REVIEW ARTICLE

APPROACHES TO THE CHEMOTHERAPY OF VIRUS DISEASES

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Antibacterial Drugs and the Chemotherapy of Infections Caused by the Largest Viruses

Many years ago, Ehrlich laid what are now generally recognised as the foundations of chemotherapy, but before 1935 positive achievements were, by modern standards, relatively modest. Syphilis could be treated slowly with the arsenicals; trypanosomiasis had responded partially to the efforts of the synthetic chemist; and malaria was controlled by that gift of nature, quinine, while plasmoquin and mepacrine were recent therapeutic introductions. Domagk's discovery of the sulphonamides radically altered the situation. Even more important than their intrinsic therapeutic value, was their effect in directing chemical effort and research into the new and hitherto inadequately explored channel. When, a year or two later, Florey was able to develop an interesting laboratory phenomenon with *Penicillium notatum* into an outstandingly active therapeutic remedy of the lowest conceivable general toxicity, he instigated an additional vast amount of research into chemotherapeutic substances produced by living organisms. The result was an impressive degree of control over many bacterial infections achieved in a total period of little more than a single decade.

The chemotherapy of virus diseases is a more recalcitrant problem. With the exception of the largest viruses of the psittacosis-lymphogranuloma group, which some workers no longer recognise as true viruses, no practical means of influencing virus diseases by chemotherapy yet exists. This is not perhaps unexpected when we reflect on the differences between a typical bacterium and one of the smaller viruses. The bacterium is at all stages a self-contained system both morphologically and biochemically. It is always recognisable with the microscope as a discrete independent organism. Biochemically it is provided with a complicated series of enzymes which convert the nutrients it encounters into bacterial protoplasm. It is true that usually these nutrients must consist of relatively simple molecules provided by the terrestial environment or by the infected host, but within these limits bacteria possess more or less complete autonomy and retain completely their individuality. When infecting a mammalian host, many of them grow extracellularly in the tissue fluids.

On the other hand, viruses exhibit a much greater degree of dependence on the infected host. All are obligatory intracellular parasites and many of them are apparently devoid of the enzymatic complement necessary to carry out even the most simple metabolic processes. There is even evidence to suggest that at one stage in their replication some of them merge so completely with the substance of the infected cell as to be

undetectable by any means known to us. As Sir Patrick Laidlaw used to say, viruses "lead a borrowed life". This very intimate degree of parasitism would make it not wholly unreasonable to suppose that the future chemotherapy of virus diseases may be influenced in no slight degree by constitutional factors, nutritional or hormonal, in the host; indeed, as far as the experimental animal is concerned, this is more than mere conjecture.

The health of the animal may enter into intended chemotherapeutic experiments in a rather different context. It has long been accepted that the state of well-being or otherwise of an animal may modify its response to infection with a virus. A diseased or emaciated rabbit gives a poor skin reaction when infected intradermally with vaccinia or infectious myxomatosis. The event is no different if the sickly condition of the rabbit has been brought about by administering one of the more toxic products of the synthetic chemist, and more than one of the alleged antiviral effects recorded against a smaller virus probably comes into this category. In attempted chemotherapeutic experiments we must ensure that dosage of toxic chemical substances is within reasonable limits, and insufficient materially to affect the vitality of the animal, if we wish to avoid false positive results.

Nearly all the clinically recognised antibacterial drugs can be shown to be bacteriostatic or bactericidal *in vitro*. This is not the expression of any fundamental principle; rather does it reflect the manner in which the substances in question have been discovered. In the search for new antibacterials, the biologist has usually examined substances by determining their action on bacterial cultures *in vitro*, and has then tested the more highly active against diseases produced by these bacteria in some suitable experimental animal such as the mouse. Many substances active under *in vitro* conditions fail to surmount subsequent hurdles. Given orally, they may be destroyed in the alimentary canal or fail to be absorbed from it. After injection, they may cause such severe local irritation as obviously to be quite unacceptable in clinical practice. They may be too rapidly metabolised to attain effective concentrations in the tissue fluids at the site of bacterial multiplication, or they may be too generally toxic to be given in adequate amount. If the infecting organism is located in the central nervous system or in certain other sites, the drug may not pass the blood-brain or similar barrier, at least until infection is well established and a severe inflammatory reaction has altered the permeability of the barrier; normally substances of a definitely acidic character, e.g., penicillin, do not pass the blood-brain barrier. Thus relatively few of the substances active *in vitro* possess all the qualifications for systemic antibacterial action. To be useful in virus diseases, where so intimate a relation exists between the virus and the metabolic processes of the host-cell, a substance would need to possess still other properties. It would need to penetrate and to act intracellularly, and in such a manner as not seriously to interfere with the essential metabolic functions of the cells—unless it were able to render the cell insusceptible to infection, or to the effect of growth within it of the virus, both of which occurrences are theoretical possibilities.

Were the mouse test rather than an *in vitro* test the initial one in the search for new antibacterial drugs, it is possible that new remedies might be discovered having little or no *in vitro* activity. Indeed, it is a fact that the initial sulphonamide, prontosil rubrum, would never have been discovered by an *in vitro* test because it is not active as such but only after the sulphonamide moiety has been released by metabolism in the animal body. The number of substances showing *in vitro* activity against tubercle bacilli is very great, but few affect the disease in the mouse, so that with this infection it is usual nowadays to proceed directly to the mouse test in the search for new antituberculous remedies. Experimentally at least, three classes of substances are known to possess substantial antituberculous activity in the mouse with no or with very low activity *in vitro*: these are (i) a purine analogue, 7438[1], (ii) various surface-active polyoxyethylene ethers[2], and (iii) certain organic sulphur compounds derived from ethyl mercaptan[3]. None of these substances would have attracted notice as an antituberculous remedy by its activity *in vitro*.

Inactivation of Viruses in vitro

With viruses we find even less evidence of a correlation of *in vitro* and *in vivo* effects; indeed, the evidence available suggests that little or none exists. This fact is not perhaps surprising. Many antibacterial drugs act on the susceptible bacteria when these are in some particular phase of their cycle of growth: outside the living body viruses are in a state of suspended animation and perhaps only susceptible to crude chemical influences which grossly disrupt their substance. Thus *in vitro* they are inactivated by acids and alkalies, by oxidising agents such as hydrogen peroxide, potassium permanganate, hypochlorite or even atmospheric oxygen, by general protoplasmic poisons, such as phenol, mercuric chloride and formalin, and by some surface-active agents, such as sodium lauryl sulphate, bile salts and saponin. Many of these substances act efficiently only at relatively high concentrations, but iodine and other halogens are remarkably destructive to the influenza virus[4] and inactivate it almost instantaneously at an atmospheric concentration of around one in ten million, provided that the virus is in the form of a wet mist; if the virus is dried rather higher concentrations of iodine and longer exposure are necessary. Similarly, viruses are susceptible to a variety of physical influences such as ultra-violet and X-ray irradiation, ultrapressure, etc. None of the chemical substances mentioned, nor a great number of others capable of inactivating viruses *in vitro* has any action against virus diseases in the experimental animal. However, the chemical or physical inactivation of viruses *in vitro* is of some interest in two connections which have nothing directly to do with chemotherapy.

One point concerns the disinfection of contaminated material. Some years ago we examined a number of modern surface-active agents and antibacterial substances such as the quaternary ammonium compounds and chlorhexidine (Hibitane) for power to inactivate viruses. To one of the fairly large viruses, that of herpes febrilis, an anionic detergent,

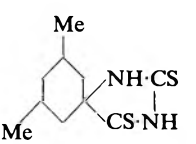
sodium cetyl-oleyl sulphate, was moderately destructive; a non-ionic detergent and the other substances were without effect. None of the substances tested inactivated two of the smallest viruses, those of mouse poliomyelitis and Rift Valley fever, at concentrations which would be at all economic to use in practice. Sodium hypochlorite, on the other hand, was exceedingly potent in this respect and superior in every way to the other compounds examined. Ultra-violet light and some chemical substances mentioned below have been used to destroy in transfusion plasma or blood the agent of infectious jaundice, with somewhat inconsistent results. The virus is apparently one of the more resistant ones, and one of the difficulties is to give an adequate exposure to the inactivating agent without causing undesirable chemical changes in the serum proteins[5–7].

Secondly, and of some topical interest at the present moment, is the use of chemical inactivating agents for the production of viral vaccines. In the past, substances such as phenol and formaldehyde have been employed almost exclusively for this purpose. They suffer from the great disadvantage of being relatively non-specific in their action and of seriously damaging all the viral proteins, including the antigenic groupings needed to stimulate antibody formation. The multiplication of viruses depends on their nucleoprotein component, and the fact that substances like phenol and formaldehyde have been used with undoubted partial success, and have apparently inactivated virus without destroying all antigenicity, may have resulted from the relatively greater lability of nucleoproteins. However this may be, it has long been recognised that to use formalin successfully in the production of a viral vaccine, the inactivating concentration is rather critical. If it is too high the vaccine loses its antigenic potency; on the other hand too low a concentration, permitting survival of living virus, presents obvious dangers. For many years discussion continued on whether a completely killed viral vaccine was, in fact, able to excite immunity, and recent American experience with the Salk poliomyelitis vaccine has underlined the great care that must be taken if disastrous events are not to follow vaccination with a formalinised vaccine. In short, it would seem that the use of formaldehyde for vaccine production is far from ideal. Only recently, however, have attempts been made to utilise some of the newer chemical substances which have much greater relative affinity for nucleoproteins, and which might, therefore, be expected to abolish infectivity while retaining unimpaired the antigenicity of the virus. Such substances are the nitrogen mustards, β-propiolactone and acetylethyleneimine. On chemical grounds the mustards are, in theory, less satisfactory than the others. All these substances are mutagenic agents and are carcinogenic, and in large doses produce agranulocytosis and radiometic effects, but as any unreacted material can readily be removed from the vaccine there need be no apprehension on this score. Our own recent experiments and those of workers in America[8–10], have suggested that vaccines produced by the agency of these substances are antigenically superior to formalinised vaccine, while the certainty of really killing the virus is guaranteed.

Large Viruses. Psittacosis-lymphogranuloma Group

Inactivation in vivo. So much for *in vitro* effects on viruses. From the point of view of chemotherapy, animal viruses may be divided into two groups—a smaller more or less effectively controlled by a number of existing remedies, and a larger for which, as yet, there exist only hints of ultimate therapeutic measures. The former includes the largest viruses of the psittacosis-lymphogranuloma group, the latter the majority of viruses.

The psittacosis-lymphogranuloma group of viruses includes the agents of psittacosis and ornithosis, lymphogranuloma venereum, trachoma and inclusion blennorrhoea together with a rather larger number of viruses naturally infecting laboratory or domestic animals. From 1938 onwards it has been known that the sulphonamides are of benefit in human cases of lymphogranuloma. Experimentally quite a number of other synthetic chemicals substances, e.g., the nitroacridines and the quinoxaline -1 : 4-oxides, have been found to show activity, but as one by one they have been discovered they have been completely overshadowed by successive emergence of new and better antibiotics, so that at the present moment the antibiotics hold the field. For the most part, the substances active against the largest viruses are also antibacterial, though we have knowledge of at least one substance, a spirothiohydantoin (20,065) (I), which is reasonably highly active against psittacosis in the chick embryo and mouse, yet is wholly devoid of antibacterial activity *in vitro* or *in vivo*. In a second series of chemical substances under examination at the moment, as the molecular structure is changed by addition or removal of various substituents, antibacterial and antiviral activity move in opposite directions, so that it is by no means invariable to find both activities in a single substance.



(I) 20,065 (*cis* α-form)

When the literature is consulted to find which of the numerous remedies available is the one of choice in a particular instance, there is far from complete agreement. This is partly because, like bacteria, the different viruses vary among themselves in their susceptibility to the various remedies, and with two distinct remedies are not necessarily arranged in the same order of susceptibility. Thus mouse pneumonitis responds well to the sulphonamides and poorly to the nitroacridines, while feline pneumonitis, meningopneumonitis and psittacosis viruses do exactly the opposite. Lymphogranuloma responds both to the sulphonamides and to some nitro compounds. Sulphonamides act on two American strains, 6BC and Gleason, of psittacosis virus, and on rare strains met with elsewhere[11], but they are useless against most strains of psittacosis isolated in America, Europe or Australia. A second difficulty arises in assessing the value of treatment in human disease, and especially in a disease such as trachoma in which the individual severity varies greatly in different parts of the world and there is no convenient animal for laboratory study. Finally, no fully comprehensive comparison of the whole range of modern antibiotics has been undertaken at one time and by the same techniques against the viruses pathogenic for man which can easily be worked with

in the experimental animal. The advantages and the limitations experienced by the experimentalist in this kind of study are best illustrated by presenting some of the results we have obtained with the virus of psittacosis.

Psittacosis Virus. Chick Embryo and Mouse Experiments

The data on eggs were obtained in three different experiments in which the virus was injected into the yolk-sac of groups of 12 or more 7-day old embryos and followed $2\frac{1}{2}$ hours later by antibiotics. The results of three experiments were virtually identical and have been combined in Table I;

TABLE I

THERAPY OF PSITTACOSIS IN THE CHICK EMBRYO

Antibiotic	Dose mg./egg	Per cent survivors	Mean period of survival in days*
Tetracycline (Achromycin)	5·0	79·2	13·5
	1·0	91·7	13·8
	0·2	79·2	13·5
	0·04	41·7	10·6
	0·008	0	7·6
Oxytetracycline (Terramycin)	5·0	75·0	13·9
	1·0	75·0	13·2
	0·2	83·3	13·3
	0·04	66·7	13·1
	0·008	0	6·6
Chlortetracycline (Aureomycin)	5·0	83·3	13·4
	1·0	79·2	13·5
	0·2	0	7·9
	0·04	0	5·5
Erythromycin (Ilotycin)	5·0	37·5	12·2
	1·0	91·7	13·7
	0·2	91·7	13·9
	0·04	33·3	12·3
	0·008	0	5·9
Penicillin	1·0	20·8	12·8
	0·2	0	10·1
	0·04	0	7·3
Chloramphenicol (Chloromycetin)	2·5	4·2	7·6
	1·0	0	9·3
	0·2	0	5·6
	0·04	0	5·0
Carbomycin (Magnamycin)	10·0	20·8	7·6
	2·0	41·7	9·4
	0·4	0	6·2
	0·08	0	5·7
None	—	0	5·0

* Eggs surviving to the time of hatching (13th day after virus) were deemed to have survived for one additional day.

they showed that, in treating yolk-sac infections with the virus of psittacosis, chlortetracycline (aureomycin) is definitely inferior to the other two tetracyclines, as we found also on a previous occasion[12]. The same has been reported for other organisms of the group[13,14]. Erythromycin is equally as good as the better tetracyclines, sodium penicillin and carbomycin are much less effective with about the same degree of activity, and chloramphenicol is relatively ineffective. The best results are not always obtained with the largest dose, a fact attributable to toxicity from excess of the antibiotic.

Four experiments were made in groups of 10 or more mice infected intraperitoneally with psittacosis (Table II). After inoculation the animals remained untreated for 48 hours to allow the infection to become established and were then dosed orally twice daily for 10 days, except with procaine-penicillin where a massive dose of the antibiotic was given subcutaneously once every third day, beginning 48 hours after virus. As

TABLE II

THERAPY OF PSITTACOSIS IN THE MOUSE

Antibiotic	Dose mg./20 g. twice daily	Per cent survivors
Tetracycline (Achromycin)	10·0	100
	1·0	100
	0·1	98 (7·4)†
	0·01	13 (7·4)†
	0·001	5 (6·7)†
Oxytetracycline (Terramycin)	10·0*	100
	1·0	100
	0·1	100†
	0·01	13 (7·6)†
	0·001	0 (6·1)
Chlortetracycline (Aureomycin)	10·0*	100
	1·0	100
	0·1	100
	0·01	95 (7·5)†
	0·001	18 (6·4)†
Erythromycin (Ilotycin)	10·0	100
	2·0	100
	0·4	93 (12·5)†
	0·1	10 (7·1)†
Procaine-penicillin	See text	100
Chloramphenicol (Chloromycetin)	10·0	100†
	2·0	90 (18·0)†
	0·4	65 (13·8)†
	0·1	3 (6·2)†
Carbomycin (Magnamycin)	10·0	100
	2·0	45 (11·2)†
	0·4	—
	0·1	5 (6·8)†
None	—	3 (6·2)†

The figures in parentheses are the mean periods of survival in days of mice ultimately dying.
* Signs of toxicity of the drug.
† Surviving mice had shown signs of infection.

we found in a similar experiment some years ago, in the mouse, chlortetracycline is the most active of the tetracyclines, thus reversing the position in the chick embryo and showing how difficult it is from work in one host to predict the effect in another. If, in spite of this, one were pressed to predict activity for man, our cumulative experience over the years would suggest that it is better to rely upon the results in the mouse rather than those in the egg. In the mouse, erythromycin also is relatively less active than in the egg and is very much inferior to chlortetracycline. Chloramphenicol and carbomycin are still less active. A massive dose of procaine-penicillin given as mentioned above will protect 100 per cent of the mice, but the doses used (30,000 units/mouse) are enormous relative to those employed in man, and smaller doses are not nearly as effective. Penicillin is also useless if virus is given intracerebrally because it does not

penetrate the blood-brain barrier, whereas under these conditions the tetracyclines are still highly active. To place these results in their fullest perspective, it should be added that against the virus used in this work, sulphadiazine or sulphamezathine possess little, if any, activity, while the most active nitroacridines have a moderate action[15–17], and quinoxaline-1:4-oxides a very considerable action[18] approaching that of the better antibiotics.

For some of the antibiotics we have prepared growth curves of the virus in treated and untreated animals. Groups of six mice were killed at various times after intraperitoneal infection, the spleens pooled and the amount of virus present estimated by titration in mice. In these experiments treatment started at 4 hours before infection with virus so as to obtain the maximum effect possible. The results indicated very considerably reduced multiplication of virus during the period of therapy in animals treated with chlortetracycline and with penicillin, whereas chloramphenicol appears only to delay virus in reaching a high titre[12].

By contrast, *in vitro* these antibiotics have seldom been found to have any effect on the viruses susceptible to their action *in vivo*; at least this holds with reasonably large doses. Using very high concentrations of penicillin, Moulder and his colleagues[19] found that the antibiotic forms a complex with the virus of feline pneumonitis with resulting loss of infectivity, but as this effect was partly reversible by treatment with penicillinase it cannot represent a true inactivation of virus.

The effect of treatment with penicillin can also be visualised in histological sections of the yolk-sac stained with Giemsa's solution or other suitable stains[18,20]. The normal colonies of tiny basophilic particles which represent the actual virus are replaced by large, deeply-staining plaques measuring up to 6 μ in diameter. In the presence of penicillin the virus particles apparently continue to grow indefinitely without subdividing as they do in the untreated egg. In the treated eggs, moreover, the number of cells infected with virus is much lower than in the untreated controls.

In spite of the excellent clinical results and the frequent failure to detect virus during the later stages of the period of therapy, none of these antibiotics certainly eradicates the virus. After treatment has ceased, virus frequently reappears and many mice carry virus for months, though they do not infect other mice placed in the same cage. In the experiments described, the spleens were removed from the treated mice and passed individually to fresh mice on the 40th day after infection, that is to say, 28 days after the close of treatment. At this time all the surviving mice looked perfectly well; nevertheless the majority carried virus (Table III). We believe that it is the immunity engendered during the period when viral growth is controlled by therapy which prevents unrestricted growth of virus after drug is discontinued. On many occasions we have noted that penicillin gives lower carrier rates than do the other antibiotics and it may be that tetracycline is less satisfactory in this respect than are chlortetracycline and oxytetracycline (terramycin).

Similar behaviour is, of course, known in man. Meyer and Eddie[21] have described a case of psittacosis in which virus was still present in the

sputum 10 years after infection, in spite of an intervening course of penicillin therapy. Recently, however, by very heavy and prolonged parenteral dosage with the tetracyclines, both Schmidt and von Sprockhoff[22] and Meyer and Eddie[23] have succeeded in freeing animals from virus. The dosage required is relatively enormous by the standards of human therapy yielding excellent clinical results, and these doses may have to be continued for up to 25 days.

TABLE III

CARRIERS OF PSITTACOSIS VIRUS AFTER TREATMENT WITH ANTIBIOTICS

Antibiotic	Dose mg./20 g. twice daily	Per cent carriers
Tetracycline (Achromycin)	10·0	80
	1·0–0·1	94
	<0·1	100
Oxytetracycline (Terramycin)	10·0	60
	1·0–0·1	86
	<0·1	100
Chlortetracycline (Aureomycin)	10·0	50
	1·0–0·1	86
	<0·1	100
Erythromycin (Ilotycin)	10·0–2·0	87
	<2·0	93
Procaine-penicillin	See text	45
Chloramphenicol (Chloromycetin)	10·0	72
	2·0–0·4	83

Regarding the possibility of drug-resistance, the available evidence is reassuring. It is true that a few resistant strains of lymphogranuloma have been isolated from patients treated with the sulphonamides and that similar resistance can be produced fairly easily experimentally. But no strains similarly made resistant to the antibiotics have been reported from the clinics, while under experimental conditions, most workers, including ourselves, have not succeeded in obtaining drug resistance in mice to penicillin or to chlortetracycline. Moulder and his colleagues[24] in the chick embryo succeeded after many passages in the presence of increasing amounts of penicillin in producing a strain of feline pneumonitis resistant to the drug, but the process does not appear to have been an easy one and it seems rather unlikely that under the conditions obtaining in human medicine, drug-resistance of the larger viruses to the antibiotics will prove a serious problem.

Status of Therapy in Man

The results of therapy in man are in fair agreement with those which have been reported in the experimental animal. As we have seen, the partial efficacy of the sulphonamides in lymphogranuloma venereum was first noted in clinical practice, and only later applied to the mouse and the chick embryo. Penicillin has been reported to be active, but apparently has seldom been used in this disease. There seems to be little doubt that the tetracyclines are the antibiotics of choice. This is undoubtedly true

also of psittacosis: against most strains of virus the sulphonamides are ineffective, and the reports of treatment with penicillin have been mixed. In the presumed virus disease or diseases passing under the name primary atypical pneumonia, therapeutic effects have been claimed with chloramphenicol or the tetracyclines after therapy with sulphadiazine or penicillin has failed. It is when we come to trachoma that opinions differ to a greater extent. By various observers, the sulphonamides have been considered ineffective in treatment, as useful adjuncts to therapy either in dealing with secondary infections or in supplementing other measures, or as curative agents in themselves. One American ophthalmologist of considerable experience[25] even maintains that they still constitute the first therapeutic choice and are demonstrably superior to the antibiotics. Penicillin certainly does not seen to have established itself in the treatment of this disease, but most recent reports suggest that once again the tetracyclines are superior to other agents, including chloramphenicol. An important limitation to their usefulness is that they are too expensive to be distributed widely in the impecunious countries in which trachoma is rampant. The extent of the need for a cheap synthetic remedy for trachoma is emphasised by facts such as the following—8 of 10 Egyptians are said to suffer from trachoma or its effects—millions of man-days are lost annually in Tunisia from trachoma—trachoma is the commonest cause of blindness in the British colonial territories and currently accounts for over 80,000 cases. Thus trachoma continues to be one of the most important targets for chemotherapy in the field of virus diseases.

