The Metabolism of Tyrosine and Phenylalanine in Premature Infants: the Effect of Large Doses

By L. I. WOOLF (I.C.I. Research Fellow)
AND MARGARET E. EDMUNDS (Cow and Gate Research Fellow)
Institute of Child Health (University of London), The Hospital for Sick Children, Great Ormond Street, London, W.C. 1

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Effects of a high-protein diet on premature infants were investigated by Levine, Marples & Gordon (1939, 1941; cf. Levine, Dann & Marples, 1943). These infants were fed diets containing 5 g. or more of milk proteins/kg. body weight/day in the absence of ascorbic acid. The excretion of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid rose with increased protein intake and continued as long as the high protein diet was fed, but was prevented or abolished by giving ascorbic acid. These authors found that full-term infants did not excrete these two acids on a similar high-protein diet, but in both premature and full-term infants the excretion of these acids could be initiated (or increased if already present) by feeding a large dose (up to 1 g./kg. body weight) of L-tyrosine or DL-phenylalanine, and thereafter the excretion of these acids continued while the child was fed a high-protein diet free from ascorbic acid.

Work along somewhat similar lines with guinea pigs has been carried out by Sealock & Silberstein (1939, 1940), Sealock, Perkinson & Basinski (1941), Basinski & Sealock (1946), Lan & Sealock (1944), Sealock & Lan (1947), Painter & Zilva (1947, 1948) (cf. also Woodruff & Darby, 1948; Swendseid, Burton & Bethell, 1943; Rodney, Swendseid & Swanson, 1947; Sealock, Ziegler & Driver, 1939; Deuel, 1948; Sealock, 1942; Rienits, 1950).

Dietary protein levels as high as 5 g./kg. body weight/day for infants in the first weeks of life are unusual, at least in Great Britain. The work reported in this communication was carried out to examine the effect of feeding large doses of L-tyrosine or L-phenylalanine to premature infants on more usual diets with and without ascorbic acid. A preliminary investigation of full-term infants is also reported.

EXPERIMENTAL

Collection of urine. The subjects used were twenty infants in a premature baby unit, and nine full-term infants in a maternity ward. For technical reasons only males were suitable. The apparatus used is described in detail elsewhere (Edmunds, 1950). The premature infants selected were normal apart from their prematurity, and the collection of urine was started when the child had reached a fairly steady level of feeding and excretion, and the nursing had become straightforward. Their ages ranged from 2 to 62 days, and weights from 2 lb. 8 oz. to 6 lb. 13 oz. Of these twenty infants, seven were given ascorbic acid 25 mg. twice daily throughout the collection and for 48 hr. beforehand. The feeds were either expressed breast milk or three-quarter strength half-cream National Dried Milk.

A preliminary 24 hr. specimen was collected before the tyrosine was given. Seven infants (nos. 1–7) had 1 g. in the feed at the beginning of the second 24 hr. collection. Six infants (nos. 10–15) had 4 g. of tyrosine; owing to its insolubility it could not all be given in one feed, but it was given instead in divided doses, 1 g./feed, in the first 12 hr. of the second 24 hr. collection. Further 24 hr. collections were made for another 4 or 6 days, depending on the dose of tyrosine; where 4 g. was given, collection of urine was continued longer to ensure complete elimination. The one infant having phenylalanine had it by gavage as he refused the feeds containing it.

The nine full-term infants were normal and breast-fed, and their weights ranged from 5 lb. 13 oz. to 10 lb. The method used was similar to that used for the premature infants, the only difference being that the breast-fed babies had the tyrosine as a suspension in a little water, given by spoon, instead of in the feed. All the collections were started 48 hr. after birth, as otherwise the children would have been discharged before the end of the experiment. Two of these infants were given ascorbic acid (25 mg. daily), starting on the first day of life. While the infant went to the breast a temporary arrangement was made so that a single voiding of the bladder, should it occur, could be collected and added to the rest of the 24 hr. specimen. Even so, the technical difficulty in making complete 24 hr. collections from full-term infants was much greater than from premature infants, owing to the greater activity of the former, and the greater risk of loss while at the breast.

Chlorbutol (2-trichloromethylpropan-2-ol) was used as a preservative. 0·5 ml. of a 50% ethanolic solution was added to each 24 hr. specimen in the ward, and a further quantity added to the larger specimens in the laboratory at the rate of 0·5 ml./100 ml. The specimens were then kept at 0°. This proved an excellent method of preservation, there being no growth of micro-organisms and no chemical changes affecting the Millon reaction even after storage for 7 months.

