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An Inborn Defect in the Metabolism of Tyrosine in Infants on a Normal Diet

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Levine and his coworkers (Levine, Marples & Gordon, 1939, 1941; Levine, Gordon & Marples, 1941; Levine, Dann & Marples, 1943) showed that premature, but not full-term, infants excrete *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid if fed on a high protein diet, but both groups of infants excrete these compounds if given a 'loading' dose of tyrosine or phenylalanine. This was confirmed and extended by Woolf & Edmunds (1950), who showed that, after a 'loading' dose of tyrosine or phenylalanine, both premature and full-term infants exhibit a temporary hydroxyphenyluria followed by a return to normal values though the peak excretion is much higher in the premature group.

Ascorbic acid promptly cured the hydroxyphenyluria in these infants (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950) and it rarely appeared in an infant that had ever been fed with ascorbic acid. Adults or infants suffering from scurvy also showed hydroxyphenyluria when fed with large doses of tyrosine (Rogers & Gardner, 1949; Morris, Harpur & Goldbloom, 1950) and here again feeding with ascorbic acid cured the hydroxyphenyluria. Independently of the above

work, Sealock & Silberstein (1939, 1940) had shown that scorbutic guinea pigs developed hydroxyphenyluria if they were fed with tyrosine. The effect of ascorbic acid on tyrosine metabolism by liver slices *etc. in vitro* has been reviewed by Knox (1955) (see also Zannoni & La Du, 1960).

Hydroxyphenyluria also occurs in advanced liver disease (Felix, Leonhardi & Glasenapp, 1951; L. I. Woolf, unpublished work); in this condition ascorbic acid has no effect.

Work *in vitro* on the foetal and neonatal development of enzymes concerned with the metabolism of phenylalanine and tyrosine has been carried out by Kretchmer, Levine, McNamara & Barnett (1956), Kretchmer & McNamara (1956), Reem & Kretchmer (1957), Kenney, Reem & Kretchmer (1958) and Auerbach & Waisman (1959).

During a survey of young infants in Cardiff to detect cases of phenylketonuria (Gibbs & Woolf, 1959), several infants were found whose urine gave a green colour with ferric chloride, but who were not phenylketonurics. As reported below, this has been found to be due to *p*-hydroxyphenylpyruvic acid and these urines have also been found

Table 1. *Details of the infants investigated*

D.M., Dried milk; B.F., breast-fed (figures in parentheses indicate the number of days that the infant was breast-fed after birth); —, not known.

Infant no.	Age (days)	Wt. (kg.)	Diet		Ascorbic acid supplement		Birth wt. (kg.)	Maturity at birth (weeks)
			Type	g./day	During pregnancy	After birth		
1	25	3.32	Cow's milk	610	Occasional	None	2.50	—
	32	3.32		(ml.)				
2	30	—	D.M. (B.F. 2)	37.2	Adequate	None	2.78	35½
3	38	—	D.M. (B.F. 2)	26.6	Adequate	From 1 month	2.04	—
	47	—		35.4				
	68	—		—				
4	54	—	D.M. (B.F. 7)	26.6	Adequate	From 6 weeks	1.68	37
5	26	3.49	D.M.	37.2	Adequate	From 1 month	3.04	38
6	37	—	D.M. (B.F. 8)	31.8	None	From 8 days	2.40	34
	41	3.14		—				
7	38	3.51	D.M.	31.8	—	From birth	2.36	38
	41	3.68		—				
8	30	—	B.F. + mixed	—	Adequate	From 1 month	3.06	40½
	33	4.31						
9	48	4.76	D.M. (B.F. 3)	35.4	Adequate	From 1 month	2.42	36
10	22	4.25	D.M. (B.F. 28)	32	Adequate	None	3.74	41½
11	39	4.65	D.M. (B.F. 14)	44	Very little	From 1 month	2.95	40
12	24	3.86	D.M.	48	Irregular	From 1 month	3.44	42½
	30	—						
	44	—						
13	26	3.97	D.M. (B.F. 9)	—	Adequate	From 1 month	3.12	40
14	35	3.70	D.M. (B.F. 2)	37.2	None	From 4 weeks	2.61	39½
	40	3.70						

to contain large amounts of *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid. This hydroxyphenyluria is discussed in relation to the maturation of enzyme systems, to diet and to prematurity.

