WALDEN INVERSIONS INVOLVING THE ISOMERIC 2,3-BUTANEDIAMINES AND RELATED COMPOUNDS

THE PREPARATION OF SPECIFIC ADSORBENTS

THE ROLE OF PEROXIDES IN MUTAGENESIS

Thesis by

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I am deeply grateful to Professor Howard J. Lucas for suggesting and guiding the study of amino compounds derived from the butylene glycols and especially for his encouragement of unusual or irregular investigations which has made possible the discovery of specific adsorption.

Abstract

It has been demonstrated that the reaction of ammonia with 1,2-dimethylethyleneimine is attended by a single Walden inversion and that the analogous reaction of ammonia with 2,3-epoxybutane very probably proceeds in the same way. This result and other evidence have been used to assign configurations to the 3-amino-2-butanols and to draw certain conclusions about the courses of reactions in which they are involved.

The theory that adsorbents formed in the presence of foreign substances will acquire, as a result of this method of formation, specific affinities for such foreign substances has been proposed. Its validity in a particular system involving silica gel and certain azo dyes has been demonstrated.

The hypothesis that x-rays, ultra-violet light, and various compounds related to mustard gas produce gene mutations through the intermediate formation of peroxides has been investigated by testing the effects of various organic peroxides on mutation rates. Several of these compounds have proved to be effective for the induction of mutations and a continuation of these studies would hold promise of uncovering fundamental information about the nature of the mutation process.

Table of Contents

Table of Contents
(continued)

PART I

WALDEN INVERSIONS INVOLVING THE ISOMERIC 2,3-BUTANEDIAMINES AND RELATED COMPOUNDS

PART I

WALDEN INVERSIONS INVOLVING THE ISOMERIC 2,3-BUTANEDIAMINES AND RELATED COMPOUNDS*

In this investigation the isomeric 3-amino-2-butanols, 1,2-dimethylethyleneimines, and 2,3-butanediamines have been prepared and their physical constants have been measured. It has been possible to draw some conclusions about the courses of the reactions by which these compounds are formed and to make plausible assignments of configurations to their respective isomers.

The methods employed are analogous to those described by Lucas and Garner (1) and others (2, 3, 4, 5, 6) for investigations of reactions interrelating the 2,3-butanediols, 2,3-epoxybutanes, 3-chloro-2-butanols and various derived compounds. Observations made on compounds derived from the 2,3-butanediols have the advantages that they can be related to a large amount of established data and special deductions can be drawn on the basis of the symmetry of some of the molecular structures involved.

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* The latter part of this investigation was carried out jointly with Mr. Wildon Fickett.

The demonstrations reported here that a single Walden inversion attends the addition of ammonia to both imine and oxide rings are not new findings. They confirm predictions that would be made on the basis of earlier work (7). However, it is important to obtain concise demonstrations of these phenomena in simple systems.

In the following section, operations and observations have been summarized, all discussion and interpretation having been reserved for the succeeding section. In conclusion, before describing experimental details, brief consideration has been given to the broader significance of the work and to some possible applications.

Reaction Schemes and Experimental Observations

Preparations of amino compounds.--The 2,3-epoxybutanes were prepared from the meso and D-2,3-butanediols by methods previously described (1, 2, 6). Reaction of the oxides with aqueous ammonia gave the 3-amino-2-butanols which were in turn converted into the 1,2-dimethylethyleneimines by treatment of their sulfuric acid esters with potassium hydroxide. The imines reacted with liquid ammonia under pressure to give the 2,3-butanediamines. These reactions are indicated in Figures 1 and 2, pages 3 and 4. Probable configurations of the compounds involved in these reactions have been included in the figures on the basis of arguments introduced in the following section.

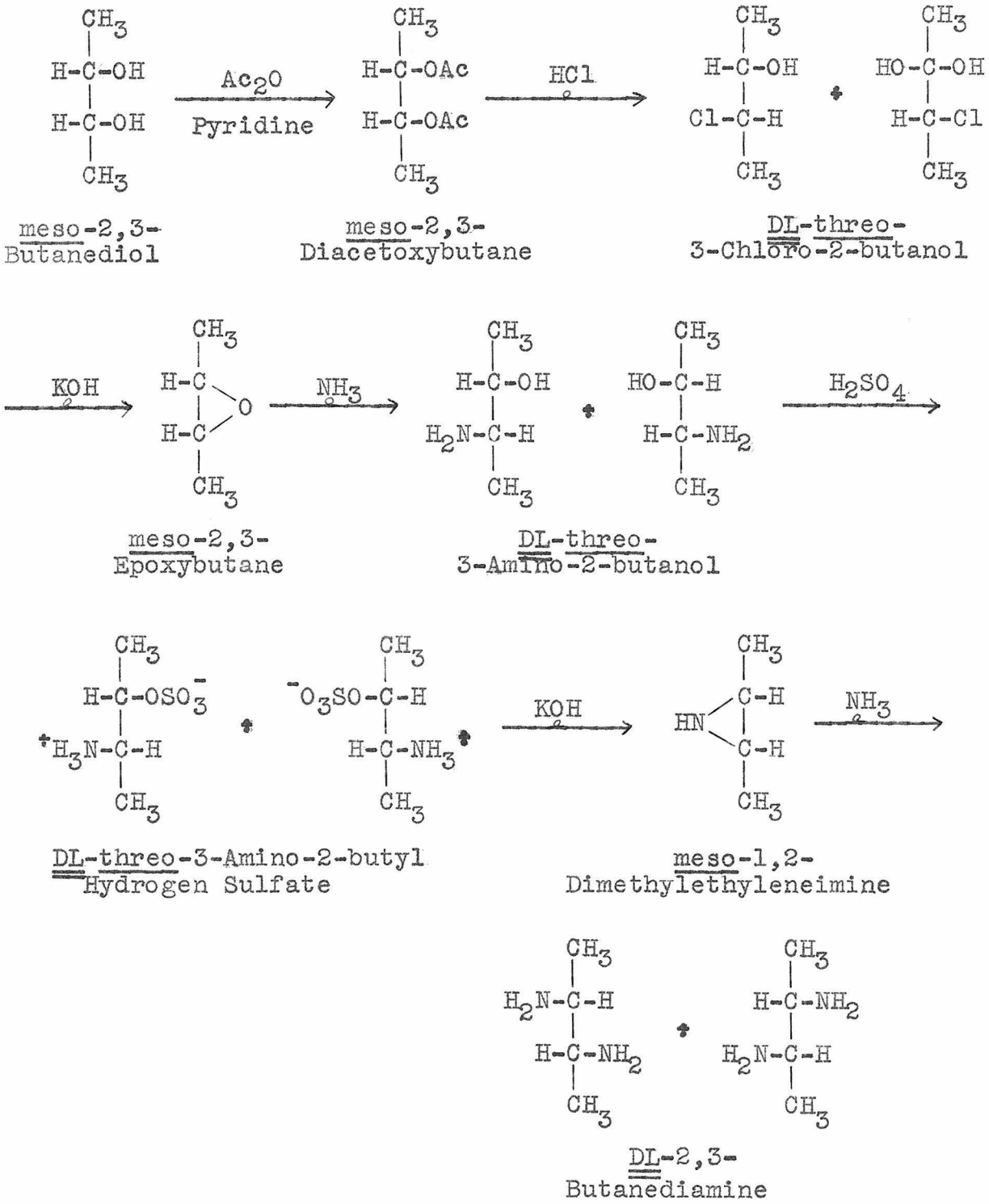

Figure 1. Configurational relationship of amino compounds derived from meso-2,3-butanediol.

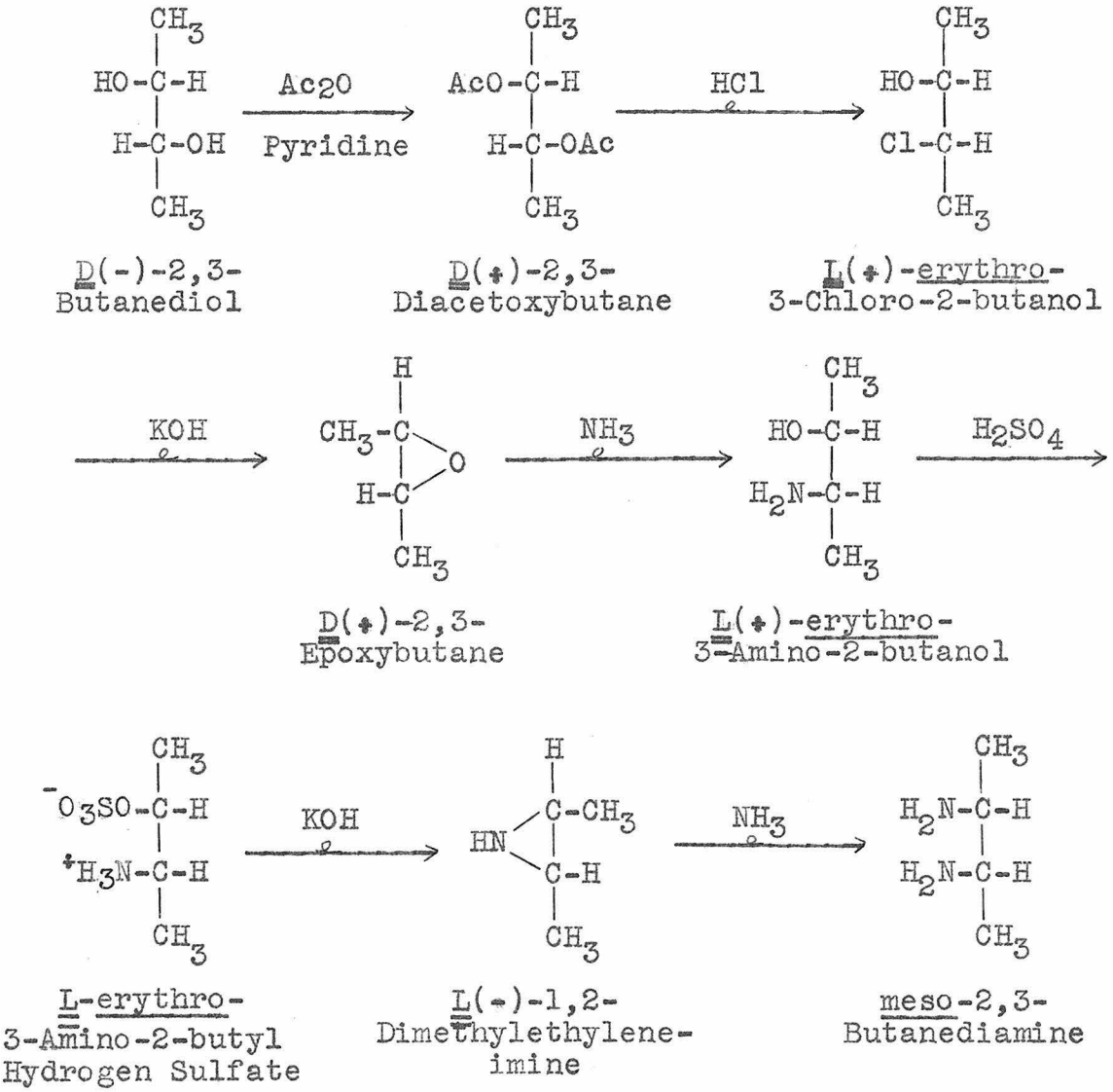

Figure 2. Configurational relationship of amino compounds derived from D-2,3-butanediol.

The amino alcohol and diamine obtained in the reaction sequence beginning with meso-2,3-butanediol were separated into optically active forms by fractional crystallization of their acid L-tartrates. Application of a similar procedure to the diamine obtained from D-2,3-butanediol effected no separation.

Physical constants.--All of the amino compounds display strong affinities for water. The dry amino alcohols and diamines fume on exposure to air and spatter like sulfuric acid when mixed with water. It was difficult to prepare dry samples and, in general, the best available preparations contained 1 to 3% water as determined (by difference) by titrating to a methyl orange endpoint with hydrochloric acid. The values given for "purity" of amino compounds throughout this discussion refer to the results of such titrations and not to configurational or optical purity.

The physical constants of the compounds were measured by conventional methods and are presented in Table 1, page 6. In order to save space, only the highest values for the melting points and boiling points have been tabulated. Boiling point and melting point ranges, and details of the determinations, will be found in the final section. Corresponding data for the analogous glycols and oxides have been included for comparison. With the exception noted below, these latter data have been taken from the reports of other investigators.

Table 1.

Physical Properties of Glycols and Oxides, and Amino Alcohols, Diamines, and Imines*

Form	Purity %	m.p. °C	b.p. °C	D^{25}	n_D^{25}	$[\alpha]_D^{25}$
Glycol						
meso		34.4	181.7^{760}	1.0003	1.4367	
DL		7.6	176.7^{760}			
D		19.7	78^{10}	0.9869	1.4308	-13.17
Amino Alcohol						
erythro-L	99.8	49.3	72^{20}	0.9378	1.4488	+ 0.85
threo-DL	97.8	20.0	70^{20}	0.9299	1.4445	
threo-D	98.7	15.8		0.9289	1.4450	-17.05
threo-L	97.0	10.8		0.9274	1.4448	+16.91
Diamine						
meso	97.1	-21.6	60^{60}	0.8564	1.4420	
DL	96.6	-21.1	58^{60}	0.8499	1.4408	
L	77.1	-30.0		0.8768	1.4311	+17.08
Oxide						
meso			59.7	0.8226	1.3802	
DL			53.5	0.8010	1.3705	
D			53.7	0.7998	1.3705	+59.05
Imine						
meso	98.9	- 6.2	82.9	0.8171	1.4172	
L	97.5	-23.0	74.8	0.7880	1.4070	-101.99

* The melting points and boiling points of the meso and DL-glycols are taken from Wilson and Lucas (2), the boiling points, densities, and refractive indices of the meso and DL-oxides from Winstein and Lucas (4). The properties of the D-glycol and D-oxide are given by Lucas and Garner (1).

The amino alcohol (L(+)-erythro) formed by the reaction of D-2,3-epoxybutane with ammonia is a solid at 25°. The density, refractive index, and rotation of this compound were measured at this temperature with the supercooled liquid. Similarly, the values for the density and refractive index of meso-2,3-butanediol are new determinations made on the material in the liquid state at 25°.

Configurational purity of reaction products.--In no instance was evidence obtained to suggest that the reactions described did not lead to configurationally pure products. The crude amino alcohol derived from D-2,3-epoxybutane melted above 45°. Fractionation of the crude imines showed no trace of the alternate isomers although their boiling points differ by 8 degrees. Further, the resolution of the diamine derived ultimately from meso-2,3-butanediol involved successive crystallizations of the acid L-tartrate until a less soluble fraction showing constant solubility was obtained. Since the acid L-tartrate of the other diamine isomer was found to be much less soluble it would have been concentrated during the fractional crystallization had any substantial amount been present.

Direct syntheses of diamines and amino alcohols.--While the reactions indicated in Figures 1 and 2 give reasonable yields, many steps are involved and some of these steps are slow and difficult to carry out on a large scale in the laboratory. Thus, the procedures are not altogether satisfactory for

preparative purposes, especially for preparation of the diamines. Various more direct syntheses of the amino alcohols and diamines have been reported. The following were tested in the present investigation and found to give poor yields: reduction of 3-nitro-2-butanol with hydrogen (8, 9), reduction of dimethylglyoxime with hydrogen (10, 11), and reduction of dimethylglyoxime diacetate with sodium and alcohol (12).