Analytical methods

Chemical estimations. Hydroxyphenyl compounds were estimated by the method of Folin & Ciocalteu (1927) as used by Levine *et al.* (1941) and Medes (1932). A photoelectric colorimeter with an Ilford 604 filter ($\lambda_{max.} = 520$ mμ.) was

used, and the value read from a standard curve drawn for tyrosine. *p*-Hydroxyphenylpyruvic acid was estimated by reduction of a strongly acid phosphomolybdate (Briggs, 1922) following the method of Medes (1932). To 2 ml. of urine (after treatment with Lloyd's reagent) were added first 5 ml. 4% (w/v) KH_2PO_4 and then 5 ml. 2·5% (w/v) sodium molybdate in 5 N-H_2SO_4. The mixture was diluted to 50 ml. The colour was read 3 hr. later in a photoelectric colorimeter with an Ilford 609 filter (λ_{max} = 710 mμ.) and the amount read from a standard curve drawn for the pure compound. This was prepared from 2-methyl-4-(4'-acetoxybenzal)-5-oxazolone by alkaline hydrolysis (Carter, 1946).

A portion of the urine was rendered acid (pH ~ 1) and extracted six times, each time with an equal volume of ether, to separate the free phenols and phenolic acids from tyrosine + tyramine + conjugated phenols. In some urines a further portion was acid hydrolysed (2 N-H_2SO_4, 100°, 30 min.) and ether extracted six times to allow separate estimation of tyrosine + tyramine and conjugated phenols. It was found in control experiments that the whole of any added *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid could be removed from normal urine by six extractions with ether at pH 1; the apparent hydroxyphenyl content of these infants' urines and of urine containing added *p*-hydroxyphenylpyruvic acid reached a maximum under these hydrolysis conditions and did not decrease on further heating.

Tyramine was estimated by difference in several urines by rendering the urine alkaline with Na_2CO_3, repeatedly extracting with amyl alcohol, and re-estimating tyrosine. About 90% of added tyramine was removed by this method.

Chromatography. Some of the urines were analysed for amino-acids by two-dimensional paper chromatography (Consden, Gordon & Martin, 1944). The urine was hydrolysed by refluxing with 6 N-HCl for 18 hr. and both the hydrolysed and unhydrolysed urines were analysed.

RESULTS

Premature infants

Feeding L-tyrosine or L-phenylalanine to premature infants usually caused a rise in the excretion of hydroxyphenyl compounds; any rise, however, was transient and excretion levels dropped to normal by, at latest, the fifth day (Figs. 1 and 2). The daily intakes of tyrosine and phenylalanine (calculated as tyrosine after multiplying by 1·1, the ratio of molecular weights) were calculated from the known amounts of milk fed using the data provided by Williamson (1944). Tables 1 and 2 give the other necessary data for each infant. Fig. 4 shows the excretion of free phenols for comparison with that of total phenols.

Excretion of *p*-hydroxyphenylpyruvic acid followed that of total hydroxyphenyl compounds, being negligible when the total was low (e.g. infant no. 12, Fig. 7). No tyramine was found in any urine examined.

Paper chromatography

All the urine specimens excreted during the experiment by infants nos. 11, 12, 23 and 24 were examined by this technique. Infant no. 12 excreted large amounts of tyrosine for several days after feeding the amino-acid, the size of the spot after chromatography and colour development with ninhydrin agreeing in a qualitative way with the level of tyrosine estimated by the Folin & Ciocalteu (1927) method. In the urine passed on the day tyrosine was fed, there was no increase in the size of the tyrosine spot after hydrolysis, thus practically all the tyrosine was present as the free amino-acid on this day. On the following days, however, more than half the tyrosine was combined in some form (probably acylated on the α-NH_2, but not necessarily by another amino-acid, see Hier, 1948; Eckhardt, Cooper, Faloon & Davidson, 1948; Eckhardt & Davidson, 1949; Wynn, 1949) from which it was released on acid hydrolysis. This is the normal proportion.

Infant no. 11 excreted a large amount of phenylalanine on the day this was fed, but on all subsequent days the amount of this amino-acid excreted was at its normal very low level. Tyrosine excretion was sharply raised on the day phenylalanine was fed, the size of the spots on a paper chromatogram again agreeing with tyrosine levels estimated for corresponding urines by the method of Folin & Ciocalteu (1927). The bulk of the tyrosine in all the urines was again released only on acid hydrolysis. Only on the day following the feeding of phenylalanine was any considerable amount of free tyrosine excreted.

In full-term infant no. 24 all the urinary tyrosine was free on the day tyrosine was fed, and about half conjugated on subsequent days. In infant no. 23 about half the urinary tyrosine was conjugated on the day tyrosine was fed and on subsequent days. In both infants the size of the tyrosine spot on a paper chromatogram agreed with the urinary tyrosine level determined by the method of Folin & Ciocalteu (1927), being normal by the third day after feeding tyrosine.

