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Review Article

A molecular basis for drug action*

E. J. ARIËNS AND A. M. SIMONIS

THE effect induced by a drug is the resultant of the interaction between the molecules of the drug and the molecules of which the biological object is composed. The higher the specificity required in the structure of the drug molecule, the more probable it becomes that the effect is based on an interaction of these molecules with certain specific molecules in the biological object. These specific molecules are called the receptors for the drug. They can be defined as those specific molecules, molecule complexes or parts of them in the biological object, with which the drug must interact in order to induce its effect. The term receptor goes back to Ehrlich (1913) who said: "Corpora non agunt nisi fixata". The notion of a specific receptive substance as a site of action for drugs such as nicotine and curare in the myoneural junction was introduced by Langley (1905). Since then the term receptor has become indispensable in reasonings on the basis of drug action. Often the model of key and lock is used for the drug-receptor interaction; reality is much more dynamic, however. Schueler (1960) defined the receptor as follows: "The drug-receptor is in general the pattern R of forces of diverse origin forming a part of some biological system and having roughly the same dimensions as a certain pattern M of forces presented by the drug molecule in such a way that between patterns M and R a relationship of complementarity for interaction exists."

Drug-receptor interaction must be seen as a mutual moulding of drug and receptor. There is mutual adaptation as far as shape and charge distribution is concerned. This adaptation plays an important role in the activation of drug and receptor and therefore is essential to drug action.

Drug-receptor interaction can have various consequences.

(*a*) The drug-receptor interaction mainly leads to changes in the charge distribution and shape of the drug molecule, in such a way that it is activated and becomes chemically more reactive, which results in chemical changes in the drug molecule. The drug is metabolised. The receptor is the "active site" on an enzyme.

(*b*) The drug-receptor interaction mainly leads to changes in the charge distribution on and in the shape of the receptor, so that as a result of this the receptor becomes activated and induces changes in the charge distribution and shape of the surrounding molecules, thus initiating the sequence of physico-chemical events leading to the effect.

(*c*) The drug-receptor interaction may take place without essential changes in the drug molecule or the receptor. No effect is then induced.

From the Department of Pharmacology, University of Nijmegen, Holland.

*The first of two articles, the second of which will be published in the May issue of the Journal.

This may be a binding to storage receptors, or, for example a binding to indifferent binding sites.

The receptors are characterised by the drugs with which they are able to interact and by the effect that can be induced on them. For drugs with low requirements for specificity in the chemical structure, sharply circumscribed receptors are less probable. In that case a more diffuse adsorption on some surface may take place. Especially then, properties such as the lipid solubility and surface activity of the compound are of importance.

Part-processes

Three groups of processes (van Rossum, 1958; Ariëns & Simonis 1961; Ariëns, 1962a) (see Fig. 1) are to be distinguished in the complicated process of drug action.

FIG 1.

RELATION BETWEEN DOSE AND CONCENTRATION

The processes which concern absorption, transport, biochemical changes, or excretion, of the drug (Brodie, 1956; Brodie & Hogben, 1957; Brodie, Gillette & la Du, 1958; Schanker, 1962; Williams, 1963). These determine the relation between the dose of the drug and its concentration in the immediate surroundings of the receptors, called the "biophase". (Furchgott, 1954, 1955.) If simple isolated organs are used as a test object, the concentration of the drug in the biophase may be supposed to be proportional to, or a simple function of, the concentration of the drug in the bath fluid.

THE DRUG-RECEPTOR INTERACTION

The processes concerned with the drug-receptor interaction and the induction of a stimulus by the drug. The interaction as such can be represented by a simple model based on the mass-action law or the Langmuir adsorption isotherm (Clark, 1937a, b). It describes the chance the molecules of the drug A have of interacting with the specific receptors R.

$$[A] + [R] \rightleftharpoons [RA]; \; k_2/k_1 = K_A \quad .. \quad .. \quad .. \quad (1)$$

The stimulus induced is supposedly proportional to the quantity of drug-receptor complex formed or present at a certain moment.

THE RELATION BETWEEN STIMULUS AND EFFECT

The processes which determine the relation between the stimulus induced and the effect obtained (Stephenson, 1956; van Rossum, 1958; Ariëns, 1962, 1964). There may be a graded response which means that the effect gradually increases with the stimulus and approaches asymptotically to some maximum value. Another possibility is the all-or-none

response in which case the effector cells respond only after the stimulus has reached a certain value, but then always with the same response per effector unit. The biological variance among the various effector units will have as a consequence a gradation in the response of the organ. In general, a certain value of the stimulus will always result in the same effect unless a change in the effector cells has taken place.

Depending on the parameter chosen as effect, a different number of steps in the sequence of events started by the drug-receptor interaction may be involved. Studying compounds which produce neuromuscular block, certain investigators use the head drop as the effect, others the decrease in contraction of the striated muscle to nervous stimulation; also the bio-electrical phenomena in the muscle fibre or at the endplate region can be measured as the effect, (Jenkinson, 1960) and so on. In all these examples the drug-receptor interaction and the stimulus induced are the same. The relation between stimulus and effect is usually complex, sometimes, however, for instance if simple isolated organs are used, it may be relatively simple.

With simple biological systems, such as isolated organs, the influence of drug metabolism can be neglected if stable compounds are used. The relationship between the concentration of the drug in the bath fluid and that in the biophase can then be assumed to be close to proportionality. For a certain type of organ and effect, the relation between stimulus and effect will be constant. The dose-response relation studied under these circumstances will mainly reflect the characteristics of drug-receptor interaction.

Affinity and intrinsic activity

As mentioned, the drug-receptor interaction results in a stimulus. This stimulus is supposedly proportional to the quantity of drug-receptor complex formed or present at a certain moment. Let us restrict ourselves first to the case when the effect is proportional to the stimulus and that the dose of the drug is large compared to the uptake capacity of the receptors. Application of the mass-action law shows that then, under equilibrium conditions, the effect E_A of the dose [A] as a fraction of the maximum effect E_m possible with the receptor-effector system concerned becomes:

E effector
|
R receptor
↑↓
A drug

$$E_A/E_m = \frac{\alpha}{1 + (K_A/[A])} \quad .. \quad .. \quad .. \quad (2)$$

K_A is the dissociation constant of the drug-receptor complex and the reciprocal of the affinity between drug and receptor. E_{Am} is the maximum effect obtainable with the drug A. With high doses of A the effect approaches the maximum value E_{Am}; then $E_{Am}/E_m = \alpha$.

If $E_A = \frac{1}{2} E_{Am}$, $k_2/k_1 = K_A = [A]$.

Besides the *affinity* between drug and receptor, the ability of the drug to interact with the receptors in an effective way, the *intrinsic activity* of the drug also is determinative for the effect (Ariëns, 1954; Ariëns, van Rossum & Simonis, 1956a, 1957; Ariëns, 1962b, 1964). The proportionality constant α in eqn 2 is a measure for the intrinsic activity of the drug.

To avoid dimensions for the intrinsic activity, it is expressed as the factor indicating the ratio between the maximum effect, E_{Am}, of the compound studied and the maximum effect possible with the receptor-effector system concerned, E_m. In practice, in a group of drugs with a common mechanism of action, the intrinsic activity of the compound producing the greatest maximum effect is taken as unity. For this reference compound, for example a compound B, the intrinsic activity has a value $E_{Bm}/E_m = 1$. The intrinsic activity of compound A then has a value E_{Am}/E_{Bm}. Once the physico-chemical processes at the basis of the intrinsic activity are known, dimensions can be applied and the term intrinsic activity can be avoided.

The effect is not necessarily proportional to the stimulus or to the fraction of receptors occupied. Where spare receptors exist, for instance, the intrinsic activity is not proportional to the maximum effect obtainable with the drug (van Rossum, 1958; Ariëns, van Rossum & Koopman, 1960; Ariëns, 1964). By definition the intrinsic activity is assumed to be always proportional to the maximum stimulus obtainable.

Active and "inactive" compounds

If log dose-response curves are studied for a homologous series of drugs in which a gradual change from active to inactive compounds takes place, the loss in activity may manifest itself in two different ways.

(i) The log dose-response curves may be shifted to higher and higher concentrations, so that finally extremely high doses of the drug are required, which implies that the compounds become practically inactive (Fig. 2).

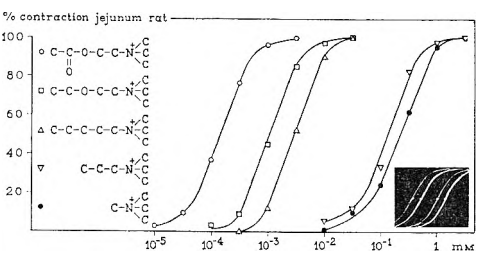


FIG. 2. Cumulative log concentration-response curves for a series of quaternary ammonium compounds. Note the decrease in activity as a result of changes in the chain manifests itself as an increase of the dose necessary to obtain the effect. Compare the experimental curves with the set of theoretical curves, inset, calculated from eqn 2.

(ii) A gradual decline in the maximal height and the slope of the log dose-response curves may take place which means that the compounds gradually become inactive within a reasonable dose range (Fig. 3).

The dose-response curves presented in the various figures are obtained with the cumulative dose-response technique described on a number of occasions (Ariëns & de Groot, 1954; Ariëns, Simonis & de Groot, 1955; van Rossum, 1958, 1963; van Rossum & van den Brink, 1963).

In terms of the model for drug-receptor interaction presented before (eqn 2), in case (i) the loss in activity may be ascribed to a loss in affinity, an increase in K_A; in case (ii) the loss in activity is possibly caused by a loss in intrinsic activity, a decrease in α. The structure-activity relation represented in Fig. 2 and 3 then indicates that the side-chain in acetylcholine mainly contributes to the affinity, while the cationic head appears to be of special importance for the intrinsic activity.

Competitive interaction

If the loss in activity, demonstrated in Fig. 3, is the result of a loss in intrinsic activity, the "inactive" compounds still have an affinity to the specific receptors and for this reason they may be expected to behave as

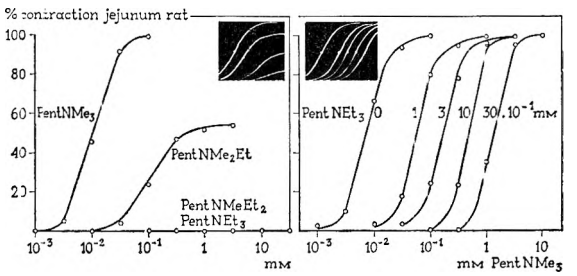
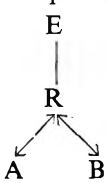


FIG. 3. (Left). Cumulative log concentration-response curves for a series of pentyl ammonium compounds. Note the decrease in activity as a result of the gradual ethylation on the ammonium group manifests itself as a decrease in the maximal effect and in the slope of the curves. Compare the experimental curves with the set of theoretical curves, inset, calculated from eqn 2.

FIG. 4 (Right). Cumulative log concentration-response curves for the agonistic compound pentylNMe$_3$ and the influence thereon of various concentrations of the "inactive" compound pentylNEt$_3$ (see Fig. 3). Note the parallel shift in the curves which indicates a competitive antagonism. Compare the experimental curves with the set of theoretical curves, inset, calculated from eqn 3.

competitive antagonists if combined with the active derivatives of the same series of compounds. Equations for the competitive interaction of two compounds can easily be derived (Ariëns, 1954; Ariëns & van Rossum, 1957; Ariëns, van Rossum & Simonis, 1957).

Eqn 3 gives the relations as derived from eqn 2 for the case when two drugs A and B, with intrinsic activities α and β, compete for common receptors.



$$E_{AB}/E_m = \frac{\alpha}{1 + [1 + ([B]/K_B)]\,(K_A/[A])} + \frac{\beta}{1 + [1 + ([A]/K_A)]\,(K_B/[B])} \quad .. \quad (3)$$

If $\alpha > 0$ and $\beta = 0$, B acts as a competitive antagonist of the agonist A. Eqn 3 then becomes identical to the well-known equation for competitive inhibition used in enzymology.

If the agonist A is combined with its competitive antagonist B, a parallel shift in the dose-response curve is expected if the curve is made in the presence of constant concentrations of the competitive antagonist. The antagonism is surmountable. An increase in the concentration of the agonist always results finally in a displacement of the antagonist from the receptors and therefore in a response. Fig. 4 gives the experimental results obtained with the combination of an active and an inactive derivative, from the series of compounds represented in Fig. 3.

In an homologous series of compounds in which the active derivatives gradually change to their competitive antagonists, transition compounds with an intermediate intrinsic activity, also called partial agonists, may be found. Take for instance the pentyl derivative bearing one ethyl group in Fig. 3 (Ariëns, 1954; Ariëns & Simonis, 1954; Stephenson, 1956; Ariëns, van Rossum & Simonis, 1957; Ariëns, 1964).

If in eqn 3, $\alpha = 1$ and $0 < \beta < 1$ is substituted, the compound B, a partial agonist, shows a dualism in action if combined with A; it acts as a synergist or antagonist depending on the value of [A]. An increase in the concentration of the partial agonist, B, if combined with constant concentrations of the agonist, A, will always result finally in the same response, independent of the concentration of A, because the partial

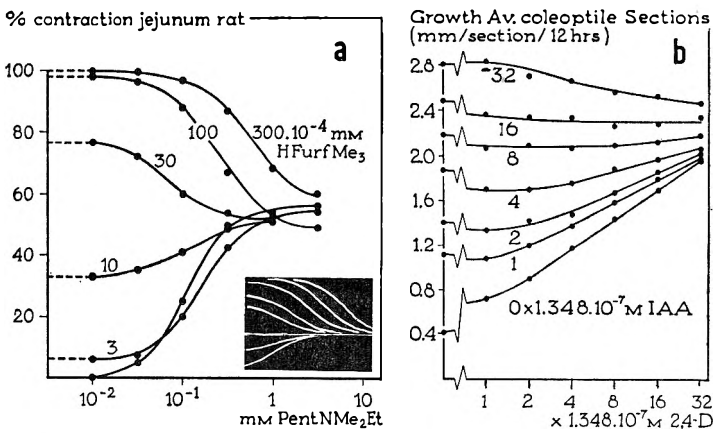


FIG. 5(a). Cumulative log concentration-response curves for the partial agonist $PentNMe_2Et$ and the influence thereon of various concentrations of the agonist furtrethonium ($HFurfMe_3$) (see Fig. 3). Note the dualistic character in the action of the partial agonist. Compare the experimental curves with the set of theoretical curves, inset, calculated from eqn 3.

FIG. 5(b). Log concentration-response curves for the partial agonist, 2,4-dichlorophenoxyacetic acid (2,4-D) and the influence thereon of various concentrations of the agonist indoleacetic acid (IAA). Both compounds act as auxins. Note the dualistic character in the action of the partial agonist. Compare the experimental curves with the set of theoretical curves, inset, calculated from eqn 3. (After McRae, Foster & Bonner, 1953).

agonist will finally always occupy all receptors. Fig. 5a gives the experimental results obtained with the combination of a partial agonist and a full agonist, from the series of compounds represented in Fig. 3. Fig. 5c

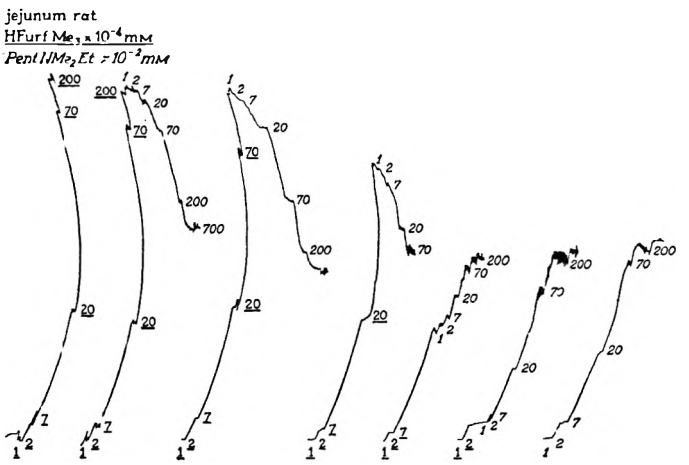


FIG. 5(c). A registrogram of cumulative dose-response curves for a combination of drugs of the type represented in Fig. 5(a). The underlined numbers concern the doses of the agonist furtrethonium ($HFurfMe_3$), the other numbers concern the doses of the partial agonist $PentNMe_2Et$. Note the dualistic character in the action of the partial agonist.

is the registrogram used for Fig. 5a. Fig. 5b gives an analogous set of curves for a combination of auxins. A gradual change from agonist to competitive antagonist via partia agonists has been described too for hormones such as polypeptides with an oxytocic action (Rudinger & Krejči, 1962). Theoretical curves which can easily be calculated from eqn 3 agree well with the experimental curves presented. If $\alpha = \beta > 0$, both A and B are agonists, and combination of these drugs will always result in a type of synergism, known as an additive action (Ariëns & Simonis, 1964).

The activity of agonistic drugs (stimulant drugs) is expressed by the intrinsic activity, which is proportional to the maximal effect obtainable with the drug and by the pD_2 value, the negative logarithm of that molar concentration of the drug that brings about an effect equal to 50% of the maximal effect obtainable with the drug, which represents the affinity (Ariëns & van Rossum, 1957). The activity of the competitive antagonists is expressed by pA_2 values according to Schild (1947); the intrinsic activity is zero (see Tables 1–3).

Molecular pharmacology and enzymology

In enzymology, a differentiation between substrates for an enzyme and specific inhibitors of the enzyme (competitive antagonists for the substrate) is well known. However, this differentiation is relative, because in a certain way each substrate will inhibit the breakdown of a related substrate

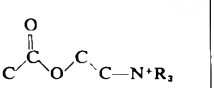
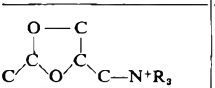
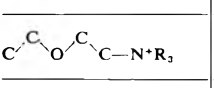
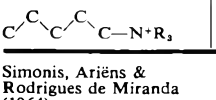
by the enzyme. Nevertheless it will not stop the enzyme action, because in protecting the related substrate it sacrifices itself. This means that the inhibitor does not necessarily act as an inhibitor as far as the formation of reaction products is concerned.

$$[E] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_3} [E] + [S'] \qquad (4)$$

A compound which has an affinity towards the active sites on the enzyme, but which is not broken down by the enzyme, ($k_3 = 0$), will act definitely as an inhibitor of the enzyme action (eqn 4). The term k_3 in enzymology is analogous to the intrinsic activity. The term $1/K_m = k_1/(k_2 + k_3)$ is analogous to the affinity.

In a series of related compounds a stepwise change from substrate to inhibitor—a gradual decrease in k_3—is possible. The intermediate

TABLE 1. INFLUENCE OF GRADUAL ETHYLATION OF PARASYMPATHOMIMETIC QUATERNARY AMMONIUM DERIVATIVES ON INTRINSIC ACTIVITY (i.a.) AND AFFINITY (pD_2 and pA_2 VALUES) (aff.) FOR THE MUSCARINIC ACTION TESTED ON THE JEJUNUM OF THE RAT

	Me_3		Me_2Et		$MeEt_2$		Et_3	
	i.a.	aff.	i.a.	aff.	i.a.	aff.	i.a.	aff.
	1	6·7 ± 0·08	1	6·1 ± 0·32	1	4·2 ± 0·3	[illegible]	4·1 ± 0·11
	1	7·1 ± 0·29	1	6·4 ± 0·32	0·3	4·0*	0	3·6*
	1	5·8 ± 0·16	1	5·3 ± 0·06	0	4·2 ± 0·17	0	4·1 ± 0 19
	1	5·4 ± 0·23	0·65 ± 0·18	4·3 ± 0·33	0	4·2	0	4·6 ± 0 30

Simonis, Ariëns & Rodrigues de Miranda (1964)

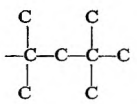
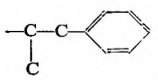
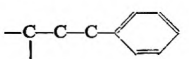
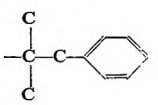
± Figures indicate standard deviations. * Van Rossum & Ariens (1959).

TABLE 2. INTRINSIC ACTIVITIES AND AFFINITIES (pD_2 AND pA_2 VALUES) OF CHOLINE ESTERS, TESTED ON THE JEJUNUM OF THE RAT

Choline esters	Intrinsic activity		Affinity	
	mimetic	lytic	mimetic pD_2	lytic pA_2
Formyl-	1		5·2	
Acetyl-	1		7·6	
Propionyl-	0·9		5·0	
Isobutyryl-	0·4		4·1	
Butyryl-	0·3		3·8	
Capronyl-		0		4·0
Lauryl-		0		5·2
Diphenylacetyl-		0		6·7
Lachesine*		0		8·4

* Benzilic ester of monoethylcholine.

TABLE 3. INTRINSIC ACTIVITIES AND AFFINITIES (pD_2 AND pA_2 VALUES) OF NORADRENALINE DERIVATIVES, TESTED ON THE VAS DEFERENS OF THE RAT

HO, HO, OH, C C—N—R	Intrinsic activity		Affinity	
	mimetic	lytic	mimetic pD_2	lytic pA_2
R = —H	1		5·4 (±0·2)	
—C	1		5·7 (±0·2)	
—C—C	0·94 (±0·05)		5·2 (±0·2)	
	0·4 (±0·1)		2·8 (±0·6)	
—C—C—C—C				<2·0
		0		3·5
		0		4·4 (±0·4)
		0		5·1 (±0·3)
		0		5·9 (±0·5)

The figures in brackets give the P_{95} for the mean value.

compounds can act as substrates as well as inhibitors; they show a dualism in action.

Fig. 6 gives log dose-response curves representing the quantity of acid formed per unit of time as a function of the concentration of the substrate for enzymatic hydrolysis of an homologous series of choline esters, including acetylthiocholine (AtCh), by acetylcholinesterase. AtCh has the highest rate of hydrolysis, therefore the largest value for k_3. Propionylcholine (PrCh) has an intermediate value, while butyrylcholine (BuCh) and laurylcholine (LCh) are hardly split at all. The substrate inhibition observed is not being considered. The Figs 7 and 8 give the experimental results for the enzymic hydrolysis of combinations of ACh and BuCh and of PrCh and AtCh. Compare the dose-response curves of Figs 6–8 with those in Figs 3–5 (van Rossum & Hurkmans, 1962).

Physico-chemical background of affinity

Besides those factors which determine the relation between the dose of the drug and its concentration in the direct vicinity of the receptors, the biophase, the dissociation constant of the drug-receptor complex in

particular, is determinative for the size of the effective dose. The affinity between drug and receptor, the reciprocal of the dissociation constant, in its turn depends on the reaction velocity constant, k_1, for the association and the velocity constant, k_2, for the dissociation. The value of k_1 is determined by the ease with which the drug molecule can detect the receptor. It will therefore depend especially on the long range electrostatic forces acting between drug and receptor. By these forces the drug

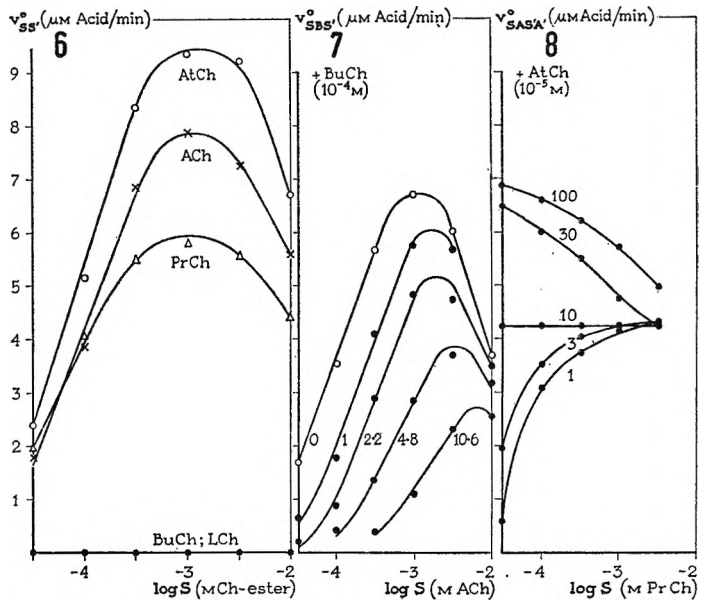


FIG. 6. Concentration-response curves for the enzymic hydrolysis (acid formation) of various choline esters. These curves were obtained by calculating the initial reaction velocity ($v^{\circ}_{ss'}$) for individual concentrations of the different substrates in a random order and under standard conditions. A 100 ml solution (0·1 M NaCl; $5{\cdot}10^{-5}$ M EDTA-Na) containing a certain concentration of a substrate was kept at pH 7·0 by an automatic titrator which regulated the flow of a 0·01 N NaOH solution. At zero time 1 ml of haemolysed human erythrocytes was added and the cumulative amount of alkali needed was recorded. The initial reaction velocity was calculated as the slope at the point of inflection of the obtained time-response curves. Note the difference in the maximal reaction velocity for the various substrates, acetylcholine (ACh), propionylcholine (PrCh) and acetylthiocholine (AtCh). Butyrylcholine (BuCh) and laurylcholine (LCh) are practically unhydrolysed (van Rossum & Hurkmans, 1962).