MATERIALS

Urine specimens from 1276 infants, aged between 3 and 8 weeks, were examined. In each case the mother collected the produce of a single voiding at home in a clean bottle containing a little chlorbutol as preservative. Fourteen infants passed urine giving a green colour with ferric chloride (excluding the one phenylketonuric detected). These specimens were investigated more fully and efforts were made to get further urine specimens from these infants. Details of the infants are given in Table 1.

Several preparations of dried milk (half-cream, full-cream, National or proprietary) were used. Only ascorbic acid taken in the form of orange juice, rose-hip syrup or some proprietary preparation is shown in Table 1. Ascorbic acid from other sources (e.g. breast milk or potatoes) is ignored. In column 9 the 'maturity' is the interval between the last menstrual period and birth, where known.

p-Hydroxyphenyl-lactic acid was prepared by reducing *p*-hydroxyphenylpyruvic acid (Sigma Chemical Co.) with sodium amalgam in acid solution. The material obtained from an ethereal extract was crystallized from ethyl acetate-benzene. The white crystals were dried over P_2O_5 , KOH and paraffin wax *in vacuo*. The material gave a single spot in the expected position on paper chromatograms giving the expected reactions with diazotized sulphanilamide and *p*-nitroaniline.

METHODS

A 5% (w/v) solution of $FeCl_3 \cdot 6H_2O$ in water was added drop by drop to a portion of the urine. A Phenistix strip (Ames Co., Nuffield House, London, W. 1) was dipped into a second portion. A saturated solution of 2:4-dinitrophenylhydrazine in 2*N*-HCl was, in most cases, added to a third portion.

Some of the specimens were so small in volume that not all the investigations could be carried out. Tyrosine (including tyramine), free phenols, conjugated phenols and *p*-hydroxyphenylpyruvic acid were estimated as before (Woolf & Edmunds, 1950; Medes, 1932). The ethereal extract of the acidified urine was re-extracted with sodium

bicarbonate solution and the phenolic acids were examined by paper chromatography. Girard's reagent P (Girard & Sandulesco, 1936) was added to a fresh portion of the urine, the solution was left for 10 min., brought to pH 2 and extracted six times with an equal volume of ether each time; the pooled ethereal extracts were re-extracted with sodium bicarbonate solution and a portion of this was tested for compounds capable of reducing Brigg's reagent (strongly acid phosphomolybdate) (cf. Berry & Woolf, 1952). Other portions were used for paper chromatography of non-ketonic phenolic acids.

Paper chromatography was used to examine the urines for amino acids (Woolf & Norman, 1957), sugars, phenolic substances (one- and two-dimensional, for solvents cf. Armstrong, Shaw & Wall, 1956) and indole derivatives (one- and two-dimensional, Jepson, 1955). The spray reagent used for phenols was generally diazotized sulphanilamide (Block, Durrum & Zweig, 1958), but in some cases duplicate chromatograms were sprayed with a *p*-nitrobenzenediazonium reagent prepared as follows: an aqueous 0.5% solution of Brentamine Fast Red GG (Imperial Chemical Industries Ltd.) was mixed with an equal volume of aqueous 50% (w/v) sodium acetate solution, the chromatogram was immediately sprayed with the mixture and resprayed, 1 min. later, with half-saturated sodium carbonate solution (cf. Harvey & Penketh, 1957).

Two specimens of urine were hydrolysed by refluxing with 6*N*-HCl for 16 hr. and the amino acids present were compared with the unhydrolysed specimens by paper chromatography. The specimen from infant no. 3, 47 days, was ultrafiltered through Visking regenerated cellulose tubing, and the unfiltered residue, about three times concentrated with respect to colloids, was hydrolysed and chromatographed as described above. The ultrafiltrate was passed through a column (10 cm. $\times$ 1 cm. diam.) of Zeo-Karb 225 (a sulphonated polystyrene resin, about 8% cross-linked, H form, beads approx. 0.5 mm. diam.) and the material unadsorbed by the acid resin was passed through a column (10 cm. $\times$ 1 cm. diam.) of Amberlite IR-45 (a weakly basic resin, OH form, beads approx. 0.5 mm. diam.). The ammonia eluate of the Zeo-Karb column was divided into two, and one half was hydrolysed as described above; both hydrolysed and unhydrolysed fractions were then chromatographed for amino acids. The N -H₂SO₄ eluate of the Amberlite IR-45 column and the material adsorbed by neither resin were hydrolysed and chromatographed as described above.