Problems of the Chemotherapy of Diseases Caused by the Smaller Viruses

With diminishing size it is obvious that the viruses smaller than those of the psittacosis-lymphogranuloma group must decrease progressively in structural complexity. By implication they must be increasingly dependent on the cell which harbours them for supplying the essentials for their reproduction. Vaccinia virus, with a diameter of about 250 mμ, has been said to have a fairly complicated structure under the electron microscope, and to contain when purified various enzymes, a co-enzyme and other substances. It is not at all certain, however, whether some of these constituents may not have been acquired during or after the process of separation from the host-cell. Influenza virus (125 mμ), which for technical reasons has been most studied in this connection, is certainly a rather complicated affair. Relatively little attention has been devoted to the still smaller viruses, though all that have been purified have been shown to contain protein and nucleic acid, the latter usually ribosenucleic acid. It is generally believed that it is the nucleic acid which is essential for reproduction of the virus.

The Eclipse Phenomenon. Influenza Virus

When many of the smaller viruses infect a susceptible tissue, a rather characteristic phenomenon ensues. Within a very short time, sometimes only a few minutes, of introduction of a suitable infecting dose, infective

virus drops sharply in amount and there follows a period of so-called eclipse during which little or none of the original virus can be recovered. After a certain time virus reappears and its concentration rapidly increases. One could imagine several reasons for this disappearance of virus. For example, much of it might be adsorbed or be inactivated by the defences of the host, leaving an undetectable amount to survive and initiate infection, and indeed since the phenomenon was first discovered, opinions regarding its significance have differed widely. No-one apparently has ever published what may be an important observation—namely of the fate of a given virus introduced into sites in which it is, respectively, capable and incapable of growth. Some years ago we performed one such experiment. Using influenza virus which grows readily in the mouse lung but not in the mouse testicle, we determined the infectivity of these tissues at intervals after introduction of virus. Within a few minutes the virus introduced into the lung fell to a level below that necessary to infect other mice intranasally, that is to say it suffered the phenomenon of eclipse—that in the testis disappeared rather slowly over about 46–60 hours without nearly as marked an immediate drop in infectivity. This of course suggested that eclipse of the virus is a phenomenon intimately associated with its multiplication. Unfortunately, we have not yet had the opportunity of confirming this observation. In a stimulating if admittedly speculative article, Bauer in 1949[26] summarised the then current knowledge of viral multiplication, and concluded that on entering a susceptible cell the virus undergoes dissolution, diffuses over a considerable area of the cytoplasm, and then, by some means or other, induces the enzymes of the cell to manufacture viral material instead of normal cellular material. Subsequent observations with at least one virus have given considerable support to this general thesis.

The studies of Hoyle and others over the last few years have provided a great deal of evidence for what happens to the influenza virus during the period of eclipse. The virus of influenza possesses a number of properties by which it may be recognised. Among these are the following:

1. The infective particle has a diameter of 125 mμ, or 1250 Å. 2. The virus is capable of attaching itself to receptor sites on the surface of the red blood corpuscles of various species and of causing these red blood corpusles to become agglutinated. 3. While the virus is attached to the red blood corpuscles, an enzyme, a mucinase, which is incorporated in the viral particles, destroys the receptor substance necessary for attachment of the virus. In consequence the virus parts company with the red blood corpuscles and these cease to be agglutinated. 4. The virus contains specific and group complement-fixing antigens.

After shaking a suspension of influenza virus with ether, the virus disintegrates, according to Hoyle[27] from disruption of a lipoid-containing surface membrane derived from the host-cell which previously harboured the virus. The suspension now is no longer infective and the particles present are much smaller than the intact virus and are mainly about 120 and 250 Å in diameter. However, some of the properties of the original virus remain. The haemagglutinin titre is higher than before. The

specific complement-fixing antigen has gone, so that Hoyle concluded that it represents the part of the virus derived from the host-cell. The group antigen persists and by suitable means can be separated from the haemagglutinin. The two activities appear to be associated with separate particles. The group, or so-called "soluble" antigen is a particle 120 Å in diameter, with the properties of a ribonucleoprotein; it contains all the nucleic acid of the virus. The agglutinin behaves like a mucoprotein with enzymatic properties. Current information of the composition of the influenza virus is summarised in Table IV.

TABLE IV

COMPOSITION OF THE INFLUENZA VIRUS

INFLUENZA VIRUS 1250 Å			
"Soluble" antigen	120 Å	14 per cent	Ribonucleoprotein (M.W. 600,000) containing 5·3 per cent ribonucleic acid—70 molecules
Haemagglutinin	120 Å or more	14 per cent	Mucoprotein with 4·2 per cent polysaccharide— 70 particles
Lipid .. .. Carbohydrate .. Protein	— — —	29-36 per cent 3 per cent 34 per cent	Derived mainly from host-cell

Inside the infected cell viral material can be sought for by several techniques, all of which have been used by one or other investigator. The virus may be labelled with radioactive phosphorus[28], it may be looked for with the electron microscope, or the antigenic material may be demonstrated by the application of antibody which has been rendered fluorescent. By labelling influenza virus with radiophosphorus, Hoyle and Frisch-Niggemeyer[29,30] were able to show that on entry into a susceptible cell much the same happens to the virus as when it is shaken with ether. Almost immediately infectivity is lost, and the virus particle as such disintegrates; at this stage and for some considerable time later, the electron microscope does not reveal any particles resembling virus in the infected cells[31]. About a quarter of the total radiophosphorus in the virus used for infection is contained in the phospholipid of the viral membrane, and this fraction is now recovered in the form of compounds of low molecular weight, not precipitated with protein precipitants, and not sedimented at a centrifugal force of 100,000 g.; they appear to be metabolic products taking part in the general metabolism of the cell, and incidentally reflect the very rapid and active metabolism which occurs at the cellular level. The remainder of the radiophosphorus incorporated in the virus is contained in the ribonucleoprotein fraction, and this is now recovered partly as free nucleic acid, and partly in association with the desoxyribonucleoprotein of the nuclei of the infected cell. Fluorescent antibody techniques have shown that new viral antigen appears first in close association with the nuclei[32,33]. The suggestion is that viral ribonucleoprotein breaks down to liberate free nucleic acid, which then enters into close relation with the nuclear material of the infected cell and somehow determines a changeover from synthesis of cellular ribonucleoprotein

to viral ribonucleoprotein; the two nucleoproteins differ considerably in the relative proportions of pyrimidine and purine bases which they contain.

When virus is about to reappear in the infected cell, it is preceded by smaller particles having the properties of the soluble antigen, and rather later by haemagglutinating activity[34,35]. Only at the very end of the process do these particles become associated with a lipoprotein envelope derived from infected cell, and does an infective particle of the size and with the properties of the complete influenza virus emerge from the cell surface.

Other Small Viruses

Information about the multiplication of other small animal viruses is much less complete, and it is not certain that the mechanism of replication is precisely the same in all instances, or even that all authorities would agree with the above interpretation of the facts about the influenza virus. However, it seems very likely that in essentials the process is similar. The recent interesting work with tobacco mosaic virus of plants shows that a non-infective protein fraction consists of little hollow rods into which, under suitable conditions, the nucleic acid can slip, so to speak, to form the infective virus[36]. Other artificial and natural ribonucleotide polymers can substitute for the nucleic acid, but the particles formed are not then infective[37]. With some bacterial viruses, it seems that the protein tail is responsible for uniting the virus to the susceptible bacterium and acts as a kind of hypodermic syringe for introducing the nucleic acid which is the component essential for multiplication[38].

To summarise, present evidence suggests that the nucleic acid is the essential component of viruses by means of which they gain control over the metabolic processes of the host-cell, and induce them to synthesise more viral nucleic acid thus ensuring their own survival. This essential requirement met, the new viral particle may be composed to quite a considerable extent of more or less unchanged material derived from the host-cell.

We thus reach the point of regarding multiplication of many of the smaller viruses as an exercise in nucleic acid synthesis. Nucleic acids are polymers of high molecular weight built up from purine and pyrimidine basis like adenine, guanine, cytosine, or uracil, a sugar which is either D-ribose or 2-deoxy-D-ribose, and phosphate. The manner in which these units are associated one with another, so as to create a structure capable of ensuring its own replication, is not yet certain—a convenient review of nucleoproteins is that of Markham and Smith[39]. However, our present knowledge falls far short of that needed intelligently to intervene in the process as it occurs in the mammalian host. The absence of this knowledge has not deterred workers from following certain lines of investigation in the hope of obtaining chemotherapeutic leads.

Chemotherapeutic Approach with Metabolic Analogues

Following a current trend in chemotherapy, various workers have sought to influence the course of events by the use of analogues of substances essential for cellular metabolism. In plants, with bacteriophages

and in animal tissue-cultures, the growth of viruses has been influenced by concentrations of chemical substances well below those seriously toxic to the cells. Thus the guanine analogue 8-azoguanine may modify infection with certain viruses of plants; it is actually incorporated into the viral nucleoprotein, production of virus is delayed, and the analogue-containing virus is less highly infective than is normal virus[40]. 2-Thiouracil acts in other virus diseases of plants. 2:6-Diaminopurine inhibits the growth of vaccinia and other viruses in animal tissue-cultures. Amino acids and their analogues have been examined in great variety. Methionine has been shown to be essential for the growth of several animal viruses, the multiplication of which may be inhibited by ethionine. Certain α-aminosulphonic acids were found to act exclusively on the earlier stages of multiplication of influenza virus, whereas DL-methoxinine affects a later stage just before the appearance of mature virus[41]. Analogues of vitamins have similarly been studied. Oxythiamine and desoxypyridoxine inhibit the growth of mumps and influenza viruses in tissue-culture. Tamm and his colleagues[42–44] have examined many derivatives of benzimidazole, which is a fragment of the vitamin B_{12} molecule, and by appropriate synthesis have increased their activity to a point several hundred times removed from that of the starting material. Both the 5:6-dichloro-ribofuranosylbenzimidazole and the 4:5:6-trichloro compound possess marked antiviral activity in tissue-culture. These are but a few selections from a much larger number of similar observations.

While studies such as these are providing valuable basic information on the requirements for viral growth in tissue-culture, none of the substances examined so far appears to have any useful activity in the experimental animal, and so to be of practical value as far as chemotherapy is concerned. Indeed, even results obtained in the chick embryo are unreliable from this point of view. Thus, my former colleague, Mr. J. Francis, examined many hundreds of compounds for activity against the virus of fowl-pox by injecting them into the yolk-sac of chick embryos which had received virus on the chorio-allantoic membrane. He found that a little more than a score possessed significant activity in reducing the lesions of the disease. When tested against fowl-pox in the hatched bird, however, none of these produced the slightest effect. In our own experience the only frequent correlation of results in the chick embryo and in the mouse has been with the largest viruses of the psittacosis-lymphogranuloma group; even here the correlation is not invariable and results produced in the egg may fail to be repeated in the mouse. In consequence, some workers have felt that the quickest route to chemotherapy may be the empirical screening of chemical substances directly in the mouse or other small experimental animal. Several small series of results have been published, dealing in all with a few hundred compounds, but it is virtually certain that the total examined in one or other commercial laboratory amounts to many thousands. As far as I am aware, these efforts also have been wholely unsuccessful in leading to the development of a practical remedy. Nevertheless, in the course of investigations devoted to other ends, one or two antiviral effects have been noted in the

experimental animal, suggesting that the problem of influencing diseases caused by the smallest viruses is not completely insoluble. We may perhaps consider a few of the more marked of these effects.

Other Observed Antiviral Chemotherapeutic Substances in Animals

Horsfall and McCarty[45] and Ginsberg and Horsfall[46,47] observed that polysaccharides derived from various bacterial species, and notably a capsular polysaccharide of Friedländer's bacillus, type B, are capable of lessening the severity of lesions and of restricting viral growth in infections with the pneumonia virus of mice. The polysaccharide is effective when given intranasally during the first two-thirds of the cycle of growth of the virus, that is to say, within the first ten hours, but not when given at twelve hours or later. However, the compound given late may inhibit the second or subsequent cycles of growth, so that a single intranasal dose of 0·02 mg. given two or three days after infection permits the animal to recover completely when all the untreated controls die. Immune serum given at a similarly late stage fails to save the animal. For this reason the polysaccharide was considered to act not on the mature viral particle but on some relatively late stage of viral synthesis by the host cell. The polysaccharide was completely inert against influenza viruses in the mouse, although these viruses are believed to grow in the same cells and produce apparently identical lesions as does the pneumonia virus of mice. This fact was taken as indication of different metabolic paths to the formation of the two types of virus.

Secondly, there are the thiosemicarbazones and phenoxythiouracils. A number of American workers[48–50] have demonstrated their activity against vaccinial infections in mice, but not against several other viruses. The compounds are not active against vaccinia in the rabbit. Some thiosemicarbazones, of course, are active in tuberculosis. When a range of these substances was examined, the antiviral activity was found not to run parallel with the antituberculosis. Working independently, Bauer[51] has reported on many of these substances and considers that isatin thiosemicarbazone is much more active than previously claimed. He found that in the presence of the drug the amount of virus needed to kill 50 per cent of the mice was four or more log units greater than in untreated controls: this he describes as a 99·99 per cent protection. 5-(2:4-Dichlorophenoxy)thiouracil is less active but acts synergically with the thiosemicarbazone, as do also some other phenoxypyrimidines even when themselves devoid of appreciable antiviral activity. We should note that these treatments do not by any means prevent the growth of virus in the mice, but merely restrict it to a level insufficient to produce clinical symptoms and death. The degree of multiplication needed to produce symptoms is considerable, and when the titres reached by virus in treated animals are taken as the criterion of antiviral activity, the results are perhaps less impressive than the figures for mortality would suggest.

The third example is of an antibiotic, helenine, which was isolated by Shope[52] from a culture of *Penicillium funiculosum*, and of an apparently similar substance described by Powell and his colleagues[53]. Given near

the time of infection, these substances prolong survival or decrease mortality in infections with a number of neurotropic viruses, including poliomyelitis in the monkey[54,55].

Antiviral Action of Mepacrine

Fourthly there is the antiviral action of mepacrine. Following a line of investigation which began with the observation in America of slight antiviral activity on the part of trypan red[56], we observed that mepacrine, the antimalarial drug belonging to the chemical class of acridines, possesses very marked protective activity against a few viruses, of which equine encephalomyelitis, Rift Valley fever and louping-ill are the chief. It has no action against the majority of viruses[57,58]. It will be sufficient here to mention the results obtained in one infection—equine encephalomyelitis.

TABLE V

ANTIVIRAL EFFECT OF MEPACRINE IN EQUINE ENCAPHALOMYELITIS

A single oral dose of 10 mg. was given at various times relative to the infecting dose of virus

Time	Deaths in 20 mice
48 hr. before virus	4 (9·0)
24 hr. " "	2 (11·1)
4 hr. " "	1 (12·0)
4 hr. after virus	6 (7·3)
24 hr. " "	11 (5·3)
48 hr. " "	16 (4·6)
No drug	19 (4·7)

TABLE VI

ANTIVIRAL EFFECT OF MEPACRINE IN EQUINE ENCEPHALOMYELITIS

A single oral dose of 10 mg. was given 24 hours before various doses of virus

Virus	Deaths in 20 mice	
	Treated	Control
10^{-3}	2 (6·0)	19 (4·4)
10^{-4}	2 (11·3)	20 (4·6)
10^{-5}	3 (14·7)	16 (4·6)
10^{-6}	0	6 (10·3)

The figures in parentheses are the mean periods of survival in days of animals ultimately dying.

Normally, this infection is transmitted by the bites of mosquitoes. If virus is introduced subcutaneously or intramuscularly into the experimental animal, it multiplies greatly outside the nervous tissue and appears in the blood in high titre without producing definite signs of infection. One of two things may then happen—uneventful recovery from this inapparent infection, or invasion of the nervous system and death. In the mouse, by using sufficiently young animals the latter event may be brought about in nearly 100 per cent of cases. If now even a single oral dose of mepacrine be given before virus, or soon after virus during the stage before invasion of the central nervous system has taken place, a very considerable number of animals is protected even when large infecting doses of virus are used (Tables V and VI). Mepacrine has no action whatsoever on the virus *in vitro*. It also has no effect against equine encephalomyelitis in the chick embryo, so that its action would never have been discovered but for the test in the mouse.

The action of mepacrine is a true antiviral one because titration of virus in the blood and organs of treated mice shows that growth of virus is wholely or partly suppressed, according to the dose administered (Fig. 1). After dosing with mepacrine, there appear in the cells of many organs deposits of tiny basophilic granules which stain blue with Giemsa's

solution. When unstained they are yellow, and they fluoresce in ultra-violet light; in other words, they have the general properties of an acridine and are presumably a metabolite of mepacrine[59]. They are particularly heavy in the cells of the reticulo-endothelial system, where of course trypan red also accumulates, and we have found that a number of high-molecular-weight polymers of very varied chemical nature, which after intravenous inoculation accumulate in the reticulo-endothelial system, also have some protective action in equine encephalomyelitis, though nothing like that of mepacrine. In other words the antiviral action of mepacrine appears somehow to be connected with the reticulo-endothelial system.

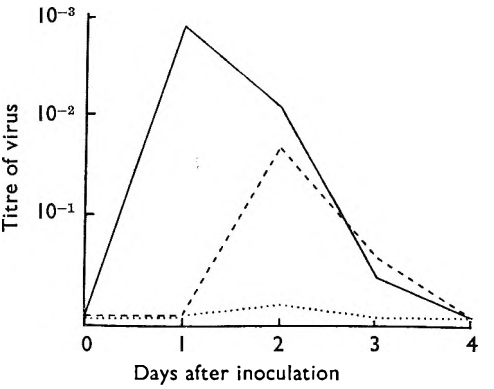
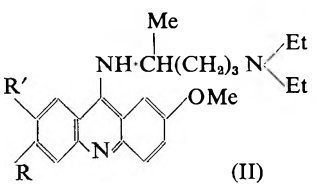


FIG. 1. The titration of equine encephalomyelitis virus in the blood of infected mice treated with two differing doses of mepacrine. The growth of virus is suppressed according to the dose given:

—— No treatment; - - - mepacrine 4 mg.; .... mepacrine 10 mg.

A number of acridines other than mepacrine have considerable antiviral activity[16], and all the most active produce basophilic granules. One of these is a nitro-acridine, and as has been seen some nitro-acridines are active against the large viruses. However, this particular one was not active against psittacosis or lymphogranuloma; in fact, we have not yet observed activity against both classes of virus in one and the same compound. How completely unpredictable these activities are may be seen from the results of varying the substituents R and R′ on formula II which is an acridine having the same basic side-chain and methoxy group. Compound II (R = Cl; R′ = H is mepacrine, which is active against equine encephalomyelitis in the mouse, but is not active against psittacosis or lymphogranuloma. Substitution of chlorine by a nitro group (II, R = NO_2; R′ = H) removes all activity for equine encephalomyelitis, but this compound is active against psittacosis and lymphogranuloma. When the nitro group is moved from the 6 to the 7 position in the acridine ring (II, R = H; R′ = NO_2) activity is regained against equine encephalomyelitis and lost against psittacosis and lymphogranuloma. None of these three compounds has any activity against viruses *in vitro*. When the nitro group at position 7 is replaced by an amino group (II, R = H; R′ = NH_2) all therapeutic activity against both large and small viruses is removed. The new compound, however, is remarkably active against all viruses *in vitro* and inactivates them in a very short time.



(II)

Metabolites of Mepacrine

Mepacrine also shows an effect against equine encephalomyelitis in the adolescent rat, but not in the guinea pig, the rabbit, the chicken or the monkey. When a drug is found to be active in one animal species but not in another, it often indicates that the metabolism of the drug differs in the several animal species, and that the therapeutic action is due wholely or principally not to the drug administered but to a metabolite of it. My colleagues and I have spent, and are spending, a great deal of time in trying to find an active metabolite of mepacrine—which might then be expected to be active in other animal species—so far without success. Of the total acridine present in the livers of mice dosed with mepacrine, about 94 per cent is unchanged drug[60]. The basophilic granules have been shown by suitable experiments not to possess appreciable therapeutic activity. However, several biological observations suggest that metabolism somehow comes into the picture.

TABLE VII

EFFECT OF SEX ON THE ACTION OF MEPACRINE IN EQUINE ENCEPHALOMYELITIS

Dose of mepacrine	Deaths in 30 mice	
	Male	Female
None	28 (5·2)	30 (5·8)
2 mg./20 g.	27 (5·9)	16 (6·8)
4 mg./20 g.	9 (7·2)	9 (8·1)

In the first place, if, after receiving a sub-optimal dose of mepacrine, the animals are dosed with SKF–525 A, the therapeutic effect is greatly diminished. SKF–525 A is a substance which interferes with the activity of certain enzyme systems in the liver which are responsible for metabolising and detoxicating drugs; its reported effect is to prolong greatly the action of drugs such as barbiturates by preventing their metabolism to inactive compounds. Of course, if it were a metabolite which was the active substance, SKF–525 A would then be expected to diminish the effect of the drug by preventing formation of the metabolite, which is precisely what it appears to do with mepacrine.

Secondly, by feeding the animals with tryptophane, which greatly increases the amount of certain enzymes in the liver, the effect of mepacrine is enhanced.

Thirdly, the effect of mepacrine is considerably affected by sex, which in itself is responsible for considerable differences in metabolism. Thus, with diminishing dosage of mepacrine, the protective effect wears off in the male at a dose which is about twice that which is minimally effective in the female (Table VII). Some of the sex and other hormones also materially affect the protective action of mepacrine. These observations are not yet complete, but they reinforce the suggestion made earlier, that in the chemotherapy of infective agents so intimately parasitic as viruses we may find that constitutional and nutritional factors play a not unimportant part.

In the United States a great deal of work has been carried out on the influence of nutritional factors in virus diseases, and particularly on the effects of depriving the animals of essential vitamins, mineral salts, amino acids, or just food in general. The results have been reviewed elsewhere[61],

and cannot be considered in detail here. Often the nutritional deficiency has apparently increased resistance to one or other virus, in the sense that the deprived animals have survived, on an average, longer than have those normally fed. In some at least of these experiments, the condition of the animals at the time of infection has been far from satisfactory, and we recall that an animal in an unsatisfactory state of health from one of several causes may be an unsuitable host for the full multiplication of a virus. That some of the experiments have no other interpretation is shown by the fact that removal of the deficiency has promptly led to development of the virus disease. There is one report, however, which if confirmed would seem to be of considerable interest. O'Dell and his colleagues[62] observed that by administering ribose or desoxyribose nucleic acids, especially in conjunction with a diet high in protein, such as one containing 60 per cent casein, they could confer a high degree of protection on mice infected with the neurotropic MM virus. They suggested that in the parasitised nerve cells of the normally fed animals the virus monopolised nucleic acid and protein and damaged the cell by leaving none available for normal cellular functions. By administering excess protein and nucleic acid they felt that they had provided a sufficient surplus to satisfy the requirements of both cell and virus and so to minimise the damage ordinarily ensuing from the presence of the latter.

