LIPID DEFICIENCIES, LEUKOCYTOSIS, BRITTLE SKIN—A LETHAL SYNDROME CAUSED BY A RECESSIVE MUTATION, EDEMATOUS (OED), IN THE MOUSE

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ABSTRACT

A new neonatal lethal mutation in the mouse with pleiotropic effects, edematous (oed), arose spontaneously in the phocomelic strain and has been shown to have an autosomal recessive mode of inheritance. The external phenotypic characteristics include a generally bloated appearance, shiny cellophane-like skin, and distal hematoma of the extremities. Internally, no gross or histological abnormalities could be identified, with the exception of a striking leukocytosis. Biochemical analysis revealed a severe disturbance of lipid metabolism. Deficiencies in the VLDL, LDL, and the HDL lipoprotein fractions have been found in the mutant plasma. In addition, all serum lipids are markedly decreased. However, in the mutant liver, only triglycerides are significantly decreased; total liver cholesterol and phospholipid values are within normal limits. The primary biochemical defect as well as the causal relationship between the striking abnormalities of lipid metabolism and those of skin and blood are unknown at this time.

THE mutation “edematous” (oed) arose spontaneously in the mouse strain “phocomelic”, in this laboratory. Among the offspring of certain parents, characteristically abnormal newborns, different from the expected phocomelics and able to survive for only a few hours, were noticed. Subsequent genetic analysis identified these as being homozygous for a new autosomal recessive mutation. The syndrome of abnormalities (Figure 1) included generally bloated appearance, shiny cellophane-like and brittle skin, protruding tongue and frequently hematoma in distal portions of tail, feet and snout. The characteristic external appearance of the homozygote was responsible for calling the mutation “edematous” (oed), although a true edema was never identified. The nature of the syndrome required the use of many diverse analytical approaches in attempts to elucidate the expression of the mutant gene.

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METHODS OF ANALYSIS

The study of "oed" homozygotes was carried out with a variety of methods.

Newborns were dissected and conventional methods were used for the preparation of material for histological studies. 1% toluidine blue in 1% borax served as the stain for thick sections of liver fixed with glutaraldehyde and imbedded in Epon. The fat soluble colorant oil red 0 was used as the stain for neutral lipids (Lillie and Ashburn 1943) in frozen sections.

Blood smears of mutant and control newborn were prepared, and hematocrits were determined with conventional methods.

Leukocyte counts were carried out on samples obtained with "unopettes" (Becton-Dickinson) from newborn normal and mutant littermates, and from adult homozygous and heterozygous normals. Conditions for counts were standardized as much as possible because of the well known effects of external factors on leukocyte numbers. In studies of genetically caused variations comparative leukocyte counts are considered most informative when carried out in newborn mice since these are exposed to more uniform environmental conditions than older animals, and thus are less subject to environmentally caused variations in leukocyte numbers (Kunze 1954).

Serum and liver for biochemical analysis were obtained from unfed newborn mutant and littermate controls which had been killed by decapitation. The blood was removed with a capillary pipette and allowed to clot in a test tube at room temperature for two hours. It was then centri-
fuged, and the serum pooled and frozen at -20°. Livers were dissected out immediately after decapitation, frozen on dry ice and stored at -90° until used.

For the biochemical study of adult heterozygous and homozygous normals, mice were starved for approximately 15 hours, then bled from the retroorbital plexus. Serum was prepared and liver obtained by the same procedures as those used for newborns. All material was analyzed within two weeks after dissection.

**Plasma for lipoprotein electrophoresis** was prepared from blood collected in capillary pipettes coated with a solution of 5% sodium EDTA, pH 7.4, and centrifuged in tubes. Subsequently, individual plasma samples were removed and 1A aliquots electrophoresed for thirty minutes using the Pfizer Pol-E system. The gels were then dried for 30 minutes at 72° in a vacuum oven, stained for lipoproteins with Pfizer Fat red 7B stain, and scanned using a Clifford densitometer, model 445.

