5 hours at the end of which time the theoretical amount of hydrogen had been absorbed. The catalyst was removed, the ethanol distilled under reduced pressure and the residue (110 g.) heated under reflux for 15 hours with 450 ml. of concd. hydrochloric acid. The hot solution was filtered and then steam distilled at constant volume until one liter of distillate was collected. The still residue was allowed to cool and 58 g. of crude product was obtained. Recrystallization from 500 ml. of boiling water (carbon) gave 35.5 g. of *cis*- and *trans*-cyclopentane-1,2-diacetic acids, m.p. 149.5°. A second crop had m.p. 138° and amounted to 3 g. From the m.p.-composition diagram,[17] the first crop contained 45% of the *trans*- and 55% of the *cis*-isomer. The second crop was 70% *trans*- and 30% *cis*-diacid. The yield was 38.5 g. (53% from XIX).

In some preparations, the intermediate reduction product was isolated. Thus, 24.2 g. of XIX yielded 17.4 g. (71%) of ethyl α-(carbethoxymethyl)-cyclopentylcyanoacetate, b.p. 150–153° (1.5 mm.), n^{25}D 1.4599 (lit.[17] b.p. 172°, 2 mm.).

cis- **and** *trans*-**Bicyclo[3.3.0]octanes.**—The *cis*- and *trans*-β-bicyclo[3.3.0]octanones were prepared as described by Barrett and Linstead.[17] The *cis*-ketone, b.p. 88° (17 mm.), n^{25}D 1.4772 (lit.[17] b.p. 78°, 10 mm., n^{18}D 1.4811) was identified as the semicarbazone, m.p. 194–195° dec. (lit.,[17] m.p. 197–198° dec.) and yielded a 2,4-dinitrophenylhydrazone, m.p. 115–116° (after crystallization from ethanol).

Anal. Calcd. for $C_{14}H_{16}O_4N_4$: C, 55.30; H, 5.26; N, 18.42. Found: C, 55.61; H, 5.29; N, 18.39.

The *trans*-ketone, b.p. 87–88° (22 mm.), n^{25}D 1.4755 (lit.[17] b.p. 61–62°, 10 mm., n^{18}D 1.4791) gave a semicarbazone, m.p. 243–244° dec. (lit.[17] m.p. 248–249° dec.), and a 2,4-dinitrophenylhydrazone which had m.p. 156.5–157.5° after crystallization from ethanol.

Anal. Calcd. for $C_{14}H_{16}O_4N_4$: C, 55.30; H, 5.26; N, 18.42. Found: C, 55.22; H, 5.32; N, 18.66.

The infrared spectra of the *cis*- and *trans*-ketones as well as that for the *cis*-α-bicyclo[3.3.0]octanone (kindly supplied by Drs. A. C. Cope and W. R. Schmitz) are given in Fig. 1.

The procedure for the reduction of the ketones follows. A mixture of 1.8 g. of *cis*-β-bicyclo[3.3.0]octanone, 1.8 g. of hydrazine hydrate, 2.5 g. of potassium hydroxide and 24 ml. of *n*-butylcarbitol contained in a 50-ml. flask equipped with a 10-cm. Vigreux column was heated with a metal-bath to 200° over 90 minutes. The distillate was taken up in ether, washed with dilute hydrochloric acid and water, dried over magnesium sulfate and the ether removed. The residue was distilled through a micro-column[22] and yielded 1.2 g. (75% of *cis*-bicyclo[3.3.0]octane, b.p. 135–136°, n^{25}D 1.4580 (lit.[17] b.p. 136–136.5°, n^{18}D 1.4629). Passage over silica gel gave material of n^{25}D 1.4598 presumably by removal of traces of ether. The infrared spectrum of the final product is given in Fig. 2.

trans-Bicyclo[3.3.0]octane was similarly prepared in 65% yield; b.p. 136–136.5°, n^{25}D 1.4568 (lit.[17] b.p. 132°, n^{18}D 1.4625). After passage over silica gel, the product had n^{25}D 1.4592. The infrared spectrum is given in Fig. 2.

