CXXXV. THE BIOLOGICAL SIGNIFICANCE OF *CIS-TRANS* ISOMERISM.

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RESEARCHES on the relations existing between stereochemical structure and physiological action appear hitherto to have been promulgated almost entirely from one aspect of stereochemistry, that which is dependent on the presence of an asymmetric carbon atom, and invariably associated with optical activity.

When we come to the important class of compounds known as *cis*- and *trans*-, or geometrical isomers, we find an almost unexplored field of research. It was with a view to developing this branch of the subject, and at the same time throwing some further light on the fundamental problems of

(a) the relationship between chemical constitution and physiological and bactericidal action,

(b) the mechanism of the action of poisonous substances upon the more important constituents of protoplasm,
that the following researches were undertaken.

Hitherto the only work on this subject of which record can be found are some experiments by Ishizuka and others by Kahlenburg and True upon the relative toxicity of maleic and fumaric acids. Ishizuka [1897], experimenting upon dogs, found maleic acid to be much the stronger poison. Kahlenburg and True [1896], using *Lupinus albus* L., found the opposite effect, fumaric acid being the more toxic. On fungi, however, maleic acid again has a stronger action, *Penicillium glaucum* growing far more readily in solutions of fumaric acid than in those of maleic acid. This was confirmed by the present authors, who found that solutions of albumin decomposed rapidly in presence of fumaric acid owing to the growth of moulds, while maleic acid preserved them from putrefaction for a much longer period.

The chief compounds used in the investigation were maleic and fumaric acids, these substances being generally taken as typical of the ethylenic compounds which exhibit geometrical isomerism. Work was commenced upon the germicidal action of the foregoing compounds and a few similarly related compounds, and extended to their precipitating action on various proteins, their action on enzymes, and influence on the imbibition of water by gelatin, and also their distribution between water and proteins.

Germicidal action.

Some unexpected results had been obtained by Cooper, Woodhouse and Forstner [1925] in the course of their studies on the bactericidal action of maleic and fumaric acids. Difficulty had been experienced in obtaining consistent results, and as the organic matter in the nutrient medium had been suspected of causing the irregularities, a culture diluted 100 times was used. With the normal culture, using the Chick-Martin method at 20°, fumaric acid, the *trans*-isomer, appeared the stronger germicide; with the diluted culture the reverse held, and maleic acid became the more active. Carrying on the experiment at 37° a reversal again took place, fumaric acid being the stronger germicide.

In the bacteriological researches embodied in this thesis where the Chick-Martin method is being used, cultures which had been freed from organic matter either by dilution or by centrifuging were usually employed, normal cultures being used only for preliminary experiments.

The first experiments were carried out with maleic and fumaric acid, using normal cultures and cultures diluted 50 and 100 times respectively. These were mainly a check upon the earlier work and confirmed the conclusion that dilution of culture caused increase in germicidal power.

The results showed:

Germicidal concentrations			
	Normal culture	Diluted culture	<i>B. coli communis</i>
Maleic acid	1 in 750	1 in 2500	37°
Fumaric acid	1 in 1000	1 in 3500	30 minutes' disinfection

The point of interest in these figures upon which most attention was concentrated was that although maleic, the *cis*-acid, was the stronger acid of the two, fumaric, the *trans*-isomer, was the more powerful germicide at 37°.

The object of diluting the cultures used has already been explained. The corresponding diminution in number of the organisms employed is an objection to the use of this method, however, and experiments were attempted with a centrifuged culture. A 24-hour culture of *B. coli* was grown in a sterilised centrifuge tube in ordinary lemco-peptone broth. This was then centrifuged for about half an hour and washed once with sterile Ringer's solution. The clear supernatant liquid was then replaced by Ringer's solution, the bacteria were well distributed and the culture thus obtained was used for the experiment. This method was found to give better results than the dilution method. The following result was obtained from a centrifuge experiment at 37°.

Germicidal concentration	
Maleic acid	1 in 1500
Fumaric acid	1 in 2500

To obtain further results from *cis*- and *trans*-compounds of this series, the methyl homologues, citraconic and mesaconic acids, were used. The same effect as with maleic and fumaric acids was observed, the *trans*-acid persistently showing higher germicidal power than the *cis*-acid.

