Role of human serum biotinidase as biotin-binding protein

Jasbir CHAUHAN and Krishnamurti DAKSHINAMURTI*

Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

Biotinidase shows two binding sites for biotin, with $K_d = 59$ and 3 nM respectively, and requires tryptophan and cysteine residues of the biotinidase protein for biotin-binding activity. Analysis of human serum by various column-chromatographic techniques indicates that biotinidase is the only protein which exchanges with labelled (+)-biotin. It was shown previously that epileptic patients receiving a high average dose of anticonvulsants (containing a carbamide group) have lower biotin concentrations than those receiving a low dose. We have shown in human serum and with purified biotinidase that these anticonvulsant drugs compete with biotin for binding to the protein moiety.

INTRODUCTION

The mechanism by which biotin is transported in mammals is still not well understood. Rogers & Lichstein [1,2] have shown that in yeast an active carrier-mediated system operates for biotin uptake. In hamster small intestine a Na^+ -dependent carrier-mediated uptake of biotin has been reported [3,4]. There is evidence for the presence of carrier protein(s) for biotin in laying-hen plasma and egg yolk [5–8].

On the basis of investigations with HeLa cells and human fibroblasts, we suggested that biotin transport in mammalian cells may occur through pinocytosis, involving the functioning of specific circulatory protein(s) [9,10]. The specific binding of protein-bound biotin (avidin–biotin complex) with liver plasma membrane was reported [10], with the suggestion that avidin–biotin complex might be mimicking a natural biotin-carrier protein for this binding. Cohen & Thomas [11], using fully differentiated 3T3-L1 cells, further substantiated this hypothesis. It has been shown that at biotin and biocytin concentration of less than 50 nM absorption proceeds by a saturable process, whereas at higher concentrations uptake by passive diffusion predominates in the rat intestine [12]. Fractionation analysis of solubilized rat intestinal brush-border preparation indicated that biotinidase is the only protein which binds biotin in this preparation [12]. Thoene *et al.* [13] suggested that in humans normal biotin uptake could be the result of two systems, one with low K_m (saturable) and the other with a high K_m for biotin absorption.

Two types of multiple carboxylase deficiencies have been described [14]. Neonatal or early-onset type of multiple carboxylase deficiency has been attributed to biotin-holocarboxylase synthetase deficiency, whereas the late-onset or juvenile form has been attributed to the deficiency of biotinidase. In biotinidase deficiency, there is a failure of recycling of endogenous biotin, a failure to liberate biotin from dietary covalently protein-bound biotin, and a probable failure of renal re-absorption of biotin. However, the low concentrations of circulating biotin in these patients would also suggest that some protein is involved in the transport of biotin [15,16]. Previous studies have indicated that human albumin, α -

and β -globulin can bind biotin [17]. A biotin-binding glycoprotein in human serum has also been reported [18,19]. Although various studies indicate the presence of carrier protein for the transport of biotin in human serum, the nature of this protein(s) is not known. In this paper we present our results of fractionation analysis of human serum for biotin-binding activity.

MATERIALS AND METHODS

Materials

[^3H]Biotin (50 Ci/mmol) was a gift from Hoffman–La Roche (Nutley, NJ, U.S.A.). Biotin, biocytin, 2-imino-biotin, desthiobiotin, N -[(+)-biotinyl]- p -aminobenzoate, human albumin, α - and β -globulin, phenobarbital, pentobarbital, phenytoin, carbamazepine and valproic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of reagent grade and were obtained from standard commercial sources.

Biotin-binding assay

The assay is essentially the same as used for biotin-holocarboxylase synthetase [20]. It contained, in a final volume of 0.3 ml, the following components (mM): potassium phosphate buffer, pH 6.0, 100; NaCl 150; EDTA 1; 2-mercaptoethanol 1; [^3H]biotin (0.1 μCi); and appropriate amount of biotin-binding protein. When more highly purified biotinidase preparations (1 – 5 munits of biotinidase) were used 1.0 mg of γ -globulin was also included in the assay to aid subsequent protein precipitation. In the control, unlabelled biotin (final concn. 1 mM) was included to correct for non-specific binding. The reaction mixture was incubated at room temperature for 5 min, and the protein was then precipitated by addition of 0.9 ml of cold saturated $(\text{NH}_4)_2\text{SO}_4$ in the phosphate buffer, pH 6.0. After standing for 10 min on ice, the precipitated protein was collected by centrifugation and washed once with 1.0 ml of cold 70% -satd. $(\text{NH}_4)_2\text{SO}_4$ in phosphate buffer. The precipitate was again collected by centrifugation and then dissolved in 0.1 ml of 0.01 M-NaOH and counted for radioactivity with 1 ml of Scintiverse II cocktail.

