</th>
</tr>
</thead>
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<tr>
<td>27-29</td>
<td>14</td>
<td>11.7</td>
<td>3.31</td>
</tr>
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<td>4.98</td>
</tr>
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<td>12.8</td>
<td>4.99</td>
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<td>17</td>
<td>13.3</td>
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<td>(40-42)</td>
<td>32</td>
<td>19.4</td>
<td>6.58</td>
</tr>
</tbody>
</table>

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Five patients had a total of 6 estimations outside the normal range.
**TABLE VIII**

Serum HSAP estimations in 39 pregnancies complicated by mild hypertension

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean P.I.U.</th>
<th>± S.D.</th>
<th>Significance of difference of mean compared with normal pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>27-29</td>
<td>7</td>
<td>9.2</td>
<td>3.22</td>
<td>NS</td>
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<td>30</td>
<td>4</td>
<td>11.0</td>
<td>5.14</td>
<td>NS</td>
</tr>
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<td>4</td>
<td>17.3</td>
<td>9.56</td>
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<td>7</td>
<td>16.3</td>
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<td>16.3</td>
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<td>NS</td>
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<td></td>
<td><strong>128</strong></td>
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</tbody>
</table>

Six patients had estimations outside the normal range. This number does not differ significantly from the normal group \(p > 0.1\).
### TABLE IX

Serum HSAP estimations in 59 pregnancies complicated by moderate to severe hypertension

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean ± S.D.</th>
<th>Significance of difference of mean compared with normal pregnancy</th>
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<tr>
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<td>18.3 ± 4.35</td>
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<td>39</td>
<td>8</td>
<td>14.0 ± 5.17</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>40-42</td>
<td>4</td>
<td>19.6 ± 13.38</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>209</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Four patients had estimations outside the normal range. This number does not differ significantly from the normal group (p>0.3).

Twenty patients had estimations carried out between 1 and 5 weeks prior to the onset of their hypertension. Only 3 of
these patients had values outside the normal range, and this number does not differ significantly from the normal group \((p>0.3)\).
TABLE X

Serum HSAP estimations in 15 non-hypertensive pregnancies complicated by the delivery of a growth retarded baby.

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean ± S.D.</th>
<th>Significance of difference of mean compared with normal pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>32-34</td>
<td>6</td>
<td>15.0 ± 5.73</td>
<td>NS</td>
</tr>
<tr>
<td>35-36</td>
<td>5</td>
<td>16.3 ± 4.42</td>
<td>NS</td>
</tr>
<tr>
<td>37</td>
<td>7</td>
<td>18.3 ± 5.92</td>
<td>NS</td>
</tr>
<tr>
<td>38</td>
<td>9</td>
<td>19.1 ± 6.04</td>
<td>NS</td>
</tr>
<tr>
<td>39</td>
<td>7</td>
<td>18.5 ± 5.20</td>
<td>NS</td>
</tr>
<tr>
<td>40-42</td>
<td>10</td>
<td>15.3 ± 5.26</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There were no estimations outside the normal range.
### TABLE XI

Serum HSAP estimations in 19 pregnancies complicated by fetal distress

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean ± S.D.</th>
<th>Significance of difference of mean compared with normal pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2</td>
<td>10.6 ± 0.49</td>
<td>NS</td>
</tr>
<tr>
<td>31</td>
<td>3</td>
<td>10.4 ± 3.83</td>
<td>NS</td>
</tr>
<tr>
<td>32</td>
<td>2</td>
<td>18.4 ± 17.40</td>
<td>NS</td>
</tr>
<tr>
<td>33</td>
<td>2</td>
<td>16.0 ± 8.84</td>
<td>NS</td>
</tr>
<tr>
<td>34</td>
<td>5</td>
<td>16.4 ± 6.81</td>
<td>NS</td>
</tr>
<tr>
<td>35</td>
<td>6</td>
<td>17.2 ± 5.62</td>
<td>NS</td>
</tr>
<tr>
<td>36</td>
<td>9</td>
<td>15.9 ± 4.70</td>
<td>NS</td>
</tr>
<tr>
<td>37</td>
<td>9</td>
<td>16.1 ± 7.67</td>
<td>NS</td>
</tr>
<tr>
<td>38</td>
<td>7</td>
<td>16.5 ± 6.01</td>
<td>NS</td>
</tr>
<tr>
<td>39</td>
<td>7</td>
<td>15.9 ± 5.95</td>
<td>NS</td>
</tr>
<tr>
<td>40-42</td>
<td>15</td>
<td>14.1 ± 4.60</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Three patients had estimations outside the normal range. This number does not differ significantly from the normal group (p > 0.3).
Fig. 5.

Serum HSAP levels in normal pregnancy.
Fig. 6.

Relationship of serum HSAP levels to fetal weight.
RELATIONSHIP OF SERUM HSAP LEVELS TO FETAL AND PLACENTAL WEIGHTS

Fetal weight and crude placental weight were related to corrected serum HSAP levels in 37 patients. Most of these patients had weekly serial estimations carried out, and it was felt that a single value of serum HSAP carried out immediately prior to the onset of labour was not necessarily representative of the preceding values, and hence not necessarily related to fetal or placental weight. Consequently, when there were three or more estimations, these were examined to determine their trend, the equation of the line best fitting the results was calculated and the last value before delivery adjusted to take into account the preceding results.

Serum HSAP levels and fetal weight.

These results are shown in Fig. 6. There is no correlation between these two parameters. The correlation coefficient is -0.053, the regression coefficient is -0.468 and as this is 0.312 times its standard error of 1.499 it is not statistically significant. (p>0.7).

Twelve of the 37 patients gave birth to growth retarded babies. The mean corrected serum HSAP value did not differ significantly from that of the normal group.
These results are shown below in Table XII.

**TABLE XII**

Serum HSAP estimations in patients delivering growth retarded and normal weight babies.

<table>
<thead>
<tr>
<th></th>
<th>Growth retarded group</th>
<th>Normal weight group</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>12</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Mean gestational age (weeks)</td>
<td>38.2 (range 34-42)</td>
<td>38.8 (range 33-42)</td>
<td>p&gt;0.4</td>
</tr>
<tr>
<td>Serum HSAP F.I.U. ± S.D.</td>
<td>15.3 ± 3.37</td>
<td>16.2 ± 6.84</td>
<td>p&gt;0.5</td>
</tr>
</tbody>
</table>

Serum HSAP levels and crude placental weight.

These results are shown in Fig. 7. There is no correlation between these two parameters. The correlation coefficient is 0.119, the regression coefficient is 5.408, and as this is 0.712 times its standard error of 7.598 it is not statistically significant (p>0.4)
In view of the positive correlation which was found between the last serum HSAP estimation and the crude placental weight by Curzen and Southcombe (1970) further analysis was carried out on 130 patients, using only the last serum HSAP estimation, provided that it had been carried out less than one week prior to the onset of labour.

The correlation coefficient between these 130 estimations and the fetal weight is 0.115, the regression coefficient is 0.63 and as this is 1.31 times its standard error of 0.48 it is not statistically significant (p>0.1).

The correlation coefficient between the 130 serum HSAP estimations and the crude placental weight is 0.04, the regression coefficient is 0.489 and as this is 0.512 times its standard error of 0.955 it is not statistically significant (p>0.6).

**RELATIONSHIP OF SERUM HSAP LEVELS TO TOTAL URINARY OESTROGEN EXCRETION**

Two hundred and eighty eight paired estimations of serum HSAP and total urinary oestrogen excretion were carried out during 65 pregnancies, both normal and abnormal. These are shown in Fig. 8. There is no correlation between these two parameters. The correlation coefficient is 0.075, the regression coefficient is 0.041, and as this is 0.837 times its standard error of 0.049, it is not statistically significant (p>0.4).
**Fig. 7.**

Relationship of serum HSAP levels to crude placental weight.
Relationship of serum HSAP levels to total urinary oestrogen excretion.
The specificity of the method used for estimating serum HSAP levels is most important for if variations in the levels of the placental iso-enzyme are to be of any prognostic value then other iso-enzymes of alkaline phosphatase must be excluded from the assay.

Establishment of the fact that alkaline phosphatase in the sera of pregnant women was heat stable was an important step in being able to measure it specifically. Initially a temperature of $56^\circ C$ for 30 minutes was suggested by McMaster et al. (1964). Subsequent work by Hunter (1969) showed that a temperature of $65^\circ C$ for 30 minutes was necessary for the complete inactivation of the non-placental iso-enzymes. The critical time and temperature were modified by Fishman et al. (1968a; 1968b) to $55^\circ C$ for 60 minutes or $65^\circ C$ for 5 minutes. The pH and substrate concentration are also important in obtaining the maximal activity of the placental iso-enzyme. Ghosh and Fishman (1968) found the critical levels of these conditions to be pH 10.7 and a substrate concentration of 72 $\text{m.mol/l.dl}$ sodium phenyl phosphate. Three studies, in addition to this work have been carried out using the same conditions. (Benster, 1970; Curzen and Varma, 1971; Curzen and Hensel, 1972).

Despite the use of different methods for estimating serum HSAP there is agreement that in normal pregnancy serum HSAP levels show a linear increase (Curzen and Morris, 1966;
Hunter, 1969; Hunter et al. 1970; Quigley et al. 1970; Watney et al. 1970; Curzen and Varma, 1971). The results of this study confirm these findings (Fig. 5). Benster (1970) recorded a distribution of normal values corresponding to a third degree polynomial curve. The reason for the difference between his findings and those of other workers using the same methods in normal pregnancy, is obscure.

However, as the method described by Fishman et al. (1968b) and Stolbach et al. (1969) seems to be the most accurate for the estimation of the placental iso-enzyme, the findings of Benster (1970) in hypertensive pregnancies are of interest. These were that in pre-eclamptic toxaemia, the values before 32 weeks gestation were high but failed to show the progressive rise found in normal pregnancy. The results of this study in cases of mild hypertension (Table VIII) are not significantly different from normal except after term. They are in agreement with the findings of Quigley et al. (1970) in cases of mild pre-eclamptic toxaemia.

The results in cases of moderate to severe hypertension (Table IX) fail to show the rise in levels recorded in normal pregnancy. All the abnormally high values occurred earlier in pregnancy than the abnormally low values. This confirms Benster's (1970) findings, but is in disagreement with other authors who found high levels of serum HSAP throughout the third trimester of pregnancy complicated by moderate to severe pre-eclamptic toxaemia (Hunter, 1969; Hunter et al. 1970; Quigley et al. 1970).
Although this work does not deal specifically with pre-eclamptic toxaemia, the majority of patients studied either did have, or probably had, this complication. Agreement with the results of Benster's (1970) specific study of pre-eclamptic toxaemia suggests that the slight difference in clinical material is not significant.

The number of patients who had abnormal serum HSAP estimations prior to the onset of hypertension does not differ significantly from the normal group and so the test seems valueless in the prediction of hypertension, this being contrary to the findings of Hunter (1969) and Hunter et al. (1970).

In cases of fetal growth retardation, the mean values were not significantly different from normal, except for a reduction after term. (Tables X and XII). No patient in the group with fetal growth retardation had an estimation outside the normal range. The absence of correlation between serum HSAP estimations, using two differently calculated values, and fetal weight (Fig. 6.) agrees with the findings of Curzen and Southcombe (1970); Curzen and Hensel (1972) and Pirani et al. (1972). The absence of correlation between serum HSAP estimations and placental weight (Fig. 7.) is in agreement with Curzen and Hensel (1972) and Pirani et al. (1972), but disagrees with the results of Curzen and Southcombe (1970).
The absence of correlation in this study between paired serum HSAP levels, and total urinary oestrogen excretion (Fig. 8.) agrees with the findings of Curzen and Hensel (1972) but disagrees with the findings of Messer (1967) and Curzen and Southcombe (1970). Messer's correlation involved only groups of results rather than actual values, while the difference between the findings of Curzen and Southcombe (1970) and Curzen and Hensel (1972) is due chiefly to a false interpretation of results in the former study. Pirani et al. (1972) showed a positive correlation in normal pregnancy between serum HSAP and urinary oestriel levels but again differences in method of estimating serum HSAP makes comparison difficult.

All the results from this study suggest that serum HSAP estimations are valueless in the prediction of a growth retarded baby and this confirms the findings of Watney et al. (1970) and Curzen and Varma (1971).

The results in cases of fetal distress are similar to those in cases of fetal growth retardation (Table XI) and again the estimations seem to be valueless in its prediction. This is in agreement with Curzen and Varma (1971).

The number of patients suffering a perinatal loss, who had abnormal serum HSAP values was significantly higher than expected. All the abnormal values were higher and occurred before 36 weeks gestation. This confirms the findings of Hunter et al. (1970) and Benster (1970), that an abnormally
high value early in the third trimester of pregnancy is associated with a poor fetal progress.

The reasons for the abnormal elevation of serum HSAP in hypertensive patients, and in those suffering a perinatal loss, is difficult to explain in the light of present knowledge. The rise may be due to excessive proliferation of trophoblast and leakage of the enzyme from damaged placental cells. Moderate sized infarcts may cause further sharp rises from the already elevated levels. The drop in serum HSAP levels in hypertensive patients may be due to a reduction in the amount of syncytiotrophoblast and the consequent reduction in placental heat stable alkaline phosphatase (Watkins and Anderson, 1971). Further rises from these altered levels may be due to acute placental infarction. However, the prospect of using these altered levels as a guide to obstetrical intervention is reduced considerably by the large overlap of values found in abnormal pregnancy, with the normal range.
All reagents used were 'AnalaR' and supplied by British Drug Houses Ltd., except for Brentamine fast garnet stain.

