The berries were frozen and thawed, and the major pigment was isolated from the hand-expressed juice by the paper chromatographic method described in a subsequent paragraph. About 8 mg of crystalline pelargonidin chloride-3-monoglucoside-$C^{14}$ was obtained.

Individual strawberry plants were covered with glass bell jars into which $C^{14}O_2$, generated by the action of HCl on $Na_2C^{14}O_3$ or on $BaC^{14}O_3$, was introduced to create an atmosphere of about 0.8 percent $CO_2$. In a preliminary greenhouse experiment, 2 μc of $C^{14}O_2$ was administered to a single plant in two doses within 3 weeks. During the time between the two doses, the plant was left uncovered for 4 days, because a few necrotic spots had appeared on its leaves. Fourteen grams of ripe berries was obtained from this plant, and about 3 mg of tagged pigment was crystallized from them.

In the subsequent field experiment, 1 mc of $C^{14}O_2$ was similarly applied over a 4-day period to four strawberry plants located in an outdoor bed. From the berries that were harvested from these plants 59.5 mg of pelargonidin-3-glucoside-$C^{14}$ was recrystallized.

Juice from the strawberries was acidified to contain 1 percent HCl, and the resulting solution was extracted with 1-butanol. On treating the alcohol extract with petroleum ether, the water was separated from the butanol in which it had been dissolved. This aqueous phase contained all of the colored matter present and could be concentrated under vacuum at temperatures below 50°C to almost one-third of the original volume. The concentrate was streaked on strips of Whatman No. 1 or 4 paper, the resulting bands being dried immediately after application. After ascending development for 12 to 16 hours, using a solution of 1-butanol, acetic acid, and water (40/10/50, respectively, by volume) as the solvent, the anthocyanin bands were fully separated (*5*). Bands corresponding to pelargonidin-3-glucoside were cut out and eluted by immersing one end of each strip in a beaker of methanol that contained a trace of HCl and allowing this solvent to descend by capillarity into a lower beaker. The methanolic eluate was evaporated under vacuum, leaving a noncrystalline residue, which was redissolved in 1-percent HCl and chromatographed by the same method as that employed for the aqueous concentrate. Methanolic eluates, obtained from pelargonidin-3-glucoside bands from the second chromatograph, were acidified with HCl and allowed to evaporate slowly at room temperature. Red, needlelike crystals of pelargonidin-3-glucoside-$C^{14}$ were obtained.

Measurements of activity were carried out using a Tracerlab Autoscaler equipped with a thin (1.4 mg/cm²) end-window Geiger-Müller tube, except for the determination of activity on the pigment resulting from the 1-mc $C^{14}O_2$ experiment. For this, a Tracerlab Superscaler equipped with a windowless, gas-flow counter was employed. Specific activities for the pigment samples recovered in the microcurie experiments were low, but counts were statistically significant at the 1-percent level. Pigment from strawberries harvested from the plants treated with glucose-$C^{14}$ showed a specific activity of 379 ± 7 disintegrations per minute, per milligram of pigment. Pigment from the plant exposed to 2 μc of $C^{14}O_2$ showed a specific activity of 951 ± 19 disintegrations per minute, per milligram. These correspond to yields, based on activity, of 0.068 percent for the glucose-$C^{14}$ method and 0.064 percent for the $C^{14}O_2$ method .

In the preparatory run, utilizing 1 mc of $C^{14}O_2$, pelargonidin-3-glucoside-$C^{14}$ with a specific activity of 1512 disintegrations per minute, per milligram was obtained. The percentage yield, based on activity for this experiment was 0.004.

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12 March 1956

## Optical Isomerism and Pharmacological Action, a Generalization

The study of the biological activity of drugs with optical isomerism has been unduly influenced by the known optical specificity of the amino acids, wherein, for example, *d*-serine is reported not only to be inactive but to produce kidney damage (*1*). *d*-Glutamic acid is reported to be cyclicized into a pyrrole compound and excreted in the urine (*2*). Such complete failure of the body to utilize the unnatural isomer might be expected with chemicals such as amino acids, which are the building blocks for proteins. Drugs, and even the naturally occurring humoral agents, probably act by modifying cellular responses and are thus not as geometrically specific as are the amino acids. Yet, one finds frequently in the pharmacological literature the statement that the active form of the drug is the *l* or *d* isomer without adequate trial of higher doses of the more inactive isomer.