**Immunodiffusion:** Double immunodiffusion (Ouchterlony) of sera was carried out using rat antisera prepared against the following: Apo VLDL (very low density lipoproteins) and VLDL subunits, an LDL (low density lipoproteins) subunit, and an Apo HDL (high density lipoproteins) and an HDL subunit, all of which had cross reactivity to mouse serum. The antisera were generously supplied by DR. P. Roheim.

**Serum lipid determinations** were carried out directly on serum fractions.

**Liver lipids:** For all determinations, frozen livers were weighed and homogenized in a Thomas tissue homogenizer at 0° in 20 volumes of a chloroform : methanol solution (2:1). Lipids were then extracted according to the method of Folch, Lees and Sloan-Stanley (1957), evaporated, and the extracts assayed directly.

**Cholesterol:** Total and free cholesterol were determined in liver extracts and serum by the methods of Leffler (1959) and Watson (1960); esterified cholesterol was calculated by subtracting the value of free cholesterol from the total value.

**Phospholipids** were determined in liver and serum by the method of Zilversmit and Davis (1950), and triglycerides by the method of Eggstein (1966).

**Incorporation of radioactively labeled precursors:** Livers were minced and incubated in tissue culture dishes containing 2.5 ml of Krebs-Ringer bicarbonate buffer as described by Kandutsch and Saucier (1969) in a 5% CO₂ incubator at 37° for the appropriate time periods. The pieces of liver were then removed and flash-frozen as previously described. For experiments using ¹⁴C-acetate the final 2.5 ml volume of Krebs-Ringer bicarbonate buffer contained 10 μc and 2.5 μM acetate. For ³H mevalonate the buffer contained 10 μc and 2.5 μM of DL-mevalonic acid, and for ¹⁴C leucine incorporation the final concentration was 5 μc and 2.5 μM of uniformly labeled L-leucine. All isotopes were diluted to the indicated concentrations with the corresponding unlabeled compounds.

In order to measure the uptake of radioactive acetate into fatty acids, lipids were extracted as described above and the extracts saponified overnight at room temperature as described by Stahl (1969). The next day, lipid soluble material was extracted with petroleum ether and the aqueous solution of potassium salts of the fatty acids was acidified and extracted with petroleum ether. An aliquot of the total fatty acids was measured to determine radioactive incorporation.

For thin layer chromatography of free and esterified cholesterol and of triglycerides, liver lipid extracts were dissolved in 50A of a chloroform : methanol solution (2:1). 10A of this solution was spotted on Brinkmann Silicia Gel G-25 plates and the lipids separated using a solvent mixture of petroleum ether : diethyl ether : glacial acetic acid (82:18:1) as described by Schiller and Woop (1965). Plates were developed with iodine and spots identified by reference standards co-chromatographed with the extracts.

**Enzyme studies**

Injections of newborn animals for purposes of enzyme induction, preparation and extraction of homogenates, as well as enzyme assays, were carried out as described by Thorndike et al. (1973).

**Hexokinase** (E. D. 2. 7. 1. 1) was assayed in the soluble fraction by the method of Purich and Fromm (1971) using glucose at a concentration of 0.05 mM.
TABLE 1

Matings of heterozygous parents inter se (+/oed x +/-oed)

<table>
<thead>
<tr>
<th>No. of experiment</th>
<th>Normal 640</th>
<th>Offspring oed/oed 168</th>
<th>Total 808</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>648</td>
<td>211</td>
<td>859</td>
</tr>
<tr>
<td>3</td>
<td>363</td>
<td>108</td>
<td>471</td>
</tr>
<tr>
<td>Total</td>
<td>1651</td>
<td>487</td>
<td>2138</td>
</tr>
</tbody>
</table>

Protein was measured by the method of Weichselbaum (1946). Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter, model #574.

14C leucine incorporation: In vitro protein synthesis was studied in mutant and normal littermate livers by measuring 14C leucine incorporation into protein (TCA precipitable material).

All chemical reagents and lipid standards were purchased from Sigma Chemical Company and all radioactive compounds from New England Nuclear Corp.

RESULTS

Genetic analysis

The first "edematous" newborn mice were observed among the offspring of a normal male mated to his normal sister, both of them heterozygous for the recessive mutation "phocomelia" (Gluecksohn-Waelsch, Hagedorn and Sisken 1956). Eight out of a total of 28 offspring were edematous, and several of the normal offspring were shown to be carriers of the new mutation.