The superiority of the *trans*-acids as germicides is of great interest when it is considered that the *cis*-acids are by far the stronger acids. Evidently bactericidal power is not determined alone by hydrogen ion concentration, but is associated with a selective action which is caused by configurational differences.

An attempt was next made to determine the inhibitory power of the acids. Maleic and fumaric acids caused so much precipitation of the peptone in the culture medium that the experiments were unsatisfactory.

The inhibitory powers of citraconic and mesaconic acids, which did not give excessive turbidity, could, however, be determined. The average results were as follows:

	Inhibitory concentration
Citraconic acid	1 in 500-750
Mesaconic acid	1 in 1000
Itaconic acid	1 in 500-750
Phenol	1 in 550

All three acids are seen to be more effective as germicides than phenol itself, and the *trans*-acid again proved to be more active than the *cis*-isomer.

The inhibitory action of the stereoisomeric citraconic and mesaconic acids on other organisms, *e.g.* *B. acidi lactici*, *B. lactis aerogenes*, *B. mesentericus*, *B. cloacae*, and *B. prodigiosus* was also examined and in each case the *trans*-acid was more efficacious than the *cis*-compound.

Zinc hydrogen fumarate was exceptional, however, in being a weaker germicide towards *B. coli* than the corresponding maleate, the latter being inhibitory in concentrations of 1 in 2500.

The germicidal action of dibromo- and *iso*-dibromosuccinic acid has also been studied with the object of throwing light on the relationship of these isomeric acids.

When maleic acid is brominated, *iso*-dibromosuccinic acid is formed, from which by loss of hydrobromic acid bromofumaric acid is obtained. Similarly, fumaric acid forms ordinary dibromosuccinic acid, which finally yields bromo-maleic acid.

The inhibitory and germicidal powers of the two acids were determined by the method already described.

	Inhibitory conc.	Germicidal conc.*
Dibromosuccinic acid	1 in 250	1 in 1000
<i>iso</i> -Dibromosuccinic acid	1 in 400	1 in 1550

* Chick-Martin method, using centrifuged culture of *B. coli*.

The *iso*-acid is thus a stronger germicide than dibromosuccinic acid, although it is formed by bromine addition from the original *cis*-acid (maleic) which has been found conversely to be less active than the *trans*-isomeride (fumaric). The results point to a similarity in chemical configuration between maleic and dibromosuccinic acids, and a corresponding similarity between fumaric and *iso*-dibromosuccinic acids, thus confirming the structures ascribed to these acids by chemists.

The fact that the *trans*-acids tend to be more powerful disinfectants than *cis*-acids is of great interest when correlated with Ishizuka's [1897] observation that maleic acid was more poisonous to higher animals than fumaric acid. It is possible therefore that *trans*-compounds may be the starting point for the preparation of a new series of germicides of high bactericidal power, but relatively low toxicity. Fumaric acid itself is a stronger disinfectant than phenol, and substitution of certain groups might still further raise its efficacy. A point of practical significance is the fact that experiments by the authors have shown that bacteria do not acquire a tolerance to the *cis*- and *trans*-acids.

With the object of offering some explanation of the superiority of the *trans*-acids as germicides, experiments were next carried out to study

- (i) the effect of the acids on enzyme action,
- (ii) their precipitating action on proteins and other colloids,
- (iii) their relative absorption by proteins.

ENZYME ACTION.

(a) *Diastase*. Mixtures of saliva, starch solution, and solutions of *cis*- and *trans*-acids were incubated at 40° and the time taken for complete hydrolysis of the starch noted and compared with mixtures of saliva and starch alone without added acid. Iodine was used as external indicator in these experiments. Tests were carried out with maleic and fumaric, also with citraconic and mesaconic acids.

It was found that the *trans*-acids inhibited the hydrolysis of starch to a greater extent than *cis*-acids, thus suggesting a parallel with the bacteriological results.

Similar observations were made in the case of the calcium and zinc hydrogen salts.

