FIG. 1. Distributions of anion-forming (left) and cation-forming (right) mineral elements in consecutive 24 hr samples of rat urine as described in text. Titrimetrically evaluated ash-TA values are shown as open circles (right).

FIG. 2. Minerals in feces samples corresponding to the urine samples of Fig. 1 (see text). The Na and Cl values (averaging 0.08 and 0.04 meq/24 hr sample, respectively) were too small for individual representation and were therefore included in the representations of the value for Ca and P, respectively. This compromise leaves the latter representations with errors from this source averaging less than 1.5% and retains original accuracy of values shown for total elements and ash-TA.

magnesium present in the samples, so that their titrimetric values could not be reconciled with corresponding values calculated on a uniform basis from mineral composition.

Summary. Titratable ash-acidity (ash-TA) is an acid-base parameter which is defined in terms of mineral composition and may be evaluated as the sum of values for Cl, S and P minus the sum of values for Na, K, Ca and Mg. A relatively simple and essentially direct titrimetric procedure for determining ash-TA in urine, feces and foods is shown to yield values which agree satisfactorily with those calculated from the mineral composition of these materials.

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Sex Related Differences in Isozymes of Serum Lactic Dehydrogenase (LDH).* (32366)

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Electrophoretic studies of the isozymes of serum and tissue LDH have made possible several contributions to the diagnosis of specific organ injury(1-3), and more recently have proved of aid in attempts to clarify the origin of serum LDH(4,5). As will be shown here, they have in addition permitted the discrimination of differences in the distribution of LDH isozymes present in the serum of men and young women. These differences were demonstrable despite the similarity in the mean total serum LDH activity in the two sexes. These new findings indicate that factors related to sex are important physiological determinants of the serum LDH isozyme composition. They also have possible implications, which will be discussed, concerning both the action of sex hormones in controlling the production or assembly of these isozymes and the use of isozymes of LDH in the diagnosis of specific organ disease.

Methods. Venous blood was collected from all subjects in the usual manner, without regard to time of day or meals. Each sample was allowed to clot in a glass tube at room temperature for one-half to one hour before the serum and clot were separated by a 15-minute centrifugation at 2400 rpm. Serum samples were kept at 4°C until used, and were assayed spectrophotometrically and electrophoretically within 24 hours of the blood collection.

The spectrophotometric method for measurement of total serum LDH activity has been described(2,3).

The electrophoretic separation, staining technique, and methods for quantitating the

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activity in each LDH isozyme have also been described(2,3). The reliability of these various techniques has been assessed previously (2,3).

As in our previous studies(1-5) the 5 LDH isozymes in the serum have been named so that each corresponds to a co-migrating serum globulin. The correspondence of this and other nomenclatures is shown in Table I.

TABLE I. LDH Isozyme Nomenclature.

Present study*	Electrophoretic migration†	Tetrameric composition‡
Alpha$_1$ LDH	LDH$_1$	H$_4$
Alpha$_2$ LDH	LDH$_2$	H$_3$M$_1$
Beta LDH	LDH$_3$	H$_2$M$_2$
Gamma$_1$ LDH	LDH$_4$	H$_1$M$_3$
Gamma$_2$ LDH	LDH$_5$	M$_4$

* Based on correspondence to serum proteins simultaneously electrophoretically separated in an agar gel(2,3).

† Based on rapidity of migration in a starch gel (6).

‡ Based on the demonstration that two different monomers (H and M) were capable of assembling into five different tetramers(7), and other studies (8,9). In the subsequent discussion, these are called the alpha and gamma monomers, corresponding respectively to the H and M monomers, to emphasize that the LDH monomeric composition of the serum LDH isozymes has not been characterized as has the LDH$_1$ in heart muscle or the LDH$_5$ in liver or skeletal muscle.

