APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 8, 215–221 (1994)

Different Acute Effects of Oral and Intratracheal Administration of Disodium Arsenate and Gallium Arsenide on Heme Synthesis in Rats

Masao Kondo*‡ and Isamu Ichikawa†
*Department of Nutrition and Biochemistry, and †Department of Industrial Health, The Institute of Public Health, Tokyo 108, Japan

The acute influences of arsenic compounds on the metabolism of porphyrins and heme in various organs of rats after oral or intratracheal administration of disodium arsenate (Na_2HAsO_4) and gallium arsenide (GaAs) were examined and compared.

For the oral administration experiments, 21 or 84 mg of Na_2HAsO_4, or 2 or 4 g of GaAs, per cm^3 saline per kg body weight of each animal was administered to Jcl:Wistar male rats and the organs were removed after exsanguination from the vein of the right axilla under anesthesia with ether, 16 h after administration. In the case of intratracheal administration, rats given 8.2 or 16.4 mg of Na_2HAsO_4, or 0.2 or 0.4 g GaAs per cm^3 saline per kg body weight were examined under the same experimental conditions as for the administration route.

Increase in the body weight of rats was suppressed after intratracheal administration of the two arsenic compounds. In these rats the hematocrit value increased significantly. These changes were not shown by the orally administered rats. Elevation in δ-aminolevulinate synthase (ALA-S, EC 2.3.1.37) activity in erythroblasts by Na_2HAsO_4 was much higher after intratracheal administration than after oral administration. Suppression in the activities of δ-aminolevulinate dehydratase (ALA-D, EC 4.2.1.24) and porphobilinogen deaminase (PBG-D, EC 4.3.1.8) in peripheral erythrocytes by Na_2HAsO_4 and GaAs were stronger by intratracheal administration than by the oral route. Influences of GaAs on the activity of PBG-D in rat liver were shown to be more effective by oral administration than by the intratracheal route. Oral administration of Na_2HAsO_4 and GaAs had a stronger suppression effect on the activities of ALA-D and PBG-D in rat kidney.

It seems from these results that the different extents of the influence of arsenic compounds might depend on the routes of intake.

Keywords: Gallium arsenide, arsenic compounds, heme biosynthesis, oral and intratracheal administration, rat

INTRODUCTION

Gallium arsenide (GaAs) and its related compounds are currently in widespread use in industry as materials for semiconductor and telecommunications applications.[1,2] It is known that GaAs has immunotoxicity causing inflammatory changes in the lung[3–5] and offers stronger toxicity by intratracheal administration rather than by the oral route.[6,7] Although GaAs has very low solubility, a very small portion of it is dissolved *in vivo* and decomposed to arsenic and gallium (Ga). The liberated arsenic is known to bind with the SH group of enzymes and thereby inhibits their activity. However, little is known about the effects of these elements on the heme biosynthetic pathway relating to abundant SH enzymes. In a recent study, Goering *et al.*[8] reported that intratracheal administration of GaAs was followed by decreased δ-aminolevulinc acid dehydratase (ALA-D) activity in peripheral erythrocytes and increased urinary excretion of ALA. They reported further that the decrease in ALA-D activity was due to direct inhibition by gallium and surmised that blood levels of ALA-D activity and of urinary ALA might be useful indicators of GaAs exposure/toxicity. The inhibition of

‡ Author to whom all correspondence should be sent, at The Institute of Public Health, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108 Japan.

CCC 0268–2605/94/030215–07

Received 10 December 1993
Accepted 19 January 1994

ALA-D activity by GaAs is noncompetitive and cannot be reversed by addition of Zinc(II).[9]

While inceased urinary excretion of uroporphyrin (URO) is known to be a manifestation of the effect of arsenic on heme biosynthesis, few reports are available to date on the effects of this element on heme biosynthetic enzymes.

The present study examined the different effects of oral and intratracheal administration of GaAs and disodium arsenate (Na_2HAsO_4) on heme biosynthesis in rats.

MATERIALS AND METHODS

Chemicals

GaAs (purity 99.999%) was obtained from Alfa Products (Danvers, MA, USA) and was prepared by the method of Yamauchi *et al.*[10] ALA were obtained from Daiichi Pure Chemicals Company (Tokyo). PBG, protoporphyrin (PROTO) and Zn-PROTO were obtained from Porphyrin Products (Logan, UT, USA). Other chemicals used were standard products of reagent grade.

