*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.

*Lt. Joseph P. Kennedy, Jr. Laboratories for Molecular Medicine,*
*Department of Pediatrics,*
*Stanford University School of Medicine,*
*Stanford, Calif.*

LESTER F. SOYKA

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

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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]

It has been reported[8] that the acute stress because of hindquarter ligation shortened hexobarbital or pentobarbital sleep time in the rat. The effects of sleep time appeared to correlate with microsomal drug meatabolizing enzyme activity since barbital, which is not extensively metabolized, elicited no difference in sleep time in stressed or non-stressed animals.[9]

Since individual caging is a common practice in toxicity studies, the examination of its effect on the activity of hepatic microsomal drug metabolizing enzyme systems appeared to be in order.

Twenty-one day-old male Sherman rats from the Wyckoff colony were used. They were placed in individual or community cages after weaning at which time they weighed approximately 50 g. The community cage was 14 × 18 × 17 in. with wire front and bottom. Up to the week 6, the rats were housed 10 per cage; thereafter the number was reduced to five. The individual cages were of similar construction with dimensions of 14 × 7 × 7 in. Purina laboratory chow and drinking water were provided *ad lib*. The rats were not handled except during experimental procedures. After 3, 6 and 12 weeks of caging, rats from the individual and community cages were examined for the duration of sleep time after the administration of various barbiturates and liver to body weight rations were determined. At the 3- and 12-week periods, hepatic microsomal enzyme activity *in vitro* was also determined.

Barbiturate sleep time was designated as the time interval, in minutes, from the loss to the regaining of the righting reflex. The barbiturates were given intraperitoneally between the hours of 10 and 11 a.m. as follows: At the end of 3 weeks of caging, pentobarbital, 30 mg/kg; hexobarbital, 125 mg/kg; barbital, 250 mg/kg. At the end of 6 and 12 weeks of caging, pentobarbital, 35 mg/kg; hexobarbital, 175 mg/kg.

Hepatic microsomal enzyme activities were determined using a 9000 *g* liver homogenate supernatant prepared from animals which were sacrificed between 10 and 11 a.m. Livers were homogenized in 3 vol. of cold 0·15 M KCl with a motor-driven Potter-Elvehjem teflon homogenizer. The liver homogenate was centrifuged at 9000 *g* in a refrigerated Sorvall centrifuge for 20 min and the supernatant fraction collected by decantation. The *N*-dimethylation enzyme activity and cortisol metabolism were

TABLE 1. BARBITURATE SLEEP TIME OF RATS INDIVIDUALLY OR COMMUNITY CAGED FOR 3 weeks

| Compund | Sleep time (min)* | |
|---|---|---|
| | Individual caging | Cmmunity caging |
| Pentobarbital (30 mg/kg) | (10) 116 ± 7·0† | (12) 201 ± 15·9 |
| | (6) 100 ± 9·8‡ | (8) 150 ± 8·1 |
| | (6) 66 ± 6·9† | (6) 147 ± 11·8 |
| Hexobarbital (125 mg/kg) | (9) 113 ± 12·3§ | (7) 170 ± 18·1 |
| Barbital (250 mg/kg) | (17) 357 ± 23·4‡ | (12) 495 ± 30·0 |

* Number in parenthesis refers to number of animals used, mean ± S.E.M.
† Significantly less than community caged ($P < 0·0025$).
‡ Significantly less than community caged ($P < 0·01$).
§ Significantly less than community caged ($P < 0·05$).

determined[10,11] and hexobarbital metabolism was measured.[12] Isoproterenol sensitivity was determined at the end of 12 weeks of caging by the subcutaneous injection of 100 mg/kg of isoproterenol hydrochloride and recording the 48-hr mortality. Statistical analysis of the data was done using the Student *t*-test.

The sleep time response to pentobarbital, hexobarbital and barbital for rats which had been caged individually or communally for 3 weeks is summarized in Table 1. The data indicate that the individually caged rats showed sleep times in response to all three barbiturates which were significantly shorter than the community caged controls. The most pronounced difference in sleep time occurred with pentobarbital.

The enzymatic activities or the 9000 *g* liver homogenate supernatants prepared from the livers of

rats which had been individually or communally caged for 3 weeks are summarized in Table 2. No significant differences were found in the enzyme activities measured or the absolute body, liver or adrenal weights of the individually and community caged animals. Calculation of liver and adrenal weights per 100 g of body weight yielded values of 4·18 g (S.E.M. ± 0·15) and 15·9 mg (S.E.M. ± 1·78) for the individually caged animals and 4·14 g (S.E.M. ± 0·11) and 17·3 mg (S.E.M. ± 0·65) for the community rats.

Upon handling, the individually caged animals were excitable and intractable by the third week of isolation, whereas the community caged rats were docile.

Pentobarbital sleep time of rats caged individually or communally for 6 weeks is given in Table 3.

