XLVII. CORRELATION OF THE SPERMICIDAL EFFICIENCIES OF AROMATIC ALDEHYDES WITH THEIR CHEMICAL REACTIVITIES, AND ELECTROMETRIC ALKALINE TITRATIONS OF GELATIN IN PRESENCE OF AROMATIC ALDEHYDES.

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IN a previous communication [Gulland, 1932] it was shown that a series of *p*-benzoquinones arranged in order of their lethal action on guinea-pig spermatozoa resembles closely a series of the same, or similar, quinones placed in order of their chemical reactivities. At the same time the results of a few experiments with aromatic aldehydes were quoted, from which it was evident that certain simple derivatives of benzaldehyde are considerably more effective than others in immobilising guinea-pig spermatozoa. These observations have now been extended to cover a series of closely related aromatic aldehydes, in order to ascertain to what extent their spermicidal efficiency, regarded as a measure of their effect on cells, may be correlated with the chemical reactivities of their aldehyde groups.

The biological tests were made essentially as described by Baker [1930; 1931, 1; 1932] but with one important alteration, which is described in the experimental section. This modification confers the following advantages. (a) Substances in pairs may be compared directly with each other, using one and the same sperm suspension. (b) Decisive results may be obtained at sub-lethal, instead of lethal, concentrations, a point which becomes important when sparingly soluble substances are examined. (c) It is possible to classify substances which differ but slightly in spermicidal efficiency. (d) Fewer animals are needed. The three former advantages were of greater value in the present investigation than in Baker's researches.

The aldehydes now examined were mono- and di-methoxybenzaldehydes; phenolic derivatives were not included, since their acidity disturbed the buffering effect of the fluid in which the sperms were suspended and consequently introduced a second variable factor into the experiments. A method has been devised, however, to enable the spermicidal efficiency of such acidic substances to be tested at the standard p_H value.

The following experimental relationships were established between the lethalities of the aldehydes, formaldehyde being included for comparative purposes: formaldehyde > 2:5-dimethoxybenzaldehyde > 2:3-dimethoxybenzaldehyde > 2-methoxybenzaldehyde $\geq$ 2:4-dimethoxybenzaldehyde $\geq$ 3-methoxybenzaldehyde > 3:4-dimethoxybenzaldehyde.

Since the carbonyl group is the only chemically reactive centre in these aldehydes, it is obvious that the amino-groups of the cell proteins or amino-acids are probable points of attack by the aldehydes. It will be shown elsewhere

that some aromatic aldehydes condense readily with glycine in presence of baryta or barium acetate, whereas others do not, and in the present connection it may be stated that the isolation of the barium salt of the arylidene glycine was found to be an indication that the aldehyde in question would prove spermidically efficient. In the same paper it is demonstrated that 2:5- and 2:3-dimethoxybenzaldehydes, both of which are of relatively high lethality towards sperms, enter into reversible condensation with *d*-phenylalanine more rapidly and to a

Fig. 1. Titration of gelatin (G) alone and in presence of formaldehyde (F) and 2-, 3-methoxybenzaldehydes, 2:3-, 2:4-, 2:5-, 3:4-dimethoxybenzaldehydes.

greater extent than does 3:4-dimethoxybenzaldehyde, which has only feeble lethality. Such condensations, however, are less closely related to those occurring in cells than are interactions of the aldehydes with a protein, and the chemical reactivities of the same aldehydes which were examined for spermidical efficiency have therefore been compared as regards their capacities for condensation with gelatin.

Birch and Harris [1930] compared the titration curve of a gelatin solution with that of a similar solution containing formaldehyde and showed that the acidic portion of the curve is unaltered by the addition of the aldehyde whereas in the alkaline range displacement occurs in the direction of decreased p_H value.

This effect is due to the combination of the aldehyde with undissociated basic groups of the gelatin anions. As a result, the effective concentration of gelatin anions is reduced, and the solution becomes more acid [Levy, 1933; Richardson, 1934]. It follows that the extents of the displacements in alkaline titrations of a protein in solutions of different aldehydes at the same concentration provide a measure of the tendencies of the aldehydes to condense with the undissociated amino-groups of the protein anion.

