REVIEW ARTICLE

THE CHOLINERGIC RECEPTOR*

BY PETER G. WASER, Prof.Dr.Med., Dr.Phil.

Department of Pharmacology, University of Zürich

SINCE the time when a knowledge of anatomical structure first offered a contribution to the understanding of the functioning of the living body, advances in physiology, and later in biochemistry, helped to further our understanding of the basic principles of life. This approach in biological research has remained the same in modern times. As we try to solve more and more problems of ultrastructural dimensions we still follow the same road that leads from morphology to function. Perhaps one day we will come to the point where we can see molecules and ions in action in a living tissue or organ. Then the two routes will meet and we will have complete understanding of a biological phenomenon. The final goal of all our endeavours in research will therefore be to link morphology with function.

Today good tools have been developed with which to attack the basic problems of this sort. Microscopy has been surpassed by electron microscopy; simple electrodes for measuring action potentials by microelectrodes, which can be placed inside single cells. Chemical compounds can be labelled with radioactive atoms and followed in the body to their site of action. Enzymes can be measured in single cells or even in microsomes. These are only a few of the many ingenious methods used in modern laboratories for biological research. In the instance I propose to discuss we have applied some of these methods to the elucidation of the nature of the cholinergic receptor.

Acetylcholine as a Neurohormone

A main problem of biology is the phenomenon of nerve activity, nerve conduction and synaptic transmission. In the last century anatomy described different nervous systems: the central nervous system, the peripheral motor and sensory nerves and the autonomic nervous system. But soon they were shown to be similar in many physiological and biochemical properties. One of these properties is the action of acetylcholine as a vital part of their functional mechanism. Early in this century it was found that choline and muscarine have many properties in common, notably the lowering of blood pressure and pulse rate, and the increase of intestinal peristalsis and glandular secretion. As different derivatives of choline were studied, Reid Hunt[1] reported in 1906 that the acetyl ester of choline has an activity many thousand times greater than choline. After the work of Sir Henry Dale[2] on the blocking of the muscarinic action of choline esters by atropine, Otto Loewi in 1921[3],

---

demonstrated the liberation of a chemical substance which caused brady-cardia after stimulation of the mixed vagus nerve, with his famous experiment using the crossed circulation of two frog hearts. This neuro-hormone was later shown to be acetylcholine. It was found in many other synapses, where it transmits the nerve impulse: in the superior ganglion cervical, the adrenal medulla, at the effector cells of the sweat glands, the myoneural junction and in motor and sensory neurones of the central nervous system.

The Cholinergic Transmitter

Acheson in 1948[4], in his review of the chemical aspects of neuromuscular junctions, summarised the quantitative results of different research workers who had determined the output of acetylcholine per nerve impulse for each nerve ending (Table I). The amount of acetylcholine

TABLE I

CALCULATION OF OUTPUT OF ACETYLCHOLINE PER NERVE IMPULSE FOR EACH NERVE END (AFTER ACHESON[4])

	Acetylcholine released per volley	Estimated number of endings of fibres	Acetylcholine released per nerve ending per impulse
Perfused organs:			
Cat tongue[35]	$10^{-4} \mu g.$	720,000	$1\cdot4 \times 10^{-10} \mu g./$ endplate
Cat cervical ganglion[38]	$10^{-4} \mu g.$	1,000,000	$1 \times 10^{-10} \mu g./$ synapse
Cut end of nerve trunk:			
Frog sciatic[37,38]	$2 \times 10^{-7} \mu g.$	1,000	$2 \times 10^{-10} \mu g./$ fibre
Quick frozen nerve trunk:			
Frog sciatic[38]	$10^{-8} \mu g.$ per μ length		$1\cdot5 \times 10^{-10} \mu g./$ endplate

released by one impulse is $1\cdot5 \times 10^{-10} \mu g.$, and what seems to be very important, it is independent of the substrate, endplate or ganglionic synapse. When we calculate the number of molecules acting in a free state at this moment, we find the relatively small number of 6×10^5. As part of the acetylcholine might be destroyed by cholinesterase in spite of neostigmine, present during the experiments, the real number would be likely to be larger, perhaps 10^6 molecules of acetylcholine.

With another method Buchtal and Lindhard, in 1942[5], found a much higher minimal dose, 5 ng., of acetylcholine applied to the endplate of a single muscle fibre of the lizard was needed to produce a contraction. But as the volume added to the endplate was a droplet of about 50 times the volume of the endplate, the need for the large amount may be explained by diffusion effects and immediate enzymatic breakdown of most of the acetylcholine. The same must be said for the results from close arterial injection. The best results, so far, have been obtained by del Castillo and Katz[6] with their elegant technique of electrophoretic application of acetylcholine to the external surface of a frog muscle endplate at a distance of $10 - 20 \mu$. With $10^{-15} - 10^{-16}$ moles of acetylcholine, or $6 \times 10^7 - 10^8$ molecules; under these conditions depolarisation was obtained. But

acetylcholine release into the interior of the muscle fibre failed to depolarise the endplate and to excite the muscle.   Here the effective number of externally applied molecules might be smaller, as the distance between nerve ending and subneural membrane is less than $0 \cdot 1 \mu$, and therefore fewer molecules may suffice for an immediate contact with the membrane. I believe an average minimal number of 10^6 molecules of acetylcholine is required for depolarisation of the membrane.   To my knowledge no measurements of this kind have been made at ganglionic synapses.

Indirect proof for the vital action of acetylcholine in the endplate was given by Nachmansohn in 1939[7] in his investigation of acetylcholinesterase in muscle.   He found a very high concentration of this enzyme right in the endplate, whereas the muscle itself had a low enzymatic activity. The endplate of frog sartorius contains enough activity to split $1 \cdot 6 \times 10^9$ molecules of acetylcholine in 1 millisecond, or a thousand times more than that needed for depolarisation.   The cholinesterase in the endplates may be stained histochemically (Koelle)[8].   The same proof is possible with ganglionic cells of the ciliary ganglion (Fig. 1), or of some regions in the brain and in many other places, as for instance in Pacinian corpuscles[9].

In addition to the splitting enzyme, the synthesising enzyme, choline acetylase, was detected at the same site[10].   Its distribution in the central nervous system was studied extensively by Feldberg and Vogt[11].   Beside acetylcholine other cholinergic transmitters, propionylcholine, butyrylcholine and murexine, are present in different animals and organs, but their action is not yet understood.

We must therefore conclude that acetylcholine plays an important role in the synaptic transmission in various anatomical substrates.   With the exception of sensory fibres and postganglionic sympathetic or adrenergic neurones, all the nerves of the mammalian peripheral nervous system are probably cholinergic, liberating acetylcholine.   Just as in the peripheral nervous system, the concentrations of acetylcholine, cholinesterase and cholineacetylase run nearly parallel to each other in different regions of the brain and spinal cord.

Even in primitive animals acetylcholine plays an important role.   The heart of the mollusc, murex brandaris, octopus, venus mercenaria, mytilus, or helix pomatia, is slowed by its action and the amplitude is diminished.   In annelids, lumbricus terrestris, it shows an exciting and tachycardic effect.   In insects the important action of organophosphorus insecticides may be explained by irreversible blocking of cholinesterase and death by maximum synaptic stimulation by acetylcholine.

The reason for the importance of acetylcholine in synaptic transmission is that this neurotransmitter depolarises the synaptic membrane.   This causes physical events which may be measured in various ways.   These include the measurement of action potentials (Fig. 2), ion movements through the membrane, especially of radioactive potassium and sodium, and even heat production and temperature changes.   The activating mechanism, may be described as a rise in permeability of the membrane. Due to this change, ions may pass freely from inside the cell to the outside and the reverse.   This happens in cholinergic neurons having synapses

with other ganglionic cells, muscle fibres, striated, smooth or cardiac muscle, or even with secretory cells. By far the largest number of the synapses in the mammalian body use this mechanism and only a small number work with other neurotransmitters like noradrenaline, adrenaline and probably other unknown neurohormones.
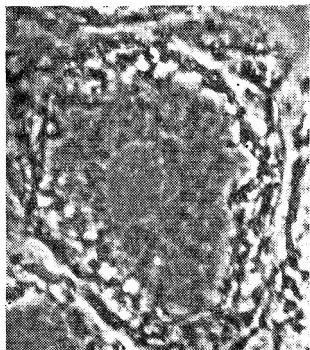

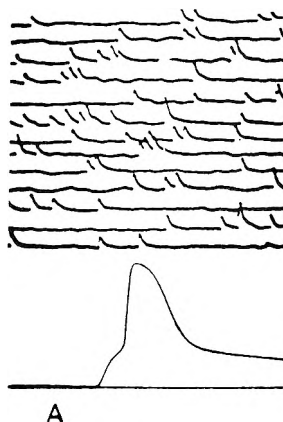FIG. 1. Ganglion-cell with acetyl-cholinesterase (phase contrast) of ciliary ganglion of cat[10].

A

FIG. 2. Spontaneous subthreshold activity at motor nerve-ending (above) and action potential after stimulation of nerve (below)[41].

The main questions to be discussed are:

(i) what is the substrate on which acetylcholine acts? and

(ii) what is the change of this substrate by depolarisation?

For our problem it is very important that a ganglionic synapse or an endplate are representative for other cholinergic synapses, even central ones. Investigations are much easier with peripheral organs which may be isolated and studied under controlled conditions.

There is excellent evidence for a specialised function of the endplate region. Langley demonstrated its stimulation by nicotine, which curare prevented. He proposed the existence of a specialised material, which he designated the "receptive substance". The same region in an isolated fibre is depolarised by very small concentrations of acetylcholine or nicotine, the rest of the surface of the muscle being at least a thousand times less sensitive to these substances.

The Use of Labelled Curarine

We are interested in the action of curare and depolarising drugs on endplates, and we hope to get more information on the cholinergic receptor substance by labelling different molecules and by tracing them to their place of action[13-15]. ^{14}C-calabash curarine (Fig. 3) was first synthesised and extensive studies of its metabolism in cats were made[16]. This alkaloid has a strictly selective action on muscle, yet the concentration in muscle of cats paralysed by the minimal effective dose was below $0·2 \mu g./g.$

Furthermore, curarine was metabolised slowly in the animal body, one-third being excreted unchanged in the urine within 3 hours. The paralysing action of the low concentration in the skeletal muscle was traced by autoradiographic analysis.

Mouse Diaphragm Autoradiographs

The biological assay system used was simple. We injected the minimal lethal dose of an aqueous solution into the tail veins of mice, which died
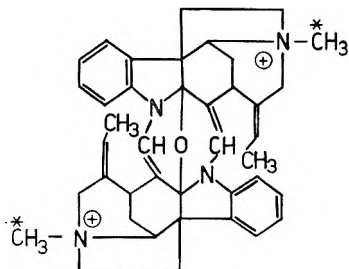

FIG. 3.   Formula of ^{14}C-calabash curarine.

of asphyxia within one to two minutes. Immediately after respiration had stopped they were decapitated and bled to diminish the interfering radioactive blood volume as far as possible. Then the diaphragms were isolated and stretched over steel rings of appropriate diameter (Fig. 4).
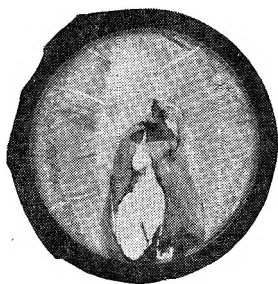

FIG. 4.   Mouse-diaphragm mounted on steel ring. Band of endplates with staining of cholinesterase[8] around centrum tendineum.
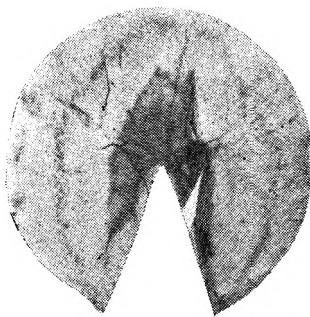
FIG. 5.   Autoradiograph with ^{14}C-curarine localised in the endplates, in the central tendon and in some central vessels. (Exposure: 5 months, Ilford film PM2.)

The $100\,\mu$ thick diaphragms were then dried in air for a few hours, cut away from the steel rings and placed on X-ray film or mounted on microscope slides and covered by stripping film. Two to six months later the contact films were developed under controlled conditions. In addition, the endplates in the diaphragms were separately stained for cholinesterase by Koelle's method[8].

The endplates are seen to be arranged in a circular band around the central tendon (Fig. 5). The details of this band, which is often split in

581

different parts, especially on the right side, show up beautifully in the autoradiographs. We can distinguish the individual endplates, the average diameter being only 8–18 μ. There is always some background fog on the film which gets stronger with long exposure. In the middle of the diaphragms blackening by radioactive blood within the vessels and in the tendon is seen. Increased radioactivity at the periphery is due to small vessels reaching the endplate band from the periphery.

The stripping film showed an accumulation of silver grains directly over the endplates (Fig. 6). Because of the heavy cross-fire from deeply situated radioactive endplates in the diaphragm there was a large number of grains in the muscle tissue between them. The radioactivity responsible for this originated mainly in the synaptic structures, because on the stripping film, four times as many silver grains were counted over the endplate band than over the endplate free central part of the muscle.

To get an idea of the distribution in a cross-section through the dia-phragm, diagrams of grain density were made (Fig. 7). Approaching the endplate region from the outside there is first some radioactivity caused by small peripheral vessels originating from the ribs. Then follows the main activity due to the endplate region, and finally a third peak due to the blood in the large vessels around and in the central tendon. The total amount of silver grains in the endplate region was integrated and the grains due to radioactivity in the muscle tissue, in the blood of capillaries and due to background fog were subtracted. The average radioactivity of one endplate was calculated by dividing this remaining activity by the number of endplates in the region considered. For this one must use an empirical formula. The radioactivity of one endplate corresponds to the number of curarine molecules reacting with the receptor surface. We have estimated in this way that 8×10^6 molecules of curarine are bound to one endplate.

With a later and much better method we have now determined this number again. Comparing for calibration purpose the autoradiographs of diaphragms with artificial discs made of gelatine of the same weight and thickness and varying concentrations of radiocurarine, the concentra-tion of curarine in one endplate can be directly measured by densitometer recordings of the film. The number of molecules may then be calculated without empirical factors as 3×10^6. This approximate calculated figure agrees fairly well with the number of acetylcholine molecules needed for synaptic transmission. Since at most one receptor can be stimulated by one molecule of acetylcholine, with the minimal paralytic dose of curarine, less than 10^6 receptors will remain free. Therefore the total number of cholinergic receptors in one endplate is probably a little larger. It might well be that one endplate has a reserve of receptors not all of which are usually needed, like the large reserve of cholinesterase for the destruction of the liberated acetylcholine. Our experiments with neostigmine may later confirm this point.

The figure of $3 - 4 \times 10^6$ for the cholinergic receptors at one endplate is in fact small when the molecular volume of curarine (mol. wt. 853) is compared with the large surface of the subneural membrane. With the

FIG. 6. Endplates of diaphragm with stripping film × 1,000 (Kodak AR10). (Exposure: 60 days.)
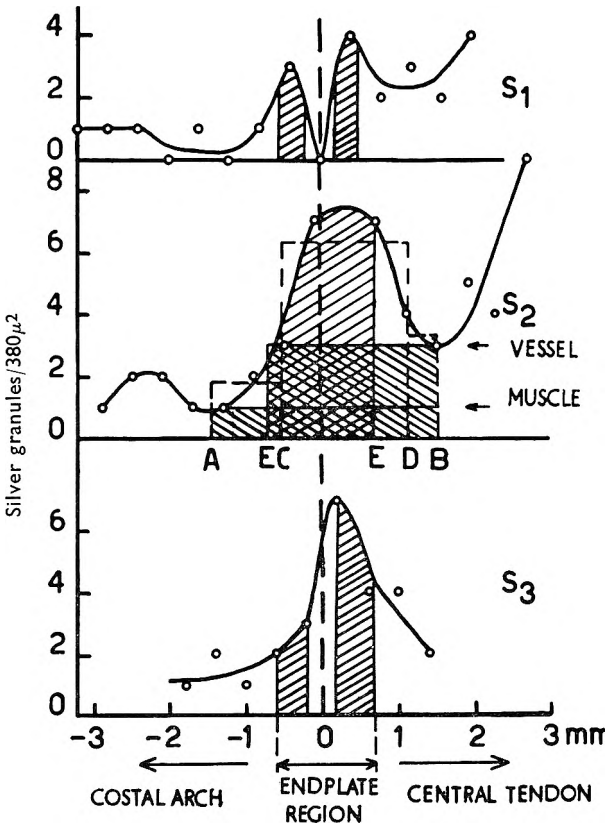

FIG. 7. Diagram of grain density at three different cross-sections through diaphragm. ^{14}C-curarine.

help of the electron microscope it was found by various authors[17] that the palisade structure of Couteaux[18] consists of many folds (Fig. 8). 6×10^{11} molecules of curarine would be present in 1 cm.^{2}, if we consider only the surface of the endplate without any folds.   But on the same surface we could place up to 10^{14} molecules side by side in a monolayer.   Therefore the coverage of the fold entrance area must be less than 1 per cent, and of the whole postsynaptic surface area considerably less than 1 per cent. Andersson-Cedergren[19] determined the percentage of fold entrance area
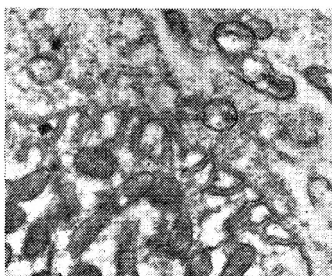

FIG. 8.   Electron micrograph of endplate in mouse-diaphragm.   Nerve axon above with mitochondria and plenty of granules, sarcoplasma of muscle with many mitochondria below, folds of postsynaptic membrane between[42].

to whole postsynaptic surface area as 10 per cent.   As we assume the subneural membrane to have a regular structure with evenly distributed receptors, this consideration shows that only a few highly differentiated and widely distributed receptors are occupied by the curarine molecules. They might be at the bottom of the folds.
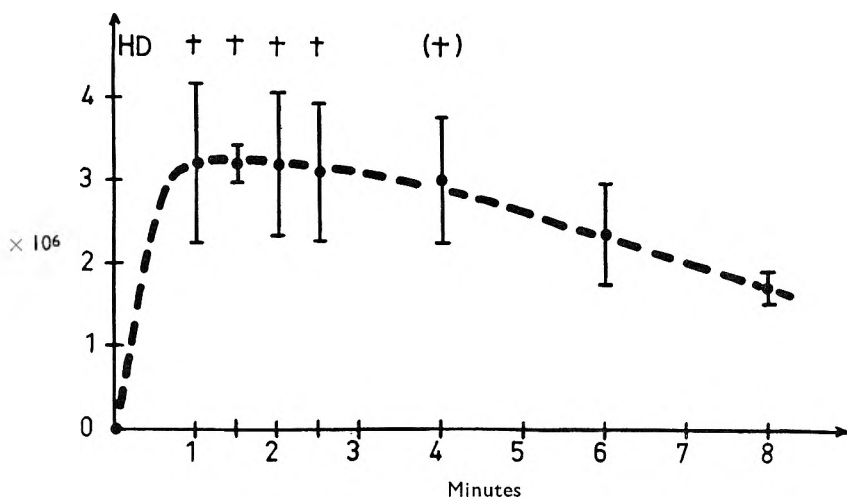

FIG. 9.   Number of curarine molecules per endplate of different mouse-diaphragms receiving the minimal lethal dose of 0·1 μg./g. intravenously.   HD: head drop, + : death by asphyxia.