RESULTS

The results of the ferric chloride and 2:4-dinitrophenylhydrazine reactions, and the concentrations of hydroxyphenyl compounds, are given in Table 2. All the specimens either gave no reaction with Phenistix or produced a fleeting green colour. After paper chromatography in butan-1-ol-acetic acid-water (4:1:1), *p*-hydroxyphenylpyruvic acid, unlike *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid, which give non-fluorescent red spots, gave only a very faint brown spot with the diazotized sulphanilamide reagent but the spot fluoresced brilliant blue in ultraviolet light (3650 Å). *p*-Hydroxyphenylpyruvic acid ran to the

same position as *p*-hydroxyphenyl-lactic acid but both could be detected, if present together, with the diazotized sulphanilamide reagent by reading in visible and in ultraviolet light. On two-dimensional chromatograms any *p*-hydroxyphenylpyruvic acid present was generally destroyed. Apart from *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid, tyrosine was generally the only phenolic substance present in abnormal amounts. However, four specimens (from infants nos. 1, 5 and 14) contained, in addition to these three substances, considerable amounts of an unidentified substance, of high *R_F*, both in butanol-acetic acid and in propan-2-ol-ammonia, that gave a yellow colour with the diazotized sulphanilamide reagent and a red colour with the *p*-nitrobenzenediazonium reagent. Incubation of *p*-hydroxyphenylpyruvic acid solution at various temperatures and pH values, with and without urea and ammonium sulphate, failed to produce this substance, but produced varying amounts of *p*-hydroxyphenylacetic acid. In 4 hr. at room temperature, most *p*-hydroxyphenylacetic acid was formed at pH 9.0-9.6; less was formed at pH 6.4 and none at pH 3.2. The amount of *p*-hydroxyphenylacetic acid found in the urines was about one-tenth of the amount of *p*-hydroxyphenyl-lactic acid. The ethereal extracts of the acidified urine contained *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid, but in the presence of Girard's reagent P only *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid were extractable, giving further evidence of the identity of the keto acid.

No abnormal indole derivatives were found in the 11 specimens examined. However, the indican content was low or very low in all and none could be detected in two (cf. Woolf & Edmunds, 1950).

The sugars in the five specimens examined were normal (Woolf & Norman, 1957), the galactose content ranging from a trace to 12 mg./100 ml., the fructose from 0 to 10 mg./100 ml. and the glucose from 0 to 10 mg./100 ml. No other sugars were detected.

Protein was absent from all the urine specimens. Of the ten specimens examined semi-quantitatively for amino acids, six had tyrosine present in amounts greatly above the normal, in two it was normal or slightly raised in amount, in one it was almost absent and in one it could not be detected at all. Taurine was present in very large amount in the last two specimens but was absent, or present in very low concentration, in the other eight. The other amino acids, including phenylalanine, were normal for infants of this age (Woolf & Norman, 1957). Homogentisic acid, which gives a characteristic brown spot, and 3:4-dihydroxyphenylalanine were not detected in any specimen. No

Table 2. *Analysis of the urine specimens*

+, Indicates formation of a precipitate with 2:4-dinitrophenylhydrazine or of a green colour with ferric chloride.

Infant no.	Age (days)	FeCl ₃ reaction	2:4-Dinitro-phenylhydrazine reaction	<i>p</i> -Hydroxy-phenylpyruvic acid (mg./100 ml.)	Free phenols (as tyrosine) (mg./100 ml.)	Conjugated phenols (as tyrosine) (mg./100 ml.)	Tyrosine (mg./100 ml.)	Creatinine (mg./100 ml.)
1	25	+	+	25.7	189	33	28	36
	32	+	+	11.8	91	10	32	17
2	30	+	+	22	215	58	18	.
	79	-	.	.	.	.	.	.
3	38	+	.	.	.	.	.	.
	47	+	+	12	97	11	7	8
	68	+	+	3.9	44	7	11	21
	103	-	.	.	.	.	.	.
4	54	+	+	9.2	66	10	15	15
	66	+	+	6	19	35	5	10
	84	-	.	.	.	.	.	.
5	26	+	+	7.5	51	23	23	42
	47	-	.	.	.	.	.	.
6	37	+	.	.	.	.	.	.
	41	-	-	0	3	2	0	17
7	38	+	+	19.2	116	63	2	54
	41	-	-	3.3	10	18	6	10
8	30	+	.	.	.	.	.	.
	33	-	-	.	.	.	.	12
9	38	+	.	.	.	.	.	.
	48	+	+	12.4	91	24	18	25
	66	-	.	.	.	.	.	.
10	22	+	-	.	.	.	.	.
	36	-	.	.	.	.	.	.
11	39	+	+	9.0	37	28	2	11
	53	+	.	.	.	.	.	.
	60	-	.	.	.	.	.	.
12	24	+	.	.	.	.	.	.
	30	+	.	.	.	.	.	.
	44	-	.	.	.	.	.	.
13	26	+	+	4.5	63	14	4	23
	40	-	.	.	.	.	.	.
14	35	+	+	24.4	152	52	39	55
	40	-	.	.	.	.	.	.