The following are the results of a typical experiment:

	Period required for hydrolysis of starch (min.)
Water control	4
Maleic acid (0.05 %)	17
Fumaric acid (0.05 %)	23
Maleic acid (0.03 %)	19
Fumaric acid (0.03 %)	30
Citraconic acid (0.037 %)	8
Mesaconic acid (0.037 %)	21

(b) *Pepsin*. The action of the acids on the rate of hydrolysis of proteins by pepsin was investigated by the Mett's tube method. The small rods of coagulated egg-white were immersed in 1 % solutions of pepsin containing varying proportions of the acids, and the lengths of the rods determined after a given period. In this way the extent of digestion could be ascertained.

	Lengths of protein digested by 1 % pepsin	
	8 hours	22 hours
In presence of:		
0.5 % maleic acid	1.7 mm.	4.2 mm.
0.5 % fumaric acid	2.2	4.6
0.5 % citraconic acid	2.0	2.3
0.5 % mesaconic acid	2.8	2.3

The results in general show that the *trans*-acids have a somewhat greater accelerating action on the rate of digestion than the *cis*-acids. This is remarkable in view of the facts that the presence of acid is necessary for digestion by pepsin, and *cis*-acids are stronger acids than the *trans*-isomerides. Evidently the *trans*-acids are again anomalous, their activity not being merely determined by their acidity.

(c) *Lipase*. A comparison was next made on the relative action of lipase on triolein and trielaidin, and ethyl esters of maleic and fumaric acids.

The lipase was used in the form of an alkaline extract of ox-pancreas. The trielaidin was prepared by the action of a solution of mercury in nitric acid on olive oil (elaidin test). 2 cc. of olein or the melted elaidin were treated with 1 cc. lipase extract, and one drop of phenolphthalein was added together with sufficient *N*/20 sodium hydroxide to impart a pink tint. The mixtures were kept at 40°. As the fats were hydrolysed, the acids liberated neutralised the alkali, and more *N*/20 alkali had therefore to be added to restore the colour. In the case of the olein, 4 cc. of sodium hydroxide were sufficient, but in the case of elaidin two further additions of alkali were necessary, indicating that olein was hydrolysed less rapidly than elaidin. In view of the greater reactivity of the *trans*-compounds already indicated, the results suggest that elaidin is the *trans*-compound and olein the *cis*-isomeride, a conclusion supported by the melting points of the corresponding acids.

In the case of the diethyl esters of maleic and fumaric acids, 1 cc. of the esters was mixed with 1 cc. of lipase and the mixture incubated at 37° and titrated at intervals with standard alkali. The results were as follows:

Ester ...	Period of hydrolysis		
	1.5 hours	18 hours	25 hours
	Amount of alkali required for neutralisation		
Ethyl maleate	6.6 cc.	9.3 cc.	11.4 cc.
„ fumarate	5.2	9.4	15.0

It is seen that initially the maleate ester is rather more readily hydrolysed than the fumarate, but as hydrolysis proceeds the *trans*-ester is broken down more rapidly than the *cis*-isomeride.

PROTEIN PRECIPITATION.

The following experiments were suggested by the observation made in the bacteriological work that maleic and fumaric acids caused precipitation of the peptone in the broth tubes, thus rendering the determination of bactericidal power very difficult.

(1) *Aqueous solutions.* Solutions of peptone and various proteins were mixed with solutions of the acids and salts of varying strength, and the effect upon the physical state of the colloids was observed.

Compound	Concentrations required to induce a maximum turbidity or precipitate				
	Peptone %	Mucin* %	Egg-white %	Caseinogen %	Serum %
Maleic acid	0.1	0.19	Acids	>0.4	0.18
Fumaric acid	0.05	0.07	caused	0.4	0.04
Citraconic acid	0.075	—	no precipi-	—	—
Mesaconic acid	0.05	—	tation	—	—
Dibromosuccinic acid	>0.3	0.25	—	—	—
<i>iso</i> -Dibromosuccinic acid	0.3	0.25	—	—	—
Normal zinc maleate	>0.01	>0.07	>0.5	0.15	—
„ fumarate	0.01	0.05	0.5	>0.15	—
Zinc hydrogen maleate	0.01	>0.07	0.5	—	—
„ fumarate	>0.1	0.05	>0.5	—	—
Sodium oleate	0.025	>0.25	>0.75	0.5	—
„ elaidate	0.025	0.25	0.75	0.05	—

* Diluted filtered saliva.