Thus we see that a few diseases caused by the smaller viruses may be influenced to the advantage of the experimental animal, though sometimes by measures far removed from those of conventional chemotherapy. It seems not impossible that further research into the precise mechanism of these effects may provide information leading to effective chemotherapy in the future.

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RESEARCH PAPERS

ANATOMICAL STUDIES IN THE GENUS *RUBUS*

PART II. THE ANATOMY OF THE LEAF OF *R. fruticosus* L.

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THE common bramble or blackberry, like the raspberry plant, has been used in medicine for many centuries. Dioscorides devotes a monograph to it in his Greek Herbal[1], stating that the leaves and tops, and the juices expressed therefrom, were used in the treatment of a wide variety of ailments; his Herbal also provides a drawing of the plant[2], with numerous prickles, illustrating compound leaves with five leaflets, flowers, fruits and the root system. In the Apuleius herbal of A.D. 600[3], there is a conventional but unmistakable drawing of the blackberry, whilst Gerard[4], whose herbal was first published in 1596, recommended both leaf and fruit as an astringent. A decoction of the leaves is recommended in Cruso's *Treasury of Easy Medicines* (1771)[5] as a fomentation for long-standing ulcers; Grieve[6] records the use of the leaves and root-bark as astringents and tonics in the treatment of dysentery and diarrhoea.

The gross morphology of the bramble leaf, like that of the raspberry, has been described in several standard botanical works[7–9], but again, only limited, and in some cases partially illustrated, histological descriptions have been published to date[11–19]; British pharmacognostical works in use at the present time contain no references to the bramble, but a survey of European works shows that blackberry leaves are more widely recognised as a medicine on the continent of Europe than are those of the raspberry.

In view of these considerations, the present investigation was undertaken to describe and depict the anatomical structure of the leaf, also to note the diagnostic characters necessary for the identification of the leaf in the broken or powdered condition and to distinguish it from the leaves of co-generic species.

MATERIAL

The literature shows considerable variation in the nomenclature of the individual species of the subgenus *RUBUS*, genus *Rubus*, family Rosaceae. In a contemporary flora[9], Warburg points out that, although Bentham[8] includes them all under one specific name, *R. fruticosus* L., the most recently available list, compiled by Watson[20], names 300 species, and even then "only widely distributed species." Warburg emphasises the difficulty of determining individual species within the subgenus—"unlike the genetically somewhat similar genus *Rosa* it does not seem possible at present to group the forms satisfactorily into a limited number of species,"

and he points out that further difficulties arise because the species appear to exhibit a more or less continuous network of forms, so that it is often doubtful to which section of the subgenus a particular species should be referred. Only a few species from each of the sections devised by Watson are described in this Flora[10].

The material used for this investigation was gathered from wild stands on Chislehurst Common, Kent, and has been assigned for the present to the section *Radulae* Focke of Watson's list. Pending a more precise identification, the specific name *R. fruticosus* is used.

Methods of Investigation

Although the leaflets are highly coloured like those of *R. idaeus*, chloral hydrate proved a satisfactory clearing agent for the examination of their epidermises, furthermore, the covering trichomes were well expanded, the spiral markings showing to advantage. Prolonged heating with this reagent accentuated the markings still further, but produced simultaneously a loss of definition of the outlines of many of the epidermal cells, similar to the effect already noted in similar preparations of the leaf of *R. idaeus*[21]. Satisfactory epidermal preparations were also obtained by heating fragments of the lamina with chlorinated lime and a little concentrated hydrochloric acid for a few minutes, subsequently mounting in 50 per cent v/v glycerol solution. Mounts made in phloroglucin and hydrochloric acid stained the covering trichomes well; the epidermis was also seen quite clearly in these mounts.

Systematic serial sections were prepared after polyethylene glycol embedding[22]; they were mounted in a solution containing glycerol 60, distilled water 30 and Delafield's Haematoxylin 10 parts by volume; the latter stained the sections to such a degree that they gave excellent contrast for camera-lucida and projection drawing.

Macerates of prickles, midrib and rachis were prepared using Schultz's maceration fluid.

Anatomical Structure

The leaf of *Rubus fruticosus*, like the leaf of *R. idaeus*, is imparipinnately compound; leaves of species of the section *Radulae* commonly possess five leaflets and the lateral, and, to a lesser extent, the basal pairs of leaflets are all stalked. Paired, adnate, subulate or linear stipules appear to arise about 3 to 5 mm. from the base of the rachis (Figs. 1, A; Fig. 6, A and Fig. 8, A).

A noteworthy sensory character for differentiation from the leaf of *R. idaeus* is that the latter has invariably a whitish underside of the leaflet lamina whereas leaflets of *R. fruticosus* are usually green or greyish-green on their undersides.

(*a*) Leaflets

Both lateral and terminal leaflets were examined, and no anatomical differences were detected between them. The following anatomical description, therefore, applies to either of these leaflets.

(*i*) *Lamina, interneural region* (Fig. 1, C and D; Fig. 2, A, B and F; Fig. 3, B and C; Fig. 5, C and D)

The UPPER EPIDERMIS is covered with a fairly thick, smooth cuticle and consists of a layer of polygonal cells having wavy, beaded anticlinal walls; they measure about* H 10 to 28 μ and Lev L and B 10 to 66 μ. Stomata are absent, but numerous prominent oval *hydathodes*, about 10 to 30 μ long and 9 to 18 μ wide are present on each of the marginal teeth (Fig. 2, A and F). *Covering trichomes* occur fairly frequently; they arise predominantly over, or in close proximity to, the veins and around the edges of the marginal teeth; a small proportion, however, arise from the interneural regions (Fig. 1, B and C; Fig. 2, C). They are unicellular, with very thick, lignified walls, tapering and acutely pointed, with heavily thickened bases commonly exhibiting fine, linear pits; they measure about 300 to 1,000 μ long and 30 to 50 μ wide at the base. The lumen is wide within the base, but narrows very sharply in most trichomes, being visible usually for a short distance only along the trichome. The bases are surrounded by about 10 to 14 radiating epidermal cells, many of which are conspicuously smaller than the normal epidermal cells and these are raised above the level of the epidermis. Double-spiral markings, extending throughout almost the whole length of the trichomes, are extremely prominent in chloral hydrate mounts and also, to a slightly lesser extent, in phloroglucin and hydrochloric acid mounts (Fig. 2, C).

The MESOPHYLL is slightly less clearly differentiated than that of *R. idaeus*; the *palisade* consists of a single layer of cells which is seen to be somewhat discontinuous in thin, transverse sections (Fig. 3, B and C). Individual cells are more or less cylindrical, but often taper slightly at the ends adjacent to the spongy mesophyll; they measure about H 14 to 30 μ, Lev 8 to 12 μ, and contain chloroplasts, about 6 μ in diameter. Scattered irregularly in the palisade are very numerous, prominent, rounded idioblasts, each containing a well-defined cluster crystal of *calcium oxalate* about 12 to 50 μ in diameter. Occasional idioblasts containing small cluster crystals of calcium oxalate, about 8 to 15 μ in diameter, also occur in the layer of the spongy mesophyll lying immediately below the palisade, but there is no well-defined crystal layer. The large, single, prismatic crystals of calcium oxalate described and figured by Gassner[22] were not observed. The spongy mesophyll consists of about 2 to 4 layers of cells, which in surface view are rounded, elongated, triangular or trabeculate and measure about H 6 to 20 μ and Lev 8 to 30 μ; they contain chloroplasts, about 3 to 8 μ in diameter; numerous air spaces occur (Fig. 3, B and C; Fig. 5, C).

The LOWER EPIDERMIS has a smooth, thin cuticle. Its cells have wavy anticlinal walls and measure about H 10 to 16 μ, Lev L and B 10 to 50 μ. *Stomata* are numerous, are of the anomocytic (ranunculaceous) type and

* The symbols H, Lev, Lev L and Lev B are suggested for the purpose of describing organs showing bilateral symmetry by Moll and Janssonius. The symbol H = height, in a direction perpendicular to the surface of the organ; Lev = in the direction of the surface of the organ; Lev L and Lev B = parallel to the surface and at the same time in a longitudinal or transverse direction respectively.

are sometimes surrounded by radiating epidermal cells; they are slightly raised above the level of the epidermis, are oval in outline and exhibit narrow but prominent ostioles; they measure about 14 to 18 μ wide and 20 to 28 μ long (Fig. 2, B).

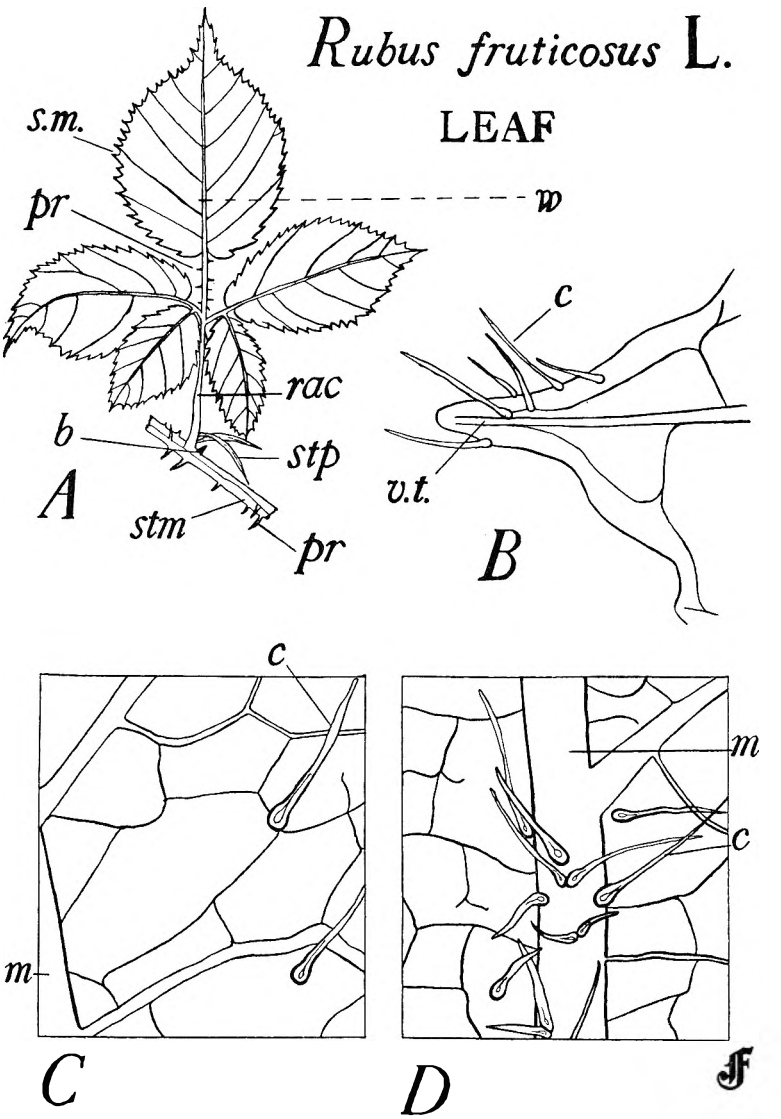

FIG. 1. Leaf of *Rubus fruticosus* L. *A*, complete, compound leaf with terminal, two lateral and two basal leaflets and paired stipules adnate to the rachis. *B*, marginal tooth of leaflet. *C* and *D*, upper and lower surfaces of the lamina to show distribution of covering trichomes. *A*, x $\frac{1}{3}$; *B*, *C* and *D*, x 33. *b*, bud; *c*, covering trichome; *m*, midrib; *pr*, prickle; *rac*, rachis; *s.m.*, serrate margin; *stm*, stem; *stp*. stipule; *v.t.*, vein termination; *w*, position at which transverse section illustrated by Fig. 3, *A* was made.

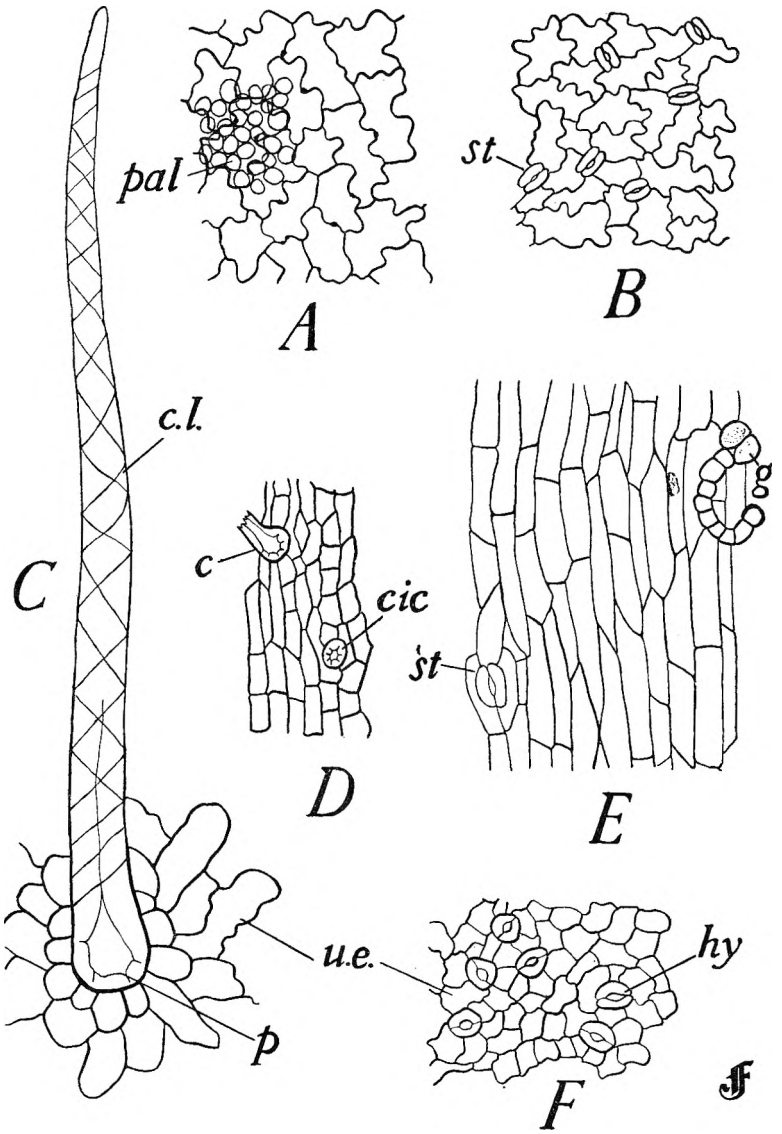

FIG. 2. Leaflet of *Rubus fruticosus* L. *A*, upper epidermis of leaflet. *B*, lower epidermis of leaflet. *C*, covering trichome on upper epidermis of lamina. *D*, upper epidermis of midrib. *E*, lower epidermis of midrib. *F*, upper epidermis of marginal tooth, showing hydathodes. All x 200. *c*, covering trichome; *cic*, cicatrix; *c.l.*, crossed-line effect; *g*, glandular trichome; *hy*, hydathode; *p*, pit; *pal*, palisade; *st*, stoma; *u.e.*, upper epidermis.

Covering trichomes occur frequently, and especially over the minute veinlets rather than on the interneural epidermis (Fig. 1, D); they are similar in character to those on the upper epidermis and frequently are also surrounded at the base by raised epidermal cells. The two- and

four-membered trichomes described by other authors[11,12] were not observed, but a few two-celled trichomes of this kind occur on the lower epidermis of the midrib—*vide infra.*

The lamina has a serrate margin, individual teeth being acutely pointed. The minute ends of the secondary and tertiary veins extend to within about 50 μ of the teeth apices and terminate in a few, very small spiral elements. Two fine veinlets, one on either side, converge towards the central veinlet and unite with it about 0·3 mm. from the tip of the tooth (Fig. 1, A and B).

(*ii*) *Midrib* (Fig. 2, D and E; Fig. 3, A and D; Fig. 4, A and B; Fig. 5, A, B and D).

The midrib has typically dicotyledonous structure; no significant variation, other than a gradual increase in amount of pericyclic fibre and of reticulated cells, was noted in transverse sections cut serially from apex to base.

The UPPER EPIDERMIS consists of a single layer of elongated, well-cuticularised, straight-walled cells measuring about H 14 to 25 μ, Lev B 7 to 15 μ and Lev L 15 to 60 μ; stomata are absent (Fig. 2, D; Fig. 3, D; Fig. 4, B). *Covering trichomes* are present in moderate numbers and are similar in character to those of the upper interneural epidermis. Frequent *glandular trichomes* about 80 to 180 μ long occur on the upper epidermis of both midrib and secondary veins; they comprise a biseriate, at times, multiseriate or, rarely, uniseriate multicellular stalk, about 3 to 7 cells long, frequently with coloured contents, and a subspherical, multicellular, glandular head about 45 to 70 μ in diameter (Fig. 5, D (1)).

The LOWER EPIDERMIS consists of strongly cuticularised, longitudinally elongated, straight-walled cells, measuring about H 10 to 20 μ, Lev B 7 to 20 μ and Lev L 42 to 120 μ; occasional stomata are present (Fig. 2, E).

Covering trichomes arise frequently; the majority are similar in structure to those of the upper interneural epidermis, but occasionally, and more particularly in the upper half of the leaflet, two-celled covering trichomes, similar to those described by other authors[11,12], occur (Fig. 1, D; Fig. 5, D (2 and 3)). Numerous *glandular trichomes* arise; they are frequently bent over at right angles with a multicellular uniseriate stalk of about 6 to 10 cells and a two- to four-celled head about 20 to 40 μ in diameter. More rarely they may somewhat resemble the glandular trichomes of the upper midrib epidermis in having a biseriate stalk (Fig. 2, E; Fig. 5, D (4)).

Laterally compressed, curved or elongated-conical *prickles* occur frequently on the lower surface of the midrib. They are about 0·5 to 2 mm. high and 80 to 600 μ long at the base, and consist of strongly lignified, fibre-like sclerotic cells, about 45 to 220 μ long and 6 to 22 μ wide, their walls being either smooth and thick, or thin with occasional small oblique or transverse pits. Towards the apex of the prickle the cells have pointed, interlocking ends, narrow lumens and thick walls (Fig. 1, A; Fig. 5, A and B; Fig. 6, A, C, D and F).

The CORTEX contains a few rows of supporting hypodermal *collenchyma* beneath both surfaces of the midrib, that towards the lower surface being greater in extent. These cells are heavily thickened, particularly in the angles, and measure about L 8 to 90 μ, R and T 6 to 24 μ; chloroplasts

are present, measuring about 6 μ in diameter. The interior of the cortex is of *parenchyma* which is often, however, slightly collenchymatous, the cells being sometimes slightly thickened at the corners and occasionally exhibiting small pits in their walls; individual cells measure about L 30 to 130 μ, R and T 10 to 60 μ.

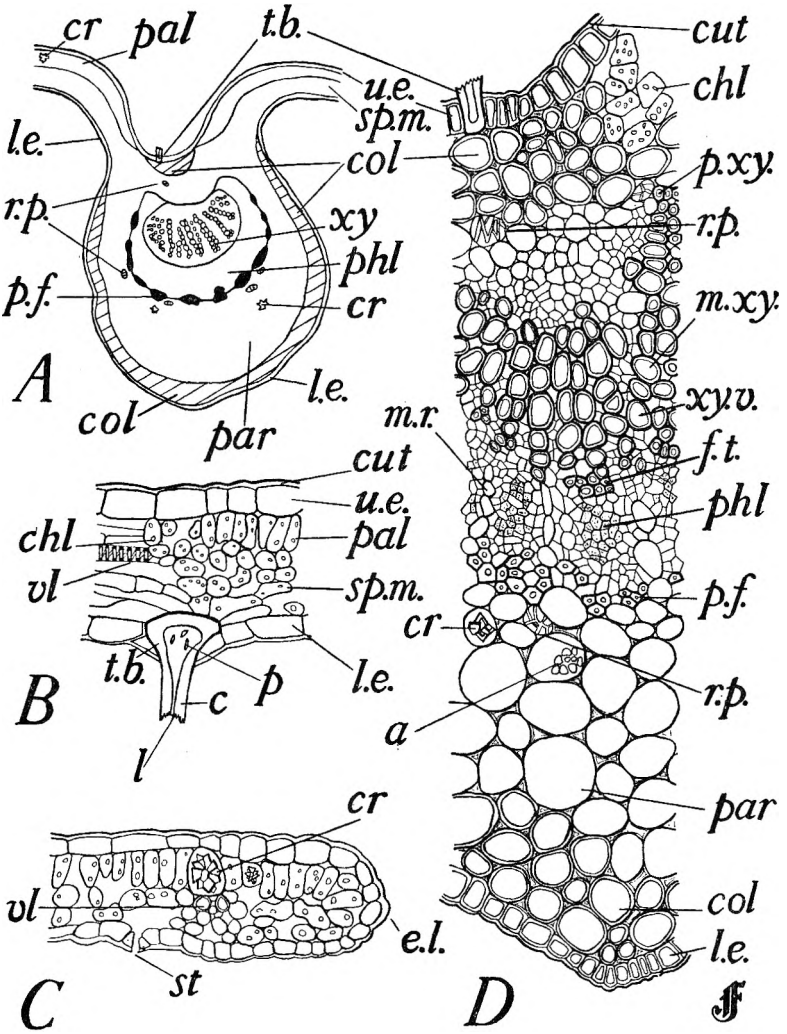

FIG. 3. Leaflet of *Rubus fruticosus* L. *A*, transverse section of midrib of terminal leaflet, cut at the position *w* (see Fig. 1, *A*). *B*, transverse section of lamina, interneural region. *C*, as *B*, but at the edge of the lamina. *D*, central region of Fig. *A*. *A*, x 55; *B-D*, x 200. *a*, starch; *c*, covering trichome; *chl*, chloroplast; *col*, collenchyma, *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *e.l.*, edge of lamina; *f.t.*, fibre-like tracheid; *l*, lumen; *l.e.*, lower epidermis; *m.r.*, medullary ray; *m.xy.*, metaxylem; *p*, pit; *pal*, palisade; *par*, parenchyma; *p.f.*, pericyclic fibre; *phl*, phloem; *p.xy.*, protoxylem; *r.p.*, reticulate parenchyma; *sp.m.*, spongy mesophyll; *st*, stoma; *t.b.*, trichome base; *u.e.*, upper epidermis; *vl*, veinlet; *xy*, xylem; *xy.v.*, xylem vessel.