CABMRIDGE 39, MASSACHUSETTS

RECEIVED NOVEMBER 15, 1951

[CONTRIBUTION FROM THE GEORGE HERBERT JONES LABORATORY OF THE UNIVERSITY OF CHICAGO]

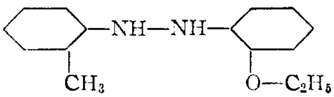
The Intramolecular Character of the Benzidine Rearrangement

BY D. H. SMITH, J. R. SCHWARTZ AND G. W. WHELAND

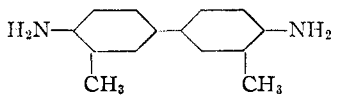
RECEIVED NOVEMBER 17, 1951

In an experiment designed to establish unambiguously whether the benzidine rearrangement is intra- or intermolecular, non-radioactive 2,2′-dimethylhydrazobenzene was rearranged in the presence of a comparable amount of 2-methylhydrazobenzene which had C^{14} in the side-chain. The radioactivity of the resulting 3,3′-dimethylbenzidine was so low that only a negligible part, and possibly none, of it could have been formed in an intermolecular reaction. It is therefore concluded that the rearrangement is intramolecular. In the search for a pair of hydrazo compounds which would be suitable for a study of the type just described, a number of hitherto unreported members of this class were prepared and rearranged. With the use of C^{14} as a tracer, the relative rates with which several different pairs of hydrazo compounds rearrange to the corresponding benzidines was measured.

The benzidine rearrangement is commonly believed to be intramolecular since it has never been found to give rise to cross products.[1] Thus, in 1922, Jacobson[2] listed all the previously reported rearrangements of unsymmetrical hydrazo compounds Ar–NH–NH–Ar′; in no one of these 65 different reactions had the formation of a symmetrical benzidine with two like radicals Ar or Ar′ been observed. Since, however, the presence of considerable quantities of such cross products might easily have been missed in this earlier work, Wheland and Schwartz[3] have more recently studied, with the aid of C^{14} as a tracer, the rearrangement of 2-methyl-2′-ethoxyhydrazobenzene (I). In this way, they showed that less than about 0.3%, and possibly none, of the cross product, *o*-tolidine (II), was formed. Such reactions as these, however, cannot provide conclusive evidence that the rearrangements are purely intramolecular, since the fact that they give no detectable cross products is not necessarily inconsistent with the belief that they proceed by a dissociation into fragments, which subsequently recombine. Indeed, if the postulated dissociation leads to two dissimilar fragments (say, a cation and a neutral molecule), and if it leads always to exactly the same two fragments, then cross products would not be expected since they could then be formed only by the combination of two like fragments. In order to avoid this difficulty, Ingold and Kidd[4] rearranged mixtures of 2,2′-dimethoxyhydrazobenzene and 2,2′-diethoxyhydrazobenzene. Here, regardless of the detailed mechanism, the cross product, 3-methoxy-3′-ethoxybenzidine, should accompany the two symmetrical benzidines if any



I



II

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(1) The term "cross product" is here used to signify a benzidine, the molecule of which contains one aryl group derived from each of two different hydrazo molecules. Clearly, a cross product can be formed only in an *inter*molecular reaction.

(2) P. Jacobson, *Ann.*, **428**, 76 (1922).

(3) G. W. Wheland and J. R. Schwartz, *J. Chem. Phys.*, **17**, 425 (1949).

(4) C. K. Ingold and H. V. Kidd, *J. Chem. Soc.*, 984 (1933).

sort of intermolecular reaction is involved. These authors were unable, however, to find evidence for the presence of this cross product.

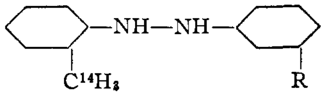
Although the procedure of Ingold and Kidd appears in principle to be completely conclusive, there is nevertheless reason to question the adequacy of the method which they employed in their search for the cross product. They made no attempt to isolate the unsymmetrical benzidine. Instead, they merely compared the melting points of the mixtures of benzidines, which they obtained from the rearrangements, with the independently determined melting point–composition curve for mixtures of the two symmetrical benzidines. The reaction products could not of course be purified before their melting points were determined, since any purification would have altered their compositions. Consequently, the method is hardly capable of high precision. Moreover, since Ingold and Kidd did not prepare the unsymmetrical benzidine, they had no way of knowing how great an effect this substance, if it had been present, would have had on the melting points of the mixtures. For these several reasons, their work probably does not exclude the possibility that as much as 5–10% of cross product may have been formed in their reactions.