(2) *Precipitation in other media than water.*

(i) Peptone dissolved in anhydrous phenol at 70° was precipitated therefrom by ethyl fumarate, but not by ethyl maleate.

(ii) Lecithin dissolved in ether was more readily precipitated by ethyl maleate.

(iii) Gliadin dissolved in alcohol was more quickly precipitated by the zinc maleates than by the fumarates. In the case of the calcium salts, the *trans*-compound was the more active precipitant.

A consideration of the tabulated results shows that almost consistently the *trans*-compounds are more efficacious precipitants than the *cis*-isomers, thus bringing out a very interesting parallel with the bacteriological observations. The exceptions to this generalisation are very few, viz. the acid zinc salts of maleic and fumaric acids in precipitating peptone, gliadin and egg-white, and precipitation of lecithin by the ethyl esters.

Furthermore, in the case of serum-proteins, not only does fumaric acid cause precipitation more readily than maleic acid, but it also induces a redispersal of the precipitated proteins in lower concentration. Dispersion of the coagula is thus not determined merely by the strength of the acid, but, like precipitation, is bound up in the configurational difference of the isomeric acids.

In a recent paper on coagulation of proteins by organic acids Isgarischev and Bogomolova [1926] have also shown that fumaric acid is more active than maleic in precipitating caseinogen.

It is of interest to note that in the case of peptone *iso*-dibromosuccinic acid is more efficacious as a precipitant than its isomer, this being consistent with the bacteriological results.

IMBIBITION.

It is well known that gelatin possesses the property of taking up water to a considerable extent when immersed therein, and that this imbibition is increased enormously when small quantities of acids or bases are dissolved in the water. Maleic and fumaric acids were therefore investigated with respect to this property. The technique was as follows.

Strips of gelatin weighing about 0.2 g. were immersed in 20 cc. of solutions of maleic, fumaric and other acids for a definite length of time. They were then removed, the surface dried with filter paper, and weighed again. The increase in weight showed the imbibition, which was then expressed as a percentage of the original weight of gelatin. The following example gives the results of one experiment.

The gelatin was allowed to remain in the acid solutions for 42 hours.

	Acid %	Weight of gelatin g.	Gain in weight g.	Percentage imbibition
Maleic	0.2	0.21	5.16	2500
"	1	0.20	4.12	2050
Fumaric	0.2	0.20	4.31	2100
"	0.5	0.20	5.76	2850
Dibromosuccinic	0.2	0.20	3.62	1800
"	1	0.20	3.38	1700
Succinic	0.2	0.20	2.52	1250
"	1	0.20	4.66	2300
Citraconic	0.2	0.20	4.81	2400
"	1	0.20	4.96	2450
Mesaconic	0.2	0.20	4.32	2150
"	1	0.19	5.52	2900
Itaconic	0.2	0.20	3.26	1600
"	1	0.19	5.08	2650
Water		0.20	1.36	660

The use of 0.5 % fumaric acid instead of 1 % was necessitated by its limited solubility.

The above results show that in the more dilute solutions the *cis*-acids caused the greater imbibition, but that in the case of stronger solutions this was reversed, the *trans*-acids having more effect.

This change with rising concentration holds also in other cases. Thus not only do the *cis*- and *trans*-acids reverse their effects, but while in dilute solutions the saturated acid (succinic) has less effect than the unsaturated one (maleic), in the higher concentrations it causes greater imbibition. (It must be noted, however, that succinic always has less effect than fumaric acid.) Itaconic acid is less effective in increasing imbibition than the *cis*- and *trans*-isomerides (citraconic and mesaconic acids) in dilute solutions, but in stronger concentrations there is little difference between the three acids.