The radioactivity in the endplates of animals surviving in spite of being given the minimal lethal dose, decreases slowly although respiration is resumed at a higher than normal rate. Autoradiographs of the diaphragms made two or four minutes after intravenous injection look nearly the same as those obtained at the moment of death in the more sensitive animals. Four minutes later the radioactivity is diminished and 12 minutes after the injection traces of radiocurarine still remain. Measurements with the densitometer show the time course of curarisation at the endplate (Fig. 9). As curarine seems to be fixed at the endplate for quite some time and metabolism of this compound is very slow, synaptic transmission must be possible with a large proportion of the receptors occupied by curarine, leaving a relatively small number of receptors free
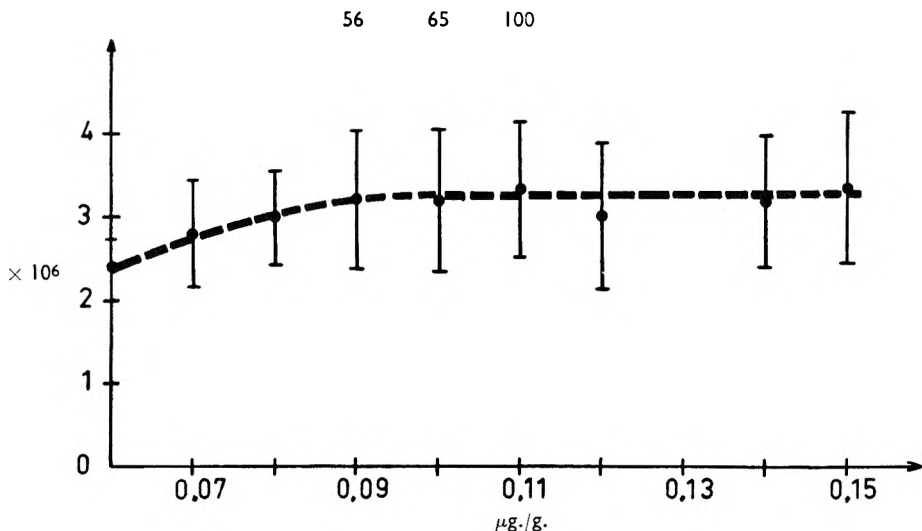

FIG. 10. Number of curarine molecules per endplate in diaphragms of mice receiving increasing doses of curarine. Saturation is reached with 0·1 μg./g. i.v. Figures above graph are per cent death by curarisation.

for acetylcholine. Curarine might on the other hand be inactivated by binding to some unspecific macromolecular structure and later slowly eliminated.

When we injected a higher than the minimal lethal dose, the mice died within 1–2 minutes. The number of curarine molecules in one endplate determined by densitometer measurements of the films does not surpass 3×10^6. With lower doses this figure is proportionally diminished, and the mice may survive the curarisation. Near the minimal lethal dose saturation of all receptor groups is reached (Fig. 10). This shows again their number to be finite. The autoradiographs after high doses are darker, due to more radioactivity in the muscle.

Anatgonism of neostigmine to curarine at the level of the endplates was shown by simultaneous injection of both substances[20]. The minimal lethal dose of radiocurarine was mixed with different doses of the

antagonist. Although nervous transmission through the endplate was restored within a minute, radioactivity in the endplates was not noticeably diminished by low doses of neostigmine when compared with the same dose of curarine alone (Fig. 11). Radiocurarine was lost by the endplates only when a lethal, ten times normal dose of neostigmine was given.
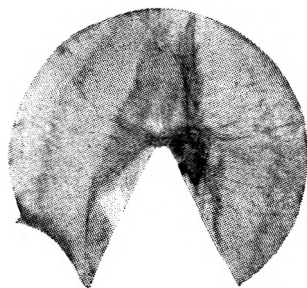
FIG. 11. Radiocurarine (0·15 μg./g. i.v.) in endplates in spite of antagonising neostigmine (2·5 μg. i.v.).

This may indicate that the cholinergic receptors blocked by curarine and the cholinesterase blocked by neostigmine are located at different sites, since both molecules cannot be at the same place. Competitive antagonism between acetylcholine and the curarising drug likewise does not

30   66

FIG. 12. Effect of phrenicotomy on left side on acetylcholinesterase (above) and curarine fixation (below) after 30 and 66 days.

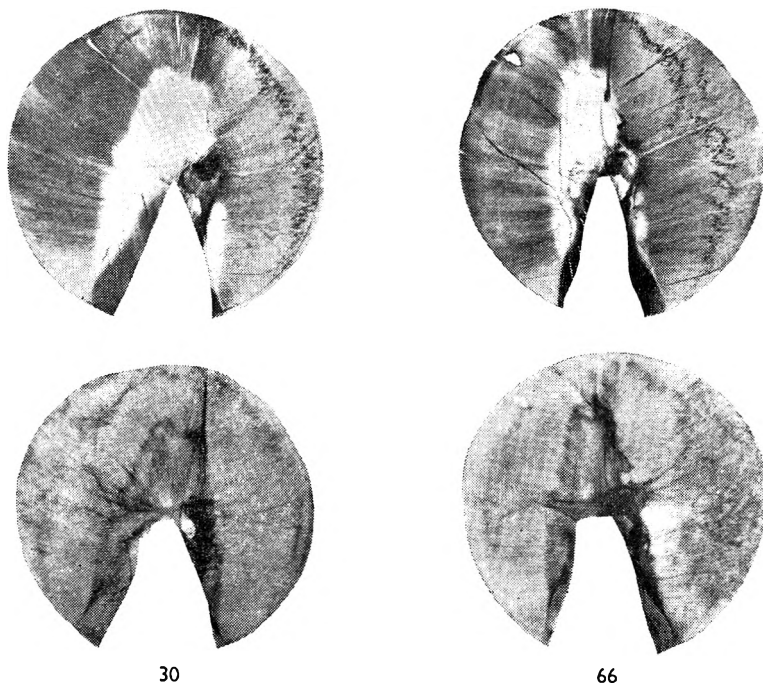explain why the radioactivity should remain in the endplates after neostigmine. If, however, there is a large reserve of cholinergic receptors for neuromuscular transmission, the reactivation of a few of them will not be easily detectable.

To show competitive antagonism between acetylcholine and curarine at the same receptor, we injected into isolated diaphragms of the living mouse by the so called close arterial injection technique through the abdominal aorta. With different combinations of both drugs or potassium chloride and curarine this antagonism is apparent by quenching the radioactivity in the endplates.

The Relation of the Receptor and Cholinesterase

What is the relationship between the cholinergic receptor and cholinesterase? To answer this question we studied the influence of denervation on binding of ^{14}C-curarine by cutting the left phrenic nerve as previously
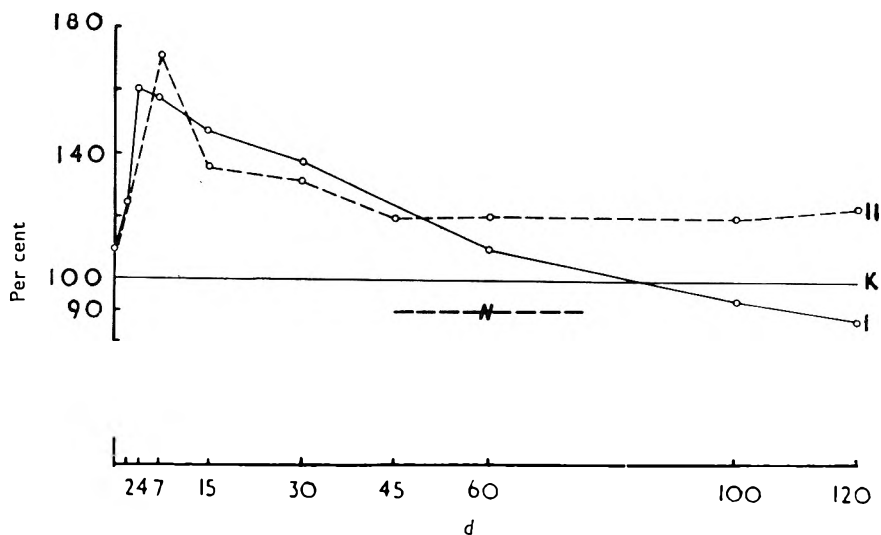

FIG. 13. Radioactivity by ^{14}C-curarine fixation after total phrenicotomy (I) and coagulation of phrenic nerve with regeneration (II). K: normal control side (right part of diaphragm), N: radioactivity of muscle.

described[15,20] Different time intervals after phrenicotomy the endplates on the severed side were compared with the endplates on the intact right side of the diaphragms (Fig. 12). In a similar group the phrenic nerve was only coagulated with dry ice (CO_2) so that regeneration was possible. In both groups an increase of curarine fixation up to the 45th–60th day, with a maximum after 4–7 days, after the operation was seen. In the denervated group the radioactivity then disappeared completely within 60–120 days (Fig. 13). In the other group regeneration set in immediately and after 45 days curarine fixation was normal. Cholinesterase, determined histochemically with Koelle's method[8], was slightly augmented in the first 7 days and then decreased continually to zero

587

120 days after denervation (Fig. 14). However, 30 days after regeneration cholinesterase was normal again. We must conclude from this corresponding behaviour that there is a close connection between the amount of active cholinesterase and the radioactivity due to bound curarine in the endplate. Differences in their concentrations may be partly due to different techniques in their determination.

Localisation of Depolarising Drugs

To study the localisation of depolarising drugs we synthesised decamethonium with six radioactive methyl groups (Fig. 15). On a molar basis the radioactivity thus obtained was 3·5 times higher than that of the labelled curarine. Decamethonium behaved similarly to curarine, and
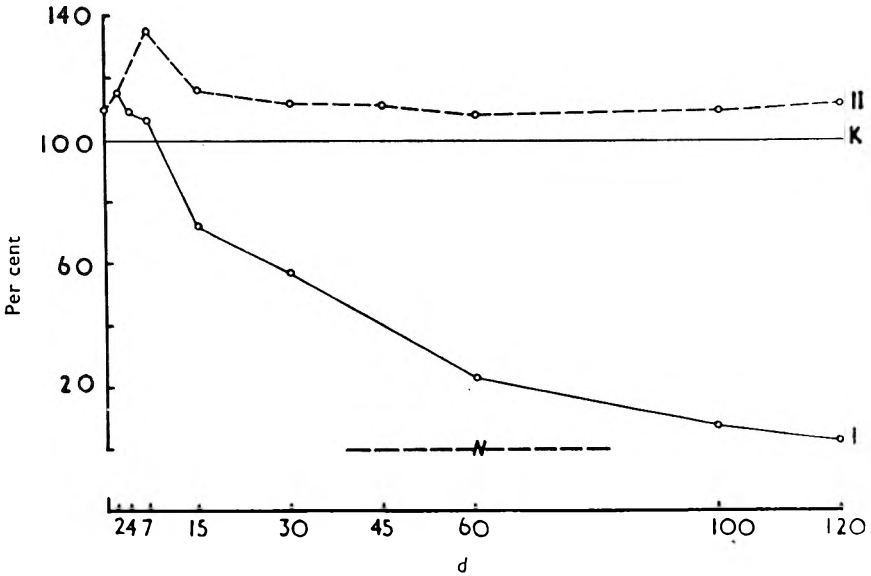

FIG. 14. Cholinesterase activity determined by histochemical staining and measurement with densitometer (I, II, K and N same as Fig. 13).

after an exposure time of only 3 days the contact films showed selective fixation in the endplates. Again the distribution of endplates identified in this way was similar to the Koelle stained diaphragm. The similarity between the autoradiographs of the two types of curarising drugs demonstrates the direct action of curarising and depolarising drugs at the endplate. But there are some important differences. On stripping films over diaphragms with decamethonium we found 10 times more grains than in films over diaphragms with curarine. On the contact films the endplate band always has a blurred appearance and the resolution of the endplates was not good (Fig. 16). We never obtained a clear picture showing individual endplates as we did with curarine. Comparing the autoradiographs with artificial diaphragms made of gelatine with different concentrations of decamethonium we found $1·4 \times 10^8$ molecules in one endplate

after the minimal paralytic dose. This amount is 50 times higher than with curarine. The diffuse appearance of the endplate band may be explained by fixation of decamethonium not only in the receptor area but farther out on the muscle membrane around the endplate. Perhaps the very different chemical structure enables decamethonium in contrast to curarine to diffuse from the post synaptic space into the muscle, which would explain the extended region of depolarisation[21].

It would be most difficult to use radioactive acetylcholine for our purpose, as this neurotransmitter acts in a very short time and is
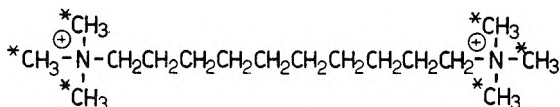

FIG. 15. Formula of ^{14}C-decamethonium.

immediately destroyed by cholinesterase. Instead of acetylcholine we have to use different cholinergic molecules resembling acetylcholine but being more stable. In the last years we have been interested in muscarine. We were able to isolate this alkaloid for the first time in a pure crystalline form from *Amanita muscaria* and to determine its pharmacological action[22,23]. Muscarine occupies the same receptors as acetylcholine but
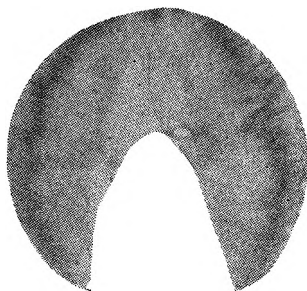
FIG. 16. Autoradiograph of diaphragm with 0·08 μg. decamethonium/g. i.v. Note diffuse fixation in endplate band.

preferably in the periphery of the autonomic nervous system[24]. Nevertheless there are marked differences in action. Muscarine is stronger than acetylcholine but much slower to act. It sensitises synapses to acetylcholine and has a prolonged action because it cannot be destroyed by cholinesterase. Endplates are not affected and ganglionic synapses are depolarised only with a high dose. Very little is known about the central effects.

The Structure of Muscarine

The structure of muscarine was found to be very similar to acetylcholine, the only difference being a tetrahydrofurane ring with a hydroxy group instead of the carbonyl in the ester group[25,26] (Fig. 17). The different isomers of muscarine are all at least 100 times less potent in action on

589

blood pressure of cats and on other cholinergic effects than the natural alkaloid[27]. The activity of the isomers depends on the position of the constituents on the tetrahydrofurane ring. Steric hindrance of the hydroxy group and of ether oxygen plays an important part in the contact

(+)—Muscarine-I (natural form)

(±)—Epimuscarine-I

(±)—Allomuscarine-I

(±)—Epiallomuscarine-I
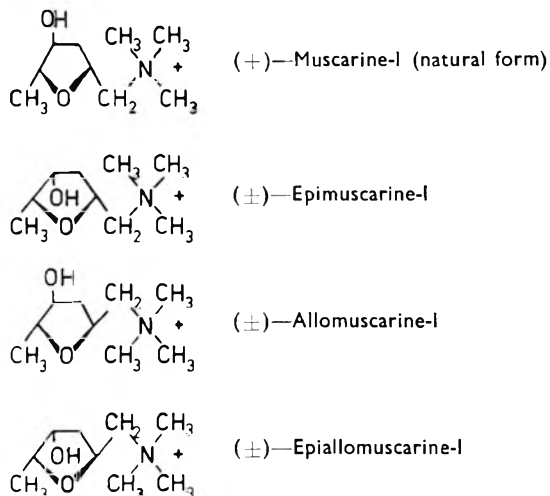
FIG. 17. Formulae of stereoisomers of muscarine.

(±)—Muscarine-I

(±)—Thiomuscarine-I

(±)—Desmethylmuscarine-I

(±)—Propylmuscarine-I

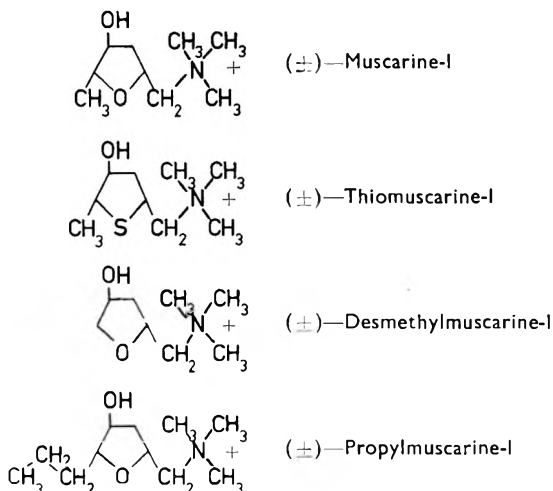FIG. 18. Formulae of derivatives of muscarine.

with the cholinergic receptor. As nor-muscarine without the quaternary nitrogen is inactive, at least three different points of chemical interaction have to be considered. This is stressed by the fact that muscarine is highly stereospecific in its action. Muscarine with a thiophene ring instead of the tetrahydrofurane loses much of its activity probably because

no hydrogen bridge may be formed with the sulphur atom in the ring[28] (Fig. 18). Three such pharmophore groups can be fixed only in one way with the corresponding receptor. Because of the tetrahydrofurane ring the positions of the hydroxy—and the methyl groups are absolutely fixed and only the trimethylammonium side chain can move. With many other synthetic muscarine-like molecules we found that the volume of the ring and the length of the side chains play important roles, as has been suggested by Ing[29] and by Pfeiffer[30].

The Application of Radioactive Muscarone

Recently muscarone with a carbonyl instead of the hydroxy group was synthesised (Fig. 19). This molecule resembles acetylcholine very closely but again it has no ester group that can be hydrolysed by cholinesterase. Its pharmacological action is astonishing[27]: in the cat it is 10 times as
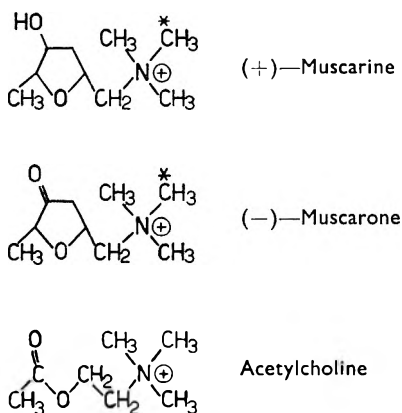
FIG. 19. Formulae of ^{14}C-muscarine and ^{14}C-muscarone.

active as acetylcholine and on intravenous injection after atropinisation it even depolarised ganglionic and neuromuscular synapses. While muscarine is highly stereospecific in its action, muscarone as compared to its isomers, is not. Therefore the configuration of this stable and rigid molecule must closely resemble the active form of the flexible acetylcholine molecule at the receptor site (Fig. 20). Hence we felt justified in using radioactive muscarone instead of acetylcholine for our investigations of endplates.

When we injected ^{14}C-muscarone with a high specific activity intravenously into mice, they were immediately killed by its strong parasympathomimetic action. The endplates of the diaphragms did not show any accumulation of radioactivity. But when we injected the labelled alkaloid into atropinised mice, they were completely paralysed and died of asphyxia in the same manner as with decamethonium. An obscure blackening of the endplate region was visible similar to the decamethonium type, but unfortunately the muscle fibres also contained much radioactive muscarone (Fig. 21).

The conclusions we can draw from these results are: The receptor sites cannot be in one plane but must have a definite three dimensional structure. The cholinergic molecules have to fit into them. For depolarisation, their pharmophore groups (quaternary nitrogen, carbonyl and ether
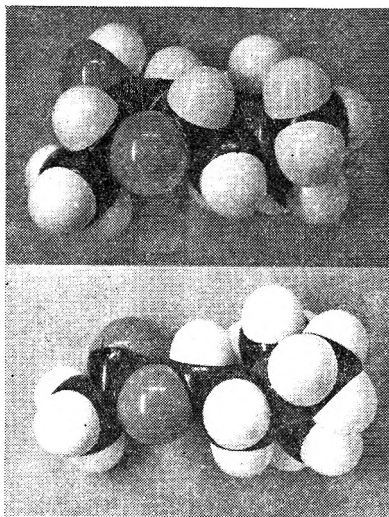
FIG. 20. Comparison of molecular models of muscarine and acetylcholine.

FIG. 21. Autoradiograph of mouse-diaphragm with 0·8 μg./g. ^{11}C-muscarone i.v. after being atropinised (50 μg. i.v.).

oxygen) have to form bonds (polar, covalent, hydrogen and van der Waalsbonds) with corresponding groups of the receptor substance.

The Nature of the Receptor Site

These and many other observations suggest that the receptor site might be a pore in the postsynaptic membrane[31,32]. This membrane pore should have an anionic wall, to which the quaternary N-groups are fixed, and an esteratic site, perhaps an imidazole group, nearby, to which the ester groups of acetylcholine becomes attached. This arrangement might possibly cause a change in the macromolecular configuration of the wall, such as the dislocation or folding of the protein and might result in the

592

development of permeability for potassium and sodium ions through the membrane pore during the short excitation period.

If these assumptions are correct, the neuromuscular blocking action of curare substances could be visualised as the covering of the pores of the endplates by the large molecules of C-curarine, tubocurarine, or gallamine thus inhibiting the access of acetylcholine to the receptors and preventing the flow of Na and K ions through the membrane. On the other hand depolarising drugs should not block this exchange of ions, but even enhance it.