We have accordingly re-examined the problem by a method which is essentially that of Ingold and Kidd, but which is made more precise by the use of C^{14} as a radioactive tracer. Moreover, as is explained below in greater detail, we found it possible to simplify the procedure, without any loss of rigor, by rearranging a mixture of one symmetrical and one suitably chosen unsymmetrical hydrazobenzene, rather than a mixture of two symmetrical hydrazobenzenes.

At the outset, we intended, like Ingold and Kidd,[4] to rearrange a mixture of two symmetrical hydrazo compounds. In order for this method to be successful, the hydrazo compounds employed must satisfy certain conditions in addition to the obvious one that all the required substances (including the possible cross product) be preparable and of rigorously established structure. (1) The two hydrazo compounds must rearrange at comparable rates, since otherwise one will be largely transformed into its benzidine before the other has started to react. (Of the two hydrazo compounds studied by Ingold and Kidd, the dimethoxy derivative rearranges approximately six times as fast as the diethoxy.[4]) (2) The major products of the rearrangements must be benzidines, and not diphenylines, semidines or disproportionation products. (3) The two benzidines and the possible cross product, or suitable derivatives of them, must be completely separable.

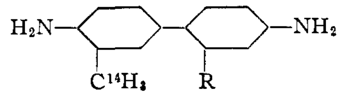
In our work, 2,2′-dimethylhydrazobenzene (hereafter called hydrazotoluene), which has been shown[5] to rearrange largely to *o*-tolidine (II), was chosen as one member of each pair of hydrazo compounds tested. The following substances, which were considered as possible reaction partners, were found unsuitable for the reasons stated: 2,2′-diethoxyhydrazobenzene rearranged about 220 times as fast as hydrazotoluene (see the Experimental part, below); 2,2′-diethylhydrazobenzene rearranged 0.67 times as fast as hydrazotoluene, but no way could be found for separating the resulting 3,3′-diethylbenzidine from *o*-tolidine; 2,2′-dichlorohydrazobenzene required 24 hours for its rearrangement, whereas hydrazotoluene was completely transformed in a few seconds; 3,3′-bis-(β-carboxyethyl)-hydrazobenzene rearranged to a product which could not satisfactorily be crystallized; 3,3′-bis-(hydroxymethyl)-hydrazobenzene rearranged 0.9 times as fast as hydrazotoluene, but the preparation of the possible cross product, 3-methyl-2′-hydroxymethylbenzidine, and its separation from the symmetrical benzidines proved to be unsatisfactory.

At this point, it was decided to pair the hydrazotoluene, not with a second symmetrical hydrazo compound, but rather with an unsymmetrical one of general structure III, in which R represents some initially undetermined substituent atom or group.

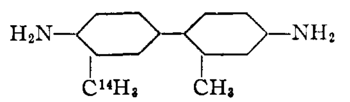


III

In view of the results previously obtained with unsymmetrical hydrazobenzenes (see above), it appears certain that the compound III cannot by itself give rise to any symmetrical benzidines. Consequently, the only benzidines which one could reasonably expect to be formed in the rearrangement of a mixture of hydrazotoluene and the substance III are the "normal" products II and IV, and perhaps also the cross product V. Moreover,



IV



V

since this possible cross product is now *chemically* identical with one of the "normal" products, namely *o*-tolidine (II), the search for the cross product is materially simplified. Indeed, all that is now required is to separate the *o*-tolidine from the one other benzidine IV (not from *two* others, as in the original procedure), and to examine it for radioactivity. Any radioactivity which cannot be removed from the *o*-tolidine by repeated purifications is then a direct measure of the amount of cross product formed in the rearrangement. (The possible formation of a second cross product, chemically identical with IV but not radioactive, in no way affects the argument.)

The conditions which must be imposed on the unsymmetrical hydrazo compound III are essentially the same as those which apply when both hydrazo compounds are symmetrical (see above). They are, however, somewhat less stringent than before, because of the decreased number of substances which must be prepared, characterized, and finally separated from one another. The following compounds were tested and were found unsatisfactory for the reasons stated: 2-methyl-3′-hydroxymethylhydrazobenzene rearranged 0.38 times

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(5) G. Schultz, G. Rohde and F. Vicari, *Ann.*, **352**, 111 (1907).

as fast as hydrazotoluene, but its preparation and rearrangement gave low yields, and the separation of the benzidines was not satisfactory; 2-methyl-3′-carboxyhydrazobenzene rearranged only 0.09 times as fast as hydrazotoluene; 2-methyl-3′-methoxyhydrazobenzene presented preparative difficulties which we were not successful in overcoming. Finally, 2-methylhydrazobenzene was found to be entirely satisfactory since (1) it rearranges approximately one-third as fast as hydrazotoluene; (2) Jacobson has shown[6] that the major product of its rearrangement is 3-methylbenzidine; and (3) 3-methylbenzidine and *o*-tolidine can be completely separated by fractional adsorption of their dibenzal derivatives on an alumina column.