1. Formaldehyde 37% (v/v)
2. Methanol 99% (v/v)
3. Brentamine fast garnet stain (Raymond A. Lamb)
4. 1 Naphthyl disodium orthophosphate
5. 2 Amino - 2 methyl - propane (1:3) diol
6. Hydrochloric Acid 0.1N
7. Methyl green 2% (w/v)
8. Chloroform

Preparation of reagents:

Fixative: Methanol formalin:

A mixture of 1 volume of formaldehyde and 1 volume of absolute methanol.

Substrate: Equal weights of Brentamine fast garnet stain and 1 naphthyl disodium orthophosphate.

Stock Buffer: Propanediol buffer 0.2M:

10.5g. 2 amino - 2 methyl - propane (1:3) diol dissolved in 500 ml. distilled water.

Working Buffer: Propanediol buffer 0.05M.

25 ml. stock propanediol buffer and 5 ml. 0.1N hydrochloric acid diluted to 100 ml. with distilled water.

Counter Stain: Purified methyl green:

Methyl green stain 2% (w/v) in distilled water is left for 48 hours with half its volume of chloroform to remove methyl violet contamination, with frequent shaking.
The purified stain is then separated from the lower chloroform layer.

**Stability of Reagents**

The substrate was made up freshly on each occasion. Stock and working buffers, and methanol formalin were stored at 4°C. The stock buffer was stable at 4°C for about 2 months. In practice it was made up freshly each month, and the working buffer at weekly intervals.
METHOD USED FOR PREPARING AND STAINING SLIDES FOR NEUTROPHIL ALKALINE PHOSPHATASE ESTIMATIONS

The preparation, staining and scoring of the slides was carried out according to the method described by Climie et al. (1962). This method is a modification of that described by Kaplow (1955) and Hayhoe and Quaglino (1958).

The principle of the method is that alkaline phosphatase catalyzes the hydrolysis of buffered naphthyl phosphate releasing naphthol, which links with a diazotised amine to form a coloured precipitate at the site of enzymic activity. Hayhoe and Quaglino (1958) found that Brentamine fast garnet was a more suitable coupling agent than the fast blue RR advocated by Kaplow (1955). Hayhoe and Quaglino (1958) and Climie et al. (1962) confirmed this, and also noted that better nuclear-cytoplasmic contrast was obtained using methyl green counter stain rather than a combination of fast blue RR and haematoxylin. Climie et al. (1962) also found that there was a tendency for smears to wash off using the more dilute fixative advocated by Kaplow (1955) and Hayhoe and Quaglino (1958). They therefore suggested the use of equal volumes of 37\% formaldehyde and absolute methyl alcohol. This fixative was found to be entirely satisfactory in this study.

Preparation of leucocytes

2.5 ml. of venous blood was anti-coagulated with EDTA. The blood was allowed to stand at room temperature until
the erythrocytes sedimented and a well defined layer of leucocytes had formed above them. A small volume of this leucocyte rich plasma was carefully aspirated with a Pasteur pipette and dropped onto a microscope slide.

**Staining**

A smear was made, air dried, fixed for 30 seconds in methanol formalin, and air dried again.

The substrate was made up of equal weights of Brentamine fast garnet stain and sodium α-naphthyl phosphate, allowing approximately 1 mg. of each substance per slide to be stained.

Immediately prior to use the substrate was dissolved in 0.05M propanediol buffer, the volume in millilitres being equal to half the total weight of the substrate in milligrammes. The substrate solution was filtered directly onto the slides and allowed to incubate at room temperature for 8 minutes. The slides were then rinsed with distilled water for 10 seconds and air dried. The purified methyl green counter stain was then applied to the slides for from 10 to 15 minutes. Further rinsing with distilled water and air drying completed the staining process. The slides, without cover slips were examined under the oil immersion lens of a microscope.
Counting

The nuclei of leucocytes stain green with the counter stain. Mature neutrophils reveal alkaline phosphatase activity in the form of a diffuse red cytoplasmic stain or granulation depending on the amount of enzyme present. One hundred consecutive mature neutrophils were examined in an area where the smear was thin and where there was no overlap of cells. Each neutrophil was given a score according to the following criteria:

0 : - colourless cytoplasm
1 : - slight diffuse red colour with occasional red granules in the cytoplasm.
2 : - diffuse red colour with a moderate number of red granules in the cytoplasm.
3 : - darker, diffuse red colour with numerous red granules in the cytoplasm.
4 : - cytoplasm a deep red and packed with coarse red granules.

The neutrophil alkaline phosphatase score is the sum of the scores of the 100 individual neutrophils which were examined.

MATERIALS AND METHOD USED FOR ESTIMATING ALKALINE PHOSPHATASE ACTIVITY IN LEUCOCYTE SUSPENSIONS

Reagents

Substrate, buffer, colour reagent and phenol standard
used were exactly as described on page 66, for the estimation of serum heat stable alkaline phosphatase.

The following reagents were used in addition:

1) Saponin - 2% (v/v) in saline. (B.D.H.)
2) Magnesium chloride 0.05M 'AnalaR' (B.D.H.)

Method

Twenty ml. of venous blood was anti-coagulated with 2 ml. of 3.8% (w/v) sodium citrate. The erythrocytes were allowed to sediment and after about 1 hour the supernatant plasma containing leucocytes and platelets was separated. This was then centrifuged for 3 minutes at 800 r.p.m., and the supernatant plasma, containing most of the platelets, removed. The leucocytes were washed in normal saline, and resuspended in a small volume of normal saline - approximately 2 to 3 ml.

The suspension was then divided into two parts and standard leucocyte counts in triplicate carried out on both parts. The mean values were used for the calculation of the final results. One part was then incubated in a water bath at 65°C for 5 minutes.

Incubation mixtures for the determination of phosphatase activity consisted of the following:
3 ml. of a mixture of di sodium phenyl phosphate $72 \times 10^{-3} \text{M}$ and 4 amino phenazone $98 \times 10^{-4} \text{M}$, buffered with sodium carbonate-bicarbonate to pH 10.7.

0.15 ml. 2% (v/v) saponin in saline

0.1 ml. 0.05M magnesium chloride

0.1 ml. leucocyte suspension – heated to 65°C or unheated.

The reaction was terminated by the addition of 1 ml. of $12 \times 10^{-3} \text{M}$ potassium ferricyanide. A blank for each leucocyte suspension was made by adding potassium ferricyanide to the incubation mixture before the leucocyte suspension was added. A standard and a standard blank were carried out on each occasion using exactly the same reagents described above, but with the addition of 0.1 ml. of phenol standard and 0.1 ml. distilled water instead of 0.1 ml. leucocyte suspension. After 5 minutes the colour reaction was read at a wavelength of 520 nm against water on an SP 600 spectrophotometer (Pye Ltd.).

The surface active substance, saponin, liberates alkaline phosphatase from the leucocytes by destroying cell membranes. Magnesium ions activate the phosphatase so liberated and this releases phenol from the substrate.

The phenol is converted to quinone as described on page 68, and it is this that is estimated colorimetrically. The definition and calculation of units of activity are described on page 69, and these are expressed as units.
per 10⁶ leucocytes.

RESULTS

Reproducibility of scoring

Duplicate slides were made from 12 patients. They were coded and mixed with other slides for scoring so that the scorer was unable to relate them to each other.

The mean difference between the duplicate slides from the 12 patients was 5.66 ± 3.53 with a range of 1 to 13.

Ten slides were made at the same time from one patient, and again coded and mixed with other slides for scoring at different times. The mean of these ten values was 130.8 ± 3.94, and the range was 125 to 137.

Ten slides were made from different patients, coded and scored by two people independently. The mean difference between the two values obtained was 7.0 ± 4.5.

NEUTROPHIL ALKALINE PHOSPHATASE VALUES IN NORMAL PREGNANCY.

Three hundred and thirty six NAP estimations were carried out on 103 patients, between 30 and 42 weeks of gestation. All cases of suspected or proven infarction were excluded from all parts of the NAP study. In many patients these estimations were carried out serially at weekly intervals. These results are shown in Table XIII.
There is no correlation between the NAP scores obtained between 30 and 42 weeks gestation and the week of gestation. The correlation coefficient is -0.07.

The normal range is taken as 2 standard deviations from the mean. Five patients each had one score below this range.

**NEUTROPHIL ALKALINE PHOSPHATASE VALUES IN PREGNANCIES COMPPLICATED BY MILD HYPERTENSION.**

One hundred and sixty NAP estimations were carried out on 48 patients with mild hypertension. These results are shown in Table XIV. There is no correlation between the NAP scores, obtained between 30 and 42 weeks gestation and the week of gestation. The correlation coefficient is 0.14. (p>0.05).

Comparison of all the NAP scores of the mildly hypertensive group with those of the normal group, irrespective of the week of gestation, shows that the former are significantly reduced. (p<0.001).

**NEUTROPHIL ALKALINE PHOSPHATASE VALUES IN PREGNANCIES COMPPLICATED BY MODERATE TO SEVERE HYPERTENSION**

One hundred and sixty-nine NAP estimations were carried out on 51 patients with moderate to severe hypertension. These results are shown in Table XV. There is a significant
negative correlation between these NAP scores, obtained between 30 and 42 weeks gestation, and the week of gestation. The correlation coefficient is -0.46, the regression coefficient is 3.397, and as this is 6.3 times its standard error of 0.539, this is significant \( p<0.001 \), indicating a downward trend as pregnancy advances. Comparison of all the NAP scores in the moderate to severely hypertensive group with those of the normal group, irrespective of the week of gestation, shows that the former are significantly reduced \( p<0.001 \).

Twenty-nine hypertensive patients had NAP scores carried out between 1 and 5 weeks prior to the onset of hypertension. Fourteen (48%) of these patients had one or more scores below the normal range, this difference from the normal group being significant \( p<0.001 \).

**NEUTROPHIL ALKALINE PHOSPHATASE VALUES IN NON-HYPERTENSIVE PREGNANCIES COMPPLICATED BY THE DELIVERY OF A GROWTH RETARDED BABY**

Forty-eight NAP estimations were carried out on 18 patients who were delivered of growth retarded babies, and who did not have hypertension. There is no correlation between these NAP scores, obtained between 30 and 42 weeks gestation, and the week of gestation \( r = 0.03 \) and their mean value is \( 113.2 \pm 18.6 \).

Comparison of these NAP scores, irrespective of the week
of gestation, shows that they are significantly lower than those found in normal pregnancy (p<0.03).

Fetal weight in normal and abnormal pregnancies, with the exception of cases of moderate to severe hypertension and of perinatal loss, was related in each case to the mean NAP score found between 30 weeks and delivery. There is no correlation between these two parameters (r = 0.03). It is possible to use the mean NAP score because apart from the two complications of pregnancy mentioned above, there is no significant trend in these NAP scores between 30 and 42 weeks gestation.

Only two patients had NAP scores outside the normal range, and this number does not differ significantly from the normal group (p<0.2).

**NEUTROPHIL ALKALINE PHOSPHATASE VALUES IN PREGNANCIES COMPLICATED BY FETAL DISTRESS**

Seventy NAP estimations were carried out on 24 patients, with fetal distress in labour. These results are shown in Table XVI. There is no correlation between the NAP scores, obtained between 30 and 42 weeks of gestation, and the week of gestation (r = 0.001). Comparison of these NAP scores, irrespective of the week of gestation, shows that they are significantly lower than those found in normal pregnancy (p<0.0001). Three patients had scores
outside the normal range and this number does not differ significantly from the normal group (p>0.3).

**NAP VALUES IN PREGNANCIES ENDING IN A PERINATAL DEATH**

There were three stillbirths and four neonatal deaths in this study. Fifteen NAP scores were carried out on these patients, their mean value being 92.9 ± 21.2. There is a strong negative correlation between these values and the week of gestation. The correlation coefficient is -0.824, the regression coefficient is 6.677 and as this is 5.249 times its standard error it is significant at the p<0.001 level.

One neonatal death was due to cerebral birth trauma. Four of the remaining six cases had one or more scores below the normal range. Comparison with the normal group reveals a significant difference (p<0.001).

**COMPARISON OF NEUTROPHIL ALKALINE PHOSPHATASE VALUES WITH TOTAL URINARY OESTROGEN EXCRETION**

Normal pregnancy

One hundred and seventeen paired estimations of NAP and total urinary oestrogen excretion were carried out during normal pregnancy. There is no relationship between these paired estimations, the correlation
coefficient being -0.053.

**Moderate to severe hypertension in pregnancy**

There is a correlation between the 99 paired estimations of NAP and total urinary oestrogen excretion. The correlation coefficient is 0.305, the regression coefficient is 0.362 and as this is 3.15 times its standard error of 0.115 this is significant at the p<0.01 level. The regression equation is \( y = 0.362x - 21.1 \), where \( x \) is the NAP score and \( y \) the total urinary oestrogen excretion. The results are illustrated in Fig. 9.

**COMPARISON OF NEUTROPHIL ALKALINE PHOSPHATASE VALUES WITH SERUM HEAT STABLE ALKALINE PHOSPHATASE LEVELS**

**Normal pregnancy**

There is no relationship between the 209 paired estimations of NAP and serum HSAP, the correlation coefficient being -0.098.

**Moderate to severe hypertension in pregnancy**

There is a correlation between the 160 paired estimations of NAP and serum HSAP. The correlation coefficient is 0.469, the regression coefficient is 0.15, and as this is 6.7 times its standard error of 0.022 it is significant at the p<0.001 level. The regression equation is \( y = 0.15x - 1.02 \), where \( x \) is the NAP score and \( y \) the serum HSAP level. (Fig. 10.)
### TABLE XIII

NAP Estimations in Normal Pregnancy

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean</th>
<th>± S.D.</th>
</tr>
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<tbody>
<tr>
<td>10-19</td>
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<td>117.8</td>
<td>29.7</td>
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<td>13</td>
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<td>121.0</td>
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336
### TABLE XIV

**NAP Estimations in Pregnancies complicated by Mild Hypertension**

<table>
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<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean</th>
<th>± S.D.</th>
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| Total              | 160                   |       |        |
### TABLE XV

**NAP Estimations in Pregnancies complicated by Moderate to Severe Hypertension**

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**Total** 169
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<th>± S.D.</th>
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</tr>
<tr>
<td></td>
<td></td>
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<td>70</td>
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</table>
Fig. 9.