A much better method for synthesizing both the amino alcohols and diamines has been developed here. It consists of reducing dimethylglyoxime in aqueous sodium hydroxide solution with Raney aluminum-nickel alloy. Schwenk et al. (13, 14, 15, 16) have reported the use of this procedure for the reduction of various compounds but not oximes. Forty per cent of the dimethylglyoxime is converted to diamine and 25% to amino alcohol, these products being easily separated by distillation.

The meso and DL-diamines can be easily, and almost quantitatively, separated by fractional crystallization of their dihydrochlorides; the former isomer comprises about a third of the diamine fraction. Separation of the erythro and threo-amino alcohols was not investigated.

Discussion of Probable Configurations

Arguments based on reaction mechanisms.--The diamine obtained as the final product of the sequence of reactions beginning with the meso-glycol was shown to be the DL form by fractional crystallization of its acid L-tartrate, which separated

it into optically active components. That the same reaction sequence starting with the D-glycol gives the meso-diamine follows from the facts that its physical constants are different, indicating that it is a distinct form, and fractional crystallization of its acid L-tartrate effects no separation.

Similarly, the imine isomers are identified by the observations that the one derived from the active glycol has a high rotation while the one derived from the meso-glycol is not only inactive but has different physical constants and is thus a different form. Since the active imine gives the meso-diamine and the meso-imine gives the DL-diamine it is established that the opening of the imine ring with ammonia is attended by a Walden inversion.

The configurations of the oxides are well-known. Thus, it can be said that the meso-oxide gives rise, in this scheme, to the meso-imine and the D-oxide to an active imine. Accordingly, zero or an even number of inversions are to be assigned to the three intervening steps.

An inversion in the reaction of the oxide with ammonia is reasonable in view of its probable mechanism (7) and the demonstrated courses of the analogous reactions of the oxide with water (1, 2) and of the imine with ammonia, above. Retention of configuration is to be expected in the reaction of the amino alcohol with sulfuric acid since this is a simple esterification

paralleling, for example, the acetylation of the glycol (17). The oxygen-carbon bond is not ordinarily broken in such reactions (18). Similarly, formation of the imine by treatment of the sulfuric acid derivative with base parallels the preparation of the oxide from the chlorohydrin and would be expected to involve an inversion (17).

Arguments based on comparisons of the physical properties.--The physical properties of the various compounds, shown in Table 1, provide an independent basis for distinguishing the erythro and threo-amino alcohols. It will be seen that the amino alcohol derived from D-2,3-butanediol has a higher boiling point, melting point, density, and refractive index than the other form. Since higher values for these constants characterize the meso form of both the glycol and the diamine (excepting the diamine melting points) it is reasonable to suppose that this is the erythro form of the amino alcohol.

From the conclusion that the high melting amino alcohol is erythro it follows that it is the L isomer (named as a derivative of 2-butanol) since it was derived in a single step from an epoxide known to be D. The only reasonable interpretation of the reaction is that the carbon-oxygen bond has survived from the oxide structure and the inversion has accompanied the formation of the carbon-nitrogen bond. Similarly, the active imine to which this amino alcohol gives rise must also be L since it is not reasonable that the carbon-nitrogen bond in the amino alcohol should have been broken.

Accepting these configurations, the signs and magnitudes of the observed rotations can be interpreted by the simple theory that replacement of an oxygen atom by N-H does not change the sign of the rotation due to that particular structure in the molecule but increases its magnitude. The low rotation of the erythro-amino alcohol results from competition between the oppositely directed actions of the two asymmetric centers. The net rotation is in the direction induced by the configuration about the third carbon atom because the amino group there enhances its effect. Similarly the magnitudes of the rotations shown by the threo-amino alcohols and the active diamine are greater than those shown by the glycols. The configurations of the respective threo-amino alcohol and active diamine isomers can then be inferred from the signs of their rotations.

Summary of arguments for assigned configurations.--Four distinct, if not altogether independent, arguments have been developed here to show that the high melting amino alcohol is the erythro form and indirectly that the configurations of the various compounds are as indicated in Figures 1 and 2. First, the reaction scheme as given is a reasonable one from theoretical considerations. Second, the demonstrations that hydrolysis of the oxides and ammonolysis of the imines are attended by Walden inversions make it quite reasonable to suppose that the very similar ammonolysis of the oxide proceeds in the same way. Third, the higher melting point,

boiling point, density, and refractive index of one form of the amino alcohol characterizes it as erythro when similar data for the glycols and diamines are considered. Fourth, the rotations of the active compounds, on the basis of a simple and plausible theory of the relative effects of amino groups and hydroxyl groups on optical activity, agree well with the assigned configurations.

Alternative interpretations.--It is not possible to effect direct demonstrations that compounds having different atoms attached to an asymmetric carbon atom have the same configuration. An alternative interpretation can always be given in which each formation or breaking of a bond to one of the atoms is assumed to proceed in the opposite way. Thus, despite the above discussion, the compounds pictured in Figures 1 and 2 could have been drawn with the positions of the nitrogen atoms reversed without conflicting with any direct experimental evidence that has been obtained here. In attempting to relate the configurations of these analogous amino and hydroxyl compounds, the object can only be to make alternate interpretations elaborately improbable.

As already indicated, the reactions necessary to support the alternate configurations of these amino compounds are improbable in the light of established theories of their mechanisms and the demonstrated course of analogous reactions. It is improbable but not impossible that the physical constants of the erythro and threo-amino alcohols should not be

related in the same way as those of the meso and active forms, respectively, of the glycols and diamines. In the same way, it is reasonable that the amino compounds should have rotations of about the same magnitude as the alcohols but rather farfetched to suppose that the replacement of an oxygen atom with an N-H group reverses the sign of rotation without substantially changing its magnitude.

Methods for obtaining additional evidence.--It is felt that the inferential evidence submitted here is so extensive that there can hardly be any doubt about the configurations of these amino alcohols. It would not be difficult, however, to enlarge the arguments by means of simple experiments suggested by the work already completed. Additional evidence could be obtained by reversing some of the reactions. The diamines might be converted to imines by heating their monohydrochlorides, the imines could be hydrolyzed to the amino alcohols, and possibly nitrous acid could be used to obtain the glycols from the amino alcohols.

The work described here could be directly related to other studies of the configurations of amino compounds by oxidizing the amino alcohols to alanines, by the reduction of threonine, or simply by the hydrogenation of the imines to give the sec.-butylamines. Also the arguments based on the physical properties of the compounds could be elaborated by extending the comparisons to different classes of compounds. However, it is not intended here to give a broad

analysis of the problem of interrelating the configurations of analagous amino and hydroxyl compounds. As explained in the introduction, the object has been to obtain a concise, self-contained demonstration of the courses of the reactions and the configurations of the isomeric compounds within the particular system selected for the study.

Applications of this Work to Other Investigations.

Some of the procedures described here might prove to be of synthetic interest since the biological importance of compounds related to 3-amino-2-butanol is attracting increasing attention. Examples of important compounds having related structures are sphingosine, the ephedrines, the amino acid threonine, and the anti-biotic Chloromycetin. Perhaps tartaric acid derivatives could be used as starting materials in the preparation of more elaborate compounds related to 3-amino-2-butanol by similar methods.

Since D-2,3-butanediol is now available these procedures permit the preparation, without the need for resolution, of a pure, inexpensive, optically active base which forms readily crystallized salts and might be valuable as an agent for the resolution of acids. The easily separated threo-amino alcohols provide a pair of such resolving agents with which both forms of optically active acids could be separated conveniently.

Many other oxide addition reactions are known, but have

not been studied with respect to the configurations of their products. Examples are the reactions of HCN, formaldehyde, alcohols, and Grignard reagents. Methods similar to those used here could be employed for the identification of the isomeric products of these reactions, and thus simple methods for the preparation of a large variety of optically active compounds might be developed.

Experimental Procedures

In general, the operations described below have been repeated many times since it was necessary to carry out a considerable amount of exploratory work in connection with the syntheses of the imines and diamines. Typical preparations are described rather than a single series of steps leading from glycol to diamine.

Preparation of 2,3-epoxybutanes.--The first three steps in the reaction scheme, Figures 1 and 2, were carried out by methods previously well described (1, 2, 6) without any important modifications. Rather higher yields were obtained, 80-90% going from glycol to oxide. The oxides were not carefully purified since the amino alcohols prepared from them were easily purified by crystallization of their salts. Typical oxide preparations had the following properties:

meso, b.p. 59.7-60.0° at 747 mm., n_D^{25} 1.3800

D, b.p. 53.2-53.6° at 748 mm., n_D^{25} 1.3704, α_D^{25} 46.73°.

Preparation of the 3-amino-2-butanols.--The reaction of 2,3-epoxybutane with aqueous ammonia has been previously reported (19, 20) to give rather poor yields of 3-amino-2-butanol mainly because the organic amino compound reacts with the oxide more rapidly than ammonia, thus producing bis-, and presumable tris-(hydroxybutyl)amines. This difficulty can be avoided by using a large excess of ammonia. To 2 *l*. (ca. 30 moles) of concentrated aqueous ammonia was added 72 g. (1 mole) of a 2,3-epoxybutane isomer and the mixture was allowed to stand at room temperature for 10 days in an ordinary screw-cap acid bottle. It is advisable to cool the ammonia to 10-15° beforehand in order to avoid rapid evolution of gas on mixing. Reasonable but smaller yields are obtained with a reaction time as short as 4 days. After 10 days the excess ammonia and most of the water were removed by distillation at atmospheric pressure through a 15 cm. column, until the flask temperature reached 105°. The remaining water was then removed under reduced pressure using a 60 cm. column packed with Raschig rings.

Purification of the DL-threo-amino alcohol, obtained from the meso-oxide, was effected by distillation through this column. Yield 68 g., 76%, b.p. 69-70° at 20 mm.

Distillation of the L-erythro-amino alcohol, obtained from the D-oxide, was inconvenient because of its high melting point. It was freed from water by distillation at 20 mm. up to 72°, the residue at this point having a melting point

above 45°. Yield 62 g., 70%. Crystallization from 100 ml. of isopropyl ether gave 46 g., 74% yield on the crystallization and 52% on the initial 1 mole of oxide. Because of the material's strong affinity for water it was not practical to recover the crystals by filtration. Instead they were washed with cold isopropyl ether and then with cold benzene. The crystals, wet with benzene, were placed in a desiccator and dried under vacuum. Recrystallized in this way the product melted sharply at 49.3°.

Resolution of DL-threo-3-amino-2-butanol.--Solutions of 17.1 g. (0.117 moles) of the amino alcohol and 26.6 g. (0.117 moles) of L-tartaric acid were combined to give a solution of 43.7 g. of DL-threo-3-amino-2-butanol acid L-tartrate. The solvent was ethyl alcohol containing 5% methyl alcohol and 5% water by volume. The salt was recrystallized successively from 3 one *l*. portions and finally from one 500 ml. portion by heating to effect solution and allowing to stand at about 22°. In this way 16.1 g. of salt having a constant solubility of 0.3 g. per 100 ml. was obtained. Reworking of the mother liquors by established fractional crystallization procedures produced an additional 4.9 g. having the same constant solubility. A total of 13 crystallizations was required. In resolving a larger batch it is convenient to increase the proportion of methyl alcohol in the solvent for the earlier crystallizations. The yield of salt, 21.0 g.,

was 96%. Treatment with aqueous sodium hydroxide liberated the free base which was recovered by distillation as described above for DL-threo-3-amino-2-butanol; b.p. 69-70° at 20 mm., α_D^{25} -15.31°, purity 97.9%.

A sample of this material was again converted to its acid L-tartrate and crystallized from the same solvent: solubility 6.4 g. in 2 ℓ. The recovered base had α_D^{25} -15.84, purity 98.7%.

The other form of this amino alcohol, recovered from the mother liquors by similar procedures, showed a lower rotation of opposite sign: α_D^{25} +13.75°, purity 97.8%. It was converted into its acid L-malate by mixing a solution of 26 g. in 100 ml. of absolute ethyl alcohol with a solution of 36 g. of malic acid in 100 ml. of absolute alchol. It was necessary to seed this mixture with crystals obtained at a higher concentration in order to induce crystallization. The product was recrystallized from 100 ml. of absolute ethyl alcohol giving 35 g. of salt. The free base recovered from this had α_D^{25} +15.69°, purity 97.0%.

Preparation of 1,2-dimethylethyleneimines.--Earlier methods for synthesizing these compounds (20, 21, 9) have been substantially improved by carrying out the reaction of the amino alcohol with sulfuric acid slowly on a steam bath and by adding the 3-amino-2-butyl hydrogen sulfate, so formed, a little at a time to hot aqueous potassium hydroxide, instead of mixing

it with the cold base and heating the whole mass.

A solution of 32 g. (0.36 moles) of 3-amino-2-butanol was neutralized to a methyl orange end point with a measured volume of 50% aqueous sulfuric acid, and then an equal volume of the acid was added in excess. This solution was evaporated on a steam bath at 15-20 mm. until a solid lump remained (8 hours). This was ground in a mortar and subjected to the same conditions of evaporation until constant weight was obtained (16 hours). Completion of the reaction was shown by the loss in weight, corresponding to the loss of one mole of water, and by the neutrality to methyl orange of the residue. The characteristics of these intermediate products were not investigated, but the yields appear to be nearly quantitative.

A 3 necked, 1-*l.* flask was fitted with a broad bladed stirrer, an exit line leading to a condenser, and a vertical, 10-mm. tube for introduction of the solid aminobutyl hydrogen sulfate. During the reaction a slow current of air, just sufficient to prevent condensation in this tube, was drawn through the system. A solution of 95 g. (1.4 moles) of potassium hydroxide in 125 ml. of water was placed in the flask, heated to about 90°, and 64 g. (0.36 moles) of the 3-amino-2-butyl hydrogen sulfate was added in small portions. The imine distilled from the mixture almost as rapidly as the 3-amino-2-butyl hydrogen sulfate was added. After the addition was complete the heating was increased and a total of about 50 ml. was taken over.

The distillate from the reaction mixture was redistilled through a 60 cm. column packed with glass helices and the products were collected at temperatures a degree or two above their proper boiling points since the fractionation was not sufficient to remove the last traces of water. These crude products were dissolved in n-butyl ether, allowed to stand over solid potassium hydroxide, and again recovered by distillation through the same apparatus.

The meso-imine was obtained from the threo-amino alcohol in 63% yield; b.p. 82.5-82.9°, m.p. -9.3 to -6.2°, purity 98.9%.

The L-imine was obtained from the L-erythro-amino alcohol in 58% yield; b.p. 74.5-74.8°, m.p., -26 to -23°, purity 97.5%.