Table 1 summarizes the breeding results from three experiments of matings of carriers of the mutation. The observed results are compatible with the assumption that "oed" is an autosomal recessive, and the ratio of normal to homozygous mutant offspring conforms to expectation. Seventy-four normal offspring from a sample of 18 litters produced by matings of heterozygotes inter se (+/oed X +/-oed) reached the age of weaning. When these were progeny tested for heterozygosity, 36 mice were found to carry the mutation "oed". Twenty-one mice did not produce any abnormal offspring in progenies from 12–20 young each and were likely not to carry "oed", while 17 remained questionable. The ratio of 36 carriers to 21 non-carriers among the normal sibs of edematous offspring is a good fit to the expected ratio of 2 to 1. This ratio, and the result of 489 normal to 0 edematous offspring from matings of heterozygotes to wild type, add support to the assumption that edematous (oed) segregates as a simple autosomal recessive. No linkage tests have been carried out other than with phocomelia (pc), of which oed is independent.

Morphological and hematological results

Dissections of newborn edematous mutants failed to reveal any gross morphological abnormalities. The only striking observation indicating a pathology was that of profuse bleeding in spite of unusual care at dissections, suggesting a possible blood vessel fragility. There was also an apparent decrease in the speed of
blood coagulation. However, neither histological studies of the blood vessel walls nor hematological examinations succeeded in finding any reason for the increased fragility of blood vessels or decreased coagulation of the blood.

Histological studies of paraffin sections of the mutant liver prepared for light microscopy failed to reveal any gross abnormalities. Lobular structure, arrangements of blood vessels, as well as morphology of parenchymal cells, macrophages, lymphocytes and mast cells were normal. Thick sections of liver fixed in glutaraldehyde, imbedded in Epon and stained with toluidine blue revealed differences between mutant and normal parenchymal cells: large blue-green staining droplets, typically present in normal parenchymal cells, were absent from mutant cells. In addition, the cytoplasm of mutant parenchymal cells appeared spotty in contrast to the homogeneously stained control cytoplasm, and frequently mutant cells contained numerous tiny unstained vacuoles.

Frozen liver sections stained with oil red 0 were found to contain a large number of bright-red droplets in the parenchymal cells of control littermates; these are presumably neutral lipid or triglyceride deposits (LILLIE and ASHBURN 1943). However, such droplets were hardly ever seen in sections of mutant liver.

In the course of hematological studies hematocrit and hemoglobin were found to be normal in the mutants; however a significant leukocytosis was observed. Blood counts in 51 homozygous newborns gave a mean value of 11645±72 WBC/mm$^3$ in contrast to a mean count of 3518±31 WBC/mm$^3$ in normal littermates. These values are primarily of comparative significance since they demonstrate a threefold increase in leukocyte numbers in homozygous mutants over normal littermates. Two sets of published values for newborn mice (4125±213, and 3250±114) agree closely with those reported here (KUNZE 1954). Leukocyte numbers determined in adults are known to vary greatly between different inbred mouse strains (RUSSELL, NEUFELD and HIGGINS 1951). The values for adult mice in the oed strain are within the range of those reported (3422±218).

Subsequently, the leukocytosis in the mutants was confirmed by scanning and counting stained blood smears in a series of 42 mutants and 42 controls. Another difference between mutants and controls was the leukocyte type observed in the stained blood smears: the differential leukocyte count revealed an increase of polymorphonuclear and band forms in the myeloid series of mutants. The neutrophil count in controls was 20%–30%, whereas, in the mutants it was found to be in the range of 80%–90%.

Biochemical results

**Plasma lipoproteins:** Electrophoresis of plasma lipoproteins showed a consistent deficiency in the mutant samples. While the decrease in the HDL fraction was small, the fraction containing VLDL and LDL showed a much larger decrease. Integration of the gel scans demonstrated a deficiency of 30% in the mutant HDL fraction, 60% in the mutant VLDL-LDL fraction, and an overall difference of 53% between mutant and normal littermate samples (Figure 2). No significant differences were found in the intensity of staining within the 6 mutant and 12 normal littermate samples analyzed.
**Immunodiffusion:** When mutant and normal littermate serum samples were tested in double diffusion studies against antibodies to VLDL, LDL and HDL samples, reactivity was observed. This result suggested that at least some of the protein moieties of lipoproteins were present also in the mutant serum. However, a specific change of an apoprotein related to the genetic defect could not be excluded.