Relationship between NAP estimations and total urinary oestrogen excretion in pregnancies complicated by moderate to severe hypertension.
Relationship between NAP and serum HSAP estimations in pregnancies complicated by moderate to severe hypertension.
RESULTS OF ALKALINE PHOSPHATASE ACTIVITY IN LEUCOCYTE SUSPENSIONS

The mean level of alkaline phosphatase activity found in 10 unheated leucocyte suspensions was $10.2 \pm 5.5$ units per $10^6$ leucocytes. Traces of alkaline phosphatase activity were found in two of the suspensions heated to 65°C for five minutes. This was of the order of $0.1$ unit per $10^6$ leucocytes, and was probably due to traces of plasma containing the heat stable iso-enzyme remaining in the washed suspension. There was no alkaline phosphatase activity detectable in the remaining 8 heated suspensions.

DISCUSSION

The method used was a minor modification of the one of the basic histochemical staining techniques for alkaline phosphatase, involving the coupling of a diazotized amine with naphthol, to form a coloured precipitate. The modifications suggested by Climie et al. (1962) were found to be entirely satisfactory.

Although scoring of the slides is an individual interpretation, the comparative results obtained from two observers are sufficiently close to discount this as a significant source of error. The method is reproducible.

In normal pregnancy this study confirms the results of Polishuk et al (1970), in that the NAP levels reach a peak
at about 30 weeks gestation and then remain relatively constant until term, after which there is a small drop in their values (Table XIII). This is shown by the absence of correlation between the NAP scores and the week of gestation from 30 to 42 weeks. However, the values are somewhat lower and the fall after term less pronounced than those recorded by Polishuk et al. (1970). This study also confirms the findings of Sadovsky et al. (1969) in that the NAP scores in pregnancies complicated by hypertension were lower than normal. (Tables XIV and XV). There is however, a large overlap of values with the normal range.

The findings of Sadovsky et al. (1969) and Zuckerman et al. (1969) have been confirmed. These are that the more severe the hypertension the lower are the NAP scores, and that this reduction may occur some weeks prior to the onset of hypertension.

From the prognostic aspect it seems that a woman with a score below the normal range has an increased chance of subsequently developing hypertension, but this must be tempered by the fact that many hypertensive patients have scores that overlap with the normal range, thus considerably reducing their significance.

Although the NAP scores in pregnancies complicated by fetal growth retardation, but not by hypertension are significantly lower than normal, the fact that there is no correlation
between NAP scores and fetal weight, and that only 2 patients had scores outside the normal range, makes the test valueless in the prediction of these fetuses.

Similarly, cases of fetal distress are associated with scores significantly lower than the normal, but as there are no values outside the normal range, the test is valueless in the prediction of fetal distress. (Table XVI).

Among patients who eventually suffered a perinatal loss, and among those who developed moderate to severe hypertension, there is a significant negative correlation between NAP scores and the week of gestation. This contrasts with the absence of correlation found in other groups. It would thus appear that low values, persistently falling, are a bad prognostic sign.

All the complications of pregnancy studied were associated with NAP scores significantly lower than normal. However, caution should be exercised in the interpretation of these results for although statistically different groups have been identified, the great overlap of scores considerably reduces the value that can be placed on an individual result.

NAP activity and total urinary oestrogen excretion.

What is the mechanism whereby NAP scores are lowered in these complicated pregnancies? It is now well established
that in many hypertensive pregnancies urinary oestrogen excretion is lower than normal and proportional to the severity of the hypertension, reflecting both diminished placental production and altered fetal metabolism of oestrogens. (Klopper, 1966; Michie, 1967). The administration of oestrone (Polishuk et al. 1970) and A.C.T.H. (Valentine et al. 1954) increases NAP activity, as do other forms of stress such as surgery or infection (Valentine et al. 1954), all presumably affecting the NAP scores through the pituitary adrenal axis.

Oestrogens increase corticosteroid binding globulin levels and reduce the catabolism of cortisol (Migeon et al. 1968), these being factors responsible for the increased total plasma corticosteroid levels found in normal pregnancy. Plasma unbound cortisol levels are also raised in pregnancy (Doe et al. 1969; Burke and Roulet, 1970), and in oestrogen treated subjects (Burke, 1969). A reduction in the placental production of oestrogens due to hypertensive damage may affect the NAP scores directly or secondarily by reducing the circulating corticosteroid levels, thus accounting for the reduced scores found in cases of moderate to severe hypertension, and for the correlation between the two parameters found in this study.

In normal pregnancy oestrogen production and plasma corticosteroid levels rise during the last few weeks
(Klopper and Billewicz, 1963; Burke and Roulet, 1970), so one would expect the same trend to occur in NAP activity. This work, and that of Polishuk et al. (1970) has shown that this is not so, perhaps the reason being that there is a maximum level to which NAP activity rises, and beyond which it will not respond to further hormonal stimulation.

**NAP activity and HSAP levels**

Serum HSAP levels have been found to be higher than normal in cases of pre-eclamptic toxaemia (Hunter et al. 1970; Quigley et al. 1970), the elevation being attributed to enzyme released from damaged placental cells. However, this work suggests a diminution of serum HSAP levels in hypertensive pregnancies, with the absence of any trend in these levels during the latter weeks of pregnancy. After 32 weeks gestation more hypertensive patients had low, rather than high, serum HSAP levels (Benster, 1970). The absence of alkaline phosphatase activity in leucocytes heated to 65°C, despite optimal conditions for detecting the placental iso-enzyme (Fishman et al. 1968b; and Stolbach et al. 1969), means that we are considering different iso-enzymes, and it is extremely unlikely that there is a direct connection between serum HSAP levels and neutrophil alkaline phosphatase activity. In the present state of our knowledge we must conclude that this positive correlation is a coincidental finding.
In normal pregnancy serum HSAP levels rise progressively from 30 weeks to term (Hunter et al. 1970; Quigley et al. 1970; Curzen and Varma, 1971) and this work confirms this finding. As NAP scores remain relatively constant from 30 to 40 weeks gestation there can be no relationship between these apparently unrelated enzyme estimations. Reduced NAP scores may reflect relatively large reductions in oestrogen output in pregnancy, suggesting that its limited value as a placental function test may be as a simple screening procedure for high risk patients, in the absence of urinary oestrogen estimations.
All reagents used were 'AnalaR' and supplied by British Drug Houses Ltd., except for glucose - 6 - phosphate.

(1) Maleic acid
(2) Tris (hydroxymethyl) methylamine
(3) Sodium carbonate
(4) Sodium bicarbonate
(5) D - glucose - 6 - phosphate, di sodium salt (Sigma Ltd., U.S.A.) 0.01M, 0.04M.
(6) Phenyl di-sodium orthophosphate 0.01M.
(7) Sodium bisulphite
(8) Sodium sulphite
(9) 1 - Amino - 2 - naphthol - 4 - sulphonic acid
(10) Beryllium sulphate
(11) Ethylene diaminetetra-acetic acid (EDTA)
(12) Ammonium molybdate 2.5% (w/v)
(13) Sucrose 0.25M.
(14) Sulphuric acid 5N
(15) Trichloracetic acid 10% (w/v)

The buffers were made up as follows:—
0.1M Tris maleate, pH range 5.0 - 8.5
0.1M Sodium carbonate - bicarbonate pH range 9.0 - 9.5
Reducing agent was prepared as follows:

1.2g. sodium bisulphite, 1.2g. sodium sulphite,
and 0.2g. 1 - Amino - 2 - naphthol - 4 - sulphonic acid.

Fifty mg. of the above mixture was dissolved in 2ml. water.
Stability and storage of reagents

The buffers, substrate and reducing agent were prepared each day. The alkaline phosphatase inhibitors and the other reagents except for 5N sulphuric acid were stored in solution at 4°C and were stable for several weeks.

The di sodium salt of D-glucose-6-phosphate was stored in a dessicated pack at -20°C.

EXPERIMENTS TO ESTABLISH AN ASSAY FOR
TRUE G-6-PASE ACTIVITY

The experiments to be described attempt to establish an assay for true G-6-Pase in serum by choosing appropriate experimental conditions. A preparation known to have G-6-Pase activity, namely the microsomal fraction of rat liver was used for comparison with serum.

MATERIAL AND METHOD

Human Serum was obtained by centrifuging the clotted venous blood of pregnant women. Care was taken to avoid haemolysis and the enzyme estimations were carried out at once.

Microsomal fraction from fresh rat liver homogenate was prepared as follows:—

The rats were killed by a blow on the head, the liver removed at once, blotted dry and then homogenised in ice cold sucrose 0.25M for 1.5 minutes at 0°C in a Potter Elvehjem homogeniser at 600 r.p.m. Fifteen ml. sucrose was used per gramme of wet liver.
The nuclei and mitochondrial fraction were removed and the microsomal fraction sedimented by centrifugation for $3 \times 10^6$ g. minutes. The microsomal pellet was washed once with 0.25M sucrose, and diluted to a convenient level of activity for use in the assay.

Substrates.

1. **Dio sodium salt of D-glucose-6-phosphate**: a final concentration of 0.01M was used for serum estimations and 0.04M for estimations involving the microsomal fraction.

2. **Phenyl di sodium ortho-phosphate**: a final concentration of 0.01M was used.

Principles of the method for G-6-Pase estimations

The enzyme G-6-Pase, under the appropriate conditions, releases phosphate ions from the substrate glucose-6-phosphate. The reaction is stopped by the addition of trichloracetic acid which precipitates the serum proteins. The phosphate ions in the supernatant fluid obtained under centrifugation are converted into phosphomolybdic acid by the addition of ammonium molybdate and sulphuric acid. A reducing agent 1-amino 2 naphthol 4 sulphonic acid is added, to bring about the reduction of the phosphomolybdic acid. This produces the characteristic blue colour which is estimated colorimetrically. The phosphorous present in serum is estimated in a second sample to which trichloracetic acid has been added first to prevent any reaction.
Technique for estimation of G-6-Pase activity

Two tubes both containing 0.4 ml. of substrate in buffer at the appropriate pH (6.5) were brought to 37°C. After the addition of 0.2 ml. of serum or diluted microsomal fraction to the first tube, both tubes were incubated at 37°C for 1 hour. One ml. of 10% (w/v) trichloracetic acid was added to both tubes, and 0.2 ml. of serum or of diluted microsomal fraction was added to the second tube. After chilling on ice for 5 minutes, distilled water was added to bring the volume to 2.5 ml. The tubes were centrifuged at 5,000 r.p.m. for 5 minutes and 2 ml. of the supernatant taken for phosphorous estimation, using the method of Fiske and Subbarow (1925).

This involved the addition of 1 ml. 5N Sulphuric acid and 1 ml. 2.5% (w/v) ammonium molybdate, to the supernatant. After mixing, 0.1 ml. of reducing agent was added, and the total volume made up to 10 ml. with distilled water. Extinction was read at 660 nm. after 10 minutes, on a Pye Unicam SP 600 spectrophotometer. The reading for the second tube was subtracted from that of the first, and the amount of inorganic phosphorous released was read off from a standard curve. One unit of G-6-Pase activity was defined as the amount of enzyme that releases 1 µg of inorganic phosphorous during 1 hour of incubation at 37°C.
**G-6-Pase** activity of serum as a function of substrate (G-6-P) concentration, in 0.1 M Tris-maleate buffer, pH 6.5 and 37°C.

**Fig. 11.**

CONCENTRATION OF GLUCOSE-6-PHOSPHATE (MOLAR)
Variation of phosphatase activity of microsomal fraction of rat liver with pH, at 37°C.
Buffers: pH 5.0 - 8.5, 0.1M Tris-maleate; pH 9.0, and 9.5, 0.1M Na HCO₃ - Na₂ CO₃ (Θ). Identical samples, pre-incubated at pH 5.0 at 37°C for 15 minutes. (▲).
Fig. 13.

Mean phosphatase activity of 3 sera as a function of pH, conditions as in Fig. 12 (♦), and pre-incubated at pH 5.0 at 37°C for 15 minutes (▲). The latter points are displaced to the right for clarity.
Fig. 14.

Mean phosphatase activity of 5 sera as a function of pH at 37°C, with (▲) and without (●) the addition of 1.4 x 10^{-2} M BeSO_4. All other conditions as in Fig. 12. The points (▲) are displaced to the right for clarity.
RESULTS

Reproducibility of Serum G-6-Pase Estimation

Four serum samples were assayed 5 times each. The results are shown below in Table XVII.

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<th>Sample</th>
<th>Mean G-6-Pase Activity</th>
<th>± S.D.</th>
<th>Coefficient of Variation</th>
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<td>0.38</td>
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</tr>
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<td>16.5%</td>
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<tr>
<td>4</td>
<td>4.8</td>
<td>0.65</td>
<td>13.5%</td>
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</table>

The coefficient of method variation is high because of experimental error, magnified by the low values, and the difficulty of reading these low values exactly from a standard curve. Adequate centrifugation was found necessary to ensure that the supernatant taken for phosphorous estimation was absolutely clear and free from traces of protein precipitated by trichloracetic acid. Failure to do this led to falsely high estimations.

The optimum substrate concentration for estimating G-6-Pase activity in serum at pH 6.5 was found to be 0.01M (Fig. 11).

