Distribution and Metabolism of Phenylalanine and Tyrosine During Tularaemia in the Rat

By MICHAEL C. POWANDA, RICHARD E. DINTERMAN, ROBERT W. WANNEMACHER, JR.
and GERALD D. HERBRANDSON

U.S. Army Medical Research Institute of Infectious Diseases,
Frederick, Md. 21701, U.S.A.

(Received 27 June 1974)

Tularaemia in rats causes a doubling of the serum phenylalanine/tyrosine ratio and a more than twofold increase in muscle phenylalanine which cannot be accounted for by decreased hepatic phenylalanine hydroxylase (EC 1.14.16.1) activity, but appear to result from a generalized movement of amino acids from muscle to liver during infection.

Various systemic infectious illnesses in man and animals are accompanied by a significant increase in the serum free phenylalanine/tyrosine ratio (Newberne, 1966; Wannemacher *et al.*, 1971, 1972a,b; Williams & Newberne, 1970), generally as a result of heightened phenylalanine concentrations and unaltered or decreased tyrosine content. The accumulation of phenylalanine in the serum is in marked contrast with the decrease in total free amino acid concentration in the serum usually noted in these circumstances. This decrease in total serum amino acid concentration has been construed to be a biochemical manifestation of an infection-induced enhanced movement of amino acids from muscle to liver which is mediated by a substance (or substances) released from phagocytic cells subsequent to and consequent on phagocytosis (Wannemacher *et al.*, 1972c; Pekarek *et al.*, 1973). The increased synthesis of serum proteins during infection (Powanda *et al.*, 1972) might well utilize a substantial portion of the amino acids taken up by the liver. Those amino acids not used in protein synthesis could be converted into carbohydrate or fat. The latter options are not available to phenylalanine, which can only be incorporated into protein, converted into tyrosine or excreted either unaltered or as a deaminated derivative. Phenylalanine hydroxylase (EC 1.14.16.1) has been considered to be the rate-limiting enzyme in the conversion of phenylalanine into tyrosine (McGee *et al.*, 1972). Certainly, children in whom this enzyme is congenitally deficient or absent are confronted with neuropathological sequelae as a consequence of markedly elevated tissue phenylalanine concentrations (Knox, 1972). Such children, even when on a low-phenylalanine diet, have shown increased serum phenylalanine concentrations during infectious illnesses (Kennedy *et al.*, 1967). It thus seems that the increase in serum phenylalanine/tyrosine ratio noted in most infections is best explained as a consequence of different increments in the movement of different amino acids from muscle to liver. To test this hypothesis we measured the

phenylalanine and tyrosine contents of the liver, muscle and serum and the hepatic phenylalanine hydroxylase activity of control rats and those exposed to *Francisella tularensis*. To assess the relative contribution of alterations in enzyme activity and amino acid flux to the increase in serum phenylalanine/tyrosine ratio we administered *p*-chlorophenylalanine, an inhibitor of phenylalanine hydroxylase (Lipton *et al.*, 1967), to both control and infected rats.

Materials and methods

In conducting the research described in this report, the investigators adhered to the 'Guide for Laboratory Animal Facilities and Care', as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

Fisher Dunning male rats weighing 150–200 g (purchased from Microbiological Associates, Walkersville, Md., U.S.A.) were housed in a room maintained at 25–28°C, and were fed on commercial rat chow (Purina) *ad libitum* until the start of the experiments, when food was removed. The live-vaccine strain of *F. tularensis* had been prepared by Merrell–National Laboratories, Cincinnati, Ohio, U.S.A., and was rehydrated, grown out on a glucose–cysteine–blood–agar slant and then transferred to a culture tube for a 24 h incubation. Infection was initiated by the intraperitoneal injection of 10⁸ organisms. Controls received 1 ml of tryptose–saline broth. *p*-Chlorophenylalanine methyl ester was obtained from Calbiochem, LaJolla, Calif., U.S.A. Phenylalanine and tyrosine were determined on a Technicon TSM amino acid analyser by using 0.3 M-lithium citrate, pH 4.2, elution buffer. Portions of liver and leg muscle, after perfusion with 0.9% NaCl, were homogenized in respectively 2 or 3 vol. of 0.9% NaCl containing 50 mg of sulphosalicylic acid/ml

by means of a Willems Polytron (Brinkman Instruments, Westbury, N.J., U.S.A.), and the supernatant was analysed for phenylalanine and tyrosine. A separate portion of liver (0.3–0.4 g) was placed in 10 vol. of ice-cold 0.12M-KCl solution and homogenized with a Willems Polytron just before assay for phenylalanine hydroxylase activity. All enzyme assays were carried out within 4 h of killing of the rats. The reaction mixture for phenylalanine hydroxylase assay contained 4 μ mol of phenylalanine, 1.6 μ mol of dithiothreitol and 0.8 μ mol of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride in a final volume of 2 ml. Each enzyme preparation was incubated for 5, 10, 15 and 20 min at 25°C, and the assay was stopped by the addition of 2 ml of 12% (w/v) trichloroacetic acid. The supernatants after addition of trichloroacetic acid and centrifugation (5000g for 10 min) were analysed for phenylalanine and tyrosine. The reactions appeared to follow linear kinetics in all instances and so estimates of enzyme activity were made by a linear regression analysis of the tyrosine values versus incubation time. Protein content was determined by an automated Lowry technique (Leighton *et al.*, 1968).