TABLE 2. HEPATIC MICROSOMAL ENZYME ACTIVITY AND BODY, LIVER AND ADRENAL WEIGHTS OF RATS INDIVIDUALLY OR COMMUNITY CAGED FOR 3 WEEKS

| Type of caging | mμmoles Hexobarbital metabolized per 0·5 g of liver 30 min† | mμmoles *p*-chloroaniline formed per 10·2 g of liver 30 min‡ | Body weight | Liver weight | Adrenal weight |
|---|---|---|---|---|---|
| Individual* Caging | (5) 260 ± 43·9 | (4) 167 ± 15.5 | (7) 132·7 ± 4·67 | (7) 5·57 ± 0·33 | (7) 22·77 ± 1·05 |
| Community | (4) 238 ± 43·0 | (5) 159 ± 9·4 | (9) 131·4 ± 4·00 | (9) 5.47 ± 0·29 | (9) 22·68 ± 0·65 |

* Number in parentheses refers to the number of animals used, mean ± S.E.M.

† In a final volume of 4 ml, 2 ml of 9000 *g* supernatant was incubated for 30 min at 37° with 1·0 μmole of hexobarbital, 50 μmoles of nicotinamide, 0·5 μmole NADP, 20 μmoles of glucose 6-phosphate, 75 μmoles of $MgCl_2$, 100 μmoles of phosphate buffer, pH 7·4, and 120 Buchner units of glucose 6-phosphate dehydrogenase.

‡ In a final volume of 2 ml, 0·6 ml of 9,000 *g* supernatant was incubated for 30 min at 37° with 3 μmoles of *p*-chloro-*n*-methylaniline, 20 μmoles nicotinamide, 1·6 μmoles NAPD, 16 μmoles glucose 6-phosphate, 37·5 μmoles $MgCl_2$, 100 μmoles phosphate buffer, pH 7·4, and 120 Buchner units of glucose 6-phosphate dehydrogenase.

TABLE 3. PENTOBARBITAL AND HEXOBARBITAL SLEEP TIMES OF RATS INDIVIDUALLY OR COMMUNITY CAGED FOR 6 OR 12 WEEKS

| Compound | Dose (mg/kg) | No. of weeks caged | Sleep time (min)* Individual caging | Community caging |
|---|---|---|---|---|
| Pentobarbital | 35 | 6 | (11) 92 ± 5·1 | (11) 90 ± 5·7 |
| | | | (8) 82 ± 6·7 | (8) 85 ± 4.6 |
| | | 12 | (4) 93 ± 5·0 | (4) 84 ± 8·0 |
| | | | (6) 88 ± 7·7 | (8) 82 ± 6·4 |
| Hexobarbital | 175 | 12 | (5) 86 ± 9·8 | (5) 85 ± 9·9 |
| | | | (11) 66 ± 3·3 | (9) 62 ± 6·3 |

* Number in parentheses refer to the number of animals used, mean ± S.E.M.

No significant differences were found, however, the excitable and intractable behavior in response to handling of isolated rats was apparent.

After 12 weeks of community or individual caging, statistically significant differences in pentobarbital and hexobarbital sleep times were not observed (Table 3). The enzymatic activities of 9000 *g* liver homogenate supernatants obtained from rats caged individually or communally for 12 weeks are shown in Table 4. No significant differences were observed, nor were the liver to body weight ratios different.

Rats individually caged for 12 weeks did show an increased sensitivity to isoproterenol. After a

TABLE 4. LIVER MICROSOMAL ENZYME ACTIVITY AND LIVER TO BODY WEIGHT RATIOS OF RATS INDIVIDUALLY OR COMMUNITY CAGED FOR 12 weeks

| Type of caging | mμmoles hexobarbital metalized/0·5 g of liver per 30 min† | mμmoles of *p*-chloroaniline formed/0·066 g of liver per 20 min‡ | μg Cortisol metabolized per 0·3 g of liver per 20 min§ | Liver weight (g) per 100 g of body/weight |
|---|---|---|---|---|
| Individual* caging | (5) 241 ± 26·4 | (5) 60 ± 4·5 | (4) 104 ± 1·1 | (6) 3·1 1·15 |
| Community caging | (5) 252 ± 30·9 | (5) 66 ± 4·5 | (4) 103 ± 1·1 | (6) 3·1 0·12 |

* Number in parentheses refer to the number of animals used, mean ± S.E.M.
† Incubating conditions identical to those of Table 2.
‡ Incubation conditions identical to those of Table 2, with the exception that 0·2 ml of supernatant used and an incubation time of 20 min.
§ In a final volume of 5 ml, 1 ml of 9000 *g* supernatant was incubated for 20 min at 37° with 500 μg of 4-$^{14}$C-cortisol (0·5 μc), 2 μmoles of NADP, 40 μmoles of glucose 6-phosphate, 50 μmoles of nicotinamide, 75 μmoles of $MgCl_2$ and 200 μmoles of phosphate buffer, pH 7·4.

single subcutaneous injection of 100 mg/kg of isoproterenol hydrochloride, five of six individually caged animals died, but no deaths supervened in six rats which had been communally caged for 12 weeks. The behavioral changes noted in response to handling at 3 weeks were still apparent at this time. Upon physical examination of the animals after 3, 6, or 12 weeks of caging, no apparent differences were noted in the incidence of respiratory disease.