The total phosphatase activities of 5 paired sera were estimated at pH 6.5, using 0.01 M glucose-6-phosphate or 0.01M phenyl ortho-phosphate as substrate. The mean value, using the former
substrate was 2.6 ± 1.3 units/ml. serum while with other latter substrate it was 2.8 ± 1.2 units/ml. serum.

Phosphatase activity was determined as a function of pH. The results are shown in Figs. 12 and 13, expressed as means for 3 portions of the microsomal fraction and 3 sera, each with and without preincubation at pH 5.0 at 37°C for 15 minutes. This latter procedure is reported to destroy true G-6-Pase activity (de Duve, 1953).

Experiments with various concentrations of the reported alkaline phosphatase inhibitors, beryllium ions, (Ashmore et al., 1954), and EDTA, (Di Bella et al., 1963), showed that the most effective was a concentration of 1.4 x 10⁻³ M BeSO₄.

The mean phosphatase activity, related to pH, of 5 sera, each with and without the addition of 1.4 x 10⁻³ M BeSO₄ is shown in Fig. 14. The activity at pH 9.0 was depressed by about 70%, while at pH 6.5 it was only depressed by about 20%.

**DISCUSSION**

The apparently almost equal amounts of enzyme activity obtained using glucose-6-phosphate and phenyl ortho-phosphate as substrates suggests that either G-6-Pase is not specific for the hydrolysis of glucose-6-phosphate at pH 6.5, or that there are other phosphatases, such as alkaline phosphatase active under these conditions, or both. The absence of a significant difference in enzyme activity using the two substrates is in agreement with Poiz (1967).
G-6-Pase is well known to be present in the microsomal fraction of liver cells (Beaufay et al. 1959). The phosphatase activity of the microsomal fraction, related to pH, shows a peak of activity at pH 6.0, this being markedly diminished by pH 9.0. Most of this activity is presumably due to true G-6-Pase. Incubation at pH 5.0 and 37°C for 15 minutes destroys G-6-Pase (de Duve, 1953). Therefore following this, little if any activity would be expected at pH 6.0 or 6.5, while residual activity at pH 5.0 or 5.5 would be due to small amounts of the more stable acid phosphatase present in the microsomal fraction. This conclusion is borne out by the results shown in Fig. 12.

Similar patterns of activity were obtained using sera (Fig. 13.), and it is reasonable to suppose that most of the enzyme activity occurring at pH 6.0 - 6.5 is due to G-6-Pase. In the present experiments, peak activity occurred between pH 6.0 (Figs 12 and 14) and pH 6.5 (Fig. 13). This is in agreement with the findings of other authors that G-6-Pase has a broad pH optimum between 6.0 and 7.0 (Swanson, 1950; Ashmore et al. 1954).

Alkaline phosphatase is active over a wide pH range and some considerable activity can still occur at pH 6.0 - 6.5. Thus the addition of beryllium ions, which partially inhibit alkaline phosphatase but not G-6-Pase (Ashmore et al. 1954), is essential. The results shown in Fig. 14 indicate that there is only a small reduction in enzyme activity at pH 6.0 and 6.5, with considerable reduction
at the more alkaline pH values. This is in agreement with
the findings of Ashmore et al. (1954) that beryllium ions
at a concentration of $1 \times 10^{-3}$M did not inhibit G-6-Pase
activity at pH 6.5.

It seems therefore, that a true measure of serum G-6-Pase
activity can be obtained using a substrate concentration
of 0.01M glucose-6-phosphate, buffered to pH 6.5, with the
addition of $1.4 \times 10^{-3}$M BeSO$_4$ to inhibit as far as possible
alkaline phosphatase activity. To assess acid phosphatase
activity, a second estimation should be carried out at
pH 6.5 on a portion of the same serum, which has been
preincubated at pH 5.0 and 37°C for 15 minutes. As this
activity is minimal, it does not seem necessary to add
beryllium ions as an inhibitor of alkaline phosphatase.
Subtraction of the second estimation from the first, would
give a true value of G-6-Pase activity.

From the present work, it appears that G-6-Pase can be
estimated in the serum distinctly from acid and alkaline
phosphatase activity.

GLUCOSE-6-PHOSPHATASE LEVELS IN
NORMAL AND ABNORMAL PREGNANCY

The method used for these estimations is that already
described on pages 117 and 118.
TABLE XVIII

Serum G-6-Pase estimations in 40 normal pregnancies.

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean</th>
<th>± S.D.</th>
</tr>
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<td>5.17</td>
<td>3.97</td>
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<td>5.74</td>
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<td>39</td>
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<td>5.59</td>
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<td>40-42</td>
<td>9</td>
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</table>

Nine patients had 11 estimations outside the normal range.
TABLE XIX

Serum G-6-Pase estimations in 22 pregnancies complicated by moderate to severe hypertension.

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean</th>
<th>± S.D.</th>
<th>Significance of difference of mean from normal pregnancy</th>
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<td>30-31</td>
<td>8</td>
<td>4.10</td>
<td>3.83</td>
<td>NS</td>
</tr>
<tr>
<td>32-33</td>
<td>18</td>
<td>6.07</td>
<td>4.88</td>
<td>NS</td>
</tr>
<tr>
<td>34-35</td>
<td>25</td>
<td>5.01</td>
<td>3.00</td>
<td>NS</td>
</tr>
<tr>
<td>36</td>
<td>21</td>
<td>5.43</td>
<td>3.42</td>
<td>NS</td>
</tr>
<tr>
<td>37</td>
<td>23</td>
<td>4.20</td>
<td>3.29</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>38</td>
<td>14</td>
<td>3.49</td>
<td>2.72</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>39</td>
<td>8</td>
<td>6.45</td>
<td>2.94</td>
<td>NS</td>
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<tr>
<td></td>
<td><strong>121</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There were no estimations outside the normal range.
TABLE XX

Serum G-6-Pase estimations in 14 pregnancies complicated by fetal distress

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean</th>
<th>± S.D.</th>
<th>Significance of difference of mean from normal pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-31</td>
<td>9</td>
<td>8.10</td>
<td>5.26</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>32-33</td>
<td>11</td>
<td>5.90</td>
<td>4.96</td>
<td>NS</td>
</tr>
<tr>
<td>34-35</td>
<td>11</td>
<td>5.40</td>
<td>4.21</td>
<td>NS</td>
</tr>
<tr>
<td>36</td>
<td>8</td>
<td>5.00</td>
<td>3.32</td>
<td>NS</td>
</tr>
<tr>
<td>37</td>
<td>8</td>
<td>4.34</td>
<td>3.70</td>
<td>NS</td>
</tr>
<tr>
<td>38</td>
<td>7</td>
<td>3.08</td>
<td>4.59</td>
<td>NS</td>
</tr>
<tr>
<td>39</td>
<td>6</td>
<td>5.88</td>
<td>5.13</td>
<td>NS</td>
</tr>
<tr>
<td>40-42</td>
<td>14</td>
<td>4.40</td>
<td>3.79</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Two patients each had one estimation outside the normal range. This number does not differ significantly from the normal group (p>0.3).
Serum G-6-Pase estimations in 16 pregnancies complicated by the delivery of a growth retarded baby.

<table>
<thead>
<tr>
<th>Weeks of Gestation</th>
<th>Number of Estimations</th>
<th>Mean ± S.D.</th>
<th>Significance of difference of mean from normal pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>32-33</td>
<td>3</td>
<td>3.66 ± 2.71</td>
<td>NS</td>
</tr>
<tr>
<td>34-35</td>
<td>7</td>
<td>3.80 ± 2.07</td>
<td>NS</td>
</tr>
<tr>
<td>36</td>
<td>5</td>
<td>1.70 ± 2.38</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>37</td>
<td>7</td>
<td>6.10 ± 5.60</td>
<td>NS</td>
</tr>
<tr>
<td>38</td>
<td>8</td>
<td>4.20 ± 4.63</td>
<td>NS</td>
</tr>
<tr>
<td>39</td>
<td>12</td>
<td>5.00 ± 4.05</td>
<td>NS</td>
</tr>
<tr>
<td>40-42</td>
<td>5</td>
<td>5.01 ± 5.17</td>
<td>NS</td>
</tr>
</tbody>
</table>

Four patients each had one estimation outside the normal range. This number does not differ significantly from the normal group (p>0.3).
Fig. 15.

Serum G-6-Pase levels in normal pregnancy.
RESULTS

The results of serum G-6-Pase levels carried out during 40 normal and abnormal pregnancies are shown in Tables XVIII to XXI, and illustrated in Fig. 15. The normal range of values is taken as 2 standard deviations from the mean.

RELATIONSHIP BETWEEN SERUM G-6-PASE LEVELS AND APGAR SCORE

In 41 pregnancies, both normal and abnormal, the G-6-Pase estimation within one week of delivery was correlated with the Apgar score of the baby at one minute.

There is no correlation between these two parameters. The correlation coefficient is -0.0146.

COMPARISON OF SERUM G-6-PASE WITH SERUM HSAP LEVELS

Normal pregnancy

One hundred and fifty-one paired estimations of serum G-6-Pase and HSAP were carried out during 40 normal pregnancies. There is no correlation between these two parameters. The correlation coefficient is 0.113, the regression coefficient is 0.137 and as this is 0.531 times its standard error of 0.258, this is not statistically significant. (p>0.5).
Abnormal pregnancy

One hundred and eleven paired estimations were carried out during 24 pregnancies complicated by moderate to severe hypertension or fetal growth retardation. There is no correlation between these two parameters. The correlation coefficient is -0.16, the regression coefficient is 0.185, and as this is 1.639 times its standard error of 0.113 this is not statistically significant (p>0.1).

Comparison of Serum G-6-Pase Levels with Total Urinary Oestrogen Excretion

Normal pregnancy

Seventy seven paired estimations of serum G-6-Pase and total urinary oestrogen excretion were carried out during 24 normal pregnancies. There is no correlation between these two parameters. The correlation coefficient is 0.083, the regression coefficient is 0.057, and as this is 0.72 times its standard error of 0.079, this is not statistically significant. (p>0.4).

Abnormal pregnancy

One hundred and nineteen paired estimations of serum G-6-Pase and total urinary oestrogen excretion were carried out during 25 pregnancies complicated by moderate to severe hypertension or fetal growth retardation. There is a negative correlation between these two parameters. The correlation coefficient is -0.303, the regression coefficient is -0.267, and as this is 3.49 times its standard error of
0.0765, the correlation is significant at the p<0.001 level.

No perinatal deaths occurred in this particular study.

SERUM G-6-PASE LEVELS IN THE NON PREGNANT

Serum G-6-Pase estimations were carried out on 10 non pregnant women. The mean value of these estimations is $4.48 \pm 2.18$. This is not significantly different from the mean values found during normal pregnancy except at 37 and 40-42 weeks gestation. (p<0.01).

DISCUSSION

Serum G-6-Pase levels in normal pregnancy show no trend from 28 weeks gestation onwards. There is a wide range of values. These facts make it unlikely that alterations in these values will provide any guide to fetal prognosis.

In pregnancies complicated by moderate to severe hypertension, the mean value of serum G-6-Pase at varying periods of gestation does not differ significantly from normal pregnancy except at 37 and 38 weeks, when the values are lower. In view of the absence of any trend in either group, and the large standard deviations from the mean value, it seems probable that these are chance findings.

There is no trend in the G-6-Pase values recorded in pregnancies complicated by fetal distress, except that the
mean value at 30 - 31 weeks gestation is higher than normal. There is again a large standard deviation, and the statistical significance of the difference between the means is not great (p<0.02), so it is probable that this finding is not of any significance. There is no relationship between the G-6-Pase value in the week prior to the onset of labour, and the infant's Apgar score at 1 minute. These estimations are of no value in predicting the state of the infant at delivery. The reason that the Apgar score is included in this study and not in the other enzyme studies is that this score was assessed routinely on infants born in Malta where most of this work was carried out.

The number of G-6-Pase estimations carried out in non hypertensive pregnancies complicated by the delivery of a growth retarded baby is small. No trend in these values is detectable, and the mean value at 36 weeks gestation that is lower than normal is of no clinical significance. Thus serum G-6-Pase estimations seem to be valueless in the prediction of the growth retarded baby. In none of the abnormal groups was there a higher number of patients than expected with serum G-6-Pase estimations outside the normal range. The fact that the mean value of serum G-6-Pase activity in 10 non pregnant women was only significantly lower than the mean values found in normal pregnancy at 37 and 40 - 42 weeks gestation is suggestive that the placenta is not contributing to the serum levels of this enzyme in pregnancy.
Curzen (1964) found that placental G-6-Pase activity increased throughout pregnancy in a manner comparable to that of serum HSAP. Therefore the serum levels of these two enzymes might be expected to bear some relationship to each other. In both normal and abnormal pregnancy however, there is no relationship between paired values of these enzyme estimations in serum. This is further borne out by the absence of an increasing linear trend of serum G-6-Pase levels throughout the later weeks of normal pregnancy, in contrast to serum HSAP levels which rise steadily throughout this period.

There may be two explanations for this. The first is that the enzyme activity recorded histochemically by Curzen (1964) was not specifically G-6-Pase, and that the conclusion of Villee (1953) that G-6-Pase is present in decreasing amounts as pregnancy advances, is correct. The second explanation is that the enzyme G-6-Pase, due to its intracellular situation in the microsomal fraction (Tice and Barnett, 1962), is unlikely to be released from placental cells damaged by senescence or infarction. Thus the serum levels of G-6-Pase recorded in abnormal pregnancy do not seem to reflect the degree of placental infarction, and as has been shown they do not reflect the clinical state of the fetus in labour or after delivery.