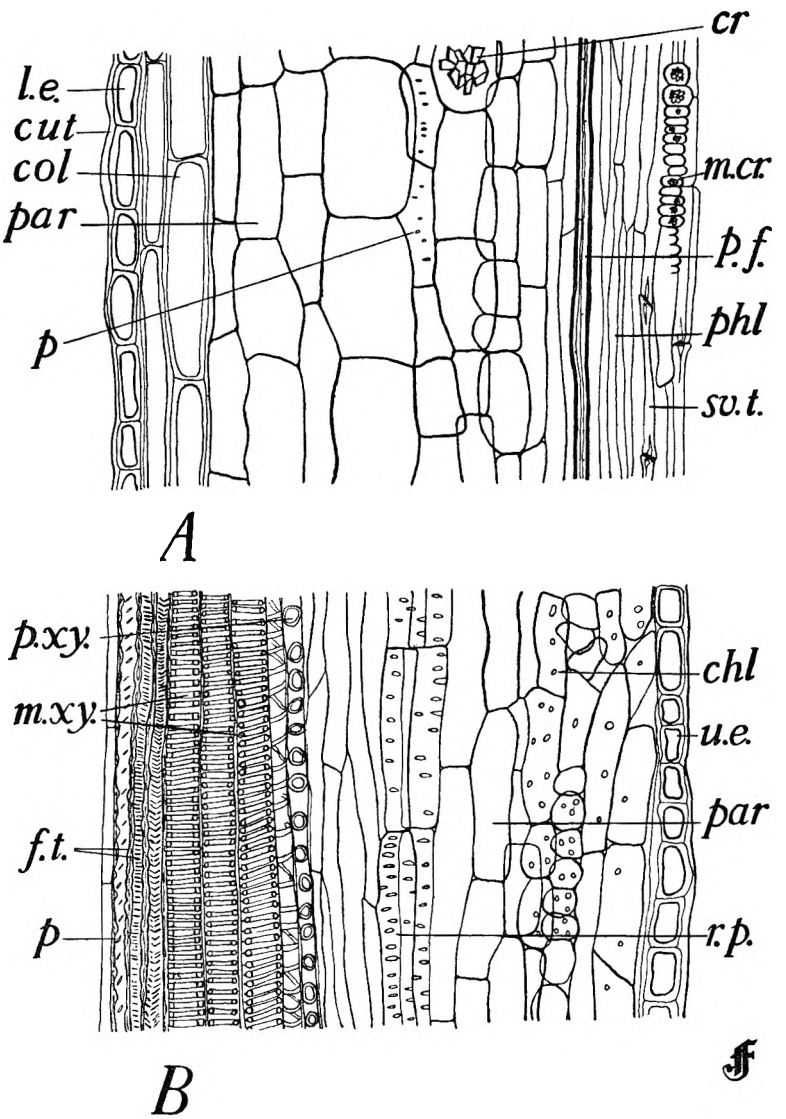

FIG. 4. Leaflet of *Rubus fruticosus* L. *A* and *B*, radial longitudinal section of midrib of terminal leaflet, cut at approximately the position *w* (see Fig. 1, *A*). Both, x 200. *chl*, chloroplast; *col*, collenchyma; *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *f.t.*, fibre-like tracheid; *l.e.*, lower epidermis; *m.cr.*, micro-cluster of calcium oxalate; *m.xy.*, metaxylem; *p*, pit; *par*, parenchyma; *p.f.*, pericyclic fibre; *phl*, phloem; *p.xy.*, protoxylem; *r.p.*, reticulate parenchyma; *sv.t.*, sieve tube; *u.e.*, upper epidermis.

Idioblasts occur frequently in this tissue; they are of two kinds. Those of the first type contain cluster crystals of *calcium oxalate* about 10 to 35 μ in diameter. The second type occurs in the inner part of this tissue and consists of slightly lignified, *reticulated cells* which are elongated

longitudinally and rounded or oval in transverse section; they measure about L 40 to 190 μ and T 18 to 36 μ, with very slightly lignified reticulate bands of thickening traversing them (Fig. 3, A and D; Fig. 4, A and B; Fig. 5, A).

Many cells of the innermost layer(s) of the cortical parenchyma are frequently lignified; in some specimens the innermost layers are non-lignified, but contain minute starch grains 2 to 7 μ in diameter; some of the cortical cells adjacent to this layer also contain similar starch grains. Owing to this irregular distribution of starch, an endodermis (or starch sheath) is not so clearly defined as it is in many other leaves, such as those of *Digitalis purpurea* and *Datura stramonium*. Engard[23] has said that the endodermis is absent from the genus *Rubus*, but it is possible to regard the innermost layer or starch-bearing cortical cells as constituting an endodermis, especially as this layer is continued over the upper side of the meristele (Fig. 3, D).

The MERISTELE is crescent-shaped in transverse section and well defined.

The PHLOEM consists of strands of sieve-tissue and small-celled parenchyma, alternating with medullary rays. The *sieve-tubes* are small; individual segments being about 100 μ long and about 3 to 7 μ in diameter, with transverse or oblique sieve-plates (Fig. 4, A). The medullary rays are clearly defined and are usually one or two cells wide (Fig. 3, D).

The XYLEM is well-developed and the conducting elements are radially arranged. The protoxylem consists of lignified, annularly and sometimes spirally thickened *tracheids* about 6 to 10 μ in diameter. The greater part of the metaxylem consists of lignified spiral, reticulate and sometimes pitted *vessels* about 14 to 26 μ in diameter; that part adjacent to the cambium consists of *fibre-like tracheids* about 100 to 600 μ long and 4 to 10 μ wide (Fig. 3, A and D; Fig. 4, B; Fig. 5, A).

In longitudinal sections, files of micro-clusters of *calcium oxalate* crystals about 3 to 8 μ in diameter are frequently seen in the parenchyma of the meristele (Fig. 4, A).

The *lateral veins* exhibit similar anatomy to that of the midrib, all features progressively diminishing towards the margin.

Sections of fresh material mounted in ferric chloride solution exhibit a dark greenish-black colouration due to tannin, most particularly in the phloem of the meristele and the mesophyll of the lamina and, to a lesser degree, in the medullary rays and stelar parenchyma. The cortical parenchyma and collenchyma of the midrib show a weak reaction, but the epidermises of the lamina are not affected.

(*b*) RACHIS

The rachis is about 4 to 10 cm. long, and 1 to 3 mm. wide; prickles are present throughout the length of the lateral and adaxial surfaces (Fig. 1, A; Fig. 6, A). Transverse sections show a shallow groove on the adaxial side in certain places, but elsewhere it is almost terete. The vascular system is more complicated than that of the rachis of *R. idaeus*; the drawings (Fig. 6, B to J) are representative of many hundreds of transverse sections cut serially from the base of the terminal leaflet to the bottom of

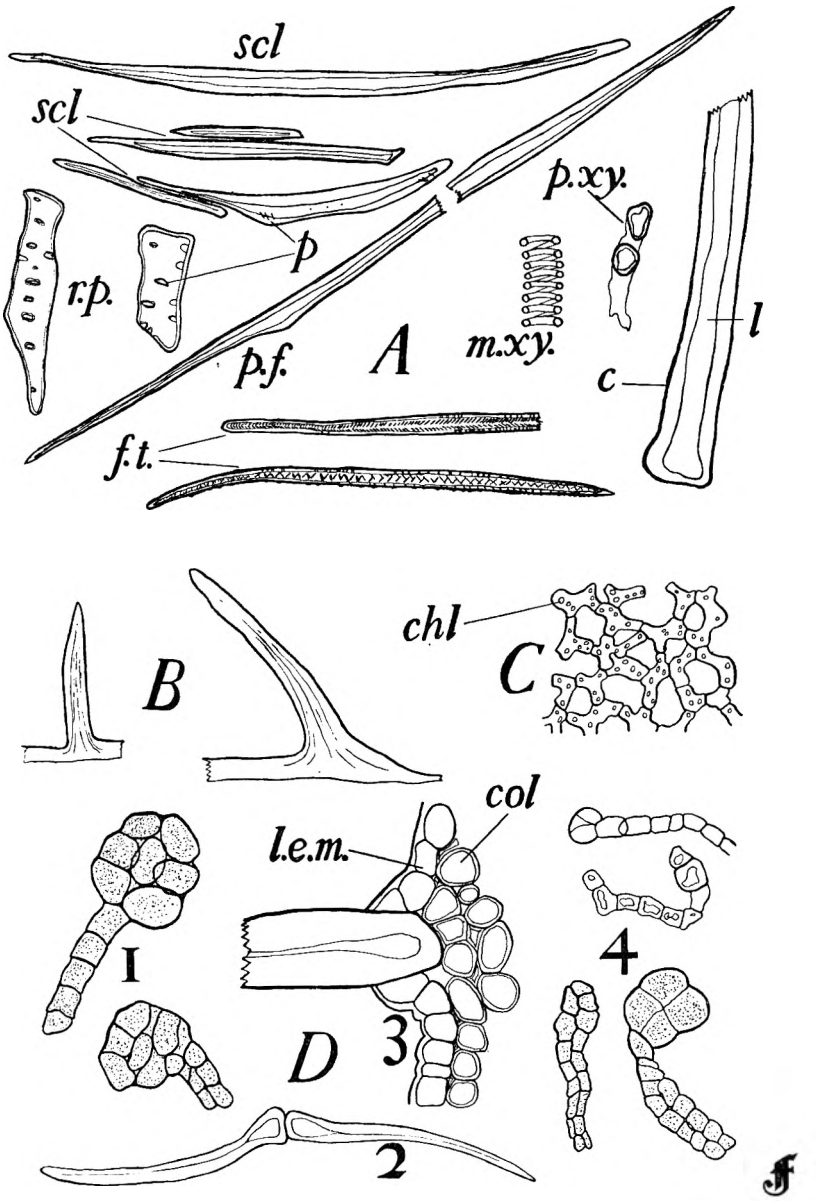

FIG. 5. Leaflet of *Rubus fruticosus* L. *A*, isolated elements obtained by maceration. *B*, prickles. *C*, spongy mesophyll in surface view. *D*, trichomes: 1, glandular trichomes from upper epidermis of midrib; 2, bicellular covering trichome from lower surface of midrib; 3, base of large covering trichome from lower surface of midrib; 4, glandular trichomes from lower surface of midrib. *A*, *C* and *D*, x 200; *B*, x 25. *c*, covering trichome (or fragment of); *chl*, chloroplast; *col*, collenchyma; *f.t.*, fibre-like tracheid; *l*, lumen; *l.e.m.*, lower epidermis of midrib; *m.xy.*, fragment of spiral vessel of the metaxylem; *p*, pit; *p.f.*, pericyclic fibre; *p.xy.*, fragment of annular tracheid of the protoxylem; *r.p.*, reticulate parenchyma; *scl*, elongated sclereids from the prickles.

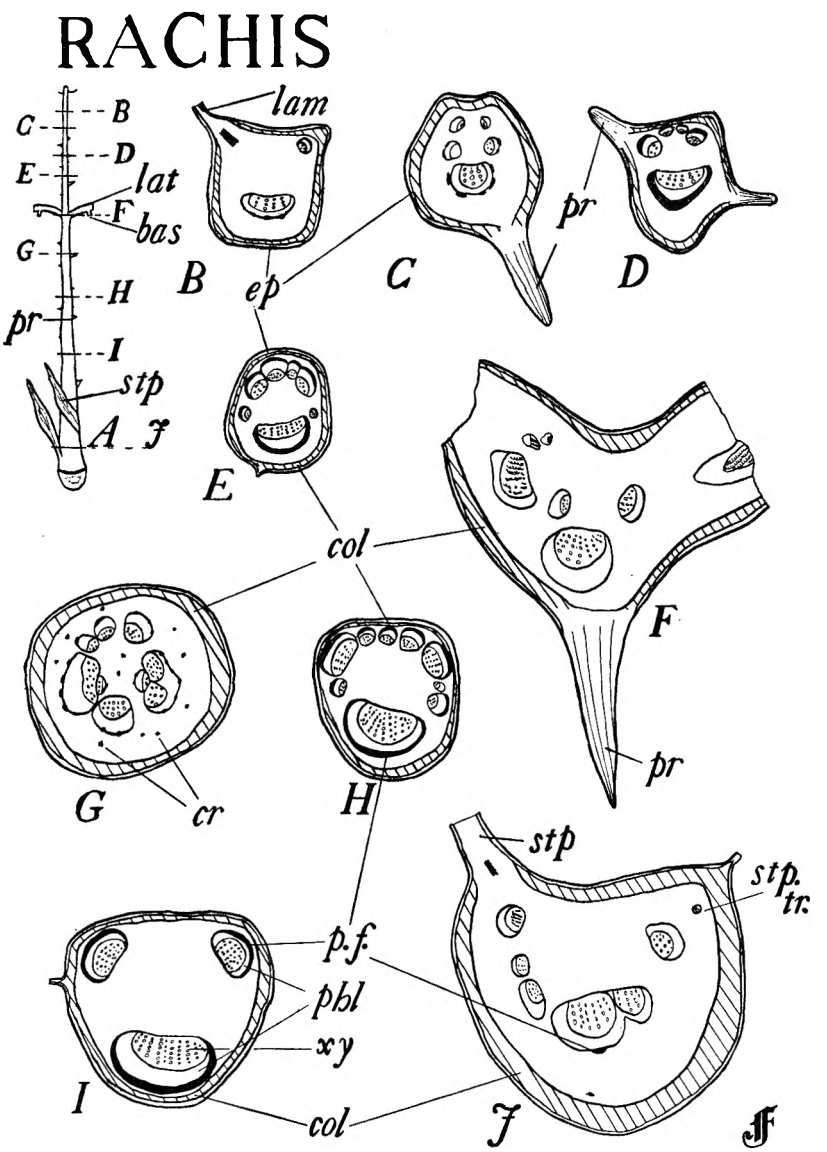

FIG. 6. Rachis of *Rubus fruticosus* L. *A*, rachis, denuded of leaflets; *B-J*, transverse sections cut at the positions indicated in Fig. *A*. *A*, x ½; *B-J*, x 14. *bas*, point of attachment of basal leaflet; *col*, collenchyma; *cr*, crystal of calcium oxalate; *ep*, epidermis; *lam*, lamina; *lat*, point of attachment of lateral leaflet; *p.f.*, pericyclic fibres; *phl*, phloem; *pr*, prickle; *stp*, stipule; *stp. tr.*, stipule trace; *xy*, xylem.

the rachis. The central, crescent-shaped meristele remains prominent throughout the length of the rachis, but the courses of the small fibro-vascular bundles on the adaxial side vary considerably; moreover, slight variations in the courses of these bundles have been observed in different

leaves. In the leaf figured, the lowest pair of lateral veins of the lamina of the terminal leaflet become the first two lateral traces of the rachis (Fig. 6, B); two more traces are abstricted from the meristele a little way further down (Fig. 6, C). Below this, the four traces, after some simple variations in their courses, become symmetrically arranged on the adaxial side (Fig. 6, D); still further down, but before the junction with the lateral leaflet traces, the meristele abstricts two further traces, whilst the other four unite to form a virtual arc of fibrovascular tissue on the adaxial side (Fig. 6, E). This arc and the two abstricted traces become gradually modified into two separate bundles just before the lateral traces enter the rachis (Fig. 6, F). Fibres are absent in the region of this junction, but below it fibres are present again and the smaller traces eventually form an almost continuous arc of fibrovascular tissue on the adaxial side (Fig. 6, H). However, a short way above the bases of the stipules and rachis, the arc divides and the section has the appearance shown in Figure 6, I. The stipule traces are finally included, just before the junction of the rachis and stem (Fig. 6, J); the pericyclic fibres are absent again in this region.

The EPIDERMIS consists of cells having similar structure to those of the epidermis of the midrib. They are heavily cuticularised, elongated longitudinally, and measure about H 9 to 16 μ, Lev B 14 to 28 μ and Lev L 16 to 60 μ (Fig. 7, A, B and C); *stomata* of the anomocytic (ranunculaceous) type are present; they are elliptical in shape and measure about 30 μ in length and 20 μ in width (Fig. 7, C). *Covering trichomes*, generally similar in detailed structure to those on the lower surface of the midrib, occur frequently, but are occasionally bi- and even tri-cellular here; they vary enormously in size—from 100 to over 1000 μ in length, and 10 to 25 μ wide at their bases (Fig. 7, F). *Glandular trichomes* occur very frequently, particularly on the upper (adaxial) epidermis; they are multicellular and frequently multiseriate, with subspherical or irregularly-shaped multicellular heads; the smallest resemble the glandular trichomes of the midribs in appearance and size, but large glandular trichomes commonly up to 500 μ long with heads about 60 μ diameter are common on the rachis. The *prickles* are frequently larger than, but have similar structure to those on the lower surface of the midrib (Fig. 7, E).

The CORTEX, like that of the midrib, consists of two layers of tissue—an outer hypodermal layer of *collenchyma*, several cells wide, similar in character to the corresponding layer of the midrib; the cells measure about L 40 to 140 μ, R and T 14 to 40 μ, and contain chloroplasts, 3 to 6 μ in diameter (Fig. 6, B-J; Fig. 7, D). The inner cortex is *parenchymatous*, consisting of cells measuring about L 20 to 140 μ and R and T 20 to 120 μ; they frequently possess slightly pitted walls. As in the midrib, many cells of the innermost layer(s) of the cortical parenchyma are frequently lignified; in specimens where the innermost layers are non-lignified, minute starch grains are often seen, about 2 to 7 μ in diameter. Frequent idioblasts containing cluster crystals of *calcium oxalate*, about 18 to 50 μ in diameter occur in idioblasts throughout the cortical parenchyma, whilst many *reticulated cells*, similar to those of the midrib, are found in the inner region and around the small ridge bundles.

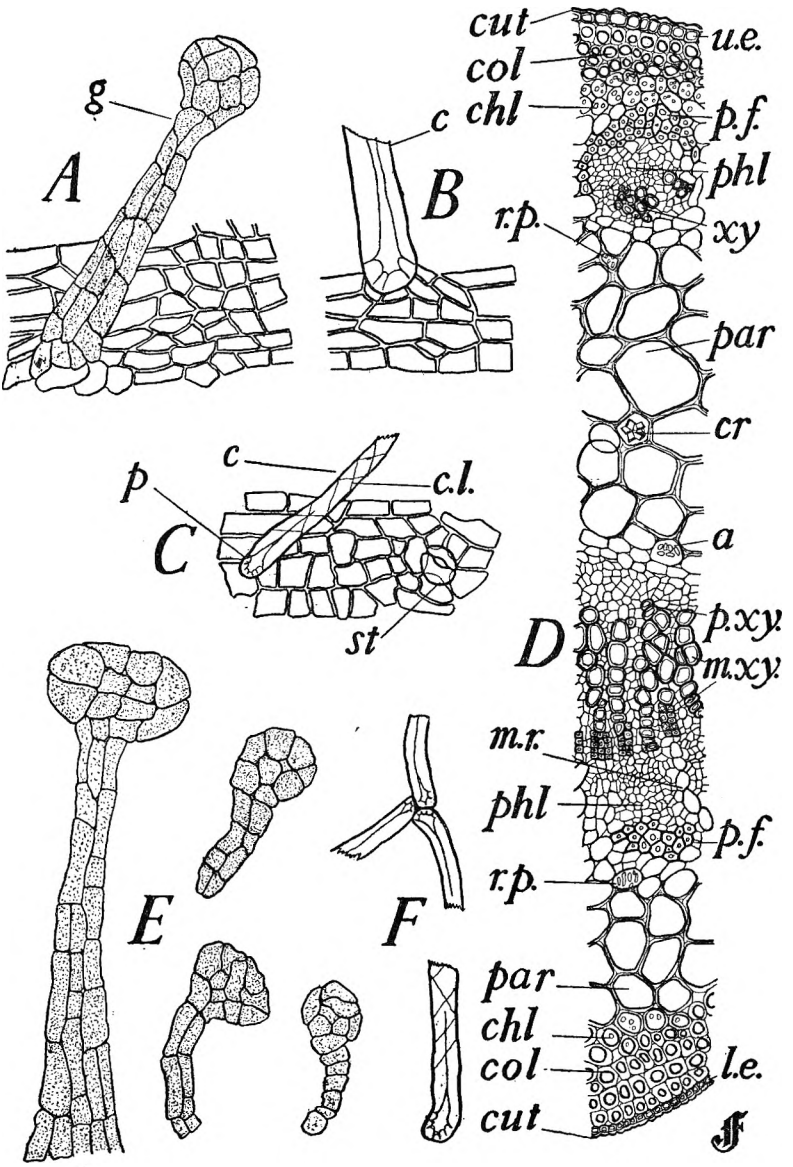

FIG. 7. Rachis of *Rubus fruticosus* L. *A*, upper epidermis with large glandular trichome. *B*, lower epidermis with base of a covering trichome. *C*, upper epidermis with stoma and base of a covering trichome. *D*, transverse section through central region of rachis at the point *D*, Fig. 6. *E*, glandular trichomes. *F*, bases of covering trichomes. *A*, *B*, *C*, *E* and *F*, x 200; *D*, x 130. *a*, starch; *c*, covering trichomes; *chl*, chloroplast; *c.l.*, crossed-line effect; *col*, collenchyma; *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *g*, glandular trichome; *l.e.*, lower epidermis; *m.r.*, medullary ray; *m.xy.*, metaxylem; *p*, pit; *par*, parenchyma; *p.f.*, pericyclic fibre; *phl*, phloem; *p.xy.*, protoxylem; *r.p.*, reticulate parenchyma; *st*, stoma; *u.e.*, upper epidermis; *xy*, xylem.