spot in the position of tyrosine *O*-sulphate was found in any specimen of urine.

In the two specimens examined (from infant no. 3, 47 days, and infant no. 4, 66 days) the conjugated amino acids released on acid hydrolysis were normal; in particular no extra tyrosine was released from the second and the tyrosine content in the first rose on hydrolysis from about 0.07 mg./ml. to about 0.1 mg./ml. On fractionation of this specimen, the residue from ultrafiltration released on hydrolysis no more tyrosine than did the original urine. Acid hydrolysis of the ammonia eluate of the Zeo-Karb released as much tyrosine, within experimental error, as was released from the original urine. The material adsorbed by the basic resin released only a trace of tyrosine on hydrolysis, but none could be detected in the hydrolysate of the unadsorbed fraction.

DISCUSSION

Where the enzyme system converting *p*-hydroxyphenylpyruvic acid into homogentisic acid (Knox & LeMay-Knox, 1951) is functioning poorly, or not at all, dietary phenylalanine and tyrosine can produce hydroxyphenyluria, especially if a high 'loading' dose is given. This can be due to absence of a cofactor such as ascorbic acid, to immaturity or to damage to the liver cells.

Zannoni & La Du (1960) have shown that the liver of scorbutic guinea pigs contains as much *p*-hydroxyphenylpyruvic acid oxidase as in non-scorbutic guinea pigs, but that in the scorbutic animals this enzyme is inactivated by excess of its substrate, both *in vivo* and *in vitro*, and that ascorbic acid prevents or reverses this inactivation. A similar mechanism would explain the results

obtained by Woolf & Edmunds (1950) in premature infants. Ascorbic acid appears to act by keeping some other compound, possibly the true coenzyme, in an active state (Zannoni & La Du, 1959, 1960).

It seems probable that infants store ascorbic acid during the last few weeks of gestation (Park, Guild, Jackson & Bond, 1935; Toverud, 1935; Steinebrei, 1939). The induced hydroxyphenyluria of prematurity (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950) is probably due to a simple lack of ascorbic acid, as shown by the extremely rapid reversion to normal on giving this vitamin. In the cases reported here, seven had received supplements of ascorbic acid (according to the mothers' accounts) for some time before passing the hydroxyphenyluric urine. Ten of the infants were breast-fed for a time, which might provide an additional source of ascorbic acid. None of the mothers showed signs of ascorbic acid deficiency during pregnancy, and seven of them took supplementary ascorbic acid. It seems improbable that these infants lacked ascorbic acid, and in any case deficiency of this vitamin will not produce hydroxyphenyluria unless the premature infant or scorbutic infant or adult is also given a high-protein diet or a 'loading' dose of phenylalanine or tyrosine (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950; Rogers & Gardner, 1949; Morris *et al.* 1950).

None of the infants' diets reached the level of 5 g. of protein/kg. body wt./day (see Table 1), and their weight gains were consistent with this level of feeding (cf. Levin, Mackay, Neill, Oberholzer & Whitehead, 1959). This level was found by Levine *et al.* to be generally necessary for establishing hydroxyphenyluria in premature infants.