**Serum lipids:** Since electrophoresis of the plasma lipoproteins showed consistent deficiencies in the amount of lipid-stainable material in mutant samples, studies were undertaken to measure various individual lipid components in mutant and normal littermate sera. Analysis of pooled serum samples revealed marked differences in all lipid fractions measured (Table 2). The level of total cholesterol was reduced by 35% in the mutant samples; this deficiency was due primarily to the reduction of free cholesterol which amounted to about 69% of normal. The ratio of free to total cholesterol in the mutants is 15%, compared to 30% in the normal newborn as well as the adult.

Furthermore, serum triglycerides of mutants were decreased by 75% and serum phospholipids by 53% compared to normal. Total serum proteins showed no quantitative difference between mutant and normal littermate samples (Table 2).

**TABLE 2**

*Serum lipids and protein in normal and mutant mice*

<table>
<thead>
<tr>
<th></th>
<th>Newborn</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oed/oed</td>
<td>Normal littermates (+/+ and +/oed)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total*</td>
<td>47.03 ± 2.01 (4)†</td>
<td>73.02 ± 3.00 (4)</td>
</tr>
<tr>
<td>Free*</td>
<td>6.87 ± 0.74 (4)</td>
<td>21.92 ± 2.49 (4)</td>
</tr>
<tr>
<td>Triglycerides*</td>
<td>10.29 ± 2.23 (6)</td>
<td>41.13 ± 3.68 (6)</td>
</tr>
<tr>
<td>Phospholipids*</td>
<td>40.54 ± 4.20 (5)</td>
<td>87.18 ± 6.18 (5)</td>
</tr>
<tr>
<td>Total serum protein†</td>
<td>3.37 ± 0.20 (5)</td>
<td>3.31 ± 0.18 (5)</td>
</tr>
</tbody>
</table>

* Concentrations are expressed as milligrams per 100 milliliters.
† Numbers in parentheses refer to numbers of pooled samples each of which was obtained from 30 newborn.
‡ Concentration is expressed as grams per 100 milliliters.
Liver lipids: Analysis of lipid fractions in the livers of newborn animals showed phospholipid concentrations and total cholesterol to be the same in mutant and normal littermate samples, whereas triglyceride concentrations were reduced by 37% in mutant livers. However, there is a barely significant increase of the fraction of free cholesterol in the mutant liver (Table 3). Once again, the measured protein values were similar for both samples (mutant: 86.87 ± 5.96 mg/gm liver vs. normal: 91.50 ± 10.19 mg/gm liver).

In vitro lipid synthesis: The ability of the mutant liver to synthesize cholesterol and fatty acids in vitro was studied in experiments with minced livers measuring the uptake of 14C acetate. Incorporation into cholesterol remained linear during the four-hour time period in both the mutant and normal littermate samples. The ability of mutant livers to synthesize free cholesterol was not significantly reduced, but synthesis of both cholesterol esters and triglycerides declined by approximately 62% and 53% respectively (Figure 3, A and B). Because of possible differences in the pool sizes of acetate in the mutant and normal littermate samples, the incorporation of 3H mevalonic acid, a specific cholesterol precursor (Wright et al. 1956), into both free and esterified cholesterol was measured. Incorporation was linear over the 4-hour time period, and the relative amount of incorporation of 3H mevalonic acid into free cholesterol was the same in mutant and normal littermate samples, but for cholesterol esters there was a decrease of 42% in the mutants as compared to the normal littermates (Figure 4A).

Similar experiments measuring 14C acetate incorporation in vitro into total fatty acids showed a 50% decrease in the amount of total fatty acids synthesized by the mutant livers (Figure 4B).