In the first study the animals were injected with 1 μ Ci of [14 C]cycloleucine/100 g body wt. 24 h before infection to assess amino acid movement (Wannemacher *et al.*, 1971).

Results

In the first study rats infected with 10⁸ live-vaccine-strain *F. tularensis* displayed a 1.1°C increase in body temperature at 22 h after exposure. Table 1 shows that more than a fourfold increase in [14 C]cycloleucine content occurred (which is indicative of enhanced amino acid uptake) in the livers of infected rats at 22 h as well as a slight but significant diminution in label in the muscle. The serum phenylalanine/tyrosine ratio increased from 0.69 in the control rats to 1.59 in the infected animals as a result of both highly significant increases in phenylalanine and markedly decreased tyrosine concentrations. Hepatic phenylalanine hydroxylase activity was diminished by about one-third whether calculated per g of liver or per mg of protein.

We repeated the study but added groups of both control and infected rats which had been injected with 300 mg of the methyl ester of *p*-chlorophenylalanine 4 h after the administration of either *F. tularensis* or tryptose-saline broth and 18 h before killing. We also measured the concentration of phenylalanine and tyrosine in the livers and leg muscles of these animals. Again infection caused an increase in the serum phenylalanine/tyrosine ratio, from 0.79 to 1.44, this time entirely as the result of an elevation in phenylalanine content. Liver phenylalanine concentration was unchanged and tyrosine concentration decreased.

Table 1. Effect of infection on [14 C]cycloleucine distribution, serum amino acid concentration and hepatic phenylalanine hydroxylase activity

There were six rats in each group. The mean $\pm$ 1 s.e.m. is shown. * $P < 0.05$ versus control; † $P < 0.001$ versus control.

	[¹⁴ C]Cycloleucine distribution (d.p.m./mg of tissue)		
	Liver	Muscle	
Control	15.4 ± 0.7	24.7 ± 0.6	
Infected	71.6 ± 0.6†	22.3 ± 0.9*	
	Serum amino acid concentration (nmol/ml)		
	Phenylalanine	Tyrosine	Ratio
Control	55.3 ± 0.4	80.3 ± 0.4	0.69 ± 0.03
Infected	87.4 ± 0.6†	55.8 ± 0.5†	1.59 ± 0.11†
	Hepatic phenylalanine hydroxylase activity		
	(μmol formed/h per g of liver)	(nmol formed/h per mg of protein)	
Control	25.5 ± 3.3	139.3 ± 13.8	
Infected	17.5 ± 1.8	102.7 ± 6.4*	

Muscle tissues, on the other hand, contained more than double the control amounts of phenylalanine and significantly increased amounts of tyrosine. Again infection only decreased hepatic phenylalanine hydroxylase activity by about one-third.

p-Chlorophenylalanine alone decreased phenylalanine hydroxylase activity by 68%, yet only managed to cause a slight rise in the serum phenylalanine/tyrosine ratios and considerably smaller increases in serum and muscle phenylalanine concentrations. *p*-Chlorophenylalanine given to infected rats caused an 86% decrement in hydroxylase activity and trebled the serum and muscle concentration of phenylalanine while raising that of liver slightly. A slight increase in muscle tyrosine was noted in these animals (Fig. 1).

Discussion

According to studies on the dissemination of *F. tularensis* in the white rat, this rodent approximates to man in its responses during tularaemia (Olsufyev & Dunayeva, 1961) and hence is a suitable model system for the study of phenylalanine and tyrosine distribution and metabolism during infection. Live-vaccine-strain *F. tularensis* (10⁸) have been shown to cause hepatic and splenic tissue damage, a massive increase in amino acid movement into liver, a dramatic increase in serum acute-phase globulins and death in rats (Powanda *et al.*, 1974). In the present studies the

Fig. 1. Distribution and metabolism of phenylalanine and tyrosine during tularaemia in the rat

There were six rats per group. □, Normal; ▨, normal plus *p*-chlorophenylalanine; ■, infected; ▩, infected plus *p*-chlorophenylalanine. The concentrations of phenylalanine and tyrosine are given (a) per ml of serum, (b) per g of liver and (c) per g of muscle. (d) Phenylalanine hydroxylase activity in liver. * $P < 0.01$ versus normal; † $P < 0.01$ versus normal plus *p*-chlorophenylalanine; ‡ $P < 0.01$ versus infected.

greater than fourfold increase in [^{14}C]cycloleucine content of the livers of infected rats and the small but significant decrease in muscle [^{14}C]cycloleucine concentration indicate that amino acids are moving out of muscle and are largely taken up by the liver. Measurement of serum phenylalanine and tyrosine concentrations showed that this infection causes a twofold rise in the serum phenylalanine/tyrosine ratio, mostly as a result of an increase in phenylalanine. Infection also appears to decrease hepatic phenylalanine hydroxylase activity somewhat.