FIG. 7. Concentration-response curves for the enzymic hydrolysis (acid formation) of ACh and the influence thereon of butyrylcholine under similar conditions as those described in Fig. 6. A different batch of haemolysed erythrocytes was used. Note the parallel shift of the ascending limb of the dose-response curves of ACh, indicating a competitive inhibition (van Rossum & Hurkmans, 1962).

FIG. 8. Concentration-response curves for the enzymic hydrolysis (acid formation) of propionylcholine (PrCh) and the influence thereon of acetylthiocholine (AtCh) under similar conditions as those described in Fig. 6. Note with higher concentrations of PrCh the rate of acid formation approaches the value obtained with PrCh alone. At lower concentrations of AtCh, PrCh acts as a synergist, with higher concentrations of AtCh, PrCh acts as an antagonist. Compare with Fig. 5(ε) (van Rossum & Hurkmans, 1962).

molecule is kept in the close vicinity of the receptor for some time, with the consequence that the chance that the molecule gets into the right position so that the short range van der Waals' forces between drug and receptor can come in action, is increased. As a result of the electrostatic attraction forces, the concentration of the drug molecules is increased in the border layer covering the surface bearing the receptors. As a consequence the ionic groups and other polar groups in drug molecule and receptor will be of special importance as far as k_1 is concerned. The reaction velocity constant for the dissociation, k_2, depends on the binding energy. Here, the van de Waals' forces which come into action once the drug molecule is adapted to the receptor, play an important role. The binding energy gained from electrostatic forces of the polar groups in drug and receptor, will remain relatively low as long as the interaction takes place in a medium of water rich in ions. The van der Waals' forces gained by the interaction of the lipophilic parts of the drug molecule and receptor, will make a large contribution to the binding and therefore to the affinity between drug and receptor.

Physico-chemical background of intrinsic activity

A wide variety of physico-chemical processes may be concerned with the induction of the stimulus. They vary with the type of stimulus induced and therefore with the type of effect studied. Some of the possibilities will be discussed in more detail.

EFFICACY OF SUBSTITUTES

The contribution to the stimulus and therefore to the effect may be constant for each individual interaction between a receptor and a molecule of a certain drug and vary for various drugs. The intrinsic activity then expresses this variation. Certain vitamin analogs may act in this way, after formation of a coenzyme analog and its binding to an apoenzyme to form a holoenzyme. The turn-over capacity of the enzyme analog will depend on the type of vitamin analog incorporated. The capacity may gradually change with its chemical structure and even decrease to zero. The compound then acts as an antivitamin. The intrinsic activity here expresses the gradation in the turn-over obtainable with the various vitamin analogs used as substitutes.

EFFECTIVE FRACTION OF COLLISIONS

It may be that only a fraction of the individual collisions between drug and receptor are effective, while the contribution to the stimulus is constant for each effective interaction. This fraction may vary for various drugs. There is an all-or-none response at receptor level. The intrinsic activity then represents the effective fraction of collisions (Ariëns, 1962, 1964).

The chance that the collision between a drug bearing a cationic group and the receptor having an anionic site will result in an ion-pair formation, will depend on the properties of the cationic group, e.g. a quaternary

ammonium group. Gradual ethylation on the onium group in acetylcholinomimetics may, because of steric hindrance, lead to an interference with ion-pair formation with as a consequence a decrease in intrinsic activity (Ariëns & de Groot, 1954; Ariëns, Simonis & de Groot, 1955; van Rossum, 1958; Ariëns, 1964) and a change to anticholinergic compounds (Figs 3, 4).

RATE OF COLLISIONS

Possibly the drug molecule contributes to the stimulus as long as it is on the receptor; as in the case of the vitamin analogs mentioned above, however, as postulated by Croxatto & Huidobro (1956) it may be that the molecule of the drug is effective only at the moment when its molecules are linked to the receptor. Durable linkage of the drug with the receptor does not then mean a lasting contribution to the effect. At the moment of linkage of the drug molecule to the receptor, free energy or an endogenous substance may be liberated, serving as, or contributing to a stimulus. Possibly the free energy must have a minimum indispensable quantal magnitude in order to be effective (Croxatto & Huidobro, 1956).

Recently, Paton (1961, 1964) combined the concept of the drug-receptor interaction based on the mass law and the postulate that only the rate at which the association between drug and receptor takes place determines the ability of a drug to contribute to the effect, or, in our terms, that the rate of association determines the intrinsic activity. As pointed out by Paton (1961, 1964), in equilibrium conditions, the rate of association is equal to the rate of dissociation of the drug-receptor complex, which is k_2 [RA]. In this model, called the rate theory, the equilibrium response obtained with a certain dose of drug A can be represented by:

$$E_A/E_m = \frac{k_2}{1 + k_2/k_1[A]} = \frac{k_2}{1 + K_A/[A]} \quad .. \quad .. \quad (5)$$

Compare this equation with eqn 2. The intrinsic activity, α, is substituted by k_2.

INDIRECTLY ACTING DRUGS

For indirectly acting drugs or liberators which act by virtue of endogenous substances, the rate of liberation and therefore the rate of drug-receptor interaction also may be determinative for the intrinsic activity. Examples of this type of action are the histamine liberators. It will be clear also that the rate of reloading of the system is then important.

In those cases in which the rate of drug-receptor interaction is essential, it is quite possible that only a fraction of the interactions are effective, which implies that the effect is determined by the product of the rate of association under equilibrium conditions, k_2 [RA], and a constant c_1 for the effective fraction of the associations. In these circumstances the equilibrium response obtained with a drug A can be represented by:

$$E_A/E_m = \frac{k_2c_1}{1 + k_2/k_1[A]} = \frac{k_2c_1}{1 + K_A/[A]} \quad .. \quad .. \quad (6)$$

where c_1 represents the effective fraction of the collisions. The intrinsic activity is determined by k_2c_1. Compare this equation with the eqns 2 and 5. The model postulated by Croxatto & Huidobro (1956) is an example. Not the rate of association, but the rate of *effective* associations—those which yield the minimum indispensable quantal magnitude of free energy—is supposed to be determinative for the intrinsic activity.

The fate of the constant α representing the intrinsic activity in eqn 2 will probably be the same as that of k_3 from the classical enzyme kinetics. It will be resolved in a variety of constants concerned with and depending on the sequence of chemical events which lead to the evocation of the stimulus (Ariëns, 1962). As long as equilibria or dose-response curves are studied, eqn 2 covers in a formal way the models represented by the eqns 5 and 6.

Fade and densensitisation

If the rate of association between drug and receptor is determinative for the intrinsic activity, a maximum will occur in the time-response curve immediately after application of the drug. This maximum is followed by a fade, a decrease, in the response to an equilibrium value. At the moment the drug is applied, all receptors are available for the interaction.

In the equilibrium state the number of receptors available is determined by the reaction velocity for the dissociation of the drug-receptor complex and/or possibly the rate of reloading in the case of liberators. The study of time-response curves may lead to a detection of those drug actions in which the rate of receptor occupation plays an essential role. Figs 9

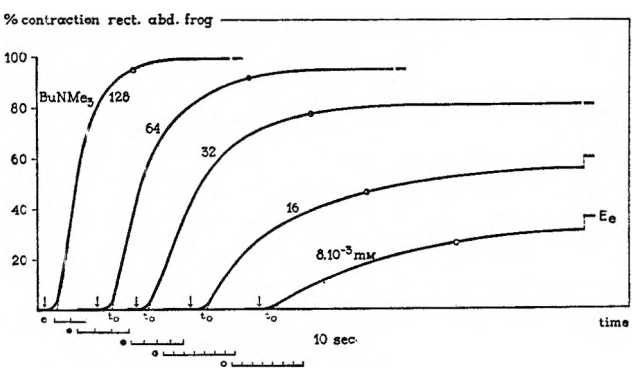


FIG. 9. Registrogram of time-response curves with various doses of $BuNMe_3$ on the isolated rectus abdominis muscle of the frog. The time scale varies for the various curves. E_e is the height of the contraction finally obtained with the dose concerned. ↓ : addition of the drug. Note there is a gradual increase of the response with the time. No fade is observed in the response.

and 10 give such experiments for the action of acetylcholinomimetics on some effector systems. In these instances no indications are found of the maximum in the response or a fade phenomenon and therefore no indications for the rate of receptor occupation as determinant factor.

One of the problems in this respect is that it is often not the rate of receptor occupation but the rate of diffusion of the drug to the receptors that may be determinative for the time-response relationship.

Another implication of the rate theory is that after elimination of the drug from the bath fluid or biophase, the response becomes zero, although a fraction of the receptors may still be occupied by the drug. This involves a decrease in the response of the second and later doses of the drug, a desensitisation. There will be a cross desensitisation with respect to other drugs inducing their effect on the same receptors (Paton, 1961).

The desensitisation observed after application of high doses of acetylcholine to the isolated gut is not specific. Not only the sensitivity to acetylcholine but also to histamine and $BaCl_2$ is decreased. This argues against the rate concept as a declaration of this desensitisation (Huidobro & Valetta, 1961; Paton, 1961). With lower doses of acetylcholinomimetics, but doses high enough to produce the maximal isotonic response, no desensitisation is observed after washing till the basal tone returns.

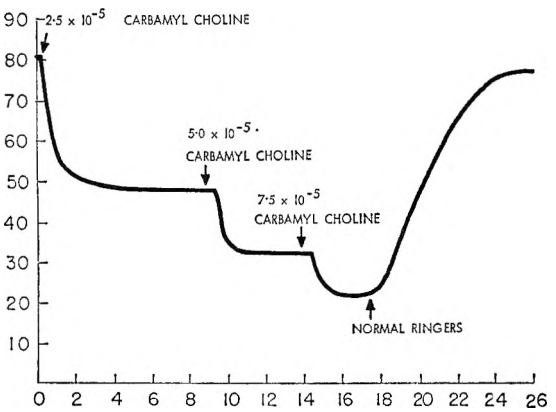


FIG. 10. Changes induced by carbamylcholine in the membrane potential of the isolated electroplax. Note after application of the drug there is a steady increase of the depolarization approaching some equilibrium state. There is no fade phenomenon in the curves (Higman, Pollewski & Bartels, 1963).

The phenomenon of the fade in the response and the specific desensitisation or tachyphylaxis has been considered earlier. The interpretation of this was the aim of the "potential" theory on drug action, a model introduced by Straub (1907), and discussed by Clark (1937a). In this model the drug is supposed to act only as long as there is a concentration gradient from the extracellular to the intracellular phase, or in other words as long as there is a net flux of drug into the cell or to the receptors. This model, too, allows for an initial maximum in the time-effect curve and for the fade in the effect with the decrease of the gradient or flux. The effect may totally fade away although the drug is still present. The model also predicts a specific desensitisation of the biological object after removal of the drug from the extracellular phase as long as there is an intracellular

residue of the drug. Mackay (1963) recently enlarged the scope of the "potential" theory by introducing specific carriers for the transportation or flux of the drug, which implies a combination of "potential" theory and receptor theory.

Structure and action

If the induction of a biological effect is the resultant of the interaction between the drug molecules and specific receptors, a relation between the chemical structure, or better the physical and chemical properties of the drug, and the activity must exist. Nevertheless, often, such a relation is not observed. There are two main reasons for this failure.

LIMITATIONS OF KNOWLEDGE ABOUT STRUCTURE

Structural formulae are a very poor means for expressing the physico-chemical properties of molecules. Drugs apparently similar on the basis of their structural formulae may differ essentially in their properties. The reverse is also true. Drugs apparently different in structure may be identical in their essential physico-chemical properties.

LIMITATIONS OF KNOWLEDGE ABOUT ACTION

The biological activities of the compounds compared may look identical from a crude phenomenological point of view; nevertheless, they may differ essentially in their mechanism of action. Consider for instance, the group of muscle relaxants, the group of convulsants or the group of diuretics. In principle, only drugs which induce their biological actions on identical receptors may be compared. Moreover, differences in transport and drug metabolism, for instance, have to be taken into account.

AFFINITY AND INTRINSIC ACTIVITY

As a consequence of the differentiation between affinity and intrinsic activity one may try to differentiate within the structure of the molecule between those moieties mainly concerned with the intrinsic activity and those concerned with the affinity. The experimental results represented in the Figs 2–5 suggest that, for the parasympathomimetic action of acetylcholine and its derivatives, the properties of the onium group are essential for the intrinsic activity, while the chain is mainly of importance for the affinity to the receptors. As a matter of fact the onium group also contributes to the affinity.

What is the significance of the ester group in acetylcholine and its equivalents in its various analogs for the muscarinic action? As a rule an equivalent is found in these analogs for the ether-oxygen but not for the carbonyl group of the ester moiety. This indicates that for muscarinic activity, the ether-oxygen is more concerned with the drug-receptor interaction than the carbonyl group.

The study of the influence of gradual ethylation of the onium group in acetylcholine and its various analogs tested on the isolated gut of the rat

as is shown in Table 1 (Simonis, Ariëns & Rodrigues de Miranda, 1964) reveals an interesting aspect. The change from agonist to competitive antagonist in the course of the ethylation is retarded by the presence of the ester moiety or its substitutes in the parasympathomimetics. In acetylcholine itself the triethyl derivative still acts as a full agonist, although the affinity is decreased. The conclusion may be that the ester moiety and its substitutes are not essential for the intrinsic activity, but nevertheless influence this parameter. It may be that a mutual neutralisation or ion-pair formation between the onium group and a complementary anionic site on the receptors is essential for the induction of the stimulus and therefore for the production of the effect. The interaction between the ester moiety and its complement on the receptor then appears to have a facilitating action as far as this neutralisation or ion-pair formation is concerned. In the absence of the facilitating ester group, the ethylation of the onium group, causing steric hindrance or changes in charge distribution, more easily interferes with a true neutralisation or ion-pair formation. The cationic head after ethylation may still be attracted by the anionic site, thus shielding this part of the receptor; the drug then acts as a blocking compound, a competitive antagonist. The ethylated molecule then takes over the place but not the function of acetylcholine.

"ATTACHING" AND "ACTING" MOIETIES

One must be aware of the fact that, as mentioned, drug-receptor interaction implies a mutual moulding of drug and receptor. The usual model of key and lock is much too static. The interaction of the ester moiety in acetylcholine and its complement with the receptor may well change the conformation or charge distribution of the receptor in such a way that the approach between the cationic head of the drug and the anionic site on the receptor is facilitated. The consequence is that, although in a study on the relation between structure and action certain moieties of the drug molecule are found to be of special importance for the intrinsic activity and other moieties for the affinity, as a rule this differentiation will not be sharp, since the various moieties are parts of the molecule as a whole and therefore interdependent.

Cavallini, Massarani, Nardi & Mauri (1961) differentiated a supporting moiety, mainly conferring to the drug affinity for the receptors, and a radical moiety determining the type of action. An analogous differentiation is made in the field of cancer chemotherapy. In the various nitrogen-mustard derivatives there can be differentiated the mustard moiety, the cytoxic group of the "war-head" and the carrier (Ross, 1962). Groups such as amino-acids, sugars and steroids (Rao & Price, 1962) may act as carrier moieties.

Especially in larger drug molecules, like the biologically active polypeptides, a distinction between moieties essential for the intrinsic activity and moieties mainly contributing to drug binding, to the affinity, seems feasible. Take for instance the differentiation in an "attachment site" and "active site" or a differentiation in "functional amino- acid residues"

and "filler sequences" in the various polypeptide hormones as postulated by Hofmann (1960) and Schwyzer (1962, 1963). The differentiation of a "Haft Gruppe" and a "Wirk Gruppe" in vitamins as postulated by Martius (1955, 1958) is also reminiscent of affinity and intrinsic activity. The various examples given have much in common with the differentiation between a haptophoric group and a toxophoric group in drugs already postulated by Ehrlich, which also implies a differentiation between affinity and intrinsic activity.

Receptors

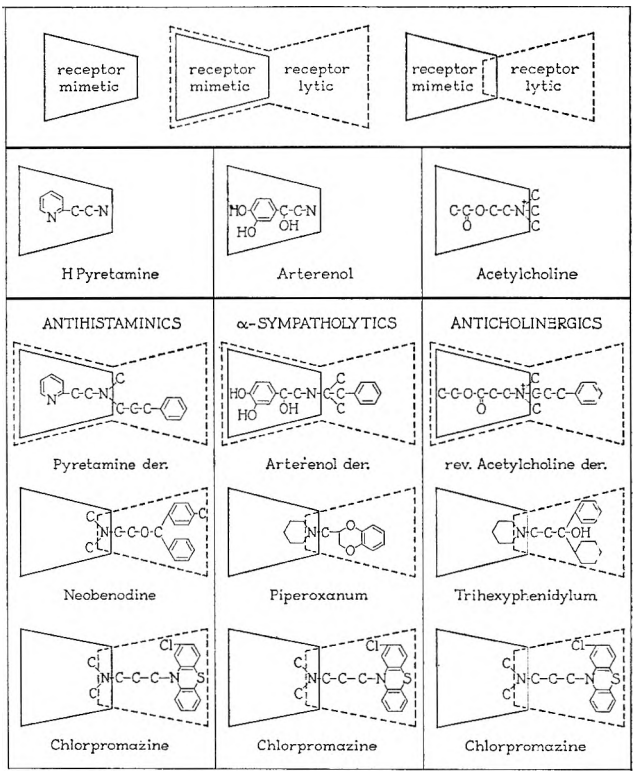
The main if not the only source of information on the properties of the receptors is the study of structure-activity relations of drugs. This study allows us, although on a basis of indirect evidence, to develop certain views on the still purely hypothetical but, for the molecular approach to drug action, indispensable receptors.

AFFINITY TO "COMMON" RECEPTORS

As mentioned before, agonist and competitive antagonist act on a common receptor. As will be shown this does not imply an action on strictly identical receptors (Ariëns & Simonis, 1960; Ariëns, 1962a, 1964). Introduction of substituents gradually increasing in length on the amino- or onium group of compounds such as histamine, arterenol (noradrenaline) and acetylcholine, or on the acetic acid group in acetylcholine results in a gradual change from agonists to competitive antagonistic compounds. The intrinsic activity, but also the affinity strongly decreases. The lytics obtained have a low affinity to the receptors. With the introduction of larger groups, especially groups with planar rings such as aralkyl groups, the affinity strongly increases again and highly active lytics are obtained. Tables 2 and 3 give examples of such series of compounds. The conclusion may be that the lytic compounds thus obtained are dependent for much of their affinity not on the original receptor for the mimetic but on additional receptor parts.

Histamine, acetylcholine and noradrenaline are stimulants bearing strong polar groups. Their receptors, being complementary to these stimulant drugs, will also present concentrations of strong polar groups. In the vicinity more indifferent, less-polar surface areas may be expected. These areas may serve for the interaction of the ring-bearing substituents mentioned above. Many of the lytic drugs probably interact slightly with the receptor area of the agonist, the mimetic, but mainly their action will be on the adjacent, additional, more indifferent receptor area. Probably histaminomimetics, acetylcholinomimetics and sympathomimetics have in common with many of their respective competitive antagonists only the anionic site in their receptors. This makes it understandable that most mimetics show little or no chemical relation to the corresponding lytics, and that certain lytics such as for example chlorpromazine, have the ability "to block the receptors" for different types of mimetics. These relations are represented in a schematic way in Table 4 (Ariëns & Simonis, 1960; Ariëns,

TABLE 4. MIMETICS (AGONISTS) AND DIFFERENT TYPES OF LYTICS (COMPETITIVE ANTAGONISTS) IN RELATION TO THEIR HYPOTHETICAL RECEPTOR SURFACES



Note: chlorpromazine has an antihistaminic, an anti-adrenergic and an anticholinergic action.

1962a, 1964). Introduction of certain, especially spatial, properties in such multipotent lytics may result in an increase in specificity again (Harms, 1956; Harms & Nauta, 1960), which is feasible, since the indifferent surface areas adjacent to the receptors for histamine, acetylcholine and noradrenaline will not be identical and may differ especially in spatial relations.

The loss of binding capacity to the original receptor areas and the acquisition of new binding capacities on additional receptor parts, as demonstrated in the series of acetylcholine derivatives (Table 2), has interesting consequences if the esters of β-methylcholine are studied. As is well-known, both stereoisomers of acetyl-β-methylcholine differ strongly in their biological activity. For the highly active competitive antagonist obtained by esterification of β-methylcholine with for instance 2,2′-diphenyl-2″-hydroxyacetic acid (benzilic acid) there is hardly any difference in potency between the two stereoisomers. If a centre of asymmetry is introduced in the acidic moiety, for instance by esterification of β-methylcholine with 2-phenyl-2′-cyclohexyl-2″-hydroxyacetic acid highly active lytics are obtained again. Now a large difference in potency

is found between the stereoisomers which differ in the steric configuration of the acidic moiety of the molecule, while only small differences in potency are observed for those stereoisomers that differ in the configuration of the β-methylcholine moiety (Ellenbroek, 1964).

Such relations are expected on a basis of the shift of the binding capacity from the cholinic to the acidic moiety of the molecule. On the other hand these results demonstrate that the significance of a centre of asymmetry for the activity of a drug molecule is strongly dependent on the degree to which the moiety of the molecule in which the centre of asymmetry is located, contributes to the binding of the drug to the receptor or to the activity of the drug. This puts restrictions on the rule that the differences in activity of stereoisomers is large for highly active and small for less active compounds, known as Pfeiffer's rule (1956) (Beckett, 1963).

ACTION ON DIFFERENT RECEPTORS

The above reasoning dealt with the interaction of drug and receptor as far as the affinity is concerned. The drug-receptor interaction is also concerned with the intrinsic activity. The catecholamines are interesting in this respect. Drugs like adrenaline induce effects on two types of receptors called α- and β-receptors (Ahlquist, 1959). Substitution of large alkyl or aralkyl groups on the amino-group of noradrenaline results in a loss of the intrinsic activity on the α-receptors. The compounds obtained act as α-sympatholytics and β-sympathomimetics simultaneously. If on the other hand the catechol configuration is eliminated such as is the case in dichloroisoprenaline and in pronethalol, the intrinsic activity on the β-receptors is lost and β-sympatholytics are obtained (Ariëns & Simonis, 1960; Howe, 1963). A close correlation of structure and action is found in the series of *N*-alkyl or -aralkyl substituted noradrenalines (β-sympathomimetics) and *N*-alkyl or -aralkyl substituted pronethalols (β-sympatholytics). In both series, branched alkyl groups such as isopropyl and t-butyl result in highly active compounds. The same obtains for the introduction of a phenyl-isopropyl group. The absolute steric configuration of the most active isomers of isoprenaline (mimetics) and dichloroisoprenaline and the isopropyl derivative pronethalol (lytics) are identical (Howe, 1963). These relations make it highly probable that these β-sympatholytics really block in a more strict sense the surface of the β-receptors. The fact that blockade of the β-receptors by β-sympatholytics such as pronethalol does not interfere with the induction of effects by α-sympathomimetics on the α-receptors strongly argues for two really different entities as far as α- and β-receptors are concerned.