In all four infants all the urines, both before and after hydrolysis, showed no abnormal increase or decrease in amount of any amino-acid (or other ninhydrin-reacting substance) other than tyrosine and (in no. 11) phenylalanine.

Full-term infants

All the infants classed as premature had a birth weight under 5·5 lb. and, in addition, the duration of pregnancy was under 40 weeks.

One infant was born at 40 weeks but weighed only 4 lb. 8 oz., and two were born at under 40 weeks but weighed over 5·5 lb. Results of investigations on these infants are recorded in Fig. 6, and Table 3. In addition seven full-term infants were investigated and the results for four of them are shown in Figs. 3 and 5 and Table 4; the remaining three showed similar results, but several of the urine specimens were incomplete. Since there was occasionally some

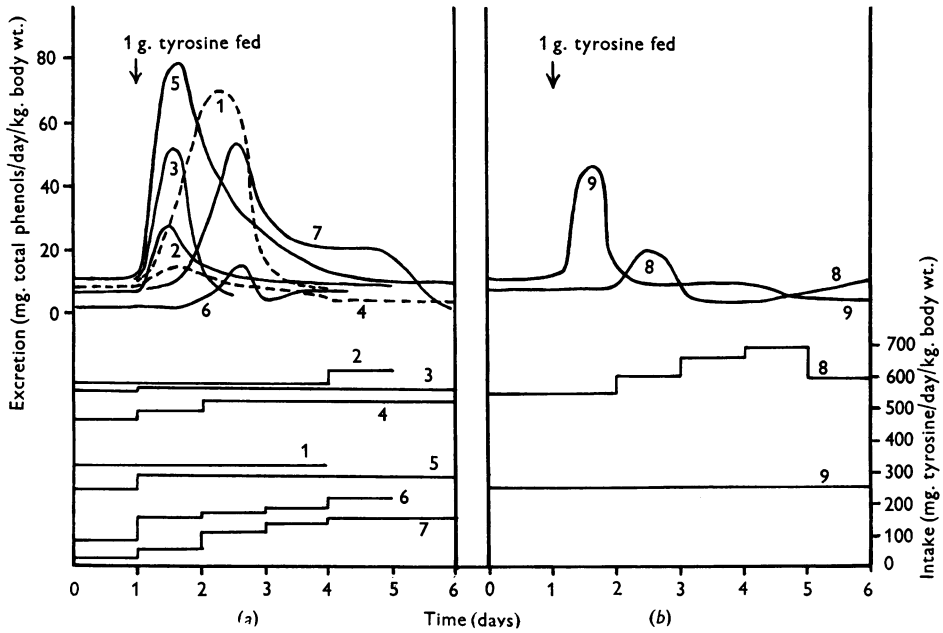

Fig. 1. (*a*) Excretion of total hydroxyphenyl compounds (as tyrosine) by premature infants nos. 1, 2 and 4–7: fed 1 g. tyrosine but no ascorbic acid. Upper curves, smooth curves drawn through histograms for daily excretions; lower curves, dietary intake of tyrosine and phenylalanine, excluding test dose, calculated as tyrosine. (*b*) Excretion of total hydroxyphenyl compounds by premature infants nos. 8 and 9 (upper curves) and dietary intake of tyrosine (lower curves, test dose excluded). Fed 1 g. tyrosine and ascorbic acid.