* To whom correspondence should be addressed.

Purification of biotinidase

Biotinidase (sp. activity 1900 units/mg of protein) was purified from human serum as previously described [21].

Biotinidase activity

Biotinidase activity was determined with *N*-[(+)-biotinyl]-*p*-aminobenzoate and biocytin as substrates as previously described [21,22].

Sucrose-density-gradient analysis of human serum

The procedure used for sucrose-density-gradient centrifugation was that of Martin & Ames [23]. Dialysed and concentrated human serum (0.35 ml) containing [³H]biotin was layered on top of a 5–20% (12.5 ml) linear gradient of sucrose containing 0.1 M-potassium phosphate buffer, pH 6.0, 1 mM-2-mercaptoethanol and 1 mM-EDTA and equal amounts (c.p.m.) of radioactive biotin along the gradient. Centrifugation was at 4 °C for 22 h at 140000 *g* in a Beckman SW 40 Ti rotor. At the end of the run, fractions were collected from the bottom of the gradient and assayed for radioactivity, and biotinidase activity was measured with *N*-[(+)-biotinyl]-*p*-aminobenzoate as the substrate.

Sephadex G-150 chromatography of human serum

Human serum (15 ml) was applied on to a Sephadex G-150 column (2.5 cm × 100 cm), previously equilibrated with 0.05 M-phosphate buffer, pH 6.0, containing 1 mM-2-mercaptoethanol, 1 mM-EDTA, 150 mM-NaCl and 0.02% NaN₃. The same buffer was used for elution of the protein at a flow rate of 20 ml/h, and 6.0 ml fractions were collected. Biotinidase activity, biotin-binding activity and biotin contents were determined.

Equilibrium dialysis

A solution (1.0 mg/0.1 ml) of various purified proteins in 0.1 M-phosphate buffer (pH 7.5) was placed inside dialysis tubing, and the ends of the tubing were secured with a thread. The dialysis tubing was placed in test tubes

containing various concentrations (0.1 nM–0.1 mM) of [³H]biotin in 10 ml of 0.1 M-phosphate buffer, pH 7.0. The test tubes were mixed overnight in an end-over-end mixer. Parallel incubations with 1000-fold excess of biotin were used to check for non-specific binding. At the end of this time period, the dialysis tubing was removed and radioactivity associated with protein as well as with the solution in the test tube was measured. Equal volumes (50 μl) of the protein solution or the diffusate were added to 10 ml of Scintiverse-II scintillation 'cocktail', and radioactivity was determined in a Beckman LS 2800 liquid-scintillation spectrometer. The difference between radioactivity (d.p.m.) associated with the protein solution and that associated with the diffusate represented the total radioactivity associated with protein. Specific

Fig. 1. Fractionation of human serum on Sephadex G-150 column

Human serum was fractionated on a Sephadex G-150 column as described in the Materials and methods section. Biotin-binding activity (▲) and biotinidase activity (■) were determined.

Fig. 2. Sucrose-density-gradient analysis of human serum

Human serum was fractionated on a sucrose density gradient with a constant amount of radioactivity along the gradient. Protein (▲), biotinidase activity (■) and [³H]biotin accumulated (●) were determined as described in the Materials and methods section.

Fig. 3. Concentration-dependent binding of (+)-[³H]biotin to purified human serum biotinidase

(a) Samples of purified biotinidase were incubated with increasing concentrations of (+)-[³H]biotin (0.1 nM–10 μ M) as described in the Materials and methods section. A parallel set contained the amount of (+)-[³H]biotin indicated above plus a 1000-fold excess of unlabelled (+)-biotin for the determination of non-specific binding. Each point is the mean of three determinations. Linear regression of the points gave K_d values of 3 and 59 nM and B_{max} values of 0.065 and 0.79 mol/mol of biotinidase. (b) Displacement-curve analysis of human serum biotinidase, alone (●) and in the presence of 10 μ M-*N*-bromosuccinimide (▲) and *p*-chloromercuribenzoate (■).

binding was determined by subtracting non-specific binding from the total binding.