In the way outlined, we have been able to show that, in the rearrangement of a mixture of hydrazotoluene and 2-methylhydrazobenzene, less than 0.03%, and possibly none, of the resulting *o*-tolidine II could have been formed in an intramolecular reaction. This work therefore provides much the most conclusive evidence which is now available for the belief that the benzidine rearrangement is purely intramolecular.

Experimental

Preparation of Materials.—Except as noted below, all the compounds which were studied in this work, and for which no analytical data are cited, have been previously reported in the literature, and the physical properties of the materials prepared by us were in satisfactory agreement with those recorded by the earlier investigators. All the symmetrical hydrazobenzenes were prepared from the corresponding nitro compounds by reduction with zinc dust and aqueous alcoholic sodium hydroxide. The unsymmetrical hydrazobenzenes were prepared from the corresponding azo compounds by reduction either with zinc dust and sodium hydroxide or with ammonium sulfide. The unsymmetrical azo compounds were themselves prepared by the condensation of primary aromatic amines with nitroso compounds in ethanol containing about 2% acetic acid. The benzidine rearrangements were carried out by adding the calculated amounts of ethyl alcoholic hydrogen chloride to solutions of the hydrazo compounds in absolute ether. The dihydrochlorides of the benzidines, which precipitated, were then separated by filtration, washed several times with anhydrous ether, and dried in a desiccator for 24 hours. The dibenzal derivatives of the benzidines were formed by the action of benzaldehyde and sodium or potassium acetate upon the benzidine dihydrochlorides. Since rigorous purifications and proofs of structure were not attempted for the benzidines which were not used in the subsequent studies, there is a possibility that these materials may have consisted of, or may have been contaminated by, diphenylines.

2,2′-Diethylhydrazobenzene melted at 103–104°. *Anal.* Calcd. for $C_{16}H_{20}N_2$: N, 11.64. Found: N, 11.44. 3,3′-Diethylbenzidine melted at 38–40°. Since Beilstein describes this substance as an "oily mass," its identity was confirmed by preparation of its N,N′-diacetyl derivative, melting at 306–307°. 2-Ethyl-2′-methylazobenzene was prepared from *o*-nitrosotoluene and *o*-ethylaniline. Since it did not crystallize, it was reduced to the hydrazo compound, which was then rearranged to 3-ethyl-3′-methylbenzidine. The dihydrochloride of this benzidine charred without melting at about 290°. Equivalent weight (by titration with base): calcd. 150, found 152. 3,3′-Bis-(β-carboxyethyl)-hydrazobenzene melted at 129–130°, but was not further characterized since its rearrangement led to products which could not be crystallized. 3,3′-Bis-(hydroxymethyl)-hydrazobenzene[7] melted at 108–110°. *Anal.* Calcd. for $C_{14}H_{16}N_2O_2$: C, 68.8; H, 6.7. Found: C, 68.9; H, 6.5. 2,2′-Bis-(hydroxymethyl)-benzidine[7] melted at 170–175°. *Anal.* Calcd. for $C_{14}H_{16}N_2O_2$: C, 68.8; H, 6.7. Found: C, 68.4; H, 6.6.

