Table 2. Effects of malonate on heme biosynthesis. Malonate concentration was $1.2 \times 10^{-2} M$. Other conditions were as described in Table 1.

Radioactivity; in heme formed in 1.0 g of liver (count/min)			
No addition	Malonate	Aceto-acetate	Malonate + aceto-acetate
	2,3-C^{14}-succinate		
384	211	443	408
	1-C^{14}-succinate		
79	82	81	69

are lost during metabolism through the cycle (*10*). Together with these observations, it should be noted that 2,3-C^{14}-succinate is incorporated into heme by passing through two NAD-dependent enzymatic reactions (malate dehydrogenase and 2-ketoglutarate oxidase). 2-Ketoglutarate is incorporated into heme by conversion to succinyl-coenzyme A through the action of the latter enzyme system. In the incorporation of radioactivity into heme by 1-C^{14}-succinate, 4-C^{14}-5-aminolevulinate, and Fe^{59}, no known NAD-dependent reactions occur. Thus, it would appear that these effects of Seconal can be explained as an inhibition of $NADH_2$ oxidation.

A consistent change observed in the livers of porphyric animals has been an increase in the acetoacetate concentration (*8*). This important metabolite is an excellent oxidant for $NADH_2$ as catalyzed by β-hydroxybutyrate dehydrogenase. Therefore, if Seconal inhibits heme synthesis indirectly in liver homogenates by blocking $NADH_2$ oxidation, then the inhibition should be reversed by acetoacetate; and this is the effect observed (Table 1). In fact, even in the absence of Seconal, acetoacetate at a concentration of $1.85 \times 10^{-3} M$ stimulated the synthesis of heme from 2,3-C^{14}-succinate by as much as 100 percent. The stimulation from 2-ketoglutarate and glycine to heme was somewhat less; and there was no effect of acetoacetate on 1-C^{14}-succinate, 4-C^{14}-5-aminolevulinate, or Fe^{59} incorporation into heme.

Acetoacetate may further influence the utilization of succinate by another mechanism. Succinic dehydrogenase is competitively inhibited by malonate, resulting in a block of the TCA cycle and inhibition of the incorporation of 2,3-C^{14}-succinate into heme. This malonate inhibition appeared to be reversed completely by acetoacetate (Table 2). The utilization of 1-C^{14}-succinate was unaffected by malonate, implying that there may be a NAD-dependent, malonate-insensitive pathway for the oxidation of succinate, a pathway that would be stimulated by acetoacetate. This, of course, is speculative since the reduction of NAD by succinate is not yet fully understood. Chance and Hollunger (*11*) have suggested several possible mechanisms by which this process might occur. Acetoacetate may be quite specific in affecting heme synthesis since no other hydrogen acceptor has yet been found which behaves similarly.

Accordingly, Seconal probably acts by inhibiting the TCA cycle, an effect that would be expected from impairment of $NADH_2$ oxidation. Acetoacetate, on the other hand, appears to behave in the opposite manner by increasing $NADH_2$ oxidation and hence perpetuation of the TCA cycle, making tenable the hypothesis that porphyrin and heme synthesis is regulated, partially at least, by the rate of turnover of the TCA cycle. A sustained high level of succinyl-coenzyme A may participate in the induction of 5-aminolevulinate synthetase (*3, 12*) in much the same way that 5-aminolevulinate induced 5-aminolevulinate dehydratase (*4, 13*). Undoubtedly the activities of these two enzymes play important roles in regulating porphyrin biosynthesis.

An apparent paradox in the effects of Seconal should be noted. In liver homogenates the drug inhibits porphyrin and heme synthesis; but after injection into an animal, it induces porphyria and thereby increases porphyrin and heme synthesis. These differing responses to Seconal can be reasonably explained by assuming that the drug in both instances still inhibits $NADH_2$ oxidation. However, the intact cell, with its compartmentalization, might behave quite differently from an homogenate in response to this inhibition (*14*).