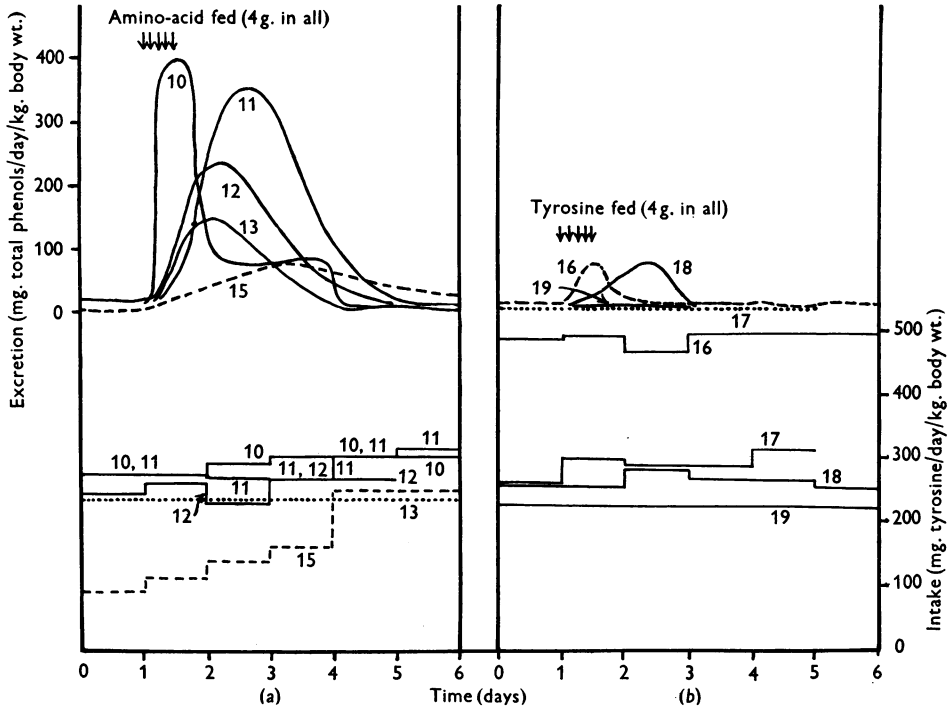

Fig. 2. Excretion of total hydroxyphenyl compounds (upper curves) and dietary intake of tyrosine (lower curves, test dose excluded). (*a*) Premature infants nos. 10, 12, 13 and 15: fed 4 g. tyrosine, and no. 11: fed 4 g. L-phenylalanine. No ascorbic acid. The curves for infant no. 14 were similar and are excluded for clarity. (*b*) Premature infants nos. 16–19: fed 4 g. tyrosine and ascorbic acid.

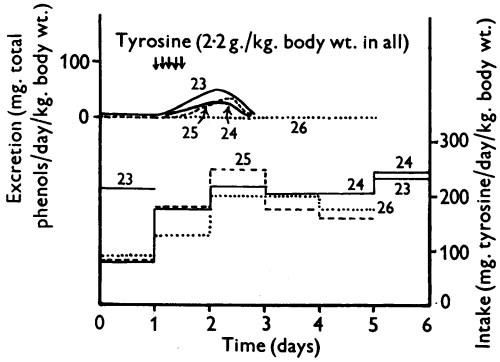

Fig. 3. Excretion of total hydroxyphenyl compounds (upper curves) and dietary intake of tyrosine (lower curves, test dose excluded) for tull-term infants nos. 23–26: fed 2·2 g. tyrosine/kg. body weight. Part of the intake curve for infant no. 23 is omitted for clarity.

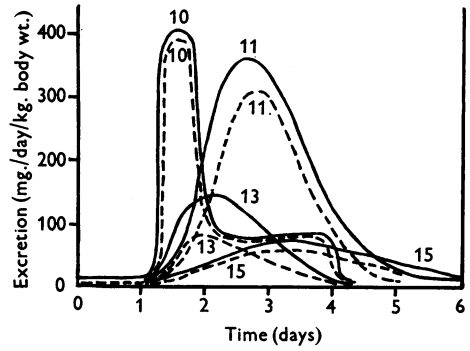

Fig. 4. Excretion of total and unconjugated hydroxyphenyl compounds by premature infants nos. 10, 11, 13 and 15. Shows relative contributions of free and conjugated forms. Total phenols, ——; free phenols, – – –.

Table 1. *Ages, weights and diets of premature infants fed no ascorbic acid since birth*

Infant no.	At birth: Maturity (weeks)	At birth: Weight (kg.)	At time of experiment: Age (days)	At time of experiment: Weight (kg.)	At time of experiment: Diet*	Test dose (mg. tyrosine/kg. body wt.)
1	28	1·13	62	2·01	E.B.M. + N.D.M.	495
2	32	1·42	38	1·95	N.D.M.	513
3	34	1·82	29	2·20	N.D.M.	454
4	34	1·80	19	2·13	N.D.M.	469
5	30	1·57	11	1·32	E.B.M.	756
6	34	2·16	4	2·10	E.B.M.	475
7	32	2·16	2	1·84	E.B.M.	542
10	35·5	1·65	17	2·10	E.B.M.	1901
11	32	1·93	15	1·73	E.B.M.	2540†
12	37	2·10	11	2·04	E.B.M.	1960
13	36	1·97	9	2·01	E.B.M.	1990
14	36	2·49	7	2·27	E.B.M.	1760
15	32	2·08	3	2·08	E.B.M.	1920

* E.B.M.: 'Expressed breast milk', i.e. boiled human milk. N.D.M.: 'half-cream National Dried Milk', i.e. powdered half-cream cow's milk.

† Calculated. Fed 4 g. L-Phenylalanine.

