Biochemical Pharmacology, Vol. 19, pp. 945–951. Pergamon Press. 1970. Printed in Great Britain

Isolation and characterization of reduced nicotinamide adenine dinucleotide phosphate:ferri cytochrome *c* oxidoreductase and identification of cytochrome b5 in the liver of human infants*†

(*Received* 20 *March* 1969; *accepted* 29 *August* 1969)

ALTHOUGH it seems certain that a mixed-function oxidase system is present in human liver, a biochemical description of its components has not yet been attempted. The enzyme, NADPH: ferri cytochrome *c* oxidoreductase (EC 1.6.2.3), is believed to be the initial step in this microsomal electron transport chain.[1] We wish to report its isolation from the liver of two infants, one of whom was full-term and the other premature. The enzyme has a molecular weight, K_m and pH optimum equivalent to that of NADPH cytochrome *c* reductase isolated from rat liver. As an incidental finding, tentative identification of cytochrome b5, but not cytochrome P-450, was made in the human microsomal fractions.

MATERIALS AND METHODS

Tissue. Specimen 1 (newborn). The patient was a full-term male infant who had palliative surgery for pulmonary atresia on the third day of life. Despite initial improvement, his condition deteriorated with closure of the ductus arteriosus. On day 14 of life, he suffered a sudden cardiac arrest at 1.30 p.m. He was resuscitated, but died at 4:30 p.m. Intracardiac epinephrine and oxygen were the only drugs used. A 5-g liver specimen was obtained 30 min postmortem and immediately placed on ice and processed.

Specimen 2 (*premature*). The patient was a premature female infant, born at 33-weeks gestation, according to dates, weighing 1385 g. She was limp and cyanotic at delivery (Apgar score of 3) and died 8 hr later during an apneic episode. Thirty minutes before delivery the mother received meperidine, secobarbital, hydroxyzine and hydroxyprogesterone caproate. The infant received oxygen and sodium bicarbonate. A number of congenital anomalies (talipes equinovarus, low set ears, facial asymmetry) were present. A 6·3-g liver specimen was obtained 10 hr postmortem and treated as above.

Rat tissue was obtained from animals of the Sprague–Dawley strain, sacrificed by decapitation. Rat livers were perfused with ice cold 1·15%KCl.

Preparation of fractions. The human liver was chopped finely with a microtome blade (not necessary with rat liver) and homogenized with 20 strokes in a smooth-walled glass homogenizer with teflon pestle (Kontes Glass Co., clearance 0·002 mm), in 5 vol. of buffered sucrose solution (0·2 M sucrose in 0·03 M Na-K phosphate buffer, pH 7·5, containing 0·001 M tetrasodium EDTA). The homogenate was centrifuged at 9000 *g* (average) for 25 min at 4°. The resulting pellet was resuspended and again sedimented. The original and second supernatant were pooled and centrifuged at 105,000 *g* for 60 min to yield a microsomal pellet. This pellet was resuspended in 4 ml of buffered sucrose solution, and the microsomes were resedimented. The resulting pellet was washed successively with water and 1·15% KCl by resuspending and sedimenting.

Solubilization and isolation. The washed microsomes were treated with crystalline bovine pancreatic trypsin (10,000 BAEE units per mg (Sigma Chemical Co.), 1 mg per 100 mg protein, for 30 min with stirring at 4°. The suspension was then centrifuged for 120 min at 105,000 *g*, and the supernatant was removed. The pellet was resuspended and resedimented, and the resulting supernatants were pooled.

The solubilized enzyme solution was concentrated by pressure dialysis, placed on a 2·5 × 45 cm column of Sephadex G-100 and eluted with 0·03 M Na phosphate buffer, containing 0·001 M EDTA, pH 7·5. Pooled fractions having a specific activity greater than 1 μmole of substrate reduced per min per mg were placed on a 1 × 10 cm column of DEAE-cellulose (Whatman Column Chromedia

* Supported in part by USPHS Grants HD-03063 and HD-02147

† Presented in part at the 39th Annual Meeting of the Society for Pediatric Research, Atlantic City N. J., May 2-3, 1969.

DE-52) and eluted with a continuous ionic gradient made from 125 ml each of 0·1 and 0·4 M Na phosphate buffer, pH 6·8.

