(7) K. M. Piafsky, *Clin. Pharmacokinet.*, **5**, 246 (1980).
(8) E. Pike, B. Skuterud, P. Kierulf, D. Fremstad, S. M. Abdel Sayed, and P. K. M. Lunde, *Clin. Pharmacokinet.*, **6**, 367 (1981).
(9) P. J. McNamara, R. L. Slaughter, J. A. Pieper, M. G. Wyman, and D. Lalka, *An. Analg.*, **60**, 295 (1981).
(10) D. Fremstad, K. Bergerud, J. F. W. Haffner, and P. K. M. Lunde, *Eur. J. Clin. Pharmacol.*, **10**, 411 (1976).
(11) B. G. Johansson, C. O. Kindmark, E. Y. Trell, and F. A. Wollheim, *Scand. J. Clin. Lab. Invest.*, **291** (suppl) 124, 117 (1972).
(12) S. J. Smith, G. Bos, M. R. Esseveld, H. G. Van Eijk, and J. Gerbrandy, *Clin. Chim. Acta.*, **81**, 75 (1977).
(13) G. Mancini, A. O. Carbonara, and J. F. Hereman, *Immunochemistry*, **2**, 235 (1965).
(14) R. L. G. Norris, J. T. Ahokas, and P. J. Ravenscroft, *J. Pharmacol. Meth.* **7**, 143 (1982).

ACKNOWLEDGMENTS

Financial support from the Lions Kidney and Medical Research Foundation is gratefully acknowledged. This work has been undertaken in partial fulfillment of the requirements of a Master of Applied Science Degree at the Queensland Institute of Technology by R.L.G.N.

The authors thank Dr. J. Cameron of Hoechst-Roussel Pty. Ltd., Castle Hill, New South Wales, Australia, for assistance with equipment and provision of disopyramide, both ^{14}C-labeled and unlabeled.

In Vitro and *In Vivo* Assessment of Hepatic and Extrahepatic Metabolism of Diazepam in the Rat

YASUTAKA IGARI, YUICHI SUGIYAMA, YASUFUMI SAWADA, TATSUJI IGA ˣ, and MANABU HANANO

Received December 30, 1982, from the *Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.* Accepted for publication April 27, 1983.

Abstract □ Since diazepam is metabolized by many organs in the rat, the microsomal fractions of the liver, kidney, and lung from male Wistar rats were assayed for NADPH-dependent metabolism of diazepam and enzymatic parameters. The predicted extraction ratios were obtained from this *in vitro* experimental system. The organ clearances of the liver, kidney, and lung were then calculated for the determination of the relative contribution of each eliminating organ to the total body clearance (CL_{tot}) of diazepam in the rat. The liver was the most effective eliminating organ, followed by the kidney and the lung, in that order. The hepatic extraction ratio of diazepam was determined *in vivo* after portal and femoral vein administrations of diazepam. The validity of the *in vitro* experimental system for the liver was demonstrated by a good agreement between the calculated hepatic extraction ratio of diazepam from *in vitro* enzymatic parameters (0.616) and that derived *in vivo* (0.648). However, the sum of organ clearances of the liver, kidney, and lung did not account for CL_{tot} of diazepam in the rat, suggesting the possible contribution of the metabolism in the other organs or tissues, or an underestimation of the pulmonary and renal metabolism.

Keyphrases □ Diazepam -metabolism, hepatic and extrahepatic, *in vitro* assessment, comparison with *in vivo* parameters in the rat □ Metabolism—diazepam in the rat, hepatic and extrahepatic, *in vitro* assessment compared with *in vivo* parameters

Diazepam is a clinically important minor tranquilizer which is extensively metabolized by all species studied. However, there are pronounced interspecies differences in the total plasma clearance (CL_{tot}) per unit of body weight that presumably reflect differences in organ intrinsic clearances, blood flow, plasma binding, and the relative contributions of eliminating organs (1). In humans the total clearance is small [26.6 ± 4.1 mL/min (mean ± *SD*; $n = 5$)] (2) and it is assumed to reflect only hepatic metabolism. In contrast, in the rat the total clearance exceeds the liver blood flow, suggesting the involvement of extrahepatic elimination. The relative contribution of different organs to the overall metabolism of a drug may be assessed *in vivo*, but this is often difficult. Instead, comparison is often made between the *in vitro* enzymatic characteristics of an organ. This approach has recently received new impetus, since it has been shown to be readily extrapolated to the whole organ.

