(7), but in each of these cases sufficient cultural and genetic evidence could be accumulated to show that discrepancies in the relationship between Diego gene frequencies and culture development were due to acculturation rather than to gene drift or natural selection. For instance, the Colla and Omaguaca, who adopted the Quechua language during the Spanish conquest, showed 3 percent of Di (a +) while the true Quechua tribe tested exhibited 25 percent. Tunebo, although linguistically classified as Chibchan, exhibited an incipient agriculture, and their physical features differ from other Chibcha Indians. The Diego frequency was only 1 percent for the Tunebo, while two other Chibcha tribes tested, Ica and Páez, demonstrated 41 and 31 percent, respectively. The Irapa, a Yupa subtribe which in turn is of Cariban affiliation, exhibited 2 percent of Di (a +), and four other Yupa subtribes tested had a range from 21 to 34 percent; also, the Rh and MNSs of the Irapa were statistically different from the other Yupa subtribes, while similar to the Southern tribe (Dobokubi) which showed a negative incidence of Diego (8). We are not proposing, of course, that a gene is to be made responsible for a certain culture pattern. However, from a parallel study of cultural characteristics and Diego frequencies, it is becoming gradually clear that different culture types go along with different genetic constitutions. In particular, Marginal Indians differ in both cultural and genetic aspects from the Tropical Forest Indians and the Central American Indians. It is obvious that this observation, if substantiated by future research, will be of great importance for the historical reconstruction of the peopling of America. At the present stage of Diego research, we propose that the Diego-negative peoples represent an early wave of immigrants in South America, and that most of them can still be identified by a Marginal type of culture. They were possibly followed by Diego-positive peoples, whose earlier waves would have had a better chance of interbreeding with the Diego-negative tribes than their later ones, thereby causing the different frequency ranges (9).

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26 June 1961

Effect of Deuteration of N—CH_3 Group on Potency and Enzymatic N-Demethylation of Morphine

Abstract. Substitution of deuterium for the N-methyl hydrogens of morphine produced a significant reduction in the potency and lethality of morphine in mice regardless of the route of administration. There was no effect on the time of onset, maximal effect, or duration of action. N-demethylation by rat liver microsomal enzymes was characterized by a smaller reaction rate constant, a higher energy of activation, and a larger Michaelis constant with respect to the deuterated morphine. These findings indicated that deuteration of the N-methyl group of morphine not only caused reduction in potency, but also a reduction in the rate of oxidative N-demethylation, and a distinct weakening of the binding to the enzyme active centers.

The role of demethylation in the action and metabolism of morphine-like analgesics has been the subject of much recent work. Beckett *et al.* (1) postulate that N-dealkylation at the central receptor site is the initial reaction in the production of analgesia, and Axelrod (2) has stressed the similarity between the receptors for these drugs and the N-demethylating enzyme present in the liver.

The process of N-demethylation appears to be an enzymatic oxidative reaction resulting in the breaking of a C—N and a C—H bond, as evidenced by formaldehyde formation (3). If the rate of demethylation is dependent on the ease with which the C—H bond is oxidized, and if the biological actions are a function of such N-demethylation, then a change in the C—H bonding force would similarly affect both phenomena. To test this hypothesis, morphine in which the N-methyl group has been completely deuterated has been prepared and studied in vivo and in vitro.

To prepare morphine—N—CD_3, normorphine was treated with excess ethyl chloroformate. The resulting N-ethoxycarbonyl group was reduced with lithium aluminum deuteride, giving morphine—N—CD_3, identical to the corresponding protium compound in melting point and ultraviolet absorption spectrum. However, the pK_a of morphine—N—CD_3 was found to be 8.17 as compared to 8.05 for morphine, thus making the deuterium compound a stronger base by 24 percent (4).

Alterations in the pharmacological activity of the resulting deuteriomorphine and/or in its oxidative N-demethylation by rat liver microsomal enzymes might suggest a relationship between the methyl group and drug action. Accordingly, the LD_{50}'s for morphine and deuteriomorphine were determined in swiss albino mice by the subcutaneous and intracerebral routes and the ED_{50}'s for analgesia as tested by the tail flick method of D'Amour and Smith (5) by the subcutaneous and the intravenous routes. It is apparent from Table 1 that deuteriomorphine is less potent than the parent compound in all categories tested. This is not due to slower absorption of the N—CD_3 compound since it is also less potent than the N—CH_3 compound when administered by the intracerebral and intravenous routes. In addition, passage through the organism does not seem to be slowed, since the onset of action and maximal effect and duration of action of ED_{95} doses was the same for both compounds. Thus, two of the several actions of morphine in mice—death by central nervous system stimulation and analgesia as measured by prolongation of reaction time to a thermal stimulus —are similarly influenced by deuterium substitution in the N—CH_3 group. A change in the potency of sympathomimetic amines upon deuteration in the α-position has recently been reported by Belleau and Burba (6).