Preparation of the 2,3-butanediamines.--The reaction of the imines with ammonia was carried out in a monel metal-lined bomb with ammonium chloride as a catalyst. The need for this catalyst was not established. Preliminary experiments showed that, in contrast to the ready reaction of epoxy compounds with ammonia, no reaction of the imine with ammonia occurred in aqueous solution, or even after long refluxing of the imine with liquid ammonia containing ammonium chloride at -33°. For the bomb reaction a mixture of 8.3 g. (0.12 moles) of imine, 15 g. (0.28 moles) of ammonium chloride, and 130 ml. (ca. 6 moles) of liquid ammonia was used. The bomb was heated to 50-55°, for 110 hours. On evaporation of the excess ammonia at the conclusion of the reaction the diamine remained, probably as

the monohydrochloride. The residue was treated with excess aqueous sodium hydroxide and distilled under reduced pressure without any attempt to obtain a dry diamine fraction since earlier experiments had shown that it is difficult to remove the last traces of water by distillation alone. The diamine was then collected as the dihydrochloride, dried thoroughly by warming under vacuum and then treated with sodium methylate in methyl alcohol. By repeated distillation of the methanol solution pure, almost anhydrous, samples of the diamines were isolated. With both isomers the yield was 2.5 g., 23%, but at least half of the material formed by the reaction was lost in the tedious purification process.

meso-2,3-Butanediamine was obtained from L-1,2-dimethyl-ethyleneimine; b.p. 59-60°, m.p. -22.- to -21.1°.

DL-2,3-Butanediamine was obtained from meso-1,2-dimethyl-ethyleneimine; b.p. 57-58°, m.p. -23.5 to -21.6°.

Resolution of DL-2,3-butanediamine.--The acid D-tartrate of the DL-diamine was prepared by mixing absolute alcohol solutions of 2.5 g. (0.028 moles) of diamine and 8.4 g. (0.056 moles) of L-tartaric acid. This salt is nearly insoluble in absolute alcohol and 10.4 g. precipitated from 100 ml. Eleven crystallizations using a mixture of 2 volumes of ethanol to 1 of water as solvent gave 3.8 g. of salt, a 70% yield, having a constant solubility of 0.6 g. in a mixture of 100 ml. of ethanol and 50 ml. of water. Because of its low solubility

in anhydrous solvents this salt could not be decomposed with sodium methylate. It was treated with aqueous sodium hydroxide, the diamine was distilled, converted again to the dihydrochloride, dried and recovered as in the original diamine preparations. The final yield was 0.4 g., 32%; b.p. 57-59°.

The other diamine isomer obtained in the series of reactions starting with D-2,3-butanediol was converted by the same procedure into a less soluble acid D-tartrate. Successive crystallizations of this salt from water showed constant solubility, 8 g. per 100 ml., and the refractive indices of the successive mother liquors were similarly constant at 1.3430.

Reduction of dimethylglyoxime.--In 3 *l.* of water were dissolved 371 g. (9 moles) of sodium hydroxide and 116 g. (1 mole) of dimethylglyoxime. This mixture was placed in a 3-necked, 5-*l.* flask fitted with a sealed, broad-bladed stirrer, a thermometer, and an exit line to dispose of hydrogen and ammonia. The mixture was heated to 50° and 248 g. of Raney aluminum-nickel alloy was added in 2 g. portions at such a rate as to maintain the temperature between 48 and 52°. Ninety minutes was required for the addition of the metal which contained 50% more aluminum than that theoretically necessary to reduce 1 mole of dioxime either to diamine or to amino alcohol.

After standing overnight the reaction mixture was separated by filtration from the residual nickel and distilled rapidly without fractionation until, after 2 *l.* of distillate, all but

traces of volatile base had been removed. The distillate was then made just acid to methyl orange with hydrochloric acid and evaporated to dryness, the last traces of water being removed by azeotropic distillation with isopropyl alcohol. The dry salt was decomposed with a solution of 34 g. (1.48 moles) of sodium in 350 ml. of methyl alcohol. This quantity of sodium was equivalent to the base present in the distillate from the reaction mixture as determined by titration. The mixture was flash distilled giving a solution of the organic bases in dry methanol which was then distilled under reduced pressure through a 60 cm. ring packed column.

The diamine fraction, taken from 40 to 45° at 30 mm., weighed 40 g., yield 41%, purity 92%.

The amino alcohol fraction taken from 70 to 74°C at 20 mm. weighed 21 g., had a purity of 102%,* a yield (on a 100% basis) of 25%.

Separation of diamine isomers.--A sample of diamine from the reduction of dimethylglyoxime was neutralized in aqueous solution with hydrochloric acid and evaporated to dryness giving 13.2 g. of 2,3-butanediamine dihydrochloride. Fractional crystallization using methyl alcohol and methyl alcohol-water mixtures as solvent gave 3.7 g. having a constant solubility in methyl alcohol at 22°C of 0.9 g. per 100 ml. The more

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* As determined by titration with hydrochloric acid to a methyl orange end point.

soluble fraction obtained from the mother liquors from this crystallization had a solubility of 7 g. per 100 ml. Approximate measurements of the solubilities of the hydrochlorides of the diamines from the imine-ammonia reactions, described above, showed that the less soluble is the meso form, the more soluble DL.

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PART II

THE PREPARATION OF SPECIFIC ADSORBENTS

PART II

THE PREPARATION OF SPECIFIC ADSORBENTS*

The functioning of enzymes and antibodies characteristically involves the loose binding of foreign molecules. These processes, the attraction of an enzyme for its substrate and of an antibody for its antigen, resemble in many ways the simple adsorption of a substance on a solid surface. However, there is a most conspicuous difference between these two sorts of phenomena. Enzymes and antibodies are specific adsorbents. The adsorption affinities which they display are directed toward narrow classes of substances or toward single substances, while ordinary adsorbents do not make such sharp distinctions.

The proposition underlying the investigation to be described here is a method for conferring on ordinary adsorbents just such specific affinities for predetermined substances. The method by which it now appears that this can be done consists simply of forming the permanent or rigid structure of the adsorbent in the presence of the particular substance for which it is desired to prepare a specific adsorbent.

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* A brief report of this investigation is in press (1).

Theoretical basis for this method.--At first thought it might seem unlikely that the mere presence of a foreign substance during the formation of an adsorbent would have a large enough effect on its structure to produce significant changes in its ultimate adsorption properties. This idea appears to be more reasonable, however, when consideration is given to certain observations that can be made on ordinary adsorption phenomena in connection with the use of chromatography. Some of the arguments that led to the initiation of this study are indicated below.

In the first place, when two substances are adsorbed on the same surface their competition for this surface is not complete. For example, Tiselius (2) reports that the most effective agent for displacing protein from the adsorbed state is a strongly adsorbed protein. In general, the more strongly adsorbed a substance is the more effective it will be as a displacing agent. But this is only one criterion for estimating displacing power. Another is that the agent should bear a structural resemblance to the substances which it is to displace.

Further, even under the most favorable circumstances, adsorbed substances are not ordinarily completely removed from the adsorbed state. When a particular fraction is eluted from a chromatographic column, traces of it remain as though there were regions on the adsorbent surface which held the substance

very strongly. These observations suggest not only that ordinary adsorption processes depend in some measure on details of the surface structure of the adsorbent, but also that there is a certain specificity associated with these structural details. The regions on the adsorbent surface that most strongly attract the molecules of one compound appear not to be identical with the regions which most strongly attract the molecules of a different compound.

If then, regions having specific adsorption affinities appear spontaneously in the structures of ordinary adsorbents, it is easier to believe that the action of small forces during the formation of an adsorbent could effect significant specific changes in its properties. The fact that significant changes in surface characteristics (although non-specific changes) are easily produced is indicated by the difficulties that are encountered in reproducing the characteristics of a given preparation of an adsorbent.

The mechanism by which the presence of foreign molecules during the formation of an adsorbent might be expected to produce a specifically attracting surface is the same as that proposed by Pauling (3) for the formation of antibodies with use of antigen molecules as a template, which formed the basis for the manufacture of artificial antibodies reported by Pauling and Campbell (4). The adsorbent in the process of formation has accessible to it a very great number of structures

which differ only slightly in stability. In the presence of a foreign molecule those structures that are stabilized through attraction for the foreign molecule are preferentially assumed. The adsorbent is thus pictured as automatically forming pockets that fit closely enough to the foreign molecule to hold it by van der Waals forces, hydrogen bonds, interionic attractions, and other types of intermolecular interaction. It should be pointed out that it is not necessary to make definite assumptions about the nature of the action of the foreign molecules on the forming adsorbent structure in order to predict that a specifically attracting structure will result. It is sufficient to assume that the presence of the foreign molecules modifies the ultimate structure of the adsorbent. Any modification so produced will be in such a direction as to increase the stability of the adsorbed state and hence to increase the affinity of the adsorbent for the particular molecules involved.

Extent of the present investigation.--The work to be described here has been directed toward obtaining a complete demonstration that it is possible to prepare specific adsorbents by means of the process discussed above. This objective has been attained but time has not permitted any organized exploration of the broader aspects of the phenomena involved. The first three sections below deal with the design of an experimental demonstration of specific adsorption, details of the

experiments actually carried out, and an analysis of their results. Some miscellaneous additional experimental information is then presented together with a discussion of its bearing on the theory of the process. In conclusion, plans for future work in this field are outlined.

Requirements for a Demonstration of Specific Adsorption

An experimental demonstration of the theory outlined above has been effected by preparing adsorbents in the presence of particular compounds, removing the compounds by prolonged extraction, and observing that this method of preparation has the following results:

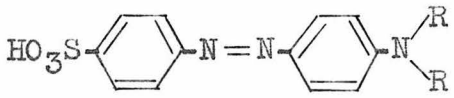
1. It increases the power of the adsorbent to bind molecules of the particular compound with which it was prepared.
2. It does not increase, or does not increase to so great a degree, the power of the adsorbent to bind molecules of different compounds.

Selection of the adsorbent.--Silica gel was selected as the adsorbent for these experiments mainly because of its convenience. Certain special qualifications of silica gel might be mentioned at this point, however, since they will be important to a later discussion of the general significance of the work.

First, silica gel can be formed under mild chemical and physical conditions so that the co-present compound for which specific affinities are to be created is not likely to be

affected by the process. Second, its structure, once formed, is reasonably stable and may be expected to survive the procedures necessary for measuring adsorption properties. Third, and perhaps most important, the natural structure of silica gel is quite amorphous and hence will offer minimum resistance to the imposition upon it of a specific configuration.

Selection of the compounds to be adsorbed.--The compounds for which specific adsorbents have been prepared are methyl orange and some of its homologues--ethyl, n-propyl, and n-butyl orange. These dyes have the following structure:



where R represents the radicals named. Strongly colored substances are useful for this sort of study because very low concentrations can be conveniently measured and compared. It now seems likely that the moderate degree of adsorption on ordinary silica gel which this particular series of compounds shows is of critical importance to the results obtained. This question will be taken up later.

Selection of experimental conditions.--Besides the choice of materials there are many other variables in the design of an experimental demonstration of specific adsorption which might profoundly influence its outcome. However, more or less arbitrary selections were made of the precise conditions for

preparing the adsorbents and for measuring their adsorption powers. A detailed description will be given of the most recent and most complete of these experiments but the reader should bear in mind that the details of the procedures are not known at this time to have any special merit.

Procedure for Preparing and Testing Specific Adsorbents

The following is an exact description only of the procedures followed in preparing and testing gel samples 34-39 for the principal experiment on which this demonstration of specific adsorption rests. However, all of the gels for which quantitative results will be reported were prepared by approximately the same method.

Materials.--Sodium silicate solution from Mefford Chemical Company had a density of 1.401 g. per ml. at 20°. It contained 3.5 equivalents of base per liter as determined by titration to a phenolphthalein end point.

Grasselli CP acetic acid was used.

Methyl and ethyl orange were obtained from Eastman Kodak Company and used without purification. Their absorption spectra agreed well with observations made on samples synthesized in this laboratory.

Propyl and butyl orange were synthesized from Eastman Kodak Company samples of di-n-propylaniline and di-n-butylaniline respectively by coupling with diazotized sulfanilic

acid according to standard procedures (5). The sulfanilic acid, of analytical grade, was manufactured by Powers-Weightman-Rosengarten (now Merck).

Combination of gel ingredients.--A diluted sodium silicate solution was prepared by mixing 192 ml. of the concentrated solution with 800 ml. of water and from this solution there were withdrawn six 168.8 g. portions (containing ca. 30 ml. of the initial concentrated solution). These weighed portions of silicate solution were placed in 600 ml. beakers numbered for identification from 34 through 39. To each was added 50 ml. of water and 130 ml. of a solution prepared by diluting 180 ml. of acetic acid to 800 ml. with water. The dilute acetic acid portions added to beakers 35, 37, 38, and 39 contained one half gram of methyl, ethyl, propyl, and butyl orange respectively. This is more dye than is soluble in the mixtures described and blue crystals of the acid forms of the dyes settled out* during the setting of the gels, which required about six hours. These operations were carried out at room temperature, 20-23°.

Processing of gels.--The above mixtures were allowed to stand in beakers for eight days (covered with watch glasses), during which time weight losses ranged from six to twelve grams. They were then expelled on paper towels on a wooden rack and allowed to dry for six days longer, losing from 304 to 317 grams, and

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* It was shown in earlier experiments, by centrifuging the gels before they set, that the suspended material did not affect the ultimate properties of the adsorbents.

leaving weights ranging from 35 to 48 grams. The gels were now quite brittle; they were ground in a mortar, sieved, and the portions between 48 and 200 mesh, comprising about 70% of the whole, were placed in Soxhlet extractors with methyl alcohol. The extraction, which was continued for 80 to 89 hours, involved contact with approximately 1000 30-ml. portions of methyl alcohol.

Methyl alcohol was used for this purpose because it was felt that the gel structure would be more stable if water were removed (the methyl alcohol was replaced several times during the extraction). Methyl alcohol boils more readily than water, and the extractors work more rapidly. Also more dye is removed with this solvent than with the same volume of water.

At the conclusion of the extraction period the remaining solvent was allowed to evaporate from the gels in air. The last traces of dye were not removed by the extraction, and gels 35, 37, 38, and 39 retained a bright pink color. Gels 34 and 36, prepared without dye, were white.

Standard dye solutions.--Standard solutions of the four dyes were prepared by dissolving 1.50×10^{-4} moles of dye in hot 5% acetic acid and making the volume up to a liter in a volumetric flask. In preparing these solutions it is important to use a large part, more than half, of the solvent to effect solution since the desired concentrations are not far from saturation and the rate of solution is very low.

The absorption spectrum of each dye was measured with a Beckman spectrophotometer from 4000 through 6000 Å and at more than one concentration (in 5% acetic acid) in order to show that Beer's law holds for the solutions. All showed a maximum at 5100 Å. Absorptions at this wave length are tabulated below.

Table 2.

Dye	Extinction x 10^3 at a concentration of 1.0 x 10^{-9} moles per ml.
Methyl Orange	8.17
Ethyl Orange	4.04
Propyl Orange	8.27
Butyl Orange	6.00

The low value for the color intensity of ethyl orange is difficult to explain. Perhaps these results are to be correlated with the basic strengths of the corresponding alkylamines as shown in the following table.

Table 3.

	K_b x 10^4		K_b x 10^4
Methylamine	4.4	Dimethylamine . . .	5.1
Ethylamine	5.6	Diethylamine . . .	12.6
n-Propylamine	3.9	Di-n-propylamine .	8.2
n-Butylamine	4.1		

Dyes of the methyl orange series are not especially stable in aqueous solution. Losses of color of the order of 2-3% per week were sometimes observed. The standard solutions used in this experiment were prepared, and their absorption spectra were taken, immediately before their use in measuring the adsorption properties of the gels as described below.