In a previous study (Woolf & Edmunds, 1950) small premature infants who had never received ascorbic acid were fed with a dose of 4 g. of tyrosine, about seven times their normal daily intake. Their mean birth weight was 1.88 kg., the gestation period ranged from 28 to 37 weeks (mean 33 weeks), and the sum of age and gestation was between 31½ and 38½ weeks (mean 35½ weeks). The concentrations of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyl-lactic acid in their urine after the test dose were no higher than in the present group of infants, who were more mature on each of the three criteria of birth weight, gestation period and age plus gestation (Table 1). About one-quarter of the dietary phenylalanine and tyrosine was excreted as hydroxyphenyl derivatives by the present group of infants (calculated from the creatinine concentrations). The infants studied by Woolf & Edmunds (1950) were plainly metabolizing *p*-hydroxyphenylpyruvic acid at a far greater rate, but in both groups either some

p-hydroxyphenylpyruvic oxidase was present or some alternative pathway of tyrosine metabolism was being used.

Kretchmer *et al.* (1956) reported that liver homogenates from the majority of very premature infants lacked the enzymes necessary for oxidizing tyrosine to maleylacetoacetic acid and that, unlike adult-liver homogenates, the addition of ascorbic acid had little effect. The hydroxyphenyluria reported here is, we suggest, also due to the failure to synthesize an enzyme, either completely or in sufficient amount, even though the infants were not nearly so premature as those investigated by Kretchmer *et al.* (1956), or even as premature as infants in whom this ability had already appeared (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950). In spite of this immaturity of one enzyme system the children were well, with no evidence of jaundice or liver disease and, in particular, phenylalanine hydroxylase and the enzymes concerned with tryptophan metabolism and galactose metabolism appear to have developed normally. Tyrosine transaminase was evidently active since *p*-hydroxyphenylpyruvic acid was formed, but it is uncertain whether the high excretion of tyrosine reflected a lower transaminase activity than normal or the accumulation of *p*-hydroxyphenylpyruvic acid leading to increased reconversion into tyrosine.

This enzyme defect can be compared with tyrosinosis, as described by Medes (1932), and the inborn errors of metabolism. Unlike these, however, the hydroxyphenyluria in these infants was transient, vanishing remarkably abruptly (Table 2). We suggest that this enzyme system suddenly becomes active at an age which varies from individual to individual with a continuous distribution from (say) 5 to 12 months from conception. Thereafter its activity probably increases more gradually since Kretchmer *et al.* (1956) found that adult liver had, weight for weight, about four times the tyrosine-oxidizing activity of normal full-term liver. It would seem that a person can live and thrive for a time without a normal amount of this enzyme. If this were not so the incidence of hydroxyphenyluria would surely be much lower since selection would operate against it unless there were some compensating genetic advantage.

Five of these 14 infants weighed less than 2.5 kg. at birth, but only 7.5 % of infants born in Cardiff during the period were premature on a weight basis. This indicates some correlation between prematurity and immaturity of the enzyme system for metabolizing tyrosine, but prematurity is neither a necessary nor a sufficient cause of the hydroxyphenyluria. The incidence of hydroxyphenyluria, even at an age greater than 3 weeks, may well be higher than is suggested by 14 cases out of 1276 infants tested, since the ferric chloride reaction is

not very reliable for detecting *p*-hydroxyphenylpyruvic acid, being very sensitive to variation in experimental conditions, and mild cases, if they occur, would probably excrete very little of the keto acid, the major metabolite being *p*-hydroxyphenyl-lactic acid (cf. Levine *et al.* 1941).

p-Hydroxyphenylacetic acid has not previously been reported to occur in hydroxyphenyluric urine. It could arise *in vivo* from *p*-hydroxyphenylpyruvic acid by oxidative decarboxylation (Kirberger & Bücher, 1952), or it could be an artifact formed after the urine is voided. As noted above, *p*-hydroxyphenylpyruvic acid solution gives rise to *p*-hydroxyphenylacetic acid on standing at neutral or alkaline pH.