In the present study, the *in vitro* experimental system using microsomal fractions was employed to evaluate enzymatic parameters of the hepatic and extrahepatic diazepam elimination systems in the rat. The kidney and the lung were selected as the representative extrahepatic eliminating organs, since these two organs are reported to contain considerable amounts of cytochrome P_{450} (3). Enzymatic parameters of the liver, kidney, and lung were extrapolated to those of the whole organs. Using these parameters, the relative contributions of individual disposing organs were evaluated and, in the case of the liver, compared with an *in vivo* assessment.

EXPERIMENTAL

Materials—Male Wistar rats[1], weighing 250–270 g, on a normal laboratory diet were used throughout. [2-^{14}C]Diazepam (40–60 mCi/mmoL)[2] was used. Unlabeled diazepam[3], demethyldiazepam[4], oxazepam[4], and 3-hydroxydiazepam[4] were gifts from commerical sources. NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained commercially[5]. All other chemicals were reagent grade.

Assay of Radioactivity—[2-^{14}C]Diazepam was diluted with unlabeled drug prior to the studies. The determination of carbon-14 and the assay of labeled drug and its metabolites were described elsewhere (4). The radiochemical purity was >98–99% for [2-^{14}C]diazepam by TLC.

Tissue Preparation and Drug Metabolism Study—Each of the 3–5 separate determinations were conducted on a separate pool of organs, each pool containing organs from 2–10 individual animals. After overnight fasting, the rats were exsanguinated *via* a carotid artery and perfused *in situ* with cold physiological saline *via* the venous trunk just inferior to the renal veins and *via* the portal vein until the effusate became colorless. The liver, kidney, and lung were excised, blotted, weighed, and pooled to obtain 10–11 g of each organ. The preparation of microsomal fractions was described elsewhere (5).

Incubation mixtures for the mixed function oxidation consisted of microsomal protein, 50 mM Tris-HCl buffer (pH 7.4), the NADPH-generating system (1 mM NADP, 10 mM glucose-6-phosphate, 2 U of glucose-6-phosphate dehydrogenase, and 5 mM magnesium chloride), and various amounts of [^{14}C]diazepam diluted with unlabeled diazepam to a final volume of 1.0 mL. The NADPH-generating system, microsomal protein solution, and [^{14}C]diazepam solution were preincubated separately for 2 min at 37°C. The

[1] Nihon Seibutsu Zairyo, Tokyo, Japan.
[2] Radiochemical Center, Amersham, England.
[3] Yamanouchi Pharm. Co., Tokyo, Japan.
[4] Banyu Pharm. Co., Tokyo, Japan.
[5] Boehringer Mannheim Yamanouchi Co., Tokyo, Japan.

0022-3549/84/0600-0826$01.00/

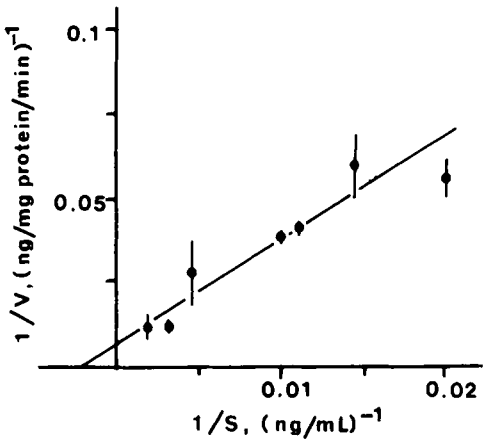

Figure 1—*Lineweaver-Burk plot of metabolism of diazepam by liver microsomes. Each point represents the mean of 3–5 separate determinations with standard error. The solid line represents the best fit to these data by a least-squares method (10).*