Titration curves were prepared under standard conditions over the range p_H 5–10 for gelatin alone and in presence of a constant fraction of a molecular proportion of each of the aldehydes (Fig. 1). Absolute accuracy was not attempted, but as the conditions of each experiment were identical, the results obtained were strictly comparable among themselves. These curves almost coincide at about p_H 6.5, and closely approach the gelatin curve. They are deflected from the gelatin curve towards increased acidity at higher and lower p_H values

Fig. 2. Titrations of gelatin alone and in presence of:

A { $M/100$ 2:5-dimethoxybenzaldehyde
2:5-dimethoxybenzoic acid

B { $M/100$ 2-methoxybenzaldehyde
2-methoxybenzoic acid

(shown more clearly in Fig. 2). These portions of the curves are considered separately below. The curve obtained using 3-methoxybenzaldehyde is apparently abnormal and is discussed later.

Consideration of the more alkaline sections of the titration curves shows that condensation occurred progressively over approximately the same p_H range as that in which the amino-groups of the lysine residues and the "terminal" amino-groups of peptide chains dissociate [cf. Jordan-Lloyd, 1933]. This suggests that these groups are involved in this condensation.

From the standpoint of the relative capacities of the aldehydes for condensation, the displacements of the titration curves may be compared at p_H 8.2,

the reaction of the fluid in which the sperms are suspended in the biological tests, or more easily at p_H 9.6, where the spatial differences are greater; the curves do not intersect, with the exception that that of formaldehyde crosses that of 2:3-dimethoxybenzaldehyde at p_H 9.1–9.2. By this means the aldehydes were arranged in the following order of decreasing capacity for condensation with gelatin anions: 2:3-dimethoxybenzaldehyde > (formaldehyde) > 3-methoxybenzaldehyde > 2:5-dimethoxybenzaldehyde > 2-methoxybenzaldehyde > 3:4-dimethoxybenzaldehyde > 2:4-dimethoxybenzaldehyde. The position of formaldehyde in the series is doubtful on account of the intersection mentioned above, and 3-methoxybenzaldehyde occupies an unexpectedly high place (see below).

3-Methoxybenzaldehyde autoxidises with great ease, and it was shown by titration that the sample used contained 0.3 % of the corresponding acid, although all possible precautions had been taken to prevent aerial oxidation during its purification. Moreover, the amount of acid may well have increased beyond 0.3 % during the preparation of the aldehyde-gelatin solution before the titration was begun. It was therefore suspected that the position of the titration curve in Fig. 1 was due to the presence of 3-methoxybenzoic acid and not to a superior capacity for condensation by this aldehyde, and confirmation of this view was obtained in two experiments. 2-Methoxybenzoic acid or 2:5-dimethoxybenzoic acid respectively was added to the gelatin solution before titration in amounts sufficient to lower the p_H to that of the corresponding aldehyde-gelatin mixture at the isoelectric point of gelatin. The subsequent titration of the mixture of gelatin and acid then followed a course parallel with the gelatin curve and quite different from the aldehyde-gelatin curve (Fig. 2).

By transposing the position of the 3-methoxybenzaldehyde-gelatin curve so that it coincides with the curves of the other aldehyde complexes at p_H 6.6, that is, by making allowance for the presence of 3-methoxybenzoic acid, it may be shown that at alkaline reactions 3-methoxybenzaldehyde has a capacity for condensation approximately equal to that of 2-methoxybenzaldehyde, and that it also reacts with gelatin at p_H values acid to p_H 6.6, in common with the other aromatic aldehydes. These aldehydes thus fall into the following order of decreasing capacity for condensation with gelatin anions at the reaction at which the spermicidal tests were made (Table I). It is also possible that in the spermicidal tests the facile autoxidation of 3-methoxybenzaldehyde reduced its true lethality slightly by decreasing its concentration as a result of conversion into 3-methoxybenzoic acid. Attention was given to these corrections in drawing up Table I, which in our opinion shows the arrangement of the aldehydes in decreasing order (*a*) of spermicidal efficiency, (*b*) of capacity for condensation with gelatin anions.

Table I.

