Table 1. Summary of experiment and results.

Group No.	Composition of diet (%)					Ingested isotopes per gram of bone ash (%)		
	Ca	P	Ca/P	Carbonate	Lactate	Sr^{89}	Ca^{45}	Sr^{89}/Ca^{45}
1	0.1	0.12	0.8		0.38	1.8 ± 0.2	8.1 ± 1.0	0.22 ± 0.02
2	0.1	0.12	0.8	0.13		2.3 ± 0.3	9.1 ± 1.0	0.25 ± 0.02
3	0.1	0.12	0.8	3.1		1.5 ± 0.3	4.4 ± 0.9	0.34 ± 0.02
4	2.0	0.12	17		8.8	1.0 ± 0.1	1.7 ± 0.2	0.60 ± 0.02
5	2.0	0.12	17	3.0		0.74 ± 0.16	1.6 ± 0.2	0.46 ± 0.07
6	2.0	2.4	0.8	3.0		0.34 ± 0.04	0.84 ± 0.19	0.41 ± 0.04
A†	0.1	0.5	0.2		0.38	0.87 ± 0.19‡	4.4 ± 0.7	0.20 ± 0.02‡
B†	2.0	0.5	4.0		8.8	0.43 ± 0.15‡	0.79 ± 0.28	0.55 ± 0.09‡

* All values are for an average of four animals, plus or minus one standard deviation.
† Results of previously reported experiment (see *1*).
‡ Strontium-90 rather than Sr^{89} was employed in these experiments (see *1*).

bonate, and lactate. The composition of the diets with respect to these variables is indicated in Table 1. For the 3 days prior to sacrifice, Sr^{89} and Ca^{45} were added to all of the diets. Other details of diet and procedure were as previously described (*1*). The rats employed were mature females of the Sprague-Dawley strain.

The concentrations of Sr^{89} and Ca^{45} in the femur, at sacrifice, expressed as percentages of total isotope fed, are recorded in Table 1. Also shown, as groups A and B, are comparable data from the previously reported experiment (*1*). Animals in groups A and B were sacrificed after 3 days on a Sr^{90}, Ca^{45} regimen; however, their period on the experimental diet prior to the addition of radioisotopes was 30 days, rather than the 5-day conditioning period employed for groups 1 through 6.

The previously reported effect of dietary calcium level on the ratio of Sr^{90}/Ca^{45} deposition in bone (see data for groups A and B) is confirmed by the results from groups 1 and 4. The animals fed a 2.0-percent calcium level diet show a Sr^{89}/Ca^{45} ratio nearly three times that of the animals fed the 0.1-percent calcium level diet. The lower phosphate content of the diets of groups 1 and 4 as compared with the diets of groups A and B had no apparent effect on the Sr^{89}/Ca^{45} ratio but did increase by a factor of two the absolute deposition of both Sr^{89} and Ca^{45}.

In groups A, B, 1, and 4, supplementary calcium was added as the lactate. Groups 2 and 5 correspond, respectively, to groups 1 and 4, except that supplementary calcium was added as the carbonate. The effect of dietary calcium level on the Sr^{89}/Ca^{45} ratio in bone is again evident in groups 2 and 5, although the difference in the ratios is somewhat less with calcium carbonate supplementation than with calcium lactate supplementation. The effect of carbonate (added as Na_2CO_3), independent of changes in calcium level, is seen in the comparison of group 3 with group 2. Added carbonate reduces deposition of both Sr^{89} and Ca^{45}, but the effect on Ca^{45} deposition is significantly greater than the effect on Sr^{89} deposition.

The effect of phosphate (added as Na_2HPO_4), independent of changes in level of calcium or carbonate, is seen in the comparison of groups 5 and 6. A 20-fold increase in phosphate reduced both Sr^{89} and Ca^{45} deposition by a factor of about two, leaving the ratio of Sr^{89} to Ca^{45} in bone essentially unchanged. At a constant phosphate level and a constant high carbonate level, the effect of variation in calcium level on the Sr^{89}/Ca^{45} ratio in bone is greatly reduced (compare groups 3 and 5), and with a constant Ca/P ratio, and high carbonate, the increase in the Sr^{89}/Ca^{45} ratio, with increased calcium level, is even smaller (compare groups 3 and 6).