A surprising finding is the statistically significant negative correlation between serum G-6-Pase levels and total urinary oestrogen excretion in abnormal pregnancies.
The simple explanation for this would be that in those complications of pregnancy in which urinary oestrogen excretion is low, such as severe hypertension or fetal growth retardation, the serum G-6-Pase levels would be high. Despite a relatively small number of serum G-6-Pase estimations it seems clear that they are not significantly raised in these complications of pregnancy, but that there are one or two serum G-6-Pase levels raised towards the upper limit of the normal range. It may be that these transient elevations caused by episodes of acute placental infarction, account for the negative correlation with urinary oestrogen excretion, without making possible the clinical differentiation of the abnormal groups.
DISCUSSION

It is well established that urinary oestriol and total urinary oestrogen levels are guides to fetal weight and to fetal prognosis in complicated pregnancies. The total urinary oestrogen studies used for comparative purposes in this work confirm previous findings, and so they can be used as a valid comparison with the enzyme estimations. A close correlation between plasma and urinary oestriol levels has been found in normal and abnormal pregnancy (Macrae and Mohamedally, 1970). Although only a few studies have been carried out using plasma oestriol levels, the use of radio-immuno assay methods will make the estimations much more readily available in the future. As this method eliminates the errors associated with a 24 hour urine collection and nullifies any change in renal function, and as no diurnal variation has been found by Macrae and Mohamedally (1970), the method would seem to offer a more accurate and reliable assessment of feto-placental function than urinary oestrogen estimations.

Placental alkaline phosphatase has been studied histochemically by several authors including McKay et al. (1958); Wachstein et al. (1963) and Curzen (1964). All these authors concluded that the enzyme activity appeared to be associated with the trophoblast, and that it increased in amount as pregnancy advanced. Von Hempel and Geyer (1969) found that in both immature and term placentae the alkaline phosphatase activity was greatest in the microvilli
and syncytiotrophoblast.

Alkaline phosphatase probably assists in the transfer of carbohydrates across the placental barrier (Wislocki and Dempsey, 1945) and so as pregnancy advances, the need for increasing amounts of the placental enzyme is obvious. The serum levels, increasing in a linear fashion during normal pregnancy, may reflect a leakage of the steadily increasing amount of enzyme from damaged syncytiotrophoblast cells.

Maqueo et al. (1964) found that syncytial degeneration was proportional to the severity of pre-eclamptic toxaemia. Watkins and Anderson (1971) have shown that in this condition both the syncytiotrophoblast volume and the levels of heat stable alkaline phosphatase are much reduced when compared with normal pregnancy, and that these two factors are related. Their conclusion was that placental heat stable alkaline phosphatase originates from the syncytiotrophoblast and is reduced in pre-eclamptic toxaemia.

High levels of serum HSAP occurring during the early part of the third trimester of hypertensive pregnancies may be caused by preceding trophoblastic proliferation with increased leakage of the enzyme, into the serum. As syncytial degeneration takes place, and the alkaline phosphatase content of the syncytiotrophoblast falls, the serum levels would be expected to fall as well. This
hypothesis would account for the results obtained by Benster (1970) and for the results of this work, namely, that in hypertensive pregnancies the serum HSAP levels fall to normal or low levels, after being abnormally high at about 30 weeks gestation. The association of high serum HSAP values and perinatal death may be due to episodes of acute and significant placental infarction, as suggested by Quigley et al. (1970). Pirani et al. (1972) estimating urinary oestriol excretion in normal pregnancy did find a correlation with serum HSAP, and they suggested that this was because urinary oestriol excretion gave a better assessment of placental function than the measurement of total urinary oestrogens. As both urinary oestriol and serum HSAP levels rise in a linear fashion during the third trimester of normal pregnancy, a statistical correlation is not surprising. Whether this correlation will persist in abnormal pregnancy requires further study.

Differences in methods used for estimating the placental iso-enzyme have created difficulties in drawing valid comparisons between results. The conditions of pH and substrate concentration under which the placental iso-enzyme records maximum activity are quite specific. Failure to comply with these criteria means that serum over or HSAP activity is under-recorded, and this leads to difficulty in the comparison of results.

Based on the assumption that a high serum HSAP value and
subsequent falling serial values are associated with a poor fetal prognosis, the present value of serum HSAP estimations would seem to be limited to a screening test, carried out at 30, 34 and 38 weeks gestation in high risk pregnancies.

Neutrophil alkaline phosphatase levels as a guide to fetal prognosis are of limited value. The simplicity of the estimation is a point in its favour, but individual variation in scoring is a possible argument against the method. It seems that changes in neutrophil alkaline phosphatase are a reflection of changes in circulating steroid levels and hence presumably changes in placental steroid production. It is uncertain which hormone has the principal effect on NAP activity. Valentine et al. (1954) related rises in NAP activity to ACTH and this is substantiated by rises found in stress situations by many authors. Polishuk et al. (1968) related rises in NAP activity to increased oestrogen levels, while O'Kell and Axon (1965) felt that progesterone levels influence NAP. It may be that any of these hormones can cause the rise in activity found in pregnancy, and which starts as early as 5 weeks gestation (Harer and Quigley, 1961). Without more knowledge, one can only conclude at this stage that a reduction in one or other, or all, of these hormones may be responsible for the reduction in NAP activity found in pregnancies complicated by hypertension, a growth retarded fetus, fetal distress in labour, or a perinatal death.
Serum G-6-Pase levels recorded in this study show neither a rising trend during the third trimester of normal pregnancy, nor a significant difference in the enzyme levels between normal and abnormal pregnancy.

The number of estimations in this study is small, and it is possible that a larger study might produce positive findings. This seems unlikely in view of the large scatter of values already found, and the fact that there is probably no significant difference between serum G-6-Pase levels in the pregnant and non pregnant patient.

These facts suggest that the placenta contributes minimally, if at all, to the serum levels of G-6-Pase. This is presumably because G-6-Pase is situated in the microsomal fraction of the cells and is not released in significant amounts into the serum during placental damage caused by senescence or infarction, in the same way as the more soluble HSAP. The absence of any relationship between paired estimations of these two enzymes confirms this. Occasional high levels of serum G-6-Pase have been recorded and these may be due to moderately severe and sudden placental infarction.

It can be concluded that serum G-6-Pase estimations are valueless in assessing the degree of placental infarction and so valueless in the prediction of the at risk fetus.
It is possible that placental G-6-Pase plays some part in maintaining the levels of available glucose in the intervillous blood. However, there is controversy about varying levels of this enzyme in the placenta at different stages of gestation (Villee, 1953; Curzen, 1964). These differences may be due to difficulty in estimating the enzyme specifically from other phosphatases. It would be valuable to repeat these histochemical studies in normal and abnormal pregnancy, using the more specific conditions for estimating G-6-Pase, described in this work. As placental G-6-Pase levels may be higher in early pregnancy (Villee, 1953), it is possible that serum G-6-Pase levels at this stage may be higher than those found in this study. This point might also be investigated.
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SERUM HEAT STABLE ALKALINE PHOSPHATASE LEVELS AND THEIR RELATIONSHIP TO URINARY OESTROGEN OUTPUT AND FETAL AND PLACENTAL WEIGHTS

BY

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Summary

One hundred and fifty-two paired estimations of serum heat stable alkaline phosphatase (HSAP) and urinary oestrogen excretion were performed on 37 patients; there was no correlation between these values. There was no correlation between the serum HSAP levels and fetal or placental weight, although there were correlations between urinary oestrogen output and fetal and placental weights. Serum HSAP levels are probably of no value in the prediction of a small-for-dates baby.

A recent report by Curzen and Southcombe (1970) suggested that there was a correlation between serum heat stable alkaline phosphatase (HSAP) and urinary oestrogen excretion, estimated concurrently during the latter weeks of pregnancy. In this study a similar series of investigations has been carried out, but using a more accurate method for estimating the placental iso-enzymes of alkaline phosphatase.

METHODS

One hundred and fifty-two paired estimations of serum HSAP and of total urinary oestrogen output were performed on 37 patients at between 20 and 42 weeks gestation. These patients had pre-eclamptic toxæmia, essential hypertension, recurrent antepartum haemorrhage, or were suspected of having a small-for-dates baby. All blood samples were collected at a constant time of day (8.30 a.m.), which coincided with the completion of the 24-hour urine collection, and the serum HSAP estimations were carried out as soon as possible after collection.

The method used was that described by Hansen (1966), with the modifications suggested by Fishman et al. (1968a), except that instead of the sera being heated to 55 °C. for one hour they were heated to 65 °C. for five minutes (Fishman et al., 1968b; Stolbach et al., 1969).

Incubation at 38 °C. for 15 minutes with 72 m mol. disodium phenyl phosphate was then carried out at pH 10·7. These are optimal conditions for the activity of variant A of the placental iso-enzyme (Ghosh and Fishman, 1968). The phenol liberated was estimated colorimetrically at a wave length of 520 mμ, and the results were expressed as placental isoenzyme units (PIU). One unit is defined as the amount of enzyme which will liberate 1·0 mg. phenol from 72 m mol. phenyl phosphate in 15 minutes at 38 °C. and pH 10·7 (Fishman et al., 1968a).

Urinary oestrogen excretion was estimated on a 24-hour urine collection by the method of Oakey et al. (1967), the results being expressed as mg. of Kober chromagen per 24 hours.

The paired estimations were examined to see whether there was any correlation between them. Fetal and crude placental weights were compared with the serum HSAP and urinary oestrogen levels. Serum HSAP levels were compared with the placental coefficients.

Twelve patients were delivered of a small-for-dates baby; the criteria used for its definition were those described by Elder et al. (1970). Serum HSAP levels and urinary oestrogen output were examined in this group and compared with those of the remaining 25 patients.
Table I

<table>
<thead>
<tr>
<th></th>
<th>Small-for-dates group</th>
<th>Normal weight group</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>12</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>Mean gestational age</td>
<td>38·2 (range 34-42)</td>
<td>38·8 (range 33-42)</td>
<td>p &gt; 0·4</td>
</tr>
<tr>
<td>Mean HSAP (P.I.U./100 ml. serum) ± SD</td>
<td>15·3 ± 3·37</td>
<td>16·2 ± 6·84</td>
<td>p &gt; 0·5</td>
</tr>
<tr>
<td>Mean urinary oestrogen output (mg, Kober chromagen/24 hours) ± SD</td>
<td>11·7 ± 7·56</td>
<td>20·4 ± 7·42</td>
<td>p &lt; 0·01</td>
</tr>
</tbody>
</table>

Most of the patients had weekly serial estimations carried out, and it was felt that a single value of either serum HSAP or urinary oestrogen excretion carried out immediately prior to the onset of labour was not necessarily representative of the preceding values, and hence not necessarily related to the fetal or placental weight. Consequently when there were three or more estimations these were examined to determine their trend, the equation of the line best fitting the results was calculated, and the last value before delivery adjusted to take into account the preceding results. This was the figure used in all the correlations except the first, and in calculating the results shown in Table I. In cases where there were less than three estimations the last value, always within one week of the onset of labour, was used.

Results

1. Serum HSAP Levels and Urinary Oestrogen Output

These results are shown in Figure 1. There is no correlation between the 152 paired estima-

\[ \text{HSAP IN PLUS} \]

\[ \text{OESTROGEN OUTPUT IN MG.} \]

Fig. 1

Relationship between urinary oestrogen and serum HSAP levels.
2. Serum HSAP Levels and Fetal Weight

These results are shown in Figure 2. There is no correlation between these two parameters. The correlation coefficient is \(-0.053\), the regression coefficient is \(-0.468\), and as its standard error is 1.499 this is not statistically significant \((p>0.7)\).

3. Serum HSAP Levels and Crude Placental Weight

These results are shown in Figure 3. There is no correlation between these two parameters. The correlation coefficient is 0.119, the regression coefficient is 5.408, and as its standard error is 7.598 this is not statistically significant \((p>0.4)\).

4. Serum HSAP Levels and Placental Coefficient

There is no correlation between these two parameters. The correlation coefficient is \(-0.197\), the regression coefficients \(-1.2\), and as its standard error is 1.01 this is not statistically significant \((p>0.2)\).

5. Urinary Oestrogen Output and Fetal Weight

These results are shown in Figure 4. There is a correlation between these two parameters. The correlation coefficient is 0.533, the regression coefficient is 7.224, and as this is 3.205 times its standard error of 1.95 this is statistically significant at the \(p<0.001\) level. The regression
equation is \( y = 7.224x - 4.065 \), where \( x \) is the fetal weight and \( y \) the urinary oestrogen output.

6. Urinary Oestrogen Output and Crude Placental Weight

These results are shown in Figure 5. There is a correlation between these two parameters. The correlation coefficient is 0.402, the regression coefficient is 27.94, and as this is 2.597 times its standard error of 10.76 this is statistically significant at the \( p<0.02 \) level. The regression equation is \( y = 27.94x + 1.36 \) where \( x \) is the crude placental weight and \( y \) the urinary oestrogen output.

7. Mean Values of Serum HSAP and Urinary Oestrogen Output

The mean values of serum HSAP and urinary output in the groups having either small-for-dates or normal weight babies are shown in Table I.

**Discussion**

These results show that there is a positive correlation between fetal weight and urinary oestrogen output at, or close to, the time of delivery. This finding is in agreement with those of Frandsen and Stakemann (1960), Coyle and Brown (1963), Beling (1967), Beischer et al. (1968), Curzen and Southcombe (1970), and Watney et al. (1970). The positive correlation between urinary oestrogen output and crude placental weight is in agreement with Curzen and Southcombe (1970), but other authors have been unable to demonstrate this relationship (Klopper and Billewicz, 1963; Coyle and Brown, 1963;
RELATION OF SERUM H.S.A. PHOSPHATASE TO URINARY OESTROGEN

It is difficult to explain this discrepancy in results, but as the correlation in this study is not a particularly strong one ($0.01 < p < 0.02$), it may be a chance finding.