Assay of reductase activity

NADPH Cytochrome c *reductase.* The method was that of Williams and Kamin,[2] using 1 μmole KCN, 34 mμmoles of cytochrome *c* (Type III from horse heart, Sigma Chemical Co.), and enzyme in 0·05 M Na-K phosphate buffer, pH 8·2, at 21°. The reaction was started by addition of 0·1 μmole of NADPH in a final volume of 1·0 ml. Increases in the optical density at 550 mμ were recorded at 3-second intervals for 3 min on a Gilford model 2000 recording spectrophotometer. Nonenzymatic reduction was negligible. Specific activity was calculated by the use of a molar extinction ratio for reduced minus oxidized cytochrome *c* of $18{\cdot}7 \times 10^6$ cm^2 mole^{-1}.[3]

Cytochrome b5 and P-450. The method was essentially that of Dallner.[4] Microsomal suspensions were placed in the cuvettes of a double-beam spectrophotometer (Beckman DB-G). A few mg of sodium dithionite ($Na_2S_2O_4$) were added to the sample cuvette and it was capped. The instrument was balanced at 500 mμ, and readings were taken at the peak and trough absorbance for b5 determinations. The cuvettes were then reversed. Carbon monoxide was bubbled through the sample for 30 sec, a

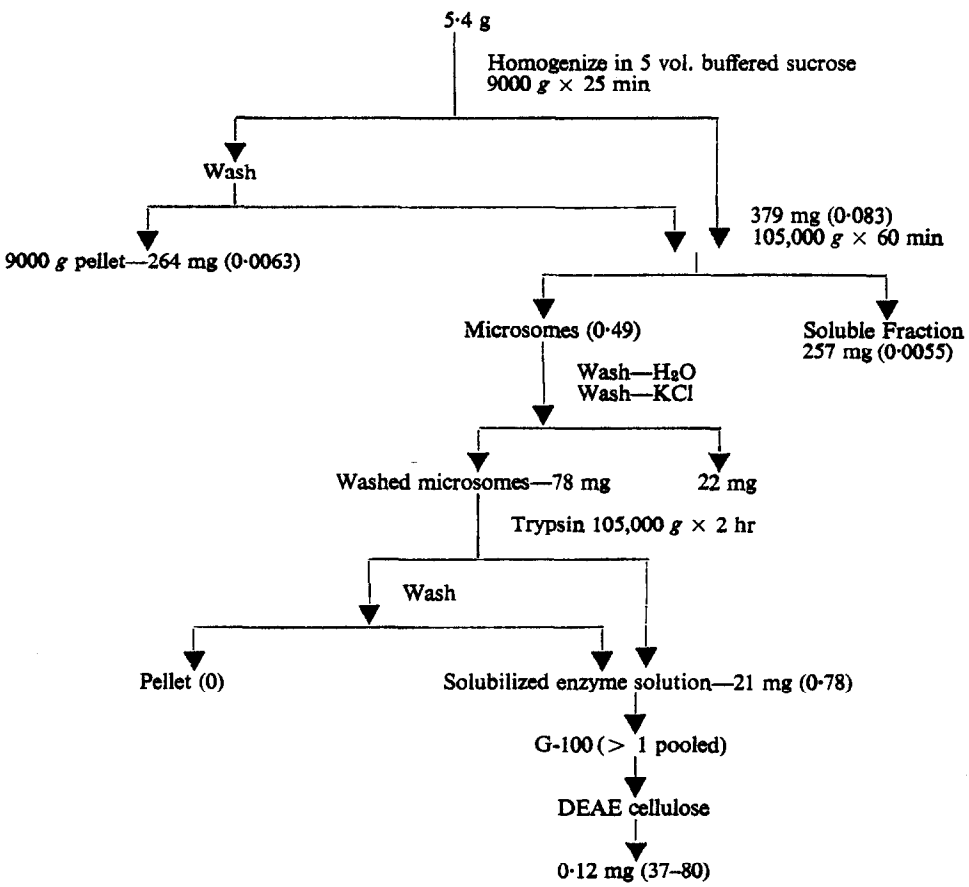

FIG. 1. Flow-sheet for isolation of NADPH cytochrome *c* reductase from human liver (specimen 1). Values in parenthesis are specific activities in μmoles cytochrome *c* reduced per min per mg protein.

few mg of dithionite added, carbon monoxide again added, and the cuvettes capped. The instrument was balanced at 490 mμ and readings made at 450 mμ for P-450 content.

Protein. The method of Lowry *et al.*[5] was used with bovine serum albumin (BSA) as the standard.