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28 January 1963

Radium-226 in Human Diet and Bone

Abstract. *The mean radium-226 content of samples of vertebrae from New York City and San Francisco is 0.032 and 0.026 picocuries of radium-226 per gram of calcium, respectively. The ratio of these two mean values is associated with a similar ratio of radium-226 to calcium found in the diet at the two locations. A plot of the radium-226 content of bone as a function of age indicates that there is no marked difference in levels of bone radium with age.*

In the past few years, perhaps spurred by studies of Sr^{90} from weapons fallout, there has been renewed interest in the Ra^{226} content of man in relation to his diet. A knowledge of the Ra^{226} content of the biosphere can lead to an understanding of a significant part of our environmental radioactivity. In addition, it is of interest in that Ra^{226} in man is probably close to being in equilibrium with the environment, as other investigators indicate and this study further corroborates. This relationship is not true of Sr^{90}.

In 1962, this laboratory determined the dietary Ra^{226} levels for three cities in the United States: New York, Chicago, and San Francisco (*1*). The average daily intake, as determined from some 180 samples of all foodstuffs including water, was found to be 2.3, 2.1, and 1.7 pc of Ra^{226} per day or 2.2, 2.0, and 1.6 pc/g of Ca, respectively.

In this current work, samples of

human bone were analyzed in an attempt to correlate the Ra^{226} content of bone with the diet. Since few bone samples were available from Chicago, the study was limited to New York and San Francisco. Although it is known that the individuals were living in either New York or San Francisco for a few years before their deaths, it is impossible to determine whether or not they were lifetime residents of either city The bone in all cases is vertebrae, since this is the specimen that can be most easily obtained.

The entire procedure was carried out in a centrifuge tube and the sample was never transferred until it was in complete solution. The initial sample treatment was to ash the samples at 600°C in silica trays. Ten grams of bone ash were dissolved in concentrated HCl after the addition of barium carrier and a known amount of Ba^{133} spike solution to determine recovery. The *p*H of the sample was adjusted with NH_4OH until a permanent white flock appeared. This was dissolved in HCl, and the sample was buffered with ammonium acetate–acetic acid.

Mixed $BaRaSO_4$ was then precipitated with $(NH_4)_2SO_4$. The small amount of $CaSO_4$ which was present was removed by dissolving the $CaSO_4$ precipitate in HCl and repeating the precipitation. During this step the $BaSO_4$ was present as a precipitate. The $BaRaSO_4$ precipitate was dissolved in hot ethylenediaminetetraacetic acid solution made basic with monoethanolamine. The sample was then filtered into a polyethylene bottle for gamma counting to determine the radiometric recovery.

Radium-226 was determined by an emanation technique where the gaseous daughter Rn^{222} was counted in a large ion chamber (*2*). Radon daughters were allowed to build up in the chamber for 5 hours, and then the sample was counted for 14 hours. The chambers have backgrounds of 6 to 10 count/hour, and 1 pc of Rn^{222} gives 225 count/hour. Each sample was measured twice, and the mean of the two emanations was used to determine the radium content of the sample.

The recovery of Ra^{226} from spiked samples averaged 95 percent for 16 test samples. The average Ba^{133} recovery was 95 percent for these test samples and 92 percent for 120 routine bone samples. The standard deviation of a single sample due to counting error was about ± 10 percent.

In order to compute the value picocuries of Ra^{226} per gram of Ca from picocuries of Ra^{226} per gram of ash, the value 0.37 ± 0.02 g of Ca per gram of ash was used. It has been determined in this laboratory (*3*) from some 115 samples that this factor is constant with age, and the error in using this one number is slight. The age categories, used in determining the calcium factor included children from 3 months to adults more than 60 years old at death.

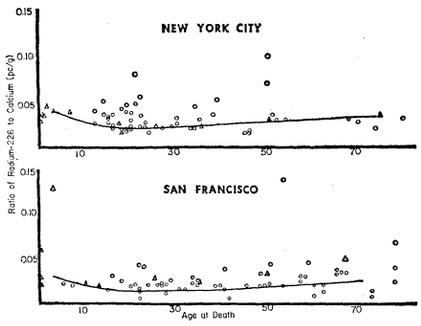

Fig. 1. Measured Ra^{226} concentration in bone as a function of age. The solid line is the derived Ra^{226} concentration as a function of age. For New York City, the metabolized Ra^{226} equals 0.23 pc/day, and for San Francisco, the metabolized Ra^{226} equals 0.17 pc/day. Circles, individual samples; triangles, composite samples.