Further, the relationships described indicate that the interaction between the cationic amino-group in the catecholamines and some complementary anionic site on the α-receptor is essential for the induction of the effect, or for the intrinsic activity there. On the β-receptors, for the induction of the effect, or the intrinsic activity, the interaction between the catechol group and the receptor appears to be essential. Analogous

reasoning can be given for the relations between muscarinic and nicotinic drugs and their receptors, and relations between the polypeptide hormones related to oxytocin and ADH and their receptors (Schwyzer, 1963).

ISOLATED RECEPTORS

Various investigators (Chagas, Penna-Franca, Hassón, Crocker, Nishie & Garcia, 1958; Hassón & Chagas, 1959; Ehrenpreis, 1962a, 1963b) tried to isolate receptors and studied the interaction of drugs with such isolated "receptor proteins" or receptor substances.

A characteristic of the receptors on which effects are induced by stimulant drugs is that the changes induced by the drug in charge distribution and shape of the receptor, are propagated to surrounding molecules. This then leads to a stimulus and consequently to the effects. This implies an intricate relationship between the receptor molecule and the adjoining molecules which is essential for the properties of the receptor *in situ*. The isolated receptor will, as a rule, be essentially changed in its charge distribution and conformation as compared to the receptor *in situ*. Although it may still bind drugs, the isolated receptor will differ in essential aspects from the receptor *in situ*. Certainly no stimulus can be induced on the "isolated receptor". Drug binding to macromolecules obtained from various tissues can serve as a model for the study of the interaction between drugs and receptors in general (Hassón & Chagas, 1959; Ehrenpreis, 1962b, 1963a).

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Research Papers

Determination of vitamin D by isomerisation

EARL B. DECHENE

Conditions have been established in which the spectral change produced by the isomerisation of vitamin D_2 and D_3 to the trans-isomers can be used as an accurate measurement of vitamin D. Vitamin D was successfully determined by isomerisation in commercial products containing only vitamin D at a relatively high potency, without the use of chromatographic procedures.

VERLOOP, Koevoet & Havinga (1955) have showed that calciferol, like other partly cis-polyenes, can be isomerised to the all *trans*-isomer. They detailed the conditions for the isomerisation of calciferol by iodine, and followed its conversion to *trans*-calciferol by the change in the absorption spectrum of the solutions.

We have studied the isomerisation of calciferol and crystalline vitamin D_3 by iodine in hexane solutions, and established conditions under which the spectral change was sufficiently characteristic and reproducible to yield a quantitative measurement of the vitamin D.

A simple method was, therefore, developed, which yielded satisfactory assays of vitamin D in some commercial products, without using chromatographic procedures for the removal of materials that usually interfere in the colorimetric measurement of the vitamin D with antimony trichloride.

This method was not successful for assaying low potency vitamin D products or those containing vitamin A. These products when diluted to suitable extinction values for spectrophotometric measurement do not provide sufficient vitamin D for an accurate measurement of the spectral change produced by the isomerisation of the vitamin D.

Experimental and results

REAGENTS

Vitamin D_2 solution. Prepared with Calciferol B.P. and spectro grade hexane to yield a concentration of 20 μg/ml and stored in low actinic glass containers.

Vitamin D_3 solution. Prepared with crystalline D_3 and spectro grade hexane to yield a concentration of 20 μg/ml and stored in low actinic glass containers.

Iodine solution. Prepared with re-sublimed iodine and spectro grade hexane to yield a concentration of 1 μg/ml.

Verloop & others (1955) reported that isomerisation of calciferol in light petroleum causes the absorption maximum of the solution to shift from 265 to 270 mμ with an increase in extinction. They specified that

From the Control and Research Laboratories, Charles E. Frosst & Co, Montreal, Quebec.

the iodine concentration should not exceed 2% of the weight of the calciferol in solution to avoid degradation of the calciferol.

We have determined the effect of iodine concentration, light, and time on the isomerisation of vitamin D in the following manner: Aliquots of the vitamin D solutions, equal to 100 μg of vitamin D, were placed in 10 ml flint glass volumetric flasks. Iodine solution was added to the flasks to yield a concentration from 0 to 5% of the weight of vitamin D, and each flask brought to 10·0 ml with hexane. Three or more solutions were prepared for each level of iodine.

EFFECT OF LIGHT AND IODINE CONCENTRATION

One set of solutions representing all levels of iodine was stored in the dark, and a duplicate set was exposed to fluorescent light. The absorption spectrum of each solution was determined in a ratio recording spectrophotometer at intervals of 15 to 20 min.

In the absence of light, no spectral change occurred over 4 hr in the solutions without iodine or containing iodine to D ratios of 0·1 : 100 or less. In solutions containing iodine to D ratios greater than 0·1 : 100, a spectral change occurred that appeared to be proportional in both magnitude and rate to the concentration of iodine. In solutions containing iodine to D ratios greater than 2 : 100, a gradual decrease in the extinction values occurred after 2 to 4 hr storage.

In the presence of light, no spectral change occurred in solutions without iodine during 16 hr exposure to light. Solutions containing iodine to D ratios of 0·1, 0·2 and 0·5 : 100 showed gradual spectral changes over a period of 30 to 60 min. After this time the extinction at 270 mμ of the solutions, reached the same values, and did not alter during 16 hr exposure to light.

In solutions containing iodine to D ratios of 1 and 2 : 100, the same spectral shift and extinction values at 270 mμ were produced in 15 to 30 min. A slow decrease in the extinction values of these solutions occurred during further exposure to light.

The spectral change occurred rapidly in solutions containing iodine to D ratios of 3 : 100 or greater. The extinction at 270 mμ of these solutions did not reach the maximum value shown by solutions containing smaller concentrations of iodine, and decreased rapidly on further exposure to light.

The spectral change produced by the isomerisation of calciferol to *trans*-calciferol in our tests is similar to that found by Verloop & others (1955). The spectral change produced by the isomerisation of vitamin D_3 is identical with that produced by the isomerisation of calciferol, except that the extinction value at 270 mμ is lower than the value obtained by the isomerisation of an equal weight of calciferol.

MEASUREMENT OF VITAMIN D

Because the extinction of solutions at a wavelength other than that at which a maximum occurs does not vary exactly in proportion with the

concentration of solute, we derived the quantitative factors for the spectral change produced by the isomerisation of vitamin D_2 and D_3 by means of several replicate determinations at one concentration of each vitamin.

Based on the results obtained in the preceding tests, we used a ratio of iodine to D of 0·2 : 100, which we judged to be the most reliable for the isomerisation of vitamin D.

5-ml aliquots of either vitamin D_2 or D_3 solution, representating 100 μg or 4000 units of the vitamin, were placed in 10 ml flint glass volumetric flasks, containing 0·2 μg of iodine, and the solution made to 10·0 ml with spectro grade hexane. Replicates of these solutions were made without iodine. All the solutions were exposed to fluorescent light and their extinction determined at 15 to 20 min intervals over the spectral range 255 to 280 mμ. The rate of spectral change varied to some extent in the solutions containing iodine. A constant extinction value at 270 mμ was reached by these solutions, which did not alter during exposure of the solutions to light during 16 hr. The solutions of vitamin D_2 gave a higher extinction value than the solutions of vitamin D_3.

The extinction at 270 mμ of the solutions treated with iodine was subtracted from that at 270 mμ of the corresponding untreated solutions of the vitamin D and the mean differences (10 estimations) in these values are for vitamin D_2, 400 units/ml, 0·127 $\pm$ 0·012 s.d., and for D_3, 400 units/ml, 0·103 $\pm$ 0·003 s.d.

The calciferol we used showed a molar extinction $\epsilon = 18{,}300$ and yielded an $\epsilon = 23{,}377$ for *trans*-calciferol after isomerisation. Our crystalline vitamin D_3 showed an $\epsilon = 17{,}731$ and yielded an $\epsilon = 21{,}692$ for *trans*-vitamin D_3 after isomerisation.

Fieser & Fieser (1959) give a value of $\epsilon = 23{,}600$ for *trans*-calciferol. Inhoffen, Quinkert, Hess & Hirschfield (1957) reported an $\epsilon = 25{,}400$ at 272 mμ for *trans*-calciferol, and an $\epsilon = 24{,}300$ at 272 mμ for *trans*-vitamin D_3.

We have, over a period of time, been able to confirm the factor 0·127 for the isomerisation of 400 units of vitamin D_2 in a number of different batches of B.P. calciferol. Only one batch of crystalline vitamin D_3 was available, and was used in deriving this factor.

APPLICATION OF THE METHOD

We measured vitamin D by isomerisation in the various products listed in Table 1. In these assays, the materials were treated by one of the following methods.

Method A. For solutions of vitamin D in vegetable oil or other solvents.

The product is diluted with spectro grade hexane to a concentration of 20 μg or 800 units/ml. This solution is diluted with spectro grade hexane to a concentration of 10 μg or 400 units/ml, and its extinction determined over the spectral range of 240 to 280 mμ. If an extinction between 0·50 and 0·90 at 265 mμ is given by the solution, transfer 5·0 ml aliquots at 20 μg/ml to 4 $\times$ 10-ml flint glass volumetric flasks. To

2 flasks add 0·2 μg of iodine and make each flask to 10·0 ml with spectro grade hexane. Expose the flasks to fluorescent light and determine their extinction over the spectral range 240 to 280 mμ at hourly intervals. When a constant extinction is not reached in 4 to 5 hr, the solutions can be exposed to light overnight (16 hr) and examined again at hourly intervals, until a constant value is reached. Should the extinction of the solutions show a decrease between consecutive readings, fresh solutions are prepared with the concentration of iodine reduced to 0·15 or 0·1 μg.

We found the isomerisation of vitamin D occurred very slowly in the assays of a number of commercial products. We believe this is due to the action of materials other than vitamin D competing for iodine. We chose to maintain the selected ratio of iodine to D and allow more time for isomerisation, rather than risk degrading vitamin D by using too high a concentration of iodine.

TABLE 1. THE VITAMIN D CONTENT OF VARIOUS PRODUCTS DETERMINED BY ISOMERISATION OF THE VITAMIN D

Product	Claim* units D	Found units D av. 2 assays	% of claim
Vitamin D_2 capsules	50,000	48,000	96·0
Vitamin D_2 capsules	50,000	54,500	109·0
Vitamin D_2 capsules	50,000	60,882	121·0
Vitamin D_2 capsules	50,000	47,410	94·8
Vitamin D_2 capsules	50,000	47,200	94·4
Vitamin D_2 oil	30,800	31,884	103·0
Vitamin D_2 oil	30,800	28,828	94·0
Vitamin D_2 oil	1,000,000	1,040,000	104·0
Vitamin D_2 oil	1,000,000	1,031,239	103·12
Vitamin D_2 oil	1,000,000	1,049,000	104·9
Vitamin D_2 oil	3,000,000	2,679,024	89·0
Vitamin D_2 powder	200,000	227,000	113·5
Vitamin D_2 powder	850,000	952,000	112·0
Vitamin D_2 powder	850,000	1,067,000	125·0
Vitamin D_2 powder	850,000	1,169,000	137·6
Vitamin D_2 powder	850,000	969,474	114·0
Vitamin D_3 oil	400,000	416,000	104·0
Vitamin D_3 oil	400,000	469,454	117·2
Vitamin D_3 oil	500,000	515,000	103·0
Vitamin D_3 oil	2,000,000	2,676,000	133·5
Vitamin D_3 oil	2,000,000	2,345,000	117·5

* The suppliers established their claim in the products by either biological or colorimetric assay with antimony trichloride.

Method B. When the products treated as described in Method A gave extinction values greater than 0·90 at 265 mμ, or for products in solid form, the following procedure was used.

Take a weight or volume of the product to yield 500 μg or 20,000 units of vitamin D. Place the sample in a 100 ml flask, add 3 ml of 50% aqueous potassium hydroxide, 40 ml of spectro grade ethanol, and reflux for 20 min. Cool the solution and transfer with 100 ml of water to a separatory funnel. Extract with 4 × 20 ml portions of hexane. Wash the combined hexane extracts with water until the last washing is neutral to litmus. Evaporate the hexane solution to dryness at 35° under vacuum, and transfer the residue to a 25 ml volumetric flask with spectro grade hexane and make to volume with the same solvent. Place 5·0 ml aliquots of this solution in four 10 ml flint glass volumetric flasks. To 2 flasks

add 0·2 μg of iodine and make to volume with spectro grade hexane. The course of isomerisation of the vitamin D is followed in the same way as described in Method A.

Calculations. The amount of vitamin D in the product as determined by either Method A or B is obtained by the following equation.

$$\frac{(AI - A) \times 400}{0{\cdot}127 \text{ or } 0{\cdot}103} = \text{units of vitamin D per ml of solution.}$$

AI: is the average extinction at 270 mμ of the duplicate solutions containing iodine.

A: is the average extinction at 270 mμ of the duplicate solutions without iodine.

0·127 or 0·103 are the factors for the increase in extinction at 270 mμ produced by the isomerisation of 10 μg or 400 units of vitamin D_2 or D_3. The appropriate factor is used depending on which vitamin is claimed to be in the product.

The value in units of vitamin D obtained by this equation is divided by the volume or weight of the product present in 1·0 ml of the solutions used in the isomerisation measurements, to yield the number of units of vitamin per gram or ml of the product.

Discussion

The isomerisation of vitamin D_2 and D_3 by iodine has been found to be reproducible, and was applied successfully to the measurement of vitamin D in a number of commercial products containing only vitamin D.

This assay method did not require the use of chromatographic procedures to remove substances that usually interfere in the determination of vitamin D by the antimony trichloride colorimetric method. It does require that a hexane solution of the saponified product containing 400 units of vitamin D per ml, be sufficiently transparent to allow measurement of the extinction. Because of this requirement, the assay was not applied to products containing vitamin A and low concentrations of vitamin D, which require chromatographic procedures to reduce the extinction contributed by materials other than vitamin D.

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The synthesis of some potential antibacterial agents

B. H. CHASE AND W. T. WELLER

The preparation of 37 potential antibacterial agents is described. Most of them possess the structure R—X—R′ where R and R′ are substituted phenyl groups and X is a short bridging group; 25 different examples of X have been studied. Minimum inhibitory concentrations against *Staphylococcus aureus* and against *Escherichia coli* are recorded.

OUR interest in new antibacterial agents lay primarily in their potential use in soap tablets for personal or domestic use. Such an objective imposes considerable restriction on the types of compound which can be considered since many bactericides are inactivated by soap over a range of relative concentrations. There are other limitations imposed by the need to avoid toxic effects like irritation, sensitisation or photosensitisation, on human skin and to avoid interference with the physical properties of the tablet, but neither of these broad aspects is considered here.

Many bactericides, particularly cationic bactericides such as the quaternary ammonium compounds, are bacteriologically incompatible with soap over almost the whole range of relative concentrations. Others, such as the halogenated cresols and xylenols, may be compatible if the soap concentration is of the same order as, or less than their own, so that they can be successfully solubilised in formulations that include relatively small amounts of soap (as in some household disinfectants) but are inactivated by the higher ratios of soap: bactericide met with in a soap tablet containing perhaps 1% of "bactericide". The nature of the soap-phenol, or in the more general case surfactant-disinfectant interaction has been studied in a few instances, notably by Alexander and by Berry, but although the picture has been much clarified it is still incompletely understood (Agar & Alexander, 1949; Alexander & Tomlinson, 1949; Bean & Berry, 1948, 1950, 1951, 1953; Berry, 1952; Berry & Bean, 1950; Berry & Briggs, 1956; Berry, Cook & Wills, 1956. See also Brudney, 1956; Cook, 1960). A measure of the interaction is given by the Soap Inactivation Coefficient (S.I.C.) which has been defined (Hurst, Stuttard & Woodroffe, 1960) as the concentration of a disinfectant in soap solution divided by its concentration in an equally potent soap-free solution. Thus an SIC of 1 shows that the compound is unaffected by the presence of soap, while a value greater or less than one shows inhibition and potentiation respectively. It may be noted that the S.I.C. value of any substance is affected by the soap concentration employed and a 10% solution, approximating to that in the lather formed in washing the hands, is normally used as a standard. Furthermore, where disinfection of the skin is the objective, the possession of a high S.I.C. value does not necessarily preclude the use of a potential disinfectant in a formulation involving soap. Provided that the disinfectant has adequate affinity for ("substantivity" to) the skin it will remain on the skin surface after rinsing

From Unilever Research Laboratory, Port Sunlight, Cheshire.

and continue to exert an antibacterial effect. Hexachlorophane, for example, despite having an S.I.C. of about 200, is an effective disinfectant and has been widely used both in a preparation for pre-operative scrubbing of surgeons' hands and in a number of toilet soap tablets.

Many of the bactericides which are known to be effective in soap tablets have broadly similar structures. Thus hexachlorophane [G11; di(3,5,6-trichloro-2-hydroxyphenyl)methane], bithionol [Actamer; di-(3,5-dichloro-2-hydroxy)phenyl sulphide], salicylanilides such as 3,5,3′,4′-tetrachloro- (TCS), 5,3′,4′-trichloro- (Anobial) and 3,5,4′-tribromo-salicylanilide (TBS), and TCC (3,4,4′-trichlorocarbanilide) possess, in common, two benzene rings linked by a short bridging group. The benzene rings are all substituted by halogen and with the exception of TCC also contain at least one hydroxyl group adjacent to the bridge. There are, of course, others such as TMTD (tetramethylthiuram disulphide) and the mercurials, which are of totally different chemical structure. Nevertheless, it seemed to us that the examination of further examples of compounds consisting of substituted benzene rings linked by short bridges might well bring to light some new antibacterial agents, particularly as Jerchel & Oberheiden (1955) in their review of disinfectants of this type have shown that activity is not restricted to the bridging groups cited above.

Thirty-seven compounds have been examined, exemplifying 25 different bridges. For convenience of discussion of both synthesis and bacteriological properties these have been classified (see Table 1) into five groups.

(1) SULPHONAMIDE BRIDGE COMPOUNDS

Compounds (I) and (II) were prepared by condensation of 3,4-dichlorobenzenesulphonyl chloride with 4-chloro- and 3,4-dichloro-aniline respectively. Alkylation of (II) proceeded smoothly to give compounds (III), (IV) and (V). The dibromosulphanilide (VI) was prepared by bromination of sulphanilide itself, which was obtained in moderate yield from aniline and sulphuryl chloride. Attempts to prepare halogenated sulphanilides directly from halogenated anilines failed. Parnall (1960) has recently described a modified procedure which is successful in this respect, but in view of his report of apparent lack of activity in this series we have not attempted to prepare further examples.

(2) UREA AND THIOUREA BRIDGE COMPOUNDS

The urea derivatives (VII–X) were prepared from *p*-chlorophenyl isocyanate and various amines. The thiourea derivatives (XI–XXI) were obtained similarly from the corresponding isothiocyanate or (in the case of symmetrical compounds) from the anilines and carbon disulphide.

(3) AMIDE BRIDGE COMPOUNDS

The hydrazine derivative (XXIV) was prepared from ethyl 2,4-dichlorobenzoate and hydrazine hydrate, and the remaining amides (XXII, XXIII, XXV–XXVIII) via acid chlorides. Compound (XXVII) melts over 100° higher than the melting-point recorded in the literature (Beaver,

Roman & Stoffel, 1957). Since our material gives satisfactory elementary analysis it appears likely that there is a typographical error.

(4) OTHER BRIDGES

The substituted benzoic, carbonic and oxalic esters (XXIX–XXXI) were prepared by standard methods. Alkylation of 2,4,5-trichlorophenol with dichlorodiethyl ether gave the ether (XXXII).

(5) HETEROCYCLIC COMPOUNDS

The benzimidazoles (XXXIII and XXXIV) were prepared from 4-chloro-*o*-phenylene diamine. Treatment of cyanuric chloride with 3,4-dichloro-aniline gave the dianilinotriazine (XXXV), which with diethanolamine gave (XXXVI). The phthalimide (XXXVII) was synthesised from tetrachlorophthalic anhydride and 3,4-dichloroaniline, via the phthalamic acid, which readily underwent ring closure.

Experimental

PREPARATION OF COMPOUNDS

3,4,4′-*Trichlorobenzenesulphonanilide* (I). To a solution of *p*-chloroaniline (5 g) in dry pyridine (10 ml) was added 3,4-dichlorobenzenesulphonyl chloride (10 g). After 15 min the mixture was cooled, poured into water and acidified with concentrated hydrochloric acid. The product was filtered, washed with water and dried *in vacuo* (13·0 g, 98%, m.p. 126–8°). Recrystallisation from aqueous ethanol afforded the *sulphonanilide*, m.p. 131–3°. Found: C, 42·8; H, 2·4; Cl, 31·6; N, 4·2; S, 9·5. $C_{12}H_8C_{13}NO_2S$ requires C, 42·9; H, 2·1; Cl, 31·9; N, 4·3; S, 9·5%.

3,4,3′,4′-*Tetrachlorobenzenesulphonanilide* (II). In a similar manner 3,4-dichloroaniline (16 g), pyridine (25 ml) and 3,4-dichlorobenzenesulphonyl chloride (25 g) gave 3,4,3′,4′-*tetrachlorobenzenesulphonanilide*, which separated from aqueous ethanol in colourless needles, m.p. 140–2°. Found: C, 38·6; H, 1·9; Cl, 37·8. $C_{12}H_7Cl_4NO_2S$ requires C, 38·8; H, 1·9; Cl, 38·2%.

N-*Methyl*-3,4,3′,4′-*tetrachlorobenzenesulphonanilide* (III). To a solution of the above sulphonanilide (20 g) in methanol (60 ml) and 10% aqueous sodium hydroxide solution (30 ml) at 25° was added dropwise with stirring dimethyl sulphate (10·2 g). When the addition was complete the temperature was raised to 40° for 1 hr and the solution cooled and filtered. N-*Methyl*-3,4,3′,4′-*tetrachlorobenzenesulphonanilide* (15·0 g) separated from ethanol in colourless needles. Found: C, 40·8; H, 2·7; Cl, 37·0. $C_{13}H_9Cl_4NO_2S$ requires C, 40·5; H, 2·4; Cl, 36·8%.

N-*n*-*Butyl*-3,4,3′,4′-*tetrachlorobenzenesulphonanilide* (IV). To the tetrachlorobenzenesulphonanilide (15 g) and butyl bromide (15 g) in methanol (60 ml) was added 2N aqueous sodium hydroxide (20 ml) and the mixture boiled under reflux for 4 hr. The N-*butyl derivative* separated out on cooling. Recrystallisation from methanol afforded colourless needles, m.p. 83–4°. Found: C, 45·2; H, 3·9. $C_{16}H_{15}Cl_4NO_2S$ requires C, 45·0 H, 3·5%.

N-*n*-*Dodecyl*-3,4,3′,4′-*tetrachlorobenzenesulphonanilide* (V). Prepared in a similar manner to the butyl derivative, the N-*dodecyl analogue* separated from methanol in colourless needles, m.p. 83–4°. Found: C, 53·2; H, 5·8. $C_{24}H_{31}Cl_4NO_2S$ requires C, 53·4; H, 5·8%.

4,4′-*Dibromosulphanilide* (VI). Prepared by the method of Wohl & Koch (1910) 4,4′-dibromosulphanilide had m.p. 122–3° (lit. 124–5°).

Urea and thiourea derivatives (VII–XXI). With the exception of (XI), (XVII) and (XX) (see below) all compounds in this class were prepared by one of the following methods (see Table 2).

Method A. To a solution of the amine (0·1M) in dry benzene (100 ml) was added dropwise with stirring 0·1M (0·2M for diamines) of the appropriate isocyanate or isothiocyanate in the same solvent (100 ml). The mixture was then boiled under reflux for 15 min, cooled, and the product filtered and recrystallised from a suitable solvent.

Method B. (Thiocarbanilides only.) The carbon disulphide-iodine method described by Fry (1913).

Thiocarbanilide (XI) was prepared by the method of Vogel (1948) and its 4,4′-diethoxy derivative (XVII) by that of von Braun & Beschke (1906). 3,4-Dichlorophenylthiourea (XX) was made by the method described (Kurzer, 1951) for the 2-chloro-analogue.