2-Methyl-3′-carboxyazobenzene, obtained in 19% yield from *o*-nitrosotoluene and *m*-aminobenzoic acid, formed red crystals melting at 164–165°. *Anal.* Calcd. for $C_{14}H_{12}N_2O_2$: N, 11.65; equiv. wt., 240. Found: N, 11.81, 11.78; equiv. wt., 240. Reduction with lithium aluminum hydride gave 2-methyl-3′-hydroxymethylazobenzene, an orange-red oil, which was reduced without purification to the yellowish white 2-methyl-3′-hydroxymethylhydrazobenzene, melting at 66.5–68.5°; yield 59%, based on the carboxyazo compound. *Anal.* Calcd. for $C_{14}H_{16}N_2O$: N, 12.28. Found: N, 12.11. 3-Methyl-2′-hydroxymethylbenzidine, obtained from rearrangement of the foregoing hydrazo compound, was an amorphous mass, forming a crystalline but hygroscopic dihydrochloride. The yield of the dihydrochloride was 64%. Equivalent weight: calcd. 150.5; found, 152. The dibenzal derivative, obtained in 70% yield, melted at 149–150°. *Anal.* Calcd. for $C_{28}H_{24}N_2O$: N, 6.93. Found: N, 7.01, 7.09. Reduction of 2-methyl-3′-carboxyazobenzene gave a 65% yield of 2-methyl-3′-carboxyhydrazobenzene, melting at 155.5–158°. *Anal.* Calcd. for $C_{14}H_{14}N_2O_2$: N, 11.57; equiv. wt., 242. Found. N, 11.72; equiv. wt., 239. 3-Methyl-2′-carboxybenzidine dihydrochloride was obtained in 77% yield from this hydrazo compound. *Anal.* Calcd. for $C_{14}H_{16}Cl_2N_2O_2$: Cl, 22.5; N, 8.88; equiv. wt., 105. Found: Cl, 22.7; N, 8.72, equiv. wt., 113. By addition of sodium acetate to the aqueous solution, continuous extraction of that solution with ether, and evaporation of the extracts: there was obtained a 72% yield of the free benzidine, melting at 230–233°. *Anal.* Calcd. for $C_{14}H_{14}N_2O_2$: N, 11.56. Found: N, 11.34.

2-Methyl-3′-methoxyazobenzene, obtained in a very low yield from *o*-toluidine and *m*-nitrosoanisole, was a liquid. It was reduced with ammonium sulfide to the hydrazo compound, a light orange-yellow solid melting at 70°. The yield, based on the nitrosoanisole, was 4%. *Anal.* Calcd. for $C_{14}H_{16}N_2O$: N, 12.28. Found: N, 12.50. Rearrangement gave the benzidine dihydrochloride in 40% yield *Anal.* Calcd. for $C_{14}H_{18}Cl_2N_2O$: N, 9.31. Found: N, 9.71. Attempts to prepare the free benzidine and its dibenzal derivative were unsuccessful.

All the radioactive compounds which were used were prepared by standard methods (see also above) from *o*-nitrotoluene, o-O_2N-C_6H_4-$C^{14}H_3$, which had been made as in the preceding paper.[3]

Measurements of Radioactivity.—Except for a few preliminary measurements, in which an end-window counter was used, all determinations of radioactivity were made with a counter of the type described by Kelsey.[8] Samples possessing the highest activities were counted with 1.0–1.5% error to a certainty of 0.99; samples of lower activity were counted with 5% error, or less, to a certainty of 0.99. The materials which were counted were the benzidine dihydrochlorides in the determinations of relative rates of rearrangement, and the dibenzal derivatives (Schiff bases) of the benzidines in the final measurements of the amount of cross product. The radioactive standards which were used were *o*-tolidine dihydrochloride in the former measurements, and the dibenzal derivative of 3-methylbenzidine in the latter measurements. Whenever possible, the samples mounted for counting were of at least "infinite" thickness, which was taken as 20 mg. per square centimeter. When a sample to be counted was of less than "infinite" thickness, the observed count was corrected to "infinite" thickness by the method described by Libby.[9] No sample containing less than 20% of the material required for an "infinitely" thick layer was counted.

Measurement of Relative Rates of Rearrangement.—As an example of the method employed, the measurement of the relative rates of rearrangement of hydrazotoluene and 2-methylhydrazobenzene may be described. A mixture of 239.2 mg. of radioactive hydrazotoluene and 248.0 mg. of inactive 2-methylhydrazobenzene was dissolved in 60 cc. of anhydrous ether, and enough alcoholic hydrogen chloride to precipitate 15% of the benzidines as their dihydrochlorides was added. The precipitated mixture of benzidine dihydrochlorides was washed several times with anhydrous ether,

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(6) P. Jacobson, *Ber.*, **28**, 2541 (1895).

(7) R. N. Sen and R. Sadasivam, *J. Indian Chem. Soc.*, **9**, 403 (1932), have reported that this hydrazo compound and its corresponding benzidine melt, respectively, at 268 and 177°. It may, however, be questioned whether these authors actually had the assumed compounds since they reported the former to be soluble in aqueous alkali.