The data for the two cities are plotted in Fig. 1 as a function of age (*4*). The mean values of vertebrae for New York and San Francisco of 0.032 pc/g Ca and 0.026 pc/g Ca may be compared with other reported data (*5–10*) summarized in Table 1. The values from the individual authors have been recalculated to the same units.

Table 1. Summary of Ra^{226} bone data.

Reference	Number in average	pc Ra^{226}/g Ca
(*5*)	56 tibias	0.032
	37 femurs	0.030
(*6*)	1	0.030 Denver
	Composite of 77	0.038 Boston
	Composite of 143	0.027 New York City
	Composite of 23	0.062 Houston
(*7*)	50	0.042
(*8*)	8 clavicles	0.025
	9 vertebrae	0.029
(*9*)	1 7-mo fetus	0.032
	1 8-mo fetus	0.054
	2 9-mo fetuses	0.011, 0.022
(*10*)	42	0.033
Present report	64 vertebrae	0.032 New York City
Present report	71 vertebrae	0.026 San Francisco

The mean value and the standard deviation of the mean for New York vertebrae is 0.032 ± 0.002 pc of Ra^{226} per gram of Ca. This value is computed by averaging all the data, assuming that there is no age dependency in the radium content of the bone. A comparison of the diet level of 2.2 pc/g Ca for New York and the bone level of 0.032 pc/g of Ca yields an "observed ratio" (*11*) for Ra^{226} of 0.015. The mean value for San Francisco vertebrae samples is 0.026 ± 0.002 pc of Ra^{226} per gram of Ca, and a comparison of the diet level of 1.6 pc of Ra^{226} per gram of Ca with this bone level yields an observed ratio of 0.016. The similarity of the two observed ratio values for the two cities is encouraging and should not be fortuitous since the error term for the mean bone values is less than 10 percent.

From the estimates of Ra^{226} in the diet, it is possible to estimate the body burden as a function of age in relation to calcium, if a few assumptions are made. First, it is assumed that the total body Ra^{226} content can be predicted as a function of time by the equation derived by Norris *et al.* (*12*), and confirmed by Miller and Marinelli (*13*)

$$R_t = C\,[1.12\,(t^{0.48} - 1) + 0.54]$$

where R is picocuries of Ra^{226} in the total body, t is days, and C is picocuries of Ra^{226} metabolized per day.

Second, it is assumed that about 10 percent of the ingested Ra^{226} is metabolized. This figure can be documented at present only by the one report of Seil (*14*), in which the urine/feces ratio for a soluble radium salt taken by mouth is 10 percent of that found when the radium was injected.

Finally, it is assumed that the calcium content of the body as a function of age is that given by Mitchell *et al.* (*15*).

The estimate of Ra^{226} per gram of calcium as computed from the above assumptions is shown as a solid line drawn through the data in Fig. 1. The predicted Ra^{226} content of bone is in reasonable agreement with the measured Ra^{226}, although there are insufficient data to test the early part of the curve.

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1 April 1963

Lactate Dehydrogenase Isozymes: Dissociation and Recombination of Subunits

Abstract. *Lactate dehydrogenase from beef tissues may be resolved electrophoretically into five isozymes each of which is a tetramer. These tetramers can be dissociated into monomers by freezing in 1M sodium chloride. On thawing, reassociation into functional tetramers occurs. On the basis of charge and amino acid composition there are two kinds of monomers. Lactate dehydrogenase-1 contains one kind of monomer and lactate dehydrogenase-5 the other kind. A mixture of equal quantities of these two isozymes, after dissociation and reassociation, leads to the production of all five isozymes in the expected proportions of 1:4:6:4:1.*

Numerous laboratories have reported that the lactate dehydrogenase (*1*) of many organisms exists in five distinct isozymic forms (*2*). These isozymes have the same molecular weight (about 135,000) but differ in net electrical charge and hence may be separated electrophoretically. Moreover, each isozyme is a tetramer (*3*) since it may be dissociated by urea or guanidine into four polypeptide subunits of equal size. These subunits exist in two distinct electrophoretic varieties, A and B. Assortment of these subunits in all possible combinations of four would yield five isozymes of the following compositions. LDH-5 = A^4B^0, LDH-4 = A^3B^1, LDH-3 = A^2B^2, LDH-2 = A^1B^3, LDH-1 = A^0B^4 (*3*, *4*). This hypothesis of LDH isozyme structure has since been supported by other investigators (*5*) who have presented additional immunochemical evidence; likewise total amino acid analyses of LDH-1, LDH-3, and LDH-5 also support the hypothesis, since, in accordance with prediction, the composition of LDH-3 is approximately the average of the quite distinct compositions of LDH-1 and LDH-5 (*6*).