Sixty-one apparently healthy persons were studied, including 15 young females with normal menstrual cycles (mean age 30.2 years, range 20 to 41 years); 10 pregnant females (mean age 25.8 years, range 19 to 41 years); 11 postmenopausal females (mean age 59.0 years, range 44 to 78 years); 13 young men (mean age 26.8 years, range 16 to 38 years); and 12 older men (mean age 55.6 years, range 41 to 75 years). The mean duration of the pregnancies at the time of these studies was 5 months (range 1½ to 8 months). In all but one instance the young women were within the first and third weeks of their menstrual cycle; one young woman was in the fourth week of her cycle.

Although the data pertinent to each of these 5 groups are presented in detail the 5 different groups were reduced to 3 for convenience in presenting the statistical analyses. This was possible because the isozyme data of the 2 male groups were statistically equivalent to one another, as were the data of the 2 groups of young women. Thus all the men comprised one group, all the young females, pregnant or not, formed the second group; and the third group consisted of the postmenopausal females.

A total of 71 serum samples were also examined electrophoretically for their non-specific dehydrogenase activities. This was performed by omitting the lactic acid substrate from the incubation medium. The "activity" demonstrated was low; as previously found(2,3) there was no (beta or gamma) activity demonstrable on the cathodal side of the origin. The activity in the alpha$_1$ area was on the average 3 times greater than that found in the alpha$_2$ area, but in contrast to the LDH isozymes the division between the two was usually poor, and of necessity often arbitrary. Most important is that the young females, males of all ages and older females were indistinguishable as concerns the levels of activity in each, and thus differences in non-specific dehydrogenase activity could not account for the differences in lactic dehydrogenase isozymes reported here.

Results. Total serum LDH activity. There was no difference of statistical significance in the total serum LDH activity between any 2 of the 5 groups studied. When all males were compared with all females, again no significant difference was noted. These data are summarized in Table II.

LDH isozymes. The 2 male groups were comparable in all the isozyme parameters measured; these included the percent of the total activity in the alpha$_1$, alpha$_2$, beta and gamma LDH isozymes, and the LDH alpha$_1$: LDH alpha$_2$ activity ratios. In every one of these measures, the postmenopausal females were indistinguishable from the males. These data are also summarized in Table II.

The two groups of younger women and pregnant women did not differ significantly from one another in any of these same parameters. Each group, however, had a significantly higher proportion of the total serum enzyme activity in the LDH alpha$_1$, and a significantly lower percentage of the total activity in the alpha$_2$ than was present in the males or older females. The beta LDH

TABLE II. Total Serum LDH Activity and LDH Isozymes in Men and Women of Various Ages.*

	Total LDH (u/ml/min)	LDH isozymes				
		$\frac{\text{Alpha}_1}{\text{Alpha}_2}$	Alpha$_1$	Alpha$_2$	Beta	Gamma$_1$ + gamma$_2$
		(%)				
Females:						
Young	226 ± 44.6	1.27 ± 0.186	44 ± 4.2	35 ± 3.7	11 ± 3.1	10 ± 3.5
Pregnant	220 ± 42.2	1.51 ± 0.214	48 ± 3.7	35 ± 4.1	10 ± 3.1	7 ± 3.5
Postmenopausal	239 ± 56.2	.89 ± 0.27	37 ± 8.7	43 ± 6.4	13 ± 4.4	7 ± 4.0
Males:						
Young	238 ± 46.7	.95 ± 0.22	37 ± 5.5	40 ± 4.5	13 ± 3.1	10 ± 4.2
Older	237 ± 34.2	.85 ± 0.14	36 ± 5.4	43 ± 5.3	12 ± 4.3	9 ± 5.2

* All values are means ± one standard deviation.

isozyme also was lower in the young and pregnant females. Only the gamma LDH activity did not differ in males and females.‡ These data are also shown in Table II. All

TABLE III. Statistical Analysis of the Serum LDH Isozyme Data.