The absence of correlations between serum SAP levels and urinary oestrogen output or crude placental weight disagrees with the findings of Curzen and Southcombe (1970). The failure to demonstrate a relationship between serum HSAP levels and fetal weight agrees with their findings. The method employed by Curzen and Southcombe (1970) and Watney et al. (1970) to estimate serum HSAP levels involved heating the serum to $56^\circ$C for 30 minutes in order to activate the nonplacental iso-enzymes. It has been shown that this temperature is inadequate and that a temperature of $65^\circ$C is necessary for the complete inactivation of these iso-enzymes. Hunter (1969) heated the sera to $65^\circ$C for 30 minutes, while Fishman et al. (1968b) and Stolbach et al. (1969) found that five minutes was an adequate length of time. Use of the higher temperature has eliminated the abnormally elevated values, due to persistent activity at the lower temperature, of iso-enzymes of alkaline phosphatase originating in the intestine and liver. The correlations therefore are not influenced by these abnormal results, and hence are presumably more accurate. This may account, to some extent, for the difference between our findings and those of Curzen and Southcombe (1970), but it certainly does not explain the complete absence, in this study, of a correlation between serum HSAP levels and crude placental weight.

Although Hunter (1969) felt that serum HSAP levels were valuable in predicting failing placental function, Watney et al. (1970) found that
these levels were either abnormally high or low in only one-third of cases of proven placental insufficiency. The failure in this study to correlate serum HSAP levels with fetal weight, placental weight or the placental coefficient suggests that they are of no value in the prediction of a small-for-dates baby. This is in direct contrast to the fact that low urinary oestrogen output is a good index of placental insufficiency (Coyle and Brown, 1963; Michie, 1967). The findings shown in Table I confirm this impression, in that patients delivering small-for-dates babies had significantly lower urinary oestrogen excretion than the group having normal weight babies (p<0.01), while the serum HSAP levels did not differ significantly between the two groups (p>0.5).

Experience in this unit at present is that a significant alteration in the trend of serum HSAP levels for a particular patient may be of value in predicting acute placental insufficiency, and there is agreement with Watney et al. (1970) that abnormal serum HSAP levels are valuable in corroborating abnormal urinary oestrogen excretion. This will be the basis of a future study.

It should be noted that as the methods used by Hunter (1969), Watney et al. (1970), and by ourselves, for estimating serum HSAP levels are all different, this to some extent invalidates a comparative study. The modifications of Fishman et al. (1968a and b) to the method described by Hansen (1966) are recommended for future studies as the most accurate.
ACKNOWLEDGEMENTS

I thank Professor A. P. Camilleri for his advice and encouragement and Mr. J. Ellul for technical assistance.

REFERENCES


Neutrophil alkaline phosphatase levels in normal and abnormal pregnancy

M. G. ELDER, M.B., Ch.B., F.R.C.S.E., M.R.C.O.G.
F. BONELLO, M.D., D.(Obst.)R.C.O.G.
J. ELLUL
Guardamangia, Malta

Seven hundred and thirty-one neutrophil alkaline phosphatase (NAP) estimations were carried out during normal and abnormal pregnancies. The normal range of activity is defined, and it is shown that in pregnancies complicated by hypertension, dysmaturity, fetal distress, or perinatal death the NAP activity is lower than normal. Despite this, the value of the estimations in the prediction of these complications is minimal.

Leukocyte alkaline phosphatase activity has been shown to be raised with infection, pregnancy, and after the administration of adrenocorticotropic hormone and estrone. It has been suggested that leukocyte alkaline phosphatase activity is lower than normal in cases of pre-eclamptic toxemia. The present study was undertaken to assess in more detail the role of NAP estimations as a test of placental function. This paper will evaluate the NAP results, while their relationship to other tests will be the basis of a subsequent paper.

Material and methods

There were 731 NAP estimations carried out on 226 women during pregnancy. The distribution of these is shown in Table I.

Smears from the buffy coat were fixed and stained according to the method described by Climie and associates, and 100 consecutive neutrophils were scored with the use of their criteria.

Statistical comparison of groups was carried out by the Mann-Whitney test.

The groups studied were those with normal pregnancies and pregnancies complicated by hypertension, dysmaturity, fetal distress, and perinatal death. Hypertension was mild—blood pressure 140-90 to 150-100 mm. Hg—or moderate to severe—over 150-100 mm. Hg. Many patients had unclassifiable hypertension, and no attempt was made to differentiate these from cases of essential hypertension or pre-eclamptic toxemia. Dysmaturity was defined according to the criteria used by Elder and associates. Fetal distress was defined as a fetal heart rate of less than 100 beats per minute, recorded on more than one occasion, or the presence of meconium in the liquor amnii. Cases of proved or suspected infection were excluded.

Results

Table II shows the results of NAP estimations obtained during normal pregnancy: Tables III and IV, during hypertensive pregnancies; and Table V, during pregnancies complicated by fetal distress.

Forty-eight NAP scores were obtained from 18 patients whose pregnancies were complicated by dysmaturity but not by hypertension. The mean value is 113.2 ± 18.6. Fifteen NAP scores were obtained from 7 cases with perinatal deaths, the mean value being 92.9 ± 21.2.

From the Department of Obstetrics and Gynaecology, Royal University of Malta.
Table I. The distribution of NAP estimations carried out during normal and abnormal pregnancies

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>No. of estimations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pregnancy</td>
<td>103</td>
</tr>
<tr>
<td>Mild hypertension</td>
<td>48</td>
</tr>
<tr>
<td>Moderate to severe hypertension</td>
<td>51</td>
</tr>
<tr>
<td>Dysmaturity uncomplicated by hypertension</td>
<td>18</td>
</tr>
<tr>
<td>Fetal distress*</td>
<td>24 (5)</td>
</tr>
<tr>
<td>Perinatal loss*</td>
<td>7 (1)</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>226</strong></td>
</tr>
</tbody>
</table>

*Figures in parentheses are the numbers not included in other categories.

There is no correlation between the week of gestation and the NAP score, except in cases of moderate to severe hypertension ($r = -0.46$, $p < 0.001$) and perinatal death ($r = -0.824$, $p < 0.001$).

All groups had NAP values significantly lower than normal, these being dysmaturity ($p < 0.03$), fetal distress ($p < 0.0001$), mild hypertension ($p < 10^{-7}$), and moderate to severe hypertension ($p < 10^{-8}$).

The normal range is taken as 2 standard deviations from the mean, and 5 patients each had one score below this range. Twenty-nine patients had NAP estimations between 1 and 5 weeks prior to the onset of hypertension. Fourteen (48 per cent) of these had scores outside the normal range, this difference from the normal group being significant ($p < 0.001$). Four of the 7 cases of perinatal death had scores outside the normal range, and this number is also significantly different from the normal group ($p < 0.001$). The number of cases of fetal distress and dysmaturity were not significantly different, being 3 ($p > 0.3$) and 2 ($p > 0.2$), respectively.

No correlation exists between fetal weight and the mean NAP score between 30 weeks and delivery ($r = 0.03$).

Comment

All the complications of pregnancy studied were associated with NAP scores significantly lower than normal. This may be due to the abnormal production or metabolism of placental steroids, perhaps estrogen.

Caution should be exercised in the interpretation of these results, for, although statistically different groups have been identified, the great overlap of scores considerably reduces the value that can be placed on an individual result.

In cases of fetal distress and dysmaturity, the almost complete overlap of NAP scores into the normal range and, in the latter group, the absence of correlation with fetal weight make the test valueless for prediction.

This study confirms that NAP scores are lower in hypertensive pregnancies and that the drop may occur some weeks prior to the

Table II. NAP estimations in normal pregnancy

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>No. of estimations</th>
<th>Mean</th>
<th>± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-19</td>
<td>5</td>
<td>117.8</td>
<td>29.7</td>
</tr>
<tr>
<td>20-29</td>
<td>13</td>
<td>136.1</td>
<td>19.3</td>
</tr>
<tr>
<td>30</td>
<td>11</td>
<td>121.0</td>
<td>12.9</td>
</tr>
<tr>
<td>31</td>
<td>20</td>
<td>127.0</td>
<td>10.9</td>
</tr>
<tr>
<td>32</td>
<td>23</td>
<td>124.0</td>
<td>12.0</td>
</tr>
<tr>
<td>33</td>
<td>22</td>
<td>126.9</td>
<td>15.3</td>
</tr>
<tr>
<td>34</td>
<td>34</td>
<td>127.9</td>
<td>13.2</td>
</tr>
<tr>
<td>35</td>
<td>30</td>
<td>121.8</td>
<td>15.7</td>
</tr>
<tr>
<td>36</td>
<td>39</td>
<td>123.3</td>
<td>19.5</td>
</tr>
<tr>
<td>37</td>
<td>37</td>
<td>120.0</td>
<td>18.4</td>
</tr>
<tr>
<td>38</td>
<td>36</td>
<td>124.6</td>
<td>18.6</td>
</tr>
<tr>
<td>39</td>
<td>28</td>
<td>125.2</td>
<td>22.3</td>
</tr>
<tr>
<td>40</td>
<td>19</td>
<td>118.4</td>
<td>17.9</td>
</tr>
<tr>
<td>41</td>
<td>9</td>
<td>112.3</td>
<td>26.0</td>
</tr>
<tr>
<td>42</td>
<td>10</td>
<td>106.8</td>
<td>18.9</td>
</tr>
</tbody>
</table>

Table III. NAP estimations in pregnancies complicated by mild hypertension

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>No. of estimations</th>
<th>Mean</th>
<th>± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>8</td>
<td>109.5</td>
<td>14.0</td>
</tr>
<tr>
<td>31</td>
<td>8</td>
<td>113.1</td>
<td>13.3</td>
</tr>
<tr>
<td>32</td>
<td>10</td>
<td>120.0</td>
<td>19.4</td>
</tr>
<tr>
<td>33</td>
<td>10</td>
<td>120.7</td>
<td>25.8</td>
</tr>
<tr>
<td>34</td>
<td>13</td>
<td>108.9</td>
<td>19.3</td>
</tr>
<tr>
<td>35</td>
<td>18</td>
<td>111.5</td>
<td>19.2</td>
</tr>
<tr>
<td>36</td>
<td>21</td>
<td>113.9</td>
<td>18.8</td>
</tr>
<tr>
<td>37</td>
<td>25</td>
<td>113.2</td>
<td>20.0</td>
</tr>
<tr>
<td>38</td>
<td>22</td>
<td>112.5</td>
<td>16.9</td>
</tr>
<tr>
<td>39</td>
<td>11</td>
<td>104.5</td>
<td>18.9</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>108.5</td>
<td>14.8</td>
</tr>
<tr>
<td>41</td>
<td>6</td>
<td>93.0</td>
<td>16.4</td>
</tr>
<tr>
<td>42</td>
<td>2</td>
<td>103.0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table IV. NAP estimations in pregnancies complicated by moderate to severe hypertension

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>No. of estimations</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>5</td>
<td>95.6 ± 9.3</td>
</tr>
<tr>
<td>31</td>
<td>6</td>
<td>101.5 ± 16.5</td>
</tr>
<tr>
<td>32</td>
<td>15</td>
<td>96.1 ± 14.3</td>
</tr>
<tr>
<td>33</td>
<td>16</td>
<td>101.1 ± 13.2</td>
</tr>
<tr>
<td>34</td>
<td>15</td>
<td>100.6 ± 22.1</td>
</tr>
<tr>
<td>35</td>
<td>21</td>
<td>105.3 ± 11.2</td>
</tr>
<tr>
<td>36</td>
<td>28</td>
<td>101.5 ± 15.9</td>
</tr>
<tr>
<td>37</td>
<td>30</td>
<td>98.2 ± 15.5</td>
</tr>
<tr>
<td>38</td>
<td>17</td>
<td>104.1 ± 30.6</td>
</tr>
<tr>
<td>39</td>
<td>9</td>
<td>98.1 ± 15.2</td>
</tr>
<tr>
<td>40</td>
<td>4</td>
<td>82.5 ± 17.1</td>
</tr>
<tr>
<td>41</td>
<td>2</td>
<td>84.0 ± 25.4</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
<td>53.0 ±</td>
</tr>
</tbody>
</table>

onset of hypertension, but there is a much greater overlap of values with the normal than was found by Sadovsky and co-workers. This is such as to make the prognostic value of the test, in these cases, minimal, despite a significant number of women having abnormal scores before the onset of their hypertension.

Table V. NAP estimations in pregnancies complicated by fetal distress

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>No. of estimations</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>4</td>
<td>112.5 ± 23.7</td>
</tr>
<tr>
<td>31</td>
<td>5</td>
<td>111.4 ± 18.9</td>
</tr>
<tr>
<td>32</td>
<td>4</td>
<td>108.5 ± 15.4</td>
</tr>
<tr>
<td>33</td>
<td>5</td>
<td>116.4 ± 13.5</td>
</tr>
<tr>
<td>34</td>
<td>6</td>
<td>109.0 ± 11.2</td>
</tr>
<tr>
<td>35</td>
<td>8</td>
<td>104.8 ± 11.1</td>
</tr>
<tr>
<td>36</td>
<td>9</td>
<td>121.3 ± 18.4</td>
</tr>
<tr>
<td>37</td>
<td>8</td>
<td>112.9 ± 20.1</td>
</tr>
<tr>
<td>38</td>
<td>5</td>
<td>115.8 ± 26.1</td>
</tr>
<tr>
<td>39</td>
<td>6</td>
<td>111.7 ± 11.5</td>
</tr>
<tr>
<td>40</td>
<td>7</td>
<td>113.6 ± 23.4</td>
</tr>
<tr>
<td>41</td>
<td>2</td>
<td>108.5 ± 36.1</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
<td>106.0 ±</td>
</tr>
</tbody>
</table>

In cases with perinatal deaths, there is a marked fall in values as pregnancy proceeds, and a significant number of women had abnormal scores. However, as nearly half of the estimations fell within the normal range the test is unlikely to be of prognostic significance. A larger number of such cases should be studied before reaching a firm conclusion.