RESULTS AND DISCUSSION

Enzymatic activity was found largely in the 9000 *g* supernatant and resultant microsomal fractions (Fig. 1.) The yield of washed microsomal protein was about 14 mg per g wet weight of liver for both specimens, which is comparable to the usual yield from adult non-fasted rats. Exposure to trypsin for 30 min effected solubilization of about 25% of the microsomal protein and virtually all the enzymatic activity. Solubilization was defined as non sedimentation after centrifugation at 105,000 *g* for 2 hr.

Sephadex column chromatography. The solubilized enzyme of both species was retarded in Sephadex G-100 and eluted after the initial protein peak (Fig. 2). The elution volume was determined by use of

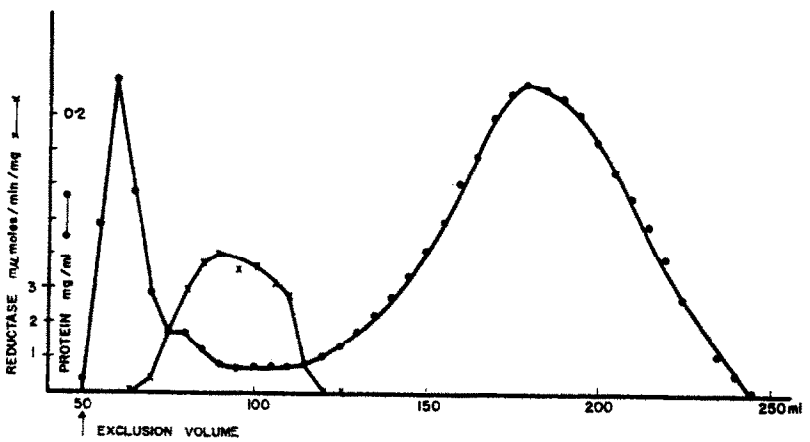

FIG. 2. Elution curve of protein and reductase activity from Sephadex G-100 for specimen 1. A 2·5 × 45 cm column was employed with 0·03 M sodium phosphate buffer (pH 7·5) containing 0.001 M tetrasodium EDTA used for equilibration and elution. The flow rate was 12 ml/hr, and fraction volume 1·9 ml. Blue Dextran 2000 was used for determination of the exclusion volume. All operations were carried out in a cold room at 4°.

Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.). With specimen 2 (premature), the peak reductase activity was eluted at 17·7 for the enzyme from human liver and 17·8 ml for the rat liver enzyme.

DEAE cellulose column chromatography. The enzyme was adsorbed onto DEAE-cellulose at pH 7·6 in a low ionic strength medium. Elution was accomplished in a linear gradient of Na phosphate buffer (pH 6·8) at a molarity of about 0·15 (Fig. 3).

The specific activities (μmoles of cytochrome *c* reduced per min per mg) of the first 2 active fractions from specimen 1 from the DEAE column were 37 and 53. The specific activity of the next fraction was 80, but because of the low protein concentration (1 μg/ml), the value must be considered only an estimate. Assuming a mean value of 50, these fractions were about 100X more active than the microsomes and represent a purification of about 600-fold over that of the 9000 *g* supernatant. The protein concentrations of all the DEAE fractions from specimen 2 were too low to permit accurate calculations of specific activities. However, the peak values were about 6 μmoles/min/mg, considerably lower than that found in the fractions from the full-term infant liver.

The values found with specimen 1 are higher than those found in the rat. The highest specific activity reported for the purified rat enzyme is 20 μmoles/min/mg.[6] In 8 isolations from rat liver, we found a specific activity of 14·4 ± 0·9 (S.E.M.). Rats pretreated with phenobarbital had significantly

higher values (25·6 ± 1·6, N = 5). Similarly, values for the non-solubilized enzyme (9000 *g* supernatants and microsomes) were 25–50 per cent lower (depending upon the age of the rats) than those noted for the full-term infant liver.