	Young plus pregnant women *vs* postmenopausal women		Young plus pregnant women *vs* young plus older men		Postmenopausal women *vs* young plus older men	
	t-value	P-value	t-value	P-value	t-value	P-value
LDH isozymes (%):						
$\frac{\text{Alpha}_1}{\text{Alpha}_2}$	4.9	<.0005	7.0	<.0005	.12	>.5
Alpha$_1$	4.0	<.0005	6.2	<.0005	.02	>.5
Alpha$_2$	4.2	<.0005	4.8	<.0005	.61	>.5
Beta	2.2	<.05	1.9	<.06	.69	>.5
Degrees of freedom	35		49		35	

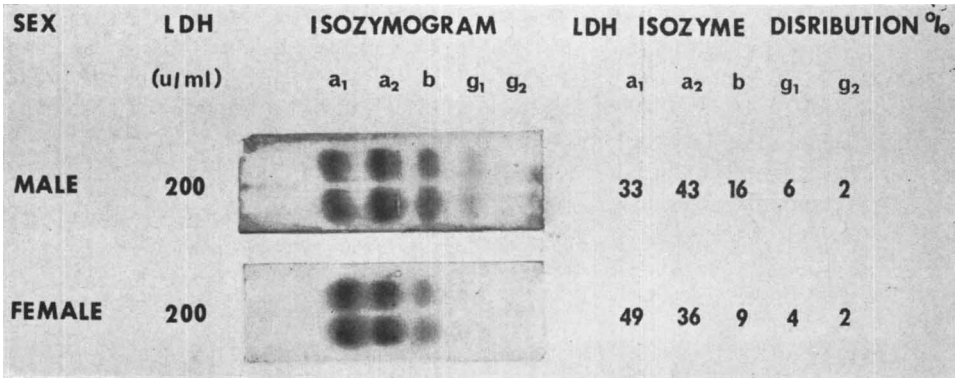

FIG. 1. Serum LDH isozymes in a man and young woman with comparable serum LDH activity. The differences in LDH alpha$_1$, LDH alpha$_2$ and LDH beta isozymes in the two sexes are visually evident in these 2 examples.

‡ The gamma LDH activites were low. Together gamma$_1$ and gamma$_2$ LDH displayed only 7 to 11% of the total serum LDH activity. The error in such low activities, as pointed out previously(2) can be high. The absence of a difference in this parameter among the groups studied therefore cannot be construed as unequivocal evidence that no difference exists.

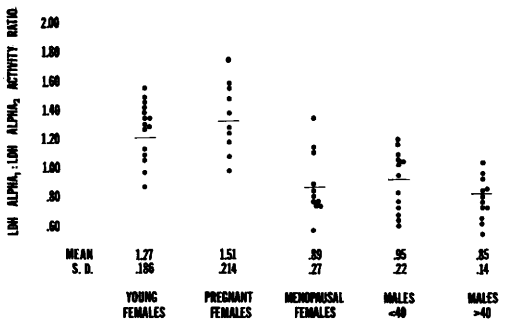

FIG. 2. Influence of sex, age and pregnancy on LDH alpha$_1$: LDH alpha$_2$ activity ratio. The similarity in distribution of this ratio between the 2 young female groups, between the 2 male groups, and between the males and older women is evident. These differences suggest that the sex hormones have an important influence on the distribution of these isozymes.

tests of significance are tabulated in Table III. An example of the typical differences in male and young female isozyme patterns at the same LDH level is shown in Fig. 1.

The mean LDH alpha$_1$:LDH alpha$_2$ ratios in the groups of young or pregnant women were significantly higher than found in either male group, or in the older women. This is displayed in Figure 2. In every instance but one, this ratio was 1.00 or greater in the pregnant or young females. On the other hand, it was less than 1.00 in 8 of 11 instances in the older women, 9 of 12 instances in the older men, and 7 of 13 instances in the younger men. In a previous study an LDH alpha$_1$:LDH alpha$_2$ ratio greater than 1.00 was seen in only 2 of 51 adult males studied (2,3). The bimodal distribution of this ratio in males and the younger women is depicted in Fig. 3 and emphasizes the importance of sex as a determinant of the magnitude of this ratio.