Spermicidal efficiency	Capacity for condensation with gelatin
2:5-Dimethoxybenzaldehyde	2:3-Dimethoxybenzaldehyde
2:3-Dimethoxybenzaldehyde	2:5-Dimethoxybenzaldehyde
2-Methoxybenzaldehyde	2-Methoxybenzaldehyde
3-Methoxybenzaldehyde	3-Methoxybenzaldehyde
2:4-Dimethoxybenzaldehyde	3:4-Dimethoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	2:4-Dimethoxybenzaldehyde

Thus, the series of aldehydes arranged in order of spermicidal efficiency closely resembles the series of the same aldehydes arranged in order of their capacity for condensation with gelatin anions.

The arguments used above are based on the assumption that in no case is the aldehyde present in such amount that all, or even most of those groups in the gelatin molecule which can react have in fact condensed with the aldehyde molecule. That the gelatin was capable of far more extensive condensation was shown (Fig. 3) by the very marked additional displacement of the curve in presence of 0.5 % formaldehyde as compared with that using $M/100$ formaldehyde.

Fig. 3. Titrations of gelatin in 0.5 % and $M/100$ formaldehyde solutions and in water.

The more acidic sections of the titration curves, except in the case of that determined in presence of formaldehyde, are deflected away from the gelatin curve towards greater acidity between p_H 6 and 4.9, the isoelectric point of gelatin. These changes are clearly shown in Fig. 2. It is improbable that these deflections are due to the presence of small amounts of the corresponding acids, partly because these aldehydes, excluding 3-methoxybenzaldehyde, do not autoxidise very readily and partly because the curves almost coincide at p_H 6.7 as mentioned above. It was shown above that the presence of the corresponding acid prevented this coincidence. It may be inferred, therefore, that the aromatic aldehydes, but possibly not formaldehyde at the same concentration, combine with gelatin between its isoelectric point and about p_H 6–7 to form compounds which may be resolved into their components as the reaction of the medium approaches neutrality. This condensation might conceivably be associated with the iminazole group of histidine residues, for which Neuberger [1934] has determined p_K 5.99 [cf. Jordan-Lloyd, 1933], and in this connection it is interesting to note that when salicylaldehyde and histidine condense in the presence of brucine, the resulting brucine salt contains salicylaldehyde and histidine in the ratio of 2:1 [Bergmann and Zervas, 1926]. We do not, however, advance any explanation of the deflections at acid p_H values, since further investigation is necessary.

EXPERIMENTAL.

Technique of biological tests.

The technique described in great detail by Baker showed the least concentration (weight/volume) in the series 1:50, 1:100, 1:200, 1:400, 1:800 *etc.*, which sufficed to immobilise in 30 minutes in four consecutive experiments all guinea-pig sperms suspended in a glucose-saline fluid buffered at p_H 8.1 at 37°. The early experiments of the present investigation were made according to this technique, but it soon became evident that modifications of Baker's procedure were necessary. These are described below, and should be regarded as supplementary to his description and not in any way in opposition to it.

Preparation of buffered glucose-saline solution. It is undesirable to store alkaline stock solution containing glucose and phosphate, owing to the instability of glucose under these conditions. In order, therefore, to avoid the necessity of frequent preparations of small quantities [Baker, 1931, 2] two solutions were employed, one containing the saline constituents, the other the glucose. The first solution consisted of disodium hydrogen phosphate dihydrate (5.96 g.), dihydrogen potassium phosphate (0.20 g.) and sodium chloride (4.00 g.) in 1 litre of aqueous solution; this was preserved at 0° and mixed as required with an equal volume of a solution of glucose (6.0 g.) in freshly prepared aqueous solution (100 ml.). Sodium phosphate dihydrate was used instead of the dodecahydrate [Baker, 1931, 2], since the latter effloresces and is therefore of inconstant composition.

Preparation of aldehyde solutions. These were prepared in terms of molarity, instead of percentage, so that the activities of aldehydes with different molecular weights could be readily compared.

Neutralisation of acidic substances before the biological test. It is well known that sperm-cells are susceptible to changes in the reaction of the medium in which they are suspended, and that the mobility of guinea-pig sperms is much reduced if the medium becomes acid. Measurements of the p_H values of control sperm suspensions made with a B.D.H. capillator before and after a number of experiments showed that an increase in the acidity of the medium from p_H 8.2 to 7.8 might be expected and did not interfere with the mobility of the cells. This change is presumably the result of cell metabolism and respiration and possibly of slight absorption of carbon dioxide from the atmosphere.