Measurement of adsorption.--One-gram samples of gels 34-39 were weighed into 10-ml. mixing cylinders to an accuracy of ±1 mg. The concentrations of aqueous solutions may be increased by contact with dry silica gel as a result of uptake of water by the gel. In order to avoid the possibility that random effects of this sort would arise from different initial degrees of hydration of these gel samples, they were first brought into equilibrium with the 5% acetic acid used as solvent for the adsorption measurements. A 10-ml. portion of 5% acetic acid was added to each cylinder, they were shaken mechanically for 15 minutes, allowed to settle and decanted. This process was repeated, allowing the gels to stand in contact with the solvent for an hour or two before decanting.

Finally a third portion of solvent was weighed into each cylinder so that the whole weight was 9 g. greater than the weight of the gel alone. Addition of 1 g. of standard, 1.50×10^{-4} formal, dye solution then gave a system consisting of 10 g. of solvent, 1.000 g. of gel and 150×10^{-9}

moles of dye.

These mixtures were shaken mechanically for 30 minutes, allowed to settle, decanted into centrifuge tubes, and after centrifuging, the nearly clear supernatant solutions were placed in Corex cells and absorption spectra taken at 200 Å intervals from 4000 through 6000 Å and at 5100 Å.

The adsorbent samples were recombined with their solutions, allowed to stand for 24 hours, again put through the shaking and centrifuging processes, and new absorption spectra taken. The data obtained in this latter set of readings were used for the calculations described below. The changes observed on standing and their significance will be taken up later.

Correction for cloudiness.--After centrifuging, the solutions retained a certain amount of cloudiness, which, especially in the determinations of adsorption of propyl and butyl orange, seriously affected the apparent color intensity. Measurements in separate experiments in which no dye was used showed that the light absorption due to cloudiness was 1.4-1.5 times greater at 5100 Å than at 6000 Å. Since the light absorption by the dyes was quite negligible at 6000 Å, it was possible to make an empirical correction of the readings at 5100 Å based on the readings at 6000 Å; the factor 1.46, the average of the results obtained in experiments without dye, was used for this purpose.

In a number of instances direct determinations of this cloudiness were obtained by destroying the dye color with a

trace of aqueous bromine. The results did not differ significantly from those obtained by the described calculation and the values for cloudiness actually used in calculating dye concentrations were derived from the readings at 6000 Å as explained above.

Analysis of Experimental Results

The ideal method for analyzing the adsorption properties of these gels would be to make a series of determinations like that described above, using different dye concentrations, and from these results to derive equations relating the distributions of the dyes between the adsorbed and dissolved states to their concentrations. The constants from such equations would then provide a basis for comparing the adsorption properties of the respective gels. It was found, however, that the data necessary for this sort of analysis could be obtained only with the expenditure of an inordinate amount of time. The principal difficulty was that the adsorption processes under study are not wholly reversible. The problems involved are discussed briefly in the following section. Since such an elaborate analysis was not required for the principal objective of the investigation, demonstration of the reality of specific adsorption, a much simpler procedure was adopted. The gels were compared on the basis of the results of the application of a single standard procedure, namely the procedure already described.

From the readings at 5100 Å, corrected for cloudiness, and the prior determinations of the dye absorptions at this wave

length, Table 2, the dye concentrations in the supernatant solutions in moles per gram x 10^9 were calculated. The corresponding concentrations in the adsorbents were then obtained by subtracting from 150, ten times the supernatant concentrations.

A determination was made of the extent of adsorption of each dye on each gel under the single set of conditions indicated above. For each dye-gel pair the ratio of the concentration of dye in the adsorbent to the concentration in the gel, termed the "adsorption power", was then calculated as shown in the following tables.

Table 4.

Adsorption of Methyl Orange.

Gel. No.	Reading	Cloudiness at 5100 Å	Corr. Reading	Conc. in Sol.	Conc. in Gel	Adsorption Power
34	67	2	65	1.60	134	84
35	20	0	20	0.49	145	296
36	65	0	65	1.60	134	84
37	30	2	28	0.69	143	207
38	40	10	30	0.74	143	193
39	47	2	45	1.11	139	125

Table 5.

Adsorption of Ethyl Orange.

34	40	7	33	1.65	134	81
35	28	6	22	1.10	139	126
36	38	4	34	1.70	133	78
37	11	7	4	0.20	148	740
38	17	10	7	0.35	146	417
39	22	9	13	0.65	144	221

Table 6.

Adsorption of Propyl Orange.

Gel. No.	Reading	Cloudiness at 5100 Å	Corr. Reading	Conc. in Sol.	Conc. in Gel	Adsorption Power
34	28	4	24	0.64	144	225
35	25	4	21	0.56	144	257
36	26	4	22	0.59	144	253
37	17	6	11	0.29	147	507
38	16	15	1	0.03	150	5000
39	11	6	5	0.13	149	1150

Table 7.

Adsorption of Butyl Orange.

34	25	9	16	0.45	145	322
35	21	7	14	0.40	146	365
36	22	6	16	0.45	145	322
37	20	13	7	0.20	148	724
38	25	22	3	0.08	149	1860
39	14	13	1	0.03	150	5000

Comparison of the adsorption powers shown by the gels for the particular dye used in their preparation with the adsorption powers shown by the control gels, as summarized in the following table, satisfies the first requirement for a demonstration of specific adsorption; the presence of a dye during the preparation of the adsorbent increases the affinity of the adsorbent for that dye.

Table 8.

	Adsorption power for:			
	Methyl Orange	Ethyl Orange	Propyl Orange	Butyl Orange
Control Gel	84	80	240	320
Specific Adsorbent	300	740	5000	5000

Tables 4-7 clearly satisfy the second requirement also since they show that in every instance the best adsorbent for a given dye is the gel that was prepared with that dye and, with one exception, each gel is a better adsorbent for the dye with which it was prepared than for any other in the series. These arguments are best presented in terms of a new quantity, the "relative adsorption power", defined as the ratio of the adsorption power shown by a given gel for a given dye to the average of the adsorption powers shown by the control gels for that dye. The relative adsorption powers calculated from

the data presented in Tables 4-7 are shown in Table 9 below.

Table 9.

Relative adsorption power for:

		Methyl Orange	Ethyl Orange	Propyl Orange	Butyl Orange
Gel prepared with:	Methyl Orange	3.5	1.6	1.1	1.1
	Ethyl Orange	2.5	9	2.1	2.2
	Propyl Orange	2.3	5	20	6
	Butyl Orange	1.5	2.8	5	15

Additional Experimental Information and Interpretations.

Earlier demonstrations.--In the preceding section the most recent experimental demonstration of specific adsorption has been described in some detail. Quite similar results were obtained in earlier experiments which were not conducted under such well controlled conditions. In the following table are presented the relative adsorption powers found for gels prepared with three of the dyes in this series in an entirely independent experiment. In this experiment the initial molar concentrations of the respective dyes varied widely and the details of the data are not comparable with other results. Nevertheless, the specific effect of the method of preparation on the characteristics of the adsorbents is clearly shown.

Table 10.

Relative adsorption power for:

		Methyl Orange	Ethyl Orange	Butyl Orange
Gel prepared with:	Methyl Orange	4.8	1.5	1.4
	Ethyl Orange	2.1	5.2	1.5
	Butyl Orange	1.4	1.6	3.9

In a still earlier experiment, Table 11, it was shown that silica gel samples prepared with this series of compounds are not better adsorbents for a structurally unrelated dye, fluorescein. In this case, the observation that the gel prepared with ethyl orange appears to be a better adsorbent for methyl orange than for ethyl orange is to be attributed in part to the fact that the methyl orange was presented at a much lower concentration where the specific effect is magnified (see page 49).

Table 11.

Relative adsorption power for:

		Methyl Orange	Ethyl Orange	Fluorescein
Gel prepared with:	Methyl Orange	4.3	1.8	0.9
	Ethyl Orange	5.0	4.5	0.8

While these data establish beyond question that, at least in this system, the prescribed method of preparation leads to the formation of specific adsorbents, they support only by inference the suggested mechanism and they shed little light on the question of the general applicability of the method. In the remainder of this section these problems will be considered, but only briefly and qualitatively since they have not been the subjects of systematic study.

Significance of dye structure.--Qualitative tests of adsorption on silica gel were made with a large number of water soluble dyes with a view to finding likely compounds for broadening this study of specific adsorption. The following general conclusions were reached: 1) that a basic group (i.e. an amino or alkylated amino group) is usually necessary in order that a dye should be adsorbed on silica gel from aqueous solution to an appreciable extent*, 2) that the presence of an acidic structure ($-SO_3H$ in the methyl orange series) greatly reduces the affinity for silica gel produced by the presence of an alkylated amino group, and 3) that the attachment of a phenyl or even a benzyl group to the basic nitrogen atom in such compounds still further reduces their affinity for silica gel.

Since the basicity of the nitrogen atom plays such a prominent role in determining the degree of adsorption of dyes

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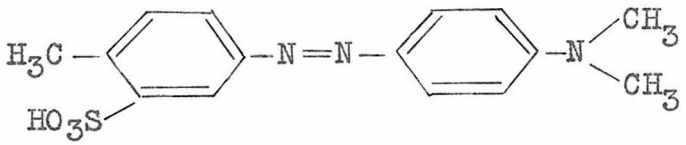
* Fluorescein is an exception.

on silica gel it is not unreasonable to imagine that this is the point of attachment of the dye to the gel. Accordingly, it might be predicted that specific affinities could not be produced in this way for structures remote from the basic nitrogen atom. The adsorption of a dye, sometimes called methyl methyl orange, having the following structure:



was tested with some of these adsorbents. Its behavior paralleled that of methyl orange, showing that the specificity of the adsorbents is not critically concerned with the configuration of the acidic end of the molecule. The quantitative tests necessary to show whether or not any distinction at all between these two dyes can be made have not been carried out.

The proposed mechanism for the formation of specific adsorbents requires that the foreign molecules be in the vicinity of the developing adsorbent structure during its preparation. In the system under study, silica occupies a very small fraction of the gel volume at the time of setting. It is perhaps unreasonable to suppose that dye molecules would be found in the right position with respect to the silica just by chance. Instead it seems likely that a mechanism is required to hold the dye molecules in the vicinity of the forming gel. This auxiliary mechanism might well be the natural or ordinary

adsorption of the dye. The picture would be that dye molecules are attracted to the silica at an early stage in the formation of the gel and that the gel then grows up around them.

On this basis, one would predict that substances that are not normally adsorbed on silica gel would fail to produce specific adsorbents. It was found that gels prepared with orange II., orange IV., and methyl benzyl orange, dyes which are very slightly adsorbed on ordinary silica gel at the pH used in the gel preparations, were not notably better adsorbents than the controls for the respective dyes.

The dyes malachite green, crystal violet, and methyl red have dimethylamino groups like methyl orange and are very strongly adsorbed on ordinary silica gel. Gels prepared with them had high relative adsorption powers for methyl orange. However, they were not tested with the dyes used in their preparation. Because of the high natural adsorption of these dyes a modified testing procedure would be required.

Adsorption equilibria.--Adsorption power values were easily reproduced when a single definite procedure was followed in determining them. When an attempt was made to reverse the process by bringing the gels in contact with fresh solvent it was found that a substantial part of the dye taken up by both the specific adsorbents and the control gels did not go back into solution so that different values for the adsorption powers at the same

supernatant concentrations were indicated. This phenomenon was not studied further.

Constant adsorption power values were reached with the control gels during the first 30-minute shaking period. The specific adsorbents took up a small additional amount of dye during the 24-hour period before the reported observations were made. A second 24-hour period of standing did not produce significant changes. It was observed that once such "equilibria" were reached the addition of more dye to the systems gave constant adsorption power values after 30 minutes shaking with both the specific adsorbents and the control gels.

The adsorption power, as defined earlier, decreases with increasing dye concentrations in all cases. However, in the range of concentrations where these measurements were made the adsorption powers shown by the control gels change but slowly with changing concentration while the adsorption powers shown by the specific adsorbents for their respective dyes change very sharply with concentration.

These observations are easily interpreted on the basis of the proposed mechanism for the formation of specific adsorbents. Slow attainment of equilibrium might reflect a requirement by the specifically attracting sites for particular orientations of the dye molecules to be adsorbed. The abrupt decrease in adsorption power at higher dye concentrations agrees with the idea that the specific adsorbents have been provided with a limited number of very strongly attracting sites.

Alternative interpretations.--While the experimental results can not be offered as a demonstration of the validity of the theory on which the investigation was based it is difficult to explain them in any other way. It is possible that certain non-specific or semi-specific effects contribute to the characteristics of the gels. For example, the presence of positively charged dye molecules during the setting process might simply distort the distribution of naturally occurring negatively charged sites in the gel so that a larger proportion of them appeared in accessible regions. This would enhance the adsorption power for basic substances as a class. If the idea is elaborated to include a growth of the gel as close to the negative site as the presence of the dye molecule permits the result could be a one-sided specificity. Gels prepared with ethyl orange would be good adsorbents for methyl orange because the basic group is small but not for propyl orange because it would not fit into the hole left by the smaller ethyl orange molecule.

Study of the tables of relative adsorption powers suggests that these considerations may have some significance although the details of the tables are really not very well established. In any case, this modified mechanism can not explain the whole specific effect observed, nor, in view of the low relative adsorption powers shown by the butyl orange gels for methyl orange, any large part of it. It must be that the gel structure is modified in such a way as to increase the forces by which particular dye molecules are held.

It should be mentioned that an explanation for the observations has been offered (6) which does not entail specific modifications of the gel structure. This explanation ascribes the results to micelle formation by the dyes and the fact that the dye is not completely removed during the preparation of the adsorbents. If the residual dye in the adsorbents were in the form of trapped agglomerates of dye molecules then the adsorbents could be pictured as presenting a dye surface as well as a silica surface. Such a dye surface might have specific adsorbing properties in the same way that a crystal can be described as a specific adsorbent for molecules like those of which it is composed.

While this alternative explanation has many minor shortcomings, and is perhaps lacking in intuitive appeal, it is not directly inconsistent with any of the data now available. It could be tested with experiments employing compounds not inclined toward micelle formation or simply by removing the last traces of dye from the adsorbents. The residual dye can be removed slowly by extraction with weak base (strong base dissolves the gel) or by oxidation with aqueous bromine. The effects of such treatments on the adsorption properties of the gels have not been studied.

Conclusion

The development of a general method for preparing specific adsorbents for predetermined substances would be of inestimable

value to many branches of chemistry. Such specific adsorption processes, even on the small scale described here, could find important applications to the analysis of complicated mixtures of organic compounds. If higher ratios of adsorbed material to adsorbent can be obtained the process might be used in the preparation of valuable compounds, especially in the isolation of such compounds from biological sources. Probably the greatest potential importance of specific adsorbents lies in the possibility that they can be made to function as specific catalysts and thus to reproduce the action of enzymes. It would seem almost to follow that if a reaction is catalyzed by a surface it will be affected in a specific way by a surface having specific affinities for one or more of the molecular species involved.

As stated in the introduction to this report, only one thing has been definitely established by the investigation. In the system involving silica gel and dyes of the methyl orange series it is possible to prepare adsorbents having strong affinities for particular details of molecular structures. It may develop that the method for preparing such adsorbents is in some ways dependent upon special features of the system in which the studies have been made. However, even if such special features are found to be responsible, it is reasonable to hope that they can be identified and studied and that when they are well understood it will still be possible to make a general application of the principles involved.