The Nature of the Receptor Substance

Finally, what is the receptor substance? We know it to be located in the postsynaptic space, forming the few receptor sites responsible for synaptic transmission. It will be very difficult to extract the small quantity present in some scarcely distributed endplates in the muscle or from ganglionic synapses. Here the electric eel, *Electrophorus electricus* comes to our help, as it has transformed many of its endplates and muscles into a powerful electric organ, which is used for hunting and self defence in his native Amazon river. The electric organ is comparable to endplates because it is full of acetylcholine, cholinesterase and has the same mechanism of depolarisation. It can be blocked by curare drugs or depolarised by decamethonium or suxamethonium.

Chagas and colleagues[33] first tried to bind radioactive gallamine to a component in an extract of electric tissue. They found strong but probably nonspecific binding to a polysaccharide. Lately Ehrenpreis[34] and Nachmansohn[7] isolated a protein from the electric tissue, which showed many characteristics of the receptor substance *in vivo*. There is striking parallelism between the ability of this protein to bind acetylcholine analogues or curare drugs in equilibrium dialysis and their effect on electrical manifestations of intact electroplates.

It will be very difficult to prove that this *in vitro* protein is the receptor substance and corresponds to the cholinergic receptor in the tissue.

At the end of my review I therefore have to admit, that we know and can prove only the existence of the cholinergic receptor. But we do not know its nature, nor do we understand its functioning.

The use of labelled curarising and depolarising drugs has perhaps permitted a new look at an old problem, which is still far from being completely solved.

REFERENCES

1. Hunt and Taveau, *Brit. med. J.*, 1906, **2**, 1788.
2. Dale, *J. Pharmacol.*, 1914, **6**, 147.
3. Loewi, *Pflügers Archiv Physiol.*, 1921, **189**, 239.
4. Acheson, *Fed. Proc.*, 1948, **7**, 447.
5. Buchtal and Lindhart, *Acta physiol.*, 1942, **4**, 136.
6. del Castillo and Katz, *J. Physiol.*, 1955, **128**, 157.
7. Nachmansohn, *Chemical and Molecular Basis of Nerve Activity*, Academic Press, 1959.
8. Koelle and Friedenwald, *Proc. Soc. exp. Biol., N.Y.*, 1949, **70**, 617.
9. Loewenstein and Mollins, *Science*, 1958, **128**, 1284.
10. Nachmansohn and Machado, *J. Neurophysiol.*, 1943, **6**, 397.
11. Feldberg and Vogt, *J. Physiol.*, 1948, **107**, 372.

12. Langley, *ibid.*, 1905, **33**, 374.
13. Waser and Lüthi, *Nature, Lond.*, 1956, **178**, 981.
14. Waser and Lüthi, *Arch. int. Pharmacodyn.*, 1957, **112**, 272.
15. Hadorn, Thesis, Zürich, 1960.
16. Waser, Schmid and Schmid, *Arch. int. Pharmacodyn.*, 1954, 96, 386.
17. Robertson, *J. Biophys. Biochem. Cytol.*, 1956, **2**, 381.
18. Couteaux, *Int. Rev. Cyrol.*, 1955, **4**, 335.
19. Andersson-Cedergreen, *J. Ultrastructure Research*, 1959, Suppl. 1.
20. Waser, *Helv. physiol. Acta*, 1958, **16**, 171.
21. Paton and Zaimis, *Pharm, Rev.*, 1952, **4**, 219.
22. Eugster and Waser, *Experientia*, 1954, **10**, 298.
23. Waser, *ibid.*, 1955, **11**, 462.
24. Kuenzle and Waser, *Helv. physiol. Acta*, 1958, **16**, 44.
25. Kögl, Salemink, Schouten and Jellinek, *Rec. Trav. chim. Pays-Bas.*, 1957, **76**, 109.
26. Eugster and Waser, *Helv. chim. Acta*, 1957, **40**, 888.
27. Waser, *Experientia*, 1958, **14**, 356.
28. Waser, *ibid.*, 1960, **16**, in press.
29. Ing, *Science*, 1949, **109**, 264.
30. Pfeiffer, *ibid.*, 1948, **107**, 94.
31. Waser, *Schweiz. Med. Wschr.*, 1957, **87**, 577.
32. Waser, *Curare and Curare-like Agents*, Elsevier, 1959, p. 229.
33. Chagas, *ibid.*, p. 327.
34. Ehrenpreis, *Science*, 1959, **129**, 1613.
35. Dale, Feldberg and Vogt, *J. Physiol.*, 1936, **86**, 353.
36. Feldberg and Vartiainen, *J. Physiol.*, 1934, **83**, 103.
37. Lissak, *Amer. J. Physiol.*, 1939, **127**, 263.
38. Scheinfinkel, *Helv. physiol. Acta*, 1943, **1**, 149.
39. von Muralt, *Pflügers Archiv ges. Physiol.*, 1942, **245**, 607.
40. Fukuda and Koelle, *J. Biophys. Biochem. Cytol.*, 1959, **5**, 433.
41. Fatt and Katz, *J. Physiol.*, 1952, **118**, 73.
42. Vogel and Waser, unpublished.

RESEARCH PAPERS

THE IMPORTANCE OF EXTINCTION RATIOS IN THE SPECTROPHOTOMETRIC ANALYSIS OF MIXTURES OF TWO KNOWN ABSORBING SUBSTANCES

By A. L. GLENN

From the Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, Brunswick Square, London, W.C.1

The accuracy obtainable from Vierordt's method largely depends upon the establishment of numerical coefficients, which apply to the analyst's own instrument at the time of analysis. For the special case of a two component mixture, the method has been formulated in terms of extinction ratios, which can be determined from solutions of unknown concentration—so facilitating the task of obtaining up-to-date numerical coefficients. A further development of the same formulation has led to a simple theoretical criterion, which enables the analyst to avoid pairs of wavelengths and concentration ratios that are unsatisfactory for precision. This criterion has been tested in an unfavourable application of the method. A procedure for choosing the best pair of wavelengths is described in detail.

The value of this new formulation and criterion of precision is discussed with special reference to a general procedure for the assay of injections which contain absorbing bacteriostatics. Any assay for a two component mixture depends upon some difference between the two components and in this respect Vierordt's method appears to be less exacting than the average quantitative separation process. For this reason, it seems to have greater scope than the latter.

THE method whereby a mixture of two known absorbing substances, A and B, may be determined spectrophotometrically is an old one. It was first applied by Vierordt[1] almost 90 years ago and involves extinction measurements at a pair of suitable wavelengths. The concentrations of A and B are then evaluated from a pair of simultaneous equations of the following form:—

$$E_1 = \alpha_1 c_A + \beta_1 c_B \qquad \cdots \qquad \cdots \qquad \cdots \qquad \cdots \qquad (1)$$

$$E_2 = \alpha_2 c_A + \beta_2 c_B \qquad \cdots \qquad \cdots \qquad \cdots \qquad \cdots \qquad (2)$$

The subscripts, "1" and "2" refer to wavelengths; E denotes an extinction of a 1 cm. layer of the solution of a mixture of A and B. c_A and c_B are the concentrations of A and B, whilst α and β are their respective extinction coefficients.

Despite its simplicity, this method seems to lack popularity among analysts, possibly for the reasons that follow. Thus, the method is far more sensitive to wavelength errors than is the spectrophotometric determination of a single absorbing substance, in which it is easy to choose a wavelength, λ, of maximum absorption (where, of course, $dE/d\lambda = O$). In Vierordt's method, on the other hand, it is almost certain that some of the extinction measurements will have to be made on the slopes of absorption curves. Here there is not only an increased source of error at the

595

time of measurement, but also a greater tendency for the extinction coefficients calculated from such measurements to go out of date—on account of changes in the instrument parameters (especially wavelength calibration) of a spectrophotometer, which inevitably occur with time. In this connection, the publication of equations involving numerical coefficients, which only apply to individual instruments at a particular time, suggests a lack of appreciation that such coefficients are impermanent. However, with regard to this important matter of calibration, the analyst is discouraged from setting up fresh coefficients by the "usual expressions" for the solution of equations (1) and (2). Thus, the expressions, which the author has seen, are all written in a form, which suggests that accurately prepared solutions of A and B are essential for the setting up of the numerical coefficients. One of the main purposes of this paper therefore is to point out that by the use of extinction ratios, the necessary coefficients can be obtained from solutions of A and B, of unknown concentration. It is hoped that this suggestion will encourage analysts to set up their own up-to-date coefficients and so obtain rather greater accuracy from Vierordt's method than hitherto. Quite apart from this however, the use of extinction ratios leads to expressions which give a much clearer idea of the conditions for precision. In particular, it is possible to set up a numerical criterion for satisfactory precision, which enables the analyst to avoid application of the method, to such examples as are doomed from the outset.

Formulation of Vierordt's method in Terms of Extinction Ratios

The formulation of equations (1) and (2) in terms of extinction ratios is readily achieved by substituting the following expressions into equation (2):

$$m = E_2/E_1 \qquad a = \alpha_2/\alpha_1 \qquad b = \beta_2/\beta_1$$

Note that m refers to the mixture, a to substance A and b to substance B. This leads to the following:

$$E_1 = \alpha_1 c_A + \beta_1 c_B \quad \cdots \qquad \cdots \qquad \cdots \qquad \cdots \qquad \cdots \qquad (1)$$

$$mE_1 = a\alpha_1 c_A + b\beta_1 c_B \qquad \cdots \qquad \cdots \qquad \cdots \qquad \cdots \qquad (3)$$

which can be solved in the usual way to give

$$c_A = \frac{E_1}{\alpha_1} \left[\frac{b-m}{b-a} \right] \qquad \cdots \qquad \cdots \qquad \cdots \qquad \cdots \qquad (4)$$

$$c_B = \frac{E_2}{\beta_2} \left[\frac{b(m-a)}{m(b-a)} \right] \qquad \cdots \qquad \cdots \qquad \cdots \qquad \cdots \qquad (5)$$

The last two expressions can each be regarded as an apparent concentration multiplied by a correction term, T. Thus, equation (4) can be written, $c_A = c_A'T_A$, where $c_A' = E_1/\alpha_1$ which is the usual Beer's Law expression for the determination of substance A in the absence of any other absorbing substance. Just as in the determination of a single component, it is of course necessary that E_1 should be obtained from an accurately prepared solution of the analytical sample and that α_1 should

be an accurately known extinction coefficient. However, the correction term, T_A, depends upon the ratios a, b and m and these involve no knowledge of the concentration of absorbing material. Although the ratio, m, must be measured in each determination, this involves no more than the second reading, E_2, in addition to E_1—that is, without removing the cells from the instrument. The ratios, a and b, are simply and rapidly determined from solutions of A and B of unknown concentration, with the sole proviso that all extinctions lie within a reasonable range (e.g., 0·2–1·0). Once having determined a and b, there is no need to re-measure them until the analyst judges that instrument parameters have changed sufficiently for a and b to need revision.

Conditions for Precision

Inspection of the correction term, T, of both (4) and (5) shows that as a approaches b, the denominator tends toward zero. Furthermore, since m always lies between a and b, the numerator of T will also tend toward zero. This means that T will become very sensitive to small errors in a, b and m and the precision of the final result will suffer accordingly. We therefore see that if the absorption curves of A and B are sufficiently similar, it may be impossible to choose any pair of wavelengths, for which a and b are sufficiently different to obtain a reasonable measure of precision. It follows that such a pair of substances cannot be determined by Vierordt's method. This situation is not always easy to appreciate from graphs of extinction coefficient against wavelength, for although the curve of A may be substantially displaced on the ordinate scale relative to that of B, the ratios associated with various pairs of wavelengths may be insufficiently different for a successful application of the method. As will be shown later, the best test involves graphs of log E against wavelength. Then, if the curves for A and B are nearly superimposable, it is useless to apply Vierordt's method.

A Numerical Criterion for the Successful Application of Vierordt's Method to a Two Component Mixture

Assuming that for a given set of instrument parameters, the coefficient of variation, u, of an extinction measurement is constant between the limits, 0·2–1·0, and that all relative extinction errors are less than 20 per cent, it has been shown theoretically[2] that in a two component analysis, the coefficient of variation of the measurement of c_A, c.v.(c_A), is $u\sqrt{H_A}$. In a similar way, c.v.(c_B) is $u\sqrt{H_B}$.

H_A and H_B are different functions of the same extinction ratios, a, b and m. Having started with so simple an assumption about u, these relationships are but crude representations of practical observation. Nevertheless, there is experimental evidence[2] that large values of H are associated with high c.v.(c), so that the relationships provide a rational basis for the setting up of a practical criterion. The equation for H_A in terms of a, b and m shows that provided the ratio, b/m, lies outside the limits, 0·1–2·0, $\sqrt{H_A}$ will not exceed $\sqrt{7}$. Under these conditions,

c.v. (c_A) should not exceed $u\sqrt{7}$.   This means that provided b/m is outside the above limits, c.v.(c_A) in the presence of B will not be more than $2\frac{1}{2}$ times c.v.(c_A) in the absence of B.   A similar criterion exists for B.   That is, c.v.(c_B) will not exceed $u\sqrt{7}$ provided the ratio, m/a, is outside the limits, 0·1–2·0.

The range of exclusion, 0·1–2·0, implies that for each ratio, b/m or m/a, there are two satisfactory ranges, that is, 0–0·1 and 2·0–∞.   Inspection of the explicit forms[2] of H_A and H_B shows that of these two ranges, the range, 2·0–∞, is decidedly the better one to use.   Sometimes, however, there is no option but to use the lower range.

This criterion limits not only the analyst's choice of wavelengths in any given case, but also the concentration ratio, c_A/c_B.   Thus, in the determination of A, there is a limit for the ratio, c_A/c_B, below which c.v.(c_A) exceeds $u\sqrt{7}$.

The Choice of Wavelengths

Although quite general, the present section is really intended for those cases where the best choice of wavelengths is not immediately obvious and the author hopes that its inclusion will not confuse what is usually a very simple operation.   Thus, in many applications of Vierordt's method, A and B have absorption peaks that are well separated in terms of wavelength.   The choice is then very simple; that is, λ_{max} of A is chosen as λ_1, whilst λ_{max} of B is chosen as λ_2.

When, however, A and B do not possess such well separated peaks, the following discussion should help toward the best choice of wavelengths. Nevertheless, *the over-riding consideration is always that the appropriate ratio (b/m for A; m/a for B) should lie outside the limits, 0·1–2·0, and preferably be in excess of* 2·0.   Thus, if the analyst chooses wavelengths that lead to unacceptable precision, then the mistake is readily shown up by the fact that the ratio for at least one of the components will lie between 0·1 and 2·0.   Since a, b and m are all concerned in the final calculation, it is just a matter of inspection to check that the ratio is satisfactory for the particular component that is being calculated.

The ratios, b/m and m/a, are overall criteria, which limit both the choice of wavelengths and the concentration ratio.   Although the latter may profoundly affect the precision of the result, it has no direct relevance to the best choice of wavelengths, which depends entirely upon the shapes of the absorption curves of A and B.   Inspection of the explicit forms[2] of H_A and H_B, shows wavelength choice to be governed by the ratio, b/a, in the case of *both* A and B.   If either b/m or m/a are to lie outside the limits, 0·1–2·0, then so also must b/a.   The latter ratio is, in fact, always optimistic.   Thus, since m always lies between a and b, the ratios b/m and m/a are always nearer to 0·1 or 2·0 than is the corresponding value of b/a (provided that the latter lies outside the limits).

It follows that when choosing wavelengths, b/a must certainly not lie within the range, 0·1–2·0.   If b/a has to be less than 0·1, then it should be as small as possible.   If, on the other hand, the *choice of wavelengths* can

achieve the more favourable condition, then in theory, b/a *should be as large as possible*. However, in practice* there is little point in making b/a much larger than about 25. It is therefore possible to pay some attention to general considerations of the kind mentioned on page 606.

The choice of wavelengths in accordance with the above requirements becomes simple when the absorption curves are plotted in the form of $\log E$ vs. λ, as in Figure 1 (*a*) and (*b*). It is then only necessary to super-impose the "B" graph upon the "A" graph, as in Figure 1 (*c*), and to slide the "B" graph along the ordinate axis, until the curves intersect at or near a λ_{max} of A. The wavelength of intersection must be designated λ_1, if the result is to be calculated by means of the expressions on p. 596. Then, to get a satisfactory value of b/a, choose λ_2 so that the distance between the "A" and "B" curves is fairly large, as in Figure 1 (*c*). It is usually necessary to try several values of λ_1 (intersection points) by sliding the "B" graph to various positions and noting the possibilities for λ_2. It is also desirable that at λ_2, the point on the "B" curve should lie above the point on the "A" curve, so that b/a comes within the range, $2 \cdot 0 - \infty$. Once the choice has been made, the value of $\log (b/a)$ can be read directly from the superimposed graphs as shown in Figure 1 (*c*). During this process, it is essential to keep the wavelength scales in register and the same ordinate and abscissa scales must be used for both graphs.

The above procedure is justified as follows. Thus,

$$b/a = \frac{\beta_2 . \alpha_1}{\beta_1 . \alpha_2}$$

$\therefore \log (b/a) = \log \beta_2 - \log \alpha_2 + \log \alpha_1 - \log \beta_1$

$\qquad\qquad = (\log \beta_2 + K) - \log \alpha_2 + [\log \alpha_1 - (\log \beta_1 + K)]$

When the "A" and "B" curves intersect, the value of K is such that $\log \alpha_1 - (\log \beta_1 + K) = O$, so that,

$$\log (b/a) = (\log \beta_2 + K) - \log \alpha_2$$

---

* The theoretical gain in precision to be expected from large values of b/a is partially offset by the large relative errors which are usually associated with the measurement of extreme values of a, b and m. Thus, the present treatment is based upon the assumption that all extinction measurements lie within the range, $0 \cdot 2 - 1 \cdot 0$. Then, if path length and solution concentration are to be kept the same during the deter-mination of one of the ratios, a, b or m, the ratio cannot fall outside the range, $0 \cdot 2 - 5 \cdot 0$, unless one of the extinctions also falls outside the assumed extinction range. This obviously restricts the minimum and maximum values of b/a that are associated with precisely measurable ratios of a, b or m. The use of different (accurately known) path lengths or dilutions would, of course, allow one to observe ratios outside the range, $0 \cdot 2 - 5 \cdot 0$, whilst keeping within the assumed limits of extinction. However, such steps are not practical in ordinary analysis.

In practice, one may have to measure quite large ratios and these will usually imply a very small value for the lower extinction. Nevertheless, although the resultant ratio will be correspondingly imprecise, the final answer for the concentration will not be greatly affected. Thus, suppose that in equation (4), a is very small and b very large, and therefore subject to considerable error. Now, since $b \gg a$, a can be neglected from the denominator, so that (4) becomes:—

$$c_A = \frac{E_1}{\alpha_1} (1 - m/b)$$

Unless the ratio c_A/c_B is very small, m will be small, so that errors in b will have little effect on c_A.