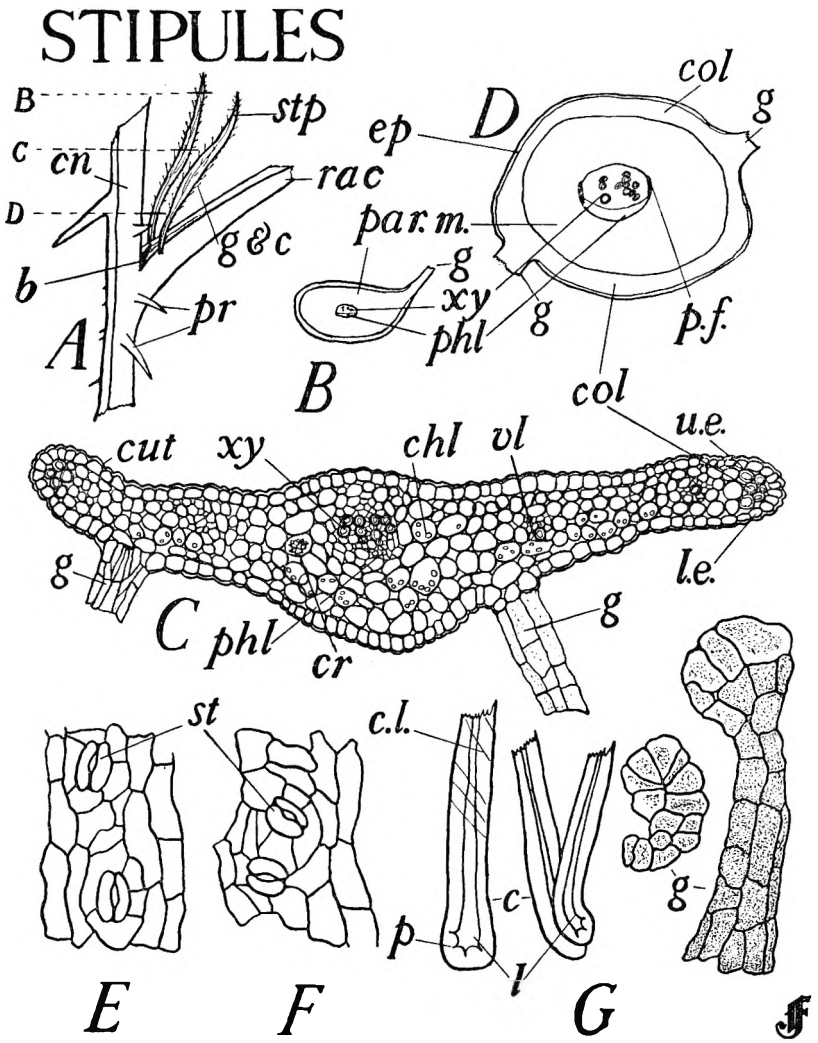

FIG. 8. Stipules of *Rubus fruticosus* L. *A*, paired stipules at base of rachis. *B*, *C* and *D*, transverse sections cut at the positions indicated in Fig. *A*. *E*, lower epidermis. *F*, upper epidermis. *G*, covering and glandular trichomes. *A*, x 2½; *B* and *D*, x 75; *C*, x 130; *E-G*, x 200. *b*, bud; c, covering trichomes (or fragment of); *chl*, chloroplast; *c.l.*, crossed-line effect; *cn*, cane; *col*, collenchyma; *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *ep*, epidermis; *g*, glandular trichome; *l.e.*, lower epidermis; *par.m.*, parenchymatous mesophyll; *p.f.*, pericyclic fibre; *phl*, phloem; *pr*, prickle; *rac*, rachis; *st*, stoma; *stp*, stipule; *u.e.*, upper epidermis; *vl*, veinlet in transverse section; *xy*, xylem.

PERICYCLIC FIBRES occur below the arc of the meristele throughout the rachis except at the junction with the lateral petioles and at the base of the rachis; they measure up to several mm. in length and 7 to 18 μ in diameter; they exhibit thick, smooth walls and pointed apices (Fig. 7, D).

The vascular tissue of the central MERISTELE is arranged in a crescent and in general the structure of the vascular elements approximates closely to those of the midrib, except that those of the rachis are all somewhat larger. The PHLOEM consists of groups of *sieve-tubes* about 80 to 100 μ long and 4 to 8 μ wide, frequently accompanied by small rectangular parenchymatous cells arranged in longitudinal files, many of which contain microclusters of *calcium oxalate* about 9 μ in diameter. Medullary rays traverse the phloem; they are usually one or two cells wide and their cells often contain minute starch grains about 3 μ in diameter. XYLEM is well-defined, the elements being arranged in radial rows. Its general structure is very similar to the xylem of the midrib, except that there is a much greater proportion of pitted and reticulate vessels. There is but little xylem parenchyma; the medullary rays are clearly seen alternating with the rows of tracheids and vessels; again, many of the cells often contain minute starch grains (Fig. 6, B-J; Fig. 7, D).

The fibrovascular bundles on the adaxial side each exhibit similar structure to that shown by the main meristele.

The reaction of sections of the fresh rachis with ferric chloride solution is similar to that observed in the midrib.

(*c*) STIPULES

The paired stipules are adnate to either side of the base of the rachis. They are about 10 to 20 mm. long and 1 to 2 mm. wide, subulate or linear, and hairy (Fig. 1, A; Fig. 6, A and Fig. 8, A).

EPIDERMAL CELLS of both upper and lower surfaces are small and elongated, sometimes with very slightly beaded anticlinal walls, measuring about H 10 to 24 μ, Lev B 8 to 30 μ and Lev L 20 to 70 μ. *Stomata* are present on both surfaces; they are usually elliptical in outline and measure about 30 μ in length and 20 μ in width (Fig. 8, E and F). Numerous *covering trichomes* occur, especially around the edges of the stipules; they measure about 600 to 800 μ in length and 12 to 22 μ wide at the bases and possess the general characters of those of the upper epidermis of the leaflets. Many large *glandular trichomes* are present, particularly around the edges; these are similar in character to those of the rachis (Fig. 8, G).

The MESOPHYLL has very simple structure, is undifferentiated and consists of rounded or somewhat elongated cells, measuring about H 10 to 30 μ, Lev B 10 to 30 μ and Lev L 10 to 30 μ, and containing chloroplasts about 4 to 8 μ in diameter. Occasional idioblasts occur, containing small cluster crystals of calcium oxalate about 10 to 35 μ in diameter. Towards the base of the stipule, the hypodermal tissue near the margin is collenchymatous (Fig. 8, B, C and D).

The VENATION is very simple, consisting of a central midrib and minute, pinnate, slightly anastomosing secondary veinlets. The midrib consists of a few xylem elements about 10 μ in diameter; there is but little phloem; the pericycle is evident and consists of a very few fibres at the base of the stipule, but of lignified parenchyma only throughout almost its whole length (Fig. 8, C).

Powder

A No. 60 powder is green in colour; it has a very slight odour and a not unpleasant, very slightly astringent taste. When some of the powder is mixed with ferric chloride solution, a greenish-black colour is observed.

To examine the powder for structural features, it was mounted in 50 per cent v/v glycerol solution, solution of chloral hydrate, or phloroglucin and hydrochloric acid. The diagnostic characters (Fig. 9) are:—

Numerous large, straight or slightly curved, lignified fragments of *covering trichomes* from both surfaces of the lamina, up to about 40 μ wide, apical fragments being acutely pointed and solid, whilst basal fragments are hollow and pitted with simple pits; most fragments show double-spiral markings very prominently; *fragments of the lamina*, showing a

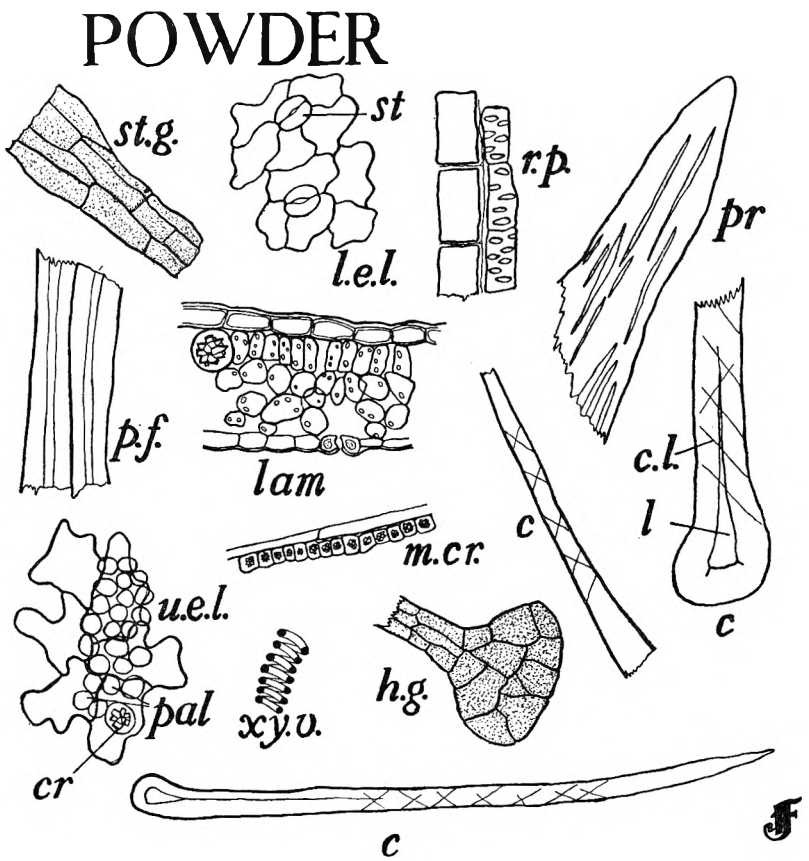

FIG. 9. Powder of *Rubus fruticosus* L. All x 200. *c*, covering trichome; *c.l.*, crossed-line effect; *cr*, cluster crystal of calcium oxalate; *h.g.*, head of large, glandular trichome; *l*, lumen; *lam*, fragment of lamina in transverse section, showing upper and lower epidermises, stoma, idioblast with cluster calcium oxalate crystal, palisade and spongy mesophyll; *l.e.l.*, lower epidermis of lamina; *m.cr.*, file of microcluster crystals of calcium oxalate, adjacent to phloem tissue; *pal*, palisade; *p.f.*, fragments of pericyclic fibres; *pr*, apex of prickle; *r.p.*, reticulate parenchyma; *st*, stoma; *st.g.*, stalk of glandular trichome; *u.e.l.*, upper epidermis of lamina; *xy.v.*, fragment of xylem vessel.

transverse sectional view, about 90 μ thick, the palisade containing rounded idioblasts, each containing a crystal of *calcium oxalate* about 12 to 50 μ in diameter; particles showing in surface view the wavy-walled cells of the *upper interneural epidermis* of the lamina and, usually, immediately beneath it, the palisade with its idioblasts; fragments showing the *lower interneural epidermis* consisting of wavy-walled cells with numerous anomocytic (ranunculaceous) stomata which are sometimes surrounded by radiating epidermal cells; fragments of the *veins* showing small lignified vascular elements showing annular, spiral, reticulate and pitted thickenings, and occasional files containing microclusters of *calcium oxalate*; *reticulated parenchymatous* cells in vertical files; fragments of prickles consisting of fibre-like *lignified sclereids* with oblique or transverse linear pits; numerous, usually broken, *glandular trichomes*, with multicellular, multiseriate stalks and yellowish-brown multicellular, glandular heads.

Discussion

We have found that the histological structure of the leaf of *R. fruticosus* L. is basically similar to that of *R. idaeus* L. Certain diagnostic characters, such as the incidence of hydathodes in the marginal teeth, of anomocytic (ranunculaceous) stomata on the lower epidermis of the lamina and of a smooth cuticle on the epidermises of the lamina, are common to leaves of both species. There are, however, several well-contrasted features; these are found in (*a*) the epidermal characters, and (*b*) the tissues of the midrib and rachis.

(*a*) *Epidermal characters*

The anticlinal walls of the cells of the upper epidermis of the leaflets of *R. fruticosus* are beaded, whilst the covering trichomes show the double-spiral markings very prominently, when mounted in chloral hydrate solution; neither of these features are normally observed in *R. idaeus*. The tomentum of hairs of the lower epidermis of the leaflets of the latter is absent from *R. fruticosus*, but the occasional two-celled covering trichomes, and the *bent* glandular trichomes found commonly on the lower surface of the midribs of the blackberry leaflets afford further differential characters; the epidermises of the rachis and stipules are also strongly characterised by the great numbers of *large*, multiseriate glandular trichomes.

(*b*) *Midrib and Rachis*

The inner cortex of the midrib and rachis of *R. fruticosus* contains many reticulated, slightly lignified, parenchymatous cells; these are absent from *R. idaeus*, but the detection of rachis in blackberry leaf powder cannot be based on the presence of pericyclic fibres, because in this case they are also present in the leaf midrib and veins; the presence of the large, multiseriate glandular trichomes mentioned above affords better evidence of the presence of rachis. The latter usually shows, moreover, an almost complete ring of vascular bundles in transverse sections.

It must be emphasised that the foregoing anatomical description applies only to the leaves taken from one particular clone of plants belonging to the section *Radulae* of the fruticose brambles. From other descriptions[11,12,22], and from our own observations on leaflets from other plants, it is apparent that considerable variations in the detailed structure are probable throughout the 300 species which have now been named. The diagnostic characters listed must not, therefore, on any account be taken as specific for all forms of *R. fruticosus* L.

Summary

Blackberry leaves collected from wild plants were used for this investigation, and, although their anatomy is described under the specific name of *R. fruticosus* L., they have been identified as belonging to the section *Radulae* Focke, described by Watson[19]. They are characterised by the following features:—

1. The *epidermises* of the leaflets. The *upper epidermis* consists of cells with wavy anticlinal walls and bears scattered, unicellular, lignified, *covering trichomes* with acute, solid apices and thickened, pitted bases. The *lower epidermis* consists of cells with slightly beaded, wavy anticlinal walls and also bears *covering trichomes* similar to those on the upper epidermis. *Glandular trichomes* are present on the lower epidermises of the midrib and main veins; they consist of a uniseriate, multicellular stalk of about 6 to 10 cells, and a two- to four-celled glandular bead, and frequently are bent over; occasional biseriate glandular trichomes also occur.

2. The epidermises of the rachis and stipules bear *large*, characteristic, multicellular, multiseriate, glandular trichomes.

3. The *lamina* of the leaflet is thin and dorsiventral, with a single row of palisade in which are rounded idioblasts, each containing a cluster crystal of *calcium oxalate*.

4. The *midrib* of the leaflet contains a meristele consisting of annularly and spirally thickened *protoxylem tracheids*, spiral and sometimes reticulate and pitted *vessels*, and fibre-like, pitted *tracheids* of the metaxylem, a phloem of simple sieve-tubes with transverse or oblique sieve-plates, and rows of parenchymatous cells in longitudinal files, each cell containing a micro-cluster of *calcium oxalate*.

5. *Prickles* of midrib and rachis are composed of lignified, fibre-like sclereids.

6. Lignified pericyclic fibres are abundant and common to both leaflets and rachis; the latter may be recognised when in powder, however, by the presence of slightly larger and more extensively thickened *vascular elements* and by the numerous, *large*, multicellular, multiseriate, glandular trichomes.

The authors are indebted to Dr. T. E. Wallis for his very helpful advice and criticisms; also to Mr. J. P. M. Brenan for his assistance in the identification of the material.

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THE EFFECT OF SOME ORGANOPHOSPHORUS AND CHLORINATED HYDROCARBON INSECTICIDES ON THE TOXICITY OF SEVERAL MUSCLE RELAXANTS

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Several investigators[1–14] have shown that certain insecticides, particularly organophosphorus and possibly chlorinated hydrocarbon compounds, are inhibitors of cholinesterases both *in vivo* and *in vitro*. It has been shown that the levels of cholinesterase in the body determine the rate of destruction of at least one muscle relaxant[15,16], and thus low levels of cholinesterase might result in a prolongation of action and enhancement of toxicity of some relaxants. The following experiments were performed to determine the effects of acute and chronic exposure of rats to some insecticides on the toxicity of representative muscle relaxants.

Experimental

Part I. Effect of Acute Exposure to Insecticides

Methods

The insecticides parathion and malathion were chosen as representatives of the organophosphorus group and aldrin as a representative of the chlorinated hydrocarbon group. Malathion and parathion were administered to male and female Wistar rats by intraperitoneal injection, and aldrin was administered by stomach tube. Doses of the insecticides corresponding to approximately $\frac{1}{4}$ of the LD50 were given as follows: aldrin—25 mg./kg., malathion—1 ml./kg. and parathion (diluted 1 : 500)

TABLE I

Muscle relaxants used

Muscle relaxant	Vehicle	Route of administration
Suxamethonium chloride ..	water	intramuscular
Mephenesin	propylene glycol, 40 per cent	intraperitoneal
Gallamine triethiodide ..	water	intraperitoneal
Benzimidazole	ethanol, 95 per cent	intramuscular
Tubocurarine chloride ..	water	intramuscular
Decamethonium iodide ..	water	intraperitoneal

—1 ml./kg. Parathion and aldrin were dissolved in peanut oil and corresponding control groups received peanut oil only. Malathion was obtained in two strengths, both in liquid form: 95 per cent malathion and commercial malathion containing 57 per cent of the 95 per cent product. There was no appreciable difference volume for volume in the toxicity of the two malathion preparations. The parathion preparation was also obtained in liquid form and contained 97·76 per cent of parathion.

After pretreatment with the insecticides, the rats received the muscle relaxants by injection eighteen to twenty-four hours later. Table I

shows the relaxants used in this study, the vehicle and the route of administration.

Three to five different doses of each relaxant were administered to groups of ten to twenty rats in both the pretreated and control groups. The per cent mortalities were converted to probits and plotted against the logarithm of the dose of muscle relaxant. The resultant linear dose responses were analysed statistically by methods proposed by Bliss[17,18], and Miller, Bliss and Braun[19], and the final results were recorded in the following Tables as toxicity of the relaxant to the pretreated rats in terms of the controls, expressed as a percentage. The results were considered significant when $P < 0{\cdot}05$.

TABLE II

THE EFFECT OF PARATHION PRETREATMENT ON THE TOXICITY OF MUSCLE RELAXANTS

Muscle relaxant	Sex of rats	Pre-treated or control	Dose of relaxant (mg./kg.)	Mortality (per cent)	Toxicity to pretreated rats in terms of controls (per cent ± S.E.M.)	S* or NS
Suxamethonium chloride	F	P	1·96 2·26 2·61	60·0 53·3 71·4	141·0 ± 12·1	S
		C	2·61 3·00 3·45	26·7 40·0 93·5		
	M	P	1·70 1·96 2·26	53·4 73·3 93·0	136·0 ± 7·0	S
		C	2·26 2·61 3·00	33·3 80·0 93·3		
Gallamine triethiodide	F	P	15·1 17·0 19·0 21·4	20·0 8·3 40·0 38·5	85·4 ± 8·3	NS
		C	17·0 19·0 21·4	25·0 66·6 66·6		
	M	P	17·0 18·0 19·0 21·4	18·2 33·3 91·0 91·0	90·3 ± 4·7	NS
		C	13·5 15·1 17·0 21·4	8·3 54·6 63·6 81·8		

* Significant or not significant from 100 per cent.

Results

Table II shows the effect of pretreatment of male and female rats with parathion followed by suxamethonium chloride or gallamine triethiodide.

There was a significant increase in the toxicity of suxamethonium to rats pretreated with parathion, but no increase in the toxicity of gallamine to the pretreated animals. There was no apparent difference in response between male and female rats. Table III demonstrates the effect on rats of pretreatment with malathion followed by the administration of several muscle relaxants.

The increase in toxicity of the relaxants to the pretreated rats was particularly striking with suxamethonium. There were also significant increases in the toxicity of mephenesin and gallamine, and a decrease in the toxicity of benzimidazole to the rats pretreated with malathion. There was no change in the toxicity of decamethonium and tubocurarine.

While parathion and malathion are well-known inhibitors of cholinesterases, aldrin has been reported to exert pharmacological activity suggestive of an anticholinesterase compound[10]. Table IV shows the effect of pretreatment of rats with aldrin on the toxicity of several muscle relaxants.

As before, suxamethonium showed the most marked increase in toxicity to the pretreated animals. This increase appeared to be more marked

in the male rats, although the difference between males and females was not significant. Gallamine and decamethonium also showed increased toxicity to the pretreated rats, but the significance of the increase was small in the case of decamethonium.

TABLE III

THE EFFECT OF MALATHION PRETREATMENT ON THE TOXICITY OF MUSCLE RELAXANTS

Muscle relaxant	Sex of rats	Pre-treated or control	Dose of relaxant (mg./kg.)	Mortality (per cent)	Toxicity to pretreated rats in terms of controls (per cent ± S.E.M.)	S* or NS
Suxamethonium chloride	F	P	1·43 1·70 1·96	46·7 80·0 85·7	169·6 ± 10·0	S
		C	2·26 2·61 3·00	33·3 40·0 100·0		
	M	P	1·43 1·70 1·96	66·7 86·7 86·7	189·2 ± 15·9	S
		C	2·26 2·61 3·00	13·3 53·3 86·7		
Mephenesin	M	P	290 325 364	13·6 54·5 72·7	132·3 ± 9·0	S
		C	364 436 488	27·3 45·4 54·5		
Gallamine triethiodide	F	P	10·8 12·1 13·6 15·2	9·1 63·7 54·5 63·7	118·4 ± 7·4	S
		C	13·6 15·2 17·0 19·0	27·3 45·4 63·7 90·9		
	M	P	15·2 17·0 19·0	18·2 59·1 72·7	119·5 ± 7·4	S
		C	17·0 19·0 21·2 23·7	18·2 36·4 63·6 81·8		
Benzimidazole	F	P	394 442 496 556	9·0 0·0 40·0 63·6	78·0 ± 7·0	S
		C	394 442 496 556	45·4 70·0 80·0 72·7		
Decamethonium iodide	M	P	3·0 3·4 3·8 4·3	35·0 50·0 55·6 70·0	99·2 ± 7·6	NS
		C	3·4 3·8 4·3	30·0 80·0 80·0		
Tubocurarine chloride	M	P	0·300 0·330 0·363 0·400	15·0 30·0 40·0 90·0	100·4 ± 3·5	NS
		C	0·300 0·330 0·363 0·400	5·0 40·0 60·0 80·0		

* Significant or not significant from 100 per cent.

TABLE IV

THE EFFECT OF ALDRIN PRETREATMENT ON THE TOXICITY OF MUSCLE RELAXANTS

Muscle relaxant	Sex of rats	Pre-treated or control	Dose of relaxant (mg./kg.)	Mortality (per cent)	Toxicity to pretreated rats in terms of controls (per cent ± S.E.M.)	S* or NS
Suxamethonium chloride	F	P	2·26 2·61 3·00	33·3 66·7 93·4	111·5 ± 5·8	S
		C	2·26 2·61 3·00	20·0 40·0 73·2		
	M	P	1·76 2·26 2·61	60·0 86·7 93·4	141·8 ± 11·7	S
		C	2·26 2·61 3·00	26·7 53·3 73·3		
Mephenesin	F	P	286 321 360	13·3 66·7 73·3	102·1 ± 3·3	NS
		C	300 321 360	6·6 46·6 93·4		
	M	P	272 305 385 432	40·0 60·0 70·0 73·7	107·1 ± 28·4	NS
		C	272 305 385 432 485	50·0 65·0 50·0 50·0 80·0		
Gallamine triethiodide	F	P	12·1 13·6 15·2	26·7 73·3 73·3	118·1 ± 6·2	S
		C	15·2 17·0 19·0	46·6 73·3 86·7		
	M	P	15·2 17·0 19·0	33·3 60·0 85·8	123·8 ± 7·4	S
		C	17·0 19·0 21·2	21·4 46·7 53·3		
Benzimidazole	F	P	413 467 525 594	0·0 18·2 54·5 45·4	78·4 ± 10·1	NS
		C	467 525 594 670	54·5 72·7 91·0 100·0		
Decamethonium iodide	M	P	3·0 3·4 3·8	40·0 65·0 70·0	121·6 ± 10·4	S
		C	3·0 3·4 3·8 4·3	20·0 40·0 50·0 60·0		

* Significant or not significant from 100 per cent.