Experiments reported by Wasserman *et al.* (*2*) were performed at a constant Ca/P ratio; this may explain in part the absence in their experiments of effects of dietary calcium level on the ratio of Sr to Ca deposited. It should also be noted that the rats in our experiments were mature, nongrowing animals, while those employed in the experiments of Wasserman *et al.* were rapidly growing animals which deposited much larger fractions of the administered radioisotopes.

Our present results, while they can hardly be said to clarify the situation, do serve to emphasize the complexity of the interrelationships involved. If, for purposes of hazard evaluation, this complexity makes expedient the temporary adoption of simplifying assumptions, such as the assumption that calcium will behave biologically as an isotopic diluent of Sr^{90}, the uncertainties introduced with such assumptions must be kept clearly in mind. Thus, in groups 2, 3, 5, and 6 of the present experiment, changes in the calcium, sodium, phosphate, and/or carbonate concentrations in the diet resulted in a nearly three-fold variation in the percentage ratio of Sr^{89}/Ca^{45} deposition in bone, and a nearly sevenfold variation in the absolute quantity of Sr^{89} deposited. Although the elevation of calcium levels in the diet did decrease Sr^{89} deposition, the effect of a 20-fold increase in calcium was only a twofold decrease in Sr^{89} deposition, and effects of similar magnitude were obtained by varying the phosphate and carbonate levels in the diet. While these are results of short-duration experiments, previous experiments involving feeding of Sr^{90} and Ca^{45} for periods of up to 24 days gave similar results with a more limited set of variables (*1*). For a reasonably adequate evaluation of this problem, additional variables must be studied over time periods embracing the life span of the experimental animals employed. Such experiments are being conducted in this laboratory (*3*).

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References and Notes

1. R. F. Palmer, R. C. Thompson, H. A. Kornberg, *Science* 127, 1505 (1958).
2. R. H. Wasserman, C. L. Comar, D. Papadopoulou, *ibid.* 126, 1180 (1957).
3. This report is based on work performed under contract No. W-31-109-Eng-52 for the U.S. Atomic Energy Commission. The technical assistance of Joan Hess is gratefully acknowledged.

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Phenylalanine Hydroxylation Cofactor in Phenylketonuria

Abstract. The enzymatic conversion of phenylalanine to tyrosine had previously been shown to require a nonprotein cofactor. It has now been demonstrated by direct assay that the cofactor is present in phenylketonuric liver samples. The lack of a functional phenylalanine hydroxylating system in phenylketonuria is not due to the absence of the cofactor.

Recent enzymological studies have strongly supported the suggestion, originally made by Jervis in 1947 (*1*), that in the disease phenylketonuria there is a block in the conversion of phenylalanine to tyrosine.

In 1953, results were reported of both in vitro (*2*) and in vivo (*3*) studies which showed that this reaction is completely missing or markedly decreased in phenylketonuria. After the system which catalyzes the hydroxylation of phenylalanine to form tyrosine was shown to require at least two protein fractions (*4, 5*), it was demonstrated by direct assay that only one of these enzymes was

missing in liver samples from phenylketonuric patients (*6, 7*).

Studies (*5*) on the stoichiometry of the enzymatic conversion of phenylalanine to tyrosine have led to the formulation of the following reaction (*8*):

$$TPNH + H^+ + O_2 + \text{phenylalanine} \rightarrow TPN^+ + H_2O + \text{tyrosine}$$

In 1958, it was reported that in addition to the two enzyme fractions already referred to, this enzyme system requires a new nonprotein cofactor which has been purified extensively from rat liver (*9*). More recently, it has been shown that tetrahydrofolic acid (*10*) and some other tetrahydropteridines (*11*) can replace the rat-liver cofactor in this reaction. Although none of the reduced pteridines which have been tested, including tetrahydrofolic acid, appears to be structurally identical with the natural cofactor, or as active in the enzyme system, their availability in relatively large amounts has made it possible to carry out experiments which have led to an understanding of the general role of the two enzymes in the over-all reaction. Evidence has been obtained from experiments in which stoichiometric amounts of tetrahydropteridines were employed which suggests that neither the sheep enzyme nor TPNH is directly involved in the hydroxylation reaction. The sheep enzyme catalyzes a reaction which serves to keep the coenzyme in an active form. In the presence of large amounts of the tetrahydropteridines, the rat enzyme alone can catalyze the conversion of phenylalanine to tyrosine (*11*).