Finally, it must be mentioned that although in the light of the simple criterion for b/a, the best choice of wavelengths for A will also be the best for B, practical considerations may favour different pairs of wavelengths for A and B.   This is because the criterion for b/a is based upon a purely photometric argument, which can accommodate wavelength setting errors only on an average basis.   That is, it cannot take account of the fact that wavelength setting errors depend upon the actual slopes of the absorption
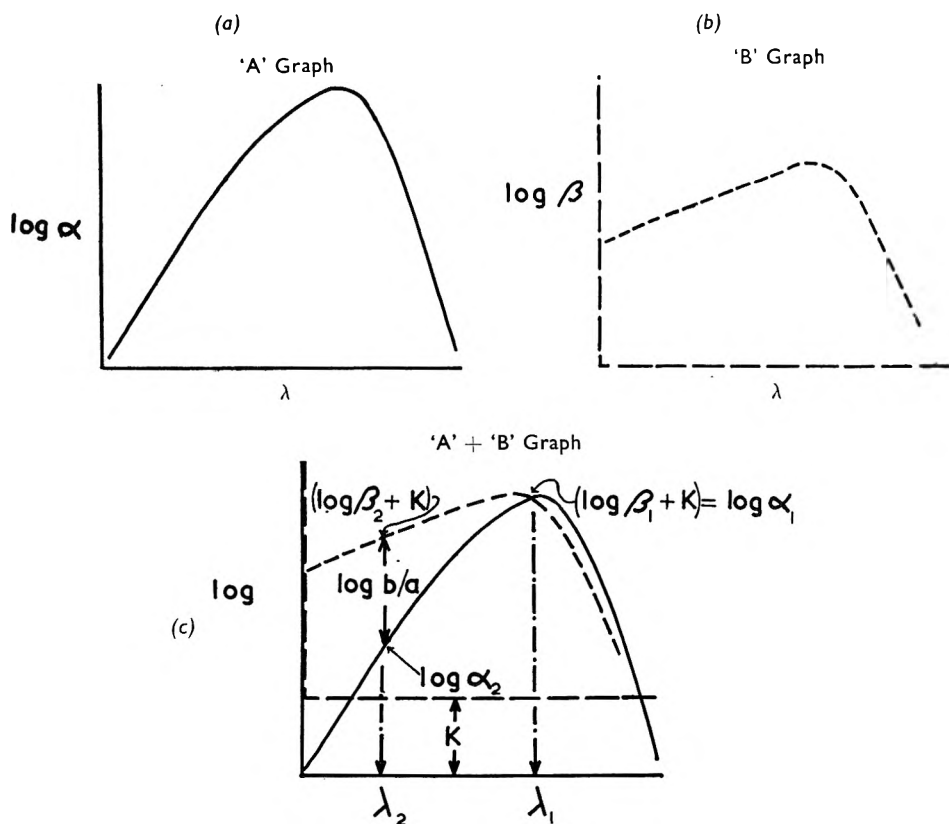

FIG. 1.   Procedure for choosing wavelengths in difficult cases.   In (c), the 'B' graph has been superimposed upon the 'A' graph and then displaced through the distance, K, along the ordinate.

curves of A and B at λ_1 and λ_2 as well as upon the ratio, c_A/c_B.   When choosing wavelengths, therefore, it is undesirable to be too rigid in applying the criterion for b/a.   However, this statement does not invalidate the importance of the ratios, b/m and m/a, as indications of the applicability of Vierordt's method.

Possible Interaction Between A and B

The validity or otherwise of Lambert's and Beer's Laws is important in any spectrophotometric method.   These considerations apply to

Vierordt's method with greater force than they do in the determination of a single substance. Thus, the latter can be determined in conditions where both laws are disobeyed, provided that the analyst is prepared to accept a non-linear calibration curve (E *vs.* c). With Vierordt's method on the other hand, such deviations require the final result for c_A and c_B to be calculated by a series of successive approximations[3], which is tedious to the point of being impractical.

Nevertheless, Vierordt's method contains an additional potential hazard in that A and B may form complexes (e.g., AB)[4] the extinction coefficients of which may not be simple sums (e.g., $\alpha_1 + \beta_1$) of those of
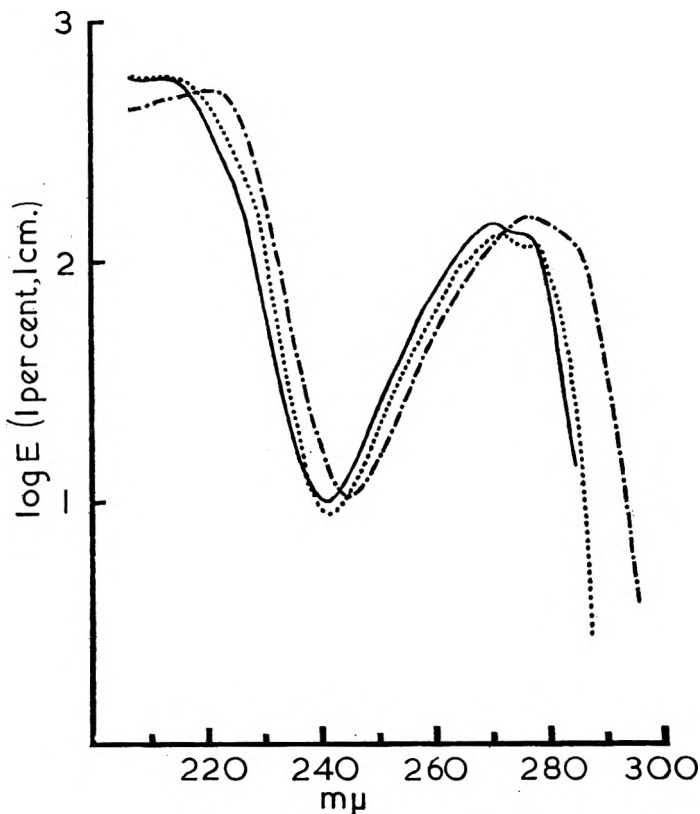
FIG. 2. The absorption curve of *o*-, *m*-, *p*-cresols in $0 \cdot 1$N aqueous H_2SO_4.
———— = *o*-cresol; · · · · = *m*-cresol; — · — · = *p*-cresol.

the uncomplexed substances. This means that although A may obey Beer's law on its own, it may not do so in the presence of B. Such inter-actions are governed by chemical equilibria, which means that in a given case their effects become more pronounced as c_A and c_B increase. Large flat aromatic systems such as dyes are especially likely to form complexes, even at the rather low concentrations used in spectrophotometry. Vierordt's method then becomes very tedious and some other method of analysis should be invoked.

The author believes that the above interactions are unlikely to be significant, where neither A nor B is a large molecule. It is, however, desirable to formulate a simple test for the absence of experimentally significant interactions. Thus, if at a particular wavelength, the E(1 cm.) of a mixture of A and B at concentrations, c_A and c_B, is the sum of (i) E(1 cm.) of A alone at concentration, c_A, and (ii) E(1 cm.) of B alone at concentration, c_B, then such interactions are absent. This is the basis of the test given in the experimental section.

An Unfavourable Application of Vierordt's Method

Mixtures of o and p-cresols in 0·1N H_2SO_4 were analysed for o-cresol in an attempt to evaluate the method under the conditions of an unfavourable application. Thus, it will be noted from Figure 2 that the

TABLE I

COEFFICIENTS OF VARIATION OF GROUPS OF EIGHT RESULTS OBTAINED IN AN
UNFAVOURABLE APPLICATION OF VIERORDT'S METHOD : THE DETERMINATION OF o-CRESOL
(SUBSTANCE A) IN THE PRESENCE OF p-CRESOL (SUBSTANCE B)

No.	$\dfrac{c_A}{c_B}$	λ_2*	a	b	m	b/a	b/m	c.v. (c_A)	Percentage error of mean value of c_A
1	3·85	272·5	0·96	1·14	0·99	1·19	1·15	0·89	−1·2
2	3·85	275	0·91	1·26	0·97	1·39	1·30	0·42	−0·4
3	3·85	277·5	0·83	1·30	0·92	1·56	1·42	1·01	−0·9
4	3·85	280	0·55	1·20	0·67	2·17	1·79	0·60	−1·2
5	3·85	282·5	0·26	1·10	0·41	4·28	2·70†	0·40	−1·0
6	3·85	285	0·10	0·96	0·25	9·76	3·81†	0·37	−0·6
7	1·44	272·5	0·96	1·14	1·03	1·19	1·11	0·57	−2·2
8	1·44	275	0·91	1·26	1·03	1·39	1·22	0·64	−1·3
9	1·44	277·5	0·83	1·30	1·00	1·56	1·29	0·74	−1·9
10	1·44	280	0·55	1·20	0·80	2·17	1·51	1·18	−3·5
11	1·44	282·5	0·26	1·10	0·57	4·28	1·93	0·48	−3·1
12	1·44	285	0·10	0·96	0·42	9·76	2·30†	0·34	−2·7
13	0·64	285	0·10	0·96	0·58	9·76	1·65	0·72	−2·3
14	0·24	285	0·10	0·96	0·77	9·76	1·25	1·25	−7·0

* $\lambda_1 = 270$ mμ throughout.

† These sets of results are the only ones that would be allowed by the rule that b/m should lie outside the limits, 0·1–2·0.

The values of a and b in rows 1–6, were used to calculate results throughout the whole table; hence, the repetitions in the columns for a, b and b/a (from row 7 onward).

absorption curves of the three cresols are similar. With o and p-cresols, it was therefore easy to select a range of wavelengths and ratios, c_A/c_B, that enabled one to explore the performance of the method in the region of the greater of the proposed limits. The results of the survey are given in Table I.

All but three of the results (that is, Nos. 5, 6 and 12) are disallowed by the criterion that b/m should lie outside the limits, 0·1–2·0. Nevertheless, the coefficient of variation of a number of these disallowed results is not large and on the whole the precision is surprisingly good.

The correlation of c.v.(c_A) and b/m was rather poor, probably because each set of results was associated with its own particular set of wavelength setting errors. As already mentioned, such errors can only be accommodated within this simple theory on an average basis. Nevertheless, the criterion for b/m was effective in disallowing all instances in which c.v.(c_A) exceeded 0·5. Note that b/m was not only effective in controlling the choice of wavelengths, but also the minimum ratio of c_A/c_B, that could be tolerated in the determination of c_A. Thus, for $\lambda_1 = 270$ mμ

and $\lambda_2 = 285$ mμ, b/m was satisfactory for Nos. 6 and 12, but not for Nos. 13 and 14, which were associated with the lowest ratios of c_A/c_B.

Since an analytical method must usually stand or fall by its accuracy rather than its precision, the matter of bias is important. The results in Table I show a consistent bias toward low values of c_A, which might be expected to arise from an interaction between A and B. Nevertheless, no significant interaction was detectable and this bias remains unexplained. On the whole, the bias became worse as c_A/c_B moved to lower values. For $c_A/c_B = 3.85$, the bias was, however, small and the ratio, b/m, did have some effect in disallowing those results, which had a large bias.

A Generalised Design for Two Component Spectrophotometric Assays

The present theory offers a generalised specification for two component spectrophotometric assays in that having decided upon limits for b/m, the conditions for satisfactory wavelengths and concentration ratios follow with substantial certainty. The particular choice of limits used in this paper is only tentative; they could be more or less stringent than the range, 0.1–2.0. The general idea might be helpful in the rational design of official assays for particular combinations of substances. Such an assay would specify equations (4) and (5), as well as λ_1, λ_2, α_1, β_2, together with the allowable limiting concentration ratios. The analyst would, however, be expected to measure the ratios, a and b, on his own instrument, using pure samples of A and B. There would be no need to mention the limits of b/m, upon which the specification was based.

By comparison with existing specifications, the main advantage of the above would reside in the improved accuracy to be gained from the use of individually determined ratios, a and b. Nevertheless, the theory offers much more scope in dealing with a situation in which a particular absorbing substance, A, has to be determined in the presence of an "absorbing nuisance", B, which, although known to the analyst, could be one of a large range of possibilities. Hitherto, this situation has posed an apparently insoluble problem to the designers of official assay processes. Thus, if substance B is quite unspecified, the official assay process must be a completely general one and it is difficult to design a process of this kind, which is neither vague nor cumbersome. However, a general specification that uses equation (4) in conjunction with suitable limits for b/m seems to provide a satisfactory answer.

A small problem arises in that any such official process would need to quote an extinction coefficient, α_0, for a certain wavelength, λ_0. However, when assaying A in the presence of a particular substance, B, it might be impossible to choose λ_1 equal to λ_0. The analyst would then need to set up a value of α_1 based on the official value, α_0, but this would only necessitate an additional extinction measurement at λ_0 when measuring a.

A Solution to the Problem of Absorbing Bacteriostatics in the Spectrophotometric Assay of Pharmacopoeial Injection Solutions

The fact that on occasions any suitable bacteriostatic can be added to a pharmacopoeial injection has undoubtedly retarded the inclusion of

spectrophotometric assays in a number of monographs that would other-
wise have constituted ideal spectrophotometric applications. Thus, a
simple spectrophotometric determination breaks down in the presence of
an appreciably absorbing bacteriostatic. However, although the range
of bacteriostatics that might be found in an injection solution is rather a
wide one[5], the answers[6,7] that have been offered so far have been restricted
in that they have referred to the particular absorbing bacteriostatics,
phenol and chlorocresol.

In an attempt to produce "an ideal method . . . by which any injection
solution containing an interfering bacteriostatic agent could be deter-
mined (spectrophotometrically)", Elvidge, Proctor and Baines[7] have
successfully used oxidised cellulose for the quantitative separation of
active constituents from either phenol or chlorocresol in the case of some
13 injection solutions. This method has given good results and appears
to be of wide application. Nevertheless, it seems doubtful whether so
general an aim can be achieved by a particular separation process. The
process, which will quantitatively separate any two substances in a
reliable and predictable manner surely does not exist? If the analyst
applies a standard separation process to a bacteriostatic, which he has not
previously met, he must presumably run a quantitative recovery experi-
ment. This is apt to be time consuming and may merely lead to the con-
clusion that the standard separation process is inapplicable. On the other
hand, the decision whether a particular combination of substances will be
feasible by Vierordt's method is a matter of simple arithmetic and it is
often possible to assess the feasibility of the method by inspecting the
molecular formulae of A and B and noting their approximate relative
concentrations, where such information is available.

It is fundamental to most quantitative separations that A should belong
to one class of substance and B to another. In merely requiring a sufficient
difference between the absorption curves of A and B, Vierordt's method
is much less stringent and therefore of correspondingly wider application
than any one separation process. The mathematical character of
Vierordt's method also leads to a precise but simple general specification,
which would be difficult to equal in the case of the separation process.
Nevertheless, both general approaches are valuable and it is to the
analyst's advantage to have two strings to his bow. Furthermore, where
the two components cannot be completely separated from one another,
and where the concentration ratio is unfavourable to Vierordt's method,
a combination of the two general approaches may succeed in circum-
stances which defeated either one alone.

Collections of Spectra of Active Constituents and Bacteriostatics

When assaying injections that contain absorbing bacteriostatics, it is
useful to build up a collection of absorption curves for all substances
encountered. The same graph scales should be used throughout and all
curves should be determined in the same solvent. In this connection, the
author has found $0 \cdot 1 \text{N}$ aqueous H_2SO_4 to be the solvent of choice, for when
A and B contain acid-base auxochromes, its high buffering power favours

the obedience to Beer's law. The concentration of H_2SO_4 should be controlled to within about ± 10 per cent, since variations of say ± 100 per cent may produce significant spectral changes, especially when the absorbing compound contains a very weakly basic auxochrome[8,9].

By way of example, a small collection is shown in Figure 3, in which log E(1 cm.) refers to the pharmacopoeial concentrations of the substances in question. Whilst facilitating the choice of wavelengths, this particular ordinate scale also gives an immediate impression of the relative absorptions of active constituent and bacteriostatic that arise from given combinations. A diagram of this kind is easy to construct by first plotting extinctions directly onto logarithmic graph paper and then tracing off the curve at the correct height on the diagram.

Unlike the bacteriostatic concentration, the concentration of a particular active constituent is variable. In this connection, Figure 3 has

TABLE II

WAVELENGTHS CHOSEN FROM FIGURE 3

Injection	Bacteriostatic	λ_1 (mμ)	λ_2 (mμ)	b/a
Aminophylline 2·5 per cent w/v	Chlorocresol	245	—	—
	Phenol	240	271	5
	Phenylmercuric Nitrate	271	—	—
Aneurine Hydrochloride 0·5 per cent w/v	Chlorocresol	249	—	—
	Phenol	246	270	12
	Phenylmercuric Nitrate	246	—	—
Atropine Sulphate 0·03 per cent w/v ..	Chlorocresol*	247	280	210
	Phenol*	247	270	31
	Phenylmercuric Nitrate	257	220	2·7
Morphine Sulphate 1·0 per cent w/v ..	Chlorocresol	285	249	0·07
	Phenol	282	265	12
	Phenylmercuric Nitrate	285	—	—
Nicotinamide 5·0 per cent w/v	Chlorocresol	261	—	—
	Phenol	238	—	—
	Phenylmercuric Nitrate	261	—	—

The absence of a figure for λ_2 means that at λ_1, the extinction due to the bacteriostatic is less than 0·5 per cent of that due to the active constituent. In such a case, the bacteriostatic's absorption can be ignored, so that a normal single component determination can be applied at λ_1. This approximation was referred to by Brealey and Proctor[6] as the "Direct Method".
* Assuming that the bacteriostatic concentration is first reduced by partial extraction.

been constructed pessimistically by using the lowest concentrations of active constituent that are likely to be met in practice. It is, of course, easy to adjust any curve in the Figure to a different concentration by simply sliding it bodily up or down the ordinate scale. For example, curve No. 7 refers to Atropine Sulphate 0·03 per cent w/v and the curve for twice this concentration could be obtained by a vertical displacement of curve No. 7 through a distance equal to log 2.

Figure 3 contains a good deal of information that is available at a glance : (i) the extent to which an injection must be diluted to bring E(1 cm.) into a measurable range at a particular wavelength, (ii) wavelengths at which the bacteriostatic absorption contributes less than 0·5 per cent to the total extinction of the mixture[6], (iii) of the three bacteriostatics shown, Phenol provides the worst problem, (iv) Phenylmercuric Nitrate only makes a significant contribution in the case of Atropine Sulphate Injection, (v) Vierordt's method cannot be applied to Atropine Sulphate Injection containing phenol or chlorocresol, unless the bacteriostatic concentration has been reduced to about 1 per cent of its nominal value by partial extraction.

The list of wavelengths given in Table II has been compiled from Figure 3 according to the considerations; (i) b/a to be as large as possible, (ii) avoidance of absorption curve slopes, particularly for E_1 (for example,
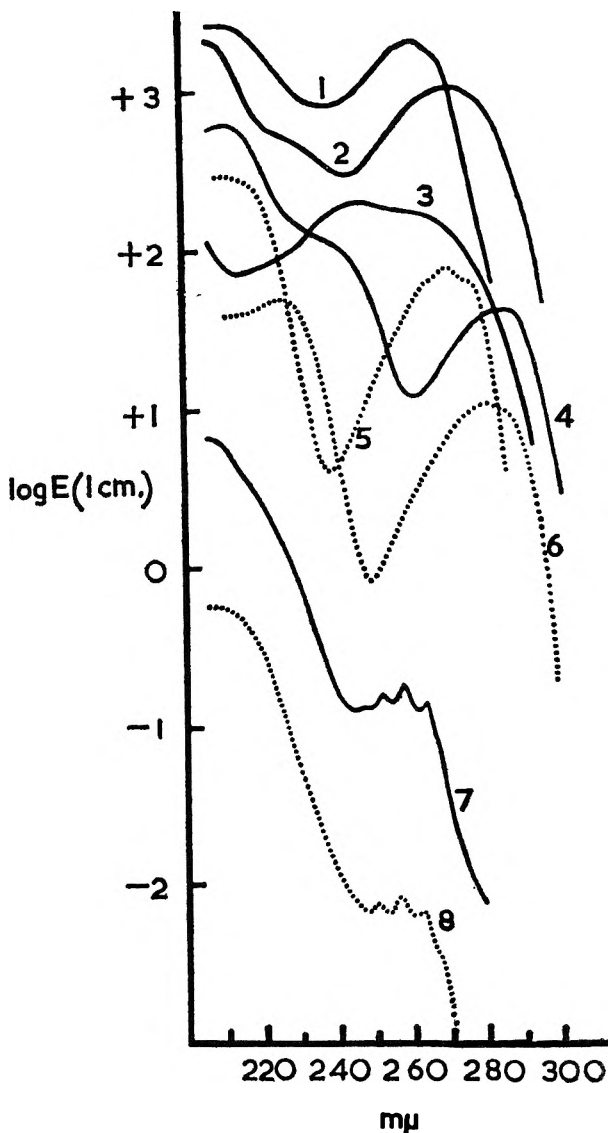

FIG. 3. Ultra-violet absorption curves of constituents of injection solutions (in $0\cdot1$N aqueous H_2SO_4).

1. Nicotinamide (5 per cent w/v); 2. Theophylline (2 per cent w/v) equivalent to Aminophylline (2·5 per cent w/v); 3. Aneurine Hydrochloride (0·5 per cent w/v); 4. Morphine Sulphate (1 per cent w/v); 5. Phenol (0·5 per cent w/v); 6. Chlorocresol (0·1 per cent w/v); 7. Atropine Sulphate (0·03 per cent w/v); 8. Phenylmercuric Nitrate (0·001 per cent w/v).