Kinetic studies

Kinetic studies were performed on DEAE fractions from human liver. Concentrations of NADPH varied from 2×10^{-4} to 1×10^{-6}M, in the presence of an excess of cytochrome *c*. The reciprocal

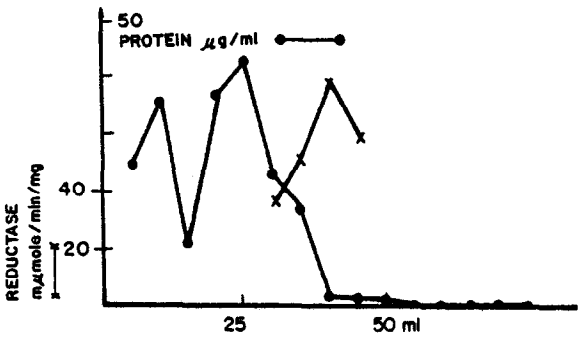

FIG. 3. Elution from DEAE-cellulose of active fractions from Sephadex G-100 column of specimen 1. Fractions with a specific activity greater than 1 μmole/min/mg were pooled and placed on a 1 × 10 cm column of DEAE cellulose (Whatman Chromedia DE-52) equilibrated with 0·03 M sodium phosphate buffer containing 0·001 M tetrasodium EDTA. The column was washed several times with 0·1 M sodium phosphate buffer (pH 6·8). For elution, a gradient was prepared by use of 125 ml each of 0·1 and 0·4 M sodium phosphate buffer (pH 6·8) in a gradient elution apparatus (Kontes Glass Co.).

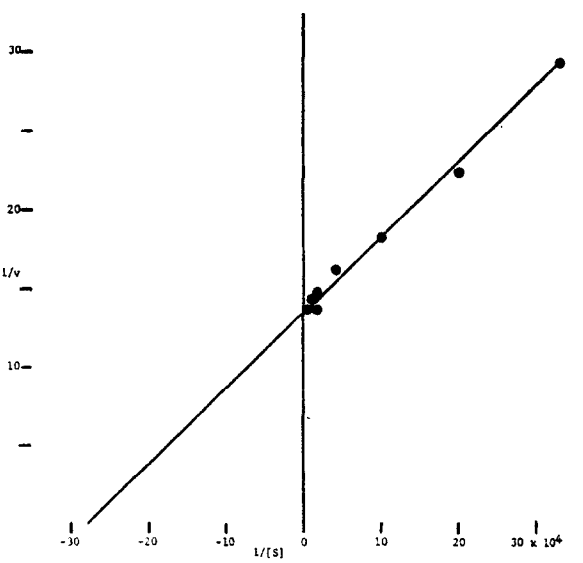

FIG. 4. Lineweaver–Burk plot of kinetic studies of human NADPH cytochrome *c* reductase. The enzyme (0·55 μg protein per cuvette) was obtained after DEAE chromatography and had a specific activity of 3·6 μmoles/min/mg. Eleven concentrations of NADPH were used, ranging from 1 to 200 μl of a 1 μmole/ml solution. Cytochrome c was present in excess. The graph is a double reciprocal plot of Δ OD 550 in 2 min vs. [NADPH].

plot of Lineweaver–Burke yielded a K_m value of $3{\cdot}6 \times 10^{-6}$M (Fig. 4). This was equal to that calculated for cytochrome *c* reductase isolated from rat liver. Phillips and Langdon[6] found a K_m of 1×10^{-6} M for the purified rat enzyme.

Molecular weight and pH optima: An estimate of molecular weight was made by determining the elution volume from columns of Sephadex G-100 and G-200, calibrated with proteins of known molecular weight. The rat reductase was eluted from both gels in the same volume as BSA (average molecular weight, 67,000). Typical data are illustrated in Fig. 5. This value is compatible with that of

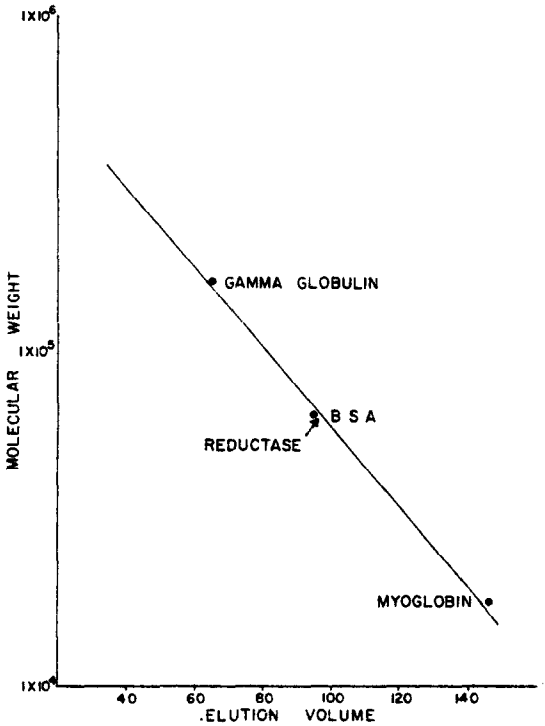

FIG. 5. Estimation of molecular weight by gel filtration. The elution volume of proteins of known molecular weight (Mann Research Laboratories) relative to that of Blue Dextran 2000 was determined on columns of Sephadex G-100 and G-200. This figure illustrates a typical experiment. NADPH cytochrome *c* reductase consistently eluted immediately before or with BSA (average molecular weight, 67,000).