Several phenolic aldehydes were examined during this work, and as these substances are acidic, it was possible that part of their spermicidal activities and those of acidic substances in general might be due to insufficient buffering by the glucose-saline medium when the higher concentrations of substances were tested. For example, at the end of an experiment with resorcyaldehyde, the sperm suspension containing this aldehyde at $M/200$ concentration was at p_H 6.8. It was thus essential to neutralise such acidic substances, and this had to be effected without disturbing the isotonicity of the solutions. The following modification of the usual procedure, namely solution of the substance in 0.9 % sodium chloride, was therefore adopted in experiments with acidic compounds. On the assumption that in dilute solution sodium chloride and sodium hydroxide are dissociated to the same extent, an aqueous solution of 6.15 g. of sodium hydroxide in 1 l. was regarded as being isotonic. A solution of the acidic substance (x g.) in 0.9 % sodium chloride (y ml.) at the strength needed for the experiment was titrated with this isotonic sodium hydroxide to p_H 8.0, and the volume of alkali required (z ml.) noted. The solution for the experiment was then prepared by dissolving the substance (x g.) in 0.9 % sodium chloride

(y - z ml.) and isotonic sodium hydroxide (z ml.). This procedure involves the assumption that the sodium salt of the acidic substance was dissociated to the same extent as sodium chloride in dilute solution. This assumption is justified for the purpose of these experiments.

Comparison of biological activities. Baker's technique involves the comparison of the lowest concentrations at which the substances being examined immobilise all sperms under the experimental conditions. There are three objections to this procedure from the standpoint of the present investigation. First, the sparing solubility of several aldehydes prevented lethal or highly active concentrations from being reached. Secondly, the recognition of small differences in activity was extremely difficult, especially when these were encountered in experiments on different days. Thirdly, sperm suspensions vary considerably in the number of cells they contain, and this might affect the results. The following method of performing the tests was therefore adopted.

The effects of two aldehydes, each at two concentrations, were compared simultaneously on one sperm suspension in each experiment. The usual concentrations were $M/200$ and $M/300$, because the stronger solution had some lethal action in each case. Baker's technique was followed closely, with the modifications described. Three slides were used for the microscopic examination, each having two hollows in it, denoted a and b respectively. Slide I a and b contained the control suspension. Slide II a contained substance x at a dilution of $M/300$, whereas II b contained substance y at $M/300$. Slide III contained the two aldehydes at $M/200$ dilution. Thus, by comparing the mobilities of the sperm suspensions a and b after the standard time of 30 minutes, a direct comparison of the relative activities of the two aldehydes could be made, without relying solely on the records of the grades of activity. Duplicate experiments were made for each pair of aldehydes, and the aldehydes could then be arranged in the order of their activities in immobilising sperms. Quotation of grades of activity recorded and dilutions used in the many experiments would occupy too much space for them to be stated here, although these data were also used in drawing up the series on p. 397. The following list, however, summarises the results of the comparative experiments.

Comparison of the spermicidal efficiencies of pairs of aldehydes.

The activity of:	was	that of:
3-Methoxybenzaldehyde	<	2-Methoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	<	2:4-Dimethoxybenzaldehyde
2:4-Dimethoxybenzaldehyde	=	2-Methoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	<	2-Methoxybenzaldehyde
3:4-Dimethoxybenzaldehyde	<	3-Methoxybenzaldehyde
3-Methoxybenzaldehyde	=	2:4-Dimethoxybenzaldehyde
2-Methoxybenzaldehyde	<	2:3-Dimethoxybenzaldehyde
2:3-Dimethoxybenzaldehyde	$\approx$	2:5-Dimethoxybenzaldehyde
2:3-Dimethoxybenzaldehyde	<	Formaldehyde
2:5-Dimethoxybenzaldehyde	<	Formaldehyde

Preparation of materials.

Aromatic aldehydes and acids were prepared by customary methods and were purified by repeated recrystallisation, with changes of solvent where possible.