2,4,3′,4′-*Tetrachlorobenzanilide* (XXII). A mixture of 3,4-dichloroaniline (17 g) and 2,4-dichlorobenzoyl chloride (10·5 g) was heated on a steam-bath for 1½ hr. The cooled residue was crushed, extracted with several portions of hot 0·1N HCl and finally with water. Recrystallisation from aqueous ethanol gave 2,4,3′,4′-*tetrachlorobenzanilide* in colourless needles, m.p. 155–6°. Found: C, 46·8; H, 2·1; Cl, 42·1. $C_{13}H_7Cl_4NO$ requires C, 46·6; H, 2·1; Cl, 42·3%.

2′-*Amino*-2,4-*dichlorobenzanilide* (XXIII). To a solution of *o*-phenylene diamine (20 g) in chloroform (250 ml) was added dropwise with stirring a solution of 2,4-dichlorobenzoyl chloride (20 g) in chloroform (70 ml). The mixture was heated under reflux for a further hour, cooled and filtered, and the product washed well with water. The residue (19·2 g, m.p. 164–172°) on recrystallisation from benzene formed colourless plates, m.p. 175–9°. Found: C, 55·8; H, 3·8; Cl, 25·2. $C_{13}H_{10}Cl_2N_2O$ requires C, 55·5; H, 3·6; Cl, 25·2%.

N,N′-*Di*(2,4-*dichlorobenzoyl*)*hydrazine* (XXIV). To hydrazine hydrate (40 g of 80%) in ethanol (100 ml) was added ethyl 2,4-dichlorobenzoate (50 g) in ethanol (100 ml) and the solution boiled under reflux for 30 min. After removal of solvent under reduced pressure the residue was recrystallised from ethanol to give N,N′-*di*(2,4-*dichlorobenzoyl*)*hydrazine* (31 g), m.p. 264–265°. Found: Cl, 37·3; N, 7·2. $C_{14}H_8Cl_4N_2O_2$ requires Cl, 37·5; N, 7·4%.

N,N′-*Di*(3,4-*dichlorophenyl*)*oxamide* (XXV). To a solution of 3,4-dichloroaniline (30 g) in dry benzene (200 ml) was added dropwise with stirring oxalyl chloride (7 g) in dry benzene (50 ml). Stirring was continued for 30 min after the addition was complete. Water (700 ml) was then added and the N,N′-*di*(3,4-*dichlorophenyl*)*oxamide* (15.9 g, m.p.

222–6°) filtered off and washed with water. Recrystallisation from benzene raised the m.p. to 226–8°. Found: C, 44·2; H, 2·3. Calculated for $C_{14}H_8Cl_4N_2O_2$, C, 44·4; H, 2·1%. (Beaver & others, 1957, quote m.p. 228·2–229·1°).

N-(3,4-*Dichlorophenyl*)-2,4,5-*trichlorophenoxyacetamide* (XXVI). A mixture of 2,4,5-trichlorophenoxyacetic acid (9·8 g) and thionyl chloride (35 ml) was boiled under reflux for 30 min. Dry benzene (35 ml) was then added and the solution evaporated under reduced pressure. A further portion (20 ml) of benzene was added and similarly removed. To the residual acid chloride in dry benzene (35 ml) was added dropwise a solution of 3,4-dichloroaniline (15 g) in 100 ml of benzene. After completion of the addition, the mixture was poured into excess of dilute HCl and filtered. The N-(3,4-*dichlorophenyl*)-2,4,5-*trichlorophenoxyacetamide* (15 g) had m.p. 202–7°, raised to 217–220° after recrystallisation from benzene and from ethanol. Found: N, 3·6. $C_{14}H_8Cl_5NO_2$ requires N, 3·5%.

N,N′-*Di*(3,4-*dichlorophenyl*)*fumaramide* (XXVII). The use of fumaryl chloride (7 g) in place of oxalyl chloride (7 g) in the preparation of (XXV) led to N,N′-*di*(3,4-*dichlorophenyl*)*fumaramide* (14·1 g), m.p. 337–340°. Found: C, 47·5; H, 2·7; Cl, 35·0. Calculated for $C_{16}H_{10}Cl_4N_2O_2$: C, 47·5; H, 2·5; Cl, 35·1%. (Beaver & others, 1957, record m.p. 227–9°).

2,4,3′,4′-*Tetrachlorocinnamanilide* (XXVIII). To a solution of 3,4-dichloroaniline (7·8 g) and pyridine (5 g) in dry benzene (70 ml) was added dropwise 3,4-dichlorocinnamoyl chloride (7·5 g) in dry benzene (100 ml). After a further 30 min at room temperature the solid was filtered off, washed with water and recrystallised from ethanol to give 2,4,3′,4′-*tetrachlorocinnamanilide* in colourless needles, m.p. 205°. Found: C, 49·9; H, 2·65. $C_{15}H_9Cl_4NO$ requires C, 49·9; H, 2·5%.

2,4,5-*Trichlorophenyl* 3,4-*dichlorobenzoate* (XXIX). To a solution of 2,4,5-trichlorophenol (10 g) in benzene (100 ml) and pyridine (10 ml) was added 3,4-dichlorobenzoyl chloride (10·5 g). After 30 min at room temperature the mixture was diluted with N HCl (200 ml), extracted with ether and the extract washed successively with 0·5N aqueous sodium carbonate and with water. Evaporation of the dried ether extract and recrystallisation from ethanol gave 2,4,5-*trichlorophenyl* 3,4-*dichlorobenzoate*, m.p. 142–3°. Found: C, 42·4; H, 1·5. $C_{13}H_5Cl_5O_2$ requires C, 42·1; H, 1·4%.

Di(4-*chlorophenyl*) *carbonate* (XXX). To a solution of *p*-chlorophenol (16 g) in a mixture of dry acetone (50 ml) and pyridine (25 ml) was added dropwise a solution of phosgene in toluene (38·5 g of 12½%). After 2½ hr at room temperature, the mixture was diluted with water, extracted with ether and the ether extract washed with 5% aqueous sodium hydroxide solution. Evaporation of the ether, followed by recrystallisation from ethanol, gave di(*p*-chlorophenyl) carbonate (6·4 g), m.p. 149–151°; British Patent 753,766 (1956), m.p. 149°.

Di(4-*chlorophenyl*) *oxalate* (XXXI). To a solution of *p*-chlorophenol (15 g) in dry benzene (100 ml) and pyridine (20 g) was added dropwise

with stirring a solution of oxalyl chloride (6·4 g) in dry benzene (25 ml) the temperature being kept below 30°. After a further 30 min at 20–30°, the mixture was diluted with ether (100 ml) and water (200 ml) and the *di*(4-*chlorophenyl*) *oxalate* (8·7 g) collected by filtration. A further quantity (0·8 g) was obtained by evaporation of the solvent. An analytical sample separated from benzene in colourless needles, m.p. 186–7°. Found: C, 54·3; H, 2·4; Cl, 22·5. $C_{14}H_8Cl_2O_4$ requires C, 54·1; H, 2·6; Cl, 22·8%.

Di[2-(2,4,5-*trichlorophenoxy*)*ethyl*] *ether* (XXXII). A mixture of 2,4,5-trichlorophenol (54·4 g), di(2-chloroethyl) ether (20 g), sodium hydroxide (11·2 g) and 50% (v/v) aqueous ethanol (60 ml) was boiled under reflux for 24 hr. Dilution of the cold solution with water precipitated *di*[2-(2,4,5-*trichlorophenoxyethyl*)] *ether* (46·3 g), which on recrystallisation from methanol had m.p. 92–3°. Found: C, 41·5; H, 2·55. $C_{16}H_{12}Cl_6O_3$ requires C, 31·3; H, 2·6%.

5-*Chloro*-2-*mercaptobenzimidazole* (XXXIII). The use of 4-chloro-*o*-phenylene diamine in the method described (Allan & Deacon, 1950) for 2-mercaptobenzimidazole gave the 5-*chloro-derivative*, m.p. 305° (decomp.). Knobloch, Winkelmann & Rintelen (1958) quote m.p. 290–292 .

5-*Chloro*-2-(2,4-*dichlorophenyl*)*benzimidazole* (XXXIV). To a solution of 4-chloro-*o*-phenylene diamine (6 g) and copper acetate (16 g) in 50% aqueous methanol (200 ml) was added 2,4-dichlorobenzaldehyde (8·4 g) in methanol (75 ml). The mixture was boiled under reflux for 30 min, cooled and filtered. The copper salt was suspended in 50% (v/v) aqueous ethanol at 50°, treated with hydrogen sulphide, filtered and the residue washed well with hot ethanol. 5-*Chloro*-2-(2,4-*dichlorophenyl*)*benzimidazole* (4·4 g) separated from the combined filtrates on cooling and had m.p. 184–5° after recrystallisation from aqueous ethanol. Found: C, 52·1; H, 2·6; Cl, 35·7; N, 9·7. Calculated for $C_{13}H_7Cl_3N_2$: C, 52·4; H, 2·35; Cl, 35·8; N, 9·4%. Subba Row & Ratnam (1958) found m.p. 184°.

2-*Chloro*-4,6-*di*(3,4-*dichloroanilino*)-1,3,5-*triazine* (XXXV). To ice-cold water (400 ml) was added dropwise with stirring a solution of cyanuric chloride (27·6 g) in acetone (120 ml), followed by a solution of 3,4-dichloroaniline (49 g) in acetone (150 ml), the temperature being maintained at 0–8°. A solution of sodium bicarbonate (25·2 g) in water (200 ml) was then added and the reaction temperature raised to 45–50° for 2 hr. The mixture was then cooled and the 2-*chloro*-4,6-*di*(3,4-*dichloroanilino*)-1,3,5-*triazine* (61·6 g) collected by filtration, washed well with water and dried *in vacuo* at 50–60°. A portion recrystallised from aqueous acetone had m.p. 243–4°. Found: Cl, 40·8. $C_{15}H_8Cl_5N_5$ requires Cl, 40·8%.

4,6-*Di*(3,4-*dichloroanilino*)-2-[*di*(2-*hydroxyethyl*)*amino*]-1,3,5-*triazine* (XXXVI). A mixture of the above chlorotriazine (9 g) and diethanolamine (9 g) was heated to 100° for 2 hr, cooled and diluted with water. The precipitated 4,6-*di*(3,4-*dichloroanilino*)-2-[*di*(2-*hydroxyethyl*)*amino*]-1,3,5-*triazine* (9·3 g) had m.p. 179–185°, raised to 188–190° after recrystallisation from ethanol. Found: Cl, 28·3. $C_{19}H_{18}Cl_4N_6O_2$ requires Cl, 28·2%.

3,4,5,6-*Tetrachloro*-N-(3,4-*dichlorophenyl*)*phthalimide* (XXXVII). To a solution of 3,4,5,6-tetrachlorophthalic anhydride (14·3 g) in dry benzene (300 ml) was added 3,4-dichloroaniline (10 g) in the same solvent (50 ml). The mixture was boiled under reflux for 15 min, cooled and filtered. The product (21·8 g), presumably 2-carboxy-3,4,5,6,3′,4′-hexachlorobenzanilide, showed a change of crystal form at 183° and melted at 290–292°. Attempted crystallisation from ethanol converted the material to 3,4,5,6-*tetrachloro*-N-(3,4-*dichlorophenyl*)*phthalimide*, a sample of which, after recrystallisation from dimethyl formamide followed by sublimation at 260–270°/15 mm, formed pale yellow needles, m.p. 296–298°. Found: Cl, 49·9; N, 3·4. $C_{14}H_3Cl_6NO_2$ requires Cl, 49·5; N, 3·3%.

SCREENING TESTS

Determination of minimum bacteriostatic concentrations and of soap inactivation coefficients was carried out as described by Hurst, Stuttard & Woodroffe (1960).

Results and discussion

(1) SULPHONAMIDE BRIDGE COMPOUNDS

The sulphonanilides (I and II) showed moderate activity against *Staphylococcus aureus* but much less against *Escherichia coli* (see Table 1). Activity was lost on alkylation (III, IV, V). It is now generally accepted that, as postulated by Woods (1940) for sulphanilamide itself, many sulphanilamide derivatives act by interfering with the utilisation of *p*-aminobenzoic acid (PABA). Bell & Roblin (1942) showed that for such compounds activity could be related to the pK_a (optimum activity at pK_a of about 6·5) and that in general the ionised form of the compound was more active than the un-ionised. The peak in activity has been accounted for in terms of the negative character of the sulphonyl group (Bell & Roblin, 1942) and in terms of penetration of the cell-wall by an un-ionised molecule, which subsequently ionises within the cell (Cowles, 1942; Brueckner, 1943; Klotz, 1944). By contrast, sulphanilanilides (i.e., *N*-phenylsulphanilamides) do not appear to act by interference with PABA utilisation, at any rate against Gram-positive organisms. Schmidt & Sesler (1946) (see also Goetchius & Lawrence, 1945) showed that the activity of the 3′,5′-dibromo-derivative and of several related compounds against a pneumococcus and against a β-haemolytic streptococcus was not antagonised by PABA, although that against Gram-negative bacteria (Friendlander's bacillus, *E. coli*) was antagonised. Evidence on the mechanism of action of the corresponding compounds without the *p*-amino-group (i.e., benzenesulphonanilides) is lacking but it would seem likely from our examples that the ionised form is again much more active than the un-ionised, since alkylation of the nitrogen atom (which precludes ionisation) destroys activity. The sulphanilide (VI) showed weak but similar activity against *Staph. aureus* and *E. coli*, in contrast to the sulphonanilides (I and II) which, like the conventional sulphonamides, showed much greater activity against the former than the latter.

TABLE 1. COMPOUNDS OF THE TYPE R—X—R′

Compound No.	R	X	R′	Minimum inhibitory concentration (μg/ml) against *Staph. aureus*	Minimum inhibitory concentration (μg/ml) against *E. coli*
Sulphonamide bridges—					
I	3,4-Dichlorophenyl	—SO_2·NH—	4-Chlorophenyl	6	100
II	3,4-Dichlorophenyl	—SO_2·NH—	3,4-Dichlorophenyl	12	>100
III	3,4-Dichlorophenyl	—SO_2·N(CH_3)—	3,4-Dichlorophenyl	>100	>100
IV	3,4-Dichlorophenyl	—SO_2·N(*n*—C_4H_9)—	3,4-Dichlorophenyl	>100	>100
V	3,4-Dichlorophenyl	—SO_2·N(*n*—$C_{12}H_{25}$)—	3,4-Dichlorophenyl	>100	>100
VI	4-Bromophenyl	—NH·SO_2·NH—	4-Bromophenyl	50	50
Urea and thiourea bridges—					
VII	4-Chlorophenyl	—NH·CO·NH—	4-Chlorophenyl	100	100
VIII	4-Chlorophenyl	—NH·CO·NH—	n-Dodecyl	50	50
IX	4-Chlorophenyl	—NH·CO·NH[CH_2]$_6$·NH·CO·NH—	4-Chlorophenyl	100	100
X	4-Chlorophenyl	—NH·CO·NH·CH_2(CHOH)·CH_2·NH·CO·NH—	4-Chlorophenyl	>100	>100
XI	Phenyl	—NH·CS·NH—	Phenyl	100	50
XII	3-Chlorophenyl	—NH·CS·NH—	3-Chlorophenyl	6	25
XIII	4-Chlorophenyl	—NH·CS·NH—	Phenyl	12	50
XIV	4-Chlorophenyl	—NH·CS·NH—	4-Chlorophenyl	3	100
XV	3,4-Dichlorophenyl	—NH·CS·NH—	4-Chlorophenyl	0·1	50
XVI	3,4-Dichlorophenyl	—NH·CS·NH—	3,4-Dichlorophenyl	0·1	100
XVII	4-Ethoxyphenyl	—NH·CS·NH—	4-Ethoxyphenyl	100	100
XVIII	4-Chloro-2-methoxyphenyl	—NH·CS·NH—	4-Chloro-2-methoxyphenyl	100	50
XIX	4-Chlorophenyl	—NH·CS·NH—	4-Carboxyphenyl	50	50
XX	3,4-Dichlorophenyl	—NH·CS·NH—	H	3	25
XXI	4-Chlorophenyl	—NH·CS·NH·CH_2·CH_2·NH·CS·NH—	4-Chlorophenyl	25	50
Amide bridges—					
XXII	2,4-Dichlorophenyl	—CO·NH—	3,4-Dichlorophenyl	50	100
XXIII	2,4-Dichlorophenyl	—CO·NH—	2-Aminophenyl	50	50
XXIV	2,4-Dichlorophenyl	—CO·NH·NH·CO—	2,4-Dichlorophenyl	50	50
XXV	2,4-Dichlorophenyl	—NH·CO·CO·NH—	3,4-Dichlorophenyl	100	100
XXVI	2,4,5-Trichlorophenyl	—O·CH_2·CO·NH—	3,4-Dichlorophenyl	100	100
XXVII	3,4-Dichlorophenyl	—NH·CO·CH : CH·CO·NH— (*trans*)	3,4-Dichlorophenyl	12	50
XXVIII	2,4-Dichlorophenyl	—CH : CH·CO·NH— (*trans*)	3,4-Dichlorophenyl	>100	>200
Other bridges—					
XXIX	3,4-Dichlorophenyl	—CO·O—	2,4,5-Trichlorophenyl	3	>100
XXX	4-Chlorophenyl	—O·CO·O—	4-Chlorophenyl	50	100
XXXI	4-Chlorophenyl	—O·CO·CO·O—	4-Chlorophenyl	50	25
XXXII	2,4,5-Trichlorophenyl	—O·CH_2·CH_2·O·CH_2·CH_2·O—	2,4,5-Trichlorophenyl	>100	>100
Heterocyclic compounds—					
XXXIII	5-Chloro-2-mercaptobenzimidazole			50	50
XXXIV	5-Chloro-2-(2,4-dichlorophenyl)benzimidazole			100	50
XXXV	2-Chloro-4,6-di(3.4-dichloroanilino)-1,3,5-triazine			50	50
XXXVI	4,6-Di(3,4-dichloranilino)-2-[di(2-hydroxyethyl)amino]-1,3,5-triazine			100	100
XXXVII	3,4,5,6-Tetrachloro-*N*-(3,4-dichlorophenyl)-phthalimide			25	100

TABLE 2. PREPARATION OF UREA AND THIOUREA DERIVATIVES

Compound No.	Method	Recrystallisation solvent	Found (%)					Formula	Required (%)					m.p. (°C)	Lit. m.p. (°C)
			C	H	Cl	N	S		C	H	Cl	N	S		
VII	A	Acetone	—	—	—	—	—	$C_{13}H_{10}Cl_2N_2O$	—	—	—	—	—	308–10	310(*a*)
VIII ..	A	Benzene	67·6	8·8	—	—	—	$C_{19}H_{21}ClN_2O$	67·4	9·2	—	—	—	126–7	—
IX	A	Dimethyl formamide	—	—	16·9	—	—	$C_{20}H_{24}Cl_2N_4O_2$			16·8	—	—	249–50	—
X	A*	Acetic acid	51·3	4·8	17·6	—	—	$C_{17}H_{18}Cl_2N_4O_3$	51·4	4·5	17·9	—	—	237–8	—
XI	See text	Aqueous ethanol	—	—	—	—	—	$C_{13}H_{12}N_2S$	—	—	—	—	—	152–3	154(*b*)
XII	B	Benzene	—	—	23·9	9·7	10·9	$C_{13}H_{10}Cl_2N_2S$	—	—	23·9	9·4	10·8	120–2	121–2(*c*)
XIII ..	A	Ethanol	—	—	—	—	—	$C_{13}H_{11}ClN_2S$	—	—	—	—	—	152–3	152(*d*)
XIV ..	B	Benzene	—	—	—	9·6	10·7	$C_{13}H_{10}Cl_2N_2S$	—	—	—	9·4	10·8	175–6	176(*e*)
XV	A	Benzene	47·1	2·8	—	—	—	$C_{13}H_9Cl_3N_2S$	47·1	2·7	—	—	—	154–5	154·2–154·9(*f*)
XVI ..	B	Benzene	—	—	—	7·3	8·7	$C_{13}H_9Cl_4N_2S$	—	—	—	7·6	8·7	160–1	162·6–163·5(*f*); 144(*e*)
XVII ..	See text	Ethanol	—	—	—	8·6	10·3	$C_{17}H_{20}N_2O_2S$	—	—	—	8·9	10·1	169–70	170(*g*)
XVIII ..	B	Benzene	—	—	—	—	—	$C_{15}H_{14}Cl_2N_2O_2S$	—	—	—	—	—	162–3	152–5(*h*)
XIX	A	Ethanol	54·8	3·4	11·4	8·95	10·5	$C_{14}H_{11}ClN_2O_2S$	54·8	3·6	11·6	9·1	10·4	200 (dec.)	—
XX	See text	Acetic acid	—	—	—	—	—	$C_7H_8N_2S$	—	—	—	—	—	203–5	203–4(*i*); 164(*e*)
XXI ..	A	Acetic acid	—	—	—	14·1	—	$C_{10}H_{10}Cl_2N_4S$	—	—	—	—	—	192–4	—

* Temperature kept below 40° during reaction. (*a*) Chattaway & Orton (1901). (*b*) Vogel (1948). (*c*) Fry (1913). (*d*) Kjellin (1903). (*e*) Dyson, George & Hunter (1926). (*f*) Beaver, Roman & Stollel (1957). (*g*) Braun & Beschke (1906). (*h*) Herold (1882). (*i*) Douglass & Dains (1934).

TABLE 3. SOAP INACTIVATION CONSTANTS (S.I.C.) AGAINST *Staph. aureus* OF SOME OF THE COMPOUNDS LISTED IN TABLE 1

Compound	Concentration in soap (μg/ml)	S.I.C.
I	2000	1·7
VI	500	1·6
XV	62·5	1·5
XVI	31·2	1·2
XIX	500	1·0

(2) UREA AND THIOUREA BRIDGE COMPOUNDS

TCC (3,4,4′-trichlorocarbanilide) is known to be a bacteriostat, but when this work was begun not many analogues appeared to have been studied. However, fairly soon afterwards Beaver & others (1957) recorded the synthesis of 205 urea, thiourea and related derivatives and showed that peak activity occurred in the 3,4,3′- and 3,4,4′-trichlorocarbanilides, both of which inhibit *Micrococcus pyogenes* var. *aureus* at 1 in 3×10^7. It is of interest that the thio-analogues, such as "Thio-TCC" (XV), show a similar order of activity, although they are unlikely to have a comparable utility owing to their relatively poor stability in mildly alkaline media such as soap. Peak activity seems to be in the compounds with a 3,4-dichlorophenyl group, not only in the carbanilides and thiocarbanilides, but also in the related isothiocyanates, dibenzylthioureas and benzyl dithiocarbamate esters (McKay & others, 1959). It is somewhat surprising that, although optimum activity in the ureas and thioureas occurs in compounds with a suitably halogenated pair of aromatic rings, significant activity can occur in compounds in which one ring is absent (XX) or replaced by a long aliphatic chain (VIII). The coupling of two urea or thiourea groups by various chains (IX, X, XXI) did not lead to enhanced activity.

(3) AMIDE BRIDGES

The only amide showing appreciable activity was the fumaramide (XXVII), which inhibited *Staph. aureus* at 12·5 μg/ml.

(4) OTHER BRIDGES

Of these, notable activity was shown only by a chlorinated phenyl benzoate (XXIX).

(5) HETEROCYCLIC COMPOUNDS

No appreciable activity was observed within this group. High activity has previously been recorded (Jerchel, Fischer & Kracht, 1952) for a phenolic benzimidazole related to XXXIV. Some anilino-triazine analogues of XXXV and XXXVI have been claimed as fungicides (Wolf, 1955).

The soap inactivation coefficients of some of the more active compounds are shown in Table 3.

Some further examples of the more promising types of compound are being studied.

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The analgesic properties of some 14-substituted derivatives of codeine and codeinone

W. R. BUCKETT, MURIEL E. FARQUHARSON AND C. G. HAINING

The effects of 14-hydroxylation and subsequent 14-acylation on the toxicity and analgesic activity of codeine, codeine-6-acetate, codeinone, and Δ^7-deoxycodeine have been examined in rats and mice. Acute toxicity was reduced in each instance by the introduction of a 14-hydroxy group and was not generally enhanced by its esterification. 14-Acetoxycodeine was approximately equal to morphine in potency and esterification at the 14-position of hydroxycodeine with other straight chain aliphatic acids containing up to 5 carbon atoms failed to enhance analgesic potency further. 14-Benzoylation of either 14-hydroxycodeine or 14-hydroxycodeinone had little effect on analgesic activity but the introduction of a methylene group between the carboxyl group and the phenyl ring enhanced potency considerably in each case. Increasing the number of carbon atoms from 2 to 5 in the 14-acyl groups of esters of 14-hydroxycodeinone and 14-hydroxy-Δ^7-deoxycodeine led to a gradual increase in analgesic activity. In rats the n-valeryl ester of 14-hydroxy-Δ^7-deoxycodeine was estimated to have 75 times the potency of morphine.