We tried to determine the onset of the isolation-induced decreased sensitivity to barbiturates. Weaned rats were isolated for periods of 2, 4, 7 and 17 days and examined for sleep time response to pentobarbital. Only those rats subjected to 17 days of isolation exhibited a singificantly shorter pentobarbital sleep time in comparison to community caged animals.

Our data confirmed the observations of previous authors that individual caging of rats resulted in observable behavioral changes and pharmacological responses. The rats individually caged for 3 weeks showed diminished sleep time responses to barbiturates. One possible explanation for this phenomenon could be an increase in the specific activity of the hepatic microsomal drug metablizing enzyme system, and/or an increase in the liver to body weight ratios of the individually caged animals. However, our findings indicated that these factors were not responsible for the observed phenomenon. The individually caged animals showed a shortened sleep time in response to barbital, a barbiturate which is not extensively metabolized. This consistent with the interpretation that an alteration in drug metabolizing activity was not responsible for the shortened barbiturate sleep time. Liver to body weight ratios were identical for community and individually caged rats. An alternate explanation could involve an alteration in central nervous system activity resulting in decreased barbiturate sensitivity or a change in the pharmacokinetic parameters in the central nervous system.

The data of our experiment indicated that individually caged rats can partially adapt to their environment. This was manifested by the fact that after 6 weeks of caging, there was no longer any difference between community and individually caged animals with respect to barbiturates sleep times. However, the behavioral changes observed at 3 weeks still persisted after 6 and 12 weeks of individualized caging. Therefore, it appears that the altered susceptibility to barbiturates is independent of the behavioral changes involved by individual caging. In addition, increased isoproterenol toxicity was observed after 12 weeks of individual caging, at which time differences in barbiturate sleep times were no longer demonstrable.

*Toxicology Research Section*
*Lederle Laboratories,*
*American Cyanamid Co.,*
*Pearl River, N.Y., U.S.A.*

WALLACE DAIRMAN*
TIBOR BALAZS†

* Present address: Roche Institute of Molecular Biology, Nutley, N.J. 07110.
† Present address: Research and Development Division, Smith, Kline & French Laboratorie Philadelphia, Pa. 19101.

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# Inhibition of 3′5′-cyclic-AMP phosphodiesterase by some platelet aggregation inhibitors

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DURING the screening of compounds as inhibitors of 3′5′-cyclic-AMP phosphodiesterase, we chose to investigate the action of 2-chloroadenosine in view of its structural relationship to 3′5′-cyclic-AMP. 2-Chloroadenosine had previously been reported to be an inhibitor of induced platelet[1] aggregation and a potent vasodilator.[2]

We found 2-chloroadenosine to be a potent inhibitor of beef heart phosphodiesterase and from the literature we found that several known inhibitors of this enzyme have also been independently reported to be inhibitors of induced platelet aggregation. These include methyl xanthines[3, 4], phenothiazines,[5–7] intensain[8, 9] and reserpine.[5–7]

We wish to report here the action of several inhibitors of platelet aggregation on 3′5′-cyclic-AMP phosphodiesterase. The enzyme was prepared from beef heart by the method of Butcher and Sutherland[4] to the end of step 2. Incubations were carried out at 30° for 10 min in an incubation mixture containing test compound, 200 μmoles Tris-buffer pH 7·5, 2 μmoles $MgCl_2$, Crotalus adamanteus venom (0·1 mg protein) and phosphodiesterase (0·5 mg protein) in a total volume of 0·85 ml. The reaction was initiated by addition of 0·1 ml 10 mm 3′5′-cyclic-AMP and terminated with 0·05 ml 100% trichloracetic acid. Compounds which interfered with the phosphate analysis were removed by charcoal treatment. The inorganic phosphate formed from 5′-AMP by nucleotidase activity of the venom was measured by the method of Fisk and Subba-Row.[10]

Table 1 shows the 50 per cent inhibition levels obtained from graphs of at least four levels of test compounds. References describing their activity as inhibitors of platelet aggregation are also indicated.

All inhibitors were also tested for their activity on the 5′-nucleotidase of Crotalus adamanteus venom. Only 2-chloroadenosine produced any inhibition. The specific activity of the 5′-nucleotidase was much greater than that of the phosphodiesterase in the assay system used for screening and under these conditions the inhibition of 5′-nucleotidase by 2-chloroadenosine was not significant.

It is interesting to note that the rank order of potency against phosphodiesterase for 2-chloroadenosine, adenosine and adenosine N-1 oxide is the same as that observed by Born *et al.*[2] for inhibition of induced platelet aggregation. The possible significance of 3′5′-cyclic-AMP in the inhibition of