—— Active constituent; · · · · Bacteriostatic.

regions of vibrational structure), (iii) preference for $\lambda_1 = \lambda_{max}$ of A, (iv) avoidance of measurements below 220 mμ, (v) avoidance of Vierordt's method where possible[6].

EXPERIMENTAL

All extinctions were measured on a Uvispek Spectrophotometer (Mark III). 0·1N aqueous H_2SO_4 was used as solvent throughout.

Coefficients of Variation of the Determination of o-Cresol in the Presence of p-Cresol (Table I)

Since pure materials were not essential to this work, the o- and p-cresols were used without further purification. Solutions containing both o- and p-cresols were prepared by mixing known weights of Solution "A" (o-cresol) and Solution "B" (p-cresol). The results, "percentage error of mean value of c_A" in Table I were calculated with reference to the observed E(1 cm.) of comparison solutions. Each comparison solution was identical to the solution of mixed isomers to which it referred except that p-cresol was omitted.

The object of the experiment was to include photometric and wavelength setting errors, but to eliminate other sources of variance including cell filling. Each solution was therefore placed in a stoppered 10 mm. cell and subjected to a series of measurements, which continued until all necessary readings had been obtained from the solution in question. The differences in radiant power that occurred over the small wavelength interval, 270 mμ–285 mμ, were insignificant and deflection sensitivity was kept constant throughout the whole experiment. Throughout each series, measurements alternated between λ_1 and λ_2, each extinction being the mean of two readings for one particular setting of the wavelength scale. With the exception of a few measurements at 282·5 mμ and 285 mμ, all extinctions were within the range, 0·2–0·6.

Test for Interaction Between o-Cresol and p-Cresol

I_0

| (1) | (3) |

| (2) | (4) |
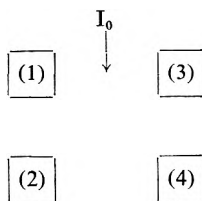
Throughout the test, four 10 mm. cells were used as shown herewith. Solutions "A" and "B" (as above) were accurately diluted or mixed and placed in the cells according to the following arrangements. ("S" = 0·1N aqueous H_2SO_4)

Series	Cell (1)	Cell (2)	Cell (3)	Cell (4)
I	"A" 50 ml. "S" 50 ml.	"B" 50 ml. "S" 50 ml.	"A" 50 ml. "B" 50 ml.	"S"
II	"A" 50 ml. "B" 50 ml.	"S"	"A" 50 ml. "B" 50 ml.	"S"
III	"S"	"A" 50 ml. "B" 50 ml.	"A" 50 ml. "B" 50 ml.	"S"

For each series, the extinction of the four cell arrangement was measured at 270, 275, 280 and 285 mμ. Series III was included to detect any path length mis-match between cells (1) and (2), which would show itself as a difference between Series II and Series III. In all three series and at all four wavelengths, the readings differed insignificantly from 0·005, which value evidently arose from absorption mis-match between the cell pair, (1) + (2), and the pair, (3) + (4). Interaction between the o- and p-cresols was therefore experimentally insignificant under the conditions, which applied to the results in Table I.

Absorption Curves (see Figures 2 and 3)

With the exception of o-, m-, and p-cresols, all substances were of British Pharmacopoeial standard and dried according to B.P. requirements. Primary solutions were prepared from quantities of the order of 0·5 g. and then diluted to produce solutions for measurement. Extinctions were plotted directly onto logarithmic graph paper (Wightman & Mountain No. 11L).

REFERENCES

1. Heilmeyer, *Spectrophotometry in Medicine*, Adam Hilger Ltd., London, 1943, p. 7.
2. To be published.
3. Lothian, *Absorption Spectrophotometry*, Hilger & Watts Ltd., London, 1949, p. 60.
4. Andrews, *Chem. Rev.*, 1954, **54**, 713.
5. Sykes, *J. Pharm. Pharmacol.*, 1958, **10**, *Suppl.* 40T.
6. Brealey and Proctor, *ibid.*, 1955, **7**, 830.
7. Elvidge, Proctor and Baines, *Analyst*, 1957, **82**, 367.
8. Doherty, Cane and Wokes, *J. Pharm. Pharmacol.*, 1955, **7**, 1053.
9. Helgren, Chadde and Campbell, *J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 644.

SURFACE INTERACTION OF LECITHIN AND LYSOLECITHIN

By N. Robinson

From the Department of Pharmaceutical Chemistry, School of Pharmacy, University of London, Brunswick Square, London, W.C.1

The lowering of the surface tension of water by lysolecithin in the presence of a small constant amount of lecithin has been investigated to examine the changes in the boundary tension during the formation of a primary phosphatide membrane. In the higher concentration range of lysolecithin (above 0·05 per cent w/v corresponding to a weight fraction of 0·4) the surface activity was not affected by the presence of lecithin. Below 0·05 per cent w/v lysolecithin the surface activity was reduced but in the very dilute region of the critical micelle concentration it was restored.

The presence of calcium chloride reduced the surface activity to a greater extent above the critical micelle concentration region of lysolecithin than below. The effects of potassium chloride differed from calcium chloride for different regions of the lysolecithin concentration range. Ageing effects due to the salts affected the surface acitivity, probably by reason of the removal of lecithin from the interface. The results were complex and only a qualitative interpretation of the behaviour was attempted. The surface tension : concentration relation for aqueous lecithin sols at four different temperatures, a precursor to the main work, showed a lowering of the surface tension of water to less than 41 dyne/cm. by 0·5 per cent w/v lecithin at 25°; reducing the concentration to 0·05 per cent w/v the surface activity of lecithin steadily diminished to zero.

Since lysolecithin is an enzymatic breakdown product of lecithin, these two substances occur together in biological systems and the surface properties of one will be modified by the presence of the other.

In 1957 Elworthy and Saunders[1] suggested that when stable boundaries were formed between an aqueous phosphatide sol and water, the structure of the interfacial film was that of an extended bimolecular leaflet, with polar groups on its outside surface. This concept bore some resemblance to Danielli and Davson's[2] general structure of a simple cell membrane. Later Saunders[3] observed that lysolecithin and lecithin interacted, when present in certain proportions, to form highly viscous sols. He suggested that if sufficient lysolecithin was present in the internal fluid of a cell the lecithin present would be stable to monovalent metal ions, but precipitation of a phosphatide membrane could still occur when the internal fluid met a solution containing divalent metal ions. At the weight fraction necessary to give precipitation the system was not lytic and hence the membrane would be stable. Robinson and Saunders[4] have reported that the strong interaction of lysolecithin and monostearin to form a viscous sol may also be indicative of typical lysolecithin-lipid cohesive forces contributory to the rigidity of a cell membrane.

The strength of the membrane will be governed in some measure by the change in surface tension of the membrane boundary according to the amount of lysolecithin present within the internal fluid. The latter will,

609

in turn, be influenced by the concentration of lecithinase catalysing the hydrolysis of lecithin, hydrogen ion concentration, ionic strength and other environmental conditions. The surface interaction of lysolecithin and lecithin has been studied to determine the extent of modification of a boundary tension by changes in some of these conditions.

Both phosphatides possess surface activities to different extents and the lowering of the surface tension of water when both components are present will depend upon interaction in solution. Lysolecithin could exert a solubilising power on lecithin thus tending to remove lecithin from the interface. It is suggested that the physical state of the mixed phosphatide aggregate in the bulk phase will not be one of lecithin solubilised within the lipophilic region of the lysolecithin micelle in the conventional manner; this is prevented by the hydrophilic phosphoryl choline headgroup of the lecithin molecule. It is more probable that the surface of a lysolecithin micelle will be impregnated with single lecithin molecules. The physical state of this mixed phosphatide aggregate will be reported later.

Lecithin sols are sensitive to very small amounts of sodium, potassium and calcium chlorides and the presence of these salts was expected to modify the surface activity of lecithin sols. Lysolecithin sols are stable to small amounts of electrolytes and the surface tension of these sols is comparatively unaffected by their presence. In studying the surface effects of sols containing both phosphatide components, a concentration of lecithin was chosen sufficient to influence the behaviour of the lysolecithin component whilst independently lecithin exerted little or no surface activity itself. An initial study of the lowering of the surface tension of water by lecithin showed that at a concentration of 0·05 per cent w/v lecithin its surface activity was negligible. This concentration was therefor chosen for the work.

The measurements in these experiments were taken one hour after preparation of the sols, but systems containing salts were re-examined after 24 and 96 hours.

<center>EXPERIMENTAL</center>

Preparation of Lecithin and Lysolecithin

Lecithin was prepared from egg yolks as previously described[4]. Lysolecithin was prepared from a sample of the lecithin using Russell viper venom according to Saunders[3] modification of the method of Hanahan[5]. Analytical figures for the two substances are given below.

		Lecithin	*Lysolecithin*
Nitrogen (per cent)	..	1·75	2·72
Phosphorus (per cent)	..	3·89	5·98
N:P ratio	..	0·99:1	1:1·02
Iodine number ..	..	72	4·2

Preparation of lecithin sols. Lecithin sols were prepared by evaporating a sample of the stock solution of lecithin to dryness, dissolving the weighed quantity of lecithin in a minimum volume of ether, adding

distilled water and evaporating the ether with a stream of nitrogen. The sols were freed from air on a filter pump, passed down a small column of mixed strong ion exchange resins and made up to volume.

Preparation of mixed sols. The mixed sols were prepared by taking measured quantities of stock solutions of the two phosphatides of known concentrations, mixing and evaporating to dryness. The film of intimately
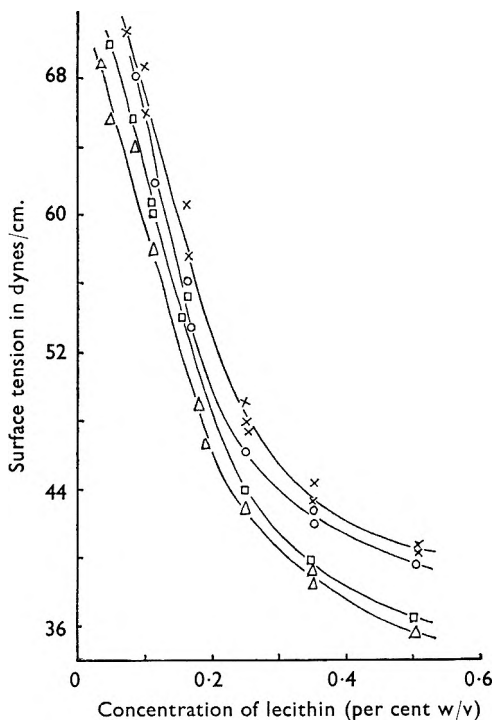

FIG. 1. Variation of surface tension of water with concentration of lecithin at × 20°, ○25°, □32·5°, △40°.

mixed phosphatides was dispersed in warm distilled water and shaken to give a clear sol. Traces of electrolytes and dissolved air were removed. Sols containing potassium chloride and calcium chloride were prepared as previously and made up to volume by addition of small calculated volumes of concentrated salt solutions.

Apparatus. Surface tensions between 20° and 40° were measured by the ring method using the chainomatic balance assembly previously described[6].

RESULTS AND DISCUSSION

Surface Tension Effects of Lecithin

The surface activity of lecithin at a concentration of 0·5 per cent w/v was marked, the surface tension of water being lowered to less than 41 dyne/cm. at the four temperatures investigated (Fig. 1). Smaller concentrations continued to produce a considerable lowering of the

surface tension but at 0·05 per cent w/v the effect became negligible except at 40° when the surface tension was 66·6 dyne/cm. The results did not show any abrupt change in the surface tension : concentration relationship indicating that the critical micelle concentration of lecithin in water was very low; the good balance between the hydrophilic and lipophilic groups in the lecithin molecule suggests that aggregates would be present below the concentrations range examined.

The surface tension effects of lecithin on water under different conditions have been previously reported, but more recent preparations of
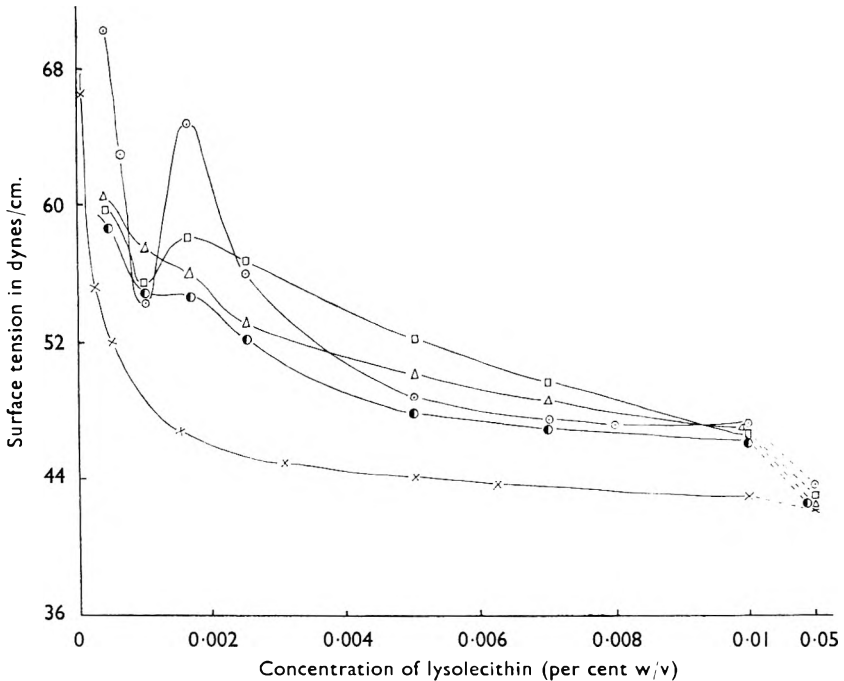

FIG. 2.   Effect of CaCl$_2$ on the surface tension of mixed lysolecithin-lecithin sols at 25°.
× Lysolecithin
⊙ Mixed sols of lysolecithin and 0-05 per cent w/v lecithin
□ Mixed sols in 10^{-4} CaCl$_2$
△ Mixed sols in 10^{-4} CaCl$_2$ after 24 hours
◑ Mixed sols in 10^{-4} CaCl$_2$ after 96 hours

lecithin by chromatography indicated that small amounts of lysolecithin and other phosphatides were probably present[7-9]. An equally successful but more rapid method for the final purification of lecithin using ion exchange resins has been reported by Perrin and Saunders[10]. The high surface activity of lysolecithin could greatly affect measurements of the surface tension of aqueous lecithin sols.

Earlier studies by Neuschloz[11], using a drop number method, showed that salts brought about a change in the lowering effects of lecithin on the surface tension of water, aluminium chloride inhibiting the surface activity in smaller concentrations than calcium, sodium and potassium

chlorides. Price and Lewis[12], using the maximum bubble pressure method, obtained a maximum in the surface tension:concentration relationship at pH 2·6 which was thought to be the isoelectric point. Fischgold and Chain[13] have since shown that the isoelectric point is in fact much higher (6·7). The experiments of Boutaric and Berthier[14] showed a lowering of the surface tension of water by 0·5 per cent w/v
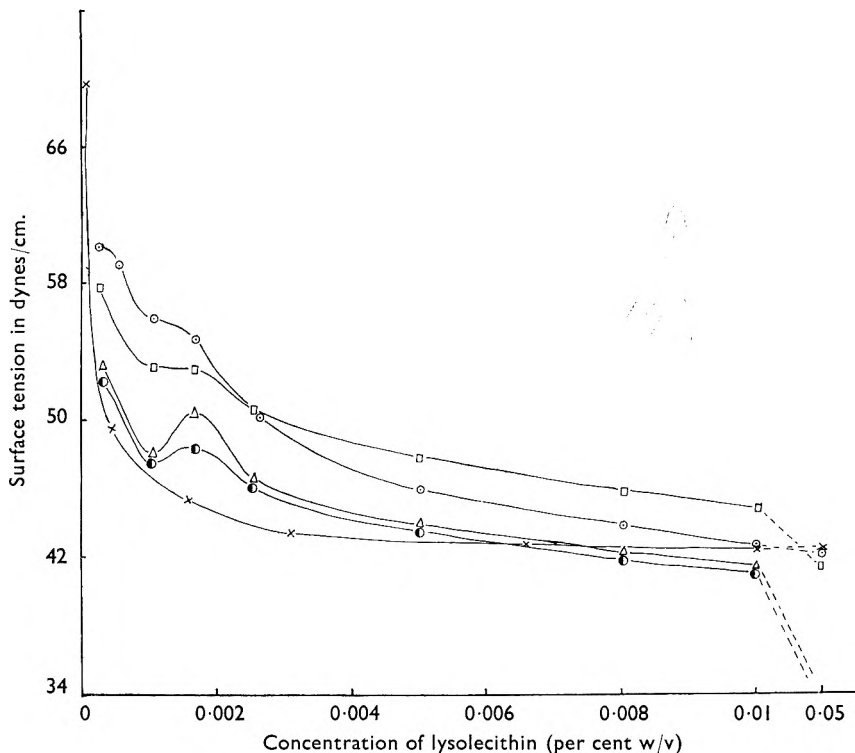

FIG. 3. Effect of CaCl₂ on the surface tension of mixed lysolecithin-lecithin sols at 40°. For key see Fig. 2.

lecithin to 32·6 dyne/cm. after 1 hour; the effect of salts on the surface tension of lecithin sols was, however, contrary to results obtained by previous workers.

Surface Interaction with Lecithin

Throughout the concentration range of lysolecithin at 25° its surface activity was depressed to different extents by the presence of lecithin. Above a concentration of 0·05 per cent w/v, sufficient lysolecithin was present to remove lecithin from the interface and the surface tension of the sol remained relatively unaltered. Below this concentratin the rise in surface tension suggested that the lecithin brought about a withdrawal of lysolecithin away from the air-water interface to participate in the solubilisation of the lecithin. In this concentration region the boundary

tension of a membrane could be lessened by removal of lecithin from the interface resulting in instability which may lead to some lysing action. At a concentration approaching the critical micelle concentration of lysolecithin (mol ratio of lysolecithin to lecithin approximately 1 : 20) a marked increase in surface activity took place which was attributed to small aggregates and single molecules, which possessed little solubilising
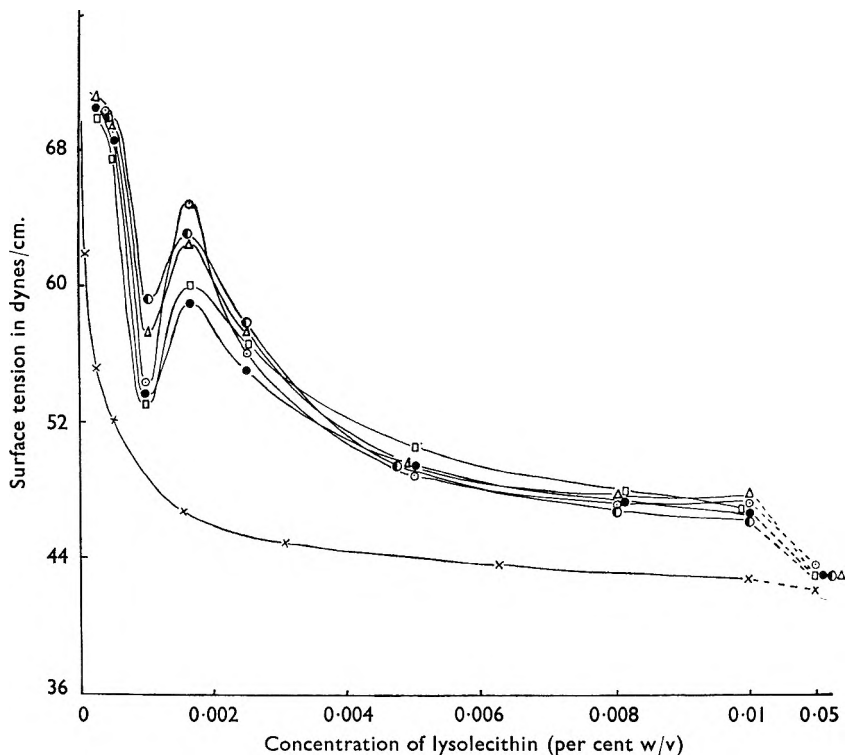

FIG. 4. Effect of KCl on the surface tension of mixed lysolecithin-lecithin sols at 25°.