RESULTS

Fractionation of human serum on Sephadex G-150 column

Human serum was fractionated on a Sephadex G-150 column. Biotinidase and biotin-binding activity both coincided (Fig. 1) in the fractions, suggesting that biotinidase is the only protein which binds biotin in human serum. These findings were corroborated by

Fig. 4. pH-dependence of biotin-binding activity of human serum biotinidase

Purified human serum biotinidase was assayed for biotin-binding activity (▲) and biotinidase activity (■) with biocytin as substrate as described in the Materials and methods section. The pH range was 3.5–11.0, with the following 0.1 M buffers: citric acid/sodium citrate (pH 3.0–3.5); acetic acid/sodium acetate (pH 4.0–5.5); sodium phosphate (pH 6.0–8.0); Na₂CO₃/NaHCO₃ (pH 9.0–10.5).

analysis of human serum on DEAE-Sephacel, hydroxyapatite and octyl-Sepharose columns (results not shown). On all these column separations biotin-binding activity and biotinidase activity completely overlapped.

Sucrose-density-gradient analysis of human serum

To confirm further that biotin binding to human serum is not an artifact of the biotin-binding assay, human serum was analysed by equilibrium sucrose-density-gradient centrifugation. As shown in Fig. 2, most of the [³H]biotin accumulated in one peak, and this peak coincided with the biotinidase activity peak. These results again indicate that biotin binding correlates with the presence of biotinidase protein in the fraction.

Biotin-binding activity of human serum biotinidase

We previously showed that biotin in the millimolar concentration range inhibited biotinidase activity [21]. However, this inhibition is of little physiological significance, as the concentration of biotin in human serum is only in the nanomolar rather than the millimolar range [24]. The binding of biotin to pure human serum biotinidase was examined. Fig. 3(a) shows the concentration-dependent binding of (+)-[³H]biotin to biotinidase and the Scatchard [25] transformation of the data. The Scatchard plot shows two linear parts, indicating that two classes of sites, with high and low affinities for biotin, are present (3 nM; 59 nM; $n = 3$). The binding sites have B_{max} of 0.065 and 0.79 mol/mol of biotinidase respectively. The findings from Scatchard analysis were corroborated by displacement-curve analysis. As seen in Fig. 3(b), the displacement curve also shows both low- and high-affinity binding sites. Similar binding characteristics were obtained when we used human serum in place of pure biotinidase.

Fig. 5. Effect of biotin analogues on biotin binding to biotinidase

Effect of biocytin (■), desthiobiotin (▲) and 2-iminobiotin (●) on biotin binding to pure human serum biotinidase.

Effect of *N*-bromosuccinimide and *p*-chloromercuribenzoate on biotin-binding activity

We have previously shown that *p*-chloromercuribenzoate inhibits the biotinidase activity [21]. At a concentration of 10 μ M, *p*-chloromercuribenzoate completely inhibited both the binding sites on the biotinidase (Fig. 3*b*), suggesting that cysteine residues are involved in biotin binding. Furthermore, both the binding sites were inhibited by *N*-bromosuccinimide (10 μ M), indicating that tryptophan residues in biotinidase are also essential for binding of biotin (Fig. 3*b*).

Effect of pH on biotin-binding activity

The effect of pH on biotin-binding activity of biotinidase was examined, as human serum biotinidase showed different pH optima with the synthetic substrate *N*-[(+)-biotinyl]-*p*-aminobenzoate (pH 6–8) and natural substrate biocytin (pH 4–6) [21]. The optimum pH for biotin-binding activity was in the range 4–6, as shown in Fig. 4. The pH profile of biotin binding was identical with that for the hydrolysis of biocytin by biotinidase.