Consideration of the specificity of the optical isomers of thyroxin is omitted from this report, since the asymmetric carbon atom occurs at the portion of the molecule that may be involved in synthesis of the thyroxin molecule into cellular proteins where the optical configuration may be as crucial as that of the amino acids. Several individual factors that may dominate in the drug action of optical isomers also have been omitted from consideration in this generalization. These factors are (i) rate of absorption or destruction of the isomers, (ii) competitive inhibition between isomers, (iii) molecular size such as *d-l* complex formation by hydrogen bonding between isomers, (iv) affinity for the receptors of the isomers, (v) differential penetration to the site of drug action, and (vi) the possible racemization by the body of the less active isomer.

Several years ago we were disappointed when the anticonvulsant *d*-atrolactamide was found to have a ratio of potency only 1.68 times greater than that of *l*-atrolactamide. This difference is easily measurable in terms of the $LD_{50}$ in the mouse and is a statistically significant difference, as is shown by the following oral $LD_{50}$'s and their standard deviations: *d,l*-atrolactamide, 1.86 ± .07 g/kg; *d*-atrolactamide, 1.39 ± .04 g/kg; and *l*-atrolactamide, 2.33 ± .06 g/kg (*3*). However, atrolactamide as an anticonvulsant is relatively nonpotent in that it must be given in a dose of 1.5 g four times daily to control the seizures of epileptic patients. In retrospect, it becomes clear that potency per se, as expressed by the $LD_{50}$, is probably a measure of the degree of reactivity of the drug with cells or enzymes. Therefore, with a nonpotent drug, such as atrolactamide, the degree of geometric conformation to the locus of action must be relatively poor and, hence, would not be greatly influenced by the optical isomerism of the compound.

Since effective dosage of a drug is a measurement of potency, the dose can be used as an approximation of this geometric conformation, and the postulate can be made that the lower the effective dose of a drug, the greater the difference in the pharmacological effect of the optical isomers. In order to document this thesis, one would wish to have $LD_{50}$'s in the same species of animal for all of the commonly used drugs that can exist as *d* and *l* isomers. However, a survey of the literature does not provide such data

Table 1. Mouse $LD_{50}$'s (in milligrams per kilogram) for isomethadone.

| Injection type | Isomer | | Isomeric ratio |
|---|---|---|---|
| | *l* | *d* | |
| Intravenous (90 sec) | 18.0 ± 1.8 | 38.8 ± 1.1 | 2.16 |
| Intravenous (120 sec) | | 54.0 ± 2 | 3.0 |
| Intraperitoneal | 25.5 ± 2.4 | 73.1 ± 5.7 | 2.9 |
| Subcutaneous | 49.0 ± 2.7 | 155.1 ± 14.2 | 3.2 |
| Subcutaneous | 60 | 150 | 2.5 (Eddy) |
| Oral | 81.1 ± 6.2 | 227.7 ± 10.8 | 2.8 |

for a single animal species by a uniform route of administration.

This scarcity of data is, in part, owing to the fact that the intravenous route of administration does not differentiate between the pharmacological effect of optical isomers (*4*). We have determined the $LD_{50}$'s in milligrams per kilogram for isomethadone in the mouse that are presented in Table 1.

The data agree with the findings of Eddy (*5*) for subcutaneous toxicity in the mouse. These data indicate that any route of administration will disclose a constant difference between the *d* and *l* isomers of isomethadone. The intravenous route approaches the discrimination of the intraperitoneal route if the rate

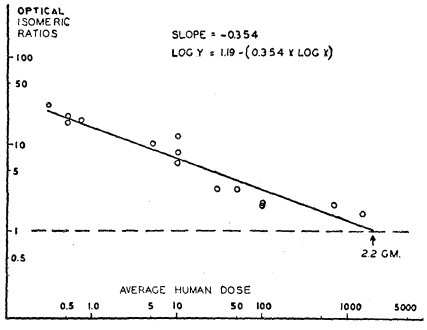


Fig. 1. Decrease in isomeric ratios with decrease in drug potency. The geometric ratio between the potency of the optical isomers is plotted in logarithmic units on the ordinate. The average human dose in milligrams is plotted in logarithmic units on the abscissa. The points for the 14 drugs plotted may be read from left to right as follows: norepinephrine 27 (*9*), atropine 20 (*11*), epinephrine 18.5 (*12*), scopolamine 17 (*11*), Dromoran 10 (*13*), methadone 6 (*14*), amphetamine 12 (*15*), and methamphetamine 8 (*16*), isomethadone 3 (*17*), ephedrine 3 (*18*), paired points Nirvanol and Mesantoin 2 (*19*), quinine (*20*), quinidine 2 (*21*), and atrolactamide 1.68 (*3*). The relationship may be expressed as log *Y* (isomeric ratio) = $1.19 - [0.354 \times \log X$ (human dose)]. The *X*-axis intercept is the human dose of a theoretical drug with no measurable difference between optical isomers. An intercept with the *Y*-axis is not drawn, since future work with more potent drugs may prove that the regression line approaches the *Y*-axis asymptotically.

of injection is slower than the usual 90-second interval. Conversely, published data on the intravenous route of administration that does not discriminate between *d* and *l* isomers may be correlated with the rapidity of injection wherein the drug affects nonspecific vital centers rather than those specific vital centers that can be affected by the optical configuration.