Phillips and Langdon[6] who, on the basis of sedimentation and diffusion coefficients, calculated a molecular weight of 57,700 for impure reductase preparations (species not indicated). Their diffusion coefficient was almost identical to that of BSA (1·04). Likewise, by sedimentation velocity, Williams and Kamin[2] calculated the molecular weight of a highly purified (beef or pork) reductase to be approximately 68,000. However, in a later report,[7] a minimum molecular weight of 40–60,000 was found, based on Kjeldahl and amino acid analyses.

The pH optimum of human enzyme lay between pH 7·9 and 8·5 and was equivalent to the pH optimum of rat enzyme.

Microsomal cytochromes. The microsomal suspension from specimen 1, upon reduction with sodium dithionite, exhibited a peak at 426–429 mμ with a trough at 409 mμ. A similar spectrum was

found with microsomes from specimen 2, except that the peak was at 432 mμ (Fig. 6). With rat microsomal suspensions, the peak after reduction was consistently at 423–426 mμ with the trough at 409 mμ.

After reduction and addition of carbon monoxide, a small peak was noted at 450 mμ only with specimen 1. Its magnitude was not increased when a more concentrated suspension was employed, making its identification as cytochrome P-450 doubtful. After carbon monoxide, microsomal suspensions from both specimens exhibited a trough at 430 mμ and a peak at 417–420 mμ (Fig. 6). The 420 mμ peak could represent hemoglobin contamination or the inactivated form of cytochrome P-450, namely P-420.[8]

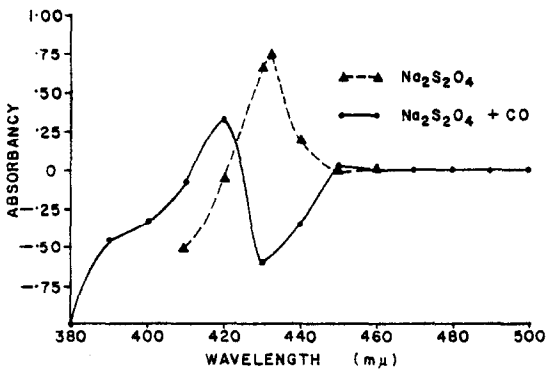

FIG. 6. Absorbance spectrum of human microsomes (specimen 2) after reduction with sodium dithionite and addition of carbon monoxide. Microsomes were sedimented from the 9000 *g* supernatant, washed with H_2O and 1·15% KCl and resuspended in buffered sucrose solution. Each cuvette contained 1 ml of a 0·73 mg/ml suspension.

Physiologic significance. The marked similarity of NADPH cytochrome *c* reductase, isolated from human and rat liver, suggests that data accumulated from animal studies as to subcellular localization, characteristics and regulation may be applicable to man. Horecker in 1950[9] first isolated this enzyme from an acetone powder of porcine liver, and subsequently it has been solubilized by various workers.[2,6,10–14] Studies indicated that this enzyme was localized in the endoplasmic reticulum,[6,15,16] and the function of it is not related to cytochrome *c*, which is confined to the mitochondria. Cytochrome *c* is utilized simply as a convenient electron acceptor in the assay of its activity. Physiologically, the enzyme participates in hepatic mixed-function oxidations of a large number of drugs, chemicals and endogenous substrates, such as adrenal and gonadal steroids, fatty acids, thyroxine, etc. The activity[17,18] and amount[18] of enzyme in the liver of experimental animals are subject to control by environmental, hormonal, genetic and nutritional factors.[1] Amount and activity are particularly sensitive to exposure to a number of drugs, polycyclic hydrocarbons and chlorinated insecticides. For these reasons, it seems possible that NADPH cytochrome *c* oxidoreductase may play a role in regulating the rate of the mixed-function oxidations.