Effect of biotin analogues on biotin-binding activity

The effect of structural analogues of biotin on the biotin-binding activity of biotinidase is presented in Fig. 5. 2-Iminobiotin and desthiobiotin were effective in displacing the biotin in the micromolar range. It was shown previously that the K_m for biocytin of serum biotinidase was 10 μ M [21,26]. However, the serum concentration of biotin is of the order of 2 nM [24], and biocytin is hardly detectable in normal serum [22,27]. As shown in Fig. 5, biocytin was able to displace biotin at about 5 nM concentration, suggesting that biocytin can bind biotinidase at low concentrations. The incubation conditions, i.e. room temperature and for 5 min, would not lead to any significant hydrolysis of biocytin, and hence the binding should essentially be as biocytin. It

Fig. 6. Effect of anticonvulsant on biotin binding to biotinidase

Effect of (a) phenobarbital (▲) and pentobarbital (■) and (b) phenytoin (▲), carbamazepine (■) and valproic acid (●) on biotin binding to pure human serum biotinidase. Reactions were done at pH 7.2. Solubility of carbamazepine in the 0.5–1 mM range was much less, and it was used in suspension.

was difficult to estimate the K_d value for biocytin, as we were unable to prepare radioactive biocytin of high enough specific radioactivity [12].

Effect of anticonvulsants on biotin-binding activity

Kraus *et al.* [28] reported that the concentration of biotin in the plasma of epileptics under long-term treatment with anticonvulsants was markedly decreased. All the anticonvulsants used (phenobarbital, carbamazepine and phenytoin) have a carbamide structure in common with biotin. As shown in Fig. 6, all of these drugs displaced biotin bound to purified biotinidase. Displacement was higher with phenobarbitone and pentobarbiton, whereas carbamazepine and phenytoin

showed slightly lower displacements. Owing to the low solubility of carbamezipine, it was essential to use 5% ethanol, and this resulted in a 20% decrease in biotinidase activity, as shown in Fig. 6(b). The order of displacement of biotin by the anticonvulsants corresponded to the dosage of drugs used in treatment of epileptics (1 equivalent unit = 30 mg of phenobarbital, 50 mg of phenytoin and 200 mg of carbamezipine [28]). The low displacement of biotin by valproic acid correlates with the report that epileptics on valproic acid treatment had normal concentrations of biotin [28]. Similar results were obtained when we used serum samples rather than pure biotinidase in the displacement experiments (results not shown).

Biotin binding to human albumin, α - and β -globulin

Each of these proteins at a concentration of 1 mg/0.1 ml of phosphate buffer was used to determine their specific binding with biotin, by using both equilibrium dialysis and the biotin-binding assay. In the range of biotin used, between 1 nM and 0.1 mM, specific binding of biotin to any of these proteins was not noted.

DISCUSSION

Although previous studies have indicated that human albumin and crude fractions of α - and β -globulins can bind biotin [17], in the present study we found this binding to be non-specific. It is likely that biotinidase contamination in crude samples might have contributed to the non-specific binding. Furthermore, it was reported that a glycoprotein present in human serum can bind biotin [18,19]. We have isolated this protein, using an agarose-biotin column under low-salt conditions [29]. This protein does not bind biotin specifically. The basis of these discrepancies is not clear. It is possible that interpretation of technical difficulties encountered in equilibrium-dialysis analysis with microgram quantities of proteins might explain this.

Baumgartner *et al.* [27] suggested that the decreased concentration of plasma biotin in biotinidase-deficient patients may be due to decreased amount of biotinidase or a specific biotin-binding protein in plasma. Other indirect evidence [28] for the existence of biotin-binding protein comes from the study of epileptic patients on long-term therapy with anticonvulsants. Significantly low concentrations of biotin were reported in these patients. Although the mechanism for the low concentrations of biotin was not indicated, Kraus *et al.* [28] suggested that the decrease in biotin concentrations could be a factor in the mode of action of anticonvulsants. The anticonvulsants used share with biotin the common structural feature of a cyclic carbamide group. It is the cyclic carbamide group of biotin that is involved in protein binding (avidin-biotin complex) [30]. We have shown that all these anticonvulsants competed with biotin for the biotin-binding activity of biotinidase. The only exception to this is the observation that valproic acid does not compete with biotin for binding to biotinidase. Our results indicate that biotinidase is the only protein which exchanges with [3 H](+)-biotin. To our knowledge, this is the first demonstration of the presence of a specific biotin-binding protein (biotinidase) in human serum.