However, none of this evidence is conclusive. A critical test of the subunit hypothesis must involve the construction of the five isozymes from the subunits. This test has now been completed and the results are shown in Fig. 1. Crystalline LDH was prepared from beef tissues containing all five isozymes. The LDH-1 and LDH-5 were then separated from the mixture of isozymes by DEAE-column chromatography and by electrophoresis through a column of cellulose (*7*). According to our hypothesis each of these two isozymes should consist of only one kind of subunit: A polypeptides in LDH-5 and B polypeptides in LDH-1. These two isozymes were mixed in equal proportions in 1*M* NaCl, frozen overnight and then resolved by starch-gel electrophoresis. The mixture of LDH-1 and LDH-5 generated all five isozymes and in approximately the expected proportions of 1:4:6:4:1 which should be attained at equilibrium if random reassociation of subunits into tetramers occurs (Fig. 1). Identical treatment of LDH-1 or LDH-5 alone produced no change in these isozymes—the dissociated subunits reassembled into the original tetramer only. These results, particularly in view of the corroborative chemical evidence, provide a satisfying demonstration of the validity of the original hypothesis of isozyme structure.

The relative amounts of the various isozymes produced depend upon the proportions in which LDH-1 and LDH-5 are mixed. The distribution may be skewed in accord with the input of subunits to generate the various isozyme patterns described in normal tissue homogenates (*5*, *8*). Since the two subunits, A and B, have different amino acid compositions they are different proteins and probably under the control of separate genes. The isozyme patterns generated in developing tissues can thus be attributed to the different relative amounts of A and B polypeptides synthesized in each cell as a result of differential gene function (*6*, *8*).

In apparent conflict with this hypothesis is the fact that under suitable conditions of electrophoresis a single isozyme may be represented by two, three, or more closely spaced bands. In the reassociation experiments multiple bands commonly appeared (although they are not apparent in Fig. 1) at the location of each of the newly generated isozymes. These multiple bands cannot represent distinct isozymes in terms of protein composition but must be produced by minor changes in molecular migration, perhaps because of combination of the isozyme with different amounts of NAD (*9*). More difficult to fit into the current hypothesis is the

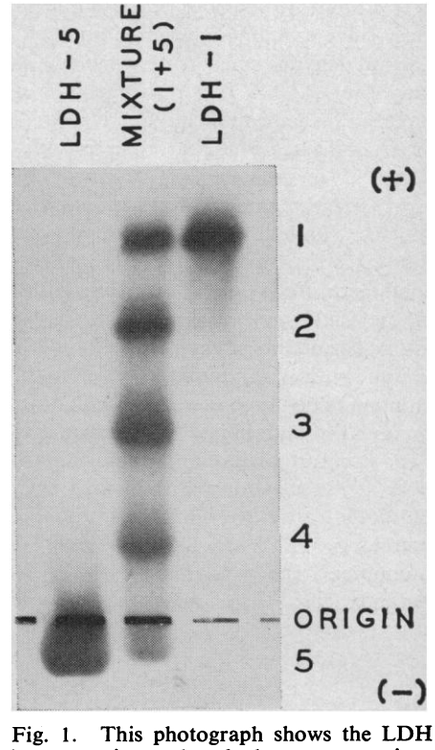

Fig. 1. This photograph shows the LDH isozymes in each of three preparations after electrophoretic resolution in starch gel. On the right is LDH–1, on the left LDH–5, and in the middle are the isozymes resulting from a mixture of equal quantities of these two preparations. All five isozymes were generated in the mixture in the approximate ratio of 1:4:6:4:1, the expected distribution after random reassociation of subunits. The total enzyme activity in the mixture was the sum of the activities of the single isozyme preparations. All three preparations were placed in 1*M* NaCl and frozen overnight before electrophoretic resolution. Electrophoretic and staining procedures have been described previously (*8*).