TABLE V

GROWTH OF MALE RATS ON A DIET CONTAINING MALATHION

Treatment	No. of days on test	No. of rats on test	Body weight mean ± S.E.M. (g.) Initial	Final	Gain	Food consumption (g./rat/day)	Food efficiency (g. gain/g. food consumed × 100)
Control diet ..	31	60*	107·5 ± 1·7	188·4 ± 1·8†	80·9	14·7	17·7
Control diet + malathion (500 p.p.m.) ..	31	60*	107·0 ± 1·5	196·0 ± 1·9†	89·0	14·4	19·9

* In 4 groups of 15 each. † $t = 2{\cdot}90$; $P < 0{\cdot}01$.

There was some indication of a decrease in the toxicity of benzimidazole, but the difference was not significant. There was no increase in the toxicity of mephenesin to the rats pretreated with aldrin.

Part II. Effect of Chronic Exposure to an Insecticide

Methods

Sixty male rats were divided into four equal groups, and fed a proprietary brand of laboratory diet. A further sixty male rats were similarly divided and fed a similar diet containing in addition 500 parts per million of malathion. All of the animals received water *ad libitum* and their respective diets for a period of thirty-one days. The rats were weighed once weekly, and the amount of food consumed in this time was recorded.

TABLE VI

THE TOXICITY OF SUXAMETHONIUM CHLORIDE TO RATS ON A DIET CONTAINING MALATHION

Pretreatment	Sex of rats	No. of rats per dose	Dose of relaxant (mg./kg.)	Mortality (per cent)	Toxicity to pretreated rats in terms of controls (per cent ± S.E.M.)	S* or NS
Control diet + malathion (500 p.p.m.) ..	M	15	2·05 2·30 2·60 3·00	26·7 20·0 40·0 46·7	98·0 ± 7·7	NS
Control diet ..	M	15	2·05 2·30 2·60 3·00	13·3 20·0 33·3 73·4		

* Significant or not significant from 100 per cent.

The diet containing malathion was prepared by dissolving an appropriate amount of 57 per cent commercial malathion in corn oil and mixing this solution thoroughly with the diet. The malathion in the diet constituted 500 p.p.m. of 95 per cent technical malathion, which is present in the commercial product at a concentration of 57 per cent. The corn oil used in this diet amounted to 5 per cent of the total. No corn oil was added to the control diet.

After thirty-one days on these diets, all rats received graded doses of suxamethonium by intramuscular injection. The deaths that occurred within one hour were recorded, and the data again treated statistically.

Results

Table V shows the effect of malathion on the body weights and food consumption of the experimental animals, and Table VI shows the effect of feeding malathion to rats on the toxicity of suxamethonium.

The rats that received the diet containing malathion were significantly heavier at the end of the experimental period than the rats that received the control diet; however, the food consumption was equal in both groups, resulting in an increased food efficiency for the group that received the insecticide. It is very likely that the increase in body weight was due to the corn oil in the diet and not to the malathion; however, it is interesting to note that Ball, Kay and Sinclair[20] reported an increase in weight in rats fed aldrin, although in this case there was an increase in food consumed.

Chronic feeding of 500 p.p.m. of malathion to the rats did not increase the toxicity of suxamethonium. This particular muscle relaxant was chosen, since it was shown previously (Table III) that a single injection of malathion greatly increased the toxicity of this relaxant to male rats.

Discussion

The administration of sub-lethal doses of parathion, malathion and aldrin to rats increased the toxicity of some muscle relaxants to these animals, presumably due to a lowering of cholinesterase levels by the insecticides. Davison[3] presented evidence that the organophosphorus compounds phosphorylate both true and pseudocholinesterase. He suggested that these compounds combine with an amino group of pseudocholinesterase, but with some other group in true cholinesterase. Fraser[15] reported that suxamethonium was hydrolysed *in vitro* by pseudocholinesterase, but not by true cholinesterase; however, he suggested that *in vivo* there are factors other than the plasma levels of pseudocholinesterase that determine the final effect of suxamethonium on the muscle. The deaths observed were due to cessation of respiration, and any reduction of cholinesterase levels by the insecticides would enhance the paralysing properties of suxamethonium, by decreasing the rate of destruction of the latter.

While only indirect evidence[10] has been given that aldrin is a cholinesterase inhibitor, these experiments with aldrin and the muscle relaxants agree with this evidence.

Pure parathion does not have anticholinesterase activity *in vitro*[6,7], but is converted to a compound having this activity when administered *in vivo*[4,7,8]. Gage[8] reported that an extract of liver, from rats treated with parathion, contained a substance identical with paraoxan by chromatographic analysis. The total amount of active substance extracted from the rat liver was about 0·1 per cent of the parathion administered. Other organophosphorus compounds, such as tetraethyl pyrophosphate, have anticholinesterase activity without undergoing conversion *in vivo*[4]. The authors have not seen any evidence for conversion of malathion to another active anticholinesterase compound.

The organophosphorus compounds are gradually destroyed by enzymes in the body. Aldridge[21] reported that rabbit serum contains an esterase

that hydrolyses diethyl *p*-nitrophenyl phosphate (E 600) rapidly, while rat serum hydrolyses it slowly. This might be a possible explanation for the prolonged action of these compounds in rats, although the rate of recovery of cholinesterases must also be taken into consideration.

Lucas and Miles[16] performed experiments similar to those described herein. Sarin, an organophosphorus compound, increased the period of respiratory paralysis in monkeys induced by suxamethonium, and decreased the periods of respiratory paralysis induced by tubocurarine and gallamine[16]. The latter observations might be attributed to a lowering of the true cholinesterase levels. Sarin had no effect on the respiratory paralysis produced by decamethonium. Lucas and Miles[16] administered Sarin at a level of 2/3 of the LD50, whereas, in the experiments in this paper the insecticide was administered at a level of approximately $\frac{1}{4}$ of the LD50. This latter dose of insecticide is considerably less than the former; therefore, it is possible that only the pseudocholinesterase levels were lowered appreciably, and true cholinesterase levels were not affected. Hazleton[11] has reported that plasma cholinesterase and red blood cell cholinesterase (true cholinesterase) are affected by different amounts of insecticide, depending on the insecticide chosen. This might explain the discrepancy in results obtained in these two laboratories with tubocurarine and gallamine.

In the present series of acute experiments, there was some discrepancy between the toxicity of a muscle relaxant to rats pretreated with different insecticides. For example, the toxicity of gallamine was increased by pretreatment of the animals with malathion and aldrin, but not by pretreatment with parathion, and mephenesin was more toxic to rats pretreated with malathion than those pretreated with aldrin. A possible explanation is that cholinesterase levels were not lowered to the same extent by the three insecticides, even though they were given at a dose in the same ratio to the LD50. It is also very likely that other cholinesterases in the body, as well as other enzyme systems may be adversely affected to a different extent by these insecticides. Denny and Hagerman[22] extracted a cholinesterase from human leucocytes that behaved in a similar fashion to the true cholinesterase of red blood cells and brain. The direct action of the cholinesterases and other enzymes on the muscle relaxants is not too well known except in the case of suxamethonium[15].

The toxicity of benzimidazole was significantly reduced in rats pretreated with malathion but not in those pretreated with aldrin. No explanation is evident at present for this observation.

Chronic feeding of 500 p.p.m. of malathion to rats did not increase the toxicity of suxamethonium to the experimental animals. Hazleton[11] reported that chronic feeding of malathion to rats for two years at a level of 100 p.p.m. had no effect on cholinesterase levels of plasma, red cells and brain, but at 1000 p.p.m. these levels were moderately depressed. Therefore, it was felt that a level of 500 p.p.m. might have a slight effect on cholinesterase levels. However, in this experiment, this level of malathion was apparently not sufficient to increase the toxicity of this relaxant.

There is the possibility that excessive exposure to these insecticides,

sometimes experienced by spray applicators and formulators of the insecticides might be sufficient to augment the toxicity of some of the muscle relaxants, should they be used on the individual. Lucas and Miles[16] have warned of this possibility, and Hansson[23] has also warned of the possible hazard to farm animals exposed to a sprayed field, and subsequently treated in surgery with a relaxant. Gage[9] has suggested that a decrease in cholinesterase in man of more than 40 per cent of the population average should be regarded as a reason for taking appropriate action.

Summary

1. Parathion, malathion and aldrin were administered to rats in sublethal doses, followed by graded doses of various muscle relaxants eighteen to twenty-four hours later. It was found that pretreatment with these insecticides increased the toxicity of some of the relaxants to the animals, had no effect on the toxicity of some, and apparently decreased the toxicity of one relaxant.

2. The change in toxicity of the relaxants was believed to be due to lowered levels of cholinesterases and possibly other enzymes in the rats when pretreated with these insecticides.

3. Feeding malathion at a level of 500 p.p.m. in the diet to rats for thirty-one days had no effect on the toxicity of suxamethonium chloride to these animals.

4. The possible hazard of insecticide-relaxant interaction to humans and farm animals has been discussed.

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ESTIMATION OF CHLORAMPHENICOL CINNAMATE

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Chloramphenicol cinnamate is a yellowish-white crystalline powder free from the bitter taste of chloramphenicol, and therefore of particular value for administration to young children. It may be prepared by reacting chloramphenicol with cinnamyl chloride. It is almost insoluble in water, but readily soluble in ethanol and in ethyl acetate. It has a melting point of 115° to 116°, and an optical rotation $[\alpha]_D$ of +55·0° to +58·0°. It is without antibacterial action *in vitro* and cannot therefore be assayed microbiologically.

Chloramphenicol cinnamate is administered in the form of an aqueous suspension ("Alficetyn" Suspension) and although the potency of this preparation when freshly made can be satisfactorily controlled by chemical methods, for example by the polarographic method of Hess[1], or by the colorimetric method of Bessman and Stevens[2], these may give misleading results when used to determine the potency of samples after storage, since certain degradation products containing a nitro group, such as 1-(*p*-nitrophenyl)-2-amino-propane-1 : 3-diol, are indistinguishable polarographically from chloramphenicol itself. Furthermore, chloramphenicol cinnamate cannot be converted into free chloramphenicol as a preliminary to microbiological assay by chemical methods of hydrolysis, as the reaction does not cease with the formation of chloramphenicol, the dichloracetyl radical also being eliminated, giving 1-(*p*-nitrophenyl)-2-aminopropane-1 : 3-diol. It seemed probable, therefore, that only an enzymic method of hydrolysis would be likely to convert chloramphenicol cinnamate quantitatively to chloramphenicol.

Similar difficulties have been reported in the assay of chloramphenicol palmitate and stearate, two other tasteless chloramphenicol derivatives which cannot be assayed microbiologically until converted into chloramphenicol. Glazko, Edgerton, Dill and Lenz[3] found that the palmitate could be quantitatively converted into chloramphenicol by incubation with a bacterial lipase preparation. Trolle-Lassen[4], using a similar method, obtained satisfactory results with the stearate. Both groups of workers reported incomplete hydrolysis with pancreatic and intestinal enzyme preparations, whilst Glazko and others[3] obtained the following figures for the percentage of chloramphenicol palmitate hydrolysed after incubation with different rat tissue extracts: liver, 10; kidney, 8; spleen, 5; duodenal contents, 95·6.

We examined the effect on chloramphenicol cinnamate of several enzyme preparations, namely, saliva, pancreatin, soya bean lipase, castor oil bean lipase, and rat liver extract. When incubated for 4 or 24 hours at 37° with saliva or pancreatin the resulting solution gave no zones of inhibition in the cup plate test, using *E. coli* or *Sarcina lutea* as test

organism. Treatment with ground soya bean powder produced only partial hydrolysis, 10 mg. of chloramphenicol cinnamate after incubation with 5 g. of soya bean meal in 100 ml. of water for 24 hours at 37° with stirring, giving a response equivalent to only 30 per cent of the theoretical amount of chloramphenicol when estimated by means of *Sarcina lutea*. Castor oil bean powder gave somewhat better results, 10 mg. of chloramphenicol cinnamate after incubation with 2 g. of castor oil bean powder in 100 ml. of water for 24 hours at 37° giving a response equivalent to 50 per cent of the theoretical amount of chloramphenicol. When the amount of castor oil bean powder was doubled the value was increased to 60 per cent after 24 hours incubation, to 80 per cent after 48 hours incubation, and to 90 per cent after 72 hours incubation. Thus chloramphenicol cinnamate would not appear to be so readily hydrolysed by lipase as are chloramphenicol palmitate and stearate.

On the other hand, chloramphenicol cinnamate, unlike the palmitate or stearate, is readily hydrolysed by rat liver extract, and satisfactory results were eventually obtained by this means. An ethanolic solution of chloramphenicol cinnamate was added to the rat liver extract, and the mixture was stirred continuously at 37°. Lower results were obtained if the stirring was only intermittent. Incomplete hydrolysis was also obtained with livers from rats that had been used for determining the toxicity of drugs, so that only livers from healthy rats should be used.

Assay of Chloramphenicol Cinnamate

One gram of fresh liver from a healthy rat (Wistar strain) was ground in a pestle and mortar and extracted with sterile distilled water to give a volume of 94 ml. Ten mg. of chloramphenicol cinnamate were dissolved in 4 ml. of absolute ethanol and the solution was added to the rat liver extract giving a colloidal solution. After adjusting the pH to 7·2 with 0·1N sodium hydroxide, the volume was made up to 100 ml. with more sterile distilled water, and 1 ml. of chloroform was added as a preservative. The mixture was immersed in a water bath at 37° and stirred continuously for 24 hours. At the end of this time the solution was assayed by the cup-plate method using *Sarcina lutea* as the test organism, and chloramphenicol as a standard.

Method

Test organism. *Sarcina lutea* NCTC 8340 was grown for 24 hours at 30° on nutrient agar slopes (Medium I) stored at 5° and renewed at monthly intervals.

Inoculum. The growth from a slope culture (Medium I) incubated at 30° for 24 hours was washed into quarter-strength Ringer solution and the suspension was adjusted to an opacity equivalent to the Wellcome Standard opacity tube No. 4. A 0·6-ml. portion was then added to 250 ml. of plate medium (Medium II).

Preparation of plates. The required amount of medium was melted by steaming, cooled to 50° and the inoculum was then added. Fifteen ml. quantities were transferred to standard 4-inch Petri dishes resting on a

level surface; when the agar had set, the plates were removed to a refrigerator. When sufficiently cooled, six 5-mm. diameter discs were cut out of each plate using a sterile cork-borer, and the plates were then returned to the refrigerator until required.

Standard and test solutions. Twenty mg. of pure chloramphenicol were dissolved in 2 ml. of ethanol and the solution was made up to 25 ml. with a phosphate buffer solution (Medium III). Dilutions were made with phosphate buffer solution to give solutions containing 20 and 40 μg. per ml. of chloramphenicol. The test solution was diluted 1:2 and 1:4 with buffer solution to give a "2 and 2" dose assay[5].

Media

Medium I. Nutrient Agar

Eupepton No. 2 (A & H)	10 g.
Sodium chloride	5 g.
Lab Lemco	10 g.
Agar	15 g.
Distilled water to	1000 ml.

Dissolve by steaming and adjust the pH to 7·2. Sterilise in 2-oz. McCartney bottles by autoclaving for 30 minutes at 10 lb. pressure.

Medium II. Plate Agar

Eupepton No. 2 (A & H)	6 g.
Lab Lemco	1·5 g.
"Yeastrel"	3 g.
"Cerelose" (Dextrose)	1 g.
Agar	2·0 g.
Distilled water to	1000 ml.

Dissolve by steaming, adjust the pH to 7·0 and autoclave for 30 minutes at 10 lb. pressure.

Medium III. Phosphate Buffer Solution

Dipotassium hydrogen phosphate	7·3 g.
Dihydrogen potassium phosphate	3·4 g.
Distilled water to	1000 ml.

Adjust to pH 7·0 and sterilise by autoclaving for 30 minutes at 10 lb. pressure.

Filling the plates. A "set" of four plates was removed from the refrigerator, and any fluid in the holes was removed by suction. Sufficient of the diluted standard and test solutions were put into the holes to fill each to the same level, with a slightly concave meniscus. The filler used for this purpose consisted of a 3-inch length of narrow bore glass tubing, with a $\frac{1}{4}$-inch platinum tube, internal diameter 0·0295 inch, external diameter 0·0365 inch, fused into the tip at an angle of 130°, and a rubber teat at the other end. The tube was rinsed between each change of sample, first with phosphate buffer solution and then with the next

sample to be filled. After being filled, each "set" of plates was incubated for 18 to 20 hours at 30°.

Evaluation of Results

After incubation, the diameters of the zones of inhibition were measured by means of calipers, and the amount of chloramphenicol, in μg. per ml., in the test solution was calculated.

The method of calculation is illustrated by the following example, in which solutions of chloramphenicol cinnamate containing approximately 10 mg. per 100 ml. were assayed (Samples P and Q). The zone diameters, less 12 mm. to simplify the arithmetic, of each sample are recorded, as in Table I (A), S_L and S_H representing the low and high doses of standard,

TABLE I

POTENCY AND FIDUCIAL LIMITS OF CHLORAMPHENICOL CINNAMATE SOLUTION SAMPLES P AND Q

The solutions were diluted 2- and 4-fold, and the standard solutions contained 40 and 20 μg. per ml. of chloramphenicol

(A)

S_L	P_H	Q_L	S_H	P_L	Q_H	Total
1·5	5·0	0·5	5·5	0·5	5·5	18·5
1·0	5·0	1·0	5·5	0·5	6·0	19·0
0·5	5·0	0·0	5·5	0·5	5·0	16·5
1·5	5·0	0·5	5·5	0·5	5·0	18·0
4·5	20·0	2·0	22·0	2·0	21·5	72·0

(B)

	L	H	Total	L_1	L_P
S	4·5	22·0	26·5	17·5	
P	2·0	20·0	22·0	18·0	−4·5
Q	2·0	21·5	23·5	19·5	−3·0
Total	8·5	63·5	72·0	55·0	

and P_L, P_H, Q_L and Q_H the low and high doses of the two samples P and Q. The sums of the responses are then computed and used to calculate L_1, the differences between the high and low dose totals, and L_P, the values of $\Sigma P - \Sigma S$ and $\Sigma Q - \Sigma S$. These values are recorded in Table I (B).

TABLE II

ANALYSIS OF VARIANCE

Correction Term: 216·0

Source	S. of S.	d.f.	M.S.	F.	P.
Regression	126·0417	1			
Preparations	1·3125	2	0·65625	7·62	<0·01 H.S.
Parallelism	0·2708	2	0·1354	1·57	>0·05 N.S.
Doses	127·6250	5			
Plates	0·5833	3			
Error	1·2917	15	0·0861		
Total	129·5	23			

An analysis of variance is then carried out to test the significance of the regression and the absence of lack of parrallelism. The results are given in Table II. The "Preparations" term is normally small, but in the present example was highly significant, indicating that the samples were not diluted to precisely the same range as the standard. Since the

"Parallelism" term was not significant, however, the assays are regarded as valid. The breakdown of the doses sum of the squares into "Regression", "Preparations" and "Parallelism" terms is best accomplished by the use of detached coefficients of orthogonal contrast[6] and the breakdown of the total sum of the squares into "Doses", "Plates" and "Error" terms by the normal rows and columns analysis. The sums of the squares are calculated as follows:

$$\text{Regression} = \frac{(\Sigma L_1)^2}{24} = 126{\cdot}0417$$

$$\text{Preparations} = \frac{(\Sigma S)^2 + (\Sigma P)^2 + (\Sigma Q)^2}{8} - \text{Correction term} = 1{\cdot}3125$$

$$\text{Parallelism} = \frac{(\Sigma L_1 S)^2 + (\Sigma L_1 P)^2 + (\Sigma L_1 Q)^2}{8} - \text{Regression S of S} = 0{\cdot}2708$$

The potency of Sample P and the fiducial limits are calculated as follows:

Log ratio of doses, $I = 0{\cdot}3010$

Error mean square, $s^2 = 0{\cdot}0861$

$$\text{Slope, } b = \frac{\Sigma L_1}{12} = 4{\cdot}583$$

$$\text{For sample P, } M' = \frac{L_p}{8b} = -0{\cdot}12272$$

Log potency ratio, $M = M'.I = -0{\cdot}0369 = \bar{1}{\cdot}9631$

Ratio of potencies, $R = \text{antilog } M = 0{\cdot}9185$

The approximate 95 per cent fiducial limits are given by $M \pm t.s_M$ (t has 15 d.f. and equals 2·131) and $t.s_M = \frac{t.I}{b}\sqrt{S^2\left(\frac{1}{4} + \frac{M'^2}{6}\right)} = 0{\cdot}0206$

(The index of significance (g) of the slope b is invariably less than 0·1 in this assay and can be ignored)

whence log potency ratios, M_L and $M_U = \bar{1}{\cdot}9425$ and $\bar{1}{\cdot}9837$

and ratios of potencies, R_L and $R_U = 0{\cdot}8760$ and $0{\cdot}9632$

equivalent to 95·4 and 104·8 per cent.

Potency = $R \times S_H \times$ dilution of $P_H = 73{\cdot}5$ μg. of chloramphenicol per ml. with limits of 70·1 to 77·0 μg. of chloramphenicol per ml.

The potency of sample Q and the fiducial limits, similarly computed, were found to be 75·6 μg. of chloramphenicol per ml. with limits of 72·1 to 79·2 μg. of chloramphenicol per ml.

Assay of Suspension of Chloramphenicol Cinnamate containing 4·5 *per cent w/v*

To 5 g. of the suspension were added 50 ml. of absolute ethanol and the mixture was thoroughly shaken and allowed to stand. Two ml. of the clear supernatant liquid were then removed and added to 90 ml. of

an extract prepared from 1 g. of finely ground rat liver. The pH was adjusted to 7·2 and the volume was made up to 100 ml. with sterile distilled water. Two ml. of chloroform were added as preservative and the suspension was immersed in a water bath at 37° and stirred continually for 24 hours. The solution was assayed as described above against *Sarcina lutea*.

Summary

1. Chloramphenicol cinnamate cannot be assayed microbiologically until converted into free chloramphenicol. Most hydrolytic agents examined either give incomplete hydrolysis or hydrolyse the substance beyond the chloramphenicol stage. Quantitative conversion to chloramphenicol was however achieved by incubation with rat liver extract.

2. A method of assaying chloramphenicol cinnamate and its suspensions is described.

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COLORIMETRIC ESTIMATION OF DIHYDROSTREPTOMYCIN AND STREPTOMYCIN

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The pharmacopoeias have recommended microbiological methods for estimating the potency of streptomycin and dihydrostreptomycin. A number of chemical methods have been described in literature[1–7], and of these, one estimates both streptomycin and dihydrostreptomycin in a combined form[2]. It consists of measuring the red colour produced by a mixture of these antibiotics with α-naphthol and sodium hypobromite in an alkaline medium under ice-cold conditions. We observed that (i) it is not possible to measure optical density at such a low temperature due to condensation of moisture on the sides of the cell; (ii) due to addition of urea, nitrogen gas is formed which interferes with the quick and accurate measurement of the optical density; the colour produced is not stable and (iii) the limit of concentration up to which Beer's law is followed is too low (0·4 mg. of the substance). With the help of a modified method, which is quick and reliable, estimates of samples of dihydrostreptomycin have been found to compare favourably with the figures obtained by the standard microbiological method of assay described in the British Pharmacopoeia 1953.