REFERENCES
Neutrophil alkaline phosphatase in pregnancy and its relationship to urinary estrogen excretion and serum heat-stable alkaline phosphatase levels

M. G. Elder, M.B., Ch.B., F.R.C.S.E., M.R.C.O.G.
F. Bonello, M.D., D.(Obst.)R.C.O.G.
J. Ellul.
Guardamangia, Malta

Two hundred and sixteen paired estimations of neutrophil alkaline phosphatase (NAP) and total urinary estrogen excretion and 369 paired NAP and serum heat-stable alkaline phosphatase (HSAP) estimations were carried out during normal and hypertensive pregnancies. In the latter, positive correlations were found between the NAP estimations on the one hand and total urinary estrogen excretion and serum HSAP levels on the other. There was no correlation between the estimations in normal pregnancy. The significance of these results is discussed.

Neutrophil alkaline phosphatase (NAP) activity is increased during pregnancy. Various complications of pregnancy reduce its activity, this being most marked in cases of moderate to severe hypertension and perinatal death.1 This study was carried out to compare the NAP results with 2 other estimations currently being used to assess placental function.

Material and methods

Serial estimations of NAP, total urinary estrogen excretion, and serum heat-stable alkaline phosphatase (HSAP) levels were carried out on 64 patients during normal pregnancy and on 48 patients during pregnancy complicated by moderate to severe hypertension. This was defined as a blood pressure reading above 150/100 mm. Hg at any stage of pregnancy.

Many of the patients had unclassifiable hypertension, and no attempt has been made to differentiate these from cases of essential hypertension or pre-eclamptic toxemia. All cases of proved or suspected infection were excluded. The estimations carried out are shown in Table I. The method used to estimate neutrophil alkaline phosphatase was that described by Climie and associates.2 The method used to estimate total urinary estrogen excretion was that described by Oakey and colleagues.3 The method used to estimate serum HSAP was that described by Hansen1 and as modified by Fishman and co-workers5 and Stolbach and associates,6 the results being expressed in placental isoenzyme units (P.I.U.).

Finally, experiments were carried out on 10 leukocyte suspensions from pregnant women to ascertain whether any of the alkaline phosphatase present was heat stable. The method used for preparing the washed suspensions of leukocytes was that described...
Table I. Distribution of paired estimations carried out during normal and hypertensive pregnancies

<table>
<thead>
<tr>
<th></th>
<th>Paired NAP and total urinary estrogen estimations</th>
<th>Paired NAP and serum HSAP estimations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pregnancy</td>
<td>117</td>
<td>209</td>
</tr>
<tr>
<td>Moderate to severe hypertension</td>
<td>99</td>
<td>160</td>
</tr>
<tr>
<td>Totals</td>
<td>216</td>
<td>369</td>
</tr>
</tbody>
</table>

by Valentine and Beck. Each suspension was divided into 2 equal parts, and white cell counts were performed to ensure approximately equal division of the leukocytes. One part was heated to 65° C. for 5 min. Incubation mixtures were made up as described by Valentine and Beck, except that 72 mmole of phenyl phosphate, buffered to pH 10.7, was used as the substrate, and all the volumes were reduced to one third of that described. Alkaline phosphatase activity was determined after incubation for 15 minutes at 37° C. and expressed as units per 10⁶ leukocytes.

Results

NAP scores compared with total urinary estrogen excretion.

Normal pregnancy. There is no relationship between the 117 paired estimations of NAP and total urinary estrogen excretion. The correlation coefficient is -0.053, and the regression equation is \( y = 27.1 - 0.03x \), where \( x \) is the NAP score and \( y \) the total urinary estrogen output.

Moderate to severe hypertension. These results are shown in Fig. 1. There is a correlation between the 99 paired estimations of NAP and total urinary estrogen excretion. The correlation coefficient is 0.305; the regression coefficient is 0.362; and as this is 3.15 times its standard error of 0.115 this is significant at the \( p < 0.01 \) level. The regression equation is \( y = 0.362x - 21.1 \).

NAP scores compared with serum HSAP levels.

Normal pregnancy. There is no relationship between the 209 paired estimations of NAP and serum HSAP. The correlation coefficient is -0.098, and the regression equation is \( y = 21.7 - 0.03x \), where \( x \) is the NAP score and \( y \) is the serum HSAP level.

Moderate to severe hypertension. These results are shown in Fig. 2. There is a correlation between the 160 paired estimations of NAP and serum HSAP. The correlation coefficient is 0.469; the regression coefficient is 0.15; and as this is 6.7 times its standard error of 0.022 it is significant at the \( p < 0.001 \) level. The regression equation is \( y = 0.15x - 1.02 \).

Alkaline phosphatase activity in leukocyte suspensions. The mean level of alkaline phosphatase activity found in the 10 unheated suspensions was 10.19 ± 5.5 U. per 10⁶ leukocytes. Traces of alkaline phosphatase activity were found in 2 of the heated suspensions, this being probably due to traces of plasma containing the heat-stable isoenzyme remaining in the washed suspension. There was no alkaline phosphatase activity in the remaining 8 heated suspensions.

Comment

NAP activity and total urinary estrogen excretion. It is now well established that in hypertensive pregnancies urinary estrogen excretion is lower than normal and proportional to the severity of the hypertension, reflecting both diminished placental production and altered fetal metabolism of estrogens.

The administration of estrone and adrenocorticotropic hormone increases NAP activity. Estrogens increase corticosteroid-binding globulin levels and reduce the catabolism of cortisol, these being factors responsible for the increased total plasma corticosteroid levels found in normal pregnancy. Plasma unbound cortisol levels are also raised in pregnancy and in estrogen-treated subjects. A reduction in the placental production of estrogens due to hypertensive damage may affect the NAP scores directly or secondarily by reducing the circulating corticosteroid levels, thus accounting for the reduced scores found in cases of moderate to severe hypertension.

In normal pregnancy, estrogen produc-
tion and plasma corticosteroid levels rise progressively until term, so one would expect the same trend to occur in NAP activity. This is not so, perhaps the reason being that there is a maximum level to which NAP activity rises and beyond which it will not respond to further hormonal stimulation.

NAP activity and HSAP levels. Serum HSAP levels have been found to be higher than normal in cases of pre-eclamptic toxemia, the elevation being attributed to enzyme released from damaged placental cells. Our own work (unpublished) suggests a diminution of serum HSAP levels in hypertensive pregnancies, with the absence of any trend in these levels during the latter weeks of pregnancy. After 32 weeks' gestation more hypertensive patients had low, rather than high, serum HSAP levels. The positive correlation in this study between NAP and serum HSAP levels in hypertensive pregnancies is difficult to explain. The absence of alkaline phosphatase activity in leukocytes heated to 65°C, despite optimal conditions for detecting the placental isoenzyme, means that we are considering different isoenzymes, and it is extremely unlikely that there is a direct connection between serum HSAP levels and neutrophil alkaline phosphatase activity. In the present state of our knowledge, we must conclude that this positive correlation is a coincidental finding.

In normal pregnancy, serum HSAP levels rise progressively from 30 weeks to term and our own work (unpublished) confirms this finding. As NAP scores remain
Fig. 2. Relationship between NAP and serum HSAP estimations in pregnancies complicated by moderate to severe hypertension.

relatively constant from 30 to 40 weeks' gestation, there can be no relationship between these apparently unrelated enzyme estimations.

Reduced NAP scores may reflect relatively large reductions in estrogen output in pregnancy, suggesting that its limited value as a placental function test may be as a simple screening procedure for high-risk patients.

We should like to thank Professor A. P. Camilleri for his advice and encouragement.

REFERENCES


A MODIFICATION OF THE SERUM GLUCOSE-6-PHOSPHATASE ASSAY

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(Received December 4, 1971)

SUMMARY

The difficulties in estimating G-6-Pase activity (distinct from non-specific acid and alkaline phosphatases) in serum are discussed. Experiments with pregnant human serum and with rat liver microsomes suggest that G-6-Pase activity can be estimated specifically in serum buffered to pH 6.5, provided $1.4 \times 10^{-3}$ BeSO$_4$ is added as an inhibitor of alkaline phosphatase. A second portion of the same serum, preincubated at pH 5.0 to destroy G-6-Pase activity, is used to estimate acid phosphatase activity. Subtraction of this from the first value results in an estimate of true G-6-Pase activity.

There is controversy as to whether a specific glucose-6-phosphatase (G-6-Pase) activity exists in serum and if it does, whether it can be estimated distinctly from non-specific acid and alkaline phosphatases. Some authors thought that it could be estimated as a specific activity$^1-3$, while others felt that this was impossible$^4-7$. Not only is the attempted estimation complicated by the presence of the non-specific phosphatases, but G-6-Pase itself seems to be far from specific in its actions.$^8$. Since it has been reported that serum G-6-Pase activity increases in liver disease$^2$, and in view of the somewhat conflicting results of placental G-6-Pase activity obtained by histochemical methods$^9-11$, it was thought worthwhile to investigate serum levels of activity of this enzyme in pregnancy, both normal and abnormal.

As a first step the work reported here attempts to establish an assay for true G-6-Pase in serum by choosing appropriate experimental conditions. A preparation known to have G-6-Pase activity, namely the microsomal fraction of rat liver was used for comparison with serum.

MATERIALS AND METHODS

* Human serum was obtained by centrifuging the clotted venous blood of pregnant women. Care was taken to avoid haemolysis, and the enzyme estimations were carried out immediately.

** The microsomal fraction from fresh rat liver homogenate was prepared by dif-

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** Present address: Dept. of Biochemistry, University of Leeds.

ferential centrifugation as described by Nordlie and Arion12, and diluted to a convenient level of activity for use in the assay.

Buffers
I. 0.1 M Tris–maleate, pH range 5.0–8.5.
II. 0.1 M Sodium carbonate–bicarbonate, pH range 9.0–9.5.

Substrates
I. Disodium salt of D-glucose-6-phosphate: a final concentration of 0.01 M was used for serum estimations, and 0.04 M for estimations involving the microsomal fraction.
II. Phenyl disodium orthophosphate; a final concentration of 0.01 M was used.

Alkaline phosphatase inhibitors
I. Beryllium sulphate was used over the range $1.4 \times 10^{-4}$ M to $5.6 \times 10^{-3}$ M.
II. EDTA was used over the range $7 \times 10^{-3}$ M to $3.5 \times 10^{-2}$ M.

Technique for estimating G-6-Pase activity
Two tubes, both containing 0.4 ml of substrate in buffer at the appropriate pH value were brought to 37°. After the addition of 0.2 ml of serum or diluted microsomal fraction to the first tube, both tubes were incubated at 37° for 1 h. One millilitre of 10% (w/v) trichloracetic acid was added to both tubes, and 0.2 ml of serum or of diluted microsomal fraction was added to the second tube. After chilling on ice for 5 min, distilled water was added to bring the volume to 2.5 ml. The tubes were centrifuged at 5000 rev./min for 3 min, and 2 ml of the supernatant taken for phosphorus estimation, using the method of Fiske and Subbarow13. The extinction at 660 nm was determined by means of a Unicam SP 600 spectrophotometer after 10 min. The reading for the second tube was subtracted from that of the first. One unit of G-6-Pase activity

![Graph](image-url)

Fig. 1. G-6-Pase activity of serum as a function of substrate (G-6-P) concentration, in 0.1 M Tris–maleate buffer pH 6.5 and 37°.
was defined as the amount of enzyme that releases 1 μg of inorganic phosphorus during 1 h of incubation at 37°.

RESULTS

The optimum substrate concentration of glucose-6-phosphate for estimating G-6-Pase activity in serum at pH 6.5 was found to be 0.01 M (Fig. 1).

Fig. 2. Variation of phosphatase activity of microsomal fraction of rat liver with pH, at 37°. Buffers: pH 5.0-8.5, 0.1 M Tris-maleate; pH 9.0 and 9.5 0.1 M NaHCO₃-Na₂CO₃ (●). Identical samples, pre-incubated at pH 5.0 at 37° for 15 min (▲).

Fig. 3. Mean phosphatase activity of 3 sera as a function of pH, conditions as in Fig. 2, (●), and pre-incubated at pH 5.0 at 37° for 15 min (▲). The latter points are displaced to the right for clarity.

The total phosphatase activities of 5 paired sera were estimated at pH 6.5, using 0.01 M glucose-6-phosphate or 0.01 M phenyl disodium orthophosphate as substrate. The mean value, using the former substrate was 2.6 + 1.3 units/ml serum while with the latter substrate it was 2.8 ± 1.2 units/ml serum.

Phosphatase activity was determined as a function of pH. The results are shown in Figs. 2 and 3, expressed as means for 3 portions of the microsomal fraction and 3 sera, each with and without preincubation at pH 5.0 at 37° for 15 min. This latter procedure is reported to destroy true G-6-Pase activity15.

Experiments with various concentrations of the reported alkaline phosphatase inhibitors, beryllium ions1, and EDTA3, showed that the most effective was a concentration of 1.4 x 10^-3 M BeSO4. The mean phosphatase activity, related to pH, of 5 sera, each with and without the addition of 1.4 x 10^-3 M BeSO4 is shown in Fig. 4. The activity at pH 9.0 was depressed by about 70%, while at pH 6.5 it was only depressed by about 20%.