Activity of hepatic enzymes not involved in lipid biosynthesis: In order to test the general physiological condition of the mutant liver, several hepatic enzymes not involved in lipid metabolism were examined, including tyrosine aminotransferase, serine dehydratase, hexokinase and NADPH cytochrome c reductase. No significant differences were observed between mutant and normal littermate controls (Table 4). Furthermore, tyrosine aminotransferase could be

### TABLE 3
Liver lipids in normal and mutant mice*

<table>
<thead>
<tr>
<th></th>
<th>Newborn</th>
<th></th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oed/oed</td>
<td>Normal littermates (+/+ and +/oed)</td>
<td>+/+ and +/oed</td>
</tr>
<tr>
<td>Cholesterol:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.32 ± 0.12 (5) (\dagger)</td>
<td>2.24 ± 0.16 (5)</td>
<td>3.09 ± 0.13 (6)</td>
</tr>
<tr>
<td>Free</td>
<td>2.05 ± 0.14 (5)</td>
<td>1.81 ± 0.09 (5)</td>
<td>2.67 ± 0.10 (6)</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>28.56 ± 0.86 (4)</td>
<td>26.88 ± 0.55 (4)</td>
<td>41.32 ± 1.07 (6)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.65 ± 0.04 (6)</td>
<td>4.20 ± 0.24 (6)</td>
<td>5.17 ± 0.27 (6)</td>
</tr>
</tbody>
</table>

* Concentration is expressed as milligrams per gram liver.
\(\dagger\) Numbers in parentheses refer to numbers of newborn tested.
FIGURE 3.—(A) Incorporation of $^{14}$C acetate into free cholesterol in mutant (•) and normal (△) littermate liver samples, and into esterified cholesterol of mutants (○) and normal littermates (△). (B) Incorporation of $^{14}$C-labeled acetate into triglycerides in mutant (•) and normal littermate (△) liver samples. Each point represents four determinations.

<table>
<thead>
<tr>
<th></th>
<th>oed/oed</th>
<th>Normal littermates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>0.632 ± 0.039 (6)†</td>
<td>0.574 ± 0.033 (6)</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase</td>
<td>26.27 ± 1.33 (6)</td>
<td>28.41 ± 1.41 (6)</td>
</tr>
<tr>
<td>Serine dehydratase</td>
<td>1.29 ± 0.05 (5)</td>
<td>1.64 ± 0.21 (5)</td>
</tr>
<tr>
<td>Tyrosine aminotransferase</td>
<td>1.30 ± 0.13 (6)</td>
<td>1.39 ± 0.11 (6)</td>
</tr>
</tbody>
</table>

* Enzyme activities are expressed in μmoles product formed per gram liver per minute.
† Numbers in parentheses refer to numbers of newborn tested.
induced in newborn mutants injected with glucagon, and the resulting enzyme level showed no differences between mutant and normal littermate liver samples.

Protein synthesis: Experiments using $^{14}$C leucine showed linear incorporation into liver proteins during the four-hour incubation period, and no significant difference in protein synthesis between mutant and normal littermates was observed.

DISCUSSION

The homozygous mutant "edematous" with its strikingly characteristic appear-
ance is unable to survive for more than a few hours after birth. Biochemical studies have revealed a severe and specific disturbance of lipid metabolism in the mutants which are able to synthesize proteins normally. Since lipid analysis of normal newborn mice has not been reported in the literature, it was necessary to obtain control values from normal littermates of mutants. Considerable effort was devoted to overcoming the experimental difficulties inherent in the material, e.g. the small size of newborn liver and serum samples. For example, one set of serum lipid and protein determinations required pooled sera from 30 newborns of each phenotype. Furthermore, all newborn and adult specimens had to be obtained from unfed (newborn) and fasting (adult) animals.

The results reveal a deficiency of plasma lipoproteins in the mutant, and reduction of total cholesterol, free cholesterol, phospholipids and triglycerides in the serum. Triglycerides are deficient also in mutant liver. In the interpretation of these observations it is interesting that in the normal rat the amounts of cholesterol, phospholipids and triglycerides released into the serum VLDL lipoproteins have been reported to be correlated with each other and with the level of liver triglycerides (Heimberg et al. 1965).