THE properties of morphine derivatives are modified by the introduction of a hydroxyl group at position 14. Reduction of acute toxicity to mice occurs with dihydrocodeine (Winder, Jones, Weston & Gajewski, 1959), dihydrocodeinone (Krueger, Eddy & Sumwalt, 1943) and dihydromorphinone (Blumberg, Carson & Stein, 1954). The effect on analgesic properties is variable; the potencies of dihydrocodeinone (Krueger, Eddy & Sumwalt, 1943) and codeine (Sargent, Schwartzman & Small, 1958) remain unchanged whilst that of dihydromorphinone (Blumberg & others, 1954) is slightly enhanced.

There is little information on effects resulting from acylating the 14-hydroxyl group; Sargent & others (1958) described only the preparation of 14-acetoxycodeine-6-acetate, but 14-acetoxycodeinone is less toxic and slightly more potent as an analgesic than 14-hydroxycodeinone (Krueger & others, 1943).

The availability of compounds listed in Table 1 (Currie, Gillon, Newbold & Spring, 1960) enabled us to study systematically the effects of both hydroxylation and subsequent acylation at the 14-position on the toxicity and analgesic properties of codeine (series I), codeine-6-acetate (series II), codeinone (series III) and Δ^7-deoxycodeine (series IV).

Methods

ACUTE TOXICITY IN MICE

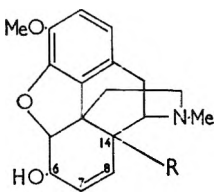
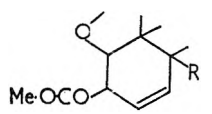
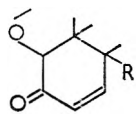
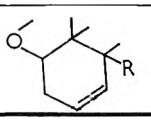
The subcutaneous LD50 of each compound was determined using albino mice weighing between 18–22 g. Compounds were dissolved in 0·9% w/v sodium chloride and administered in a volume of 0·2 ml/20 g body weight. Mortalities were recorded 24 hr later.

From the Research Department, Edinburgh Pharmaceutical Industries Limited, Wheatfield Road, Edinburgh, 11.

ANALGESIA IN RATS

Analgesia was determined by a modification of the method described by Green & Young (1951), in which pressure is applied to the tail by the plunger of a hypodermic syringe. The barrel of the syringe is connected to a reservoir almost completely filled with an aqueous solution of glycerol 50% v/v connected to a source of compressed air. Normally the air is allowed to leak away through a small hole at the top of the reservoir but when the hole is closed with the finger the rise in pressure forced the plunger of the syringe downwards on to the tail. Inflow of air is adjusted so that the pressure within the reservoir rose at a rate of approximately 2 cm Hg/sec.

TABLE 1. COMPOUNDS INVESTIGATED

Series	No.	Name
I 	1	Codeine phosphate
	2	14-Hydroxycodeine hydrochloride
	3	14-Acetoxycodeine hydrochloride
	4	14-Propionoxycodeine hydrochloride
	5	14-n-Butyryloxycodeine hydrochloride
	6	14-n-Valeryloxycodeine hydrochloride
	7	14-Benzoyloxycodeine hydrochloride
	8	14-Phenylacetoxycodeine hydrochloride
	9	14-Nicotinoyloxycodeine dihydrochloride
II 	10	Acetylcodeine acid tartrate
	11	14-Hydroxycodeine-6-acetate hydrochloride
	12	14-Acetoxycodeine-6-acetate
	13	14-Propionoxycodeine-6-acetate hydrochloride
	14	14-Benzoyloxycodeine-6-acetate hydrochloride
III 	15	Codeinone
	16	14-Hydroxycodeinone
	17	14-Propionoxycodeinone
	18	14-n-Butyryloxycodeinone
	19	14-n-Valeryloxycodeinone
	20	14-Phenylacetoxycodeinone
	21	14-Nicotinoyloxycodeinone
IV 	22	14-Hydroxy-Δ^7-deoxycodeine hydrochloride
	23	14-Acetoxy-Δ^7-deoxycodeine
	24	14-n-Butyryloxy-Δ^7-deoxycodeine
	25	14-n-Valeryloxy-Δ^7-deoxycodeine

The analgesic effect of compounds was determined 30 min after subcutaneous injection of drug in normal saline or 60 min after oral administration of a solution in water. Control animals received the appropriate solvent only. In all estimates of potency at least three dose levels of drug were employed and compounds were compared with morphine on the same day.

Female Wistar rats weighing between 30 and 60 g were distributed randomly into groups of 10. The mean pressure required to elicit a squeak was determined for control animals and the proportion of animals in each treated group which failed to respond at twice this pressure was recorded.

ANALGESIA IN MICE

Estimates of analgesic activity were made using the method of Bianchi & Franceschini (1954). Albino mice weighing 18–22 g were tested for sensitivity to a bulldog artery clip covered with rubber tubing, which was applied to the base of the tail and only those attempting to remove the clip within 15 sec were used. Each compound was administered subcutaneously at three of four dose levels to groups of 10 animals. Animals were tested 30 min later and the proportion in each group which made no attempt to remove the clip within 30 sec was determined.

RESPIRATORY RATE

The respiratory rate in unanaesthetised Wistar rats weighing 40–50 g was determined before and 30 min after subcutaneous injection of drug solution. Groups of five animals were used at each dose level, the animals being loosely restricted in a rigid plastic tube attached to a membrane micromanometer (Infra Red Development Co.) and pen recorder. The dose needed to reduce the respiratory rate by 50% was estimated graphically.

GASTROINTESTINAL EFFECTS

The influence of drugs on intestinal motility in mice was studied using groups of ten animals isolated in individual cages with a wire mesh floor. The faecal weight of drug-treated groups was compared with that of a control group receiving normal saline. The percentage reduction in faecal weight obtained during 2 hr after subcutaneous administration of drug was determined, and relative potency calculated quantally with respect to morphine.

The antagonism of the compounds to acetylcholine, histamine and barium chloride on the isolated guinea-pig ileum was studied using the superfusion method of Adam, Hardwick & Spencer (1954).

Calculations of the LD50, the ED50 and relative potency in experiments giving quantal results were carried out by the method of Litchfield & Wilcoxon (1949).

Results

ACUTE TOXICITY

Death generally occurred within 3 hr after subcutaneous administration, and appeared to be due to respiratory failure. Except in the instance of codeinone and 14-hydroxycodeinone, which were convulsants, the symptoms at near toxic dose levels consisted of catalepsy without loss of righting reflex and acute respiratory depression. The LD50 was determined from mortalities recorded 24 hr after drug administration (Table 2).

Hydroxyl substitution at position 14 caused more than a fivefold reduction in the acute toxicity of codeine or codeine-6-acetate and the effect on Δ^7-deoxycodeine appeared to be similar. No estimate of the LD50 of Δ^7-deoxycodeine was obtained in the present work but the calculated subcutaneous LD50 of the base in mice determined by Karrer

& Widmark (1951) was 60 mg/kg compared with 700 mg/kg for 14-hydroxy-Δ^7-deoxycodeine. The introduction of a 14-hydroxy group into the codeinone molecule was much less effective, the LD50 increasing only from 11 to 28 mg/kg.

Esterification of the 14-hydroxyl group did not produce marked changes in the acute toxicity of 14-hydroxycodeine. When esters of the following acids were examined; nicotinic, benzoic, phenylacetic and the homologous series from acetic to valeric the range of LD50s determined varied only from 400 to 640 mg/kg. Similar results were obtained with

TABLE 2. THE ACUTE TOXICITY OF CODEINES AND CODEINONES IN ALBINO MICE AFTER SUBCUTANEOUS ADMINISTRATION

[All values are calculated in terms of anhydrous base. Limits of error (P = 0·95) shown in parentheses]

	Codeine derivatives		Codeine-6-acetate derivatives		Codeinone derivatives		Δ^7-Deoxycodeine derivatives	
14-Substituent	Compound No.	LD50 mg/kg	Compound No.	LD50 mg/kg	Compound No.	LD50 mg/kg	Compound No.	LD50 mg/kg
None	1	120 (100–150)	10	140 (120–170)	15	11·0 (9·8–12·3)		60†
-OH	2	880 (730–1050)	11	760 (570–1000)	16	28 (25–30)	22	700 (600–830)
–OCOMe	3	560 (480–660)	12	630 (530–750)		127*	23	>500
–OCOEt	4	420 (360–470)	13	430 (340–560)	17	150 (110–190)		
–OCOPr	5	640 (580–750)					24	180 (160–210)
-OCOBu	6	490 (390–610)			19	495 (410–600)		
–OCO  ..	7	400 (340–460)	14	>1000				
–OCOCH$_2$  ..	8	500			20	300		
-OCO  ..	9	500			21	100–300		

Morphine (estimated as hydrochloride) = 330 (280–390)

† Karrer & Widmark (1951) * Krueger, Eddy & Sumwalt (1943)

the esters of 14-hydroxycodeine-6-acetate examined, but increasing the size of the 14-substituent in the codeinone series reduced toxicity. In the case of 14-hydroxy-Δ^7-deoxycodeine however the results suggest that enhanced toxicity might be expected with increasing length of the alkyl chain.

ANALGESIA

The effects of substitution at position 14 of codeine-6-acetate, codeinone and Δ^7-deoxycodeine on analgesic activity in rats at 30 min after subcutaneous injection are most clearly seen in relation to the results obtained with derivatives of codeine (Table 3). 14-Hydroxycodeine did not differ

in potency from codeine but acylation of the 14-alcoholic hydroxyl group generally enhanced activity. Those derivatives in which the acyl group contained an unbranched alkyl chain equalled morphine in potency but it was clear that the enhanced activity was independent of chain length since increasing the number of carbon atoms in it from one to four did not produce any marked alterations in potency. The presence of an aromatic or heterocyclic ring adjacent to the carbonyl group was not necessarily advantageous since both the benzoyl and the nicotinoyl esters of 14-hydroxycodeine were only slightly more potent than 14-hydroxycodeine itself, but introduction of a single methylene group

TABLE 3. THE ANALGESIC ACTIVITY OF CODEINES AND CODEINONES IN RATS 30 MIN AFTER SUBCUTANEOUS ADMINISTRATION

[The figures represent relative potency (morphine = 1·0) of compounds in terms of base. Limits of error (P = 0·95) are shown in parentheses]

14-Substituent	Compound No.	Codeine derivatives	Compound No.	Codeine-6-acetate derivatives	Compound No.	Codeinone derivatives	Compound No.	Δ⁷-Decxy-codeine derivatives
None	1	0·17 (0·09–0·31)	10	0·13 (0·08–0·23)	15	*		
–OH	2	0·13 (0·07–0·23)	11	0·20 (0·13–0·22)	16	*	22	0·3[illegible] (0·21–0·42)
–OCOMe	3	1·3 (0·9–2·4)	12	0·11 (0·06–0·19)			23	2·7 (1·7–4·2)
–OCOEt	4	1·3 (0·8–2·3)	13	1·0 (0·7–1·6)	17	2·5 (1·4–4·7)		
–OCOPr	5	1·7 (1·1–2·7)			18	16 (11–25)	24	7·4 (4·6–12·0)
–OCOBu	6	1·0 (0·6–1·7)			19	66 (41–105)	25	75 (37–152)
–OCO  ..	7	0·19 (0·12–0·31)	14	0·05				
–OCOCH₂  ..	8	3·4 (1·9–5·9)			20	**45** (29–68)		
–OCO  ..	9	0·33 (0·21–0·57)			21	0·36 (0·23–0·56)		

* Could not be determined because of toxic excitation

between the carbonyl group and the phenyl ring of 14-benzoyloxycodeine led to a considerable increase in activity.

Similar substitution in codeine-6-acetate did not produce any striking increase in analgesic action. Codeine-6-acetate itself, 14-hydroxycodeine-6-acetate, 14-acetoxycodeine-6-acetate, 14-benzoyloxycodeine-6-acetate and codeine were almost equi-potent but 14-propionoxycodeine-6-acetate equalled morphine in activity.

Extending the chain length of the 14-acyloxy group in the case of codeinone derivatives modified the properties of the resulting compounds considerably. Altering the number of carbon atoms in the straight alkyl chain from two to four increased potency relative to morphine from 2·5 to 66 but, as in the case of codeine derivatives, nicotinoyl substitution was

ineffective whereas phenylacetyl gave a compound estimated to have 45 times the potency of morphine.

Acylation of 14-hydroxy-Δ^7-deoxycodeine by groups containing straight alkyl chains also enhanced analgesic activity. A twentyfive-fold enhancement resulted when the number of carbon atoms in the chain was increased from one to four, 14-n-valeryloxy-Δ^7-deoxycodeine being 75 times as potent as morphine.

It is interesting to note that the minimum number of carbon atoms in the acyloxy group at the 14-position necessary to produce an increase in analgesic potency was not the same in each series. In the case of codeine and Δ^7-deoxycodeine derivatives it occurred with acetoxy and with codeine-6-acetate and codeinone with propionoxy substitution.

Those compounds tested 60 min after oral administration generally exhibited similar potency to that obtained 30 min after subcutaneous injection (Table 4) indicating good intestinal absorption.

TABLE 4. THE ANALGESIC ACTIVITY OF CODEINES AND CODEINONES IN RATS 1 HR AFTER ORAL ADMINISTRATION

[The figures represent relative potency (morphine = 1·0) of compounds in terms of base. Limits of error (P = 0·95) are given in parentheses]

14-Substituent	Compound No.	Codeine derivatives	Compound No.	Codeinone derivatives
–OCOMe	3	0·5 (0·2–1·1)		
–OCOEt	4	0·7 (0·4–1·2)		
–OCOPr	5	1·8 (1·0–3·4)	18	59 (24–117)
–OCOBu	6	0·9 (0·4–2·0)	19	59 (31–114)
–OCOCH$_2$  ..			20	60 (33–111)

Results obtained after subcutaneous injection in mice are given in Table 5. The potencies of compounds relative to morphine were generally estimated to be less than those found in rats by the same route and the length of the alkyl chain in the 14-position necessary to obtain a large increase in potency was greater. Thus in the codeine series acetylation of the 14-hydroxy group gave a ten-fold increase in potency in rats but no change in mice and an approximate five-fold increase was obtained only with the 14-propionic ester. Similarly on esterifying 14-hydroxycodeine-6-acetate with propionic acid the marked increase in potency found in rats was not observed with mice. The 14-n-valeryloxy derivatives of codeinone and Δ^7-deoxycodeine were much less analgesic in mice.

RESPIRATORY RATE

14-n-Butyryloxycodeine was compared with morphine for its effect on respiratory rate in unanaesthetised rats. A linear relationship was obtained between log dose and percentage reduction in rate. It produced

relatively greater depression of the respiratory rate than an equi-analgesic dose of morphine (Table 6). The respiratory depression could be reversed by nalorphine or levallorphan.

CARDIOVASCULAR SYSTEM

14-n-Butyryloxycodeine, 1 mg/kg, produced a transient fall in blood pressure when injected intravenously into dogs anaesthetised with pentobarbitone in contrast to the more prolonged fall obtained after morphine,

TABLE 5. THE ANALGESIC ACTIVITY OF CODEINES AND CODEINONES IN MICE AFTER SUBCUTANEOUS ADMINISTRATION

[The figures represent relative potency (morphine = 1·0) of compounds in terms of base. Limits of error (P = 0·95) are given in parentheses]

14-Substituent	Compound No.	Codeine derivatives	Compound No.	Codeine-6-acetate derivatives	Compound No.	Codeinone derivatives	Compound No.	Δ⁷-Deoxycodeine derivatives
–OH	2	0·12 (0·05–0·28)	11	0·35 (0·20–0·62)	16	*	22	0·34 (0·18–0·62)
–OCOMe	3	0·11 (0·04–0·29)				About ⅓ potency of codeine†	23	0·88 (0·45–1·73)
–OCOEt	4	0·7 (0·3–1·5)	13	0·41 (0·25–0·66)				
–OCOPr	5	0·6 (0·3–1·2)						
–OCOBu	6	0·47 (0·24–0·92)			19	7·8 (3·7–16)	25	4·8 (2·5–9·2)
–OCOCH₂–  ..	8	7·7 (4·2–14·4)			20	26 (13–52)		
–OCO–  ..	9	0·32 (0·17–0·61)			21	0·15 (0·07–0·29)		

* Could not be determined because of toxic excitation
† Sargent, Schwartzman & Small (1958)

TABLE 6. THE EFFECT OF 14-n-BUTYRYLOXYCODEINE ON RESPIRATORY RATE IN RATS AND GASTROINTESTINAL MOTILITY IN MICE

[Values are expressed in terms of base. Limits of error (P = 0·95) are shown in parentheses]

Compound	Respiratory rate		Gastrointestinal motility	
	ED50 mg/kg s.c.	Relative potency	ED50 mg/kg s.c.	Relative potency
Morphine hydrochloride	15	1	1·5 (1·0–2·1)	1
14-n-Butyryloxycodeine hydrochloride	4·9	2·6	5·1 (3·3–7·9)	0·3

1 mg/kg. Rapid tolerance was acquired to both drugs, the third successive dose in each case failing to produce any effect on the blood pressure. No changes in the electrocardiogram record were observed, nor were the normotensive responses to adrenaline, acetylcholine, histamine, carotid occlusion and vagal stimulation affected in any way.

GASTROINTESTINAL EFFECTS

The stimulant actions of acetylcholine (5×10^{-8}), histamine (5×10^{-8}) and barium chloride (2×10^{-5}) were not antagonised by any of these derivatives given in a concentration of 10 μg/ml. However 14-n-butyryloxycodeine exhibited a constipating activity in mice (Table 6), its relative potency compared with morphine being similar to its analgesic potency in mice.

Discussion

The results extend the previous observations of Krueger & others (1943), Blumberg & others (1954), and Winder & others (1959), that substitution of the hydroxyl group at position 14 in alkaloids of the morphine group usually reduces acute toxicity. This action was most marked in the case of codeine and Δ^7-deoxycodeine where hydroxylation resulted in more than a five-fold lowering of acute toxicity in mice. It was much less so in the case of codeinone where the subcutaneous LD50 was only increased from 11 mg/kg to 28 mg/kg.

Winder & others (1959) have discussed the importance of the 6-position of morphine derivatives in influencing toxicity and stress that any interference with the alcoholic function at this point is likely to enhance toxic excitation. Such increased toxicity was found with codeinone and 14-hydroxycodeinone, but was absent or much attenuated in the instances of codeine-6-acetate and Δ^7-deoxycodeine and their 14-hydroxy derivatives. The reduction of toxicity induced by the 14-hydroxy group was maintained after its esterification and with 14-hydroxycodeinone this esterification further reduced toxicity. Thus acylation of 14-hydroxycodeinone to give 14-n-valeryloxycodeinone increased analgesic potency more than a hundred-fold and reduced acute toxicity by a factor of more than a hundred. This change in acute toxicity is unlikely to be of much significance and may only relate to a change from stimulant to depressant properties in mice.

Our results indicate that the effects of esterifying the 14-hydroxyl group of codeine and related compounds depends upon both the nature of the esterifying acid and upon the groups present at positions 6, 7 and 8 of the molecule. Analgesic potency is mainly unaffected by acetylating the 6-hydroxy group (Table 1, series II) but greatly enhanced by oxidising it to a ketone (series III) or by removing all substituents at position 6 (series IV). The general observation of Braenden, Eddy & Halbach (1955), that a free alcoholic hydroxyl at position 6 interferes with analgesia in the morphine group is supported by the failure to obtain potent analgesics in the codeine series similar to those obtained from codeinone and Δ^7-deoxycodeine.

Preliminary results suggest that 14-n-butyryloxycodeine has no obvious advantages over morphine. The greater potency of codeinone and Δ^7-deoxycodeine derivatives is of interest in attempting to relate structure to analgesic activity but such compounds have no advantages over those

in current clinical use unless their analgesic properties can be dissociated from undesirable effects.

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The anti-anaphylactic activity of theophylline and some related xanthine derivatives

A. FIRTH AND W. G. SMITH

Theophylline and theobromine, but not caffeine and xanthine, protect sensitised guinea-pigs against anaphylactic shock induced by aerosolised antigen. There is an inhibition of release of slow reacting substance of anaphylaxis and some reduction in histamine release. The lung lipid changes associated with anaphylactic shock are prevented by pre-treatment with theophylline.

THE effectiveness of theophylline in the treatment of severe bronchial asthma was first reported by Hermann & Aynesworth (1937) although previously Efron (1936) was able to arrest severe attacks with aminophylline injected intravenously. Numerous authors have since confirmed that the intravenous injection of aminophylline gave prompt, effective, reliable and safe relief from allergic bronchospasm (Brown, 1938; Rowe, 1938; Huber, Kahn, Maytum, Ratner & Piness, 1939; Carr, 1940; Rackemann, 1940).

It is still commonly assumed, as by Urbach & Gottlieb (1946), that the beneficial effect of theophylline is due to an antispasmodic or bronchodilator action. Young & Gilbert (1941), by direct microscopical observations of freshly isolated bronchi and bronchioles, demonstrated a protective action of aminophylline against the bronchoconstrictor action of histamine.

Sollman & Gilbert (1937) and Gilbert & Goldman (1940) have shown aminophylline to be an effective dilator of bronchiolar sections which had been contracted by histamine. Osgood & Ehret (1943) suggested that the principal action of aminophylline in relieving asthma is by increasing the blood flow through the pulmonary circulation by vasodilatation and that its bronchodilating effect is of secondary importance. We set out to assess the anti-anaphylactic activity of theophylline and related xanthine derivatives and to further elucidate the underlying mechanisms of their actions.

Experimental

ANAPHYLAXIS *in vivo*

Actively sensitised guinea-pigs were exposed to aerosolised antigen using the technique of Herxheimer (1952) as described by Smith (1961). Three weeks after sensitisation, groups of nine guinea-pigs were exposed to an aerosol of 1% antigen (egg albumin) and the time to onset of dyspnoea and cough noted. This procedure was repeated at weekly intervals for 3 weeks during which it was found that the time to onset of dyspnoea and cough became relatively constant for each animal. This was termed the "normal collapse time" (Smith, 1961). Fifteen min before the fourth weekly exposure to antigen, each animal was injected intraperitoneally with a solution of drug in Water for Injection, B.P. The "treated collapse time" of each animal was expressed as a ratio to its "normal collapse time" and

From the Research Laboratory in Biochemical Pharmacology, School of Pharmacy, Sunderland Technical College, Co. Durham, England.

the figure termed the "protection ratio" for each animal. Animals recording a protection ratio of 20 were considered to be fully protected.

DETERMINATION OF ANTIHISTAMINE EFFECT *in vivo*

This was carried out in the same manner described in the section on anaphylaxis *in vivo* except that the aerosoliser contained a 5% solution of histamine acid phosphate instead of 1% egg albumin. Protection ratios were calculated in the same way as in the anaphylaxis experiments.

ANAPHYLAXIS *in vitro*

Anaphylactic shock was induced in intact sensitised guinea-pig lungs undergoing perfusion through the pulmonary artery with Tyrode solution at 37° by the technique of Brocklehurst (1960). The perfusate (60 ml) was collected over 30 min and then centrifuged to remove blood cells. The released histamine and slow reacting substance of anaphylaxis (SRS-A) were assayed on guinea-pig ileum as described by Goadby & Smith (1963a).

EFFECT OF THEOPHYLLINE ON CHANGES IN LUNG LIPIDS AFTER ANAPHYLAXIS

The procedure was identical with that of Goadby & Smith (1962), except that instead of hydrocortisone being administered 18 hr before exposure to antigen, theophylline was administered intraperitoneally at a dose level of 80 mg/kg 15 min before exposure.