![Graph](image)

**DISCUSSION**

The apparently almost equal amounts of enzyme activity obtained using glucose-6-phosphate and phenyl orthophosphate as substrates suggest that either G-6-Pase is not specific for the hydrolysis of glucose-6-phosphate at pH 6.5, or that there are other phosphatases, such as alkaline phosphatase active under these conditions, or both. The absence of a significant difference in enzyme activity using the two substrates is in agreement with Foz7.

G-6-Pase is well known to be present in the microsomal fraction14. The phosphatase activity of the microsomal fraction, related to pH, shows a peak of activity at pH 6.0, this being markedly diminished by pH 9.0. Most of this activity is presumably due to true G-6-Pase. Since incubation at pH 5.0 and 37° for 15 min destroys G-6-Pase15, therefore following this, little if any activity would be expected at pH 6.0.
or 6.5, while residual activity at pH 5.0 or 5.5 would be due to small amounts of the more stable acid phosphatase present in the microsomal fraction. This conclusion is borne out by the results shown in Fig. 2.

Similar patterns of activity were obtained using sera (Fig. 3), and it is reasonable to suppose that most of the enzyme activity occurring at pH 6.0-6.5 is due to G-6-Pase. In the present experiments peak activity occurred between pH 6.0 (Figs. 2 and 4) and pH 6.5 (Fig. 3). This is in agreement with the findings of other authors\(^1-\)\(^6\) that G-6-Pase has a broad pH optimum between 6.0 and 7.0 (refs. 1, 16).

Alkaline phosphatase is active over a wide pH range and some considerable activity can still occur at pH 6.0-6.5. Thus the addition of beryllium ions, which partially inhibit alkaline phosphatase but not G-6-Pase\(^4\), is essential. The results shown in Fig. 4 indicate that there is only a small reduction in enzyme activity at pH 6.0 and 6.5, with considerable reduction at the more alkaline pH values. This is in agreement with the findings of Ashmore et al.\(^1\) that beryllium ions at a concentration of \(1 \times 10^{-3}\)M did not inhibit G-6-Pase activity at pH 6.5.

It seems, therefore, that a true measure of serum G-6-Pase activity can be obtained using a substrate concentration of 0.01 M glucose-6-phosphate, buffered to pH 6.5, with the addition of \(1.4 \times 10^{-3}\)M BeSO\(_4\) to inhibit as far as possible alkaline phosphatase activity. To assess acid phosphatase activity a second estimation should be carried out at pH 6.5 on a portion of the same serum, which has been preincubated at pH 5.0 and 37 \(^\circ\) for 15 min. As this activity is minimal, it does not seem necessary to add beryllium ions as an inhibitor of alkaline phosphatase. Subtraction of the second estimation from the first, would give a true value of G-6-Pase activity.

It seems, from the present work, that G-6-Pase as distinct from acid and alkaline phosphatase activity can be estimated in the serum.

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15. C. de Duve, in G. E. W. Wolstenholme and J. S. Freeman (Eds.), Ciba Foundation Colloquia on Endocrinology, 6 (1953) 22.
Serum heat-stable alkaline phosphatase levels in normal and abnormal pregnancy

Guardamangia, Malta

To ascertain the prognostic value of serum heat-stable alkaline phosphatase estimations in abnormal pregnancy, 698 estimations were carried out during 196 pregnancies. It appears from this study that serum HSAP estimations are of no value in the prediction of hypertension in pregnancy, dysmaturity, or fetal distress. In moderate to severe cases of hypertension, the values are initially high but tend to fall gradually as pregnancy advances. Sudden elevations from this trend may indicate a poor fetal prognosis and are possible indications for obstetric intervention. Cases of perinatal death are associated with high values, occurring in the earlier part of the third trimester.

Several methods have been used for the detection of serum heat-stable alkaline phosphatase (HSAP), and so comparison of results is difficult. Recently, specific conditions for detecting maximal activity of the placental isoenzyme have been reported by Fishman and colleagues, and Ghosh and Fishman. As this appears to be the most accurate method, the findings of Benster and Curzen and Varma, who used this method, are of particular interest. This study was undertaken to evaluate further the role of serum HSAP estimations as a guide to fetal prognosis, with the use of the most accurate method available.

Materials and method

Six hundred and ninety-eight serum HSAP estimations were carried out on 196 patients. The distribution of these is shown in Table I.

The method used was that described by Hansen, with modifications already mentioned, the results being expressed as placental isoenzyme units (P.I.U.). One unit is defined as the amount of enzyme which will liberate 1.0 mg. of phenol from 72 minutes of phenyl phosphate in 15 minutes at 38° C. and pH 10.7.

Statistical comparison of mean values was carried out by Student's t test.

Normal pregnancy was defined as one in which there were no antenatal complications and which terminated in the delivery of a single, normal weight-for-dates baby, at between 37 and 42 weeks' gestation, with no fetal distress.

Mild hypertension was defined as a blood pressure reading of between 140/90 and 150/100 mm. Hg at any stage of pregnancy.

Moderate to severe hypertension was defined as a blood pressure reading above 150/100 mm. Hg at any stage of pregnancy.

Many of the patients had unclassifiable hypertension, and no attempt has been made to differentiate these from cases of essential hypertension or pre-eclamptic toxemia.

Dysmaturity was defined according to the criteria used by Elder and co-workers.

Fetal distress was defined as a fetal heart rate of less than 100 beats per minute on
more than one occasion or the presence of meconium in the liquor amnii.

Results

Normal pregnancy. Table II shows the results of serum HSAP estimations obtained during 72 normal pregnancies. These are illustrated in Fig. 1. The normal range is taken as two standard deviations from the mean. Five patients had a total of 6 estimations outside this range.

Abnormal pregnancy. Tables III and IV show the results obtained in cases of mild and moderate-to-severe hypertension; Table V, those in cases of fetal distress; and Table VI, those in cases of dysmaturity.

The significance of the difference of the mean values compared with those of the same week in normal pregnancy is indicated.

In cases of mild and moderate-to-severe hypertension, dysmaturity, and fetal distress, the numbers of patients having serum HSAP values outside the normal range are 6, 4, 0, and 3, respectively. None of these numbers differs significantly from those in the normal group.

Twenty patients had serum HSAP estimations carried out between 1 and 5 weeks prior to the onset of the hypertension. Only three of these patients had values outside the normal range, and this number does not differ significantly from those in the normal group.

Twenty-four serum HSAP estimations

Table I. The distribution of HSAP estimations carried out during normal and abnormal pregnancies

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>No. of estimations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pregnancy</td>
<td>72</td>
<td>277</td>
</tr>
<tr>
<td>Mild hypertension</td>
<td>39</td>
<td>128</td>
</tr>
<tr>
<td>Moderate-to-severe hypertension</td>
<td>59</td>
<td>209</td>
</tr>
<tr>
<td>Dysmaturity uncomplicated by hypertension</td>
<td>15</td>
<td>41</td>
</tr>
<tr>
<td>Fetal distress*</td>
<td>19 (11)</td>
<td>67 (40)</td>
</tr>
<tr>
<td>Perinatal loss*</td>
<td>10 (0)</td>
<td>24 (0)</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>196</strong></td>
<td><strong>698</strong></td>
</tr>
</tbody>
</table>

*Figures in parentheses are the numbers not included in other categories.
Table II. Serum HSAP estimations in normal pregnancy

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>No. of estimations</th>
<th>Mean</th>
<th>± S.D.</th>
<th>Significance of difference of mean compared with normal pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>27-29</td>
<td>14</td>
<td>11.7</td>
<td>3.31</td>
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<tr>
<td>30</td>
<td>9</td>
<td>11.0</td>
<td>4.88</td>
<td></td>
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<tr>
<td>31</td>
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<td>12.8</td>
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<td>13.8</td>
<td>6.21</td>
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</tr>
<tr>
<td>34</td>
<td>27</td>
<td>16.6</td>
<td>6.80</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>23</td>
<td>17.5</td>
<td>5.87</td>
<td></td>
</tr>
<tr>
<td>36</td>
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<td>17.5</td>
<td>4.91</td>
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</tr>
<tr>
<td>38</td>
<td>24</td>
<td>20.9</td>
<td>6.61</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>16</td>
<td>19.1</td>
<td>6.73</td>
<td></td>
</tr>
<tr>
<td>40</td>
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<td>19.3</td>
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<tr>
<td>41</td>
<td>7</td>
<td>20.3</td>
<td>8.32</td>
<td></td>
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<tr>
<td>42</td>
<td>7</td>
<td>19.4</td>
<td>6.38</td>
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</tbody>
</table>

Table III. Serum HSAP estimations in pregnancies complicated by mild hypertension

<table>
<thead>
<tr>
<th>Weeks of gestation</th>
<th>No. of estimations</th>
<th>Mean</th>
<th>± S.D.</th>
<th>Significance of difference of mean compared with normal pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>27-29</td>
<td>7</td>
<td>9.2</td>
<td>3.22</td>
<td>N.S.</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>11.0</td>
<td>5.14</td>
<td>N.S.</td>
</tr>
<tr>
<td>31</td>
<td>4</td>
<td>17.3</td>
<td>9.98</td>
<td>N.S.</td>
</tr>
<tr>
<td>32</td>
<td>7</td>
<td>16.3</td>
<td>5.68</td>
<td>N.S.</td>
</tr>
<tr>
<td>33</td>
<td>7</td>
<td>16.3</td>
<td>8.80</td>
<td>N.S.</td>
</tr>
<tr>
<td>34</td>
<td>10</td>
<td>13.5</td>
<td>5.57</td>
<td>N.S.</td>
</tr>
<tr>
<td>35</td>
<td>13</td>
<td>16.0</td>
<td>6.17</td>
<td>N.S.</td>
</tr>
<tr>
<td>36</td>
<td>19</td>
<td>13.7</td>
<td>7.45</td>
<td>N.S.</td>
</tr>
<tr>
<td>37</td>
<td>21</td>
<td>16.6</td>
<td>5.41</td>
<td>N.S.</td>
</tr>
<tr>
<td>38</td>
<td>20</td>
<td>18.3</td>
<td>5.85</td>
<td>N.S.</td>
</tr>
<tr>
<td>39</td>
<td>9</td>
<td>17.3</td>
<td>6.28</td>
<td>N.S.</td>
</tr>
<tr>
<td>40-42</td>
<td>7</td>
<td>14.5</td>
<td>5.39</td>
<td>&lt; 0.05</td>
</tr>
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</table>

N.S. = Not significant.

were carried out in 10 cases of perinatal death, these data being inadequate for comparison on a weekly basis with the normal group. Five patients had 6 estimations outside the normal range, and this number is significantly higher than that found in the normal group (p < 0.001).

In cases of hypertension, fetal distress, and perinatal loss, there were 24 serum HSAP estimations outside the normal range. Eighteen were higher than normal and occurred at 36 weeks’ gestation or earlier, while
6 were lower than normal and occurred at 36 weeks or later.

Comment

The results in normal pregnancy show a linear increase (Fig. 1), which is in agreement with the results of Curzen and Varma, who used the same method, and with those of many other authors, who used different methods.

The results in cases of mild hypertension (Table III) are in agreement with previous findings in cases of mild pre-eclamptic toxemia.

The results in cases of moderate-to-severe hypertension (Table IV), together with the fact that all the abnormally high values occurred earlier in pregnancy than the abnormally low values, confirm previous findings. This result is in disagreement with other authors who found high levels of serum HSAP throughout pregnancy complicated by moderate and severe pre-eclamptic toxemia.

Although this paper does not deal specifically with pre-eclamptic toxemia, the majority of patients studied either did have or probably had this complication. Agreement with the results of a specific study of pre-eclamptic toxemia suggests that the slight difference in clinical material is not significant.

The number of patients who had abnormal serum HSAP estimations prior to the onset of hypertension does not differ significantly from those in the normal group, and so the test seems valueless in the prediction of hypertension, this being contrary to the findings of others.

In cases of dysmaturity, the mean values were not significantly different from the normal, except for a reduction at 40 to 42 weeks' gestation. No patient in the dysmature group had an estimation outside the normal range. These facts confirm previous results that serum HSAP estimations are valueless in the prediction of a small-for-dates baby.

The results in cases of fetal distress are similar to those in cases of dysmaturity, and again the estimations seem to be valueless in its prediction, confirming the findings of Curzen and Varma.

The number of patients suffering a perinatal loss, who had abnormal serum HSAP values, was significantly higher than expected. All the abnormal values were high and occurred before 36 weeks' gestation. This confirms the findings of Hunter, and Pinkerton, and Johnston, and Benster that an abnormally high value relatively early in pregnancy is associated with a poor fetal prognosis.

Several methods have been used for estimating serum HSAP, and this makes the comparison of results difficult. Fishman and his colleagues, Stolbach and associates, and Ghosh and Fishman have shown that the conditions of maximal activity of the placental isoenzyme are quite specific. From this point of view, these results and those of a preceding study are directly comparable and in agreement with the findings of Curzen and Varma and Benster.

The reason that high serum HSAP values occur during the early part of the third trimester in hypertensive pregnancies and those terminating in a perinatal death may be due to excessive proliferation of the trophoblast. Thereafter, a falling trend in values may reflect premature ageing of the placenta, with a reduction in its enzymatic activities, and placental insufficiency. Sudden elevations from this trend may be due to acute episodes of placental infarction, and, being suggestive of a poor fetal prognosis, are possible indications for obstetric intervention.

However, as the placenta has large functional reserves, these infarctions may not affect the fetus.

In all the groups studied, there is a considerable overlap of values within the normal range, markedly reducing the significance that can be attached to any individual value.

I would like to thank Professor A. P. Camilleri for his advice and encouragement.
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