These workers, however, observed an intensification as well as prolongation of effect on the nictitating membrane. Since the morphine molecule has been changed only by alteration of the

N—CH_3 group and is in all other respects essentially unaltered, the pharmacological differences noted should be related to the characteristics of the N—CD_3 group. Since the unionized alkaloid base preferentially enters the cell, difference in basicity might explain the difference in potencies, but this consideration seems less likely on the basis of the in vivo data relative to the routes of administration and the duration of action as well as by the in vitro data relative to the energy of activation of N-demethylation.

The effect of deuteration on N-demethylation in vitro was tested by measurement of formaldehyde evolved from a fortified incubate of rat liver microsomes and morphine or deuteriomorphine (*7*). The reaction showed the characteristics of a zero-order reaction for at least 15 min. For routine assay of the enzyme, incubation periods of 15 min were chosen. Substitution of D for H in the N—CH_3 group resulted in (i) a slower rate of demethylation since the ratio of the velocity constants $k_H/k_D = 1.4$, (ii) a higher energy of activation by 5 kcal/mole for the deuterated compound, and (iii) a weaker binding of the drug to the N-demethylating enzyme as evidenced by the difference in the Michaelis constants ($K_m^D/K_m^H = 1.43$). The velocity constants are measured rates of reaction at given substrate concentrations. The Michaelis constants (K_m's) were determined by incubating varying concentrations (S in the equation below) of the substrates with constant amounts of the enzyme. The amount of formaldehyde evolved in 15 min (v) was determined and fitted into the equation

$$\frac{S}{v} = \frac{1}{V_m}S + \frac{K_m}{V_m}$$

where V_m stands for the maximum velocity. Since this is a regression equation of S/v on S, the constants were determined by statistical methods in order to eliminate any bias that may be involved in drawing the curve.

The isotope effect of approximately 1.4 on both the Michaelis and velocity constants is considerably lower than 6.9, the maximum value of the deuterium isotope effect to be expected for the C—H bond (*8*). However, this ratio is observed only if the breaking of the C—H bond is the rate-limiting step. In N-demethylation of morphine, however, the simple oxidation of the C—H bond is apparently not the rate-limiting step.

The ratio of the relative rates of demethylation in vitro is comparable to the ratios of the analgesic potencies and lethalities in vivo. These findings are consistent with but do not prove a relationship between N-demethylation and analgesic action. Beckett's theory is based on interrelated considerations of stereochemical configuration, physicochemical properties, and N-dealkylation of narcotic analgesics at the receptor sites (*1*, *9*). The composite effects of these factors should be reflected in the magnitude of the Michaelis constant. Since deuteriomorphine is a stronger base than morphine, at the intracellular pH more of it will exist in the ionized form which is believed to be involved in binding to the receptors. Furthermore, the larger van der Waals forces associated with deuteriomorphine by virtue of the larger mass of its methyl group would lead one to expect a greater affinity of the enzyme for this drug than for morphine. The finding that the K_m^D is actually larger than the K_m^H implies a greater affinity of the enzyme for the more potent of the two homologous compounds. Therefore, the difference in potencies between morphine and deuteriomorphine could as easily be due to differences in affinities for the receptors as to differences in rates of demethylation.

Table 1. Comparison of the lethal and analgesic effects of morphine and deuteriomorphine in Swiss albino mice. Figures in parentheses are the 95-percent confidence limits. At dose levels near the 16, 50, and 84-percent response, 20 to 25 mice per dose level were used. At other dose levels, 10 to 15 mice per dose level were used.

Effect	Route	Dose (mg/kg)		$f_{P.R.}$*	P.R. of morphine to deuteriomorphine
		Morphine	Deuteriomorphine		
LD_{50}	Subcutaneous	256.0 (208–315)	400.0 (318–488)	1.32	1.56 (1.20–2.06)
LD_{50}	Intracerebral	6.9 (5.8–8.2)	11.4 (9.8–13.2)	1.26	1.65 (1.31–2.08)
ED_{50}	Subcutaneous	2.6 (2.2–3.0)	4.2 (3.5–5.2)	1.31	1.62 (1.25–2.15)
ED_{50}	Intravenous	1.1 (0.9–1.3)	3.3 (2.6–4.0)	1.30	3.00 (2.3 –4.9)

* P.R., potency ratio. For definition of $f_{P.R.}$, see (*11*). The value of P.R. must exceed the value of $f_{P.R.}$ for the two substances being compared to differ significantly in potency.

Other data relative to the rates of N-demethylation and the K_m of the N-demethylating enzymes with respect to other narcotic analgesics will be presented in a detailed publication (*10*).

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12 May 1961