Animals

Male SPF Jcl:Wistar rats, 200–220 g in weight, were used.

Oral administration

Five rats in each group were assigned to treatment with 2 or 4 g of GaAs, or 21 or 84 mg of Na_2HAsO_4, per cm^3 physiological saline per kg body weight. The compounds were given respectively as a single oral dose. Control rats were provided with the same volume of saline without arsenic.

Intratracheal adminstration

Five rats in each group were assigned to treatment groups to receive 0.2 or 0.4 g of GaAs, or 8.2 or 16.4 mg of Na_2HAsO_4, per cm^3 physiological saline per kg body weight as a single peroral intratracheal dose. Control rats were provided with the same volume of saline without arsenic.

The animals were anesthetized with ether 16 h after administration of the compounds and their blood was collected in heparinized plastic tubes from the vein of the right axilla. The hematocrit value was determined via capillary tube methods. Liver and kidney after perfusion through the portal vein were carefully removed, rinsed in cold saline, blotted and weighed.

Enzyme preparation and assay from bone marrow cells and blood

Bone marrow cells from the femora of rats were collected and suspended in saline. The cells were precipitated by centrifugation at 3000*g* for 10 min, washed twice with cold saline, and hemolyzed by adding 2 cm^3 of ice-cold water. Isotonicity was restored by adding 9% NaCl solution, and the hemolysate was centrifuged at 48 800*g* for 30 min at 4 °C. The supernatant was used for assay of ALA-D[11] and PBG-D.[12] The precipitate was washed two or three times with cold saline to exclude hemoglobin. The washed precipitate was homogenized in 1.0 cm^3 of 0.25 M sucrose solution (containing 0.05 M Tris, 0.01 M potassium phosphate, 0.01 M $NaHCO_3$, pH 8.0) and used to determine the ALA-S activity.[13] Whole-blood lysates were also used for assay of the enzymes.

Enzyme preparation from liver, kidney, and spleen

Liver and kidney were homogenized in 0.25 M sucrose solution. The mitochondria and supernatant fractions were prepared from the homogenate by centrifugation at 800*g* for 10 min, 10 000*g* for 15 min, and 105 000*g* for 1 h, successively. The mitochondrial fraction was further washed twice with 0.25 M sucrose, resuspended in the same solution and used for the assay of ALA-S. The 105 000*g* supernatant fraction was used for the assay of ALA-D and PBG-D.

Other procedures

Protein was determined by the method of Lowry *et al.*[14] with minor modification.[15]

Colorimetric determination of ALA in kidney tissue was carried out on equal volumes of the supernatant fractions. The fractions pooled from five rats in each group were deproteinized with trichloroacetic acid using ion-exchange resins of Dowex 50W-×8 H^+ form, 200–400 mesh, and Dowex 1×8 CH_3COO^- form, 200–400 mesh.[16] Erythrocyte porphyrins were determined by the method reported previously.[17]

The means and SD were calculated and the statistical significance of the differences between

Table 1 Effects of oral and intratracheal administration of gallium arsenide (GaASs) and sodium arsenate (Na_2HAsO_4) on the body weight and hematocrit value (Ht) in rats

Rat group	Dose ($g\,cm^{-3}\,kg^{-1}$)	Body weight (% change)[a]	Ht (%)[a]
Oral administration			
Control		106 ± 3	39.6 ± 0.64
GaAs	2	103 ± 1	39.2 ± 0.28
	4	104 ± 2	41.1 ± 2.39
Na_2HAsO_4	21×10^{-3}	103 ± 1	39.0 ± 1.03
	84×10^{-3}	103 ± 1	40.5 ± 2.07
Intratracheal administration			
Control		101 ± 1	36.1 ± 1.95
GaAs	0.2	91 ± 1**	38.7 ± 1.07**
	0.4	99 ± 2	38.6 ± 0.77*
Na_2HAsO_4	8.2×10^{-3}	94 ± 1**	42.4 ± 0.98**
	16.4×10^{-3}	102 ± 1	40.7 ± 0.88**

[a] Values represent means ± SD for five rats. Statistically significant from control at: $*P<0.01$ and $**P<0.05$.

the treated and the control groups was determined using Student's *t*-test.

RESULTS

Body weight and hematocrit value

In rats administered arsenic compound intratracheally, body weight decreased and the hematocrit value was elevated, whereas these effects were not observed in orally treated rats (Table 1).