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PART III.

THE ROLE OF PEROXIDES IN MUTAGENESIS

PART III.

THE ROLE OF PEROXIDES IN MUTAGENESIS*

The process by which genes occasionally undergo reproducible changes can be regarded as being fundamental to the very nature of life. Modern theories of the origin of life (1,2) suggest that it was not the development of a self-duplicating mechanism that marked the transition from inanimate matter to living things but the appearance of the capacity to reproduce random variations. Only when this mysterious property had been acquired could the units of incipient life progress and elaborate through the familiar mechanisms of evolution.

Studies of the mutation process are not pursued only because of this direct interest in the phenomenon itself, however. Mutant genes have provided the raw material for the investigations on which virtually all of our knowledge of genetics is based. The discovery by Muller in 1927 (3) of artificial means for producing mutations has enormously accelerated these investigations. New findings in the mutagenesis field, the work of Auerbach (4,5,6,7,8,9,10), for example, and that reported here, may eventually provide valuable tools for other genetical studies as well as advance our understanding of the mutation process itself.

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* This investigation has been conducted jointly with Mr. George H. Cleland and Miss Carol Lotz.

The present investigation has not been directly concerned with the nature of the actual change in gene structure which constitutes a mutation. Between the application of a mutation producing agent to an organism and the ultimate alteration of a gene a number of steps may intervene. It is the earlier steps in this process that form the subject of the investigation described here.

The investigation has been based upon the hypothesis (11) that established mutagenic agents, radiation, mustard gas, etc., function through the intermediate formation of organic peroxides. The effects of many different chemical substances on mutation rates have been examined with a view to lending substance to this speculation and some substantial progress has been made. It has been found that several organic peroxides are powerful mutagenic agents, perhaps more powerful than any chemical agents known heretofore. The possibility that certain substances, anti-oxidants and reducing agents, which could destroy or prevent the formation of peroxides, would oppose the mutagenic action of ultra-violet light and nitrogen mustard has also been studied but without obtaining conclusive results. It is felt that these investigations might be profitably continued because certain aspects of the original hypothesis now appear to have real significance, and also because the way has been cleared for the definition of a broad class of mutagenic substances.

The availability of a large number of mutation producing agents would make possible new approaches to the problem of the nature of mutations including perhaps, the discovery of substances producing specific mutations.

The following topics will be taken up in this report: first, the hypothetical role of peroxides in the mutation process, second, the principal experimental procedures and the results obtained, and then a theoretical interpretation of these results and of data recently obtained by other workers in the field. Finally, the prospects, mentioned above, for further work along these lines are considered in more detail.

The reader should be advised that the experimental work described here is not offered as a demonstration of the validity of the hypothesis on which it is based. From this standpoint the investigation is in a very early stage. Also, some of the experiments that have been included are incomplete in the sense that they require more work for proper interpretation. It was decided not to limit this treatment to the principles that have been thoroughly established and the experiments which support them because the whole field of chemical mutagenesis is in such an uncertain state at this time that even fragmentary information is of value.

A Hypothetical Mechanism for the Induction of Mutations.

The discovery of the mechanism of the mutation process is a problem of unique difficulty. It is a search for the mechanism of a process that is not well defined. One has no real understanding of the change that has occurred when a mutation is observed from which to proceed to ascertain exactly what steps have brought it about. Rather one hopes that a study of the earlier steps in the process will shed some light on the nature of the gene and of the reproducible changes to which it is susceptible.

While these difficulties can not be avoided, an interpretation of available information on mutagenesis might profitably be restricted to well defined, or what one might call "conventional" mutations. Similarly, attention should be confined to processes producing substantial changes in mutation rates. Thus one may avoid confusion with secondary effects of hopeless complexity.

Known mutagenic agents.--On this basis there are very few well known methods for inducing mutations. Such activity has long been established for various high energy radiations, especially x-rays and ultra-violet light (3,12,13). More recently the similar effects of mustard gas (10), the nitrogen mustards (8,14), and to a lesser degree, allyl isothiocyanate (9) have been clearly demonstrated. Tests by many investigators (15,17) over several decades have failed to uncover other chemical

mutagens. Thus, this property is at least uncommon.

Of course, mention should be made of the fact that certain unidentified substances produced by living organisms regularly bring about changes which can only be described as mutations (18,19). These are special effects, however, and will not be considered here. Similarly, mention is made in the following paragraphs of a number of reports of compounds showing weak mutagenic activity. This material has been included to give an idea of the nature of earlier work in the field of chemical mutagenesis. While some of these reports will be taken up later in the light of the results of the present investigation, in general, no attempt will be made to interpret them in the broader discussions of the theory of the mutagenic process.

A survey of the literature of chemical mutagenesis reveals much disagreement and uncertainty. Timofeef-Ressovsky (17) has pointed out such shortcomings as insufficient sampling, uncertain purity of stock, and undetermined spontaneous mutation rates in a large part of this work. Nevertheless, certain instances of weak chemical mutagenic activity appear to be established.

Significant increases in mutation rates have been observed with Drosophila on treatment of the eggs or larvae with copper sulfate solutions (22), with formaldehyde (23), and with various sulfonamides (25).

Hadorn (24) treated naked larval ovaries of D. melanogaster with various chemical agents, implanted the ovaries in untreated hosts, and analyzed the offspring. He found that scattered high mutation rates were produced by a $1:10^4$ solution of phenol.

Demerec (26,27,28,29) has used aerosols for the application to Drosophila of several carcinogenic compounds and compounds of related structure which were not carcinogens. Mutagenic activity was found for certain members of both of these classes.

Stubbe (30) reported mutagenic activity for both phenol and potassium thiocyanate in experiments with plants. Phage resistance in E. coli appears to be induced by alkyl carbamates (31) and by acriflavin, sodium desoxycholate, or caffeine (29). Acriflavin has also been reported by Ephrussi (32) to produce morphological mutants in yeasts.

The theory of direct action on the gene.--During the nearly twenty years that radiation was the only established method for inducing mutations it was generally supposed that it acted directly or nearly directly on the gene. Not only was this the simplest interpretation of the effect, it also fitted into an explanation for the failure to discover other means for producing mutations. The argument was that nearly all treatments applied to a cell either affected all parts equally or affected the peripheral parts preferentially. Thus, such treatments failed to affect genes without destroying the

cell at the same time. Radiation, on the other hand, offers the possibility for applying severe treatment to small regions distributed more or less at random through the cell. Thus, only radiation could alter genes and leave the cell intact.

While these arguments are not wholly satisfying they certainly provide a reasonable interpretation of the information that was available up to about five years ago. Even at the present time one may feel that this explanation of the action of radiation has some significance although the work of Wyss and Stone (33) shows beyond question that it is not a complete or necessary explanation.

Discovery of the first chemical mutagens complicated but did not upset the theory of direct action on the gene. Mustard gas and the nitrogen mustards possess the unusual property of reacting only slowly with water but readily with certain organic amino and hydroxyl compounds. Thus they could be pictured as diffusing through a cell and reacting with interior parts nearly as readily as with those near the cell wall. However, other compounds are known which have these chemical properties but do not affect mutation rates. Very recent investigations, especially the work to be described here, have produced information that seems to make it necessary to abandon this theory, at least as a criterion for predicting mutagenic activity.

One phenomenon which is extremely difficult to reconcile with the idea that all that is necessary for producing mutations

is to bring the agent into contact with the gene is the induction of back mutations. It is found that the same agents which cause genes to change from their normal behavior, generally to a less active or inactive state, are effective in restoring them to their original condition. Radiation acting directly on the gene could do this by "activating" it so as to facilitate its shifting from one configuration to another, but this argument in itself represents a departure from the idea that it is the ability of radiation to reach the gene that wholly accounts for its mutagenic activity. Further, the production of back mutations by mustard gas and the nitrogen mustards can hardly be interpreted as a result of direct action on the gene. It is reasonable to ascribe the mutagenic activity of the mustard compounds entirely to their ability to reach the gene only when this contact with the gene can be pictured as producing the effect in an obvious or automatic way.

Other recent discoveries provide more direct contradictions of the theory of the direct action of mutagenic agents. Thus, Wyss and Stone have shown that ultra-violet light need not fall on the gene or even on the organism to produce mutations. Irradiation of the substrate immediately before plating Staphylococcus aureus brings about mutation rates comparable with those obtained by direct irradiation. Similar results have been obtained by treating the substrate with nitrogen mustard or with hydrogen peroxide (34) under conditions where the effect

can not be ascribed simply to persistence of these agents. In the present investigation several organic peroxides have been found to be powerful mutagens although they have no chemical properties which would permit one to predict that they would be more likely to react with the remote parts of cells.

At the present time the idea that mutagenic agents act directly on genes has been abandoned by many persons directly concerned with their study. It seems still to be accepted, however, by a number of workers and it is for this reason, as well as to provide a background for presentation of the peroxide or free radical theory, that the above discussion has been prepared.

The peroxide or free radical theory.--The substrate irradiation studies of Wyss and Stone show that at least a part of the mutagenic action of ultra-violet light is produced by the products of light-induced reactions in relatively simple substances. These products have only a modest degree of stability since the effect of irradiating the substrate persists for only a few hours. Activated molecules might be postulated to explain these results or some sort of luminescence effect but if one stays within the realm of established phenomena the best guess would seem to be that organic* peroxides are formed and are responsible for the mutagenic effect (11).

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* Wyss and Stone (33) at one time supposed that hydrogen peroxide was the initial product.

Certain organic compounds having labile hydrogen atoms are readily converted into peroxides by reaction with molecular oxygen. The course of this reaction may be pictured as follows:

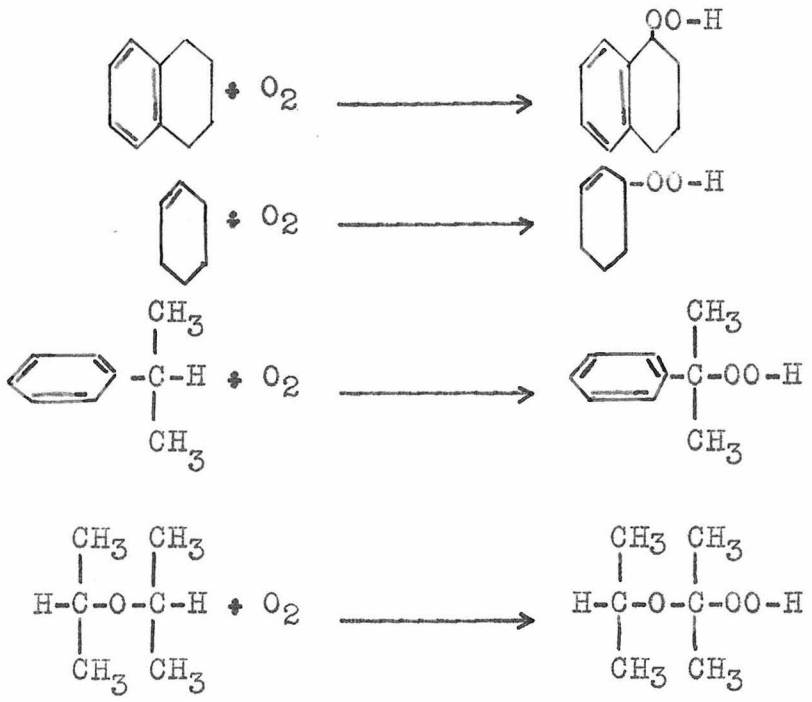
$$R\text{-}H \longrightarrow R\text{-} + H$$

$$R\text{-} + O_2 \longrightarrow R\text{-}OO\text{-}$$

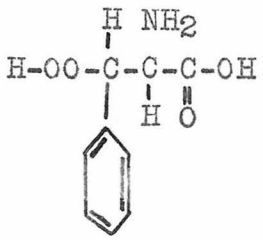
$$R\text{-}OO\text{-} \xrightarrow{R'\text{-}H} R\text{-}OO\text{-}H$$

Structures which favor this reaction may be described in terms of the carbon atom which bears the reacting hydrogen (35). Thus, when this carbon atom is attached to ether or carbonyl oxygen, is allylic to a double bond or attached to a benzene ring, is tertiary (that is, not carrying any other hydrogen atoms), or is involved in a cyclic structure the replacement of the hydrogen with -OOH is facilitated. In general, two such configurations must be simultaneously present in order to activate the hydrogen to a point where peroxide formation proceeds extensively. This is seen by considering the structures of the best known spontaneous peroxide formers. Thus, the point of attack in tetralin is cyclic and adjacent to an aromatic ring, in cyclohexene it is cyclic and allylic, in cumene, tertiary and adjacent to an aromatic ring, and in diisopropyl ether, tertiary and adjacent to ether oxygen. The overall reactions by which these compounds

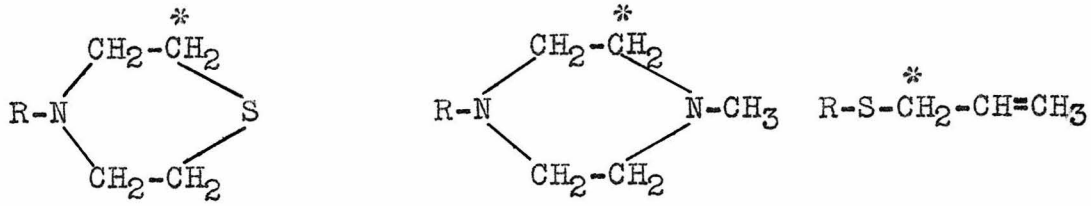
are believed to form peroxides are indicated below.



However, otherwise refractory compounds may react under the influence of ultra-violet light which strongly accelerates the reactions of the specific compounds listed above. Apparently the light provides the energy for the initial displacement of the hydrogen atom. This would then be the first step in the mutagenic action of radiation. Presumably the site of this reaction would be some structure that was moderately activated according to the above scheme, for example, the methylene group in tyrosine or phenylalanine. Phenylalanine might form:



The mutagenic activity of the mustards and allyl isothiocyanate can be explained by means of a less substantial extension of this hypothesis. These compounds might react with organic amino groups to form sulfur or nitrogen analogues of cyclic or allylic ethers as shown below.



These hypothetical products could then form peroxides directly at the doubly activated points marked by asterisks just as dioxane and allylic ethers react with molecular oxygen without the catalytic action of ultra-violet light.

This hypothesis provides a unified interpretation of the more prominent established facts about mutagenesis. It explains the observations that radiations and the mustards produce the same mutations with roughly the same relative frequencies.** As already indicated, it explains the effectiveness of treatment of the substrate rather than the organism and the ready decomposition of the postulated peroxides accounts for the transient nature of substrate irradiation.

** Small differences are observed between the effects of these agents especially with respect to the degree to which chromosomal aberrations are involved (36).

While the new hypothesis does not contribute anything toward our understanding of the nature of the steps involved it is at least consistent with the observation that mutagenic agents in general promote the occurrence of so-called back mutations as readily as forward ones. A theory would be that peroxides interfere in some random way with the gene duplication process.