The discovery that a cofactor is intimately involved in the enzymatic formation of tyrosine raised the possibility that the absence of a functional phenylalanine hydroxylating system in phenylketonuria might be due to the absence of this cofactor rather than to the absence of one of the enzymes. The present report deals with the question of whether the cofactor or the enzyme is missing in the disease.

Table 1. The cofactor activity of phenylketonuric and "normal" human liver.

Subject	Age of subject (yr)	Cofactor activity [units*/mg (dry weight)]
Human phenylketonuric biopsy liver		
J. P.	3	0.027
P. H.	5	0.033
Human nonphenylketonuric biopsy liver		
B. S.	8	0.015
S. M.	15	0.007
Rat liver		0.018–0.022

* A unit of cofactor activity has been defined as the amount which leads to the formation of an additional micromole of tyrosine over a blank without any added cofactor under the conditions of the standard assay.

Table 2. Activity of phenylalanine hydroxylase in liver homogenates from phenylketonuric and nonphenylketonuric children. Homogenate (0.3 ml) was added to reaction mixtures which contained the following components (in micromoles): phosphate buffer (*p*H 6.8), 100; L-phenylalanine, 2.0; glucose, 75; TPN, 0.3; DPN, 0.3; nicotinamide, 5.0; glucose dehydrogenase in excess. Purified rat or sheep enzymes and cofactor were added where indicated. The final volume was 1.0 ml, and the tubes were shaken in air at 25°C for 1 hour. All values are expressed as micromoles of tyrosine formed. Although this is not shown in the table, the purified rat enzyme with or without added cofactor is essentially inactive. The same is true of the sheep enzyme. The whole experiment on each liver sample was completed in 1 day. In each case the figure on the bottom line represents the activity of the purified rat and sheep enzymes which were used in that particular experiment.

Additions	Enzyme activity (Δ μm of tyrosine/60 min)			
	Phenylketonuric subjects		Nonphenylketonuric subjects	
	M. C. (3 yr)	R. R. (5 yr)	B. S. (8 yr)	S. M. (15 yr)
Liver homogenate	0.020	0.018	0.069	0.059
Liver homogenate + cofactor	0.022	0.026	0.165	0.177
Liver homogenate + rat enzyme	0.310	0.078		
Liver homogenate + rat enzyme and cofactor	0.618	0.480	0.661	0.560
Liver homogenate + sheep enzyme	0.020	0.020	0.070	0.068
Liver homogenate + sheep enzyme + cofactor	0.021	0.018	0.169	0.157
Rat enzyme + sheep enzyme + cofactor	0.700	0.550	0.448	0.355

Human liver biopsy samples of approximately 1 g were obtained during laparotomy (*12*) and immediately frozen. The tissue could be kept frozen without apparent loss of cofactor activity for several weeks before being used. For the cofactor assay, the liver sample was partially thawed and homogenized with 2 volumes of cold glass-distilled water in a glass homogenizer. The mixture was then placed in a boiling-water bath for 1½ minutes, cooled, and centrifuged. A suitable aliquot of the yellow, opalescent supernatant fluid was then assayed for cofactor activity (*9*). For the determination of enzyme activities, a separate liver sample was homogenized with three volumes of cold 0.9-percent KCl, and a sample of the homogenate was then assayed by a procedure similar to that which has previously been used for sheep- and rat-liver extracts (*5*), the only modification being the addition of nicotinamide and of both DPN and TPN. Because of the relatively low activity, the time of incubation was increased to 60 minutes.

The results of the cofactor determinations are shown in Table 1. For purposes of comparison, the activity of a boiled extract of rat liver is also included. It is clear that the cofactor is not missing in the phenylketonuric liver samples. Indeed, from this limited series it seems as though there may be more cofactor present in phenylketonuric liver than in the control samples.