Developmental aspects. Our data indicate that NADPH cytochrome *c* reductase is present in human liver as early as the 33rd week of gestation. Since this premature infant was severely depressed and hypoxic and died within a few hours of birth, it is unlikely that postnatal synthesis or activation account for its presence. In the rat, Lang[19] found reductase activity in whole liver homogenates at 12 days of gestation. Dallner *et al.*[20] also detected activity in hepatic microsomes 3 days before delivery. We have found that the activity in hepatic 9000 *g* supernatants from rats 4 days prior to delivery equals that on the day of birth.* Moreover, reductase activity was present in whole homogenates of chick embryo liver as early as 6 days of incubation.† These data suggest that NADPH cytochrome *c* reductase is present and presumably active *in utero* in several mammalian species.

*† L. F. Soyka, unpublished observations.

Note added in proof—Alvares and coworkers [*Clin. Pharm. Ther.* **10**, 655 (1959)] have recently demonstrated the presence of cytochromes P-450 and b5 in human liver microsomes from three adults.

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REFERENCES

1. J. R. GILLETTE, *Adv. Pharmac.* **4**, 219 (1966).
2. C. H. WILLIAMS, JR. and H. KAMIN, *J. biol. Chem.* **237**, 587 (1962).
3. E. MARGOLIASH and N. FROHWIRT, *Biochem. J.* **71**, 570 (1959).
4. G. DALLNER, *Acta path microbiol. scand.* **166**, 1 (1963).
5. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
6. A. H. PHILLIPS and R. G. LANGDON, *J. biol. Chem.* **237**, 2652 (1962).
7. B. S. S. MASTERS, H. KAMIN, Q. H. GIBSON and G. H. WILLIAMS, JR., *J. biol. Chem.* **240**, 921 (1965).
8. T. OMURA and R. SATO, *J. biol. Chem.* **239**, 2370 (1964).
9. B. L. HORECKER, *J. biol. Chem.* **183**, 593 (1950).
10. J. J. KAMM and J. R. GILLETTE, *Life Sci.* **2**, 587 (1963).
11. H. NISHIBAYASHI, T. OMURA and R. SATO, *Biochim. biophys. Acta* **67**, 520 (1963).
12. P. H. HERNANDEZ, P. MAZEL and J. R. GILLETTE, *Pharmacologist* **7**, 147 (1965).
13. H. JICK and L. SHUSTER, *J. biol. Chem.* **241**, 5366 (1966).
14. T. OMURA, P. SIEKEVITZ and G. E. PALADE, *J. biol. Chem.* **242**, 2389 (1967).
15. G. L. SOTTOCASA, B. KUYLENSTIERNE, L. ERNSTER and A. BERGSTRAND, *J. Cell Biol.* **32**, 415 (1967).
16. D. S. BEATTIE, *Biochem. biophys. Res. Commun.* **31**, 901 (1968).
17. H. REMMER and H. J. MERKER, *Science, N.Y.* **142**, 1657 (1963).
18. S. ORRENIUS and L. ERNSTER, *Biochem. biophys. Res. Commun.* **16**, 80 (1964).
19. C. A. LANG, *Biochem. J.* **95**, 365, (1965).
20. G. DALLNER, P. SIEKEVITZ and G. E. PALADE, *J. Cell. Biol.* **30**, 97 (1966).

Biochemical Pharmacology, Vol. 19, pp. 951–955. Pergamon Press. 1970. Printed in Great Britain

Comparison of liver microsome enzyme systems and barbiturate sleep times in rats caged individually or communally

(*Received* 3 *July* 1969; *accepted* 29 *August* 1969)

PROLONGED individual caging of Wistar Strain rats has been reported[1,2] to induce behavioral and physiological changes. After 4 weeks of individual caging, the rats were observed to be irritable and aggressive in comparison with community caged animals. After a period of 13 weeks, individually caged rats had larger adrenal and thyroid glands but smaller spleens and thymus glands when compared with community caged controls. This phenomenon has been referred to as isolation stress.[3,4] The toxicologic significance of isolation has been demonstrated by Balazs *et al.*[5] Rats individually caged for 13 weeks showed an increased sensitivity to the cardiotoxic effects of isoproterenol. In subsequent studies, other investigators[6] reported shorter pentobarbital sleep time in male rats which had been caged individually from 6 to 14 weeks. They suggested the possibility that an induction of liver microsomal drug metabolizing enzyme activity might account for this phenomenon. Similar effects of isolation on the response to barbiturates have been observed in the mouse.[7]