The summary of available information on the induction of mutations at the beginning of this section suggests one other broad question which a hypothetical explanation of the nature of the process should answer. Why is it that chemical mutagens have been so difficult to find? Of course, organic peroxides have not been readily available until recently and there is no report of their having been tested as mutagens. Besides this simple answer, however, it can be said that organic peroxides possess an unusual property which might very well account for a special effect on the gene duplication process. This is the property of catalyzing free radical reactions. Organic peroxides are manufactured, in large quantities in recent years, for use in promoting polymerization in the manufacture of rubbers, adhesives, plastics, etc. They are also effective as combustion accelerators in Diesel fuels. All of these applications depend upon the characteristic spontaneous decomposition of peroxides into fragments that serve to initiate radical chain reactions. Certain azo compounds have recently been found to function like peroxides in this way but they are still very little known. If

the production of free radicals is an essential step in the induction of mutations it is not surprising that so few chemical mutagens have been reported.

The experimental work described in the following sections was based largely on the above hypothesis. The hypothesis itself has certain shortcomings: it is rather farfetched in some of its details. Its value lies in the facts that the broader arguments are quite plausible and many aspects are open to direct experimental test. At the least, it provides a much needed theme for the systematic study of the action of chemical agents on mutation rates.

Experimental Procedure.

A considerable number of compounds have been tested for activity in promoting mutations. The method chosen for carrying out these tests consists of determining the effects of the various compounds on the back mutation rate of an adenineless strain of Neurospora, following essentially a procedure developed by Westergaard and Mitchell (37). Conidiospores were exposed to the hypothetical mutagens and the effects calculated from the fraction of these spores which gave rise to colonies on a minimal medium.

This procedure has a great advantage over older procedures for measuring or detecting mutagenic activity. It does not involve the problems of a subjective evaluation of the results, as in the counting of morphological mutants. Neither does it involve the estimation of a small number of organisms

that do not grow in the presence of a large number that do, as in the counting of lethal mutations or biochemically deficient mutations. In this method each colony that grows represents a mutation back to the original, adenine independent form, and a count of the colonies is a direct count of the mutants.

Of course, the data obtained apply only to a particular mutation in a particular gene and further tests are required to establish their general significance. Also for this reason, the fraction of spores which will show such a particular mutation will be very small under the best conditions. This is not a real problem, however, since the number of spores tested in a single experiment can be adjusted to give a convenient number of mutants.

Treatment of the spores.--Most of the compounds have been tested in aqueous solution by approximately the following procedure. A batch of 100-200 million spores from two-day cultures is suspended in water, thoroughly mixed, and then divided into a series of roughly equal samples in centrifuge tubes*. These samples are then centrifuged down and the water decanted. Ten-ml. portions of the appropriate solutions are added to the samples to be treated and fresh portions of water to the others which

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* These spore batches were numbered chronologically and the individual samples were designated by letters. This is the significance of the entries under the heading "Exp. No." in the tabulations of data presented later. Samples having the same numerical designation as 132A and 132C were made up from a homogeneous batch of spores.

serve as controls. At the conclusion of the period of treatment, usually 30 minutes, the spore samples are again centrifuged and washed with water and re-suspended in 10 ml. of water, and the spore concentration is determined with a hemocytometer. At this point a 1.0-ml. sample is withdrawn for measuring mortality and checking the spore count. For this purpose the samples are diluted 10^6 times in five stages and volumes, estimated to contain about 30 spores (per plate), are plated out on an adenine supplemented medium. The rest of each spore sample is plated on a minimal medium in order to determine the fraction that does not require adenine for growth. This is most efficiently done by reducing the 9-ml. suspension volume to 2 ml. by a final centrifugation and then using a dropper to distribute this dense suspension on four plates.

A few tests have been made with vapor-phase treatments of conidiospores. In these cases the spore batches were mixed and divided into samples in the dry state. Treatment was applied in a Thunberg tube and after treatment the spore samples were suspended in water and the experiment was completed in the regular way.

The use of n-heptane as the spore suspension medium in the application of compounds insoluble in water and insufficiently volatile for the other methods was tested with rather unsatisfactory results. Spore samples were made up in water suspensions as usual and were then centrifuged down and dried under vacuum.

After standing in contact with the solution of the chemical in heptane the spores were washed with fresh heptane, freed from this solvent under vacuum and resuspended in water for plating. The following difficulties were encountered: the spore clumps did not readily break up in the presence of the hydrocarbon so that there was doubt that uniform treatment was obtained, contact with the solvent killed a substantial fraction of the spores, and other spores were lost, apparently by being carried away in the vapor stream during the two evaporation steps. It was concluded that the method is quite inefficient but might be valuable for agents that can not be applied in any other way. It was used here to show the mutagenic activity of 2,2-bis(tert.-butylperoxy)butane.

Since the primary object of these experiments was to make comparisons between the effects of different chemical agents, the treatment procedures were kept as uniform as possible. Differences in individual experiments which might be significant will be discussed in connection with these experiments. The effects of certain general conditions of treatment such as temperature, intensity of visible light, and concentration of spore suspensions have not been explored.

Most of the treatments were carried out at room temperature, 20-23°. In certain cases series of experiments were carried out with the temperature held at 25° but there is no evidence that small temperature differences have any measurable effect. Under special circumstances the density of the spore suspension during

treatment may be significant: with ultra-violet light the denser suspensions receive a smaller mean dose, and similarly with hydrogen peroxide the agent is destroyed by the spores so that its average concentration during the treatment is an inverse function of the spore concentration. This factor has received but little attention except that the spore concentrations in the individual experiments were recorded.

Colony counts.--Both the adenine supplemented and minimal plates, prepared as described above, were allowed to develop at 25°. Colonies on the supplemented plates appeared after about 40 hours. They were uniform, sharply defined, and easily counted. However, the mortality data calculated from these counts proved to be erratic. A considerable amount of time was spent trying to improve the procedure without marked success. The errors most probably arose during the dilution process where the initial spore concentration was reduced by a factor of 10^6. They can not be ascribed to irregularities in the treatment of the spores, since mutation rates obtained for the same spore samples showed much more consistent results.

The per cent apparent mortalities entered in the later tabulations of data were calculated in the following way. First the germination fraction was obtained from the untreated control samples: simply the ratio of the number of colonies counted to the number of spores planted as determined with the hemocytometer. This fraction averaged about 0.9. Higher and more regular germination rates might be obtained with older

spores but it was believed that two-day spores would be more susceptible to mutagenic agents. The ratio of the germination fraction shown by the treated spores to that shown by the controls was then a measure of the fraction of the spores surviving the treatment, and --

$$\text{percent apparent mortality} = 100 \left(1 - \frac{\text{germination fraction--treated}}{\text{germination fraction--control}} \right)$$

The mutant colonies on minimal plates appear only after 60 to 70 hours. This delay, in comparison with the colonies growing on adenine supplemented plates, may be explained in the following way. The spores are multinucleate and only one nucleus in a given spore is likely to back mutate to a condition where adenine can be synthesized. Thus only a fraction of the nuclei in a mutant colony are actually producing adenine and the colony is still suffering from a deficiency in this substance.

Mutant colonies also appear at different times and grow at different rates, presumably a response to the toxic effects of the various treatments. The edges of these colonies tend to be somewhat indistinct and, especially when a large number of spores are planted per plate, flecks of cloudiness appear in advance of the growing colonies. A possible interpretation of this phenomenon is that the growing colonies release into the medium enough adenine to stimulate slight growth from some of the enormous number of spores that surround them.

Another phenomenon has been observed which may have a similar explanation. There have occasionally appeared on the

minimal plates large numbers of faint, feebly growing colonies which usually produce no spores and stop growing while still very small. At first, it was supposed that they were some contaminant growing under unfavorable conditions. Study of the more substantial of these colonies has shown that they are really a colonial Neurospora strain quite like that used in the experiments. Consideration of the circumstances under which these colonies have appeared--exceptionally dense spore suspensions subjected to severe chemical treatment effecting 99.9% mortality--suggests the following explanation. Nearly all of the spores were killed by the treatment which was so severe as to break down the structure of many spores. In this way a trace of adenine appeared in the medium sufficient to support the feeble growth of the few, one or two per million, surviving spores.

Whether or not the above explanations are correct the colonies are difficult to count when too many spores (that is, dead or inactive spores) are plated and 10 or 15 million per plate seems to be a practical limit. In spite of these problems reliable counts of as many as 30 or 40 mutant colonies per plate can usually be obtained. Contamination is more likely to affect the minimal plates because there is more time for foreign organisms to develop. Elaborate precautions are not necessary, however, since the colonies are counted at a stage when they can be recognized by their growth habit. It is only on rare occasions

when plates are actually overgrown with wild type Neurospora or with bacteria that experiments must be discarded.

Calculation of mutation rates.--Since the measurements of mutation rates were found to be much more reliable than the mortality determinations it was not reasonable to present the mutation rates in terms of the number of spores surviving the treatment. Accordingly they were not corrected for the killing effect of the treatment*. Numbers tabulated under the heading "Observed Mutation Rate" are simply the ratios of the number of mutant colonies counted to the number of spores planted as determined with the hemocytometer. The "Induced Mutation Rates" were obtained by subtracting from the mutation rates shown by the treated samples, the rates shown by the control samples from the same spore batch. The necessity for this particular method of evaluating the data when searching for small mutagenic effects is indicated in the following discussion.

It would seem to be a logical method for applying this system to the detection of chemical mutagens to determine the average rate at which mutants appear spontaneously in the particular strain and then to use this well-established average as a standard for comparing the behavior of treated spores. However,

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* It is for this reason that the mutation rates reach a maximum at quite modest mortalities instead of the very high mortalities used in other methods for detecting mutagenesis. Failure to obtain good mortality data limits the sensitivity of this method. The theory that "pure" mutagens should tend toward the same maximum rate is discussed in a later section.

there are theoretical objections to this procedure which in some measure have been borne out by experiment. The spontaneous mutation rate appears to vary in such a way that it is futile or even misleading to attempt to average it.

The term "spontaneous mutation rate" refers to the incidence of mutants in a given batch of untreated spores. When one inquires into the origin of these mutants it is seen that there are several possibilities. One is that they represent a perpetuated contamination in the line, another that they are the product of mutations in the formed spores. If either of these views were correct the distribution of observed rates would be expected to have a sharp maximum and an average rate could be easily obtained. However, there are definite objections to both of them.

In the first place, when a new batch of spores is to be raised for a subsequent experiment, not more than about a million spores are planted in each of ten test tubes, and of these probably only a very small fraction actually contribute nuclei to the batch of spores which is collected after two days' growth. Since the proportion of mutant spores ordinarily ranges from 0 to 2×10^{-7} it is not reasonable to regard the spontaneous mutation rate as simply a perpetuated contamination in the line.

Thus, the mutants appearing in each fresh batch of spores must be considered to be the products of mutations occurring during the preceding generation. But these mutations need not

have occurred exclusively or even predominantly in the formed spores. It may be roughly estimated that twenty-five divisions intervene between two generations of spores. Clearly, mutations in the earlier stages of development will be rarer because the number of nuclei will be less at that time, but such rare mutations will make large contributions to the true average "spontaneous mutation rate". This true average will always be beyond the reach of direct experimental determination because, in the first place, a preposterous number of experiments would be required to get the proper contribution from the infrequent early mutations and secondly because when an early mutation does occur it will produce such a high proportion of mutant spores that it will not be possible to count the colonies. The same problem has been described by Delbrück (38) in connection with mutations in bacteria.

Table 12 summarizes observations on the spontaneous mutation rate in nearly four billion untreated spores. The last column gives totals of observed mutants up to that point in the table. It will be seen that more than two thirds of the mutants were found in a single spore batch. If this experiment (No. 41, the colonies were too numerous for an accurate count) is eliminated it is seen that more than half of the remaining mutants still occurred in batches in which the rate was extraordinarily high and which numbered less than 15% of the whole. The experimental results alone show that the averaging process is impractical.

Table 12.

Spontaneous Mutation Rates

Tabulated in Order of Increasing Rate

No. of Independent Spore Batches	Total No. of Spores x 10^{-6}	Total No. of Mutants	Rate x10^{7}	Summation of Mutants
4	279	0	0.0	0
3	411	4	0.1	4
3	333	8	0.2	12
5	520	14	0.3	26
3	235	9	0.4	35
3	161	8	0.5	43
2	199	12	0.6	55
5	229	16	0.7	71
3	227	18	0.8	89
1	28	3	1.0	92
1	63	7	1.1	99
2	325	52	1.6	151
1	78	21	2.7	172
1	108	30	2.8	202
1	15	6	4.0	206
1	229	>320	>13.0	>528

In demonstrating mutagenic action of a particular substance, relatively small scale observations on an untreated control sample from the same batch of spores will be of much more value than averages obtained from tests with billions of spores from different batches.

It should be pointed out that the induced mutation rates so calculated do not represent the frequencies with which the gene in question reverts to normal under the various conditions of treatment*. The fact that the spores are multinucleate substantially increases the obtainable mutation rates since the killing of many nuclei by the induction of lethal mutations does not obscure a favorable mutation in one and even the appearance of new deficiencies in the particular nucleus in which the adenine gene has reverted may not prevent the growth of a heterocaryotic colony. These are advantages which this system has over the use of single nuclei, as microconidial Neurospora strains, for the detection of mutagenic activity. It will be seen later that they are disadvantages for certain other tests of interest to this investigation.

Demonstration of the Mutagenic Action of Peroxides.

The essential contribution made in the work to be reported here is the demonstration that organic peroxides induce mutations.

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* The rather unlikely possibility that the changes studied represent a suppressing mutation rather than reversion of the particular gene to its original state has been studied recently by Westergaard (39).

Other aspects of the mutagenesis problem have been studied but this work will be discussed only very briefly since it has not led to decisive results. In order to show the activity of peroxides it was necessary to make comparative tests with known mutagenic agents and also to try some miscellaneous compounds as a sort of control on the method. These background experiments will be presented first.

Radiation effects.--Treatment with ultra-violet light was effected simply by exposing the original spore suspension samples, 15 to 25 million spores in 3 or 4 ml. of water, to a Westinghouse sterilizing lamp. Typical results are shown in Table 13.

A single test was made with x-rays in which an induced mutation rate of 15.5 x 10^7 was obtained on exposure of spores to 1000 R units per minute for 30 minutes.

Table 13.

Mutagenic Action of Ultra-Violet Light

Treatment: exposure for the indicated period of time to ultra-violet lamp.

Exp. No.	Time Sec.	Spores x 10^{-6}	Mutants	Mutation Rates x10^7 Obs.	Induced	Mort. %
108C	60	26	65	25	25	-31
108B	75	18	80	44	44	4
112C	90	19	51	27	27	64
108A	100	14	45	31	31	72
112B	150	19	26	14	14	95
112A	210	24	8	3.3	3.3	100

Tests with mustard gas.--Sulfur mustard, bis(β-chloroethyl) sulfide, was tested by Westergaard and Mitchell (37) by this method, using a saturated aqueous solution, and showed definite evidence of mutagenic activity. This result has been confirmed in the present investigation with tests employing the compound in vapor phase. However, in none of these experiments was a control from the same spore batch used, and although the observed rates are large enough to leave little doubt that the compound is active, the magnitude of its effect is quite uncertain.

In one reported experiment Westergaard and Mitchell found 19 mutants in 14.6 million spores on treatment with a saturated aqueous solution for 30 minutes. This is a rate of 13×10^{-7}. The mortality was 81%. Data obtained in the present study are tabulated below.

Table 14.

Mutagenic Action of Sulfur Mustard.

Treatment: exposure to vapors of bis(β-chloroethyl) sulfide for the indicated time in a Thunberg tube.