To study the variation of imbibition with concentration, ranges of concentrations from 0.1 % to 0.5 % for maleic and fumaric acids and 0.1 % to 1 % for citraconic and mesaconic acids were used, with results embodied in the following table:

Percentage imbibition.

Concen- tration %	Maleic acid	Fumaric acid	Concen- tration %	Citraconic acid	Mesaconic acid
0.1	4800	4700	0.1	4850	4600
0.2	4550	4500	0.3	4550	4800
0.3	4100	5000	0.5	—	4750
0.4	4050	4500	0.7	4100	4650
0.5	3500	5050	1	3600	4900

It is seen that, while the *cis*-acid in each example caused a steady diminution of imbibition with increase of concentration, the *trans*-acid gave irregular results, although imbibition tended on the whole to increase as in former experiments.

The effect of the *cis*- and *trans*-acids on imbibition was studied in the first place with the object of throwing light on the conditions of protein precipitation. The results, although of interest, are of some complexity, and it is difficult at the present time to understand their real significance. It is remarkable that the *trans*-acids, although the weaker acids, again behave anomalously in increasing imbibition under certain conditions to a greater extent than the *cis*-compounds, and for this reason it may be considered likely that the process of imbibition is involved in the mechanism of disinfection and precipitation.

In a recent paper Isgarischev and Pomeranzeva [1926] have shown that maleic acid increases the imbibition of water by caseinogen to a greater extent than fumaric. The foregoing results obtained with gelatin are, however, more complex, and indicate that *trans*-acids sometimes induce greater imbibition.

THE DISTRIBUTION OF *CIS*- AND *TRANS*-ACIDS BETWEEN
WATER AND PROTEIN.

The object of the following experiments was primarily to ascertain in what degree the germicidal and precipitating power of the *cis*- and *trans*-acids depended on the relative absorption by, or chemical action with, the bacterial proteins. The method adopted was to allow solutions of the acids free access to a protein, so that the acids could distribute themselves between the water and the protein phases, the changes in concentration in the one phase (water) being followed by titration methods.

In connection with this work the investigations of Hermans [1924] are interesting. His experiments were concerned with the absorption of *cis*- and *trans*-isomers by charcoal and, using maleic and fumaric acids, he found the former to be the less absorbed. He also showed that if the acids were dissolved in dilute (0.05 %) HCl, the relative absorbabilities were unaffected, although the hydrogen ion concentrations were now equal. Hence the strength of the acids, maleic being the stronger, does not determine the relative extent of their absorption.

The procedure in the protein experiments was as follows:

1 g. of gelatin, in strips, was immersed in a solution of each acid and allowed to remain at room temperature therein until equilibrium had been reached.

The length of time required for equilibrium to be established was determined by preliminary titration experiments, and it was found that 48 hours was easily sufficient.

The influence of concentration upon the absorption of acids by gelatin.

1 g. gelatin in 100 cc. solution of acids.

Acid	Initial concentration	Final concentration	Amount of acid absorbed	Distribution ratio. Amount of acid held by 1 g. protein.
	%	%	%	Amount in 1 g. water
(1) Maleic	2.38	2.20	0.18	8
	1.00	0.88	0.12	14
	0.44	0.38	0.06	16
	0.087	0.044	0.04	91
Fumaric	0.50	0.46	0.04	9
	0.10	0.044	0.05	114
(2) Maleic	0.99	0.93	0.06	6
	0.51	0.42	0.09	22
	0.20	0.14	0.06	43
	0.099	0.058	0.04	70
Fumaric	0.47	0.44	0.03	7
	0.19	0.16	0.03	19
	0.095	0.064	0.03	47

A survey of these results indicates that in the case of maleic acid, the amount of acid absorbed by the gelatin increases absolutely, but not relatively, with ascending concentration, thus causing an actual diminution in the value of the distribution ratio. This relationship is suggestive of an adsorption process.

In the case of fumaric acid, however, the amount taken up is practically constant at all concentrations. Furthermore, it will be noticed that the *cis*-acid is absorbed in greater amount than the *trans*-acid, the results thus being exactly opposite to those obtained by Hermans [1924] in the case of charcoal.

Further experiments with other acids also bring out this point, the same technique being employed as before.