ALA-S, ALA-D and PBG-D activities in bone marrow cells

The activity of ALA-S in the groups receiving orally administered Na_2HAsO_4 (ALA-S is an enzyme participating in the first step in heme synthesis) increased by an average of approximately 2.1-fold compared with the control level. It was also increased by 1.9 and 2.3-fold in the groups treated with intratracheal GaAs and Na_2HAsO_4, respectively, compared with the control level. No significant changes in the ALA-D and PBG-D activities were noted in any treated groups (Table 2).

ALA-D and PBG-D activities in peripheral blood

ALA-D and PBG-D activities in erythrocytes were found to be significantly decreased in the groups of rats that had received Na_2HAsO_4 by oral administration, compared with the control group. On addition of DTT (10 mM) or Zinc(II) (0.1 mM) to the reaction mixture there was some recovery, though not complete, toward the control level; ALA-D activity in the groups treated with intratracheal GaAs was 77% of the control value and this decrease was not recovered by addition of DDT or Zinc(II), in striking contrast with the recovery of the ALA-D and PBG-D activities from intratracheal Na_2HAsO_4-induced impairment in the presence of DDT or Zinc(II) (Table 3).

Table 2 Effects of oral and intratracheal administration of gallium arsenide (GaAs) and sodium arsenate (Na_2HAsO_4) on ALA synthase, ALA dehydratase and PBG deaminase activities in bone marrow cells of rats

		Activity		
Rat group	Dose ($g\,cm^{-3}\,kg^{-1}$)	ALA-S (nmol ALA $mg^{-1}\,h^{-1}$)[a]	ALA-D (nmol PBG $mg^{-1}\,h^{-1}$)[a]	PBG-D (nmol URO $mg^{-1}\,h^{-1}$)[a]
Oral administration				
Control		1.54 ± 0.27	10.1 ± 0.68	1.02 ± 0.10
GaAs	2	1.43 ± 0.09	11.5 ± 1.12	1.06 ± 0.14
	4	1.27 ± 0.11	10.8 ± 0.32	1.00 ± 0.05
Na_2HAsO_4	21×10^{-3}	3.29 ± 0.30**	11.4 ± 1.69	1.08 ± 0.18
	84×10^{-3}	3.03 ± 0.54**	9.8 ± 0.54	0.97 ± 0.06
Intratracheal administration				
Control		1.46 ± 0.19	10.6 ± 0.80	0.97 ± 0.12
GaAs	0.2	1.09 ± 0.13**	11.0 ± 0.95	0.92 ± 0.12
	0.4	2.76 ± 0.34**	10.3 ± 0.06	0.93 ± 0.12
Na_2HAsO_4	8.2×10^{-3}	3.03 ± 0.20**	10.6 ± 1.72	1.09 ± 0.23
	16.4×10^{-3}	3.36 ± 0.38**	9.5 ± 0.76	1.03 ± 0.16

Abbreviations: ALA, δ-aminolevulinate; PBG, porphobilinogen.

[a] Values represent means ± SD for five rats. Statistically significant from control at: $*P<0.01$ and $**P<0.05$.

Table 3 Effects of oral and intratracheal administration of gallium arsenide (GaAs) and sodium arsenate (Na_2HAsO_4) on ALA dehydratase and PBG deaminase activities in peripheral blood of rats

Rat ALA-D group	Dose PBG-D ($g\,cm^{-3}\,kg^{-1}$)	ALA-D [nmol PBG $(cm^{-3}$ RBC$)^{-1}\,h^{-1}$][a]		PBG-D [nmol URO $(cm^3$ RBC$^{-1})\,h^{-1}$][a]
		No addition	+ DTT, Zn^{2+}	
Oral administration				
Control		469 ± 58.0	523 ± 72.2	31.8 ± 2.2
GaAs	2	447 ± 83.4	520 ± 92.9	32.2 ± 4.1
	4	411 ± 16.4	473 ± 38.0	29.1 ± 2.9
Na_2HAsO_4	21×10^{-3}	396 ± 40.5*	461 ± 28.9	28.3 ± 0.7**
	84×10^{-3}	366 ± 45.3*	430 ± 64.5	26.8 ± 2.3**
Intratracheal administration				
Control		460 ± 41.0	540 ± 58.3	32.4 ± 3.9
GaAs	0.2	353 ± 55.5**	426 ± 52.4*	29.9 ± 3.3
	0.4	352 ± 33.0**	430 ± 28.0**	30.0 ± 1.5
Na_2HAsO_4	8.2×10^{-3}	382 ± 44.5*	559 ± 61.3	25.7 ± 1.4**
	16.4×10^{-3}	400 ± 42.8	522 ± 41.3	25.2 ± 1.0**

Abbreviations: ALA, δ-aminolevulinate; PBG, porphobilinogen.
[a] Values represent means ± SD for five rats. Statistically significant from control at: $*P<0.01$ and $**P<0.05$.