Table 2. *Ages, weights and diets of premature infants fed ascorbic acid*

Infant no.	At birth: Maturity (weeks)	At birth: Weight (kg.)	At time of experiment: Age (days)	At time of experiment: Weight (kg.)	At time of experiment: Diet*	Ascorbic acid given (mg./day)	Test dose (mg. tyrosine/kg. body wt.)
8	33	2·18	14	1·98	N.D.M.	50	504
9	30	1·62	11	1·44	E.B.M.	50	693
16	36	2·98	42	3·09	N.D.M.	50	1295
17	32	1·52	17	2·10	E.B.M.	50	1901
18	37	2·16	11	2·13	E.B.M.	50	1879
19	36	2·12	9	2·10	E.B.M.	50	1901

* As in Table 1.

Table 3. *Ages, weights and diets of three infants who met only one of the criteria of prematurity*

Infant no.	At birth: Maturity (weeks)	At birth: Weight (kg.)	At time of experiment: Age (days)	At time of experiment: Weight (kg.)	At time of experiment: Diet*	Ascorbic acid given (mg./day)	Test dose (mg. tyrosine/kg. body wt.)
20	40	2·16	11	2·04	E.B.M.	50	1960
21	39	2·64	2	2·64	E.B.M.	—	1516
22	38	3·34	0	3·34	B.F.	—	1197

* As in Table 1. B.F. = Breast fed, i.e. unboiled human milk.

Table 4. *Ages, weights and diets of infants with maturity of 40 weeks*

Infant no.	Age (days)	Weight (kg.)*	Diet†	Ascorbic acid (mg./day)	Test dose (mg. tyrosine/kg.)
23	3	2·77	B.F.	—	2162
24	2	3·54	B.F.	—	2260
25	3	3·66	B.F.	50	2180
26	2	2·77	B.F.	50	2162

* Birth weight. † B.F. = Breast fed, i.e. unboiled human milk.

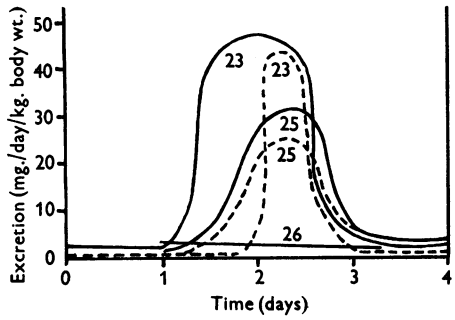

Fig. 5. Excretion of total and unconjugated hydroxyphenyl compounds by full-term infants nos. 23, 25 and 26. Total phenols, ——; free phenols, – – –.

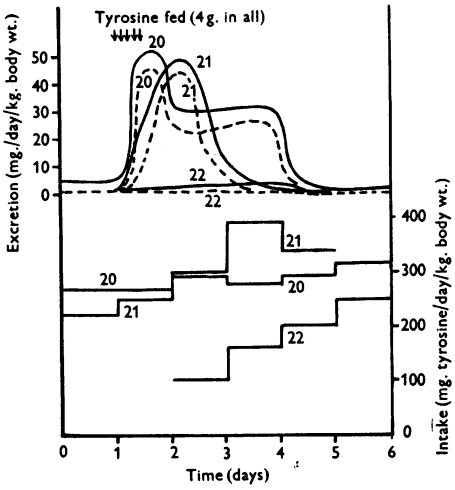

Fig. 6. Excretion of total and unconjugated hydroxyphenyl compounds (upper curves) and dietary intake of tyrosine (lower curves, test dose excluded) for infants nos. 20–22. Total phenols, ——; free phenols, – – –.

slight loss of urine in the four cases considered, some of the results shown in Figs. 3 and 5 may be up to 10% low.

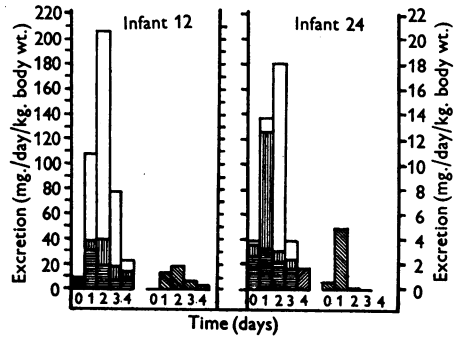

Fig. 7. Daily distribution of excreted phenols for premature infant no. 12 and full-term infant no. 24. The tyrosine test dose was fed on day 1. □, free phenols; ▥, conjugated phenols; ▤, tyrosine; ▦, tyrosine plus conjugated phenols; ▧, *p*-hydroxyphenylpyruvic acid; ▨, tyrosine plus total phenols.

Phenylpyruvic, homogentisic and benzoquinone-acetic acids

Of several urines tested with ferric chloride, only one (infant no. 11, urine of the day L-phenylalanine was fed) gave a green colour persisting over a number of minutes, indicating the presence of phenylpyruvic acid. The colour was very faint, corresponding to less than 5 mg. phenylpyruvic acid/100 ml. urine.