Discussion. The *total serum LDH activity* was found to be uninfluenced by sex, pregnancy or the age-ranges studied. This observation agrees with those of others(10,11).

The lack of influence of these variables on total serum LDH activity is in contrast to their apparent influence on *LDH isozyme distribution* in the serum. The LDH isozyme distribution in young women, pregnant or not, is conspicuously different from that in men, whether young or old, and that in postmenopausal women. This is believed to be the first demonstration of such differences in the human. The factor(s) responsible for this difference is apparently more age-related in women than in men. The several differences in the serum LDH isozyme pattern of postmenopausal women and young females suggests that they may be importantly related to a fundamental change accompanying the menopause, such as a reduced estrogenic activity. This possibility is further supported by the fact that in 2 groups of persons with high estrogenic activity, namely 10 pregnant females and 15 men receiving 5 mg of diethylstilbesterol daily for treatment of prostatic cancer, the LDH alpha$_1$: LDH alpha$_2$ activity ratio has been found to be >1 with only two exceptions.

Speculation as to the mechanism by which these sex and age related differences are expressed would be more meaningful if the exact source of the serum LDH in normal persons were known. Despite it being generally assumed that the sources of the LDH isozymes normally present in the serum are several peripheral tissues, there is no direct evidence that supports this. In fact, to the contrary, substantial circumstantial evidence suggests that serum LDH arises not from peripheral sites but rather from a small fraction of the plasma platelets(4,5).

Assuming that the LDH isozymes derive from a single (cellular) source, and that the 2 LDH monomers which comprise the LDH isozymes have equal activity(12), and with the knowledge that each isozyme has a

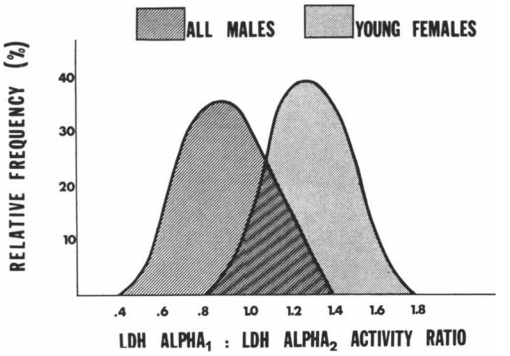

FIG. 3. Frequency distribution of LDH alpha$_1$:LDH alpha$_2$ activity ratio in men and young women. The distribution is essentially bimodal suggesting that the magnitude of this ratio is sex influenced.

characteristic tetrameric composition (Table I), it is possible to calculate the amount of enzyme activity contributed by each monomer.§ If the proportion of activity contributed by each monomer is different in the males and young females this would suggest that the *production* of each monomer is under the influence of the sex related factors causing the isozyme differences noted here. If, on the other hand, the activity contributed by each of the two monomers is the same in both sexes, and only the distribution of each monomer among the 5 tetrameric forms of these isozymes is different, this would suggest that the *assembly* of the tetramers and not the production of the two LDH monomers, was being influenced by these sex-related factors.

By calculation, 75 to 80% of the enzyme activity is attributable to the alpha (H) monomer and 20 to 25% to the gamma (M) monomer. It appears to be about the same in all 5 groups. This suggests, given the assumptions noted above, that it is primarily the tetrameric assembly of the two monomers, rather than their differential production that is being affected by these sex-related factors. Indeed, stimulated by these observations we have recently been able to demonstrate that diethylstilbesterol can alter the *in vitro* assembly of LDH isozymes(13).

The effect on the assembly of LDH isozymes noted here and demonstrated *in vitro* (13) does not preclude effects on LDH monomer production. Indeed, others have noted that administration of estrogen to the female rat stimulates the production of the monomers in the rat uterus(14,15).

These observations imply that study of the influence of the sex hormones on the serum and tissue LDH isozymes may provide a useful means for clarifying the manner in which these hormones influence at least one gene-enzyme relationship in man. They also suggest that hormonal influences on the multiple forms of other serum proteins should be more generally sought, and that the absence of differences in the concentrations or activity of a given serum protein in men and women may be obscuring more subtle differences in the multiple forms in which a protein may exist. This may be important, as the role of protein polymorphism in susceptibility and resistance to disease is poorly understood(16).