The results of studies on the enzyme activity of the human liver homogenates are shown in Table 2. The phenylketonuric liver homogenates appear to have some ability to catalyze the conversion of phenylalanine to tyrosine. Although this would be consistent with the in vitro studies, which indicated that the enzymatic block is not a complete one in the disease (*3*), it should be pointed out that this low level of activity approaches the sensitivity limits of the methods which were used. This slight activity is not significantly increased by the addition of the cofactor or the sheep enzyme, or both (*13*). The addition of the purified rat enzyme, even without any additional cofactor, leads to a large increase in activity, which also indicates that the phenylketonuric liver contains the cofactor (*14*).

These results are in agreement with those of previous studies (*6*) which have led to the conclusion that it is the rat enzyme which is almost completely nonfunctional in phenylketonuria; the sheep enzyme and the cofactor are present. In the light of our present understanding of the role of these two enzymes in the conversion of phenylalanine to tyrosine, it may be emphasized that the enzyme which is not active in phenylketonuria is that one which is intimately involved in the hydroxylation reaction. The relationship between this enzymatic defect and the pathophysiology of the disease, however, remains obscure.

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References and Notes

1. G. A. Jervis, *J. Biol. Chem.* 169, 651 (1947).
2. ———, *Proc. Soc. Exptl. Biol. Med.* 82, 514 (1953).
3. S. Udenfriend and S. P. Bessman, *J. Biol. Chem.* 203, 961 (1953).
4. C. Mitoma, *Arch. Biochem. Biophys.* 60, 476 (1956); S. Kaufman, *Biochim. et Biophys. Acta* 23, 445 (1957).
5. ———, *J. Biol. Chem.* 226, 511 (1957).
6. H. W. Wallace, K. Moldave, A. Meister, *Proc. Soc. Exptl. Biol. Med.* 94, 632 (1957); C. Mitoma, R. M. Auld, S. Udenfriend, *ibid.* 94, 634 (1957).
7. Although the two enzyme systems which have been described [by C. Mitoma, *Arch. Biochem. Biophys.* 60, 476 (1956), and S. Kaufman, *J. Biol. Chem.* 226, 511 (1957)] which are capable of catalyzing the conversion of phenylalanine to tyrosine are similar, they do differ in several respects. The first appears to be specific for DPNH, while in the second, TPNH is more active. More pertinent to the present discussion are differences in nomenclature. The two enzymes in the system described by Kaufman were purified from different sources, one from rat liver and the other from sheep-liver, extracts. Throughout the text of this report these are referred to as the rat and sheep enzymes, respectively. In all probability, the purified rat enzyme of Kaufman is equivalent to Mitoma's rat fraction I (the labile enzyme), while the enzyme purified from sheep-liver extracts by Kaufman is equivalent to Mitoma's rat fraction II (the stable enzyme).
8. The following abbreviations are used in this report: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively.
9. S. Kaufman, *J. Biol. Chem.* 230, 931 (1958).
10. ———, *Biochim. et Biophys. Acta* 27, 428 (1958).
11. ———, in preparation.

12. I would like to thank Dr. C. Everett Koop of the Children's Hospital of Philadelphia for the liver biopsy samples which made this study possible.

13. Although this is not shown in Table 1, in a few experiments the addition of THF did not restore the activity when added to the phenylketonuric liver homogenates.
14. It may be noted that the group of phenylketonurics used in this study is younger than the group of normals. Supplementary, unpublished data indicate that the age of the subjects (within the limits described in Tables 1 and 2) has no effect on the results which have been obtained.

28 July 1958

A Consideration of the Metabolic Rates of Some Shrew Tissues

Abstract. Metabolic rates of certain tissues of a shrew, *Cryptotis,* were lower than had been expected in view of the high total metabolism characteristic of shrews. Similar trends were shown in the rodent *Reithrodontomys.* The depression of metabolism of some tissues that was observed in these very small mammals may aid them in conserving energy during periods of inactivity.

The general inverse correlation between metabolic rate of an intact animal and body weight has been well demonstrated (*1*). Among the mammals whose respiratory rates have been measured, shrews of the family Soricidae appear to occupy a unique position (*2*). The asymptotic nature of the body-weight–metabolic-rate curve calculated for shrews suggests that the smallest species measured lie close to the theoretical lowest limit of adult mammalian size. Even the larger species exhibit a metabolic rate well above that of rodents of equal weight (*2*). In view of these findings, the relationship between the total metabolic rates and those of isolated tissues in shrews is of interest.