None of the urines examined darkened from above down when made alkaline, nor did they reduce neutral or weakly acid silver nitrate. The addition of a very small amount of homogentisic acid led to positive results with both these tests. Thus homogentisic acid was absent from all the urines examined. The ferric chloride test could not be relied on since

p-hydroxyphenylpyruvic acid gives an ephemeral green colour similar to that given by homogentisic acid.

Iodometric investigations (cf. Neuberger, 1947) revealed no substance with the properties of either homogentisic or benzoquinoneacetic acid in any urine examined except one (infant no. 12, urine of the third day after feeding tyrosine). Since the acidified urine released iodine from potassium iodide, the substance present had properties more in keeping with benzoquinoneacetic than homogentisic acid, but obviously this reaction is not specific. Assuming the oxidation of acid potassium iodide to have been due to benzoquinoneacetic acid, the amount present was only 4 mg./100 ml.

Conjugated phenols

Glucuronides (Tollens naphthoresorcinol test) were found in only one urine (infant no. 25, first urine after feeding tyrosine) and here only in barely detectable traces. Ethereal sulphates (determined gravimetrically as barium sulphate after removing inorganic sulphate) were present in all of several urines tested, and were in some cases equivalent in amount to the conjugated phenols present, in others rather lower (e.g. infant no. 11, Table 5). Indican (Obermayer's test) was absent from all the urines examined.

Table 5. *Distribution of excreted phenols for infant no.* 11

(Fed 4 g. L-phenylalanine on day 1.)

Day	Tyrosine (mg./day)	*p*-Hydroxyphenylpyruvic acid (mg./day)	Free phenols (mg./day)*	Conjugated phenols (mg./day)*	Conjugated phenols (μmol./day)	Ethereal sulphates (μmol./day)
1	45·2	31·1	72·0	15·4	85	65
2	44·8	64·6	518·7	13·3	73·5	78
3	41·4	57·0	348·8	28·2	156·0	109
4	19·0	9·5	63·5	1·7	9·4	—†

* Calculated as tyrosine. † Not determined.

DISCUSSION

The compounds *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid are generally considered to be intermediates in the metabolism of tyrosine. Phenylalanine, it is generally assumed, is converted to tyrosine as the first step in its metabolism (compare infant no. 11, Table 1).

In the normal individual practically all the tyrosine is further metabolized (probably mostly through homogentisic acid) in the liver, and probably elsewhere; the aromatic ring is split, producing β-keto acids which are further metabolized. Only a very small proportion of the tyrosine escapes into the urine as hydroxyphenyl compounds.

In the infants considered here and by Levine *et al.* (1941) and in scorbutic guinea pigs, there is a defect in the further metabolism of tyrosine and a considerable proportion is excreted as the intermediate metabolites, *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylpyruvic acid. There seem to be no ill effects apart from a small loss of potential calorie-providing substances.

Our results and those of Levine *et al.* (1941) agree in many important respects. However, we find the effect of a single large dose of tyrosine or phenylalanine to be transient, while Levine found that similar large test doses initiated this defect in metabolism and it persisted as long as a high protein diet was fed. Similarly, we find no spontaneous excessive excretion of phenolic substances by premature infants fed a high protein diet and no ascorbic acid (e.g. infants nos. 2–4, Fig. 1*a*); Levine *et al.* (1939, 1941) found that in similar circumstances an excessive excretion of phenolic substances occurred nearly always.

Ascorbic acid obviously occupies a key position in the metabolism of tyrosine and it is possible that the pre-natal nutrition of the infants we consider here was different from those of Levine *et al.*, owing to the supply of orange juice available to all expectant mothers in Great Britain.

Levine *et al.* (1941) found that their premature infants had to be fed a high protein diet for several days, or given a large dose of tyrosine or phenylalanine, before the intermediate metabolites appeared in the urine. This suggests either (*a*) that the system needed to be flooded with a large excess of tyrosine before the defect appeared, or (*b*) that a large amount of tyrosine could combine with or cause the destruction or elimination of ascorbic acid, permitting the defect to appear. The second alternative is disproved by the transient nature of the raised excretion of intermediate metabolites after feeding tyrosine in our experiments. Further evidence that tyrosine does not affect ascorbic acid destruction to any marked degree is provided by the finding that the onset of scurvy in guinea pigs, fed less than the minimum protective dose of ascorbic acid, was not significantly accelerated by feeding regular large doses of L-tyrosine (Payne & Woolf, 1949).

The marked effect of ascorbic acid on the metabolism of tyrosine in the premature infant can be

compared with the effect of aneurin on the metabolism of pyruvic acid in various organisms. The foetus stores ascorbic acid during the last few weeks of gestation and, if full-term, is born with a sufficient store to produce no signs of scurvy on an essentially scorbutic diet for about 6 months. This is, however, not necessarily true for premature infants (cf. Park, Guild, Jackson & Bond, 1935; Toverud, 1935). This can be correlated with the full-term infant's greater ability to metabolize tyrosine.