Exp. No.	Time Min.	Spores x10-6	Mutants	Mutation Rate x10^7 Obs.	Mort. %
3	45	40	4	1.0	0
7	60	31	1	0.3	100
8	45	47	15	3.2	0
13	53	35	12	3.4	30
17	56	34	14	4.1	0

Tests with nitrogen mustard.--Bis(β-chloroethyl)methylamine was available as its hydrochloride. Solutions were mixed with sodium acetate immediately before use in order to free the base, which is the active form of the compound. An extensive series of tests was carried out with this nitrogen mustard in the hopes of providing a sort of standard of mutagenic activity but the results were not as consistent as those subsequently obtained with certain peroxides. In Table 15, the data obtained at the most effective concentration are presented. The observation that higher rates are not given by higher concentrations has been carefully checked as shown in Table 16.

Table 15.

Mutagenic Action of Nitrogen Mustard

Treatment: 30 minutes exposure to 0.08%, 0.004 molar, bis(β-chloroethyl)methylamine in 0.02 molar aqueous sodium acetate.

Exp. No.	Spores $x10^{-6}$	Mutants	Mutation Rates $x10^{7}$ Obs.	Induced	Mort. %
16A	15	7	4.7	3.9	97
19A	15	10	6.7	6.0	57
21A	30	15	5.0	4.7	75
21B	30	14	4.7	4.4	75
21C	29	17	5.9	5.6	83
37A	91	35	3.8	2.2	100
37B	69	23	3.3	1.7	100
37C	90	20	2.2	0.6	91

Table 16.

Action of Nitrogen Mustard at Higher Concentrations

Treatment: 30 minutes exposure to bis(β-chloroethyl)methylamine at the indicated concentrations in 0.02 molar aqueous sodium acetate.

Exp. No.	Conc. Formal	Spores $x10^{-6}$	Mutants	Mutation Rates $x10^{7}$ Obs.	Induced	Mort. %
148A	0.0043	29	6	2.1	1.2	95
148B	0.0043	35	5	1.4	0.5	93
151A	0.0052	40	22	5.5	5.0	89
151B	0.0052	35	14	4.0	3.5	77
153A	0.0073	35	6	1.7	1.6	86
153B	0.0073	40	10	2.5	2.4	88
157A	0.0090	19	0	0.0	-0.3	100
157B	0.0090	16	2	1.3	1.0	100
155A	0.0104	26	1	0.4	0.0	100
155B	0.0104	30	1	0.3	-0.1	96

Miscellaneous inactive compounds.--Asa check on this particular method for detecting mutagens it was necessary to obtain some data with compounds which have no activity. The literature abounds with evidence that miscellaneous chemical treatments are not likely to produce mutations so that only a few examples are required to establish the point in this system. Two or three series of experiments have been carried out for

just this purpose and many other inactive compounds have been tested in connection with special phases of the investigation. Tables 17, 18, and 19 present the results of typical experiments of this sort with potassium permanganate, phenol, and formaldehyde. All of these compounds have been reported (22, 23,24) to have weak mutagenic activity when tested by other methods.

Table 17.

Potassium Permanganate

Treatment: 30 minutes exposure to an aqueous 0.00052 molar solution.

Exp. No.	Spores $x10^{-6}$	Mutants	Mutation Rates $x10^{7}$ Obs.	Induced	Mort. %
63A	83	28	3.4	1.6	24
65AB	91	17	1.8	-0.6	50
67AB	61	1	0.2	0.0	17
71AB	68	0	0.0	-0.3	40
76AB	80	0	0.0	-0.3	46

Table 18.

Phenol

Treatment: 30 minutes exposure to an aqueous 0.077 molar solution.

Exp. No.	Spores $x10^{-6}$	Mutants	Mutation Rates $x10^{7}$ Obs.	Induced	Mort. %
77B	38	0	0.0	-0.3	85
80AB	203	0	0.0	-0.4	91
82AB	66	1	0.2	-0.2	78
84AB	158	0	0.0	-0.6	93

Table 19.

Formaldehyde.

Treatment: 30 minutes exposure to formaldehyde at the indicated concentrations

Exp. No.	Conc. Formal	Spores x10^{-6}	Mutants	Mutation Rates x10^{7} Obs.	Induced	Mort. %
122C	.0009	27	0			
122B	.0056	30	3	1.0	1.0	
127C	.0056	29	4	1.4	-0.3	-1
122A	.0244	37	2	0.5	0.5	
127B	.0244	34	9	2.6	0.9	86
131AB	.0244	122	6	0.5	0.4	82
135AB	.0244	58	4	0.7	0.4	58
127A	.048	38	0			100
120C	.0767	61	0			100
120B	.268	69	0			100
120A	.457	75	0			100

The following compounds have also been tested under various conditions without showing any tendency to induce mutations: tert.-butyl alcohol, hydroquinone, quinone, hexamethylenetetramine, diethyl ether, n-heptane, and ferrous ammonium sulfate. Westergaard and Mitchell have reported inactivity for a larger variety of substances (37) using the same system for detecting mutagens but the sensitivity of their procedure is uncertain.

Three organic peroxides, tert.-butyl hydroperoxide, hydroxymethyl tert.-butyl peroxide, and bis(hydroxymethyl) peroxide have been found to have profound effects when tested in this way. The work with these compounds is described below followed by shorter discussions of peroxides showing less or uncertain activity. Finally the case of di-tert.-butyl peroxide, which appears to have no mutagenic action at all, will be considered.

tert.-Butyl hydroperoxide.--The preparation of this compound was first described by Milas (40). It is easily prepared in the laboratory by mixing at zero degrees equal volumes of tert.-butyl alcohol, 60-65% sulfuric acid, and 30% hydrogen peroxide. After standing for two or three days in an ice bath, the product (in yields of 50-60%) is collected in petroleum ether and purified by reduced pressure distillation. An impure preparation of the material is available commercially (Union Bay State Company, Massachusetts).

Highly purified preparations of tert.-butyl hydroperoxide decompose rather rapidly into tert.-butyl alcohol and oxygen. Its pure aqueous solutions, however, are stable over a period of years. Careful tests at low concentrations have shown that the spores of Neurospora and other organisms gradually destroy this peroxide. Under the conditions of the tests described below (Table 20), however, this effect is not significant. The peroxide concentrations at the end of the respective treatments were essentially the same as those at the beginning so that there is no reason to suppose that the spore concentrations affected the outcome of the experiments.

Table 20.

Mutagenic Action of tert.-Butyl Hydroperoxide

Treatment: 30 minutes exposure to an aqueous solution of tert.-butyl hydroperoxide at the indicated concentration.

Exp. No.	Conc. Formal	Spores x10-6	Mutants	Mutation Rates x10^7 Obs.	Induced	Mort. %
23C	0.004	48	6	1.2	0.5	5
23B	0.007	53	8	1.3	0.6	26
23A	0.009	50	13	2.6	1.9	50
30ABC	0.010	318	154	4.9	2.1	35
36ABC	0.011	277	69	2.5	2.3	
46AB	0.0124	103	32	3.1	3.0	18
104AB	0.019	62	34	5.5	5.0	18
105AB	0.0267	80	56	7.0	6.9	-14
106AB	0.0444	66	143	21.7	21.2	26
117AB	0.0666	37	69	18.8	7.2	49
118AB	0.0666	39	60	15.4	8.8	9
107AB	0.0888	142	246	17.3	17.2	34
115B	0.0888	40	51	12.8	10.5	80
114AB	0.111	61	6	1.0	0.8	84
116AB	0.111	134	136	10.2	4.7	59
113AB	0.133	29	5	1.7	-10.8	100
111AB	0.177	21	0	0.0	0.0	100
109AB	0.266	77	0	0.0	0.0	100
110AB	0.266	30	0	0.0	0.0	100

While some of the high rates in this table, particularly those observed in experiments 106 and 107 may be in error, it appears that tert.-butyl hydroperoxide at concentrations ranging from 0.02 to 0.09 formal has a greater effect on the mutation rate than does nitrogen mustard at any concentration. The substantial negative rate recorded for experiment number 113 simply reflects the result of killing on a high spontaneous mutation rate.

Hydroxymethyl tert.-butyl peroxide.--Dickey (41,42) first reported this compound. The preparation involves simply mixing tert.-butyl hydroperoxide and formalin in equimolar quantities in aqueous solution and distilling under reduced pressure (b.p. 48-50°). It presents a substantial explosion hazard and the distillation should be undertaken cautiously with shielded apparatus.

The material used for the experiments described in Table 21 was contaminated with formaldehyde, which may have prevented discovery of the maximum rates obtainable with the compound. Hydroxymethyl tert.-butyl peroxide must be regarded as being in equilibrium with the compounds used in its preparation according to the following equation.

$$HO\text{-}CH_2\text{-}OO\text{-}C_4H_9 \rightleftharpoons CH_2O + C_4H_9\text{-}OO\text{-}H$$

Thus, results obtained with it are not altogether distinct from results of treatment with tert.-butyl hydroperoxide.

However, simple dissociation can not explain the effect entirely since the equivalent formaldehyde concentration would have killed virtually all of the spores (see Table 19, page 84).

Table 21.

Mutagenic Action of Hydroxymethyl tert.-Butyl Peroxide. Treatment: 30 minutes exposure to an aqueous 0.089 formal solution.

Exp. No.	Spores 10^{-6}	Mutants	Mutation Rates $x10^{7}$ Obs.	Induced	Mort. %
165B	69	47	6.8	6.5	-12
169A	24	40	16.7	16.5	76
169B	27	35	13.0	12.8	76
173A	13	38	29.2	29.2	82
173B	14	31	22.1	22.1	73
177A	29	44	15.2	15.2	87
177B	30	35	11.7	11.7	84

Bis(hydroxymethyl) peroxide.--This compound has been known for many years (43). It is one of the species present in aqueous mixtures of hydrogen peroxide and formaldehyde as shown by the following equations.

$$H_2O_2 + CH_2O \rightleftharpoons HO\text{-}CH_2\text{-}OO\text{-}H$$

$$HO\text{-}CH_2\text{-}OO\text{-}H + CH_2O \rightleftharpoons HO\text{-}CH_2\text{-}OO\text{-}CH_2\text{-}OH$$

Thus, like the preceding compound, bis(hydroxymethyl) peroxide is not entirely independent of the compounds used in its

preparation. In this case, however, the compound described has a much higher mutagenic activity than the hydrogen peroxide from which it is formed and therefore some significance must be ascribed to its particular structure.

The name bis(hydroxymethyl) peroxide is not an accurate description of the material used in the tests described in Table 22. The actual solutions used in the tests were prepared by mixing aqueous solutions of formaldehyde and hydrogen peroxide in the molar ratio 3:2. This proportion was selected in order that the concentrations of free formaldehyde and of free hydrogen peroxide should both be low. Since the equilibria involved strongly favor the combined forms the test solutions were probably essentially equimolar mixtures of hydroxymethyl hydroperoxide and bis(hydroxymethyl) peroxide.

Table 22.

Bis(hydroxymethyl) Peroxide.

Treatment: 30 minutes exposure to an aqueous solution prepared as described in text with a peroxide concentration of 0.022 moles per liter.

Exp. No.	Spores x10-6	Mutants	Mutation Rates x10^7 Obs.	Induced	Mort. %
175C	30	32	10.7	10.7	58
183AB	134	102	7.6	7.5	96
193AB	58	82	14.1	14.1	58
196AB	125	169	13.5	13.2	54

Hydrogen peroxide.--Treatment with hydrogen peroxide produces a small but definite increase in the mutation rate. There is reason to believe, however, as indicated in the later discussion section, that this may be a secondary effect. Two factors probably contribute to the erratic character of the mutation rate data obtained with hydrogen peroxide. One of these, destruction of the agent by the spores, has already been mentioned (page 71). The other results from the fact that the mutation rates are calculated from the number of spores treated rather than from the number of surviving spores. It has already been shown (Exp. No. 113, Table 20) that the coupling of high mortalities with high spontaneous rates leads to a low value for the induced rate. This error will be significant when the true induced rates are low and will lead to erratic results because the spontaneous mutation rates are inherently erratic. These explanations, however, may be inadequate to account for the irregularities actually observed with hydrogen peroxide.

Table 23.

Mutagenic Action of Hydrogen Peroxide

Treatment: 30 minutes exposure to 0.64%, 0.21 molar, aqueous hydrogen peroxide.

Exp. No.	Spores x10^{-6}	Mutants	Mutation Rates x 10^{7} Obs.	Induced	Mort. %
15A	5	0	0.0	-0.4	96
18A	8	1	1.2	0.4	94
28A	96	21	2.2	1.4	13
28B	38	9	2.4	1.6	0
28C	78	15	1.9	1.1	20
34A	64	16	2.5	2.4	23
34B	128	21	1.6	1.5	39
34C	72	23	3.2	3.1	46
40A	27	3	1.1	1.0	0
40B	102	11	1.1	1.0	7
42A	118	35	3.0	1.4	43
42B	114	40	3.5	1.9	54
47A	66	19	2.9	2.7	0
47B	63	14	2.2	2.0	0
49A	26	2	0.8	-0.1	65
49B	37	3	0.8	-0.1	71
54A	42	22	5.2	5.2	41
54B	64	35	5.5	5.5	67
57A	22	7	3.2	2.6	48
57B	42	7	1.7	1.1	66

Two other peroxides, 2,2-bis(tert.-butylperoxy)butane and mono-tert.-butyl persuccinate, showed weak but definite mutagenic action. They have not been tested thoroughly enough to justify an extended discussion.

The preparation of the former, 2,2-bis(tert.-butylperoxy)-butane, has been described by Dickey (42,44). It is a water insoluble liquid of low volatility and was applied to the spores in solution in n-heptane. Mono-tert.-butyl persuccinate is a new compound. It was prepared by reacting tert.-butyl hydroperoxide with succinic anhydride in pyridine solution. The product, a crystalline, water-soluble acid, identified by its oxidizing and neutralization equivalents, was used in solution with sodium acetate to control the pH.

Brief mention may be made of four other peroxides for which mutagenic activity is uncertain or improbable. Benzoyl peroxide, applied as a saturated aqueous solution, gave occasional, weakly positive tests but reproducible results were not obtained because of the difficulty of eliminating suspended peroxide particles from the treating solutions. A small amount of suspended material killed virtually all of the spores. The peroxide formed in diisopropyl ether on standing in contact with air, believed to be (43) 2-hydroperoxy-2-isopropoxypropane, gave results which can be best described as positive but much too weak to be certain. The same observation can be made about the data obtained with sodium perborate. Study of these last

two compounds was not continued because of the possibility that their aqueous solutions contain hydrogen peroxide. Potassium persulfate in saturated aqueous solution, 3-5%, neither killed spores nor produced mutations. It is possible that more soluble persulfates would be effective but the molar concentration here is already 5 to 10 times greater than the optimum for the active organic peroxides.

Inactivity of di-tert.-butyl peroxide.--This compound is best prepared by the method described by Rust, Dickey, and Bell (45). It is supplied by the Shell Development Company (Emeryville, California). The effect of di-tert.-butyl peroxide, applied like sulfur mustard in the vapor phase in a Thunberg tube, has been very thoroughly studied. It is of special importance because it is the only compound tested here which neither has an -OOH group nor could reasonably be degraded in the living cell to a compound having an -OOH group. Thus if it were a mutagen the mutagenic activity of peroxides could be said to lie in the oxygen-oxygen bond.