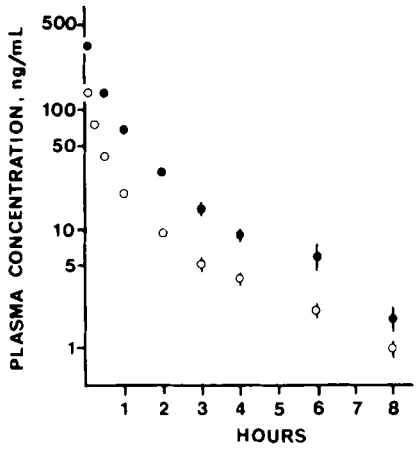

Figure 2—*Concentration-time profiles of diazepam after an injection of diazepam (1.2 mg/kg) intravenously (●) or intraportally (○) in rats (250–270 g). Each datum point represents the mean of 3–5 separate determinations ± SE. The data points without the vertical bars include the* SE *within the symbol mark.*

reaction was initiated by mixing these three solutions. The final concentration of diazepam ranged between 0.05–0.5 μg/mL. The reaction for 2 min at 37°C was stopped by transferring 0.5 mL of the incubation mixture into a stoppered glass tube containing 6 mL of cold benzene and subsequent mixing on an automatic mixer. After extraction and evaporation in boiling water (6), the amount of diazepam metabolized during 2 min was determined as previously described (4).

There are two pathways for diazepam metabolism, and the metabolites are further metabolized (7–9). Therefore, the rate of diazepam metabolized instead of the product formed was used for the enzyme assays. The spontaneous degradation of diazepam in the incubation mixture without the NADPH-generating system during incubation at 37°C did not occur. The recoveries from the extraction were determined and used for the correction. The enzymatic parameters (K_m and V_{max}) of the rat liver, kidney, and lung for diazepam metabolism were determined by Lineweaver-Burk analysis of the data of microsomal metabolism by a least-squares method (data were weighted with reciprocals of their variance) (10) using a digital computer[6].

Animal Experiments—Under light ether anesthesia, the femoral vein and artery were cannulated with polyethylene tubing[7] for intravenous drug administration and blood sampling, respectively. For intraportal venous administration, the portal vein was cannulated with polyethylene tubing[7] attached to a 27-gauge injection needle for drug administration, and the femoral artery was cannulated with polyethylene tubing[7] for blood sampling. Rats were then restrained in a Bollman cage. After 2 h of recovery from anesthesia, [^{14}C]diazepam diluted with unlabeled diazepam (1.2 mg/kg) in 0.25 mL of polyethylene glycol 300 (11) was injected, followed by 0.5 mL of physiological saline. At various times postinjection, a blood sample (0.3 mL) was withdrawn *via* a femoral artery using the cannula into a heparinized polyethylene tube followed by subsequent centrifugation[8]. Plasma samples were treated as described above.

Calculation of the Metabolic Capacity of each Disposing Organ—The enzymatic parameters (K_m and V_{max}) determined in the *in vitro* experimental system using microsomal fractions were extrapolated to those of whole organs as described previously (11–13), and the following calculations were performed. The intrinsic clearance (CL_{int}), related to the enzymatic parameters, is given as follows, assuming a single-enzyme system:

$$CL_{int} = V_{max}/(K_m + C_u) \quad \text{(Eq. 1)}$$

where C_u is the concentration of unbound drug in the water surrounding the enzyme. The CL_{int} is reduced to the following under nonsaturable conditions, *i.e.*, $K_m \gg C_u$:

$$CL_{int} \simeq V_{max}/K_m \quad \text{(Eq. 2)}$$

Assuming rapid equilibrium between organs and the effluent venous blood, the whole organ clearance (CL) of the liver or kidney is given as follows (14, 15):

$$CL = \frac{Qf_BCL_{int}}{Q + f_BCL_{int}} \quad \text{(Eq. 3)}$$

where f_B is the fraction of the drug in blood that is unbound, the Q is the blood flow supplied to the organ. The whole organ clearance of lung (CL_{Lu}) is given as follows, assuming rapid equilibrium (16):

$$CL_{Lu} \simeq f_BCL_{int(Lu)} \quad \text{(Eq. 4)}$$

The capacity of an eliminating organ to metabolize the drug can also be expressed as the extraction ratio (E). The extraction ratio of liver (E_H) derived from *in vitro* or *in vivo* experiments (17) is as follows:

$$E_H = \frac{f_BCL_{int(H)}}{Q_H + f_BCL_{int(H)}} \quad \text{(Eq. 5)}$$

$$E_H = 1 - \frac{AUC_{ip}/Dose}{AUC_{iv}/Dose} \quad \text{(Eq. 6)}$$

where AUC_{ip} and AUC_{iv} are the area under concentration-time curves of diazepam from zero to the infinity after portal venous and intravenous administration, respectively.