There was great variability in response to the tyrosine test dose among both full-term and premature infants; it would be pointless to try to assign infants nos. 20–22, to either full-term or premature group on the basis of their ability to metabolize the test dose. There is no correlation among the premature infants between peak excretion value reached and maturity at birth, age or birth weight; the duration of excessive excretion is also not correlated with maturity, age or birth weight. The tyrosine and phenylalanine in the normal diet exerted only a small effect on the level of excretion reached. In any one infant, however, a rise in the amount of protein fed during or soon after giving the test dose increased the duration of excessive excretion (cf. nos. 15, 10 and 7, Figs. 1 and 2).

The remarkable efficiency of the human organism in converting L-phenylalanine to L-tyrosine is well illustrated by infant no. 11 (Table 5), who excreted excessive amounts of phenylalanine on only the first day after feeding this amino-acid, but excreted large amounts of tyrosine and its intermediate metabolites on this and the three subsequent days. The very small amount of phenylpyruvic acid excreted compared with the values reported by Levine *et al.* (1941, 1943) is probably connected with our use of L-phenylalanine and their use of DL-phenylalanine (cf. Penrose & Quastel, 1937).

Conjugated phenols contributed a considerable proportion of the total hydroxyphenyl compounds excreted after feeding L-tyrosine to either premature or full-term infants. This was not the result of intestinal bacterial action on the tyrosine and subsequent conjugation of the primary products, since the gut in these infants is virtually sterile (cf. the absence of tyramine and indican from the urine). No explanation can be offered for the conjugation of nearly all the phenols excreted by infants nos. 23 and 24 on the day tyrosine was fed, and of practically none the next day though total phenol excretion was not very different (Figs. 5 and 7). The virtual absence of glucuronides from any urine examined and the presence in several urines of ethereal sulphates in amounts roughly equivalent to the conjugated phenols present (in the absence of indican), indicate that sulphuric acid was the conjugating agent (contrast Blazso, 1935, 1937).

The technical difficulties in studying full-term infants were due to the shortness of their stay in hospital, about 8 days. During this period their intake of protein was varying very rapidly and the volume of urine excreted rose from under 10 ml./day to 150 ml. or more. The premature infants, on the other hand, had had time to become relatively stabilized.

SUMMARY

1. Premature infants on various dietary protein levels have been fed L-tyrosine or L-phenylalanine with and without ascorbic acid. Their excretion of phenolic intermediate metabolites was determined.

2. This excretion reached a peak value soon after feeding the amino-acid, but fell to normal values within a few days. The effect of ascorbic acid was greatly to reduce the excretion of intermediate metabolites.

3. A similar investigation of full-term infants is not yet complete, but there seems as much variation as in the premature infants, especially in response to ascorbic acid.

4. By paper chromatography it has been shown that the excretion of amino-acids other than tyrosine (or phenylalanine where this was fed) was not affected by the test dose of the amino-acid.

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REFERENCES

Basinski, D. H. & Sealock, R. R. (1946). *J. biol. Chem.* **166**, 7.
Blazso, S. (1935). *J. Physiol. Path. gén.* **33**, 1076.
Blazso, S. (1937). *Mag. orv. Arch.* **38**, 378.
Briggs, A. P. (1922). *J. biol. Chem.* **51**, 453.
Carter, H. E. (1946). *Organic Reactions*, **3**, p. 198. New York: John Wiley.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Deuel, H. J. (1948). In Sahyun, M. *Proteins and Amino-acids in Nutrition*, p. 119. New York: Rheinhold Publ. Corp.
Eckhardt, R. D., Cooper, A. M., Faloon, W. W. & Davidson, C. S. (1948). *Trans. N. Y. Acad. Sci.* **10**, 284.
Eckhardt, R. D. & Davidson, C. S. (1949). *J. biol. Chem.* **177**, 687.
Edmunds, M. E. (1950). *Arch. Dis. Childh.* (in the Press).
Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 632.