These observations also have an important implication as concerns the diagnostic uses of LDH alpha$_1$: LDH alpha$_2$ ratio. As noted elsewhere, alterations in this ratio from <1 to >1 are the rule in renal cortical and myocardial injury as well as in many forms of hemolytic anemia when each was associated with increased serum LDH activity(1-3). This is explained by the enrichment of the serum with both alpha forms of LDH (alpha$_1$ $>$ alpha$_2$) in which heart, renal cortex and red cell are rich. The present data in no way alter these previous observations. However, in the presence of normal LDH levels, the presence of an alpha LDH ratio > 1, especially in young persons, must not be construed as an indication of subtle injury of the myocardium, renal cortex or red cell.

Summary. Differences in the distribution of the LDH isozymes in the serum of men and young women have been found, despite the fact that the mean total serum LDH activity is the same in men and women. These differences were found to disappear after the menopause. The theoretical significance of these observations in relation to the site of estrogen action and their importance in the diagnostic use of serum LDH isozymes are discussed.

§ For each of the 5 groups of persons studied, the proportion of total LDH activity residing in the alpha LDH monomer = 4/4 (LDH alpha$_1$) + 3/4 (LDH alpha$_2$) + ½ (LDH beta) + ⅛ (LDH gamma$_1$ + LDH gamma$_2$). Each isozyme listed in parenthesis in the preceding equation represents the proportion of total activity in that isozyme. These proportional values are shown in Table II for each group of persons studied. The proportion of total activity represented by the gamma monomer is calculated by difference.

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Chemical Changes in Epiphyseal Growth Zone of Rats Induced by Excess and Lack of Estrogen.* (32367)

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There are numerous reports describing the morphologic and histochemical changes induced in the epiphyseal growth zones of young rats by administration of estrogens(1, 2). Chemical studies have usually been limited to changes in mineral content or in some single organic component of this area(1,3).

In the present study the major constituents—water, total nitrogen, collagen, mucopolysaccharides, and calcium—have been assayed in the proximal tibial growth zones of young rats and compared with similar data obtained from rats of identical age but experimentally subjected to an excess or deficit of estrogen.

Materials and methods. The experimental subjects were weanling Sprague-Dawley rats purchased from Sprague-Dawley, Inc., Madison, Wisc. For each experiment equal numbers of control and experimental rats were separately caged in clean airconditioned animal quarters. Each rat was weighed daily, and all were fed Purina rat chow and allowed water *ad lib* throughout the experiments.

In the study of the responses to excess estrogen 8 separate experiments were run using 6 controls and 6 treated rats in each. Male weanlings were used in 6 of these experiments and females in 2 of the 4 studies at the 9 dose level. Estrogen injections were started when the rats were 27-30 days old. The estrogen-treated groups received 5, 9, or 15 daily intramuscular injections of 0.1 mg estradiol benzoate in sesame oil. Both the treated rats and their controls were killed on the day following the last injection.

The effects of estrogen deficit were examined in 30 female rats ovariectomized at the age of 45-50 days and sacrificed, together with an equal number of intact female controls of the same age, at 2, 4, and 6 weeks after ovariectomy.

At termination of any given experiment each rat was exsanguinated by decapitation in a guillotine and immediately the two proximal tibial heads were taken for analysis. These samples were obtained by a uniform dissection which involved disarticulation from the femur, careful removal of adhering soft tissue, periosteum, perichondrium, and cutting of the head from the tibia at the point where the metaphysis narrows to form the shaft. Such samples, which in the present paper we have called the "epiphyseal growth zone," include the epiphysis plus the epiphyseal plate and its growing cartilage and the calcifying trabeculae of the metaphysis; *i.e.*, the epiphysis plus the components of the area which Ross and McLean have called the

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