Results

ANTI-ANAPHYLACTIC EFFECT OF XANTHINE DERIVATIVES

The results obtained with these four compounds are given in Table 1. All the compounds are insoluble in water but can be solubilised by the addition of suitable amounts of ethylenediamine or sodium salicylate.

TABLE 1. THE ANTI-ANAPHYLACTIC ACTIVITY OF XANTHINE AND ITS METHYL DERIVATIVES

Drug	Dose mg/kg	No. of animals	No. fully protected	Mean protection ratio*	Standard deviation
Theophylline with ethylenediamine ..	40	9	1	2·02	1·26
	80	9	6	10·74	
Theobromine Na salicylate	40	9	Nil	1·25	0·53
	80	9	9		
Caffeine Na salicylate.	40	9	Nil	1·55	0·23
	80	9	1	2·55	0·97
Xanthine Na salicylate	80	9	Nil	1·02	0·25
Na salicylate	80	9	Nil	1·00	0·09
Ethylenediamine	15	9	Nil	1·33	0·27

* Of animals not fully protected.
All doses in terms of base. All drugs were administered intraperitoneally 15 min before exposure to antigen.

Control observations were made with both these solubilising agents at the maximum dose levels used. Both were without anti-anaphylactic effect. Of the xanthine derivatives examined, both theophylline and theobromine had marked anti-anaphylactic activity at a dose level of 80 mg/kg. Caffeine and xanthine were without significant activity at the same dose

level (except caffeine in one animal only). Thus the anti-anaphylactic effect observed with both xanthines having common dimethyl groups is absent in trimethylxanthine (caffeine) and also the parent ring structure (xanthine). The last observation was confirmed in further experiments using aqueous suspensions of xanthine and hypoxanthine. A group of 9 animals which had received xanthine in aqueous suspension at 80 mg/kg 30 min before exposure to antigen, recorded a mean protection ratio of 1·49 with a standard deviation of 0·33. A corresponding group of animals that had received the same dose of hypoxanthine recorded a protection ratio of 1·41 with a standard deviation of 0·28.

ANTIHISTAMINE EFFECT OF THEOPHYLLINE, THEOBROMINE AND CAFFEINE

The antihistamine effect induced after a dose of 80 mg/kg of these compounds is shown in Table 2. When compared with the effect of

TABLE 2. ANTIHISTAMINE ACTIVITY *in vivo* OF XANTHINE DERIVATIVES IN GUINEA-PIGS EXPOSED TO AEROSOLISED HISTAMINE SOLUTION

Drug	Dose mg/kg	No. of animals	No. fully protected	Mean protection ratio*	Standard deviation
Theophylline with ethylenediamine	80	9	Nil	3·28	0·91
Theobromine Na salicylate	80	8	Nil	1·51	0·48
Caffeine Na salicylate	80	8	Nil	1·56	0·23
Mepyramine maleate	1	8	8		

* Of animals not fully protected.
All doses in terms of base. All drugs were administered intraperitoneally 15 min before exposure to antigen except for mepyramine which was given intramuscularly 1 hr before exposure.

1 mg/kg of mepyramine, all the compounds exhibited negligible amounts of histamine antagonism at the dose level used. Thus the anti-anaphylactic activity of theophylline and theobromine is probably not due to pharmacological antagonism of the histamine released during anaphylactic shock.

EFFECTS OF THEOPHYLLINE, THEOBROMINE AND CAFFEINE ON RELEASE OF HISTAMINE AND SRS-A DURING ANAPHYLAXIS

The amounts of histamine and SRS-A released by anaphylaxis *in vitro* from the lungs of animals pretreated with 80 mg/kg of a xanthine derivative by the intraperitoneal route 15 min before being killed were compared with the amounts released from the lungs of control animals not so pretreated. The results are in Table 3. The amounts of SRS-A released

TABLE 3. EFFECT OF XANTHINE DERIVATIVES ON THE RELEASE OF HISTAMINE AND SRS-A DURING ANAPHYLAXIS IN GUINEA-PIG LUNG

Drug	No. of animals	Histamine µg/ml	Standard deviation	No. of animals	SRS-A units/ml	Standard deviation
Nil	6	0·93	1·42	4	20·06	5·26
Theophylline with ethylenediamine	6	0·775	1·32	6	13·02*	4·24
Theobromine Na salicylate	6	0·438	0·105	6	14·37†	4·207
Caffeine Na salicylate	6	0·968	1·296	6	22·35	12·37

* Statistically significant from control value in Student's *t* test at P = 0·95
† Statistically significant from control value in Student's *t* test at P = 0·90

after pretreatment with theophylline or theobromine are significantly smaller than the amounts released by the control animals. Whilst the mean

histamine release in these groups of animals is less than the mean histamine release from the controls, the differences are not statistically significant. Histamine and SRS-A were released in substantially the same amounts from the lungs of animals pretreated with caffeine as from the controls.

EFFECT OF PRETREATMENT WITH THEOPHYLLINE ON THE LIPID CONTENT OF GUINEA-PIG LUNGS SUBJECTED TO ANAPHYLAXIS

The changes in lung lipids of animals exposed to both aerosolised distilled water and aerosolised antigen were determined 15, 30 and 60 min after exposure to aerosol. They are summarised in Table 4. The most

TABLE 4. LIPID CONTENT (MG/G) OF FREEZE-DRIED LUNGS FROM SENSITISED GUINEA-PIGS EXPOSED TO AN AEROSOL OF DISTILLED WATER OR AEROSOLISED ANTIGEN (EGG ALBUMIN) EXPRESSED AS MEAN ± STANDARD DEVIATION

	Distilled Water			Antigen		
	15 min	30 min	1 hr	15 min	30 min	1 hr
Phospholipid	117 ± 14·75	103 ± 17·25	82 ± 12·25	119 ± 41·25	108 ± 4·25	83 ± 10·75
Cholesterol	27·98 ± 9·86	18·81 ± 1·67	26·43 ± 6·82	21·21 ± 12·32	16·95 ± 1·1	18·7 ± 1·25
Glyceride	19·1 ± 4·43	77·4 ± 10·44	22·9 ± 7·92	23·8 ± 4·55	64·8 ± 29·78	19·75 ± 9·32

prominent changes are a fall in phospholipid content and an increase in glyceride content which is pronounced 30 min after exposure to aerosol. One hr after exposure to aerosol, the glyceride content had returned to a normal level, but the amount of phospholipid appeared to be still falling.

The administration of theophylline itself produced no substantial change in the lipid content of guinea-pig lungs as shown in Table 5. In this

TABLE 5. LIPID CONTENT (MG/G) OF FREEZE-DRIED LUNGS FROM CONTROL ANIMALS COMPARED WITH THAT OF LUNGS FROM ANIMALS PRETREATED WITH THEOPHYLLINE EXPRESSED AS MEAN ± STANDARD DEVIATION

	Controls	Theophylline-treated
Phospholipid	117 ± 8·25	111·05 ± 5·4
Cholesterol	20·25 ± 1·52	18·11 ± 1·38
Glyceride	19·0 ± 5·0	15·62 ± 5·96

table the lipid content of a group of animals pretreated with an intraperitoneal injection of 80 mg/kg theophylline (solubilised with ethylenediamine) is compared with the control group of animals.

The changes in lung lipids in animals pretreated with theophylline and then subsequently exposed to aerosolised distilled water or antigen were determined at 15, 30 and 60 min after exposure to aerosol (Table 6).

TABLE 6. LIPID CONTENT (MG/G) OF FREEZE-DRIED LUNGS FROM ANIMALS EXPOSED TO AN AEROSOL OF DISTILLED WATER OR AEROSOLISED ANTIGEN 15 MIN AFTER PRETREATMENT WITH THEOPHYLLINE EXPRESSED AS MEAN ± STANDARD DEVIATION

	Distilled water			Antigen		
	15 min	30 min	1 hr	15 min	30 min	1 hr
Phospholipid	116·5 ± 14·35	108·8 ± 4·32	107·6 ± 1·98	101·15 ± 1·95	99·0 ± 2·2	99·37 ± 18·35
Cholesterol	16·25 ± 0·77	15·17 ± 1·92	20·9 ± 1·47	17·28 ± 0·67	16·83 ± 0·46	18·38 ± 0·79
Glyceride	34·3 ± 17·8	26·02 ± 16·2	32·08 ± 19·8	9·22 ± 3·74	19·94 ± 12·0	16·23 ± 4·15

A comparison of Tables 5 and 6 shows that animals pretreated with theophylline do not exhibit any marked change in their lung lipids when subsequently exposed to an aerosol of distilled water or antigen. The changes in lung lipids associated with anaphylactic shock are not observed in animals pretreated with an anti-anaphylactic dose of theophylline.

Discussion

The results have an interesting similarity to earlier observations obtained in this laboratory using anti-inflammatory steroids (Goadby & Smith, 1962, 1964) and ethanolamine (Smith, 1961). Theophylline, hydrocortisone and ethanolamine have all been shown to inhibit the release of SRS-A during an anaphylactic reaction in sensitised guinea-pig lung tissue, and theophylline and hydrocortisone have prevented changes in the lipid content of sensitised guinea-pig lungs associated with anaphylactic shock *in vivo*. *In vitro* studies have shown a similar effect with ethanolamine.

Although all three substances inhibit the release of SRS-A and prevent the changes in lipid metabolism invoked by anaphylaxis, the times required for these effects to become manifest differ markedly. These effects are induced by theophylline 15 min after administration. With ethanolamine the effects are maximal 2 hr after administration (Goadby & Smith, 1963b); whereas with hydrocortisone, 18 hr must elapse between dose and maximal anti-anaphylactic effect (Goadby & Smith, 1964).

The exact role of SRS-A as a chemical mediator of allergic bronchospasm in both guinea-pigs and the human asthmatic has yet to be elucidated. Nevertheless there is evidence pointing to its involvement in this condition in both guinea-pigs and man (Brocklehurst, 1956, 1960, 1962, 1963). Thus the beneficial effect of theophylline, hydrocortisone and ethanolamine in the treatment of allergic asthmatics is most probably due to their ability to reduce the SRS-A induced component of bronchospasm initiated by an antigen-antibody reaction. There is no evidence of other pharmacological activities possessed by these molecules which would themselves account for their anti-anaphylactic activity. For instance, in guinea-pigs, none of these substances has bronchodilator, anti-SRS-A or antihistamine properties (Smith, 1961; Goadby & Smith, 1964).

The observation that all three substances prevent the changes in lipid metabolism normally induced in anaphylaxis in sensitised guinea-pig lung, is itself one of profound significance. Since these agents protect the tissue from changes in lipid metabolism which in unprotected animals represent a marked distortion of the normal intermediary metabolism (Smith, 1964), their effects are beneficial at a biochemical level as well as the purely pharmacological level of bronchospasm.

Since inhibition of SRS-A release and the prevention of changes in lipid metabolism invoked by anaphylaxis are effects which follow pretreatment with theophylline, hydrocortisone or ethanolamine, it is possible that these two events are related. The differences in times that must elapse between administration and maximal anti-anaphylactic effect can be interpreted as manifestations of a biochemical mechanism of action and it is profitable to

speculate that these two events have some common biochemical cause. If this is so, the effects are not caused by histamine release or the release of SRS-A (Marquis & Smith, 1963). It is more likely that protection is achieved by blocking a biochemical event common to both lipid metabolism and the metabolism of SRS-A.

Besides incurring substantial losses of phospholipid as a result of anaphylactic shock (Goadby & Smith, 1962), guinea-pig lung also loses large amounts of neuraminic acid (Anderson, Goadby & Smith, 1963). Up till the present time, there is no chemical evidence contradictory to the suggestion that SRS-A is a mixture of neuraminyl glycosides (Smith, 1962). Thus a metabolic reaction common to the metabolism of SRS-A and lipid metabolism might be one situated in the intermediary metabolism of the tissue somewhere between the reactions of mucopolysaccharide synthesis on the one hand and the reactions of lipid synthesis on the other.

Neuraminic acid has recently been shown to be an important constituent of cell membranes (Wallach & Eylar, 1961). It seems probable that any biochemical effect capable of modifying the ability of cells to withstand the biochemical disorganisation attendant upon the formation of an antigen-antibody complex on or within the surface structure of their cytoplasm may inhibit the release of SRS-A and prevent the loss of other pharmacologically inert neuraminyl glycosides and phospholipid. The latter is also richly distributed in membrane structures.

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The antibacterial activity of a complex of iodine and a non-ionic surface-active agent

W. B. HUGO AND J. M. NEWTON*

The antibacterial activity of a complex of iodine and cetomacrogol against *Escherichia coli* and *Staphylococcus aureus* has been compared with a system prepared by diluting an iodine solution in ethanol and potassium iodide. Known numbers of bacteria were mixed with known concentrations of iodine and viable counts were made at intervals thereafter. The activity of both preparations was the same at equal iodine concentrations. Killing, when it occurred, was rapid, the relation between the iodine concentration and the dry weight of the bacterial suspension apparently being the controlling feature. The presence of serum reduced the activity, the reduction being greater for *Staph. aureus* than for *E. coli*. Temperatures from 20–37° had no effect whilst reducing the pH to 4, or below, caused a marked increase in the activity.

IODINE may be prepared in a homogeneous aqueous system using a non-ionic surface-active agent (Hugo & Newton, 1963). The antibacterial properties of the aqueous system have now been compared with those of weak iodine solution B.P.

Allawala & Riegelman (1953) related the sporicidal activity of a complex of iodine and a non-ionic surface-active agent to the thermodynamic activity of the iodine in the aqueous phase, rather than to the total iodine content of the complex, while Moore & Hardwick (1957) found by varying the proportions of iodine to non-ionic surface-active agent that there was a ratio giving a peak antibacterial value, although the activity of the ratios examined varied less than those of phenols solubilised in anionic surface-active agents. We have used a counting technique to compare the iodine preparations and the factors affecting the antibacterial activity.

Experimental

MATERIALS

The test organisms were *Escherichia coli* Type 1, formerly NTCT 5934 and *Staphylococcus aureus*, NTCT 6571. Oxoid "Bacteriological" grade materials were used in the preparation of the nutrient agar. The serum was horse serum (Burroughs Wellcome and Co.), containing no chemical preservatives. Other chemicals were of analytical reagent grade. The two iodine systems were a complex of iodine and cetomacrogol (Hugo & Newton, 1963) and a solution of iodine in ethanol and potassium iodide (iodine solution).

The nutrient agar contained %: Lab Lemco 0·5, peptone 1·0, sodium chloride 0·5, agar No. 3, 1·8; distilled water to 100 ml. The pH, after adjustment and sterilisation, was 7·2. The solution of serum was prepared by dilution with sterile distilled water, followed by heating at 98–100° to destroy vegetative organisms. The buffer solutions were prepared according to McIlvaine's formula and sterilised by membrane filtration.

From the Department of Pharmacy, The University, Nottingham
*Present address: The School of Pharmacy, Sunderland Technical College, Sunderland.

METHODS

A suspension of the organisms in sterile distilled water was prepared from 24 hr cultures grown on nutrient agar in Roux bottles, removing agar and bacterial clumps by centrifugation. The opacity of the suspension was adjusted to a given value by means of a photoelectric nephelometer and the dry weight determined by drying to constant weight at 105°.

To carry out the tests, 1 part of a bacterial suspension was mixed with 2 parts of water or water containing 20% serum. To this, 1 part of iodine solution was added. Activity in a buffered system was studied by adding 1 part of bacterial suspension to 3 parts of iodine solution in the appropriate buffer. The procedure was carried out in a thermostatically controlled water-bath after allowing time for the systems to reach temperature equilibrium before mixing the iodine system and bacteria. After the required time, a 1 ml sample was removed from the mixture and inactivated with 9 ml of a sterile 0·01N sodium thiosulphate solution, or if acid buffers were present, with a sterile 0·01N sodium thiosulphate and 0·1M disodium hydrogen phosphate solution. After serial dilutions with $\frac{1}{4}$ strength Ringer, dilutions were plated out.

TABLE 1. EFFECT OF TIME ON THE KILLING OF *E. coli* AND *Staph. aureus* BY IODINE FORMULATIONS AT 20° C

Serum absent						10% serum present					
Initial count × 10^8		Iodine conc. µg/ml	% Survivors after 2 min	30 min	24 hr	Initial count × 10^8		Iodine conc. µg/ml	% Survivors after 2 min	30 min	24 hr
E. coli											
9·10	A	15	63·8	48·4	27·7	12·76	A	25	60·8	69·5	94·0
	B	15	72·0	99·0	90·1		B	25	71·3	71·3	87·8
	A	20	3·1	2·3	0·9		A	50	1·5	0·3	23·0
	B	20	14·2	7·2	1·86		B	50	0·8	2·3	25·5
							A	100	0·0009	0·0	0·0
9·74	A	20	3·8	2·7	0·4		B	100	0·0008	0·00009	0·0
	B	20	48·8	35·40	15·8						
	A	25	0·008	0·003	0·017	9·95	A	25	59·3	61·3	
	B	25	9·25	4·2	4·4		B	25	48·2	43·2	
							A	50	0·7	0·2	
9·75	A	10	103·6	87·2	82·1		B	50	1·4	2·0	
	B	10	98·0	98·5	127·2		A	100	0·000001	0·0	
	A	15	68·5	70·8	64·6		B	100	0·0008	0·00004	
	B	15	79·0	113·8	101·0						
Staph. aureus											
11·12	A	10	87·2	88·6	59·0	9·02	A	53	86·8	89·0	
	B	10	87·0	88·4	28·3		B	51	85·0	78·8	
	A	26	2·9	1·4	0·01		A	106	57·7	67·6	
	B	27	1·2	1·2	0·01		B	103	65·4	65·4	
	A	53	0·0025	0·0	0·0		A	265	71·7	0·00015	
	B	52	0·000009	0·0	0·0		B	275	1·02	0·000005	
7·64	A	11	99·5	75·3	72·4	9·74	A	50	102·0	96·5	
	B	11	93·3	84·5	54·7		B	49	78·1	73·6	
	A	27	21·8	10·9	0·001		A	101	56·6	66·3	
	B	27	19·8	16·3	0·0		B	98	58·5	52·4	
	A	55	0·0004	0·0	0·0		A	252	0·18	0·12	
	B	54	0·0	0·0	0·0		B	245	0·00002	0·007	

A = Iodine-cetomacrogol complex B = Iodine solution

Results

To assess the bacterial counting technique a calculation of the index of dispersion, (Fisher, 1958) was made for the initial counts. As the

value $\sqrt{2\chi^2}-\sqrt{2n-1}$ did not exceed 2 for either organism (Newton, 1962), it was assumed that the technique was not faulty, and that the population from which the counts were derived, was homogeneous.

THE KILLING OF *E. coli.* AND *Staph. aureus* BY IODINE FORMULATIONS

The effect of time. The results in Table 1 illustrate that for both *E. coli* and *Staph. aureus*, any killing which occurred happened within 2 min, there being no apparent period of bacteriostasis. This effect was not modified by the presence of 10% serum.

The effect of iodine concentration. Initial experiments showed a correlation to exist within an experiment between the iodine concentration and the bacteria remaining, but between experiments, there was marked variation. For example for iodine, 20 μg/ml, the percentage survivors ranged from 0·0004 to 89·5%. These variations occurred with both organisms and both iodine preparations. Wide variations have previously been reported for iodine-treated bacteria (Chang & Morris, 1953; Carroll, 1955). These variations between experiments could be related to the dry weight of the bacterial suspensions. Thus Fig. 1 illustrates that as the

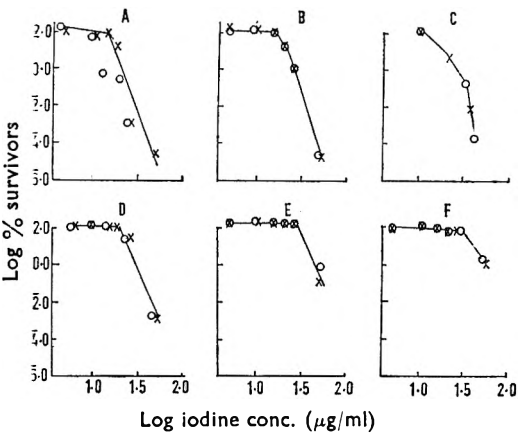

FIG. 1. The effect of iodine concentration on the killing of *Staph. aureus*, after 2 min. at 20° C. ○ Iodine-cetomacrogol complex. × Iodine solution. The initial number of organisms × 10^8 and the dry weight of suspension, (μg/ml) for the experiments are respectively A, 5·85, 450; B, 8·24, 720; C, 10·06, 780; D, 7·07, 1185; E, 9·56, 1530; F, 8·15, 1860.

dry weight of suspension of *Staph. aureus* increased, the concentration of iodine required to produce noticeable killing also increased. In the presence of 10 % serum, the between-experiment variation decreased. But, whereas in the absence of serum, the 2 types of bacteria, at the same dry weight, were affected equally by the same iodine concentrations, in the presence of serum, a higher iodine concentration was required to kill *Staph. aureus* than *E. coli* (Figs 2 and 3).

The effect of large differences in inoculum size. Table 2 illustrates how the antibacterial activity of iodine increased as the inoculum size decreased,

although the presence of 10% serum afforded the smaller inoculum considerable protection.

The effect of temperature. The percentage of *Staph. aureus* surviving after 2 min at 20° and 37° shown in Table 3 indicates that, in this range,

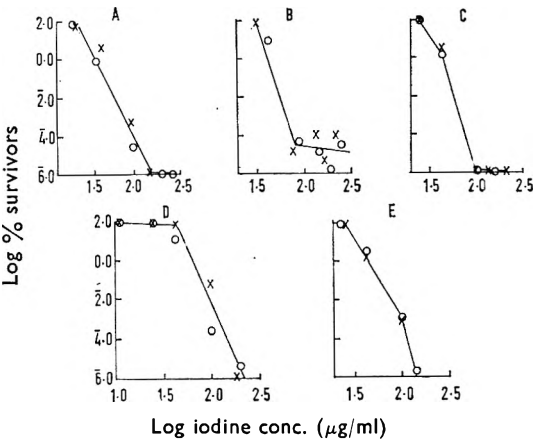

FIG. 2. The effect of various iodine concentrations on the killing of *E. coli*, after 2 min at 20° C in the presence of 10% serum. ○ Iodine-cetomacrogol complex. × Iodine solution. The initial numbers of organisms × 10^8 for the experiments are respectively A, 9·95; B, 10·50; C, 10·70; D, 10·78; E, 12·76.

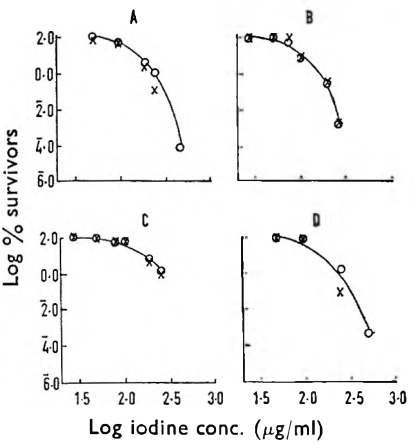

FIG. 3. The effect of various iodine concentrations on the killing of *Staph. aureus*, after 2 min at 20° C in the presence of 10% serum. ○ Iodine-cetomacrogol complex. × Iodine solution. The initial number of organisms × 10^8 for the experiments are respectively A, 9·74; B, 9·02; C, 7·26; D, 6·96.

temperature does not influence the killing of the bacteria by either preparation.