(8) F. E. Kelsey, *Science*, **109**, 566 (1949).

(9) W. F. Libby, *Anal. Chem.*, **19**, 2 (1947).

TABLE I

SEARCH FOR CROSS-PRODUCT *o*-TOLIDINE IN THE REARRANGEMENT OF A MIXTURE OF INACTIVE HYDRAZOTOLUENE AND ACTIVE 2-METHYLHYDRAZOBENZENE

No. of run	1	2	3
Wt. hydrazotoluene, mg.	610.1	900.0	1119.9
Wt. 2-methylhydrazobenzene, mg.	583.8	572.6	556.2
First purification, activity of standard	26,600 ± 245	27,250 ± 350	27,000 ± 300
activity of sample	41 ± 2	27.3 ± 1.9	72 ± 2.7
Second purification, activity of standard	28,400 ± 350	27,100 ± 250	27,100 ± 250
activity of sample	37.7 ± 1.6	25 ± 1.8	20.1 ± 1.5
Third purification, activity of standard	....	27,000 ± 350	27,000 ± 350
activity of sample	....	15.5 ± 0.75	21.2 ± 1.0
Fourth purification, activity of standard	....	27,000 ± 350	27,000 ± 350
activity of sample	....	16.6 ± 1.1	15.9 ± 0.8
Final count corrected for impurities	23	7	8
Per cent. of cross-product *o*-tolidine	0.08	0.03	0.03

dried in a desiccator for 24 hours, and then counted. The activity of the sample prepared in this way was found to be 384 ± 14 cts./min. whereas that of the standard *o*-tolidine dihydrochloride, similarly prepared from the same radioactive hydrazotoluene, was 534 ± 15 cts./min. If the two rearrangements are of the same order (presumably of second order[10]) with respect to hydrogen ion, if each is of first order with respect to hydrazo compound, and if each gives a quantitative yield of the corresponding benzidine, then the ratio of rate constants can be calculated from the equation

$$\frac{k_{\text{hydrazotoluene}}}{k_{\text{2-methylhydrazobenzene}}} = \frac{\log(1 - t/T^0)}{\log(1 - m/M^0)}$$

where t is the number of moles of *o*-tolidine dihydrochloride formed, T^0 is the number of moles of hydrazotoluene originally taken, m is the number of moles of 3-methylbenzidine dihydrochloride formed and M^0 is the number of moles of 2-methylhydrazobenzene originally taken. The sum $t + m$ is of course equal to half the number of moles of hydrogen chloride added; the ratio t/m can be calculated in an obvious way from the measured activities of the mixed benzidine salts and of the standard; and the values of T^0 and M^0 are known. From the data cited above, the ratio of the k's is found to be 3.2. In a second run, a value of 3.0 was obtained. The major source of uncertainty in these two essentially indistinguishable results doubtless does not reside in the measurements of radioactivity, but rather in the assumption that the rearrangements are quantitative and give only the respective benzidine dihydrochlorides. It is, however, unlikely that the error introduced by this assumption is sufficient to invalidate the conclusion that the two reactions go at not widely different rates, since the yields of the benzidines, although certainly not quantitative, are nevertheless rather good. In separate runs with the pure hydrazo compounds, it was in fact possible actually to isolate 80% of the theoretical amount of *o*-tolidine and 67% of the theoretical amount of the dibenzal derivative of 3-methylbenzidine.

The Search for the Cross Product.—The procedure which was adopted can be illustrated by a description of one run. To 50 cc. of anhydrous ether were added 583.8 mg. of radioactive *o*-methylhydrazobenzene and 610.1 mg. of inactive, hydrazotoluene. When this material had dissolved, 7.5 cc. of 1.7 *N* hydrogen chloride in absolute ethanol was added, and the mixture was vigorously swirled. After addition of another 50 cc. of ether, the precipitate was collected on a filter, washed several times with anhydrous ether, and dried in a vacuum desiccator for 24 hours. The thus-obtained mixture of benzidine dihydrochlorides was dissolved in 45 cc. of absolute ethanol and treated with 1.0 cc. of benzaldehyde and 1.4 g. of potassium acetate. The solution was then refluxed for 20 hours. The precipitate was collected on a filter, washed twice with 3-cc. portions of cold ethanol, several times with 5-cc. portions of distilled water, and finally once with 3 cc. of cold ethanol.