Formaldehyde was prepared by distilling in a slow current of steam a mixture of pure hexamethylenetetramine (14 g.) and dilute sulphuric acid (7 % weight/volume; 300 ml.). Six fractions (40 ml. each) were collected, and the first three (formaldehyde concentration greater than 4 %) were mixed, shaken with calcium carbonate and redistilled in presence of calcium carbonate. The first 40 ml. to

be collected were boiled gently in a long-necked flask and cooled *in vacuo*. The residual solution was neutral, was free from carbon dioxide and contained 4.8 % of formaldehyde, estimated by oxidation with neutralised perhydrol in alkaline solution and back-titration of the excess of alkali with standard hydrochloric acid, using phenolphthalein as indicator.

Water free from carbon dioxide was obtained by distilling laboratory distilled water in an apparatus fitted with efficient spray-trap and silica condenser, the first third of the distillate being rejected. The water was collected and stored in a Jena-glass bottle which had been soaked in similar water for some weeks and which was fitted with a soda-lime tube and siphon.

Sodium hydroxide solution. A solution of hydroxide in its own weight of water was left in a resistance glass vessel until the carbonate had separated. The solution was filtered through prepared filter-paper, and diluted with carbon dioxide-free water to give a 0.2*N* solution of sodium hydroxide. This solution was preserved in a wax-lined bottle connected to the burette (see later) and protected by a soda-lime tube; its strength was checked at intervals by titration with 0.1*N* hydrochloric acid.

Gelatin. Preliminary titration experiments with different samples of "Gold Leaf" gelatin gave inconsistent results, and the gelatin ultimately used was a commercial powder purified as described by Smith [1921]. The final product, a readily soluble powder, was kept in a tightly-stoppered bottle and did not change in composition during the period of the experiments. It contained 9.5 % of moisture when dried to constant weight for 11 hours at 100–105°, and a sample weighing 0.4065 g. left less than 0.1 mg. of residue after ignition.

Purified hydrogen. Hydrogen was obtained from a cylinder connected with a small gas-holder which helped to maintain a steady flow. The gas was scrubbed by passing up two towers, the first filled with glass beads over which flowed concentrated sodium hydroxide, the second filled with beads over which passed water free from carbon dioxide. Traces of oxygen were removed by passing the hydrogen over copper gauze heated at a dull red heat in an electric furnace.

Electrometric titrations of gelatin alone and in presence of aldehydes.

Apparatus. The use of rubber tubing in conveying the hydrogen supply was avoided as far as possible, connections between glass tubes being arranged so that the tubes met in the joint. All rubber stoppers and tubes were purified by treatment with sodium hydroxide and water in the usual way.

The cell consisted of a pyrex boiling-tube, 5 cm. in diameter and 12.5 cm. long, having a working capacity of 100 ml. The tube was closed by a rubber bung pierced with seven holes, through which passed a glass stirrer in a tight sleeve, a thermometer, inlet and exit tubes for hydrogen, and closely fitting glass sleeves through which passed the tip of the burette, the agar potassium chloride bridge and the hydrogen electrode respectively. These sleeves enabled the easy insertion or withdrawal of the parts in question. The stirrer was operated manually and could be raised to remove drops adhering to the tip of the burette.

The hydrogen electrode vessel was of the Bunker type [1920], so designed that the entire platinum surface (about 1 cm. of wire) was bathed in hydrogen as each bubble escaped, and was then covered with liquid before the next bubble formed. The electrodes were replatinised before each titration, and occasionally a fresh electrode was inserted during a titration in order to make certain that poisoning was not occurring. After a suitable interval for saturation, the fresh electrode was always at the same potential as that which it had replaced.

The reference electrode was a 3.5*N* potassium chloride-calomel half-cell connected to the hydrogen electrode cell by a vessel containing saturated potassium chloride and the agar-potassium chloride bridge. Fresh surfaces of the bridges were exposed before use, and bridges gave concordant results when interchanged.

The electrodes were connected to a Tinsley potentiometer by unsupported wires, and the null point was determined by a capillary electrometer. Two determinations of E.M.F. were made, the null point being approached from both sides.