Porphyrin contents in peripheral erythrocytes

Coproporphyrin (COPRO) and Zn-PROTO in erythrocytes of rats given by Na_2HAsO_4 oral administration were five- and two-fold, respectively, higher than the control level. An increasing tendency for these porphyrins was evident in the groups treated orally with GaAs, whereas any group which had had intratracheal administration of arsenic compounds showed no influence on the level of the porphyrins in the blood (Table 4).

Table 4 Effects of oral and intratracheal administration of gallium arsenide (GaAs) and sodium arsenate (Na_2HAsO_4) on the erythrocytes porphyrin content of rats

Rat group	Dose ($g\,cm^{-3}\,kg^{-1}$)	Concn of porphyrin in erythrocytes [μg (dl RBC)$^{-1}$][a]		
		COPRO	F-PROTO	Zn-PROTO
Oral administration				
Control		1.95 ± 0.59	27.5 ± 19.1	49.8 ± 16.6
GaAs	2	2.26 ± 0.26	25.2 ± 2.5	65.2 ± 10.2
	4	2.38 ± 0.61	18.9 ± 1.4	67.1 ± 15.8
Na_2HAsO_4	21×10^{-3}	2.93 ± 0.24**	24.9 ± 3.0	81.6 ± 4.1**
	84×10^{-3}	2.73 ± 0.41*	25.7 ± 4.0	99.3 ± 26.0**
Intratracheal administration				
Control		2.62 ± 0.61	24.2 ± 4.0	83.7 ± 15.8
GaAs	0.2	2.44 ± 0.44	25.4 ± 5.7	86.0 ± 12.5
	0.4	2.58 ± 0.43	22.5 ± 4.4	66.4 ± 14.6
Na_2HAsO_4	8.2×10^{-3}	2.83 ± 0.35	28.0 ± 6.4	87.2 ± 18.3
	16.4×10^{-3}	2.87 ± 0.59	23.8 ± 2.6	79.6 ± 9.6

Abbreviations: ALA, δ-aminolevulinate; PBG, porphobilinogen; COPRO, coproporphyrin; F-PROTO, free protoporphyrin; Zn-PROTO, zinc-protoporphyrin.
[a] Values represent means ± SD for five rats. Statistically significant from control at: $*P<0.01$ and $**P<0.05$.

Table 5 Effects of oral and intratracheal administration of gallium arsenide (GaAs) and sodium arsenate (Na_2HAsO_4) on ALA synthase, ALA dehydratase and PBG deaminase activities in rat liver

Rat group	Dose ($g\,cm^{-3}\,kg^{-1}$)	ALA-S (pmol ALA $mg^{-1}\,h^{-1}$)	ALA-D (nmol PBG $mg^{-1}\,h^{-1}$)	PBG-D (pmol URO $mg^{-1}\,h^{-1}$)
Oral administration				
Control		19.3 ± 5.91	16.4 ± 1.68	152 ± 22.5
GaAs	2	30.8 ± 12.3	16.3 ± 1.57	138 ± 12.4
	4	23.2 ± 13.5	15.4 ± 1.79	122 ± 10.6*
Na_2HAsO_4	21×10^{-3}	87.0 ± 30.5**	16.7 ± 1.77	139 ± 8.0
	84×10^{-3}	62.3 ± 49.6	19.2 ± 3.58	156 ± 14.0
Intratracheal administration				
Control		26.5 ± 5.5	19.2 ± 2.90	128 ± 23.2
GaAs	2	16.3 ± 1.3**	17.4 ± 1.75	120 ± 10.8
	0.4	20.5 ± 4.8	16.4 ± 2.04	126 ± 15.8
Na_2HAsO_4	8.2×10^{-3}	29.5 ± 3.4	15.6 ± 0.59*	131 ± 16.4
	16.4×10^{-3}	51.5 ± 29.8	16.9 ± 1.14	141 ± 8.8

Abbreviations: ALA, δ-aminolevulinate; PBG, porphobilinogen.
[a] Values represent means ± SD for five rats. Statistically significant from control at: *$P<0.01$ and **$P<0.05$.