RESULTS

Drug Metabolism Study—The Lineweaver-Burk plot of the disappearance rates of diazepam *versus* the initial diazepam concentrations using liver microsome is shown in Fig. 1. The enzymatic parameters (K_m and V_{max}) derived from *in vitro* experiments and those extrapolated to the whole organs for the liver, kidney, and lung are listed in Table I. Since the unbound diazepam concentration in plasma was sufficiently small compared with the values of K_m of the three disposing organs (see footnote to Table I), Eq. 2 was used to

Table I—Enzymatic Parameters for Metabolism of Diazepam by Microsomes of Rat Liver, Kidney, and Lung

Organ	K_m[a], μg/mL	V_{max}, μg/min/g of Microsomal Protein	V_{max}[b], μg/min/g of Organ	CL_{int}[c], mL/min/g of Organ	CL_{org}[d], mL/min/0.25-kg rat
Liver	0.409	125.0	6.08	14.87	8.95
Kidney	0.198	18.5	0.33	1.68	0.22
Lung	0.343	27.8	0.26	0.76	0.10

[a] Michaelis–Menten constant for the unbound drug. Nonspecific binding of the drug to each microsomal suspension was low, determined by an equilibrium dialysis technique at 37°C in the absence of an NADPH-generating system (5), and was used for the correction. [b] Grams of microsomal protein per gram of liver was calculated as reported by Igari *et al.* (5) according to the methods of Lin *et al.* (12) and Smith and Bend (13), and that of kidney or lung was calculated according to the methods of Lin *et al.* (12) and Smith and Bend (13) by using the data rreported by Litterst *et al.* (3). [c] The fraction of unbound drug in plasma and the blood-to-plasma concentration ratio were 0.14 and 1.04, respectively, taken from the report by Igari *et al.* (4). The free diazepam concentrations in the systemic blood postinjection of 1.2 mg/kg iv of diazepam into the rat were below ~60 ng/mL and adequately smaller than K_m values over time, since diazepam concentration in the systemic blood declined very rapidly with time. Therefore, since the nonsaturable condition approximately holds, CL_{int} can be described by Eq. 2. [d] The values of CL_{org} of liver and kidney were calculated by Eq. 3, and that of the lung was calculated by Eq. 4. The blood flow rates of the liver and kidney were 14.7 mL/min/0.25-kg rat and 11.4 mL/min/0.25-kg rat, respectively, taken from the reports of Sasaki and Wagner (32), Dedrick *et al.* (33), and Lutz *et al.* (34).

[6] HITACHI M200-H; Hitachi, Tokyo, Japan.
[7] PE-50; Clay Adams, N.J.
[8] Beckman Instruments, Fullerton, Calif.

calculate CL_{int}. The calculated CL_{int} and CL are also listed in Table I. The liver is, as expected, the most potent contributor to total clearance, followed by the kidney and the lung in that order.

Evaluation of the Hepatic Extraction Ratio of Diazepam—After intravenous or intraportal administration of 1.2 mg/kg of [^{14}C]diazepam into the rat, the concentrations of diazepam in plasma declined biexponentially, as shown in Fig. 2, and could be fitted using a digital computer program (10). The AUC values are 15.4 ± 0.6 μg/mL·min (mean ± *SE*) following intravenous administration and 5.4 ± 0.7 μg/mL·min (mean ± *SE*) following intraportal administration, respectively. The extraction ratio of the liver, calculated according to Eq. 6 using the mean AUC values of postintravenous and intraportal injection, was 0.648, while that calculated according to Eq. 5 using enzymatic parameters derived from the *in vitro* experiments was 0.616 (for the hepatic blood flow rate and the blood free fraction, refer to the footnote to Table I).

The observed total clearance, calculated using CL_{tot} = Dose/AUC_{iv}, was 19.6 mL/min/0.25-kg rat.