Variation in the bacterial counts in the presence of iodine. Employing the same statistical analysis used previously it was found that, where killing of the bacteria exceeded 99%, variations in the counts were far in

TABLE 2. EFFECT OF DIFFERENCES IN INOCULUM SIZE ON THE KILLING OF *E. coli* AND *Staph. aureus* BY IODINE FORMULATIONS, 2 MIN AT 20° C

Serum absent				10% Serum present			
Inoculum size	Iodine conc. µg/ml	% Survivors A	% Survivors B	Inoculum size	Iodine conc. µg/ml	% Survivors A	% Survivors B
E. coli							
$11{\cdot}74 \times 10^8$	4·5	40·0	56·2	$10{\cdot}78 \times 10^8$	10·0	107·1	104·8
$11{\cdot}40 \times 10^6$	4·5	0·0	0·0	$10{\cdot}78 \times 10^5$	10·0	94·5	103·8
$11{\cdot}74 \times 10^8$	10·0	0·01	0·01	$10{\cdot}78 \times 10^8$	25·0	102·0	90·4
$11{\cdot}40 \times 10^6$	10·0	0·0	0·0	$10{\cdot}78 \times 10^5$	25·0	73·3	77·9
				$10{\cdot}78 \times 10^8$	45·0	14·3	46·4
$10{\cdot}78 \times 10^8$	4·5	98·8	96·4	$10{\cdot}78 \times 10^5$	45·0	13·6	46·4
$10{\cdot}78 \times 10^6$	4·5	0·002	0·4	$10{\cdot}78 \times 10^8$	102·0	0·003	0·05
$10{\cdot}78 \times 10^8$	10·0	90·6	92·8	$10{\cdot}78 \times 10^5$	102·0	0·0	0·0
$10{\cdot}78 \times 10^6$	10·0	0·0008	0·002	$10{\cdot}78 \times 10^8$	200·0	0·00005	0·000001
				$10{\cdot}78 \times 10^5$	200·0	0·0	0·0
Staph. aureus							
$9{\cdot}00 \times 10^8$	5·0	104·5	102·2				
$8{\cdot}50 \times 10^7$	5·0	61·2	48·1				
$9{\cdot}97 \times 10^8$	5·0	0·04	0·001				
$10{\cdot}13 \times 10^6$	5·0	0·003	0·003	$6{\cdot}96 \times 10^8$	50·0	85·3	90·5
				$7{\cdot}86 \times 10^8$	50·0	102·1	95·1
$9{\cdot}00 \times 10^8$	10·0	83·4	95·9	$6{\cdot}96 \times 10^8$	100·0	68·2	75·2
$8{\cdot}50 \times 10^7$	10·0	0·006	0·0006	$7{\cdot}86 \times 10^5$	100·0	15·5	34·1
$9{\cdot}97 \times 10^8$	10·0	0·0	0·0	$6{\cdot}96 \times 10^8$	250·0	1·31	0·09
$10{\cdot}13 \times 10^6$	10·0	0·0	0·0	$7{\cdot}86 \times 10^6$	250·0	0·07	0·0
				$6{\cdot}96 \times 10^8$	500·0	0·0006	0·0
				$7{\cdot}86 \times 10^5$	500·0	0·0	0·0

A = Iodine-cetomacrogol complex B = Iodine solution

TABLE 3. PERCENTAGE OF *Staph. aureus* SURVIVING AFTER 2 MIN AT 20° OR 37° C

Initial count $\times 10^8$	Dry weight µg/ml	Iodine conc. µg/ml		Temperature ° C 20	Temperature ° C 37
9·10	1,500	A	25	43·5	55·0
		B	24	54·8	63·1
		A	53	0·043	0·0001
		B	51	0·0011	0·039
11·28	2,050	A	5	93·5	92·7
		B	5	96·3	96·0
		A	25	76·7	65·5
		B	25	73·3	73·0
		A	50	0·62	3·76
		B	50	0·14	5·18
12·20	2,363	A	26	98·2	79·7
		B	27	79·4	70·1
		A	52	2·00	0·27
		B	51	0·60	0·80

A = Iodine-cetomacrogol complex B = Iodine solution

excess of those which could be expected from biological sources, using the 5% level of significance for both types of iodine preparation. This variation at high mortality levels has been observed for phenol by Withel (1942) and Jordan & Jacobs (1944). When killing did not exceed 99%, variations were within normal limits, and therefore it seems reasonable to assume that the clumping of the bacteria was not induced by the presence of iodine or the two solvent systems, nor could clumping be observed in a hanging drop preparation.

The effect of solvents. The concentrations of the solvents used in an iodine system containing 50 µg/ml iodine were tested, by the same general method, for their antibacterial activity. With a contact time of 2 min and a temperature of 20°, neither solvent system affected the viability of *E. coli* nor *Staph. aureus.*

The effect of pH. The iodine systems were only stable up to a pH of 7·0, and thus, to ensure that the iodine was present in the molecular form, acid buffered solutions of pH 2·2, 4·0 and 6·0 were used, plus an unbuffered system. The final pH of the iodine-bacterial suspension mixtures was determined electrometrically on the larger volumes of the same proportions used in the bactericidal investigations. The percentage survivors after treatment at 20° for 2 min with iodine concentrations are shown in Figs 4

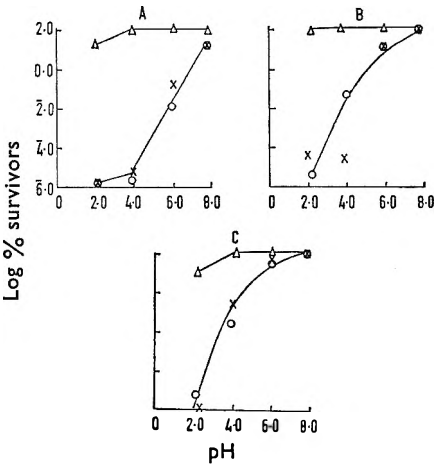

FIG. 4. The effect of pH on the killing of *E. coli* by iodine systems, after 2 min at 20° C. ○ Iodine-cetomacrogol complex. × Iodine solution. △ Buffer solution. The initial number of organisms × 10^8, the dry weight of suspension (μg/ml) and the iodine concentration (μg/ml) for the experiments are respectively A, 11·36, 890, 11; B, 11·28, 1085, 9; C, 10·88, 1260, 9.

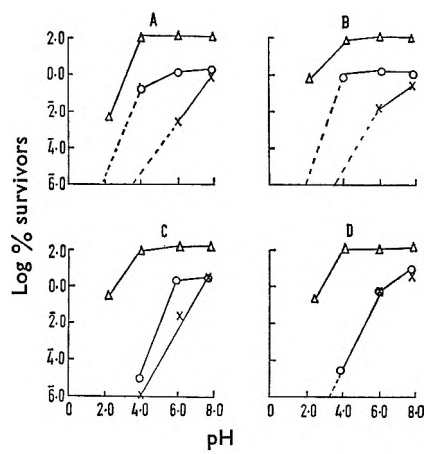

FIG. 5. The effect of pH on the killing of *Staph. aureus*, by iodine formulations, after 2 min. at 20° C. ○ Iodine-cetomacrogol complex. × Iodine solution. △ Buffer solution. The initial number of organisms × 10^8, the dry weight of suspension (μg/ml) and the iodine concentration (μg/ml) for the experiments are respectively A, 9·03, 1130, 25; B, 9·19, 1110, 27; C, 12·00, 1215, 25; D, 11·62, 1315, 26.

and 5. Data illustrating the effect of the buffers themselves are also included in these graphs.

Discussion

The main outcome of this investigation is that, when the antibacterial activities of the iodine-cetomacrogol and the iodine solution are compared at the same available iodine concentration by a counting method, there is no significant difference between the two systems. This finding is in agreement with those of Witlin & Gershenfeld (1956, 1958) who used the method of Weber & Black (1948). However, Gershenfeld & Witlin (1955, 1958) and Terry & Shelanski (1952a, b) using the capacity test of Cantor & Shelanski (1951) claimed a superior activity for an iodine-surface-active agent complex when compared with a conventional iodine solution.

We found that a number of variables affected the antibacterial activity of both systems equally. These are now considered.

Killing took place within 2 min and thereafter, the increased contact time produced little further effect. In fact, there was often an increase in the count after 24 hr if all the bacteria were not killed within 2 min. Increasing the temperature from 20 to 37° did not increase the killing of *Staph. aureus*. The antibacterial effect of a given concentration was related to inoculum size with inoculae ranging from 10^6–10^9 organisms/ml when it was found that a much lower iodine concentration was required to kill the smaller inoculum. At inoculum levels from 6×10^8 to 11×10^8 organisms/ml, however, no relationship could be demonstrated between inoculum size and concentration of iodine required to kill. The controlling feature of the killing of both *E. coli* and *Staph. aureus* was found to be the relation between the dry weight of the bacterial suspension and the concentration of iodine. Thus, Fig. 1 shows that for a given dry weight there was a given iodine concentration below which the bacteria were not killed. Conversely, Fig. 6 shows that for a given

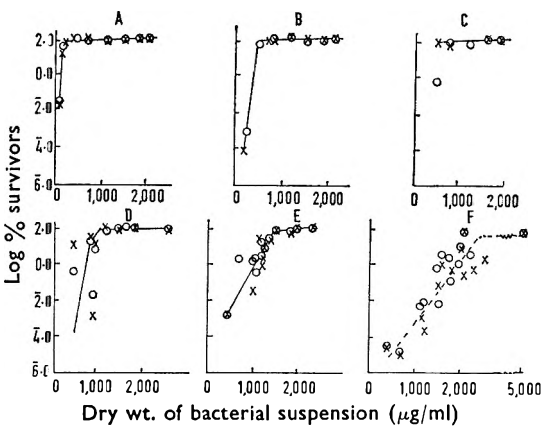

FIG. 6. The percentage of *Staph. aureus* surviving after treatment with A, 5; B, 10; C, 15; D, 20; E, 25 and F, 50 μg/ml iodine for 2 min at 20° C, with varying dry weights of bacterial suspensions. ○ Iodine-cetomacrogol complex. × Iodine solution.

iodine concentration, there was a limiting dry weight above which no killing occurred, and that this value of the dry weight increased as an approximately linear function of the iodine concentration.

This relationship between dry weight and iodine concentration suggests that an adsorption process is operative. Previous experiments (Hugo & Newton, 1964), indicated that the bacteria were far from saturated at iodine concentrations which killed the inoculum. It was also found that iodine could be completely removed from dilute solutions by the bacteria without any lethal effect upon them; it is thus possible that there are certain sites in the bacterial cell which can remove iodine from solution without an adverse effect on viability. This would suggest that these sites have a high affinity for iodine and the point at which killing commences represents the attachment of iodine to essential sites, after the non-essential sites are saturated. The lethal action of iodine is considered by Dunn (1952) to be similar to chlorine, being one of halogenation and oxidation of susceptible groups in the cell.

There appears to be little difference in the two types of bacteria in their resistance to iodine, which agrees with the lack of selectivity observed by McCulloch (1945). Indeed, no difference in the shape of the adsorption isotherms had been observed (Hugo & Newton, 1964). In the presence of 10% serum, however, *Staph. aureus* did appear to have a greater resistance. Why this should be so may be related to the relative affinity of iodine for the bacteria and serum, or differences in the total dry weight of the systems.

The addition of 10% serum required an increase in the concentration of iodine to kill the bacteria. There was a slight reduction in the "all or none" effect of a concentration producing killing, but killing still occurred rapidly. It is notable that the effects of both kinds of iodine preparation were equally reduced by the serum. This is not what would be expected from Terry & Shelanski's (1952) definition of an iodophor, which states that the carrier reduces the reactivity of iodine to materials other than microorganisms.

The increased activity of both systems as the pH is decreased is in accordance with findings of Gershenfeld & Fox (1949), Gershenfeld & Witlin (1949) and Chambers & others (1952) with vegetative cells and of Wyss & Stranskov (1946) with spores. In the controls in buffer solutions, some killing by the more acid system was observed, but this did not account for the total increase in killing. Study of the uptake of iodine at different pH values (Hugo & Newton, 1964) has shown that uptake increases as the pH increases, which is in direct contrast to the effect of pH on antibacterial activity. The lower pH of the iodine-cetomacrogol complex due to the presence of hydrogen iodide, might be expected to increase its antibacterial activity. That this did not occur is no doubt due to the fact that both types of bacterial suspension could neutralise unbuffered iodine systems of lethal concentration, within 2 min.

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The action of reserpine and α-methyl-*m*-tyrosine on the analgesic effect of morphine in rats and mice

M. MEDAKOVIĆ and B. BANIČ

The actions of α-methyl-*m*-tyrosine (MMT) and reserpine upon the analgesic effect of morphine has been studied in rats and mice. In rats, reserpine antagonised the effect of morphine, while MMT did not cause any appreciable change of the effect of morphine, injected either 2 or 24 hr after MMT. Reserpine also produced its antagonistic action to morphine in rats which were previously (24 hr) treated with MMT. Both MMT and reserpine potentiated the effect of morphine in rats pretreated (24 hr previously) with iproniazid. In mice, both MMT and reserpine antagonised the effect of morphine. Reserpine failed to do so in MMT pretreated animals. The effect of morphine was restored 24 hr after the injection of MMT. It is suggested that the inhibitory action of reserpine upon the analgesic effect of morphine is due to the antagonistic action of brain 5-HT, which is activated after being released from its stores by reserpine. The pattern of reserpine—MMT—morphine interactions in rats probably differs from that in mice.

THE relationship between the actions of reserpine and morphine-like analgesics has been widely examined (Schneider, 1954; Raducco-Thomas & Le Breton, 1957; Tripod & Gross, 1957; Sigg, Caprio & Schneider, 1958; Banić & Medaković, 1964) with the object of gaining a better understanding of the mechanisms of action of both reserpine and morphine-like analgesics and because there exists the possibility that the nature of pain itself may at least be partially explained by such studies.

The effects of reserpine (primarily sedation) have been explained by the ability of this drug to release the brain amines 5-hydroxytryptamine (5-HT), according to one concept (Brodie & Shore, 1957; Brodie & Costa, 1960) or noradrenaline according to the other (Carlsson, Lindquist & Magnusson, 1957; Kärki & Paasonen, 1959; Pletscher, Besendorf & Gey, 1959; Schaumann, 1958). Unfortunately, both views were based on indirect data. A closer approach to the problem was hindered by the fact that reserpine is equally active in releasing both 5-HT and noradrenaline.

Hess, Connamacher, Ozaki & Udenfriend (1961) have recently found that one amino-acid, α-methyl-*m*-tyrosine (MMT), releases noradrenaline from brain stores without appreciably depleting brain 5-HT stores. Thus MMT may be a "most effective means for obtaining experimental animals which are depleted of tissue noradrenaline, but still contain normal amounts of tissue 5-HT" (Hess & others, 1961).

Some controversy concerning the mechanism of reserpine action might also be due to species differences. Thus, while in rats reserpine antagonises the action of analgesics, both antagonism (Schneider, 1954; Schaumann, 1958) and potentiation (Tripod & Gross, 1957; Leme & Rocha e Silva, 1961) of the effects of morphine have been produced by reserpine in mice.

From the Department of Pharmacology, Medical Faculty, Belgrade, Yugoslavia.

The experiments now presented were made on rats and for comparison some were repeated on mice. Effects of MMT on the analgesic action of morphine and on the inhibitory action of reserpine on morphine analgesia were studied. The interaction between these drugs was also studied by means of a monoamine oxidase inhibitor (iproniazid).

The evidence suggests that the effect of reserpine on the analgesic action of morphine in rats is produced through brain 5-HT rather than through noradrenaline. This was less convincing in experiments on mice.

Methods

Rats, of both sexes, weighing approximately 180 g were used in groups of 10 animals. Each animal was placed in turn in a cylindrical cage, with the tail extending from the end of the cage. A beam of heat, from a 12 V 50 W bulb was focused on the tip of the tail of each rat, according to the method of D'Amour & Smith (1941), and the time until the heat induced movement of the tail was measured. To prevent the damage of the tail by heat stimulus in animals with total analgesia, the stimulation was applied for not longer than 15 sec. Drugs were injected intraperitoneally, except iproniazid, which was injected subcutaneously.

In experiments on mice, males weighing approximately 22 g were used. Analgesia was tested by the hot plate method of Woolfe & MacDonald (1944). A glass chamber was immersed into water, held at the thermostatically regulated temperature (53°). Each mouse in turn was placed into this chamber and the reaction time until the appearance of the paw-licking reflex was determined. Each group contained at least 15 animals. All drugs were injected intraperitoneally. To prevent paw tissue damage in mice with total analgesia, they were removed from the hot plate before this could occur. An arbitrary interval of double the control mean reaction time of each given group was selected for this purpose. The values were plotted on the graphs as percentages of this "cut-off time".

The results are presented graphically. But some of the animals did not react until the cut-off time had expired. They were given the reaction time of 15 sec in experiments in rats, and the maximum cut-off time in those in mice. Thus, the analgesic effect as plotted is not always exact and represents low values. This applies to curves showing the potentiation of the analgesic effect. It can be assumed therefore that the real difference between the control effect of morphine and the potentiated one is always larger than shown in the graphs.

The drugs used were morphine hydrochloride, reserpine (Serpasil, CIBA), iproniazid (Hoffmann-La Roche) and α-methyl-*m*-tyrosine.

Results

EFFECT OF RESERPINE ON THE ANALGESIC ACTION OF MORPHINE IN RATS

Reserpine abolishes the analgesic action of morphine in rats (Schneider, 1954; Schaumann, 1958; Banić & Medaković, 1964). This effect has been studied again, because it had to be compared with the effect of MMT on the action of morphine.

First, the analgesic action of morphine injected intraperitoneally (4 mg/kg) was established (Fig. 1). Then, it was found that, in accordance with Sigg & others (1958) and Schaumann (1959), reserpine, also injected intraperitoneally (1 mg/kg), did not affect the control mean reaction time of animals to the heat stimulus.

The effect of reserpine on the analgesic action of morphine, injected 3 hr later, is shown in Fig. 1. The analgesic action of morphine is

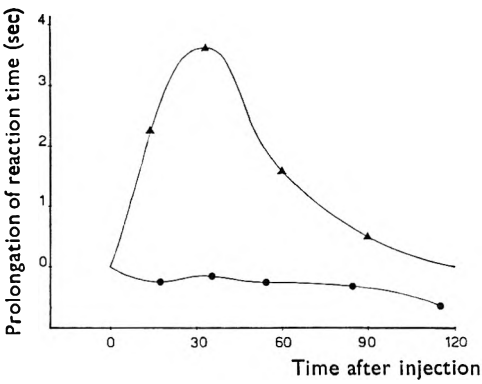

FIG. 1. Analgesic effect of morphine in control rats (–▲–) and the inhibition of this effect by the previous injection of reserpine (–●–).

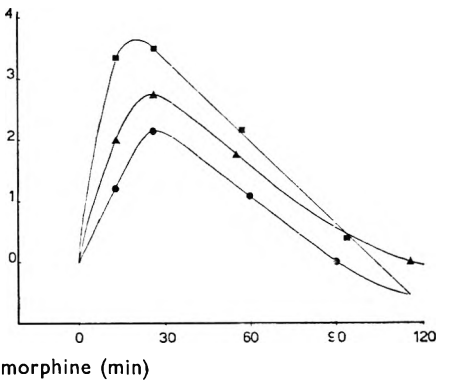

FIG. 2. The effect of pretreatment with MMT on the analgesic action of morphine in rats. Action of morphine in control animals (–▲–), in rats treated with MMT 3 hr (–■–) and 24 hr (–●–) respectively, before morphine.

abolished by the previous injection of reserpine. This finding is in accordance with the results of previous reports on the interaction between reserpine and morphine in rats (Schneider, 1954; Banić & Medaković, 1964).

EFFECT OF MMT ON THE ANALGESIC ACTION OF MORPHINE IN RATS

First the effect of morphine was determined in a group of animals. Similarly, it was established that MMT on intraperitoneal injection did not affect the control mean reaction time. However, contrary to reserpine, if MMT (400 mg/kg) is injected 90 min before morphine, the analgesic effect of the morphine was not inhibited (Fig. 2). Moreover, the effect of morphine seemed to be slightly potentiated by the previous injection of MMT. This potentiation was, nevertheless, very weak.

The effect of morphine was again assessed 24 hr after the injection of MMT and was found to be equal to the effect obtained in the first control experiment.

EFFECT OF RESERPINE ON THE ACTION OF MORPHINE AFTER MMT

The experiments above on the effects of reserpine and of MMT indicated a substantially different mode of action for these two drugs on the analgesic action of morphine. Since MMT, which releases only brain noradrenaline, was without effect on the action of morphine, it is reasonable

to suppose that the effect of reserpine, which releases 5-HT as well as noradrenaline, is due to its action on brain 5-HT stores. To obtain additional evidence, the effect of reserpine on the analgesic action of morphine was tested in animals which were pretreated with MMT, with aim of depleting their brains of noradrenaline.

The action of morphine (4 mg/kg) was determined first, then MMT (400 mg/kg) was injected. On the following day (21 hr later) the animals received reserpine (1 mg/kg i.p.) and 3 hr. later morphine (4 mg/kg). As Fig. 3 shows, the analgesic action of morphine was strongly inhibited.

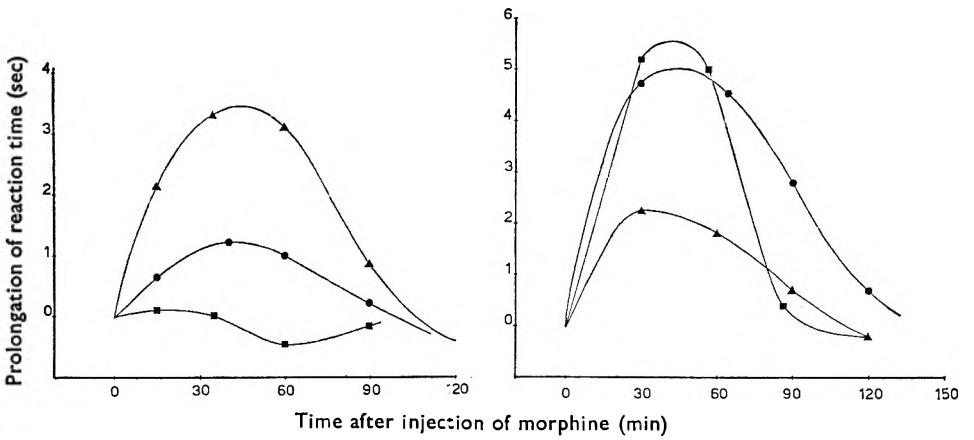

FIG. 3. The effect of reserpine on the analgesic action of morphine in rats pretreated with MMT. MMT (400 mg/kg) was injected once and reserpine twice, done 24 hr (– ●–) and 48 hr (– ■–) after MMT (always 3 hr before morphine). Control effect of morphine is indicated by (–▲–).

FIG. 4. The effect of reserpine (– ■–) and MMT (– ●–) on the analgesic action of morphine in rats, pretreated with iproniazid, 24 hr before morphine. Control effect of morphine is indicated by (–▲–).

The experiment was continued on the following day. Reserpine was reinjected (1 mg/kg), and 3 hr later the animals received morphine. The action of morphine was then completely abolished.

ACTION OF IPRONIAZID ON THE EFFECT OF RESERPINE AND MMT

Iproniazid may antagonise the effect of reserpine, and a reversal of the effect of reserpine may result in iproniazid-pretreated animals (Schaumann, 1958). The effects of iproniazid on the action of reserpine and MMT respectively were examined in relation to the analgesic action of morphine.

In the experiment shown in Fig. 4, iproniazid (100 mg/kg i.m.) was injected on the first day. On the next day MMT was injected first and morphine 2 hr later. As can be seen, the effect of morphine was strongly potentiated and prolonged. The experiment was continued, and iproniazid was re-injected on the same day. On the next day reserpine (1 mg/kg)

was injected and 2 hr after, morphine. The analgesic effect of morphine was potentiated and prolonged to the same extent as on the previous day after iproniazid and MMT (Fig. 4).

ACTION OF MMT AND RESERPINE ON THE EFFECT OF MORPHINE IN MICE

Our previous experiments (Banić & Medaković, 1964) suggested that the action of reserpine on the analgesic effect of morphine in mice might differ from that in rats. Therefore, some experiments were repeated in mice.