× Lysolecithin
⊙ Mixed sols of lysolecithin and 0·25 per cent w/v lecithin
□ Mixed sols in 10^{-2}M KCl
● Mixed sols in 10^{-1}M KCl
△ Mixed sols in 10^{-1}M KCl after 24 hours
◑ Mixed sols in 10^{-1}M KCl after 96 hours

power, present at the interface. The surface tension at high dilution was slightly less than that of a pure lysolecithin sol.

At 40° a similar lowering of the surface tension of water took place except below the region of 0·001 per cent w/v. The plateau in this region contrasted strongly with the behaviour at 25°, the higher temperature favouring greater solubilisation with a small reduction of phosphatide in the surface layer. Although this reduction in surface activity appeared to continue into the most dilute region examined at 40°, there was no marked change compared with that observed at 25°.

Effect of Calcium Chloride

The lecithin and lysolecithin molecules have two sites of charges at the phosphoryl and the cholyl groups. Adsorbed molecules such as soaps must also be considered since a primary ageing effect will be hydrolysis at the ester linkages.

Above 0·002 per cent w/v lysolecithin the lowering effect was slightly less than in the absence of calcium chloride (Fig. 2). The calcium chloride would normally be expected to lower the critical micelle concentration of lysolecithin and it was probable that above 0·002 per cent w/v lysolecithin the salt assisted aggregation of molecules and consequently increased solubilisation of the lecithin. From the small change in surface behaviour
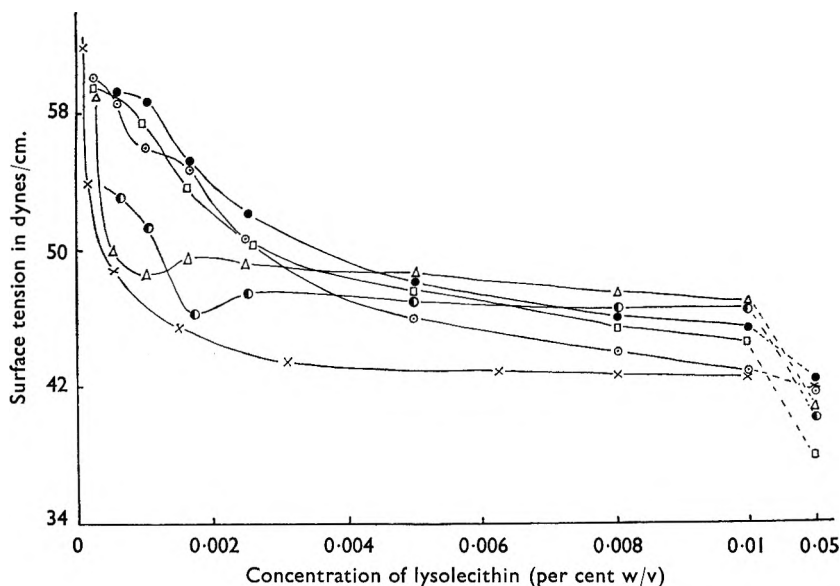

FIG. 5. Effect of KCl on the surface tension of mixed lysolecithin-lecithin sols at 40°. For key see Fig. 4.

it appeared that sufficient lysolecithin was present to prevent substantial removal of the lecithin component from the surface layer by calcium chloride.

The instability of the sols over a period of time was believed to be brought about by the electrolytes affecting the charge on the colloidal particles resulting in a tendency to coagulation.

On standing for 24 hours the calcium chloride appeared to interact with the system in this concentration region of lysolecithin (0·002 per cent) resulting in an increase in surface activity of the sols. After another 72 hours this behaviour spread throughout the concentration range of lysolecithin examined. At 40° (Fig. 3) the effect after 24 hours was more pronounced but a further 72 hours showed little change. The effect of calcium chloride with time on the general behaviour of the system was to be expected from the sensitivity of lecithin to this electrolyte. Calcium

chloride was active in much smaller concentrations than potassium chloride in producing instability of the sols which was thought to be due to the divalent calcium ions linking the phosphoryl groups of two single molecules or molecules within the aggregates.

Effect of Potassium Chloride

The effects of potassium chloride differed from those of calcium chloride due largely to the degree of sensitivity of lecithin to these two electrolytes. Above a weight fraction of lysolecithin of 0·1 the mixed sols were fairly stable to potassium chloride.

At 25° (Fig. 4) and concentrations greater than 0·0025 per cent w/v lysolecithin, addition of potassium chloride produced only a slight change in surface tension, whereas between 0·001 and 0·002 per cent w/v lysolecithin the surface tension lowering was considerably increased. This deviation was unexpected and thought to arise from ionic forces suppressing the aggregation process of lysolecithin molecules. Below this concentration region the lowering effect was unchanged. After standing, concentrations above 0·002 per cent w/v lysolecithin showed little change but below this value the surface tensions steadily increased.

At 40° (Fig. 5) the effect of potassium chloride was to depress the surface activity of the mixed phosphatide system containing more than 0·003 per cent w/v lysolecithin but below this concentration there was little change. After standing, however, the system showed a considerable lowering in the surface tension indicating that removal of lecithin from the surface layer took place resulting in small lysolecithin particles producing increased surface activity.

The surface activity of the system is thus very sensitive to both calcium chloride and potassium chloride particularly in the region of the critical micelle concentration of lysolecithin where changes are likely to be emphasised. Here the presence of electrolytes shows a tendency to increase the surface activity of the mixed phosphatide system, especially at 40°, compatible with changes in metabolism within the environment of a cell.

REFERENCES

1. Elworthy and Saunders, *J. chem. Soc.*, 1957, 330.
2. Danielli and Davson, *J. Cell. Comp. Physiol.*, 1935, **5**, 495.
3. Saunders, *J. Pharm. Pharmacol.*, 1957, **9**, 830.
4. Robinson and Saunders, *ibid.*, 1959, **11**, 346.
5. Hanahan, Rodbell and Turner, *J. biol. Chem.*, 1954, **206**, 431.
6. Robinson and Saunders, *J. Pharm. Pharmacol.*, 1958, **10**, 384.
7. Lea, Rhodes and Stoll, *Biochem. J.*, 1955, **60**, 353.
8. Lea Proc. 2nd International Conference on Biochemical Problems of Lipids, 1955.
9. Rhodes, *Chem. and Ind.*, 1956, **75**, 1010.
10. Perrin and Saunders, *J. Pharm. Pharmacol.*, 1960, **12**, 253.
11. Neuschloz, *Kolloid Z.*, 1920, **27**, 292.
12. Price and Lewis, *Biochem. J.*, 1929, **23**, 1031.
13. Fischgold and Chain, *ibid.*, 1934, **28**, 2044.
14. Boutaric and Berthier, *C.R. Acad. Sci. Paris*, 1940, **211**, 100.

FURTHER STUDIES ON THE BIOSYNTHESIS OF 5-HYDROXYTRYPTAMINE

By Shirley A. P. Price and G. B. West

From the Department of Pharmacology, School of Pharmacy, University of London, Brunswick Square, London, W.C.1

The factors affecting the 5-hydroxytryptophan decarboxylase activity of the brain of animals have been studied. By altering the conditions of incubation and the amounts of the constituents of the incubation mixture, the activity as determined by the amount of 5-hydroxytryptamine formed has been increased about 10-fold. Intramuscular injections of cortisone and prednisolone did not reduce the enzyme activity although such treatment lowers the 5-hydroxytryptamine content of several tissues.

KNOWLEDGE of the distribution of 5-hydroxytryptamine (5-HT) in the tissues may give an indication of the part it is likely to play in the normal functioning of the body, but knowledge of its formation and destruction is equally important. Studies on the biosynthesis of 5-HT have been reported by various workers[1-3]. Whereas 5-HT is concentrated in the spleen and gastrointestinal tract of most mammals, the highest activity of the enzyme decarboxylating its precursor, 5-hydroxytryptophan (5-HTP), has been found in the kidney, liver and gut, with less in the brain[3]. When the activity of the decarboxylating enzyme in rat brain was measured using methods employed by the earlier workers very low values were obtained. The present experiments were therefore carried out to study the optimal conditions for the conversion of 5-HTP to 5-HT by the enzyme present in brain tissue.

METHODS

Preparation of the Tissues for Determining their Content of 5-HTP Decarboxylase

Brains from freshly-killed animals were weighed, cut into small pieces, and ground in a mortar with a little sand and phosphate buffer (2 ml./g. tissue) of varying pH values. Such treatment extracts the enzyme but not the 5-HT. Aliquots of the homogenates containing the desired quantity of tissue were measured into specimen tubes containing varying quantities of the co-enzyme, pyridoxal-5-phosphate, and of an inhibitor of monoamine oxidase, iproniazid, to prevent the destruction of the 5-HT formed from 5-HTP. Phosphate buffer was then added to the desired volume and the substrate, DL-5-HTP, was added last. Immediately after its addition, the mixture (final volume 5 ml.) was shaken and incubation allowed to proceed at 37° for varying times. The reaction was then stopped by reducing the pH of the solution to 4·0 with N HCl, and the 5-HT content of the solution assayed. The amount of 5-HT formed per gram of tissue may be used as an indication of the 5-HTP decarboxylase activity of that tissue. Each value in Table I represents the mean of three
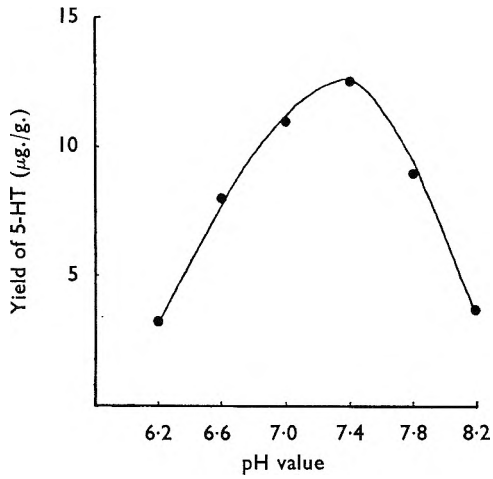
FIG. 1. Effect of pH on the 5-HTP decarboxylase activity of rat brain (as indicated by the yield of 5-HT).
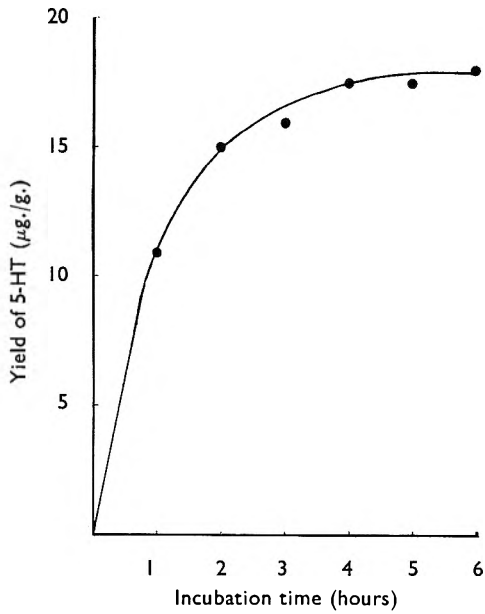

FIG. 2. Effect of incubation time on the 5-HTP decarboxylase activity of rat brain. Incubation at pH 7·4.

separate experiments. In all estimates, control tubes containing the boiled enzyme preparation were incubated and assayed; none showed 5-HT activity.

In a small series of further experiments, the brains from freshly killed rats which had received nine daily doses of cortisone or prednisolone (10 mg./kg. intramuscularly) were used to determine the effect of corticosteroid treatment on the 5-HTP decarboxylase activity in the brain.

Estimates were also made of the decarboxylase activity of mouse, guinea pig and rabbit brains.

Bioassay Procedure

Bioassays were made on the isolated uterus of the oestrus rat. An aerated 20 ml. bath of de Jalon's fluid containing atropine (10^{-7}) at

TABLE I

EFFECT OF VARYING THE CONDITIONS OF INCUBATION ON THE 5-HTP DECARBOXYLASE ACTIVITY OF RAT BRAIN (AS INDICATED BY THE YIELD OF 5-HT)

pH	Time of incubation (hr.)	Contents of the incubation mixture				Yield of 5-HT (μg./g.)	5-HTP decarboxylated per cent
		Pyridoxal (μg.)	Iproniazid (μg.)	Homogenate (mg.)	5-HTP (μg.)		
8·0	1	100	100	800	400	3–7	3
7·4	1	100	100	800	200	9–13	8
7·4	5	100	100	800	200	16–20	16
7·4	5	200	100	800	200	25–30	24
7·4	5	200	800	800	200	60–75	58

TABLE II

THE 5-HTP DECARBOXYLASE ACTIVITY OF THE BRAINS OF DIFFERENT SPECIES (AS INDICATED BY THE YIELD OF 5-HT IN μG./G.). THE RESULTS OF PREVIOUS AUTHORS ARE COMPARED WITH THOSE FOUND IN THE PRESENT STUDY

Species	Previous work[1,3]	Present work
Rat	7	7
Mouse	9	155
Guinea pig	13	110
Rabbit	5	73

28° was used. On occasion, the extracts were also assayed on the rat colon suspended in a similar bath at 20°. Usually, both preparations were sensitive to 0·01–0·02 μg. of 5-HT (i.e., approximately 10^{-9} g.). The specificity of the reaction was checked by using the 5-HT antagonist, 2-bromolysergic acid diethylamide. The standard 5-HT was used as its creatinine sulphate, but values given in the text refer to the base.

RESULTS

The 5-HTP decarboxylase of rat brain. With a phosphate buffer of pH 8·0 and an incubation time of 1 hour, the yield of 5-HT was only 3–7 μg./g. of brain tissue. This is shown in Table I where the amounts of pyridoxal, iproniazid, homogenate and 5-HTP in the incubation mixture are recorded. As only the laevo form of the amino-acid is converted into 5-HT by the brain enzyme, the rate of conversion was about 3 per cent.
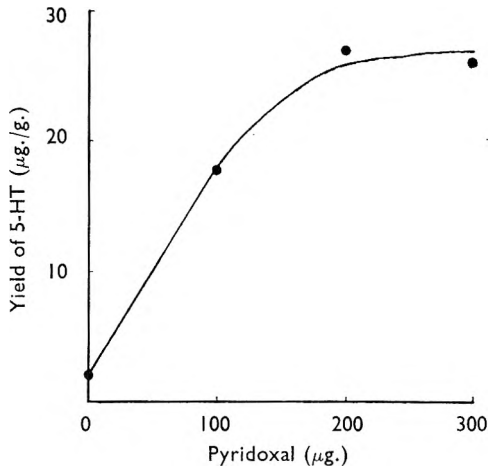
FIG. 3. Effect of pyridoxal on the 5-HTP decarboxylase activity of rat brain. Incubation at pH 7·4 for 5 hr.
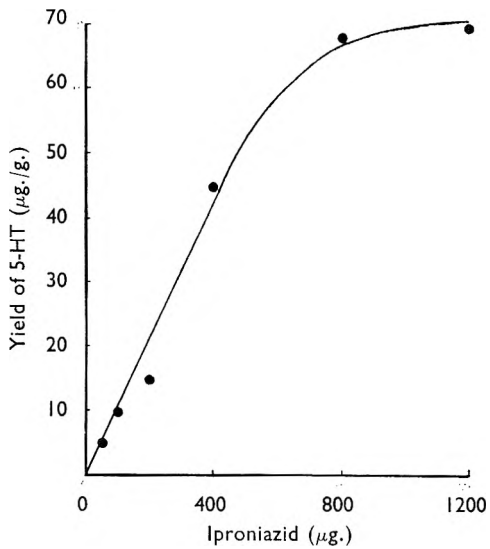

FIG. 4. Effect of iproniazid on the 5-HTP decarboxylase activity of rat brain. Incubation at pH 7·4 for 5 hr. with 200 μg. pyridoxal.

Efforts were therefore made to increase the conversion rate by altering the conditions of incubation.

Effect of pH and incubation time on the enzyme activity. Using a wide range of pH values and an incubation time longer than 1 hour, it was possible to increase the yield of 5-HT about four-fold. The optimal pH value was found to be 7·4 (see Fig. 1), incubation at this value for 1 hour yielding 9–13 μg. 5-HT per g. tissue. When incubation was extended to 5 hours the yield was 16–20 μg./g. corresponding to a conversion rate of about 16 per cent (see Fig. 2).

Effect of pyridoxal and iproniazid on the enzyme activity. By doubling the amount of pyridoxal in the incubation mixture, the yield of 5-HT was still further increased to 25–30 μg./g. (see Fig. 3). It was then possible to double this yield again by using 8 times as much iproniazid as in the original mixture (see Fig. 4). This large increase in the amount of iproniazid was necessary to prevent completely the action of the highly active monoamine oxidase known to be present in brain tissue.

Effect of homogenate and substrate on the enzyme activity. By using a wide range of amounts of both homogenate and substrate, it was not possible to increase the yield of 5-HT above 60–75 μg./g. The optimal value of homogenate was 800 μg. (Fig. 5) whilst the substrate value was 200 μg. (Fig. 6). The percentage of 5-HTP converted into 5-HT was also optimal when these amounts were used. Thus, in the present experiments, the yield of 5-HT (and therefore the 5-HTP decarboxylase activity of rat brain) has been increased 10-fold by altering the incubation conditions; the amount of 5-HTP converted has likewise been increased from 3 to 58 per cent.

The 5-HTP decarboxylase of mouse, guinea pig and rabbit brains. Using the optimal conditions for incubating rat brain, an estimate was then made of the decarboxylase activity of the brains of three other species. The results are shown in Table II. The activity has been increased about 10 times.

The effect of cortisone and prednisolone on the 5-HTP decarboxylase of rat brain. Treatment of rats with either corticosteroid failed to alter significantly the 5-HTP decarboxylase activity in the brain, the maximal change being an increase of 10 per cent in the activity. This result is surprising as treatment with glucocorticoids results in a considerable reduction in the 5-HT content of many tissues[3].

Discussion

The 5-HTP decarboxylase of brain has been estimated as the amount of 5-HT formed by 1 g. of tissue. By altering the conditions of incubation, it has been possible to increase the yield about 10-fold in the brains of rat, mouse, guinea pig, and rabbit. The work also illustrates that the conditions for optimal enzyme activity vary widely from tissue to tissue. For example, the brains of these species show optimal activity at a pH value of 7·4, with much less activity at pH values of 8·0 and 6·2, whereas the kidney and liver produce maximal yields of 5-HT when the pH of the incubation mixture is kept at 8·0[1].
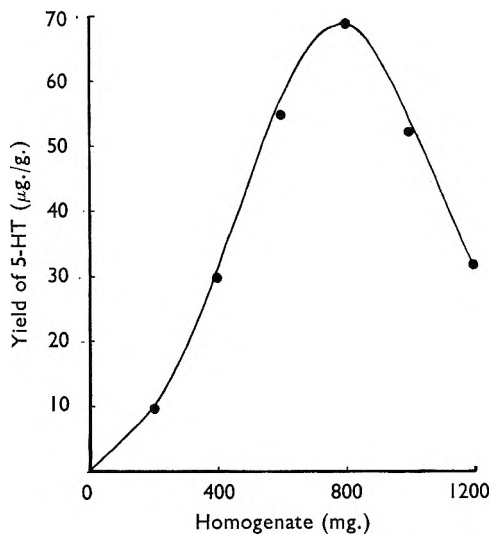
FIG. 5. Effect of varying the amount of homogenate on the 5-HTP decarboxylase activity of rat brain. Incubation at pH 7·4 for 5 hr. with 200 μg. pyridoxal and 800 μg. iproniazid.
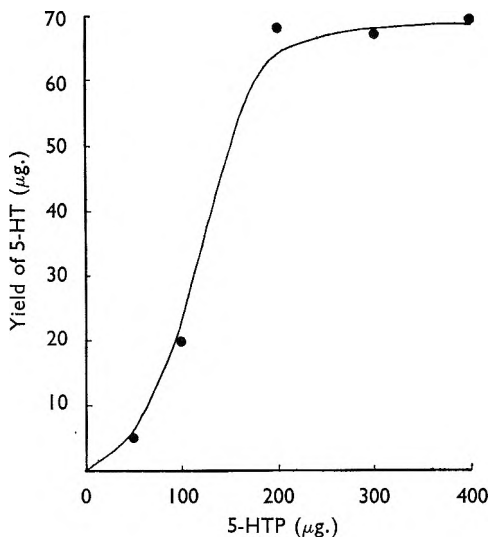

FIG. 6. Effect of varying the amount of 5-HTP on the 5-HTP decarboxylase activity of rat brain. Incubation at pH 7·4 for 5 hr. with 200 μg. pyridoxal, 800 μg. iproniazid and 800 mg. homogenate.