The absence of 3:4-dihydroxyphenylalanine from these urines is a further difference from tyrosinosis (Medes, 1932). In one of the two specimens examined, a considerable fraction of the total tyrosine was in conjugated form, as found before (Woolf & Edmunds, 1950). The tyrosine conjugate was not appreciably held back by ultrafiltration, and therefore it is not a protein or high-molecular-weight peptide. Almost the whole of the tyrosine conjugate was adsorbed by the strong acid resin; it follows that it had basic properties, unlike tyrosine *O*-sulphate, *N*-acyl derivatives of tyrosine or tyrosine glucuronide. These strongly acid conjugates are adsorbed by basic resins, but they must have been present only in traces in the urine. The bulk of the conjugated tyrosine is probably present as a peptide (or peptides) of low molecular weight. Tyrosine *O*-sulphate has been found in human urine by Tallan, Bella, Stein & Moore (1955), who suggest that it may be a conjugate formed from tyrosine as other phenols form ethereal sulphates. However, no tyrosine *O*-sulphate was found in any of the urines examined by us, including the specimen which contained much bound tyrosine. Although the method we used would not detect very small amounts of tyrosine *O*-sulphate, such as are present in normal adult urine, it is clear that at most only a small fraction of the total tyrosine was present as the ethereal sulphate, in spite of the very large amounts of tyrosine that must have accumulated in these infants and that were being excreted in free and bound forms. This is in accordance with the work of Dodgson, Rose & Tudball (1959) and of Grimes (1959), who showed that tyrosine is not sulphated by various tissue preparations *in vitro*.

The absence of indolyl-lactic acid and indolyl-acetic acid from the urine can be contrasted with the findings in phenylketonuria (Armstrong & Robinson, 1954) and 'maple-syrup urine' disease (Mackenzie & Woolf, 1959), other conditions in which large amounts of an α -oxo acid accumulate in the body. It suggests that *p*-hydroxyphenyl-

pyruvic acid may have less effect on general metabolism than some other α -oxo acids, since tryptophan metabolism is not interfered with, and this may, perhaps, be correlated with the good health of the infants reported here.

SUMMARY

1. Fourteen infants, out of 1276 tested, were found to excrete large amounts of *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-lactic acid and tyrosine. *p*-Hydroxyphenylacetic acid was present in the urine but may be an artifact. Tyrosine *O*-sulphate was not detected. A variable proportion of the tyrosine was conjugated as a low-molecular-weight compound with basic properties.

2. In contrast with previous reports of hydroxyphenyluria, these infants were fed on a normal diet with only a moderate protein intake and (in seven cases) supplementary ascorbic acid. They were well, with no evidence of liver dysfunction. Some were premature and some full-term, and they were aged up to 68 days. The hydroxyphenyluria ceased abruptly after a time without any treatment.

3. It is suggested that these infants lacked an enzyme concerned with oxidizing *p*-hydroxyphenylpyruvic acid to homogentisic acid and that the ability to carry out this reaction first appears at different absolute ages in different individuals.

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A Tissue Homogenizer

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For the separation and isolation of the particulate fractions existing within cells, the cells must be disrupted. In studies of the action of toxic substances upon mitochondria it was important that we should use a method of homogenization which was both efficient and reproducible. It was also essential that if the homogenizer was broken it could be readily replaced by one with an identical performance. The above criteria are not all fulfilled by previously described homogenizers.

The homogenizer described has been designed on the general principles of that of Potter & Elvehjem (1936) and the constructional details are given in Fig. 1. A Perspex pestle rotates in a smooth precision-bore glass tube. Since Perspex is easy to machine a precise gap between pestle and mortar may be readily obtained. Instead of the usual spherical end, the end of the pestle is a cone of 90° angle; this fits accurately into the base cavity and also allows the shape to be accurately repeated when required.

It is essential to use a motor with sufficient power ($\frac{1}{4}$ h.p.) to prevent slowing of the pestle when the tissue and fluid is being forced through the gap between the pestle and mortar. Under

these conditions the gap between pestle and mortar is likely to remain constant, thus maintaining constant the shearing forces in the fluid. The lack of contact between the two surfaces is indicated by the very slight wear in the pestle; after use for 18 months the diameter of the pestle had decreased by only 0.001 in. at the bottom and less at the top.

For the preparation of mitochondria the pestle has been rotated at 1100 rev./min. and the gap between the pestle and mortar varied for different tissues (0.01 in. for liver and 0.005 in. for brain). The properties of the liver and brain mitochondria so prepared have been previously described (Aldridge, 1957, 1958; Aldridge & Parker, 1960). Preliminary experiments have shown that with a gap of 0.005 in. mitochondria may be isolated from rat kidney. The oxidation of pyruvate and fumarate by this preparation is stimulated threefold by 2:4-dinitrophenol and potato apyrase and has an activity of 300 μ l. of O_2 /mg. of protein/hr. (for experimental details cf. Aldridge, 1958). Heart mitochondria could only be isolated (though in poor yield) when the tissue had been chopped (McIlwain & Buddle, 1953) before homogenization with a gap of 0.005 in. Such preparations were stimulated