This report is concerned with presentation of some preliminary data on O_2 consumption of isolated liver, diaphragm, kidney, and lung tissues of the shrew *Cryptotis parva,* together with comparative information on similar tissues from the harvest mouse (*Reithrodontomys humulis*), white mouse, mole (*Scalopus aquaticus*), white rat, and domestic rabbit. Determinations were made on the tissues of seven shrews of both sexes, weighing from 4.1 to 5.0 g. The specimens were wild-caught and were maintained from 1 to 6 wk in the laboratory before sacrifice. The animals were killed by crushing the cervical vertebrae; the organs were immediately removed and placed in cold Ringer's phosphate-glucose (0.1*M*) solution (*3*). The tissues were prepared by the hand-slicing technique (*3*). Respiratory rates were measured by the direct Warburg method at a temperature of 37° ± 0.03°C, air being utilized as the gas phase. Three milliliters of Ringer's phosphate-glucose solution constituted the vessel medium. Flasks were shaken at 120 cy/min, and 15 minutes of equilibration time were allowed, after which readings were taken at 15-minute intervals for 1 hour.

The results are presented in Fig. 1. Although differing absolutely to a considerable extent, the relative metabolic rates of the various tissues of white mouse, white rat, and rabbit appear to present the same qualitative relationship to body weight as does metabolism of the intact animal. A similar trend was obtained by Kleiber (*4*) for liver slices over a size range of larger species, although his values for rat and rabbit liver tissue were higher than those obtained in the present study. With the exception of the value for kidney, the values for shrew tissues fall below the extrapolated curve of the three species mentioned above. This effect is most marked in the case of liver. In view of the small size and high metabolic rate of the intact shrew, this departure is rather striking and would seem to indicate that the high respiratory rates exhibited by shrews must be due to "extrinsic" factors such as nervous stimulation, hormone levels, or concentrations of metabolites in blood or tissue fluids rather than to generally higher "inherent" rates of tissue metabolism. Because of the significantly greater metabolic rates for shrews as compared with those

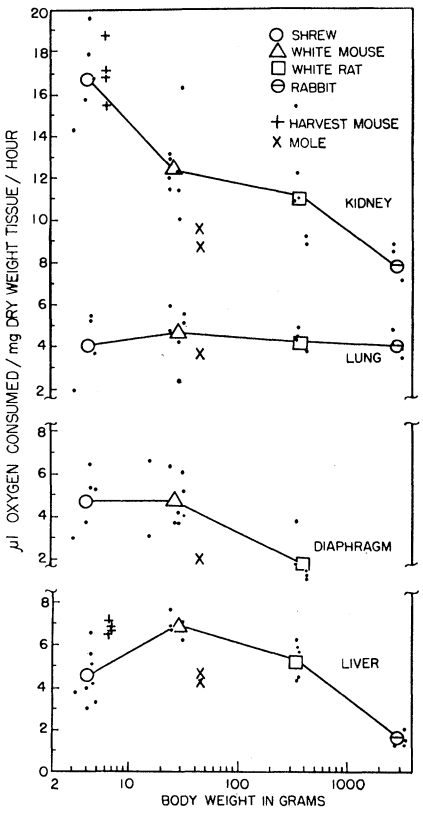

Fig. 1. Tissue metabolic rates of several small mammals.

for other mammals of similar size, the preceding statement would be valid even if the observed rates for shrew tissues fell in the position on the curve that would be predicted on the basis of size alone. The fact that they actually lie below this curve is even more unexpected.

These observations suggest two alternative explanations. Both are highly speculative in view of the limited data available for shrew and other mammalian tissues and for total respiratory rates measured under comparable conditions. On the one hand, the observed rates for lung, diaphragm, and liver tissues of the shrew may be a reflection of the relatively primitive status of the insectivores among mammals, the high metabolic rate exhibited by the intact shrew representing an adaptation of controlling mechanisms to elevate metabolic processes in order to compensate for heat loss or other factors in the physiology of these small creatures. Alternatively, the relatively low respiratory rates exhibited by three of the four tissues measured may in themselves be an adaptation from a "primitive" condition in which higher rates existed. In any case, the functional significance of this situation would seem to lie in a possible marked lowering of metabolism when the animals are inactive, thus resulting in a considerable