Further, the quantity of monoamine oxidase inhibitor needed in the kidney and liver experiments is some eight times less than that needed for brain incubation experiments. The values obtained for the 5-HTP decarboxylase activities of brain homogenates using the modified conditions of incubation and amounts of constituents show that the brain must be considered, together with the gut, kidney and liver, as one of the chief sources of this enzyme. It is known that 5-HT does not readily cross the blood-brain barrier although 5-HTP does, and the results of the present work indicate that the 5-HT-forming enzyme is particularly active in some animal species. This conclusion does not oppose the hypothesis that the 5-HT content of the brain may be an important factor concerned with the activity of nerve cells in the brain. Further work along this line is indicated, particularly as compounds which deplete the peripheral tissues of their 5-HT fail to modify the activity of the brain 5-HTP decarboxylase enzyme.

REFERENCES

1. Gaddum and Giarman, *Brit. J. Pharmacol.*, 1956, **11**, 88.
2. Bogdanski, Weissbach and Udenfriend, *J. Pharmacol.*, 1958, **122**, 182.
3. West, *J. Pharm. Pharmacol.*, 1958, **10**, *Suppl.*, 92*T*.

THE COLORIMETRIC DETERMINATION OF PHENACETIN IN TABLET MIXTURES

BY LEE KUM-TATT AND CHAN CHIAN-SENG

From the Government Department of Chemistry, Outram Road, Singapore

Phenacetin may be quantitatively converted to nitrophenetidine by nitration and subsequent saponification with sodium hydroxide. The nitrophenetidine thus formed is soluble in alkaline solution and may be determined colorimetrically at 445 mμ. The method may be applied to samples containing 1 to 5 mg. of phenacetin. This method possesses several advantages over existing methods in that it is simple, sensitive and accurate and does not require the tedious separation of caffeine when applied to the determination of phenacetin in aspirin, phenacetin and caffeine mixtures. Recovery data of phenacetin in these mixtures are given. A simple and rapid procedure for the analysis of the components of aspirin, phenacetin and caffeine is also described.

THE rapid determination of the components of the time-honoured A.P.C. tablets has always been the goal of many analytical chemists. While satisfactory results can be obtained for the determination of aspirin in this mixture by the B.P.C.[1] or the A.O.A.C.[2] methods, the procedures recommended for the separation and determination of caffeine and phenacetin are time-consuming, tedious and lack specificity and reproducibility. Daoust[3] described a simple colorimetric method for the determination of caffeine as its phosphomolybdate derivative in A.P.C. tablets, and the main difficulty seems to be the lack of a reliable method for the estimation of phenacetin in this mixture.

Many methods for the determination of phenacetin have been reported. Wollish, Colarusso, Pifer and Schmall[4], described a procedure for the determination of phenacetin by non-aqueous titration. Casini[5] recommended the conversion of phenacetin to tetra-iodophenacetin by the addition of standard iodine solution, the excess of which can be determined with thiosulphate solution. Higuchi and Patel[6] have described a spectrophotometric method for the determination of phenacetin in A.P.C. mixture. Although satisfactory results can be obtained by these methods all require time-consuming extraction for the separation of phenacetin from caffeine before its determination. Horn[7] described a colorimetric method for the determination of phenacetin as 3-nitro-4-acetamidophenetole and he reported its applicability to A.P.C. mixtures. However, Casini[5] has studied this procedure in detail and reported the method to be neither sensitive nor reliable. Degner and Johnson[8] estimated phenacetin from the colour produced with concentrated hydrochloric acid and chromic acid. The reaction is dependent on temperature, time, and the concentrations of the chromic acid and ammonium citrate used. The colour goes through a maximum intensity and then fades, the time and intensity of maximum colour formation and rate of fading depends on the amount of chromic acid, hydrochloric acid and ammonium citrate used as well as

the temperature. Because of these limitations the need for a rapid and accurate method for the determination of phenacetin for control work is apparent.

<div align="center">EXPERIMENTAL</div>

Reagents. Phenacetin standard solution—Recrystallised phenacetin, 20 mg., is dissolved in about 70 ml. of chloroform and diluted to 100 ml., 5 ml. contains 1 mg. of phenacetin. Nitric acid (50 per cent v/v). Sodium hydroxide solution (10 per cent) and chloroform, reagent grade.

Preparation of a standard curve. Aliquots of 5, 10, 15, 20, and 25 ml. of the standard solution of phenacetin are pipetted consecutively into each of five 50 ml. beakers on a steam bath. After evaporation of the chloroform 2 ml. of nitric acid is added and the solution allowed to stand for 30 minutes at room temperature. Sodium hydroxide solution, 10 ml., is then added to each beaker and the solutions heated on the bath for 30 minutes. Each solution is transferred to a 100 ml. volumetric flask and diluted to mark with distilled water. The optical density of the nitrophenetidine may be measured directly at 445 mμ on a Hilger spectrophotometer using distilled water as the blank. The calibration curve of phenacetin plotted on a linear scale at 1 mg. increments in the range for 1 to 5 mg. indicated that the method obeys Beer's Law with good agreement.

Determination of Phenacetin in the Presence of Caffeine

Known amounts of phenacetin and caffeine are weighed and dissolved in chloroform in a standard volumetric flask. Aliquots containing about 1 to 5 mg. of phenacetin are taken and evaporated in 50 ml. beakers on a water bath. Continue as described in the paragraph on the preparation of a standard curve, beginning with the words: "After evaporation of the chloroform . . ." to "measured directly at 445 mμ on a Hilger spectrophotometer." The quantity of phenacetin is calculated from the calibrated curve. Table I shows the results obtained using this procedure.

<div align="center">TABLE I</div>
<div align="center">RECOVERY OF PHENACETIN IN MIXTURES WITH CAFFEINE</div>

Phenacetin used, mg.	Phenacetin recovered, mg.	Recovery per cent
200	198	99·0
200	197	98·5
200	200	100·0
200	200	100·0
200	203	101·5
200	201	100·5

Determination of Phenacetin in the Presence of Aspirin and Caffeine

A mixture containing 0·230 g. of aspirin, 0·160 g. of phenacetin, and 0·030 g. of caffeine is dissolved in 500 ml. of chloroform. Portions of 10 ml. are taken and extracted once with 15 ml. of 6 per cent sodium bicarbonate solution. The sodium bicarbonate solution is then extracted thrice with 10 ml. portions of chloroform. The chloroform extracts are combined, washed once with 3 ml. of 6 per cent sodium bicarbonate

solution and evaporated to dryness in a 100 ml. beaker. After evaporation 2 ml. of nitric acid is added to the residue followed by 10 ml. of sodium hydroxide solution after 30 minutes. The phenacetin is determined as described previously. The recoveries of phenacetin using the above procedure are shown in Table II.

Determination of Aspirin, Phenacetin and Caffeine in Commercial A.P.C. Tablets and Mixtures

The average tablet weight is calculated from the weight of 20 tablets accurately weighed. A sample of about 0·5 g. of the pulverised tablet is transferred into a separating funnel. To this is added 10 ml. of water and 15 ml. of cold 6 per cent sodium bicarbonate solution together with 2

TABLE II

RESULTS OF ASSAYS FOR PHENACETIN ON KNOWN A.P.C. MIXTURES

Phenacetin present in 10 ml. CHCl₃ solution, mg.	Phenacetin recovered, mg.
3·20	3·15
3·20	3·17
3·20	3·22
3·20	3·16
3·20	3·17

TABLE III

RESULTS OF ASSAYS FOR ASPIRIN, PHENACETIN AND CAFFEINE IN COMMERCIAL A.P.C. TABLETS

Samples	Aspirin		Phenacetin		Caffeine	
	Amt. labelled per tablet (mg.)	Amt. found per tablet (mg.)	Amt. labelled per tablet (mg.)	Amt. found per tablet (mg.)	Amt. labelled per tablet (mg.)	Amt. found per tablet (mg.)
A	226·8	214 217	162·0	156 157	32·4	32·4 31·6
B	226·8	216 220	162·0	156 156	32·4	32·4 32·0
C	226·8	220 224	162·0	160 162	32·4	31·1 30·6
D	226·8	223 218	162·0	158 158	32·4	31·3 32·8

drops of dilute hydrochloric acid. The phenacetin and caffeine present are extracted with five 30 ml. portions of chloroform. Each chloroform portion is passed through a second separating funnel containing 3 ml. of cold 6 per cent sodium bicarbonate solution. The chloroform fractions are filtered through cotton wool and combined for later treatment. The sodium bicarbonate solutions are combined, acidified with 1:1 hydrochloric acid and the aspirin present is extracted and determined by the procedure described in A.O.A.C.[2]

The chloroform extracts are combined and transferred to a 200 ml. volumetric flask and made up to mark. Aliquots of 5 ml. are taken and evaporated in 50 ml. beakers and the phenacetin content is determined by the procedure already described. For the determination of caffeine 10 to 15 ml. aliquots of the chloroform solution are taken and evaporated

and the amount of caffeine is determined according to the procedure described by Daoust[3]. The results obtained for the analyses of four commercial samples of A.P.C. are shown in Table III.

Effects of Time and Heat on the Stability of the Colour of Nitrophenetidine Solutions

A known amount of phenacetin is treated with 2 ml. of nitric acid and the solution is allowed to stand for 30 minutes. Ten ml. of sodium hydroxide solution is added and after 30 minutes the solution is transferred to a 100 ml. volumetric flask and the optical density of this solution

TABLE IV

EFFECTS OF HEAT AND TIME ON COLOUR OF THE SOLUTIONS

Time in hours	Optical density		
	Solution 1	Solution 2	Solution 3
0	0·40	0·425	0·886
1	0·430	0·480	0·880
2	0·505	0·555	0·880
3½	0·580	0·625	0·884
4	0·595	0·645	0·882
5	0·640	0·695	0·878
6	0·662	0·715	0·880
18	0·874	0·878	0·882
19	0·880	0·878	0·880
24	0·878	0·876	0·882

No heat was applied to solutions 1 and 2. Solution 3 was heated for 30 min. after addition of sodium hydroxide solution before dilution.

is measured at 445 mμ at hourly intervals. No heat is applied in this procedure and the results obtained are shown in Table IV, Columns II and III.
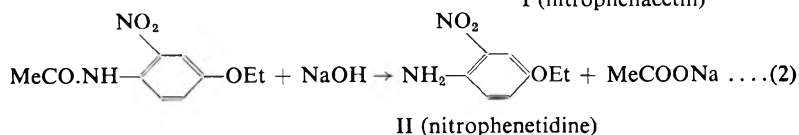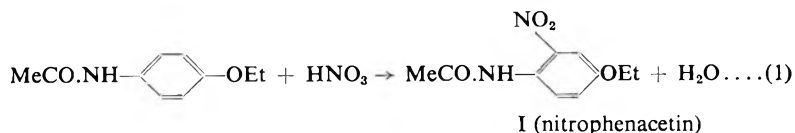
A similar quantity of phenacetin is treated with 2 ml. of nitric acid. After standing at room temperature for 30 minutes 10 ml. of sodium hydroxide solution is added and the alkaline solution is heated on a water bath for 30 minutes. The solution is transferred to a 100 ml. volumetric flask and diluted to mark with distilled water. The optical density of the solution was measured at 445 mμ at one hour intervals. There was no difference between the various readings as shown in Table IV, Column IV. It was observed that when no heat is applied the complete hydrolysis of nitrophenacetin to nitrophenetidine requires about 18 hours.

Effects of Amount of Alkali Used

Five 15 ml. aliquots of the standard solution of phenacetin are pipetted into each of five 50 ml. beakers on a water bath. After evaporation of the chloroform 2 ml. of nitric acid is added and the solution allowed to stand for 30 minutes after which 10, 15, 20, 25 and 30 ml. of sodium hydroxide solution is added to each beaker respectively. The resulting solutions are warmed on the water bath for a further 30 minutes and transferred to a 100 ml. volumetric flask and diluted to mark. The optical densities of these solutions were measured at 445 mμ and no differences between the readings were observed.

RESULTS AND DISCUSSION

When phenacetin is reacted with nitric acid followed by warming with sodium hydroxide, nitration and hydrolysis take place in accordance with the following equations:

$$MeCO.NH\!-\!\!\langle\ \rangle\!-\!OEt + HNO_3 \rightarrow MeCO.NH\!-\!\overset{NO_2}{\langle\ \rangle}\!OEt + H_2O \ldots(1)$$

I (nitrophenacetin)

$$MeCO.NH\!-\!\overset{NO_2}{\langle\ \rangle}\!-\!OEt + NaOH \rightarrow NH_2\!-\!\overset{NO_2}{\langle\ \rangle}\!OEt + MeCOONa \ldots(2)$$

II (nitrophenetidine)

These two reactions form the basis of the proposed method.

Nitrophenacetin (I) when recrystallised from 20 per cent ethanol precipitates in the form of yellowish needles with a melting point of 97°. Nitrophenetidine (II), under suitable conditions is obtained as orange
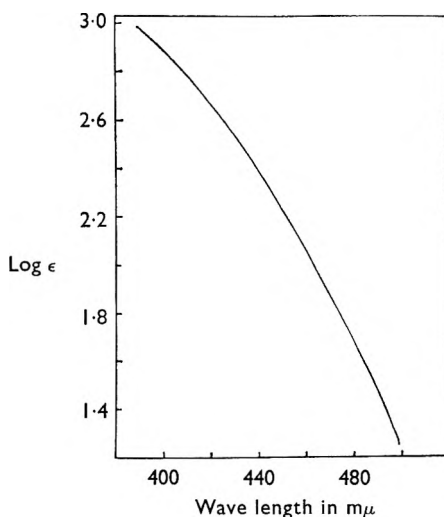

FIG. 1. Light absorption curves of nitrophenacetin in water.

red crystals having a melting point of 78° after recrystallisation from light petroleum. Nitrophenacetin has very low absorption at 465 mμ and its absorption spectra possesses no maximum between 400 mμ and 500 mμ. A typical absorption spectrum of nitrophenacetin is shown in Figure 1. It is therefore not unexpected that the determination of phenacetin as its nitro derivative at 465 mμ as proposed by Horn[7] lacks both sensitivity and reliability as reported by Casini[5]. Nitrophenetidine on the other hand exhibits very strong absorption between 400 mμ and 500 mμ and its absorption spectra has a maximum at 445 mμ as shown in Figure 2. This compound is very stable in alkaline solution and its absorption

spectra is not affected by the amount of alkali present. However, when nitrophenetidine is dissolved in an acid media a light yellow solution results. The spectra of the yellow solution is shown in Figure 3.

Heat is essential for the rapid and complete hydrolysis of nitrophenacetin to nitrophenetidine. Hydrolysis in the cold requires about 18 hours to
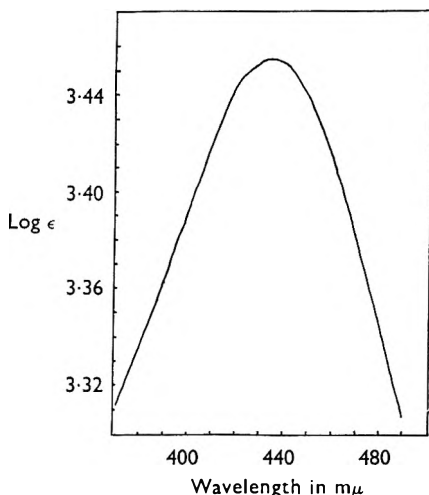

FIG. 2. Light absorption curves of nitrophenetidine in 10 per cent sodium hydroxide solution.
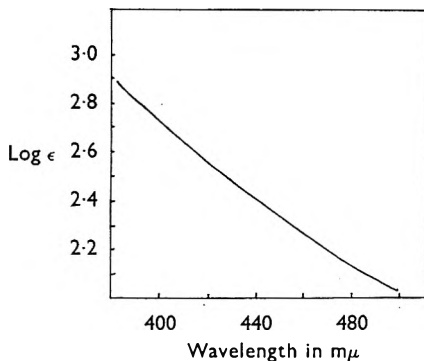

FIG. 3. Light absorption curves of nitrophenetidine in 10 per cent nitric acid.

reach completion. The necessity for heating the alkaline solution for 30 minutes before dilution cannot therefore be over-emphasised for reproducible results.

Recovery results by the proposed method are presented in Tables I to III. The values obtained showed that caffeine does not interfere in the procedure and the recoveries of phenacetin were good indicating satisfactory precision.

The proposed method fulfils the requirements for a routine procedure for the determination of phenacetin in tablet mixtures. The procedure

for the determination is simple and of general applicability and the colour developed is independent of time and the amount of alkali used. The tedious separation of caffeine from phenacetin is not required. The method is sensitive and reliable and may be advantageously used for the determination of phenacetin in A.P.C. mixture. Together with the A.O.A.C.[2] procedure for aspirin and the Daoust[3] method for caffeine, it makes feasible the rapid determination of the components in A.P.C. This proposed method for the analysis of the components of A.P.C. gave satisfactory results as shown in Table III. One analyst can make 6 to 8 complete assays of A.P.C. mixtures in an 8-hour day.

Acknowledgements. The authors thank Dr. Loke Kwong Hung, Biochemistry Department, University of Malaya, for his valuable discussions and the Chief Chemist, Mr. Chia Chwee Leong for his interest.

REFERENCES

1. *British Pharmaceutical Codex*, The Pharmaceutical Press, London, 1954, p. 1161.
2. *Methods of Analysis*, A.O.A.C., 8th Edn, Association of Official Agricultural Chemists, Washington, 4, D.C., p. 619.
3. Daoust, *J. Amer. pharm. Ass., Sci. Ed.*, 1953, **42**, 744.
4. Wollish, Colarusso, Pifer, and Schmall, *Analyt. Chem.*, 1954, **26**, 1753.
5. Casini, *Annali Chim.*, 1951, **41**, 611.
6. Higuchi and Patel, *J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 171.
7. Horn, *Pharm. Zentralh.*, 1951, **90**, 296.
8. Degner and Johnson, *Analyt. Chem.*, 1947, **19**, 330.

DETERMINATION OF XENOPUS INDEX AND HAEMOLYTIC INDEX IN FRUITS OF *SAPINDUS MUKOROSSI* GAERTN. (SAPINDACEAE) AND SEEDS OF *ENTADA SCANDENS* BENTH. (MIMOSACEAE)

BY MARTHE BLYBERG

From the Department of Pharmacognosy, Institute of Pharmacy, University of Oslo, Norway

Two drugs, known from the Burmese and Indian folk medicines, the fruits of *Sapindus mukorossi* and the seeds from *Entada scandens*, were examined for their effect on Xenopus larvae. As a comparative test the haemolytic index of the drugs was also determined.

The investigations showed that parts of both drugs contained substances which were toxic for Xenopus larvae and which haemolysed blood. The fruit wall from *Sapindus mukorossi* and the seed kernel from *Entada scandens* had a Xenopus index of approximately 7,000 and 400 respectively. The corresponding haemolytical indexes were approximately 7,000 and 150.

THE fruits of *Sapindus mukorossi*, Indian soap nuts, and the seeds of *Entada scandens* have for a long time been used in Burma and India as saponin drugs and fish poisons. It has also been claimed that the entada seeds possess anthelmintic properties.

According to the available literature both drugs contain saponins[1,2], and as East-Asiatic drugs in recent years have attained increasing importance in Europe, it was felt to be of interest to determine the content of saponin-like substances in the two drugs.

The drugs were examined according to the Xenopus method of Karma and von Schantz[3–5]. The haemolytical index of both drugs was also determined.

EXPERIMENTAL

Determination of Xenopus Index

The investigations were aimed at finding the drug concentration at which the average time of death of the Xenopus larvae is exactly 1 hour. This concentration is termed the hour-concentration. The Xenopus index is then defined as the amount of water, expressed in g., with which 1 g. of crude drug or of pure saponin must be diluted to obtain the hour-concentration.