DISCUSSION

Drug metabolism systems exist not only in the liver, but also in the kidney, intestine, lung, skin, brain, testes, placenta, adrenals, spleen, and plasma (11, 16–22); each enzyme system may have different enzymatic parameters. Since total clearance cannot be ascribed to only the liver for drugs which are metabolized by multiple organs, an accurate assessment of metabolic capacities of individual disposing organs would be indispensable in describing the drug kinetics in plasma and various tissues or organs using the physiologically based pharmacokinetic model developed by Dedrick *et al.* (23, 24). Some authors have dealt with the drug metabolism study using experimental systems prepared from the liver and the other organs. For example, Lake *et al.* (25) and Litterst *et al.* (3) reported the hepatic and extrahepatic *in vitro* metabolism of some drugs using subcellular fractions. Hayes and Brendel (26) and Devereux *et al.* (27) reported the metabolism of drugs using isolated liver and lung cell preparations, respectively. However, to incorporate the *in vitro* data into a physiologically based pharmacokinetic model, an appropriate scaling is needed to extrapolate the *in vitro* data to the *in vivo* situation, and comparison should be made between the capacity of the whole organ predicted from the *in vitro* data and observed to validate the scaling. On this point, Rane *et al.* (11) have successfully predicted intrinsic clearance and hence the extraction ratio of the liver of a wide spectrum of compounds using the values of V_{max} and K_m determined from liver homogenates, compared with those observed in the isolated liver perfusion study. Furthermore, Collins *et al.* (28) and Lin *et al.* (29) also reported the *in vitro* and *in vivo* correlation of the drug metabolism using isolated liver preparations.

In the present study, the enzymatic parameters K_m and V_{max} for the microsomal oxidation of diazepam in the liver, kidney, and lung were estimated using *in vitro* enzyme assays. It was demonstrated that the kidney and lung in addition to the liver contributed to the total clearance of diazepam in the rat. As expected, the liver was the most efficient organ for diazepam metabolism, followed by the kidney and the lung in that order. This is because there were differences in the values of V_{max} among three organs, as shown in Table I. The sum of the predicted organ clearances of the liver, kidney, and lung (9.3 mL/min/0.25-kg rat; Table I), however, was still below the observed total clearance (19.6 mL/min/0.25-kg rat). Since the predicted liver organ clearance (CL_{li}) in the present study was probably an estimate, the microsomes of the kidney and lung might not be prepared with enzymatic activity matching those of the intact kidney and lung.

Recently, the extrapolation of the *in vitro* enzymatic parameters to those *in vivo* has been demonstrated also in the lung. For example, Smith and Bend (13) and Law (30) succeeded in the extrapolation using benzo[*a*]pyrene 4,5-oxide and phencyclidine as substrates, respectively, both of which are metabolized mainly by microsomal enzymes. In the case of 5-hydroxytryptamide, which is mainly metabolized by mitochondrial monoamine oxidase, the prediction of its clearance from the *in vitro* enzymatic parameters was successful for the liver, but underestimated the observed clearance in the lung (31). Our present study using diazepam showed results similar to those for 5-hydroxytryptamine (31), although the intracellular localization of the enzymes is different.

Thus, the estimation of the enzymatic parameters of the metabolism from the *in vitro* assay systems of the liver and an appropriate scaling to the whole organ V_{max} value proved to be a useful tool for the estimation of the metabolic occurrences of diazepam *in vivo*. Since diazepam is metabolized by the kidney and lung in addition to the liver, the preparation of microsomes of the kidney and lung or experimental conditions such as components and concentrations of cofactors to yield appropriate enzymatic degradation rates should be further studied to assess the extrahepatic metabolism of diazepam.

REFERENCES

(1) U. Klotz, K. H. Antonin, and P. R. Bieck, *J. Pharmacol. Exp. Ther.*, **199,** 67 (1976).

(2) U. Klotz, G. R. Avant, A. Hoyumpa, S. Schenker, and G. R. Wilkinson, *J. Clin. Invest.*, **55,** 347 (1975).

(3) C. L. Litterst, E. G. Mimnaugh, R. L. Reagan, and T. E. Gram, *Drug Metab. Dispos.*, **3,** 259 (1975).

(4) Y. Igari, Y. Sugiyama, Y. Sawada, T. Iga, and M. Hanano, *Drug Metab. Dispos.*, **10,** 676 (1982).

(5) Y. Igari, Y. Sugiyama, S. Awazu, and M. Hanano, *J. Pharmacokinet. Biopharm.*, **10,** 53 (1982).

(6) I. A. Zingales, *J. Chromatogr.*, **75,** 55 (1973).

(7) M. A. Schwartz, R. A. Koechlin, E. Postman, S. Palmer, and G. Krol, *J. Pharmacol. Exp. Ther.*, **149,** 423 (1965).