ALApS, ALA-D and PBG-D activities in liver

In the livers of rats treated with Na_2HAsO_4, ALA-S activity was increased. The extent of this increase was much greater after intratracheal administration than after the oral route. ALA-D activity exhibited a declining tendency in the intratracheally treated groups. PBG-D activity decreased to a significant extent in the orally GaAs-treated groups (Table 5).

ALA-D and PBG-D activities and ALA contents in kidney

ALA, ALA-D and PBG-D levels in kidney of the group receiving an oral $84\,mg\,kg^{-1}$ dose of Na_2HAsO_4 were 51, 41, and 60% respectively, of

Table 6 Effects of oral and intratracheal administration of gallium arsenide (GaAs) and sodium arsenate (Na_2HAsO_4) on ALA, ALA dehydratase and PBG deaminase activities in rat kidney

Rat group	Dose ($g\,cm^{-3}\,kg^{-1}$)	ALA-S [ng (mg protein)$^{-1}$]	ALA-D (nmol PBG $mg^{-1}\,h^{-1}$)[a]	PBG-D (pmol URO $mg^{-1}\,h^{-1}$)[a]
Oral administration				
Control		61.7	8.90 ± 0.42	89.8 ± 4.73
GaAs	2	56.4	8.21 ± 1.41	88.6 ± 14.6
	4	52.1	7.49 ± 0.76**	81.0 ± 2.6**
Na_2HAsO_4	21×10^{-3}	42.1	5.71 ± 1.00**	55.4 ± 3.5**
	84×10^{-3}	36.5	3.62 ± 0.30**	53.9 ± 2.3**
Intratracheal administration				
Control		51.7	7.42 ± 1.11	81.2 ± 6.11
GaAs	0.2	52.3	7.52 ± 0.96	84.1 ± 9.87
	0.4	48.7	6.48 ± 0.80	83.7 ± 4.24
Na_2HAsO_4	8.2×10^{-3}	36.6	6.22 ± 1.12	59.5 ± 2.72**
	16.4×10^{-3}	45.6	6.27 ± 0.50	60.3 ± 7.79**

Abbreviations: ALA, δ-aminolevulinate; PBG, porphobilinogen; ALA-D, ALA dehydratase; PBG-D, PBG deaminase.
[a] Values represent means ± SD for five rats. Statistically significant from control at: *$P<0.01$ and **$P<0.05$.

the control levels. A significant reduction in PBG-D activity was also noted in rats with intratracheal administration of Na_2HAsO_4 (Table 6).

DISCUSSION

An investigation of the effects on porphyrin metabolic enzymes of GaAs and Na_2HAsO_4 which were administered by two different routes, i.e. oral and intratracheal, has been carried out. The results indicated that the effects of GaAs and of Na_2HAsO_4 varied depending upon the organs and enzymes examined.

In bone marrow cells, ALA-S activity increased after oral or intratracheal administration of the arsenic compounds in rats. This enzyme response to Na_2HAsO_4 was more conspicuous than to GaAs. That there was no consistent response of ALA-S to orally or intratracheally administered GaAs is probably due to the different solubilities of the compounds in different types of tissue. There are few metals known to elevate ALA-S activity in bone marrow cells. In this context, arsenic poisoning through the intratracheal route could provide a model of interest for studying the regulatory mechanism of heme synthesis in erythroblasts.

On the other hand, ALA-D activity in peripheral erythrocytes was depressed in the groups treated with intratracheal GaAs and did not recover toward its normal level even after addition of DTT or Zinc(II). Decreased ALA-D activity was noted also in the Na_2HAsO_4-treated groups. Complete recovery from impairment of ALA-D activity in the intratracheally treated groups by addition of DTT or Zinc(II) suggests that gallium and arsenic have different inhibition mechanisms for this enzyme. This may be because ALA-D is a heat-stable protein containing zinc and is activated by protectors with SH groups (such as reduced GSH and DTT) and by Zinc(II) ion.[13]

A Na_2HAsO_4-induced decrase in erythrocyte PBG-D activity, like that in kidney PBG-D activity, was observed for both of these administration routes of this arsenic compound. Reduction in this enzyme activity is a new finding. Further investigation into the inhibition mechanism of PBG-D (which is not an SH enzyme) will be needed.