Acid	Distribution ratio
Maleic	14
Fumaric	10
Citraconic	20
Mesaconic	14
Succinic	7
Dibromosuccinic	12
<i>iso</i> -Dibromosuccinic	5

It is seen that the *cis*-compounds are consistently absorbed to a greater extent than the *trans*-isomerides, although the *trans*-derivatives were previously found to be the more efficacious as germicides and protein-precipitants. Bactericidal and precipitating action are thus not determined merely by selective absorption of the agent by the proteins, but are intrinsic properties independent of solubility or adsorption.

Work was next put in hand to ascertain whether any evidence could be obtained of a chemical action between the unsaturated acids and amino-acids or proteins.

The experiments with maleic acid and gelatin pointed to adsorption, but in the case of fumaric acid the amount absorbed was constant over a range of concentration, suggestive of chemical action. There was a possibility that amino-groups in either amino-acids or proteins could add on at the double-linkage of the unsaturated acids thus:

By titrating amino-acids, *e.g.* glycine with standard alkali in the presence of either maleic or fumaric acids, analogously to Sørensen's formalin method, no evidence, however, could be obtained of such a reaction. The amino-groups of gelatin were next substituted by various methods, and the effect of this change upon the absorptive capacities of the proteins for the acids was studied.

The following products were employed.

1. Gelatin treated with formalin for 2 days

2. Gelatin treated with 0.25 % benzoquinone solution for 2 days, aromatic nuclei being thereby introduced into the NH_2 groups.

3. Desamino-gelatin was obtained by treating gelatin with nitrous acid, the NH_2 groups being replaced by OH.

In all cases the protein was finally washed in running water and dried at 100° before use.

The absorption of maleic and fumaric acids by the foregoing materials, with ordinary gelatin as control, was next determined by the methods already described.

	Distribution ratios	
	Maleic acid	Fumaric acid
Gelatin	3	2
Formalised gelatin	12	8
"Quinone" gelatin	16	12
Desamino-gelatin	10	10

A consideration of the figures shows that in nearly every case the *cis*-acid is taken up by the protein to a greater extent than the *trans*-compound, thus confirming earlier observations. Moreover, by introducing substituents into the amino-groups of gelatin, and even replacing them by hydroxyl groups (as in the desamino-product) absorptive power of the protein for the two acids is actually in all cases considerably increased. There is thus no evidence of chemical action between the unsaturated acids and the amino-groups of proteins or glycine. In the case of maleic acid, the uptake would appear to be of the nature of adsorption, and in the case of fumaric acid adsorption is probably the main factor, although it happens that a maximum uptake is indicated at an unusually low concentration.

SUMMARY.

The dibasic unsaturated *trans*-acids are superior to the *cis*-isomerides in bactericidal action, although they are weaker acids. Their germicidal power is therefore not determined by hydrogen ion concentration, but must depend upon the stereochemical configuration of the molecule.

The *trans*-compounds are also generally more efficacious as protein-precipitants and more reactive towards enzymes than the *cis*-isomers. Notwithstanding, the *cis*-acids are absorbed by protein in larger amount than the *trans*-acids from aqueous solution.

The higher bactericidal power of the *trans*-compounds is therefore apparently due not to their selective absorption by the bacterial proteins, but may be associated with their greater precipitating action on proteins, or inhibitory effect upon enzymes.

The uptake of the *cis*- and *trans*-acids by protein is probably an adsorption process, and there is no evidence of chemical interaction with protein or amino-acid by addition of amino-groups through the unsaturated linkage.

The observations bring out very clearly the analogy between *cis*- and *trans*-compounds, and *ortho*- and *para*-isomers in the aromatic series. In each case the more symmetrically constituted compounds (*trans*- and *para*- respectively) are in general the more powerful germicides and precipitants.

Finally, the results suggest a problem of practical interest. Whilst fumaric acid is a weaker poison than maleic acid to the higher animals, it is conversely a stronger disinfectant. As fumaric acid is more bactericidal than phenol, *trans*-derivatives may thus be useful as germicides, possessing relatively low toxicity.

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