The present study with human serum biotinidase and previous studies with egg-yolk biotin-binding protein [31] suggest that both of these proteins require tryptophan residues in the active centre. Green [30] has reviewed avidin-biotin interactions. Importance has been attached to both tryptophan residues (four per subunit) [30,32-34] and hydrogen bonding to the ureido ring [35] in the binding process. Biotin binding to streptavidin results in changes in the absorption spectrum, and tryptophan reactivity of streptavidin is similar to that of avidin [36]. The sequence for streptavidin has recently been published, and shows a considerable homology to avidin around tryptophan residues [37]. The requirement for tryptophan residues in the active centre for biotin binding by all these proteins would suggest a homology in the amino acid sequence around the tryptophan residues in all the known biotin-binding proteins.

Biotinidase showed low-affinity high-capacity and high-affinity low-capacity biotin-binding sites. The high-affinity low-capacity biotin-binding site represents about 8% of total binding sites on the purified biotinidase. The explanation for this binding site is speculative at present. It is unlikely that purified biotinidase is contaminated with some other protein. Both purified biotinidase and serum samples showed identical binding sites, and the ratio of the two sites remained constant. Secondly, it could be due to the formation of an enzyme-acyl complex. This has been shown for biotinidase with *N*-[(+)-biotinyl]-*p*-aminobenzoate as substrate and hydroxylamine as an enzyme acceptor [38]. Thirdly, it might be due to dimerization of biotinidase, which we routinely observed on non-denaturing gels with purified biotinidase.

The K_d value (5.9×10^{-8} M) obtained for the binding of biotin to the purified human serum biotinidase is in close agreement with the values reported for the binding of other vitamins to their respective binding proteins. Thiamin-binding protein binds thiamin with a K_d for thiamin of 5.5×10^{-7} M [39]. Biotin-binding proteins such as avidin and streptavidin (10^{-15} M) [40] and yolk biotin-binding protein (10^{-12} M) [8] have much lower K_d values for biotin. Biotin-holocarboxylase synthetase from *Escherichia coli* [20] has about the same affinity for biotin ($K_d = 1.3 \times 10^{-7}$ M) as does human serum biotinidase. However, with biotinyl 5'-adenylate, this value decreased to 1.1×10^{-9} M, indicating that the activated form of biotin binds much more tightly to the holocarboxylase synthetase [20].

The primary role of biotinidase is to hydrolyse biocytin or biotinylated peptides, so that biotin can be reutilized in the system. However, an examination of the properties and distribution of biotinidase raises some questions about this function. The ubiquitous distribution of biotinidase [38] and its high K_m (10 μ M) for biocytin [21,26] might preclude a primary hydrolytic role unless the K_m is decreased by modifier. It has been shown previously that biotinidase-deficient patients have low circulating concentrations of biotin [27]. Furthermore, by using polyclonal antibodies against biotinidase, it has been shown that biotinidase protein was completely absent in biotinidase-deficient patients [41]. Results presented here indicate that biotinidase binds to biotin with K_d values of 3 and 59 nM, which is in the physiological range of concentration of biotin. It is possible that biotinidase is the major carrier of biotin in plasma, and as such functions in biotin transport.

This work was supported by research grants from the Medical Research Council of Canada and the Children's Hospital of Winnipeg Research Foundation.