To determine if the decrease observed in hydroxylase activity could account for the changes in serum phenylalanine/tyrosine ratio noted during infection we treated rats with *p*-chlorophenylalanine. The dose of *p*-chlorophenylalanine used was twice as effective as infection in decreasing hydroxylase activity yet only elicited an insignificant rise in the serum phenylalanine/tyrosine ratio.

By looking at the liver and muscle concentrations of phenylalanine and tyrosine it became readily apparent that infection not only increased the serum phenylalanine/tyrosine ratio but also significantly increased the concentration of both amino acids in the muscle while only slightly decreasing that of tyrosine in the liver. Again, *p*-chlorophenylalanine only caused a slight increase in muscle phenylalanine.

Infection and *p*-chlorophenylalanine appeared to have a synergistic or at least additive action in that the rats in this group had the lowest enzyme activities and the highest serum phenylalanine/tyrosine ratios, serum and muscle phenylalanine concentrations and even an elevated liver concentration of phenylalanine. The fact that only these animals had increased amounts of phenylalanine in the liver may be evidence that phenylalanine hydroxylase is not rate-limiting in the conversion of phenylalanine into tyrosine except when enzyme activity is decreased below a certain value. Other investigators have suggested that it is the dihydropteridine reductase that may be rate-limiting rather than phenylalanine hydroxylase (Brand & Harper, 1974).

The foregoing observations are consistent with the data indicating a breakdown in muscle tissue (Lust, 1966; Powanda *et al.*, 1972; Reiss, 1959) and a movement of amino acids from muscle to liver during infection (Wannemacher *et al.*, 1971). The slight decrease in hydroxylase activity which occurs during this infection is unlikely to account for the marked increase in serum and muscle phenylalanine concentrations and consequent rise in the serum phenylalanine/tyrosine ratio. These data appear to be pertinent to the clinical situation in which patients with phenylketonuria exhibit increases in serum phenyl-

alanine/tyrosine ratio during an infectious illness even while being maintained on a low-phenylalanine diet (Kennedy *et al.*, 1967).

We gratefully acknowledge the excellent technical assistance of Thomas Cartwright and the secretarial aid of Ann Clabaugh.

- Brand, L. M. & Harper, A. E. (1974) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **33**, 652
- Kennedy, J. L., Jr., Wertelecki, W., Gales, L., Sperry, B. P. & Cass, V. M. (1967) *Amer. J. Dis. Child.* **113**, 16-21
- Knox, W. E. (1972) *Metabolic Basis of Inherited Diseases*, pp. 266-295, McGraw-Hill, New York
- Leighton, F., Poole, B., Beaufay, H., Bandhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482-513
- Lipton, M. A., Gordon, R., Guroff, G. & Udenfriend, S. (1967) *Science* **156**, 248-250
- Lust, G. (1966) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **25**, 1688-1694
- McGee, M. M., Greengard, O. & Knox, W. E. (1972) *Biochem. J.* **127**, 675-680
- Newberne, P. M. (1966) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **25**, 1701-1710
- Olsufyev, N. G. & Dunayeva, T. N. (1961) *J. Hyg. Epidemiol. Microbiol. Immunol.* **400**, 409-422
- Pekarek, R. S., Wannemacher, R. W., Jr., Powanda, M. C. & Beisel, W. R. (1973) *Clin. Res.* **21**, 608
- Powanda, M. C., Wannemacher, R. W., Jr. & Cockerell, G. L. (1972) *Infec. Immunity* **6**, 266-271
- Powanda, M. C., Canonico, P. G., Moe, J. B. & Cockerell, G. L. (1974) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **33**, 620
- Reiss, E. (1959) *Metab. Clin. Exp.* **8**, 151-159
- Wannemacher, R. W., Jr., Powanda, M. C., Pekarek, R. S. & Beisel, W. R. (1971) *Infec. Immunity* **4**, 556-562
- Wannemacher, R. W., Jr., Pekarek, R. S., Bartelloni, P. J., Vollmer, R. T. & Beisel, W. R. (1972a) *Metab. Clin. Exp.* **21**, 67-76
- Wannemacher, R. W., Jr., DuPont, H. L., Pekarek, R. S., Powanda, M. C., Schwartz, A., Hornick, R. B. & Beisel, W. R. (1972b) *J. Infec. Dis.* **126**, 77-86
- Wannemacher, R. W., Jr., Pekarek, R. S. & Beisel, W. R. (1972c) *Proc. Soc. Exp. Biol. Med.* **139**, 128-132
- Williams, C. & Newberne, P. M. (1970) *Brit. J. Exp. Pathol.* **51**, 253-264