The biochemical data show the decrease of cholesterol in the mutants to be distributed unevenly among the various fractions. In the serum, total cholesterol is decreased by 35% but free cholesterol by 69%, resulting in a calculated decrease of only 21% for esterified cholesterol. In the liver, total cholesterol levels are normal and free cholesterol levels are possibly even slightly elevated in the mutants. Synthesis of esterified cholesterol is deficient in the mutant liver in contrast to the normal synthesis of free cholesterol. Also reduced in the mutant liver are the synthesis of triglycerides and of total fatty acids.

For the possible interpretation of these results it is significant that esterification of free cholesterol in solid tissues appears to be accomplished by microsomal enzymes that transfer fatty acids from their acyl coenzyme A thiol esters (Goodman 1965), whereas in plasma esterification of cholesterol involves the transfer of fatty acids from lecithin through the action of the plasma enzyme lecithin: cholesterol acyltransferase, LCAT (Glomset 1968). In view of these differences in the esterification mechanisms it is conceivable that the oed mutation interferes differentially with the two enzyme systems. Alternatively, it might be possible that deficiencies of fatty acids in the liver limit the esterification of liver-free cholesterol. The site of effect of the "oed" mutation in these complex mechanisms cannot be determined at present, and its identification requires further studies.

The histological observations, e.g. the absence of large toluidine blue stained droplets in thick sections of mutant liver cells and of oil red O droplets in the mutant parenchymal cells stained for lipids, indicate deficiencies of lipids, similar to those reported in the biochemical studies of the mutant liver.

In order to detect a possible heterozygous effect of "oed", heterozygous adults had to be used since the genotype of newborns could not be ascertained. Lipid values of homozygous and heterozygous adults were found to be within the range reported in the literature (Yamamoto et al. 1963), and no gene dosage effect was apparent.
Other aspects of the phenotype, e.g. the striking abnormal appearance of the skin, may be correlated with lipid deficiencies of the mutant since a particular composition of fatty acids has been shown to characterize the normal skin (Nicolaides 1974).

The marked leukocytosis in homozygous mutants is of particular interest in view of recent reports of the controlling role of cholesterol in the regulation of leukocyte function and behavior. In the case of human leukemia a reduced level of serum cholesterol was shown to be correlated with a deficiency of cholesterol in the leukocyte cell membrane that in turn affects membrane microviscosity (Inbar and Shinitzky 1974). Although cholesterol levels in the mutant leukocytes themselves were not measured, it is possible that an abnormality of the leukocyte membrane due to cholesterol deficiency actually exists and interferes with the active processes of leukocyte migration into the tissues (Marchesi and Florey 1960); this might account for the increased number of circulating leukocytes observed in mutants. Alternatively, alterations of the cell surface membranes of capillary vessels due to lipid deficiencies might cause a block. This would also serve to explain the bleeding diathesis observed in the mutant homozygotes. On the other hand, the excess bleeding might be due to deficiencies of lipids since these have been shown to be important components of the blood clotting mechanism (Didisheim and Mibashian 1963).

A complete understanding of the correlation between the pleiotropic effects in the oed homozygote, i.e. bloated external appearance, brittle skin, disturbances of lipid metabolism and leukocytosis, is not possible at this time. Developmental studies may provide further information, and preliminary results of such investigations show that the mutant phenotype is expressed in fetuses as early as two days before birth, when mutant fetuses can be identified by external appearance as well as high leukocyte counts and low liver triglyceride levels. At a fetal age of three days before birth, the homozygous mutant cannot be distinguished from its normal littermate by its external appearance, but a proportion of fetuses corresponding to the expected 25% with high leukocyte counts and low liver triglyceride levels are likely to represent the homozygous genotype. These studies are being continued with the hope of identifying the primary error responsible for the pleiotropic syndrome in the homozygous lethal mutants.

The authors are greatly indebted to Dr. Paul S. Roheim, without whose continued advice, criticism and suggestions the biochemical analysis could not have been carried out. Thanks are due also to Estelle Ellis for her help with the lipoprotein electrophoresis.

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LITERATURE CITED


GENETICS AND MORPHOLOGY OF A RECESSIVE MUTATION IN THE HOUSE MOUSE AFFECTING HEAD AND LIMB SKELETON.