REFERENCES

1. Rogers, T. O. & Lichstein, H. C. (1969) *J. Bacteriol.* **100**, 557–564
2. Rogers, T. O. & Lichstein, H. C. (1969) *J. Bacteriol.* **100**, 565–572
3. Berger, E., Long, E. & Semenza, G. (1972) *Biochim. Biophys. Acta* **255**, 873–887
4. Spencer, R. P. & Brody, K. R. (1964) *Am. J. Physiol.* **206**, 653–657
5. Mandella, R. D., Meslar, H. W. & White, H. B., III (1975) *Biochem. J.* **175**, 629–633
6. White, H. B., III, Dennison, B. A., Della Fera, M. A., Whitney, C. J., McGuire, J. C., Meslar, H. W. & Sammelwitz, P. H. (1976) *Biochem. J.* **157**, 395–400
7. Meslar, H. W., Camper, S. A. & White, H. B., III (1978) *J. Biol. Chem.* **253**, 6979–6982
8. White, H. B., III, & Whitehead, C. C. (1987) *Biochem. J.* **241**, 677–684
9. Dakshinamurti, K. & Chaulifour, L. E. (1981) *J. Cell. Physiol.* **197**, 427–438
10. Chaulifour, L. E. & Dakshinamurti, K. (1982) *Biochem. Biophys. Res. Commun.* **104**, 1047–1053
11. Cohen, N. D. & Thomas, M. (1982) *Biochem. Biophys. Res. Commun.* **108**, 1508–1516
12. Dakshinamurti, K., Chauhan, J. & Ebrahim, H. (1987) *Biosci. Rep.* **7**, 667–673
13. Thoene, J. G., Lemons, R. & Baker, H. (1983) *N. Engl. J. Med.* **308**, 639–642
14. Wolf, B., Grier, R. E., Allen, R. J., Goodman, S. I. & Kien, C. L. (1983) *Clin. Chim. Acta* **131**, 273–281
15. Baumgartner, E. R., Suormala, T., Wick, H., Bausch, J. & Bonjour, J.-P. (1985) *J. Inherited Metab. Dis.* **8**, Suppl. 1, 59–64
16. Wolf, B., Heard, G. S., Secor McVoy, J. R. & Grier, R. E. (1985) *Ann. N.Y. Acad. Sci.* **447**, 252–262
17. Frank, O., Luisada-Opper, A. V., Feingold, S. & Baker, H. (1970) *Nutr. Rep. Int.* **1**, 161–168
18. Vallotton, M., Hess-Sander, U. & Leuthardt, F. (1965) *Helv. Chim. Acta* **48**, 126–133
19. Gehrig, D. & Leuthardt, F. (1976) *Abstr. 10th Int. Congr. Biochem.* p. 208
20. Eisenberg, M. A., Prakash, O. & Hsiung, S. (1982) *J. Biol. Chem.* **257**, 15167–15173
21. Chauhan, J. & Dakshinamurti, K. (1986) *J. Biol. Chem.* **261**, 4268–4275
22. Ebrahim, H. & Dakshinamurti, K. (1987) *Anal. Biochem.* **162**, 319–324
23. Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
24. Baker, H. (1985) *Ann. N.Y. Acad. Sci.* **447**, 129–132
25. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
26. Craft, D. V., Goss, N. H., Chandramouli, N. & Wood, H. G. (1985) *Biochemistry* **24**, 2471–2476
27. Baumgartner, E. R., Suormala, T., Wick, H., Bausch, J. & Bonjour, J.-P. (1985) *Ann. N.Y. Acad. Sci.* **447**, 272–287
28. Krause, K.-H., Bonjour, J.-P., Berlit, P. & Kochen, W. (1985) *Ann. N.Y. Acad. Sci.* **447**, 297–313
29. Dakshinamurti, K., Chalifour, L. & Bhullar, R. P. (1985) *Ann. N.Y. Acad. Sci.* **447**, 38–55
30. Green, N. M. (1964) *Biochem. J.* **89**, 599–609
31. Ramana Murthy, C. V. & Adiga, P. R. (1985) *Biochim. Biophys. Acta* **786**, 222–230
32. Green, N. M. (1964) *Biochem. J.* **90**, 564–568
33. Green, N. M. (1962) *Biochim. Biophys. Acta* **59**, 244–246
34. Green, N. M. & Toms, E. J. (1970) *Biochem. J.* **118**, 67–70
35. Fraenkel-Conrat, H., Ward, W., Snell, N. S. & Ducay, E. D. (1950) *J. Am. Chem. Soc.* **72**, 3826–3827
36. Green, N. M. & Melamed, M. D. (1966) *Biochem. J.* **100**, 614–621
37. Argarana, C. E., Kuntz, I. D., Birken, S., Axel, R. & Cantor, C. R. (1986) *Nucleic Acids Res.* **14**, 1871–1882
38. Pispas, J. (1965) *Ann. Med. Exp. Biol. Fenn.* **43**, Suppl. 5, 5–39
39. Nishimura, H., Yoshioka, K. & Iwashima, A. (1984) *Anal. Biochem.* **139**, 373–376
40. Chalet, L. & Wolf, F. (1964) *Arch. Biochem. Biophys.* **106**, 1–5
41. Wolf, B., Miller, J. B., Hynes, J., Secor McVoy, J., Ishikawa, Y. & Shapira, E. (1987) *Clin. Chim. Acta* **164**, 27–32

Received 29 February 1988/23 May 1988; accepted 23 June 1988