Experimental

Reagents. 10 per cent NaOH solution, 0·02 per cent α-naphthol solution in absolute ethanol, carbon tetrachloride (B.P.), absolute ethanol (B.P.), and sodium hypobromite.

The sodium hypobromite reagent was prepared by dissolving 20 g. of NaOH in 75 ml. of distilled water, cooling and adding 5 ml. of bromine; after solution, distilled water was added to 100 ml., 10 ml. of this was diluted to 100 ml. with distilled water.

Preliminary experiments. To remove the difficulties encountered in the procedure recommended by Buch and colleagues[2], excess bromine was removed by organic solvents. Ten ml. of carbon tetrachloride for each reaction helps to stabilise the colour; but the absorption values obtained with the aqueous layer, though found to observe Beer's Law, were slightly low, indicating that the reaction product might be partially retained by the carbon tetrachloride. Also the aqueous layer was slightly turbid, but this could be clarified with an equal volume of absolute ethanol which also restored the colour to the carbon tetrachloride layer.

Minimum percentage transmission. The minimum percentage transmission was obtained at 530 mμ and was found to remain stable for about 10 minutes after the addition of NaOBr reagent. Percentage transmissions of blank, three concentrations of the standard and one sample can be measured well within this period.

Procedure. Solutions of dihydrostreptomycin or streptomycin (known standards) were made in distilled water, the strengths of the different solutions varied from 0·25 to 2·5 mg./ml. One ml. of each solution was taken in a 50 ml. stoppered cylinder; 1 ml. of 10 per cent NaOH and 2 ml. of α-naphthol were added to each cylinder. The contents of the cylinder were shaken and allowed to stand for 15 minutes; 1 ml. of NaOBr reagent was then added and within half a minute 10 ml. of carbon tetrachloride was added; the contents were again shaken well; 5 ml. of absolute ethanol was subsequently added to each cylinder and the contents shaken well. The liquids were allowed to separate; without disturbing the layers, a portion of the aqueous layer from each was pipetted into 1 cm. cells and the percentage transmission measured by using a blank (prepared in the same way as the standard with 1 ml. of distilled water in place of the sample) kept at 100 per cent transmission at 530 mμ. The entire procedure was carried out at room temperature (30°).

This procedure was employed for estimating the potency of samples of dihydrostreptomycin by intrapolating the percentage transmission values of the samples with a standard curve drawn from values obtained with known strengths of a standard preparation. The microbiological method of assay employed for estimating the potency of the samples is that of the British Pharmacopoeia 1953.

Results and Discussion

Beer's Law. Percentage transmission of 0·5, 1·0, 1·5, 2·0 and 2·5 mg./ml. of dihydrostreptomycin was measured using the same reagents. Values obtained are given in Table I. Six estimations were made for each concentration. Similarly, 0·5, 0·75, 1·0, 1·25 and 1·5 mg./ml. of streptomycin sulphate was taken and the values of percentage transmission in relation to these are also recorded in Table I. The reaction was found to follow Beer's Law up to a concentration of 1·5 mg./ml. of dihydrostreptomycin and streptomycin sulphate.

TABLE I

PERCENTAGE TRANSMISSION OF THE TWO ANTIBIOTICS IN MG./ML. CONCENTRATIONS (AVERAGE OF SIX READINGS)

Dihydrostreptomycin sulphate mg.	Per cent transmission	Streptomycin sulphate mg.	Per cent transmission
0·5	92·5	0·5	94·0
1·0	84·8	0·75	91·05
1·5	77·5	1·0	88·0
2·0	71·25	1·25	85·1
2·5	65·5	1·5	82·15

After establishing the range of concentration within which the reaction follows Beer's Law, values of percentage transmission were obtained with 0·25, 0·50, 0·75, 1·0, 1·25 and 1·50 mg./ml. of dihydrostreptomycin sulphate. Three estimations were made for each concentration using different sets of reagents on different days. Although the values of percentage transmission obtained on different days were found to follow Beer's Law

independently, they tended to differ slightly among themselves. It is, therefore, advisable to draw a fresh standard curve, whenever this method is to be employed for the estimation of dihydrostreptomycin or streptomycin sulphate.

Comparison of Proposed Colorimetric Method with the B.P. Bioassay Method

To assess the suitability of the colorimetric method for the estimation of the potency of samples of dihydrostreptomycin, six samples of dihydrostreptomycin sulphate manufactured by a reputable firm were selected; these samples were found to conform to the B.P. test for "limit of streptomycin".

TABLE II

COMPARATIVE FIGURES OF DIHYDROSTREPTOMYCIN CONTENT BY THE COLORIMETRIC METHOD AND THE B.P. MICROBIOLOGICAL METHOD

Sample No.	Dihydrostreptomycin sulphate content units/mg.		Deviation from the microbiological method, per cent
	By the suggested method	By B.P. microbiological method	
1	658	675	−2·5
2	665	663	+0·3
3	675	673	+0·3
4	658	670	−1·8
5	658	661	−0·45
6	658	681	−3·4

The potencies of these samples were estimated by intrapolating the percentage transmission values, obtained with a known concentration of the sample with a standard curve drawn with the percentage transmission values plotted against known potency of the standard in terms of units/mg. The same samples were assayed by the microbiological method of assay and the results obtained by the colorimetric method compared with those obtained by the microbiological method. These results are tabulated in Table II. From this, it will be seen that there is good agreement between the values obtained by the proposed colorimetric and the standard microbiological methods; the deviations in values between the two methods fall within a narrow range of experimental error.

SUMMARY

1. A colorimetric method for the estimation of dihydrostreptomycin and streptomycin sulphate has been described.

2. The values obtained compare favourably with those of the B.P. microbiological assay.

3. As the colour reaction is equally applicable both to streptomycin and dihydrostreptomycin it has been recommended that in the case of samples of dihydrostreptomycin sulphate, the proposed colorimetric method should be used in conjunction with the B.P. test standard for the limit of streptomycin in dihydrostreptomycin.

Guidance during the course of this investigation from Dr. T. S. Subramanian is gratefully acknowledged.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Barbiturates, Detection and Identification of. L. Levi and C. E. Hubley. (*Analyt. Chem.*, 1956, **28**, 1591.) Advantage is taken of the formation of the characteristic dark purple-coloured complexes when barbiturates react with aqueous copper sulphate-pyridine solutions. The sensitivity is increased when the copper sulphate-pyridine ratio in the reagent is increased so that as little as 0·1 mg. would form a precipitate on standing. The melting of the complexes is accompanied by decomposition but the melting points are sharp enough to be used as diagnostic features. The compounds have the general formula $(Bb)_2Cu(Py)_2$ where Bb is the barbituric acid ion and Py is pyridine. Infra-red curves of a number of complexes are compared with those of the parent barbiturates, and the curves of both the barbiturates and their complexes show unique features throughout the region 4000 to 650 $cm.^{-1}$, and hence the method affords a high degree of specificity for the detection and characterisation of these drugs.

D. B. C.

Chlortetracycline and Tetracycline, Simultaneous Determination of. J. Doskočil. (*Československ. Farm.*, 1956, **6**, 321.) A sample of a mixture of chlortetracycline and tetracycline is dissolved in water to give a concentration of 1 mg./ml. and this solution is diluted 10-fold with (*a*) 0·2M trisodium phosphate solution, and (*b*) 0·2M phosphate buffer solution having a pH of 6·0. The solutions are set aside for 30 minutes at 20° to 25°; the chlortetracycline in (*a*) decomposes almost completely while the tetracycline remains practically unchanged. Both samples are then boiled with 2N HCl for 5 minutes to convert the antibiotics to their anhydro derivatives, and the extinction of the solutions is measured at 440 mμ. A control experiment is carried out to determine the degree of decomposition of pure tetracycline and chlortetracycline under the same conditions and the amount of each present in the test sample can then be calculated.

E. H.

Neomycins B and C in Neomycin Sulphate, Determination of. A. A. Brooks, A. A. Forist and B. F. Loehr. (*Analyt. Chem.*, 1956, **28**, 1788.) Three methods of simultaneous assay are compared. In the first method, the optical rotation of a solution is measured, an aliquot part is then taken and degraded with sulphuric acid under strictly controlled conditions of time and temperature to split off furfural or a derivative of it the absorption of which is measured. From the known behaviour of pure neomycins B and C under these conditions and their optical rotations, two simultaneous equations may be built up from the data obtained from a mixture, and solved to find the percentage of each in the mixture. Formulae are included. The second method depends upon the variations of the optical rotations of the two isomers with temperature, and consists of measuring the rotation of a solution at two different temperatures. In the third method, the optical rotation is measured as in method I and a neutral equivalent is found on an aliquot of the same solution by mixing with an excess of saturated barium hydroxide solution and back-titrating with standard

sulphuric acid. The total neomycin is calculated from the normality (N) while the percentage of B is read from a plot of $[\alpha]_D^{25}/N$ against percentage B, which was shown to be linear. The quantities required are large for the measurement of optical rotation, viz., 5 g. of neomycin sulphate in 25 ml. of 0·1N sulphuric acid. The first method is preferred and has an accuracy on pure mixtures within about 1 per cent on total neomycin, 3 per cent on neomycin B and up to 10 per cent on neomycin C (when small percentages of C are involved).

D. B. C.

Phenothiazine Derivatives, Polarimetric Determination of. J. Blažek. (*Československ. Farm.*, 1956, **4**, 210.) Phenothiazine derivatives are determined by titration with silicotungstic acid, the end point being determined polarimetrically; in solutions containing a suitable amount of hydrochloric acid the derivatives form an almost insoluble precipitate with this reagent. For the determination of promethazine hydrochloride in tablets, a weighed sample of ground tablets, containing 20 to 50 mg. of promethazine, is placed in a 100-ml. beaker and 1 ml. of 35 per cent solution of HCl is added; the mixture is diluted to 20 ml. with water and titrated against 0·01M silicotungstic acid. A Heyrovsky type polarograph with a galvanometer having a sensitivity of 4×10^{-9} is used to determine the end point; the test-solution is connected to a saturated calomel electrode, forming the anode, by means of a potassium nitrate solution bridge, and a dropping-mercury electrode is used as the cathode. An E.M.F. of 0·65 V is supplied by a 2-volt accumulator with a potential divider. With this E.M.F. an excess of silicotungstic acid gives a diffusion current. Other phenothiazine derivatives are determined in the same way. Results on various proprietary preparations are within ± 4 per cent of those calculated from Kjeldahl nitrogen determinations.

E. H.

Pyrethrins, New Colorimetric Method of Estimation of. H. L. Williams, W. E. Dale and J. P. Sweeney. (*J. Assoc. off. agric. Chem., Wash.*, 1956, **39**, 872.) This method claims greater sensitivity (down to 7 μg. of pyrethrins), greater reproducibility (2 per cent) and greater specificity than methods heretofore used. The colour-developing reagent used was a mixture of 80 per cent by volume of 85 per cent orthophosphoric acid and 20 per cent by volume of reagent-grade ethyl acetate. The standard curve was prepared using a solution containing 25 μg./ml. of pyrethrins in a hydrocarbon fraction b.p. 30 to 60°, prepared from a pyrethrum concentrate analysed by the A.O.A.C. method. Suitable volumes were carefully evaporated to dryness on a water bath, 5 ml. of colour reagent added, and the resulting solutions shaken for 1 minute mechanically, and immersed in a boiling water bath for exactly 3 minutes. After transference to suitably matched test-tubes, the solutions were centrifuged for 15 minutes at a medium speed and the absorbance at 550 mμ read against a blank of 5 ml. of colour reagent. Samples of cotton wool etc. containing pyrethrins were extracted with the hydrocarbon solvent and treated similarly, the blank being prepared by carrying a sample of untreated material, equivalent in weight to that of the sample being tested, through the analytical procedure. When materials such as grains, burlap and cardboard contained substances which suppressed the pyrethrum colour, standards were prepared by the addition of a known amount of pyrethrin standard to extracts of untreated samples. The colour produced in the reaction was stable indefinitely and pyrethrins I and pyrethrins II were found to give colours which had identical absorbtion spectra. Good recoveries of pyrethrins from wool, kraft paper, botany wool, muslin cloth and raisins were obtained, the errors being from about 3 to 6 per cent.

A few substances (e.g. piperonyl butoxide) used as synergists suppressed the colour reaction when present in large concentration compared with that of the pyrethrins. Chromatographic methods of separation enabled good recoveries of pyrethrins to be obtained. D. B. C.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Anticholinesterase Activity of Ethyleneimines and certain other Cytotoxic Agents. K. Bullock. (*Biochem. J.*, 1956, **63**, 484.) A method for the examination of drugs for antiacetylcholinesterase activity at pH 6·3 by the use of insoluble erythrocyte envelopes (stromata) is described. Suspensions of stromata, as a source of acetylcholinesterase, are mixed with inhibitor solutions either with (pH 8·3) or without (pH 6·3) added sodium bicarbonate, maintained at 37° for times varying between 2 and 3 hours, and the residual acetylcholinesterase (AChE) activity determined. *S*-Mustard ($CH_2Cl.CH_2.S.CH_2CH_2Cl$) is shown not to be a potent inhibitor of AChE. The ethyleneimines, TEM, CB 1263, CB 1289 and the nitrogen mustard CB 1348, unlike HN 2, were considerably more active at pH 6·3 than at 7·4 or 8·3. It is pointed out in this connection that cancerous tissues, unlike normal tissue, produce large quantities of lactic acid, and injections of glucose can be used to reduce the pH of such tissues to around 6·3. The anticholinesterase activity of the ethyleneimines is progressive and irreversible. Only very small quantities of TEM are taken up by erythrocyte stromata. J. B. S.

Reduced Diphosphopyridine Nucleotide Derivative, Isolation and Properties of. S. Chaykin, J. O. Meinhart and E. G. Krebs. (*J. biol. Chem.*, 1956, **220**, 811.) A reduced diphosphopyridine nucleotide derivative, DPNH-X, the formation of which from reduced diphosphopyridine nucleotide (DPNH) is catalysed by triose phosphate dehydrogenase, has been isolated and its structure and properties studied. A high concentration of triose phosphate dehydrogenase is used in the formation of the derivative, the reaction being buffered at pH 6·0, rather than at the optimum of 5·0, because of the instability of the product at lower pH. Incubation at 25° was continued until 85 per cent of the ultra-violet absorption at 340° had disappeared. More prolonged incubation gave a product contaminated with an acidic substance. The product was isolated as the barium salt by precipitation with barium bromide and absolute ethanol at −15°. DPNH-X shows an absorption maximum at 265 mμ, and the point of maximum difference between the absorption of DPNH-X and DPN or DPNH is approximately 290 mμ. Analysis of DPNH-X corresponds to a compound with the composition $C_{21}H_{27}O_{14}N_7P_2Ba.4H_2O$ and of the same general structure as DPNH. There is no loss of nitrogen, or addition of phosphate, and all the various moieties of the DPNH structure are retained. On paper electrophoresis at pH 8·0 DPN, DPNH and DPNH-X migrated as single spots 0·70, 2·55 and 2·75 cm. respectively towards the positive electrode, indicating that the net charge on the molecule is the same as that for DPNH. DPNH-X has a spectrum almost identical with that of the primary acid modification product formed in the acid-catalysed reaction of DPNH at pH 4·0, though differences are apparent with the lower peak of the latter at 265 mμ, and an elevated shoulder at 290 to 300 mμ. DPNH-X is actually converted to the primary acid modification product by incubation at pH 3·0 to 4·0, but it has not been detected as an intermediate in the reaction of DPNH with acid.

Catalytic hydrogenations suggest that DPNH-X and the primary acid modification product each contain only one readily reducible double bond, compared with three in DPN, and two in DPNH. J. B. S.

Thiamine (Aneurine), a New Antagonist of. T. L. V. Ulbricht and J. S. Gots. (*Nature Lond.*, 1956, **178**, 913.) 4-Methyl-5-(2-hydroxyethyl)-*N*-(4-amino-2-methyl-thiopyrimidyl-(5)-methyl)-thiazolium chloride hydrochloride has been prepared, and found to be an inhibitory antagonist of thiamine in *E. coli*. The molar ratio of competition (inhibitor/thiamine) was of the order of 100. G. F. S.

BIOCHEMICAL ANALYSIS

Alkaloids in Biological Material, Determination of, by Compound Formation with Indicators. Z. I. El Darawy and S. L. Tompsett. (*Analyst*, 1956, **81**, 601.) A method is described for the determination of alkaloids in urine, plasma and tissues, e.g., liver and muscle, based upon the formation of a compound with an acidic indicator which is soluble in an organic solvent. An aliquot part of the organic solvent containing the base-indicator complex is then taken, and the latter decomposed with an aqueous solution of a strong acid or base. The absorption is then determined with a suitable spectrophotometer against a blank. When the amount of alkaloid is small, concentration is best achieved by passing the urine, diluted plasma, or tissue extract, adjusted to a suitable pH, through a column of Florisil, a synthetic silicate obtainable in various standard particle sizes (60 to 100 mesh used in this work) from the Floridin Co., U.S.A. After elution with a solution of 5 per cent sodium carbonate in 75 per cent ethanol and acidification, the ethanol is removed completely by evaporation to dryness and the assay performed on a solution of the residue, using the bromothymol blue-benzene procedure:—A suitable volume is adjusted to pH 8·0 to 8·5, extracted with benzene, centrifuged, and an aliquot portion of the benzene layer shaken with a buffered (pH 7·4) bromothymol blue solution. After further centrifuging another aliquot of the benzene layer is shaken with a definite quantity of 0·1N sodium hydroxide solution. Readings on the coloured aqueous solution are taken against a blank at 510 mμ. Without preliminary concentration, recoveries of strychnine from about 90 to 100 per cent were obtained from urine and plasma containing 5 μg./ml., and from liver and muscle containing 10 μg./ml. With preliminary concentration, amounts of the order of 0·5 μg./ml. in urine and 2 μg./ml. in plasma and liver could be determined. Further work is described using paper chromatography to increase the specificity of the method, strychnine and brucine being successfully separated and assayed. The recoveries were almost quantitative. D. B. C.

Glutethimide and a Metabolite in Dog Urine, Detection of. H. Sheppard, B. S. D'Asaro and A. J. Plummer. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 681.) The method is based on the reaction of glutethimide or α-phenylglutarimide with hydroxylamine to form a hydroxamic acid derivative, which yields a purple coloured complex with ferric ions. A sample of urine is treated so as to obtain a residue containing the glutarimide derivative. This is dissolved in 1 ml. of dehydrated methanol and 1 ml. of 2M hydroxylamine hydrochloride and 1 ml. of 3·5N sodium hydroxide added. After allowing the solution to stand for 30 minutes, 1 ml. of 3·5N hydrochloric acid and 1 ml. of 0·37M ferric chloride in 0·1N hydrochloric acid are added, and the light absorption is measured in a colorimeter with a suitable filter. Known amounts of glutethimide or α-phenylglutarimide are added to urine samples which are treated in

the same way, a standard curve being prepared from which the assay results are calculated. The conditions specified have been shown experimentally to be the most satisfactory. The colour reaction may also be used to indicate the position of the substances on paper chromatograms. When glutethimide is administered orally to dogs, it is excreted in the urine in the form of α-phenylglutarimide and a conjugated form which yields α-phenylglutarimide on acid hydrolysis. The free α-phenylglutarimide reaches its maximum rate of excretion later than the conjugated form, which may be a precursor of the free form.

G. B.

Oestriol, Oestrone and Oestradiol-17β in Human Urine, Determination of. W. S. Bauld. (*Biochem. J.*, 1956, **63**, 488.) A method is described for the determination of oestriol, oestrone and oestradiol-17β in human urine. The method involves acid hydrolysis of the urine, extraction with ether, separation of the ether extract into oestriol and oestrone-oestradiol fractions by partition between water and benzene. The aqueous extract, after saponification with sodium hydroxide, and extraction with ether, gives impure oestriol. This is chromatographed on a column of Celite using ethylene dichloride as the mobile phase and 70 per cent methanol as the stationary phase to give a purified fraction which can be determined colorimetrically by an improved Kober reaction. The procedure includes a photometric correction for non-oestrogen chromogenic material. The benzene solution, containing oestrone and oestradiol-17β, submitted to column partition chromatography, followed by saponification of the appropriate fractions, yields the two components in fractions, which can also be determined colorimetrically by means of the Kober reaction. Four urine specimens can be analysed in 9 to 10 man hours by this procedure. Results obtained on urine excreted during the menstrual cycle are reproducible and the method appears to be specific for oestrogens. The method is satisfactory for quantities of the order of 5 to 10 μg./day. J. B. S.

CHEMOTHERAPY

***p*-Aminophenoxyalkane Derivatives, Activity of, Against *Schistosoma mansoni*.** A. G. Caldwell and O. D. Standen. (*Brit. J. Pharmacol.*, 1956, **11**, 367.) A study of the schistosomicidal activity of derivatives of *p*-aminophenoxyalkane in mice infected with *S. mansoni* has shown that diphenoxyalkanes with different primary, secondary or tertiary amino groups in the *p*-positions have an activity similar to that of the symmetrical *pp'*-diaminodiphenoxyalkanes. Diphenoxyalkanes, having a *p*-amino group on only one ring, and any of a variety of substituents on the other ring were less active than the diaminodiphenoxyalkanes, but where the other group was acetamido-, acetmethylamido-, ethoxycarbonamido-, ethoxycarbonmethylamido-, or cyano-, there was a high activity. *p*-Aminophenoxyalkoxyalkanes had a moderate activity. *p*-Aminophenol, and a few of its derivatives, which are possible metabolites of these compounds, had no activity. The relationship between structure and activity is briefly discussed.

G. F. S.

Thiacetazone (Amithiozone, Tibione) Analogues. H. C. Caldwell and W. L. Nobles. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 729.) A series of thiosemicarbazones was prepared, including mainly monosubstituted derivatives of benzaldehyde 3-thiosemicarbazone or of acetophenone 3-thiosemicarbazone, and these compounds modified by the introduction of a vinyl group between the aromatic ring and the substituted aldehyde or keto group. The thiosemicarbazones of certain heterocyclic compounds and their vinylogues were also

prepared. The method of preparation of *p*-dimethylaminocinnamaldehyde and of 6-(2-thenyl)-3 : 5-hexadien-2-one, required as intermediates, is reported. When the thiosemicarbazones were examined for antituberculosis activity *in vitro*, in all cases the vinylogue was found to be at least as active as its parent compound. The thiosemicarbazones were also tested for activity in schistosomiasis, and as amoebicides and carbonic anhydrase inhibitors. 4-(3-Thenyl)-3-buten-2-one semicarbazone showed marked antituberculosis activity, inhibited the growth of *Streptococcus pyogenes* and *Staphylococcus aureus* and showed appreciable amoebicidal activity. G. B.