However, it must be concluded from this prolonged study that if di-tert.-butyl peroxide possesses mutagenic activity it is of a very low order and it is not probable that it has any such activity whatsoever. In the earlier tests a small but significant induced mutation rate was observed (about 0.8 x 10^{7}). Although the material used was of high quality and had been thoroughly washed with water it was felt that the results might simply reflect contamination with traces of

hydroperoxide used in the preparation of the dialkyl peroxide. Accordingly, a second series of experiments was carried out using di-tert.-butyl peroxide that had been repeatedly extracted with 6-normal sodium hydroxide, which removes hydroperoxide more efficiently by converting it into its sodium salt. In this second series, presented in Table 24, it will be seen that a trend toward a positive effect is still discernible. The reason that this evidence has been rejected is that the trend is no larger than in the earlier series but the exposure times are much longer. In the earlier series high mortalities were produced with six-hour exposures and no spores appeared to survive a twelve-hour treatment. Accordingly, one must conclude that the agent responsible for the observed effect is not the di-tert.-butyl peroxide. While this work does not constitute a conclusive demonstration that di-tert.-butyl peroxide is inactive, it seems safe to say that any activity that it does have is too small to be detected by these methods.

Table 24.

Hydroperoxide-Free Di-tert.-butyl Peroxide.

Treatment: exposure in Thünberg tube for indicated period of time at 25°.

Exp. No.	Time Hours	Spores x10^{-6}	Mutants	Mutation Rates x 10^{7} Obs.	Induced	Mort. %
128	8	16	2	1.3	-0.4	53
134	10	58	9	1.6	0.3	21
136	14	35	13	3.7	3.7	20
139	16	32	1	0.3	-0.8	67
144	16	48	7	1.5	1.3	78
150	20	38	1	0.3	0.3	99

Miscellaneous Experiments.

Two other aspects of the mutagenesis problem have been studied but generally without conclusive results. The nature of these experiments is indicated briefly below.

Confirmation of peroxide activity in different systems.--Drosophila males were exposed to the vapor of di-tert.-butyl peroxide and a Muller-5 test was used to detect lethal X chromosome mutations. The mutation rate observed was an excellent check with the spontaneous rate in the Canton S strain used. This experiment was carried out before it was realized that di-tert.-butyl peroxide is probably quite inactive.

Markert has reported (46) that a very high incidence, 30 to 50%*, of visible mutants can be obtained by exposing spores

* These mutation rates, of course, are based on survivors. Mortalities were greater than 99%.

of the mold Glomerella to large doses of ultra-violet light. This would thus seem to be a very simple system for detecting mutagenesis but it was found that the effect could not be produced with tert.-butyl hydroperoxide or with nitrogen mustard.

Anti-mutagenic agents.--As indicated earlier the peroxide theory of mutagenic action suggests that so called anti-oxidants or reducing agents might suppress the mutation inducing action of ultra-violet and the mustards. Hydroquinone, one of the simplest and best known anti-oxidants, has been thoroughly studied with both ultra-violet and nitrogen mustard. In the latter case no effect of the copresence of hydroquinone on the mutation rates arising from treatment with nitrogen mustard was observed. The results of these experiments in condensed form are presented in Table 25.

Hydroquinone alone had no effect on the mutation rate and these data have been omitted from the table in order to save space. The mortalities have been tabulated in order to show that it was used at the highest practical concentration. It is clear that the results are not sufficiently consistant to uncover a small suppressive action by hydroquinone. The conclusion is simply that nitrogen mustard continues to function as a mutagen when this particular anti-oxidant is added to its solutions.

Table 25.

Effect of Hydroquinone on Action of Nitrogen Mustard.

Hydroquinone Alone			Nitrogen Mustard* Alone			Nitrogen Mustard* and Hydroquinone			
Exp. No.	Conc Formal	Mort. %	Exp. No.	Mort. %	Mutation Rate x10^7	Exp. No.	Hydroquinone Conc.-Formal	Mort. %	Mutation Rate x10^7
							Successively		
83C	0.003	8	16A	90	3.9				
83B	0.032	15	19A	73	6.0				
85C	0.061	-3	21	81	4.9	93A	0.08	89	7.8
93C	0.08	-16	37	94	1.5	93B	0.08	89	5.0
93D	0.08	33	97C	50	4.6	94A	0.08	88	1.7
94C	0.08	28	97D	77	2.3	94B	0.08	77	4.3
94D	0.08	40	99C	48	2.4				
85D	0.10	17	99D	55	2.4				
87C	0.12	42	100C	53	8.0				
87A	0.16	90	100D	55	11.9		Simultaneously		
87B	0.16	90				97A	0.12	100	3.5
90A	0.16	38				97B	0.12	100	11.4
90B	0.16	36				99A	0.036	98	2.2
85A	0.18	100				99B	0.036	96	1.7
83A	0.36	100				100A	0.007	66	4.9
						100B	0.007	71	4.0

*In all of these experiments nitrogen mustard was used at a concentration of 0.004 moles per liter. Both chemicals were used in aqueous solution with an exposure time of 30 minutes.

The results with ultra-violet light are not so easily interpreted. The mutagenic action of ultra-violet was almost completely abolished when the spores were suspended in 0.10 molar hydroquinone during the irradiation, it was reduced by half if the spores were treated with the hydroquinone immediately (10-20 seconds) after the irradiation, and little or no effect was produced by hydroquinone treatment administered 2 hours after the irradiation. These results are exactly what would be predicted from the peroxide hypothesis and the hypothesized processes may indeed be the correct explanation for them. Unfortunately other explanations are possible and long series of experiments have failed to eliminate them.

In the first place, the action of hydroquinone present during the exposure may be a simple shielding of the spores from the light. This effect has not been studied quantitatively but it has been shown that hydroquinone solutions absorb strongly in the wave length region (2537 Å) where the lamp used produces most of its energy. For this reason more attention was paid to the effects of hydroquinone treatments following irradiation.

Induced mutation rates and per cent mortalities for a series of experiments in which the effects of ultra-violet alone, ultra-violet followed immediately by hydroquinone, and ultra-violet followed after 2 hours by hydroquinone were determined are given in Table 26.

Table 26.

Effect of Hydroquinone on Action of Ultra-violet.

	Ultra-violet alone		Ultra-violet followed immediately by 0.10 molar hydroquinone		Ultra-violet followed after 2 hours by 0.10 molar hydroquinone	
Exp. No.	Mutation Rate x10^7	Mort. %	Mutation Rate x10^7	Mort. %	Mutation Rate x10^7	Mort. %
126	45.8	30	21.1	28		
129	76.9	51	13.4	85		
130	56.3	58	27.9	89		
137	26.2	33	13.9	38	25.2	35
138		18		27		16
140	28.1	10	12.6	38	21.3	8
141	25.1	37	15.7	57	21.1	60
142	29.6	44	12.8	63	30.4	20
143	78.6	71	25.0	92	83.3	43
Averages:						
	45.8	39	17.8	57	36.3	25

It will be seen that the mortality figures, while erratic as in many of these experiments, still tend to indicate that the effect of hydroquinone is due to killing of the spores rather than to suppression of the appearance of mutations.

Since the mortality determinations are known to be unreliable this explanation of the data can not be considered certain. Further, it entails the rather complicated theory that ultra-violet produces a temporary sensitivity to the killing action of hydroquinone. Nevertheless, this is exactly what the data seem to show and it must be concluded that if hydroquinone prevents the appearance of a significant fraction of ultra-violet induced mutants these experiments have failed to demonstrate it.

A number of other methods for demonstrating anti-mutagenic activity have been considered and a few experiments have been carried out. Treatment of spores with x-rays in the presence of hydroquinone is not open to the objection that the action is only shielding. In a single test of this sort no effect was observed but the hydroquinone concentration (0.05 molar) was only half that used in the ultra-violet experiments.

Cysteine reduces some hydroperoxides but had no effect on the mutagenic action of ultra-violet light. Ferrous ion, as another peroxide reducer, gave positive results in a very few experiments but these are open to the same objections as those raised against the tests with hydroquinone.

Interpretation of New Mutagenesis Data.

The demonstration given here that organic peroxides produce mutations together with the previously known facts that radiation

can produce such peroxides and that ultra-violet functions at least in part by producing chemical changes in rather simple substances constitute a strong argument for that part of the original hypothesis that states that peroxide formation is an essential step in the mutagenic action of radiation. Little progress has been made toward showing whether or not the mustards act in the same way but experimental approaches to this problem have been opened.

Some new speculations can be offered regarding what might be looked upon as the next stage in the study of mutagenesis, the question of what the peroxide does to produce the mutation. As already indicated, the best a priori guess would be that peroxides function through their ability to decompose into free radicals and catalyze reactions involving radical chains. This idea would be supported by experiments showing that the peroxide structure itself is responsible for the effect rather than its oxidizing action or other possible special characteristics, or showing that mutagenic activity characterized non-peroxides which had catalytic activity of the peroxide sort. The inactivity of di-tert.-butyl peroxide, however, makes it necessary to consider other aspects of the possible relationship between the structure of a peroxide and its activity as a mutagen.

It might be that peroxide action is really of an oxidizing nature which would mean that only hydroperoxides would qualify. Another possibility is that the -OOH group is

necessary for attaching the peroxide to some critical cell structure, even to the gene itself. At the present time, however, it seems more reasonable to stay with the theory that peroxides produce mutations by a process that is closely related to their characteristic catalytic activity. This catalytic activity depends upon breaking of the oxygen-oxygen bond and its rate is strongly dependent on the nature of the radicals attached to the peroxide oxygen.

In connection with mutagenic activity it is more pertinent to consider the relationship of structure to the temperature at which the decomposition of peroxides proceeds at an appreciable rate. This is not a subject that has received very thorough study because of the limited number of organic peroxides that have been available up until very recently. In general, though, it can be said that additional oxygen atoms near the peroxide link weaken the oxygen-oxygen bond or permit its rupture at a lower temperature. Thus di-tert.-butyl peroxide decomposes at a rate useful for catalysis in the temperature range 115 to 125°, bis(tert.-butylperoxy)butane from 90 to 100°, benzoyl peroxide, 65 to 75°, and di-tert.-butyl per-oxalate 25 to 35°. It might be, therefore, that di-tert.-butyl peroxide is simply too stable to be effective while the other compounds tested here either decompose at a sufficient rate at 25° or react with compounds in the cell to form structures of still lower stability.

Very recently Westergaard, in a private communication, has reported that diazo methane has strong mutagenic activity when tested by the same procedure on which most of this work has been based. It is a curious fact that the activity of this compound, while of great interest, does not shed much light on the question of the relationship between the mutagenic effects of the mustards and the peroxides. Diazo methane was tested by Westergaard because like nitrogen mustard it is moderately stable in water but reacts readily with certain organic structures, especially carboxyl groups. On the other hand, it is one of those unusual compounds, to which reference was made earlier, that can be substituted for peroxides as catalysts for polymerization reactions. As a matter of fact, it is in commercial use for this purpose in the manufacture of adhesives by the polymerization of conjugated olefinic compounds.

Conclusion.

A hypothesis has been presented here interrelating the actions of known mutation producting agents and explaining thereby, the fact that these agents produce qualitatively similar mutations. Data obtained in this investigation as well as the recent results of other workers have been shown to give direct support to certain phases of this hypothesis. One important part of these arguments, the formation of peroxides by compounds of the mustard type, remains in a dubious state. Even here, however, recent information provides indirect support by stripping away alternate interpretations. Thus it can not be argued now that the mustards act by penetrating to the gene, or by reacting readily with protein or, in fact, that there is anything about the known chemical properties of these compounds that is essential for mutagenesis. Perhaps even this aspect of the hypothesis may be said to have acquired sufficient stature to justify a thorough investigation*.

While a large amount of speculative material has been included in this discussion it is felt that it is justified by the fact that it suggests practical, simple experiments. Thus, the activity of a compound of the mustard type having only one β-chloroethyl group would be a critical test of the

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* When you have eliminated the impossible, whatever remains, however improbable, must be the truth.--Sherlock Holmes.

hypothesized peroxide formation by the mustards; the action of diazomethane when applied to the substrate rather than the organism would distinguish between the two possible interpretations of its activity; and direct tests for peroxide in the irradiated or mustard treated substrates described by Wyss and Stone could be made. Substantial steps toward clarification of the problems of induced mutations can be anticipated for the immediate future.

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Propositions Submitted by Frank H. Dickey

1. The demonstrated procedure for preparing specific adsorbents may require that the adsorbent in its ordinary form should attract the substance for which a specific affinity is to be created. This limitation might be avoided by:
   a. Forming the adsorbent by polymerization of a compound to which the substance to be adsorbed was chemically bound and subsequently removing this substance by breaking this chemical bond which might, for example, be an ester linkage.
   b. Using a third material which would attract the substance to be adsorbed and would itself be trapped in the structure of the forming adsorbent.
   c. Depositing the adsorbent as a film on top of a surface layer of the substance to be adsorbed.

   and in other ways.

2. An instrument for measuring the concentrations of particular dissolved substances might employ an electrode having a surface specially prepared so as to function as an adsorbent for the substance, the concentration of which is to be measured. Resistance between the electrode and the solution would vary with the concentration of the solution.

3. The pure L isomer of sec.-butyl alcohol might be prepared by reduction with Raney aluminum-nickel alloy of L-erythro-3-chloro-2-butanol obtainable from available D-2,3-butanediol.

4. Treatment of 2,3-butanediol isomers with SO_3-dioxane should give configurationally pure preparations of the 3-hydroxy-2-butyl hydrogen sulfates which would be useful for:

   a. direct preparations of related amino compounds,

   b. inversion of one hydroxyl group in the glycol,

   c. preparation of miscellaneous derived compounds in particular isomeric forms.

5. Slow, basic hydrolysis of the 3-amino-2-butyl hydrogen sulfates should give amino alcohols in which the configuration about the second carbon atom would be inverted with respect to the structure from which they were derived and thus permit the preparations of isomeric forms of various amino compounds which could not be conveniently obtained in any other way.

6. Studies of adaptive enzymes suggest that their mode of formation may be related to the mechanism of formation of antibodies. This view suggests a particular theory for the mechanism of the formation of antibodies which could be tested by studying inheritance of the ability to form antibodies to particular antigens.

7. The discovery that peroxides produce mutations suggests that these compounds should be tested in the treatment of cancer. Not only might miscellaneous toxic effects be avoided in this treatment as compared with treatment with radiation or the mustards but also the large number of peroxides potentially available would permit search for particularly effective agents.

8. The theory that certain plant tumors are caused by over-production of an enzyme converting tryptophane to indole-acetic acid might be tested with a fluorotryptophane. On the basis of this theory, tumor tissue grown in a medium containing a suitably adjusted concentration of a fluorotryptophane might be expected to revert to normal growth habits.

9. Since it has been shown that certain mutagenic agents, ultra-violet light and nitrogen mustard, produce their effects indirectly, consideration should be given to possible hazards arising from:

   a. exposure of food to radiation,

   b. bleaching of food with peroxides,

   c. sterilization of blood with nitrogen mustard.

10. I propose an interpretation of nuclear dimorphism in the ciliates.

11. I propose that the functioning of certain enzymes or enzyme systems may be difficult to reconcile with the second law of thermodynamics.