First, the action of MMT on the effect of morphine was studied, and compared with the action of reserpine on morphine analgesia. MMT (400 mg/kg) was injected 2 hr before morphine (10 mg/kg). As Fig. 5

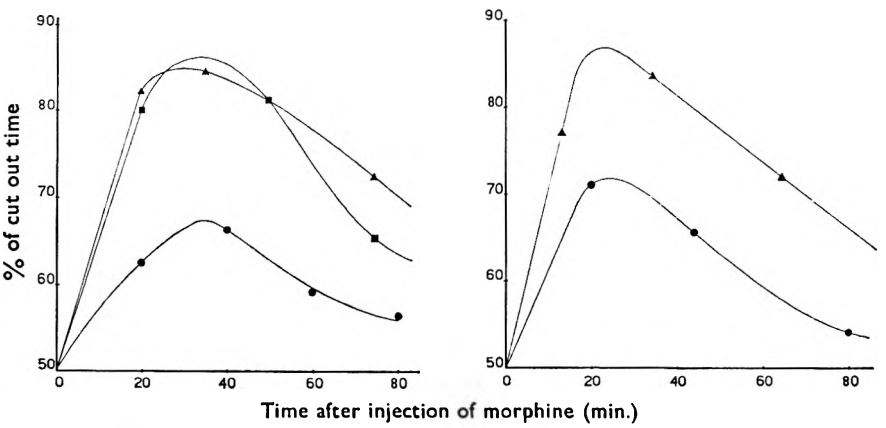

FIG. 5. The effect of MMT on the analgesic action of morphine in mice. The action of morphine in control animals (–▲–), in animals treated with MMT 3 hr (–●–) and 24 hr (–■–) before morphine.

FIG. 6. The effect of reserpine on the analgesic action of morphine in mice. The action of morphine in control animals (–▲–) and in those treated with reserpine 3 hr before morphine (–●–).

shows, the effect of morphine was significantly inhibited in MMT pretreated mice, as compared with the effect of morphine in untreated controls. The same effect on morphine analgesia was obtained with reserpine (Fig. 6). It is noteworthy that the effect of morphine was restored 24 hr after the treatment with MMT (Fig. 5).

In the second experiment mice first received MMT (400 mg/kg) and 24 hr later reserpine (2 mg/kg). This treatment was followed, 2 hr after reserpine, by the injection of morphine (10 mg/kg). Fig. 7 shows that reserpine did not antagonise the effect of morphine in mice which were pretreated with MMT on the previous day.

Discussion

At least three points can be discussed on the basis of the present results: (1) the evaluation of the concepts about the participation of noradrenaline and 5-HT respectively on the action of reserpine, (2) the

mode of action of brain amines on the analgesic effect of morphine, and (3) the mechanism of morphine analgesia.

Firstly, the effects of reserpine and of MMT on the analgesic action of morphine were studied in rats. According to the data presented, reserpine could affect the action of morphine in two ways: by direct action on the CNS or, indirectly, by its action on the stores of biologically active amines in the brain. Some actions of reserpine on the CNS have been explained by its direct action (Kobinger, 1958). However, the assumption that the effect of reserpine on morphine analgesia is produced indirectly, by its action in releasing the stores of biologically active brain amines, is favoured by the fact that those rauwolfia alkaloids which do not release these amines from the brain (e.g., rescinamine, serpentine, raubasine—Brodie, Shore & Pletscher, 1956) do not affect the analgesic effect of morphine (Schaumann, 1958). Therefore, the present results will be

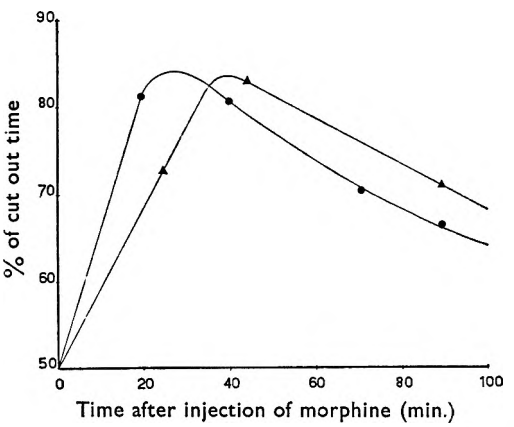

FIG. 7. The effect of reserpine on the analgesic action of morphine in mice treated with MMT (24 hr before morphine). The action of morphine in control animals (-●-) and in those pretreated with MMT and with reserpine (-▲-).

discussed on the assumption that the effect of reserpine is caused mainly indirectly, by its action on the amines, stored in the brain. The question now arises as to which of the actions, that on the stores of noradrenaline, or that on the stores of 5-HT, is responsible for the effect of reserpine.

Both MMT (Hess & others, 1961; Brodie & Costa, 1962) and reserpine (Holzbauer & Vogt, 1956) release noradrenaline from the stores in the brain. Consequently, if reserpine inhibits the action of morphine by acting on the stores of noradrenaline, MMT should produce a reserpine-like effect on morphine analgesia. However, we found the effects of these drugs to differ: while reserpine abolished the action of morphine, MMT did not do so. Hence, it is not likely that the inhibitory effect of reserpine was caused by its action on noradrenaline stores.

This assumption was further tested in the second experiment. First, the noradrenaline releaser MMT was injected and the effect of reserpine on morphine analgesia was examined in these animals on the following

day when reserpine also produced its inhibitory effect on the action of morphine in these animals. This fact favours the assumption that the action of reserpine on the stores of noradrenaline is not of primary importance for the inhibition of the effect of morphine. Reserpine releases 5-HT as well as noradrenaline and the action of reserpine on the stores of brain 5-HT has been repeatedly claimed to be responsible for the central effects of this drug (Brodie & Shore, 1957; Holtz, Balzer, Westermann & Wezler, 1957; Garratini Valzelli, 1958; Costa, Gessa, Kuntzmann & Brodie, 1962). Therefore, the fact that reserpine antagonised the action of morphine in rats pretreated with a high dose of MMT which depletes the brain stores of noradrenaline, but not those of 5-HT (Hess, 1961; Brodie & Costa, 1962), argues in favour of the assumption that the antagonism of reserpine to morphine was due to its releasing 5-HT. This agrees with the result on animals kept in a cold environment (Banić & Medaković, 1964), which suggested that reserpine acted on the analgesic effect of morphine through 5-HT rather than noradrenaline. It should be added that the relation between the actions of 5-HT and morphine has been much studied (Kosterlitz & Robinson, 1955, 1958; Gaddum & Picarelli, 1958; Medaković, 1957; 1958 b, c, d, 1959) and a highly specific antagonism between the actions of 5-HT and morphine has been found. Of special importance is the fact that the target tissue of this antagonism was always the nervous tissue, e.g., nervous elements in the isolated ileum of the guinea-pig. This is important since this isolated organ has been proposed and used as a suitable paradigm for studying central effects of analgesic drugs (Schaumann and others, 1952; Paton, 1957; Medaković, 1958). The reports on the antagonism between 5-HT and morphine on this isolated organ suggested that the competition between the two drugs takes place in the range of low concentrations (Medaković, 1958a). It seems reasonable to try to explain the antagonism between the actions of morphine and reserpine in the brain on a similar basis. This explanation is furthered by the concept of Brodie & his co-workers (1960, 1962), who explained the sedative effect of reserpine by its ability to release 5-HT from the pools onto the receptor sites in the brain. Hence, the hypothesis that the antagonistic action of reserpine to morphine is elicited by the presence of high concentrations of 5-HT at the receptor sites, and not because the brain stores of this amine are depleted by reserpine seems plausible. Our finding, that the second injection of reserpine in animals, which, after MMT, had already received a relatively small dose of reserpine on the preceding day (Fig. 3), still inhibited the action of morphine is in favour of the hypothesis. This second injection could be supposed to produce an additional effect by releasing those amounts of 5-HT which were not released by the first injection on the preceding day.

It may seem difficult to explain the findings obtained in experiments with iproniazid. As was shown, both MMT and reserpine potentiated the effect of morphine in iproniazid-pretreated animals. Hence, the effect of MMT was not qualitatively changed after iproniazid. However, the effect of reserpine was reversed. The same reversal by iproniazid of the

effect of reserpine was also obtained in experiments on animals which had received MMT 24 hr previously. As it can be assumed that brain stores in these animals were depleted of noradrenaline by MMT, the potentiating effect of reserpine must be ascribed to brain 5-HT. The assumption that 5-HT may exert two opposite effects, i.e., to antagonise and potentiate the action of morphine, may appear confusing. The inhibition of the brain monoamine oxidase by iproniazid should preserve brain 5-HT, and provoke its accumulation in high concentrations in the brain. Hence, one would now expect a more pronounced antagonism between reserpine and morphine than without iproniazid. The controversial finding that potentiation of the effect of morphine was obtained, instead of the expected deep inhibition, cannot be explained completely, but a feasible working hypothesis can be based on the fact that agents with high biological activity, for instance, acetylcholine, may cause opposite effects, depending on the actual concentrations. The same fact has already been established for 5-HT in experiments on the isolated guinea-pig ileum, where high concentrations of 5-HT antagonised the effect of smaller concentrations of the same drug (Rocha e Silva & Picarelli, 1953). It is noteworthy that a dual response can be obtained in rabbits given 5-hydroxytryptophan after a monoamine oxidase inhibitor; synchronisation is followed by desynchronisation of the brain EEG patterns (Costa, Pscheidt, Van Metter & Himwich, 1960). This finding prompted the hypothesis that monoamine oxidase inhibitors block 5-HT receptors through the presence of a large excess of 5-HT in the brain (Costa, Morpurgo & Revzin, 1961).

The experiments on mice showed that the mechanism of the action of reserpine on the effect of morphine might differ in this species from the action in rats. Contrary to the findings in rats, the previous injection of MMT antagonised the action of morphine, and reserpine failed to inhibit the action of morphine in mice which had received MMT 24 hr before reserpine.

The results of the present experiments in rats and mice do not seem to support the concept that the analgesic action of morphine is accomplished indirectly through its releasing brain noradrenaline stores. In rats, the analgesic action of morphine was not substantially changed by MMT pretreatment.

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Note on the detection of hexoestrol, stilboestrol, dienoestrol and the *p*-hydroxy metabolites of phenobarbitone and phenytoin in urine

S. L. TOMPSETT

A procedure for the identification of the synthetic oestrogens, *p*-hydroxyphenobarbitone and 5-(*p*-hydroxyphenyl) 5-phenylhydantoin in urine by use of two dimensional paper chromatography is described.

THE examination of the phenolic acids of human urine by two dimensional paper chromatography has been described previously (Tompsett, 1961). The results of such an examination could be confused by the presence of certain drugs, for example the synthetic oestrogens or of the *p*-hydroxy metabolites of drugs such as phenobarbitone (Butler, 1954, 1956; Curry, 1955; Algeri & McBay, 1956) and phenytoin (Butler, 1956, 1957) which may be included in treatment schedules.

Experimental

Essentially the same procedure was used as that described previously (Tompsett, 1961). Before ether extraction of the urine, the conjugates were hydrolysed by adding hydrochloric acid to give a N solution; the mixture then being placed in a boiling water-bath for 1 hr. Two dimensional paper chromatography using two systems (1) isopropanol ammonia, 0·88 : water (8:1:1) and (2) benzene: propionic acid: water (2:1:1) was employed and the same detecting agents used.

Since the substances under examination are *p*-hydroxylated compounds, the 1-nitroso 2-naphthol-nitric acid reaction described for the detection of tyrosine on paper chromatograms (Block, Durran & Zweig, 1955) was also used. The paper chromatograms were sprayed with a 0·1% solution of 1-nitroso 2-naphthol in ethanol and allowed to dry. After spraying with an aqueous 10% solution of nitric acid, the chromatograms were heated at 100° for 3 min. A positive reaction is indicated by the development of a red colour.

Experimental results are shown in Table 1.

Results and Discussion

In the first solvent system, the synthetic oestrogens show Rf values very much different from those shown by the phenolic acids. Little confusion can therefore result and the use of a second solvent system is unnecessary. Two dimensional paper chromatography is however necessary to indicate the presence of *p*-hydroxyphenobarbitone and 5-(*p*-hydroxyphenyl) 5-phenylhydantoin.

From the Biochemical Laboratory, Northern General Hospital, Edinburgh.

TABLE 1. CHARACTERISTICS OF SUBSTANCES EXAMINED

	Rf values solvent (1)	Rf values solvent (2)	Colour reactions on paper chromatograms *A*	*B*	*C*	*D*
Stilboestrol	0·85	—	yellow	brown	brown	negative
Dienoestrol	0·85	—	purple	brown	brown	negative
Hexoestrol	0·85	—	red-brown	brown	brown	positive
p-Hydroxyphenobarbitone.. ..	0·60	0·00	orange	deep purple	purple	positive
5-(*p*-Hydroxyphenyl)-5-phenyl-hydantoin	0·65	0·25	yellow	brown purple	orange	negative

A Pauly Reaction
B Diazotised *p*-Nitroaniline
C Diazotised Diethylaminoethyl *p*-aminophenylsulphone
D 1-Nitroso 2-naphthol/Nitric Acid Reaction

The use of differential detecting agents is of value in the detection of these substances. By the use of the Pauly reaction, at least 5 μg of each of these substances can be detected on paper chromatograms. This reaction can also be used for approximate determination, using the technique previously described (Tompsett, 1961). 5, 10, 20, 40 and 80 μg represent a useful range of standards. When positive, the 1-nitroso 2-naphthol-nitric acid reaction can detect 5 μg of these substances on paper chromatograms. Approximate determination can be made by comparison against a series of standards, 5, 10, 20, 40 and 80 μg representing a useful range. Amongst the phenolic acids, only *p*-hydroxyphenylacetic acid gives this reaction.

It has been suggested (Kolzelka & Hine, 1943) that phenytoin is also metabolised to α-aminodiphenylacetic acid (10 to 27%). In this investigation, no evidence to support this could be found.

Acknowledgements. I wish to express my thanks to Dr. A. S. Curry for a gift of *p*-hydroxyphenobarbitone and to Dr. T. C. Butler for a gift of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin.

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Letters to the Editor

Growth of *Pseudomonas aeruginosa* in solutions of esters of *p*-hydroxybenzoic acid

SIR,—During experiments with preservatives for eye-drops we have found that *Pseudomonas aeruginosa* NCTC 7244, originally isolated from an infected human eye, will grow readily, without previous adaption, in solutions of esters of *p*-hydroxybenzoic acid.

Two categories of experiment were performed, all at 37°. In one, rates of growth, as measured by changes in optical density of the culture, were made in a medium containing the esters in the same final concentration as in solution for eye-drops B.P.C. but supplemented with salts as % final concentration thus, $(NH_3)_2HPO_4$ 0·06, NaCl 0·1, $FeSO_4 \cdot 7H_2O$ 0·001, KH_2PO_4 0·04, $MgSO_4 \cdot 7H_2O$ 0·04, and compared with the growth rate in a similar medium containing 1% glucose in place of the esters (Table 1). In the second experiment growth in solution for eye-drops (methyl *p*-hydroxybenzoate, 0·0229%, propyl *p*-hydroxybenzoate, 0·0114% in purified water B.P.) was followed by means of viable counts. Thus at time intervals 0, 24, 48, 72 and 96 hr, the counts were 6, 24, 68, 120 and 270 × 10^4 organisms/ml.

TABLE 1. GROWTH OF *Pseudomonas aeruginosa* IN MINERAL MEDIUM WITH GLUCOSE OR ESTERS OF *p*-HYDROXYBENZOIC ACID AS SOLE CARBON SOURCE

Carbon source %	Optical density at 420 mμ after (hr)						
	0	18	24	40	50	62	72
Glucose 0·1	0	0·155	0·225	0·40	0·57	0·64	0·64
Methyl *p*-hydroxybenzoate 0·0229 Propyl *p*-hydroxybenzoate 0·0114	0	0·020	0·040	0·19	0·36	0·43	0·43
None	0	0	0	0	0	0	0

In the first experiment the rate of growth in the benzoate medium during the logarithmic phase was equal to that obtained with glucose as a carbon and energy source, the differences being that whereas with glucose a lag phase of 8 hr ensued before logarithmic growth commenced, with the benzoates, the lag phase was 26 hr; also the final optical density (cell crop) was greater with glucose.

The second experiment, which more nearly represented conditions likely to be met in practice, showed that this organism, even without mineral supplementation, grew in solution for eye-drops B.P.C.

In view of the current interest in eye-drop formulation (Editorial, 1963) we felt that these experiments might be of interest.

Department of Pharmacy, W. B. HUGO
The University,
Nottingham.
School of Pharmacy, J. H. S. FOSTER
Bristol College of Science and Technology.
January 9, 1964

Reference

Editorial (1963). *Pharm. J.*, **191**, 575–576.

Method for the determination of iodine value

SIR,—We have discovered that certain peroxides have an abnormally enhancing effect on the addition of bromine to sterically hindered double bonds. Using this we propose a method for the determination of unsaturation in oils and fats.

The procedure was tried on several oils and fats and the results, obtained after only 1 min contact between the oil or fat and the reagent, were identical with those obtained by using the method of Hanus (1901).

Reagent. To 500 ml of chloroform, add 4·5 g of dioxan and 6 ml of bromine, mix thoroughly and make up to 1 litre with chloroform. Store in a cool place in well-stoppered dark containers.

Procedure. Weigh accurately a suitable weight of the oil or fat in a dry glass-stoppered Erlenmeyer flask of about 500 ml capacity, Add 25 ml of the reagent, insert the stopper, previously moistened with potassium iodide solution, and swirl the contents. Allow to stand for 1 min, add 10 ml of 20% potassium iodide solution, mix, wash the stopper and the neck of the flask with water and dilute to about 200 ml. Titrate the liberated iodine with 0·1 N sodium thiosulphate solution, using starch as an indicator.

Carry out a blank and calculate the iodine value in the usual manner.

We find that samples weighing about 1 g for a fat, 0·3–0·4 g for a non-drying oil, 0·2–0·3 g for a semi-drying oil and 0·1–0·2 g for a drying oil were the most suitable.

Results. The results obtained by applying the above procedure to different oils and fats are given in Table 1.

TABLE 1. COMPARISON OF IODINE VALUES

Oil	Proposed method	Hanus	Wijs	Kaufmann
Linseed	180·9	182·6	183·4	181·2
	181·2	185·2	184·8	181·6
Cod liver	138	138·2	140·1	139·2
	138	140	140·3	140·1
Safflower	138·6	139·7	140·3	138·6
	140·2	140·2	140·7	139·4
Lettuce seed	127·0	126·7	127·8	125·9
	127·3	128·1	128·5	126·4
Maize	107·5	107·2	107·6	107·3
	107·7	107·7	108·2	107·4
Sesame	108·7	107·8	108·2	107·4
	108·7	108·1	108·6	107·8
Cotton seed	101·4	102·8	103·5	101·5
	102·2	103·4	104·1	103·2
Archis	75·3	74·6	74·9	73·8
	74·9	75·3	75·1	75·1
Olive	80·6	81·7	81·5	80·0
	81·3	82·8	82·3	81·1
Butter fat	32·9	33·4	33·7	33·1
	33·7	33·9	34·1	33·4
Depot fat	37·5	38·2	37·9	37·6
	38·2	38·5	38·5	38·3
Coconut fat	8·4	8·6	8·7	8·3
	8·5	9·0	9·0	8·7
Tung	154·3	154·2	—	—
(I.V. by hydrogenation = 229)	155·1	154·9	—	—

Analytical Chemistry Department,
Faculty of Pharmacy
Cairo University,
Kasr-El-Ainy, Cairo,
U.A.R.

F. SAID
M. M. AMER
A. K. SAYED AHMED
A. A. SAID

December 15, 1963

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Book Reviews

CHEMISTRY OF THROMBOLYSIS: HUMAN FIBRINOLYTIC ENZYMES. By Kurt N. von Kaulla. Pp. xvi + 333 (including Index). Charles C. Thomas, Springfield, Ill., U.S.A., 1963. $12.75.

Although the phenomenon had been recorded before, Denis is usually credited with being the first to describe in 1838 the spontaneous dissolution of a fibrin clot on storage. This phenomenon was termed fibrinolysis by Dastre in 1893, but remained a relatively neglected field of research. The attentions of haematologists were chiefly confined to the mechanisms of clot formation rather than the subsequent history of the fibrin clot. The past few decades have seen renewed interest in fibrinolysis and thrombolysis (the latter term denoting the intra-vascular dissolution of a thrombus). The current theory of fibrinolysis holds that circulating blood contains an enzyme precursor, plasminogen, which may be activated by a variety of activators or activating procedures to produce the enzyme, plasmin. The proteolytic enzyme, plasmin, then hydrolyses the polymer, fibrin, into fibrinopeptides and fibrinolysates, thus the fibrin meshwork of a clot is broken down. In the circulating blood under normal conditions there is an excess of an anti-plasmin which inhibits plasmin so that when blood is shed the normal prothrombin-thrombin-fibrin mechanism produces a clot.

Dr. von Kaulla of the University of Colorado School of Medicine has written the first comprehensive monograph on the physiological and pathological aspects of fibrinolysis. His own research work in this field, covering many aspects, such as the measurement of the excretion of urokinase in man or an extensive investigation into non-enzyme compounds which increase fibrinolysis, makes him well qualified for the authorship of such a book.

After an interesting account of the historical development of the study of fibrinolysis, the text proper begins with a consideration of the basic components of the fibrinolytic system which is a very clear account of current fact and theory backed up by a comprehensive bibliography.

The next three chapters are devoted to methods of measuring fibrinolytic activity and its component factors; there is a mixture of the description of principles of methods with detailed technical procedures. The plasminogen activator urokinase, which can now be extracted from human urine, is given a chapter on its own which includes a full account of the author's technique for urokinase estimation and a consideration of the significance of the results, with case history illustrations. There is a comprehensive tabular review of diseases, observations and references, in the chapter on fibrinolysis and diseases, which will prove to be an invaluable reference list. The account of maternal and neo-natal fibrinolytic systems is shorter than the importance of this aspect of fibrinolysis would seem to warrant.

The discussions on the influence of dietary lipaemia and cholesterolaemia of the fibrinolytic system and on the interactions of the clotting and fibrinolytic systems are made in an unbiased and scientific manner.

The remainder of the book is devoted to the therapeutic uses to which knowledge of fibrinolytic mechanisms may be put in thrombolytic therapy in man and animals and in the treatment of fibrin deposits in extra-vascular sites. Chapter 21 is forward-looking in being an account of a study of synthetic non-enzyme compounds which increase fibrinolysis. The first to be investigated was ethylurethan. The hydrotropic activity of such compounds is suggested as a probable mechanism of action. Of 147 hydrotropic chemicals tested, 53 were found to induce fibrinolysis.

Dr. von Kaulla's book is easy to read and its usefulness as a book of reference enhanced by separate Author and Subject Indices. The high reputation enjoyed by the publisher is well maintained by this volume. It is a book which may be studied with profit by all concerned with the study of a subject which promises to be of increasing importance.

A. T. BIRMINGHAM

GENERAL MICROBIOLOGY. Second Edition. By R. Y. Stanier, M. Doudoroff and E. A. Adelberg. Pp. xiii + 753 (including Index). Macmillan & Co. Ltd., London, 1963. 50s.

One of the most impressive aspects of this very good book is the wealth of illustrations. The tables and figures are well conceived and executed. The general layout also shows much evidence of care and attention in the communication of ideas. The book is to be recommended to students requiring a general, broad introduction to microbiology.

Many students now study microbiology without the benefit of a background in general biology. Such students are considered by the authors who stress general biological principles at appropriate points and in fact make little assumption of prior biological knowledge.

All the basic aspects of microbiology are adequately covered and in particular the sections on metabolism and genetics are models of clear, logical presentation. In addition most topics of special interest to students of the medical sciences are considered in reasonable detail. There are some exceptions, however. The section on antibacterial agents is too scanty and could well be expanded to cover dynamics of disinfection, dilution and temperature coefficients. Nevertheless it is pleasing to see these agents classified according to their effects on the cell rather than on their chemical structure.

Methods of sterilisation are treated inadequately for pharmaceutical microbiologists, particularly in view of the recent advances made in this field. An improvement would be to treat sterilisation as part of the chapter dealing with the effect of environment on growth and death rather than as part of a chapter on History. The lethal effects on micro-organisms of ionising radiations is allotted only one paragraph and this is insufficient from a pharmaceutical standpoint. From the same point of view the principles of antibiotic production are almost completely neglected. It is a pity that some elementary material such as the diagrams relating to refraction of light and focusing by lenses should be included at the expense of less elementary material. But it is clear that the authors do not set out completely to cover every aspect of microbiology and the omissions mentioned are comparatively minor ones.

M. R. W. BROWN

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Review Article

Research Papers

Letters to the Editor