Hier, S. W. (1948). *Trans. N.Y. Acad. Sci.* **10**, 280.
Lan, T. H. & Sealock, R. R. (1944). *J. biol. Chem.* **155**, 483.
Levine, S. Z., Dann, M. & Marples, E. (1943). *J. clin. Invest.* **22**, 551.
Levine, S. Z., Marples, E. & Gordon, H. H. (1939). *Science*, **90**, 620.
Levine, S. Z., Marples, E. & Gordon, H. H. (1941). *J. clin. Invest.* **20**, 199.
Medes, G. (1932). *Biochem. J.* **26**, 917.
Neuberger, A. (1947). *Biochem. J.* **41**, 431.
Painter, H. A. & Zilva, S. S. (1947). *Biochem. J.* **41**, 511.
Painter, H. A. & Zilva, S. S. (1948). *Biochem. J.* **43**, 398.
Park, E. A., Guild, H. G., Jackson, D. & Bond, M. (1935). *Arch. Dis. Childh.* **10**, 265.
Payne, W. H. & Woolf, L. I. (1949). Unpublished observations.
Penrose, L. & Quastel, J. H. (1937). *Biochem. J.* **31**, 266.
Rienits, K. G. (1950). *J. biol. Chem.* **182**, 11.
Rodney, G., Swendseid, M. E. & Swanson, A. L. (1947). *J. biol. Chem.* **168**, 395.
Sealock, R. R. (1942). *Fed. Proc.* **1**, 287.
Sealock, R. R. & Lan, T. H. (1947). *J. biol. Chem.* **167**, 689.
Sealock, R. R., Perkinson, J. D. & Basinski, D. H. (1941). *J. biol. Chem.* **140**, 153.
Sealock, R. R. & Silberstein, H. E. (1939). *Science*, **90**, 517.
Sealock, R. R. & Silberstein, H. E. (1940). *J. biol. Chem.* **135**, 251.
Sealock, R. R., Ziegler, B. & Driver, R. L. (1939). *J. biol. Chem.* **128**, lxxxix.
Swendseid, M. E., Burton, I. F. & Bethell, F. H. (1943). *Proc. Soc. exp. Biol., N.Y.*, **52**, 202.
Toverud, K. U. (1935). *Arch. Dis. Childh.* **10**, 313.
Williamson, M. B. (1944). *J. biol. Chem.* **156**, 50.
Woodruff, C. W. & Darby, W. J. (1948). *J. biol. Chem.* **172**, 851.
Wynn, V. (1949). *Nature, Lond.*, **164**, 445.

Sodium Ethanemonothiophosphonate. A Weak Antidote to Mustard Gas

By E. R. HOLIDAY, J. ST L. PHILPOT and L. A. STOCKEN
Department of Biochemistry, University of Oxford

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The following work was done in 1940 as part of an antigas programme directed by Prof. R. A. Peters on behalf of the Ministry of Supply. The objective was to discover an antidote to mustard gas (*H*) which could act at a late stage when the vesicant had soaked into living tissue. A review by Peters (1947) gives the references to the original reports to the Ministry and the contribution made by the Oxford group to this problem.

It was assumed as a working hypothesis that the toxicity of *H* was due to the chlorine replacement reaction, the *in vitro* kinetics of which were studied as part of the same programme by Ogston (1948). The interesting feature of this reaction is that the slow disappearance of *H* in aqueous media cannot be accelerated by reagents which replace the chlorine, but that such reagents compete with each other to form *H* substitution products, and that an active reagent may almost completely suppress an inactive one. The power to compete is quantitatively expressible by the 'competition factor' which was measured by Ogston for about eighty substances.

Only the most hopeful of these substances were tested biologically because of shortage of animals at that time. The few rats available sufficed to show that none of the selected substances was a sufficiently good antidote to be of practical importance; but by using forty rats on one substance (sodium ethanemonothiophosphonate) a weak protective action was demonstrated statistically. This seems to be the only substance which has been proved to give any internal protection against *H*. It was prepared by a new method which is described below together with such biological tests as could be done in the circumstances.

EXPERIMENTAL

Preparation of mono- and di-thiophosphonates

In 1940 three methods were available for the preparation of thiophosphonates. Hofmann & Mahla (1892) treated substituted phosphines with sulphur; Guichard (1899) obtained $RPSCl_2$ by the action of S on $RPCl_2$ and Strecker & Grossmann (1916) prepared R_2PSOH from $PSCl_3$ and Grignard reagents. Since none of these methods was satisfactory, the reaction between Grignard reagents and P_2S_5 was investigated and found to be convenient for the preparation of alkyl or aryl dithiophosphonates ($RPOS_2Na_2$). All the thiophosphonates were made by the same process, the ethanethiophosphonates being typical examples.

A more complete investigation of the reaction between P_2S_5 and Grignard reagents was subsequently carried out by Malatesta & Pizzotti (1946). These authors claim that quantitative yields of dithiophosphates can be obtained by a suitable choice of experimental conditions.

Disodium ethanedithiophosphonate. Ethyl bromide (327 g.) was converted to the Grignard reagent by dropping on to Mg (72 g.) in dry ether (600 ml.). The solution was then slowly added to a suspension of finely powdered (60 mesh sieve) P_2S_5 (426 g.) in dry ether (400 ml.) at such a rate that the ether was kept gently boiling. This took about 2 hr.