The resulting dibenzal derivatives were separated chromatographically on a column of alumina, 28 inches long and 15 mm. in diameter. The alumina (Alcoa #80 mesh) was previously treated with ethyl acetate, then washed several times with methanol, and dried under vacuum. After this treatment, it was activated by being heated to 400° under vacuum. While hot, it was placed in bottles, which were then tightly capped. In the preparation of the chromatographic column, the tube was first filled with ligroin, the alumina was poured in, and finally the ligroin was replaced by benzene.

The mixture of Schiff bases was dissolved in benzene, and the solution was allowed to pass through the column. The adsorbed material was eluted with a mixture (9:1 by volume) of benzene and ether. No sharp separation of the chromatogram into distinct bands was observed. Consequently, the entire column was yellow. The dibenzal derivative of *o*-tolidine, which was the first Schiff base to come off the column in the elution, was isolated by evaporation of the solvent. Its activity was found to be only 41 ± 2 cts./min. A second purification on a shorter column reduced the activity to 37.7 ± 1.6 cts./min. For comparison, the activity of the standard (the dibenzal derivative of the same radioactive 3-methylbenzidine) was 26,600 ± 245 cts./min. in the first measurement and 28,400 ± 350 cts./min. in the second. If all the residual radioactivity were due to cross-product *o*-tolidine, then the amount of such cross product would be about 0.15% of the total *o*-tolidine. This figure must, however, be decreased for two reasons. In the first place, the purification to which the sample had been subjected was doubtless insufficient to remove the last traces of the Schiff base derived from the highly radioactive 3-methylbenzidine. Indeed, in other runs, in which the sample could be chromatographed two additional times, the residual activity was further reduced to about 16 cts./min., corresponding to about 0.06_5% cross product (*cf.* Table I). In the second place, the radioactive 2-methylhydrazobenzene apparently contained a trace of radioactive hydrazotoluene, which had arisen from an extremely small amount of disproportionation during the original condensation of active *o*-nitrosotoluene and aniline

$$2o\text{-CH}_3\text{—C}_6\text{H}_4\text{—NO} + 2\text{C}_6\text{H}_5\text{—NH}_2 \longrightarrow o\text{-CH}_3\text{—C}_6\text{H}_4\text{—NH—NH—C}_6\text{H}_4\text{—CH}_3\text{—}o + \text{C}_6\text{H}_5\text{—NH—NH—C}_6\text{H}_5$$

In order to determine the extent of this disproportionation, 484 mg. of the Schiff base of the radioactive 3-methylbenzidine (activity 28,000 cts./min.) was mixed with 1566 mg. of the Schiff base of inactive *o*-tolidine. The mixture was then separated on a 28-inch alumina column. The activity of the dibenzal *o*-tolidine obtained in this way was 614 ± 21 cts./min. Further purification on a 10-inch column reduced the activity to 5.7 ± 1.4 cts./min., and finally to 4.9 ± 0.8 cts./min. Before this last purification, 3.0 mg. of the Schiff base of *inactive* 3-methylbenzidine was added to the sample as a hold-back carrier. The quantity of this material was such that no measurable activity due to the unremoved dibenzal 3-methylbenzidine could have remained. From these data, it follows that about 0.06% of the total activity found for the Schiff base of the *o*-methylbenzidine was actually due to the Schiff base of *o*-tolidine. When the corrections are made for this impurity, the amount of cross-product *o*-tolidine is decreased to about 0.08% of the total in the first run and to about 0.03% in the subsequent ones

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[10] G. S. Hammond and H. J. Shine, THIS JOURNAL, **72**, 220 (1950).

(see Table I). Whether the small residual activity is significant or is instead merely a measure of the experimental error in the determinations is not clear. In any event, however, there can be no doubt that the only *important* mechanism for the rearrangement is purely intramolecular.