As a less suitable alternative to the use of $LD_{50}$'s, the dosage of the racemate, as used in man, is plotted in Fig. 1 against the observed isomeric ratio of the *d* and *l* isomers as tested in animals or animal organ systems. This use of the effective human dose of the racemate is further justified by the fact that some drugs, such as amphetamine, LSD-25, and the methadone series of analgesic drugs, affect the higher centers of the brain, which are poorly developed in the mouse compared with those of man.

Figure 1 shows that there is a steady decrease in the isomeric ratios with the increase in the total dose needed to produce an effect in man. In each instance the optical isomeric ratio is obtained by dividing the effective dose of the less active isomer by the effective dose of the more active isomer without regard to actual optical rotation.

In choosing isomeric ratios for Fig. 1, preference was given to activity in pharmacodynamic tests and to oral or subcutaneous $LD_{50}$'s. The straight line resulting from a log-log plot is calculated by the method of least squares. The difference in pharmacological activity of the isomers is greatest with norepinephrine, epinephrine, and atropine, where the dose used in man is in the neighborhood of 0.25 to 1 mg. (*l*-LSD-25 has been tried in man in a total dose of only 0.150 mg.) Atrolactamide, a nonpotent drug, in a dose of 1.5 g, has the smallest isomeric ratio. The arrow indicates the intercept of the regression line with unity, which is at a dose of 2.2 g. This is the dose of a theoretical drug that would show no detectable effect of isomers.

Another nonpotent drug, camphor, is reported to have no difference in the pharmacological effect of its isomers. This observation should be tested by more accurate modern methods. In the case of the sympathomimetic amines, sufficient data are not available to provide a separate graphing of their isomeric ratios. When data are available, these amines must, of necessity, be divided into those that are mainly pressor and those that are mainly stimulant. Cobefrine with an isomeric ratio of 200 (*6*) and isopropylarterenol with an isomeric ratio of 1100 (*7*) appear to be exceptions to the foregoing generalization. However, Furchgott (*8*) estimates a fourfold greater affinity of isopropylarterenol for depressor receptors compared with epinephrine. Stimulant pressor amines, such as amphetamine and methamphetamine, which have the brain as their principal locus of action, show a greater difference in isomeric ratios for the central nervous system (approximately 6 to 12) than for the blood pressure response (approximately 2). If one accepts the foregoing premise, we can now postulate that the geometric conformation of a central nervous system stimulant pressor amine is more critical for the brain than it is for the vascular smooth muscle.

The study of Luduena *et al.* (*9*) is another interesting example of this greater geometric conformation in selected tissues of the body. In their studies on *d*- and *l*-norepinephrine they found a 1/15 ratio of vasoconstrictor potency for the perfused rabbit's ear and a 1/60 ratio for inhibition of the rabbit ileum. If the isomeric ratio is a measurement of geometric conformation to the site of action, these data might indicate that the molecular configuration of norepinephrine is much more specific for the smooth muscle of the intestine than for the smooth muscle of the arterioles. Similarly, the isomeric ratios of the action of epinephrine and norepinephrine at sympathetic nerve endings might determine which of these two sympathins has the more critical geometric conformation.

This generalization might be used in the future to determine the relative degree of geometric conformation of a drug to the specific tissue under test. In other words, the greater the difference between the pharmacological activity of the *d* and *l* isomers the greater is the specificity of the active isomer for the response of the tissue under test (*10*).

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2 February 1956

## Photoreactivation of Ultraviolet-Inactivated Diphosphopyridine Nucleotide

Available information suggests that the site of photoreversible ultraviolet injury may be in a nucleotide, or in a compound containing a nucleotide, or in more than one such compound (*1*). It is further suggested that the substance is associated in some basic way with a diversity of cellular activities. The phosphopyridine nucleotide coenzymes, which are associated with a variety of hydrogen-transfer reactions, meet these specifications. The fact that a triosephosphate dehydrogenase-diphosphopyridine nucleotide complex, which loses activity in preparation, may be reactivated by light (*2*) suggests that diphosphopyridine nucleotide (DPN) may be involved. This report deals with a study of photoreactivation *in vitro* of DPN that has been partially inactivated by ultraviolet radiation (*3*). Some of the experiments discussed have been reported previously in abstract form (*4*).