The contents of COPRO and Zn-PROTO in the erythrocyte of oral Na_2HAsO_4-treated groups were significantly higher than the respective control values. These changes in the erythrocyte porphyrins being similar to those in iron-deficiency anemia and lead poisoning,[18] suggest that heme production might be impaired directly or indirectly.

Results regarding ALA-S activity in the liver showed similar findings to those for the erythroblast enzyme. Since ALA-S is the rate-limiting enzyme in heme biosynthesis,[19] an increase of this enzyme activity can be interpreted as implying decreased heme production, increased heme utilization, and enhanced heme decomposition. ALA-D activity was decreased in the intratracheally Na_2HAsO_4-treated groups, but remained unchanged in the orally treated groups. The influence of Na_2HAsO_4 upon heme biosynthesis in the liver will be important for elucidation of the mechanism of development of hepatic impairment by arsenic poisoning.

ALA-D and PBG-D activities and ALA content in the kidney were shown to decrease following administration of arsenic. Furthermore, the decrease in these enzyme activities was more marked in the kidney than in the liver, bone marrow erythroblasts and peripheral erythrocytes, strongly suggesting nephrotoxicity of arsenic. From this finding and from the decreased ALA content in the kidney (suggesting decreased ALA-S activity) it is surmised that exposure to arsenic compounds might produce severe damage to heme biosynthesis.

The results of the present study clearly indicate that exposure to arsenic compounds altered the enzyme activities participating in heme biosynthesis in various organs of rats. Before utilization of the changes in the activity of these enzymes as biological indicators of exposure to GaAs and other arsenic compounds or their toxicity, further in-depth pharmacological studies of administration, absorption, dose–response relationships and subacute toxicity need to be conducted. Investigations of the influence of these materials on heme biosynthesis will give an insight into the regulatory mechanisms of heme synthesis operating in various tissues.

REFERENCES

1. A. L. Robinson, *Science* **219**, 275 (1983).
2. P. O. Dupuis, *Science* **226**, 623 (1984).
3. E. E. Sikorski, K. L. McCoy, K. L. White, S. G. Bradly

and A. E. Munson, *Fund. Appl. Toxicol.* **13**, 843 (1989).
4. E. E. Sikorski, L. A. Burns, K. L. McCoy, M. Stern and A. E. Munson, *Toxicol. Appl. Pharmacol.* **110**, 143 (1991).
5. L. A. Burns, E. E. Sikorski, J. J. Saady and A. E. Munson, *Toxicol. Appl. Pharmacol.* **110**, 157 (1991).
6. T. A. Roschina, *Grif. Tr. Prof. Zabol.* **10**, 30 (1966).
7. D. R. Webb, I. G. Sipes and D. E. Carter, *Toxicol. Appl. Pharmacol.* **76**, 96 (1984).
8. P. L. Goering, R. R. Maronpot and B. A. Fowler, *Toxicol. Appl. Pharmacol.* **92**, 179 (1988).
9. P. L. Goering and A. Rehm, *Env. Res.* **53**, 135 (1990).
10. H. Yamauchi, K. Takahashi and Y. Yamamura, *Toxicology* **40**, 237 (1986).
11. M. Kondo, M. Kajimoto, H. Kimura, T. Suzuki, A. Sasaki, M. Niwa and G. Urata, *Arch. Biochem. Biophys.* **208**, 189 (1981).
12. M. Kondo, M. Mori and Y. Aoki, *Acta Haematol. Jpn* **53**, 851 (1990).
13. M. Kondo, M. Kajimoto and G. Urata, *Exp. Hematol.* **11**, 324 (1983).
14. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
15. M. Kondo, H. Kimura and G. Urata, *Bull. Inst. Publ. Health* **29**, 131 (1980).
16. M. Kondo, G. Urata and Y. Shimizu, *Clin. Sci.* **65**, 423 (1983).
17. M. Kondo and M. Hirosawa, *Jap. J. Clin. Chem.* **17**, 36 (1988).
18. A. Kappas, S. Sassa, R. A. Galbraith and Y. Nordmann, *The Metabolic Basis of Inherited Disease*, pp. 1305–1365. McGraw-Hill, New York (1989).