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Effect of Catalysts on the Hydrolysis of Acetyl Phosphate. Nucleophilic Displacement Mechanisms in Enzymatic Reactions[1]

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The effect of catalysts on the non-enzymatic hydrolysis of acetyl phosphate has been studied to provide a basis for interpreting the enzymatic mechanisms of this and related substrates. The *p*H-rate profile was determined over the *p*H range 0 to 12. Strong acid and base catalysis was shown at the extremes of *p*H with a broad flat minimum in the region of neutrality. From these data, it was concluded that nucleophilic attack by water on the phosphorus atom is the predominant reaction at neutrality whereas attack at the carbonyl carbon atom is the major pathway of hydrolysis in the strongly acidic and strongly basic solutions. Magnesium ion was found to catalyze the hydrolysis of the dinegative acetyl phosphate ion but not of the mononegative ion or of the uncharged acid. One mole of magnesium ion and one mole of acetyl phosphate are present in the activated complex. Pyridine was shown to catalyze the hydrolysis in neutral solutions and evidence is presented that the catalysis proceeds by formation of an acetylpyridinium ion. By analogy to the non-enzymatic reactions, it is suggested that nucleophilic displacements play an important role in the enzymatic reactions of acetyl phosphate and other substrates. A series of two successive displacements, each involving an inversion, is postulated to explain the over-all retention of configuration observed in certain enzymatic reactions which occur by attack at an asymmetric carbon atom. The biological evidence is shown to be consistent with these hypotheses.

To obtain a detailed picture of an enzymatic reaction, it is necessary to know the chemical identity of the active sites on the enzyme surface, their spatial arrangement and their effect on the substrate molecules. One approach to this problem is a study of the mechanism of the non-enzymatic reactions of the substrate molecules and the effect on these reactions of catalysts analogous to those active on the enzyme surface. In some cases the enzymatic and non-enzymatic mechanisms may be very similar; in others, the steric factors and concerted action of the groups on the enzyme surface may occasion qualitative as well as quantitative changes in mechanism. In either case, the non-enzymatic mechanism provides information essential to the interpretation of the enzymatic mechanism.

In the present study the effect of catalysts on the mechanism of hydrolysis of acetyl phosphate has been investigated. The catalysts, chosen because of their relationship to potential active groups on an enzyme, were: (a) acid and base as determined through the effect of *p*H, (b) a metal ion, magnesium and (c) a nucleophilic reagent, pyridine.

In addition to the specific information obtained about the individual catalysts, it was found that the non-enzymatic reactions of the acetyl phosphate all could be classed in the general category of nucleophilic displacement mechanisms.[3] This led to the suggestion that the same mechanisms might be operating in the biological reactions. The available information on the biochemistry of acetyl phosphate, and of some other substrates as well, was examined and found to be consistent with this hypothesis.

Experimental

The solution containing the appropriate buffer was allowed to stand in a 39.00 ± 0.02° thermostat until temperature equilibrium was attained. A few milligrams of powdered lithium acetyl phosphate were added, and the decrease in the concentration of acetyl phosphate followed as a function of time by removing two-ml. aliquots at appropriate intervals. The acetyl phosphate was assayed by a slight modification of the method of Lipmann and Tuttle.[4]

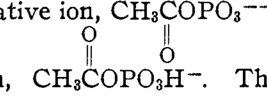
All solutions were brought to an ionic strength of 0.6 by adding the calculated amount of sodium perchlorate. The *p*H measurements were made at 25°, using a Beckman Model G instrument calibrated by the standard buffers recommended by Bates, Pinching and Smith.[5] All kinetic experiments to determine rate constants were run to greater than 75% disappearance of acetyl phosphate.

Results and Discussion

Effect of *p*H.—The rate of disappearance of acetyl phosphate was first order in all cases. Some typical results are shown in Fig. 1.

To clarify the effect of *p*H on the rate of hydrolysis, the first order rate constants were plotted as a function of *p*H. The results are shown graphically in Fig. 2. Each value of k_1 listed is the average of two kinetic runs which differed from each other by less than 5%.

It is seen that the hydrolysis is relatively unaffected by changes in *p*H in the region near neutrality but is greatly accelerated in strongly acidic and strongly basic solutions. The asymmetric hump in the *p*H 2–5 region resembles a titration curve and corresponds to the change in acetyl phosphate from the dinegative ion, $CH_3\underset{\underset{\displaystyle O}{\|}}{C}OPO_3^{--}$, to the mononegative ion, $CH_3\overset{\overset{\displaystyle O}{\|}}{C}OPO_3H^{-}$. The



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[1] Presented at the Spring Meeting of the American Chemical Society, Boston, Mass., April, 1951.

[2] Brookhaven National Laboratory, Upton, N. Y.

[3] L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 131.

[4] F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).

[5] R. G. Bates, G. D. Pinching and E. R. Smith, *J. Research Natl. Bur. Standards*, **45**, 418 (1950).