For the decoctions, coarse powder of the drugs (Pharm. Fennic. VII, screen No. 4) and for the tests, chlorine-free water was used. The decoctions were made according to the original papers[3,4], but the extraction was in a boiling water bath.

For the experiments a larva strain was used for which the hour-concentration of a decoction of the standard drug "Cortex Qvillajae XV" was determined as 0·038 per cent. The corresponding hour-concentration of the normal strain is 0·025 per cent[4]. Accordingly the correction factor is 0·65.

The Fruit of Sapindus mukorossi

The fruit was divided into the fruit wall, the seed coat and the seed kernel.

The fruit wall. Preliminary tests made with solutions of different strength prepared from a 5 per cent decoction showed that the hour-concentration lay between 0·1 and 0·01 per cent. A subsequent experiment with dilutions of a 0·1 per cent decoction (pH 6·3) showed that the
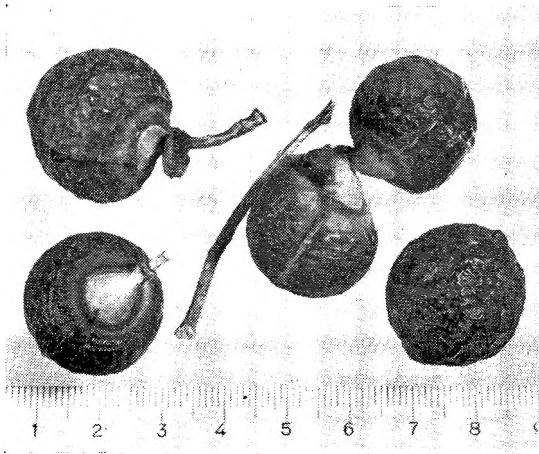

FIG. 1. The fruits of *Sapindus mukorossi* Gaertn. (Sapindaceae).
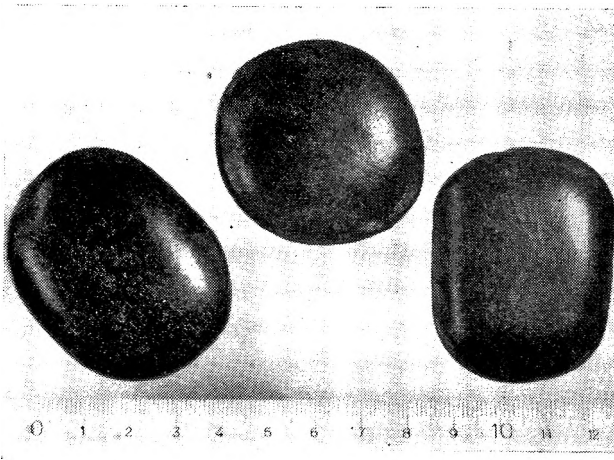

FIG. 2. The seeds of *Entada scandens* Benth. (Mimosaceae).

hour-concentration lay between 0·03 and 0·02 per cent. By reducing the concentration intervals gradually (Table I) the hour-concentration was determined as 0·022, corresponding to 0·014 when adjusted to the normal strain which gives a Xenopus index of approximately 7,000.

The seed coat. Preliminary tests showed that the larvae survived 1 to 2 hours in 5 per cent decoction, which indicates that saponins cannot be detected by this means.

The seed kernel. Preliminary tests showed that the larvae were not injured in a 5 per cent decoction. Accordingly no saponins could be demonstrated by this means.

The Seed of Entada scandens

The seeds were divided into seed coat and seed kernel.

The seed coat. Preliminary tests with solutions of different strength prepared from a 5 per cent decoction showed that the time of death of the

TABLE I

DEATH TIMES OF LARVAE TO WHICH DECREASING CONCENTRATIONS OF THE DECOCTION OF THE FRUIT WALL OF *S. mukorossi* WERE ADDED

Concentration of the decoction in per cent	Number of larvae	Mean value of death time in min.
0·0300	9	29
0·0275	6	40
0·0250	17	45
0·0225	13	55
0·0200	13	75

larvae varied greatly and that there was no clear connection between the concentration of the decoctions and the time of death of the larvae. Accordingly there is no clear evidence that saponins are present in the seed coat.

The seed kernel. Preliminary tests made with dilutions of different strength prepared from a 5 per cent decoction showed that the hour-concentration lay between 1 and 0·1 per cent.

TABLE II

DEATH TIMES OF LARVAE TO WHICH DECREASING CONCENTRATIONS OF DECOCTION OF THE SEED KERNEL OF *E. scandens* WERE ADDED

Concentration of the decoction in per cent	Number of larvae	Mean value of death time in min.
0·400	4	43
0·375	5	48
0·350	5	69
0·325	5	70

A subsequent experiment with dilutions of a 2 per cent decoction (pH 5·6) showed that the hour-concentration lay between 0·4 per cent and 0·3 per cent. By reducing the concentration intervals gradually (Table II) the hour-concentration was determined as 0·36, corresponding to 0·24 when adjusted to the normal strain which gives a Xenopus index of approximately 400.

Determination of the Haemolytic Index

The experiments were made according to a modification of Büchi, Hippenmeyer and Dolder[6]. The sodium citrate solution and phosphate buffer were prepared according to Sandberg[7].

The decoctions were prepared with buffer solution. Otherwise the method described under determination of Xenopus index was followed. Sheep blood was used.

Under the same conditions the haemolytical index of pure saponin Merck was determined: 0·08 mg. pure saponin produced full haemolysis in 2 ml. of 1 per cent blood-suspension, which gives a haemolytic index of approximately 25,000.

The Fruit of Sapindus mukorossi

The fruit wall. 0·3 mg. of drug produced full haemolysis in 2 ml. of 1 per cent blood-suspension. HI = 7,000 (approx.).

The seed coat and the seed kernel. 10 per cent decoctions of the seed coat and the seed kernel gave no haemolysis, and thus no saponins were demonstrated.

The Seed of Entada scandens

The seed coat. A 10 per cent decoction gave no haemolysis.

The seed kernel. 14 mg. of drug produced full haemolysis in 2 ml. of 1 per cent blood-suspension. HI = 150 (approx.).

REFERENCES

1. Chopra, Nayar, Chopra, *Glossary of Indian Medical Plants*, New Delhi, 1956, p. 221.
2. Chopra, Nayar, Chopra, *ibid.*, p. 107.
3. Karma and von Schantz, *Medd. Norsk Farm. Selsk.*, 1955, **17**, 173.
4. Karma and von Schantz, *Sci. Pharm.*, 1959, **27**, 6.
5. Von Schantz, *Farm. Notisblad*, 1956, **65**, 247.
6. Gstirner, *Prüfung und Verarbeitung von Arzneidrogen*, I, Berlin, 1955, p. 49.
7. Sandberg, *Svensk Farm. Tidskr.*, 1948, **52**, 173.

PHARMACOPOEIAS AND FORMULARIES

THE PHARMACOPEIA OF THE UNITED STATES OF AMERICA
SIXTEENTH REVISION*

REVIEWED BY A. D. MACDONALD

There is something inexorable, even relentless, about Pharmacopoeias and especially in these days when they aim at a "five year period of effectiveness". U.S.P. XVI takes over from U.S.P. XV on October 1. It adds 224 new monographs, deletes 159 and advances for the first time a list of 81 Interim Admissions "for which monographs could not be completed by press time", usually because "creditable and objective standards of purity were still beyond attainment". It is hoped that monographs on these will become official by means of Supplements to U.S.P. XVI. Incidentally, these Supplements are supplied without additional charge to all who purchase the main book and return the official order form. Only 12 monographs were added by Supplement to U.S.P. XV—drugs like chlorpromazine, prednisone, prednisolone and reserpine and their preparations. Dried Torula Yeast, added in 1956, has now been deleted. Looking at the list of Interim Admissions, it is safe to predict that many more are likely to be added to the present volume. The turnover in a total of 908 monographs is very substantial and reflects the changing face and fashion of medicine today, the prodigious energy of the synthetic chemists and the manufacturers. In scanning the monographs one finds few containing more than one active ingredient —no opium or codeine compound powders—but paregoric and aromatic spirit of ammonia survive. Most monographs indicate a therapeutic Category—e.g., Antibacterial, Antinauseant, Flavoured vehicle, and both a "Usual Dose" and a "Usual Dose Range". The sub-titles do not always include those of the B.P. Abbreviations for titles are not provided. There is much more detailed information on Packaging and Storage, both in the monographs and in the General Notices, than in B.P. and B.P.C.

U.S.P. XVI is substantially thinner and lighter than B.P. 1958, but has 136 pages more. The main type is larger, the small type much smaller than in B.P. The paper resembles the cream of B.P.C. 1959 rather than the blue-white of the B.P.—experts maintain that off-white is easier on the eye. The "General Tests, Processes and Apparatus" and "Reagents, Indicators and Solutions" which we here call Appendices are printed in double column to save space—even so, they run to nearly 300 pages, even though matter such as the Assay of Digitalis is dealt with in the monograph, not in an Appendix.

Federal recognition of the standards of U.S.P. dates back only to 1906. though the book has been maintained since 1820. But this book is much more than a book of legal standards, guaranteeing the quality of therapeutic substances. It is indeed a therapeutic guide, "the soundness of

which is tempered only by that of the judgement of those who select the articles recognised". The responsibility for this book rests upon a Revision Committee of 60 with an Executive of 10 with Dr. Lloyd C. Miller as Chairman and Director of Pharmacopeial Revision. There are 10 Sub-committees, 22 Additional Committees and 19 Advisory Panels, each dealing with a medical speciality. Tribute is paid to the increasing responsibilities and work of the chemists on these Committees. A list of 240 names of participants in the Revision who are not otherwise mentioned includes the Secretary of the B.P. Commission, Mr. T. C. Denston, and five others outside the U.S.A. It is a vast team and it has done a fine job.

BOOK REVIEW

PHARMACOLOGY AND THERAPEUTICS. Fourth, revised and enlarged, edition. By Arthur Grollman. Pp. 1079 (including Index and 217 illustrations). Henry Kimpton, London, 1960. 93s. 6d.

The author of a text on pharmacology is faced with difficulties not of his own making. Not only must he make a selection of material on the basis of current utility in medical practice and in the laboratory, but must also consider if a compound should be retained on historical grounds, and whether a drug currently acceptable and perhaps enthusiastically received, will have been consigned to therapeutic oblivion by the time his book is in print. He has to face criticisms of not being up to date on the one hand and of including misleading information on the other. The form and emphasis of the book will depend largely upon the meaning the author gives to his definition of pharmacology, and he must face thoughtless criticism for not having written a textbook of endocrinology, of medicine, of mathematical statistics or of chemistry or biochemistry, as applied to pharmacology. As never before, pharmacology has expanded and ramified and is subdivided into separate disciplines all of which apply themselves to the study of the mode of action of drugs. With these observations in mind the reviewer must decide what sort of book the author has decided to write and read it in this light.

Dr. Grollman's fourth edition is apparently aimed primarily at medical students and medical practitioners. It is a tribute to its value and popularity that only two years have elapsed between the third and fourth editions. The book is up-to-date, accurate and comprehensive in its treatment of the subject matter, and each chapter contains a useful bibliography and a list of official preparations. The illustrations are usually very good, particularly those which show drug side effects or toxic reactions, and there are a number of useful tables which list the official names of drugs and their proprietary counterparts. Proprietary names and alternative approved (non-U.S.P.) names are given throughout the text. Some criticism must be levelled at the chemical formulae in which there are a number of minor errors and inconsistencies and at a few of the reproductions of the black and white tracings, which are poor in quality and need replacing in the next edition. The text is, however, up-to-date and comprehensive in its inclusion of data (including dosage) upon new drugs and older well-established ones, whilst the classic pharmacological laboratory tools, such as nicotine and cocaine, are also dealt with. In contrast to the two hundred or more pages which deal with hormones, vitamins, water and inorganic salts, mechanisms of action and structure-action relationships get very little space, nor are there adequate descriptions of practical methods or apparatus.

The book is well printed and bound and, unlike some of the other American texts, of an easily manageable size. There is a sixty page index, a therapeutic index and a brief appendix on prescription writing. Without doubt it will appeal strongly to medical practitioners and medical students, but science students may find the need to supplement it.

<div align="right">J. J. Lewis.</div>

LETTERS TO THE EDITOR

Effects of Gentisate on the Urinary Excretion of Salicylate in the Rat

SIR,—Salicylates are among the commoner causes of poisoning either taken for suicidal purposes by adults or after accidental ingestion by children. The treatment of salicylate intoxication attempts either to control the more dangerous symptoms (for example, by using external cooling for hyperthermia) or to remove salicylate from the body. In the latter category are included such mechanical measures as gastric lavage, exchange transfusion, the use of the artificial kidney, and the administration of alkaline fluids to promote an alkaline urine because the renal excretion of salicylate is significantly increased as the urine pH rises above 7.

After salicylate has entered the cells it may react with a number of enzyme systems particularly those concerned with oxidative phosphorylation reactions, and many of the toxic effects of the drug are explicable in terms of its uncoupling action[1]. The use of a non-toxic substance which could displace salicylate from its intracellular combination would therefore be a valuable adjunct in the treatment of salicylate poisoning. A possible candidate for this role appeared to be

TABLE I

CUMULATIVE ^{14}C EXCRETION IN URINE EXPRESSED AS PERCENTAGES OF THE INJECTED DOSE OF SODIUM [carboxy-^{14}C] SALICYLATE

Group	Urine collection (hr.)				
	0–8	8–15	15–24	24–32	32–48
Saline (6)**	36·2*	61·4	75·9	80·5	85·9
	24·9–42·1	52·6–70·8	68·1–86·7	72·4–91·5	76·8– 96·7
Gentisate (6)	40·8	61·0	78·4	83·2	87·3
	34·0–44·0	56·3–72·9	73·0–83·8	77·5–98·4	79·4–102·5

* Results are given as means and ranges.
** Number of animals in each group.
Comparison of the results of the saline and gentisate groups by the *t*-test showed that the values of P exceeded 0·2 in every case.

the closely related gentisic acid (2,5-dihydroxybenzoic acid) which is devoid of uncoupling activity in mitochondrial suspensions[2] and does not produce toxic symptoms in large doses in man[3]. We have therefore studied the effects of the repeated administration of gentisate on the urinary excretion of radioactivity after the injection of [carboxy-^{14}C] salicylate in the rat.

6 male Wistar rats (280–310 g.) were given an intraperitoneal injection of 1 ml. of 10 per cent (w/v) sodium salicylate containing about 7 μc of sodium [carboxy-^{14}C] salicylate at 0 hours. Three rats subsequently received intraperitoneal injections of 0·5 ml. of 20 per cent (w/v) sodium gentisate at 2, 4, 6 and 8 hours, and the remaining three animals were given injections of 0·5 ml. of 5 per cent (w/v) sodium chloride at similar times. Urine collections were made at the following time intervals: 0–8; 8–15; 15–24; 24–32 and 32–48 hours. No fluid or dietary restrictions were imposed during the experiment. After measurement of the volume of each urine specimen, 1 ml. samples were counted in dished aluminium planchettes (1·0 mm. deep; 2·45 cm. diameter) using an end-window Geiger-Muller tube[4], which was flushed with helium containing 1·6 per cent (v/v) ethanol, and connected to a scaler. The distance between the end-window of the counting tube and the bottom of the planchette was 3·75 mm. Counts (5 minutes) were recorded and the background (60 counts/minute) was deducted. Under these conditions 1 ml. of sodium salicylate used

for the initial injection gave 40,515 counts/minute. The experiment was repeated after an interval of 4 weeks except that the rats which had received the gentisate injections in the previous experiment now received the saline, and vice versa.

The results are given in Table I and show that the administration of the gentisate produced no significant effects on either the rate or the total cumulative excretion of ^{14}C in the urine. The rats receiving gentisate showed an initial diuresis during the first 15 hours of the experiment but this did not alter the urinary excretion of radiocarbon. Thus, it must be concluded that despite the close chemical similarity of salicylate and gentisate it is unlikely that the latter substance displaces salicylate from its intracellular binding sites.

<div align="right">

M. J. H. SMITH,
M. SANDIFORD,
V. MOSES.*

</div>

Department of Chemical Pathology,
King's College Hospital Medical School,
Denmark Hill,
London, S.E.5.
August 8, 1960.

REFERENCES

1. Smith, *J. Pharm. Pharmacol.*, 1959, **11**, 705.
2. Brody, *J. Pharmacol.*, 1956, **117**, 39.
3. Smith, *Postgrad. med. J.*, 1952, **28**, 179.
4. Fuller, *Science*, 1956, **124**, 1253.

New Possibilities for the Biological Assay of Digitalis

SIR,—In the research of cardiotonic-glycoside plants it is essential to determine the biological value of preparations containing glycoside mixtures or glycosides in the pure state, and it often happens that only a minimal quantity is available. Thus, biological titrations on larger animals or animals of a relatively high resistance to cardiotonic glycoside activity may be impossible.

Presuming that birds other than pigeons could be used to evaluate cardiotonic glycosides, attempts were made to use turtle-doves (*Streptopelia roseogrisea*) and sparrows (*Passer domesticus*). Since both the turtle-doves and especially the sparrows weigh less than pigeons, smaller quantities of the active materials have been thought to be needed.

The titrations on turtle-doves and sparrows were made by the method prescribed in the B.P. 1953 for pigeons, ignoring the sex of the birds. The solution to be examined was injected into sparrows by means of a micro-syringe connected to a plastic tube and intracutaneous canula. The weight of the turtle-doves varied between 133 and 205 g., whereas that of sparrows varied between 12·5 and 18·5 g.

Experiments were carried out with *Digitalis purpurea* from two different sources (Preparations A and B) on pigeons, turtle-doves and sparrows comparing the results with the Yugoslav Digitalis standard preparation. Results of these experiments are given in Table I. As can be seen, these were not significantly different for the three species used.

Titrations of the Yugoslav Digitalis standard preparation were repeated at intervals of 3–6 months to check the reproducibility of the results. These results are given in Table II.

* John Wyeth Research Fellow of the Empire Rheumatism Council.

As can be seen from Table II, it seems that the average lethal dose undergoes practically no changes during the stated period of time, the variations between the three results obtained on the same animal being not significantly different.

It is seen that the biological titration on turtle-doves can be made with smaller quantities of material than with pigeons, and the range of the results

TABLE I

TITRATION OF TWO COMMERCIAL PREPARATIONS

Preparation	Int. unit	Limits of error per cent (P = 0·05)	Number of animals used	
			Standard preparation	Sample
Pigeons A	10·8	91·9–108·8	12	6
B	12·9	90·9–110·1	12	6
Turtle-doves A	10·6	97·7–102·4	8	6
B	12·7	98·2–101·8	8	6
Sparrows A	10·3	86·0–116·2	8	6
B	10·6	85·5–117·9	8	6

TABLE II

DETERMINATION OF THE LD OF THE YUGOSLAV DIGITALIS STANDARD PREPARATION

	Number of animals	Mean of weights of animals in g.	Mean of log's of lethal doses	Σd^2	Coefficient of variation
Pigeons	12	273·5	1·22246	0·01609	3·13
	12	299·7	1·24510	0·21603	3·56
	12	284·7	1·20897	0·02011	3·54
Turtle-doves ..	6	150·8	1·23890	0·00727	3·08
	5	161·2	1·21965	0·00563	3·08
	7	183·1	1·21045	0·00933	3·26
Sparrows	9	17·2	1·12448	0·03188	5·61
	8	17·4	1·14281	0·15470	13·01
	8	16·9	1·11992	0·09001	10·11

was found to be even smaller than for pigeons. Turtle-doves are as easy to breed as pigeons.

Sparrows are relatively resistant to digitalis, and the concentration of the solution to be injected should be 3–4 times higher than for pigeons or turtle-doves. On the other hand, the weight of the sparrows being nearly 10 times smaller than that of the pigeons, the determination can be made with quantities of material so small that they can be eluted from a paper chromatogram.

Although the range of the results obtained is larger than that obtained with turtle-doves and pigeons, they can be considered satisfactory. Sparrows are not so easily bred as pigeons and turtle-doves, special conditions having to be established.

V. KUŠEVIĆ,
J. PETRIČIĆ.

Institute for the Control of Drugs,
Zagreb,
Yugoslavia.
July 18, 1960.