(8) J. A. F. de Silva, R. A. Koechlin, and G. Bader, *J. Pharm. Sci.*, **55,** 692 (1966).

(9) J. A. F. de Silva and G. Bader, *Fed. Proc.*, **25** (part 1), 532 (1966).

(10) T. Nakagawa, Y. Koyanagi, and H. Togawa, "SALS, a Computer Program for Statistical Analysis with Least-Squares Fitting," Library Program of the University of Tokyo Computer Center, Tokyo, Japan, 1978.

(11) A. Rane, G. R. Wilkinson, and D. G. Shand, *J. Pharmacol. Exp. Ther.*, **200,** 420 (1977).

(12) J. H. Lin, Y. Sugiyama, S. Awazu, and M. Hanano, *Biochem. Pharmacol.*, **29,** 2825 (1980).

(13) B. R. Smith and J. R. Bend, *J. Pharmacol. Exp. Ther.*, **214,** 478 (1980).

(14) M. Rowland, L. Z. Benet, and G. G. Graham, *J. Pharmacokinet. Biopharm.*, **1,** 123 (1973).

(15) G. R. Wilkinson and D. G. Shand, *Clin. Pharmacol. Ther.*, **18,** 377 (1975).

(16) L. W. Wattenberg and J. L. Leong, *J. Histochem. Cytochem.*, **10,** 412 (1962).

(17) J. R. Gillette and K. S. Pang, *Clin. Pharmacol. Ther.*, **22,** 623 (1977).

(18) L. W. Wattenberg, J. L. Leong, and P. J. Strand, *Cancer Res.*, **22,** 1120 (1962).

(19) E. Schlede and A. H. Conney, *Life. Sci.*, **9,** 1295 (1970).

(20) G. Feuer, J. C. Sosa-Lucero, G. Lumb, and G. Moddel, *Toxicol Appl. Pharmacol.*, **19,** 579 (1971).

(21) M. R. Jeuchau, *Fed. Proc.*, **31,** 48 (1972).

(22) M. Rowland, S. Riegelman, P. A. Harris, and S. D. Sholkoff, *J. Pharm. Sci.*, **61,** 379 (1972).

(23) R. L. Dedrick and K. B. Bischoff, *Chem. Eng. Prog. Symp. Ser.*, **64,** 32 (1968).

(24) R. L. Dedrick, D. D. Forrester, and D. H. W. Ho, *Biochem. Pharmacol.*, **21,** 1 (1972).

(25) B. G. Lake, R. Hopkins, J. Chakraborty, J. W. Bridges, and D. V. W. Parke, *Drug Metab. Dispos.*, **1,** 342 (1973).

(26) J. S. Hayes and K. Brendel, *Biochem. Pharmacol.*, **25,** 1495 (1976).

(27) T. R. Devereux, G. E. R. Hook, and J. R. Fouts, *Drug Metab. Dispos.*, **7,** 70 (1979).

(28) J. M. Collins, D. A. Blake, and P. G. Egner, *Drug Metab. Dispos.*, **6,** 251 (1978).

(29) J. H. Lin, M. Hayashi, S. Awazu, and M. Hanano, *J. Pharmacokinet. Biopharm.*, **6,** 327 (1978).

(30) F. C. P. Law, *Drug Metab. Dispos.*, **10,** 361 (1982).

(31) D. A. Wiersma and R. A. Roth, *J. Pharm. Exp. Ther.*, **212,** 97 (1980).

(32) Y. Sasaki and N. Wagner, *J. Appl. Physiol.*, **30,** 879 (1968).

(33) R. L. Dedrick, D. S. Zaharko, and R. J. Lutz, *J. Pharm. Sci.*, **62,** 882 (1973).

(34) R. J. Lutz, R. L. Dedrick, H. B. Mathews, T. E. Eling, and M. W. Anderson, *Drug Metab. Dispos.*, **5,** 386 (1977).

ACKNOWLEDGMENTS

Portions of the paper appeared in a dissertation submitted by Y. Igari to the Graduate Division, University of Tokyo, Tokyo, Japan, in partial fulfillment of the requirements for the Ph.D. degree.

This study was supported by a grant-in-aid for Scientific Research provided by the Ministry of Education, Science and Culture of Japan. The authors thank Dr. G. R. Wilkinson for his review of this manuscript.