Solutions of DPN (*5*) were prepared at concentrations of 1/5000 or 1/10,000 in 0.1*M* phosphate buffer at *p*H 7. The source of ultraviolet radiation (UV) was a Westinghouse Sterilamp (emitting mainly $\lambda = 2537$ A), and the source of visible light (VL) was a General Electric CH-4 spotlamp. A Corning No. 3060 glass filter, which transmits no short ultraviolet waves, and 3 in. of water were always interposed between the CH-4 lamp and solutions being exposed to VL. In typical experiments, samples were withdrawn from stock DPN solutions and were exposed to UV, to VL, or to UV and VL or were retained as unirradiated controls. Samples were irradiated in flat glass dishes with quartz covers (Vaseline sealed to prevent evaporation). Solutions were stirred with glass-enclosed magnetic fleas. The ability of DPN to accelerate the reduction of hog- or rat-liver extracts was used as the criterion of the activity of samples.

Four series of experiments are reported here. For series I, II, and III, hog liver was ground and mixed with equal parts phosphate buffer in a Waring Blendor, filtered through a clean laboratory towel, and stored frozen until used. For series IV, rat liver was prepared in the same way, except that, after being filtered through cloth, it was filtered twice through analytic-grade filter paper prior to freezing, and again after thawing, just prior to use.

In series I, irradiation periods of 2 hours were used for UV and for VL. In series II, exposure periods were 4 hours for UV and 2.5 hours for VL. The series-III samples were exposed to UV for 22 hours and to VL for 2 hours, and series-IV samples were exposed for 75 hours to UV and for 2 hours to VL. The experiment was repeated four times in series I, III, and IV and three times in series II. The direction and magnitude of result were consistent in each series of experiments.

Liver preparations were placed in Thunberg tubes. Equal amounts (1 or 2 ml) of methylene-blue solution (1/3000 in series I, II, and III; 1/5000 in series IV) and DPN solutions (variously treated) were placed in the caps. Control tubes were always run with buffer substituted for the added DPN solution. Tubes were evacuated of air, and their contents were mixed. Then the mixtures were incubated in a water bath at 30°C while achromic times were being determined. The average values for achromic times are reported in Table 1. Thunberg tubes from an experiment in series IV are shown in Fig. 1 as they appeared after 20 minutes of incubation.

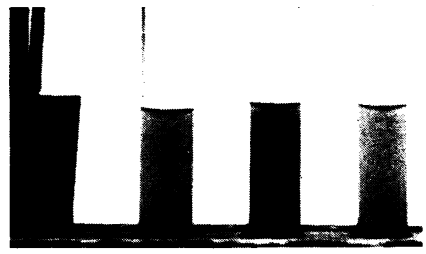

Fig. 1. A point of interest in a representative experiment. Thunberg tubes from an experiment in series IV, photographed after 20 minutes of incubation. Tubes were immediately returned to the water bath for the remainder of the incubation period. The tubes, from left to right, respectively, contain: No added DPN (achromic time, 100 min.+); untreated DPN (16 min.); UV-irradiated DPN (29 min.); DPN irradiated with UV + VL (20 min.). Photoreactivation is demonstrated by accelerated methylene-blue reduction in the fourth tube.

Liver preparations that received no added DPN reduced methylene blue slowly (series I, II, and III) or not measurably (series IV). In each experiment, the rate of decolorization was greatly accelerated by the addition of unirradiated DPN. Partial inactivation of DPN by UV was indicated by reduction of this acceleration in comparison with unirradiated controls. Visible light alone did not alter the activity of DPN. Photoreactivation of DPN samples that had been partially inactivated by ultraviolet radiation was apparent when UV treatment was followed by VL treatment. The photoreactivation of ultraviolet-inactivated diphosphopyridine nucleotide is of considerable interest as a possible model for interpretation of photoreactivation *in vivo*.

Table 1. Data for experiments on photoreactivation of DPN *in vitro*. All values are averages of several experiments.

| Experiment series | Achromic times (min) | | | | | Photoreactivation (min), UV − (UV + VL) |
|---|---|---|---|---|---|---|
| | Buffer only | Treatment of DPN | | | | |
| | | None | With VL | With UV | With UV + VL | |
| I | 16.50 | 3.69 | 3.63 | 4.88 | 4.63 | 0.25 |
| II | 17.67 | 7.83 | 7.83 | 8.83 | 7.92 | 0.91 |
| III | 21.25 | 6.31 | 6.44 | 10.75 | 9.72 | 1.00 |
| IV | 78+ | 20.25 | | 31.13 | 24.00 | 7.13 |

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