Before reaching the cell, the hydrogen passed through a presaturator containing pure water for all experiments except those with solutions containing formaldehyde, when the presaturator contained a formaldehyde solution of the same strength as that in the cell. Condensation in the tube between the presaturator and the cell was prevented by an adjacent carbon-filament lamp.

The cell, presaturator, saturated potassium chloride vessel and calomel electrode were immersed in a paraffin thermostat at $37^{\circ} \pm 0.1^{\circ}$.

The burette was protected by soda-lime tubes and was fitted with two jets, one which could be lowered into the cell, the other arranged outside the thermostat for adjusting the zero level and for checking the strength of the sodium hydroxide.

Procedure. Purified gelatin (1 g.) and the aldehyde (0.1 millimol) in water (100 ml.) were used for each experiment, except in one instance when more concentrated formaldehyde solution was used. The total volume of 0.2*N* sodium hydroxide required in each titration was 2.4 ml., and the range p_H 5–10 was covered. The aldehyde and gelatin were either dissolved simultaneously by warming their suspension in the water to 90° and then cooling to 38° , or the gelatin was dissolved first in this way and the aldehyde was added to the warm solution. The results were not affected by these changes in procedure, and the latter alternative was adopted in the cases of 3-methoxybenzaldehyde and formaldehyde.

The calomel electrode and potassium chloride vessel were placed in the thermostat 30 minutes earlier than the cell, the contents of which were saturated by a slow stream of hydrogen through the inlet tube. Ten minutes later, the tip of the burette was arranged to deliver just above the surface of the liquid in the cell, the hydrogen electrode and its vessel were inserted, and the hydrogen supply was diverted from the inlet tube to the electrode. Twenty minutes later the agar-bridge was inserted, and equilibrium had usually been attained after a further period of 10 minutes.

When the E.M.F. was constant, the titration was begun by the addition of sodium hydroxide solution in portions of 0.1 ml., the new equilibrium being reached within 2 minutes. The contents of the cell were stirred after each addition and between each measurement of potential. Steady values were obtained between p_H 5–6 and 8–10, but occasionally drifts towards the acid side were observed near the neutral point. Since these were observed to the same extent with gelatin alone, it was concluded that they were not due to increased time taken for the aldehyde-gelatin system to reach the new equilibrium. An accuracy of 0.01 p_H unit is probable for the values obtained in the more acid and alkaline regions, but this accuracy is slightly less in the case of values about the neutral point on account of the drifts of potential.

The p_H values were calculated from the observed potential differences by the Nernst expression for the E.M.F. of a concentration cell.

In the titrations of gelatin in presence of small amounts of aromatic acids, a 0.02 *N* solution of the required acid was added to the gelatin solution in the cell until the p_H value had fallen to the same extent as had been observed in titrations of gelatin-aldehyde mixtures where small amounts of the corresponding acid were suspected. The titration of the acid-gelatin mixture was then carried out with 0.02 *N* sodium hydroxide in the usual way. In these experiments 4.2 mg. of 2:5-dimethoxybenzoic acid and 3.6 mg. of 2-methoxybenzoic acid were used.

SUMMARY.

1. A method is described for comparing directly on the same material the spermicidal efficiencies of two substances. This is specially valuable when sparingly soluble compounds are to be examined.

2. This method has been used to arrange a series of mono- and di-methoxybenzaldehydes in the order of their spermicidal efficiencies.

3. Titration curves of gelatin in presence of the same mono- and di-methoxybenzaldehydes are deflected from the gelatin curve towards a more acid reaction over the range p_H 8–10. This deflection is a measure of the extent to which the aldehydes have condensed at equilibrium with the gelatin anion between these p_H values.

4. A series of mono- and di-methoxybenzaldehydes arranged in order of their capacities for condensation with the gelatin anion closely resembles the series of the same aldehydes arranged in order of their spermicidal efficiencies.

5. Titration curves of gelatin in presence of mono- and di-methoxybenzaldehyde are deflected from the gelatin curve towards a more acid reaction between the isoelectric point and p_H 6–7.

6. A method is described for testing the spermicidal efficiencies of acidic substances which disturb the buffer effect of the media in which the sperms are suspended.

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