PHARMACY

Decanol-1 in Soap Solutions, The Solubility of, below the CMC. P. Ekwall and T. Vittasmäki. (*Acta chem. scand.*, 1956, **10**, 1177.) The solubility of decanol in dilute aqueous solutions of sodium caprate, sodium laurate, sodium myristate in concentrations below the critical micelle concentrations (CMC) at 40° has been determined, making use of the fact that the system becomes turbid when a new phase appears. With all three soaps decanol solubility starts to increase in the region of the limiting concentration of the soap. This increase in solubility slows as soap concentration increases, and actually begins to decrease until just below the CMC of the pure soap, when solubility again begins to increase. A second and rapid increase in solubility starts at soap concentrations about 30 per cent lower than the CMC of the pure soap, corresponding to the point at which alcohol-soap micelle formation also commences to increase rapidly. Above this point the increase in solubility is rapid. The minimum points thus give the CMC value for soap solutions containing decanol, and this is supported by the fact that 0·018M and 0·023M laurate solutions begin to solubilise *p*-xylene only after decanol has been added to them. Estimates are derived of the amount of soap bound in the mixed micelles above the critical concentration. Solubility curves show that the maximum ratio of decanol to soap in the mixed micelles remains constant above the CMC.

J. B. S.

Water, Sorption of, by Rubber Closures for Injections; Effect of Inorganic Salts. G. Milosovich and A. M. Mattocks. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 758.) Rubber stoppers of several types were dried to constant weight and placed in vials which were filled with the solution under examination and closed. Air bubbles adhering to the stoppers were removed by giving each vial a sharp tap before placing it in a water bath. After a specified period the stoppers were removed, deprived of surface moisture with a blast of compressed air and weighed. The stoppers were then returned to the vials, fresh solution added and the experiments continued. Inorganic salts were shown to depress the absorption of water by the stoppers. Some variation was observed between individual stoppers of the same type, owing largely to differences in density, and this made interpretation of the results more difficult. The effects of the various ions were compared by an analysis of variance, and it was shown that the results could be explained on the basis of the lowering of the vapour pressure of the solutions relative to water, divalent ions depressing the water absorption more than monovalent ones. The bisulphite ion was an exception, greatly increasing the penetration of water into the rubber. It is suggested that the bisulphite ion or a derivative of it penetrates the rubber and renders it more permeable to water. G. B.

PHARMACOLOGY AND THERAPEUTICS

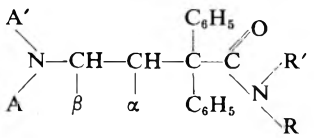
Acetazolamide, an Inhibitor of Carbonic Anhydrase, Effect of, on Gastric Secretion. L. Poller. (*Brit. J. Pharmacol.*, 1956, **11**, 263.) A high concentration of carbonic anhydrase has been demonstrated in the oxyntic cells of the stomach (Davenport, *Amer. J. Physiol.*, 1940, **128**, 725) and the enzyme has been shown to be intimately concerned in gastric acid secretion. The author undertook a study of the effects of acetazolamide on acid secretion to assess whether the inhibitor could be used in the treatment of hyperacidity associated with peptic ulcer. The subjects were twelve normal healthy adults and, after being kept on a fixed weight diet for three days with additional fluid intake restricted to 1500 ml., they were given 250 mg. or 500 mg. acetazolamide by mouth. Gastric, blood and urine samples were collected before and after administration of the drug. The rate of gastric secretion, acid production and rate of pyloric evacuation were all slightly diminished after acetazolamide. Atropine (2 mg. subcutaneously) had a much greater effect under similar conditions. Marked diuresis, increased sodium and potassium excretion and increased urinary pH were seen in all subjects in the first twenty-four hours after acetazolamide administration. It was concluded that, although acetazolamide inhibited gastric acid secretion, its other effects, such as systemic acidosis, would forbid prolonged therapy for this purpose. G. P.

Alphaprodine, Ro-1-7780, a Potent Antagonist of. C. W. White, Jr., R. Megirian and P. S. Marcus. (*Proc. Soc. exp. Biol., N.Y.*, 1956, **92**, 512.) Ro-1-7780 (1 : 3-hydroxy-*N*-propargyl morphinan tartrate) antagonized respiratory depression caused by alphaprodine and morphine in the dog and by alphaprodine in man. In the dog the dose ratio of alphaprodine to antagonist, at which no depression of respiration occurred when the two drugs were given simultaneously, was 75 to 1. When the two drugs were given to man in this dose ratio the antagonism was only partial, some degree of respiratory depression still being experienced. In dogs, Ro-1-7780 given either after or at the same time as alphaprodine gave an initial increase in ventilation rate and/or volume above control values, in spite of the fact that the antagonist given alone had no significant effects. This agrees with similar findings for other opiate antagonists. The narcotic effects of the alphaprodine in man were judged to be unaltered by the antagonist. G. P.

β-Aminoethyl*iso*thiuronium Bromide, The Pharmacology of, in the Cat. V. Di Stefano, D. E. Leary and D. G. Doherty. (*J. Pharmacol.*, 1956, **117**, 425.) In a screening test for drugs affording protection against the lethal effects of radiation the most promising was β-aminoethyl*iso*thiuronium bromide (AET). The pharmacology of this drug was studied further. Low doses (2·5 mg./kg.) caused a fall in blood pressure, bradycardia and apnoea in cats under barbiturate anaesthesia; these effects were all abolished by cutting the vagus nerves, and, except for the apnoea, by atropine. Gut contractions caused by the drug were also abolished by atropine. With larger doses of AET there was a diphasic blood pressure response, the initial fall being followed by a rise. The nature of this rise was obscure as it was still present after the spinal cord had been destroyed in the spinal cat, after bilateral adrenalectomy and after dibenamine or tolazoline. With doses of AET above 10 mg./kg. the contractions of the nictitating membrane of the cat were inhibited, partly by ganglion blockade and partly by a direct action. With these doses there was a slight augmentation of

skeletal muscle contractions. Doses above 25 mg./kg. caused convulsions and death. G. P.

Analgesics, A New Series of Potent. P. A. J. Janssen and J. C. Janssen. (*J. Amer. chem. Soc.*, 1956, **78**, 3862.) A series of over 100 new basic amides of structure I were prepared and some proved to have high analgesic activity in mice, rats, cats, guinea pigs, dogs and man.



(I)

The relation between chemical structure and analgesic activity in this series is briefly outlined. In the α-CH_3 series, one of the optical isomers of each enantiomorphic pair is twice as active as the racemic mixture; the other optical isomer is devoid of significant analgesic activity. The (+)-isomer of I; $\alpha = CH_3$, β = H, $-N\begin{matrix}A'\\A\end{matrix}$ = morpholino, $-N\begin{matrix}R'\\R\end{matrix}$ = 1-pyrrolidinyl is an analgesic 60 to 100 times more active than pethidine, 10 to 40 times more active than morphine, 5 to 20 times more active than methadone and about 4 times more active than heroin in various experimental conditions. In animals it had a higher oral activity and a better therapeutic ratio than any other analgesic tested. Preliminary experiments with the racemic compound in man indicate an analgesic potency of about 3 times that of morphine; no side effects were observed after subcutaneous injections of up to 12 mg. A. H. B.

Benactyzine, Pharmacology of. F. M. Berger, C. D. Hendley and T. E. Lynes. (*Proc. Soc. exp. Biol., N.Y.*, 1956, **92**, 563.) Benactyzine, an antispasmodic which has been used in the treatment of psychoneuroses, caused hyperexcitability and clonic convulsions in mice after intraperitoneal injection of one-half of an LD50 (155 $\pm$ 9 mg./kg.). In adult Rhesus monkeys intravenous doses ranging from 1 to 6 mg./kg. caused only minor changes in behaviour. After 1 mg./kg. there was mydriasis and some decrease in locomotion, but no demonstrable taming effect. Ataxia and occasional convulsive jerks occurred with 2 mg./kg. and with 6 mg./kg. one monkey went into frank convulsions for about 10 minutes, followed by post-ictal depression lasting one hour. The duration of the anaesthesia with 100 mg./kg. hexobarbitone given intraperitonially to mice was more than doubled when 10 mg./kg. benactyzine was given with the barbiturate. This was markedly greater than the prolonging effect of diphenhydramine. Benactyzine also increased mortality in mice subjected to electroshock seizures. The drug antagonized the actions of acetylcholine, 5-hydroxytryptamine and histamine on guinea pig and rat smooth muscle. In chloralosed cats the pressor effects of adrenaline were potentiated, 1 mg./kg. benactyzine almost doubling the effect of 5 μg./kg. adrenaline. The toxicity of adrenaline in mice was also increased. The knee jerk and flexor reflexes in chloralosed cats were unaffected by 3 mg./kg. benactyzine; increasing the dose to 10 mg./kg. abolished both reflexes and caused respiratory arrest. The effects on the EEG of curarized cats were to block EEG arousal from sensory or thalamic stimulation, while leaving unaffected recruiting responses evolved in the cortex; the EEG records obtained were

indistinguishable from those obtained after suitable doses of atropine under similar conditions. G. P.

Brom-lysergic Acid Diethylamide (BOL), Blockade by, of the Potentiating Action of 5-Hydroxytryptamine and Reserpine on Hexobarbitone Hypnosis. G. C. Salmoiraghi, L. Sollero and I. H. Page. (*J. Pharmacol.*, 1956, **117**, 166.) The hypnotic effect of hexobarbitone in mice is potentiated by 5-hydroxytryptamine (5-HT) and by reserpine; this potentiation is blocked by (+)-lysergic acid diethylamide (LSD). The 2-bromo derivative of LSD, known as BOL, has been shown to inhibit the action of 5-HT on the rat's uterus, but otherwise to differ in its actions from LSD in that it has no hallucinogenic action It also blocks 5-HT on the guinea pig's ileum, where LSD has no effect. In the present experiments the authors have shown that BOL (10 mg./kg.) antagonized the potentiation of hexobarbitone sleeping time by 5-HT and reserpine in mice and rats. By itself, BOL decreased spontaneous activity in the rats and mice, but did not prolong hexobarbitone hypnosis. These results support the view that LSD does not owe its hallucinogenic actions to blockade of normally occurring 5-HT in the brain. G. P.

Chemical Radio-Sensitizer (Synkavit), Protection by Cysteine Against the Acute Toxicity of. A. F. Phillips and D. B. Cater. (*Brit. J. Pharmacol.*, 1956, **11**, 128.) Toxic doses of the vitamin K substitute, tetrasodium 2-methyl-1:4-naphthohydroquinone diphosphate (Synkavit), caused hyperexcitability and convulsions on intravenous injection into rats. These effects were not due to hypoglycaemia and were presumably on the central nervous system; oxidation-reduction potential measurements in the brain give some support to this view. Death usually occurred within 45 minutes of administration: LD50 for rats was approximately 0·9 mM/kg. Cysteine in doses of 1·3 and 3·9 mM/kg. injected at the same time, or up to three hours before, protected a high proportion of rats against the immediate toxic effects of Synkavit, and increased the average survival times of animals receiving doses of Synkavit from 1 mM to 2 mM/kg. Cysteine by itself had no toxic actions in doses up to 4·7 mM/kg. Oxidation-reduction potentials in the brain rose after cysteine and fell after Synkavit. Glutathione had a similar protective effect, giving an increase in survival time comparable with that of cysteine at the same molar dose. G. P.

Chlorpromazine, Effect of, on Adrenaline Vasoconstriction in Man. J. Ginsburg and R. S. Duff. (*Brit. J. Pharmacol.*, 1956, **11**, 180.) The effects of intra-arterial and of intravenous infusions of chlorpromazine on the blood vessels of the hands of healthy men were assessed by venous occlusion plethysmography. The drug was infused into the brachial artery just above its bifurcation at the elbow, or into a superficial vein of the other arm. The blood flow through the hand increased by an average of about 50 per cent after intra-arterial (1·2 mg.) and by about 400 per cent after intravenous (50 mg.) chlorpromazine. The intravenous dose was calculated so as to give the same local concentration of chlorpromazine. The results indicate the degree of direct vasodilator action (with intra-arterial infusion) and total vasodilator action (intravenous infusion). The proportional reduction in blood flow with intra-arterial adrenaline (0·5 μg./min.) was 75 per cent before and 27 per cent after the infusion of 1·2 mg. chlorpromazine into the brachial artery; however, when the increased control level of flow during chlorpromazine administration, before the adrenaline was given, is considered, the total reduction in flow with intra-arterial adrenaline was not significantly different after chlorpromazine. The average proportional reduction in flow in the hand with intravenous infusion of adrenaline, was

slightly, but significantly, decreased by intra-arterial infusion of 1·2 mg. chlorpromazine. There was no reversal of adrenaline vasoconstriction even after the intravenous infusion of 50 mg. chlorpromazine. The results were discussed in relation to the antagonism between adrenaline and chlorpromazine. G. P.

5-Hydroxytryptamine, Potentiation of, by Phenylethylamine Derivatives with Central-stimulant Actions. J. Delay and J. Thuillier. (*C. R. Acad., Paris*, 1956, **242**, 3138.) Phenylethylamine, amphetamine, dexamphetamine, methylamphetamine and mescaline potentiated the action of 5-hydroxytryptamine (5-HT) on the isolated oestrous uterus of the rat. Two other phenylethylamine derivatives, ephedrine and neosynephrine, had no action on, and antagonized, respectively, the action of 5-HT. The potentiation may result from inhibition of amine oxidase. This may also account for the mental disturbances in man caused by potentiation of 5-HT by mescaline and large doses of central-stimulating amines. (E. Costa, *Proc. Soc. exp. Biol., N.Y.*, 1956, 91, 39.) G. P.

Local Anaesthetics, a Simple New Quantitative Method for Testing. C. Bianchi. (*Brit. J. Pharmacol.*, 1956, **11**, 104.) Two methods for determining local anaesthetic activity are commonly used: inhibition of the corneal reflex by local application of the drug and inhibition of the reaction to pin-prick stimulus of the skin by intracutaneous injection of the drug. The first method measures surface anaesthetic activity, the second also the power of infiltration. A new method for determining nerve trunk anaesthesia has been developed. It depends upon the reaction of a mouse to pressure applied to the tail by a small artery clip with the blades covered with rubber tubing. Mice failing to respond to the stimulus were eliminated from the test and the remainder received subcutaneously 0·1 ml. of a solution of the local anaesthetic, about 1 cm. from the root of the tail. 15 minutes after injection the pain reflex in the tail was again tested and the proportion of animals failing to react, noted. A linear relation was shown to exist between the logarithm of the concentration of anaesthetic and the probit of the proportion of mice showing anaesthesia. By this method the activity of cocaine was nearly half that of cinchocaine, approximately four times that of lidocaine and seven times that of procaine. The anaesthetic activity of all the compounds was of short duration and wore off within 90 minutes of injection. G. P.

Mecamylamine, Ganglion-blocking Properties of. C. A. Stone, M. L. Torchiana, A. Navarro and K. H. Beyer. (*J. Pharmacol.*, 1956, **117**, 169.) The ganglion-blocking properties of mecamylamine (3-methylamino-*iso*camphane hydrochloride) are characterized by a high order of potency, specificity and long duration of action. In the chloralosed cat the ratios of potency of mecamylamine: pentolinium: hexamethonium were:—for blockade of transmission through the superior cervical ganglion, 1:4:1; blockade of the pressor response to nicotine, 4:4:1; and increase in pupillary diameter, 2:4:1. The duration of action of the secondary amine was ten to twenty times longer than that of hexamethonium and three to four times that of pentolinium. In the dog under vinylbarbitone anaesthesia mecamylamine decreased the vasopressor responses to carotid occlusion and to nicotine and the fall in B.P. with stimulation of the peripheral vagus nerve. The effects of injection of acetylcholine were not altered and those to adrenaline, 5-hydroxytryptamine and angiotonin were increased. The drug had no atropine-like, antihistamine, or local anaesthetic properties, but in high doses had a neuromuscular blocking action; the ratio between minimally effective ganglion-blocking doses and skeletal muscle paralyzing doses was of the order of one to one hundred. The

relatively low ratio between the oral and intravenous LD50's, as determined in mice, indicate that mecamylamine was considerably better absorbed after oral administration than were tetraethylammonium, hexamethonium, pentolinium and chlorisondamine, whose ratios were four to six times greater. G. P.

Narcotic Drugs, Possible Mechanism of Tolerance to. J. Axelrod. (*Science*, 1956, **124**, 263.) Several striking similarities have been observed between the receptors for narcotic drugs and the enzymes which *N*-demethylate these drugs. They have in common, substrate specificity, stereospecificity and antagonism by nalorphine. Since changes in enzyme activity during the development of tolerance might reflect similar changes in the receptors, rats were made tolerant to morphine and the changes in enzymic activity of the liver observed. Tolerance was induced by daily intraperitoneal injections of morphine, starting with a dose of 20 mg./kg. and increasing over a period of 35 days to 150 mg./kg. A second group of rats had in addition a daily dose of nalorphine equal to a quarter of that of morphine given. The effects of withdrawal were studied on a third group, in which the dose regimen was the same as the first group, but the drug was abruptly withdrawn for 12 days after tolerance to 150 mg./kg. had been established. 24 hours after the test period the animals were killed and their livers examined for ability to *N*-demethylate morphine, dilaudid, pethidine and cocaine. In the case of the morphine-treated animals a marked decrease in demethylating activity of the livers occurred, both with morphine and dilaudid as substrates; demethylation of pethidine, a drug which exhibits less cross-tolerance to morphine than does dilaudid, was decreased to a less degree and demethylation of cocaine was no different from that of controls. Where the animals received both nalorphine and morphine, the reduction in enzymic demethylation of the three narcotic drugs was significantly less than in those receiving morphine only. The enzyme activity had returned to the control level or above in the animals where the morphine had been withdrawn. In none of the animals were enzymic *O*-demethylation of codeine, hydrolysis of diacetylmorphine or conjugation of morphine affected. From these results it would appear that the continuous interaction of narcotic drugs with enzymes inactivates the enzymes. Similarly, the development of tolerance may be due to an inactivation of receptors. G. P.

Nalorphine, a Potent Analgesic in Man. A. S. Keats and J. Telford. (*J. Pharmacol.*, 1956, **117**, 190.) The analgesic potency of nalorphine was determined in post-operative patients and compared with that of morphine. Morphine and nalorphine were never given to the same patient, thus avoiding any residual antagonistic effects the one might have had on the other. Nalorphine was at least as potent as morphine as an analgesic. Subjective side actions noted included drowsiness, dizziness, coloured dreams, visual hallucinations and disorientation. Respiratory depression caused by nalorphine was equal to that caused by morphine. It has been postulated that the antagonism of narcotic drugs by nalorphine is the result of the substitution of a weak narcotic (nalorphine) for a potent narcotic (e.g., morphine) at cell receptor sites, by competition for these sites. The results obtained with nalorphine cannot support this, since in no respect can it be considered a weak narcotic. G. P.

Rauwolfia Alkaloids, Anticonvulsant Action of Some Anti-epileptic Drugs in Mice Pretreated with. C. Bianchi. (*Brit. J. Pharmacol.*, 1956, **11**, 141.) The anticonvulsant activity of soluble phenytoin, troxidone, phenacemide reserpine and a preparation containing mixed rauwolfia alkaloids, was evaluated in mice against convulsions induced by camphor, leptazol or strychnine. The

anticonvulsant activity of combinations of the anti-epileptic drugs with reserpine or mixed rauwolfia alkaloids was also tested. The convulsant threshold to camphor, strychnine and leptazol was lowered by reserpine and rauwolfia alkaloids. These alkaloids also had the ability to nullify the anticonvulsant activity of phenytoin, troxidone and phenacemide against leptazol-induced convulsions, but had no effect on the activity of phenacemide against camphor or strychnine-induced convulsions. The anticonvulsants decreased the effects of reserpine and rauwolfia alkaloids in lowering the convulsant threshold to doses of camphor, leptazol and strychnine. The convulsions induced in mice by camphor were always clonic under normal conditions, but when reserpine or the rauwolfia alkaloids had been given previously they became tonic; this action was also reduced by the anticonvulsant drugs. As the adrenal ascorbic acid is known to be depleted by reserpine this was investigated as a possible site of the convulsion-facilitating action; adrenalectomized mice, however, did not show any significant change in their susceptibility to the convulsant effect of camphor. G. P.

Reserpine, Release of Blood Platelet 5-Hydroxytryptamine by, and Lack of Effect on Bleeding Time. P. A. Shore, A. Pletscher, E. G. Tomich, R. Kuntzman and B. B. Brodie. (*J. Pharmacol.*, 1956, **117**, 232.) The potent vasoconstrictive action of 5-hydroxytryptamine (5-HT) has led to the postulation that with disruption of platelets during clotting, the released 5-HT might participate in the mechanism of haemostasis. In the present experiments reserpine was shown to release 5-HT from the platelets of rabbits, rats and guinea pigs. The 5-HT content of the platelets was determined by extracting whole blood or a suspension of isolated platelets, first adjusted to pH 10, with butanol, returning the 5-HT to formate buffer at pH 4. The 5-HT content was then estimated in a spectrophotofluorometer by activating at 295 mμ and measuring the resulting fluorescence at 330 mμ. In rabbits injected intravenously with 5 mg./kg. reserpine, the 5-HT content of the blood declined progressively to 50 per cent four hours after injection. At 16 hours the 5-HT level was about 5 per cent of normal, this low level persisting for some 48 hours, normal values being regained only after 6 days. These results are similar to the effects of reserpine on the 5-HT content of the intestinal tract. Similar results were obtained for depletion of platelet 5-HT in rats and guinea pigs. There was no disruption of the platelets to effect the release of 5-HT. Bleeding times were, however, unchanged by the depletion of platelet 5-HT, thus making it unlikely that 5-HT has a role in haemostasis. G. P.

Substance Resembling Kallidin and Bradykinin, a Delayed Slow Contracting Effect of Serum and Plasma Due to the Release of. M. Schachter. (*Brit. J. Pharmacol.*, 1956, **11**, 111.) During experiments on a delayed slow contracting effect of serum and plasma on the isolated guinea pig's ileum, it was found that this slow contraction was not caused by any substance originally present, but had been the result of the release or formation of a substance from the serum or plasma in the organ bath. This substance could be produced by dilution of ox, guinea pig, rat, dog, cat or human serum or plasma. The release of the substance by dilution of ox or guinea pig serum was greatly reduced or abolished by soya bean trypsin inhibitor and by heating serum at 56° for 3 hours before dilution, to destroy kallikreinogen. The smooth-muscle stimulant resembled kallidin and bradykinin in that it contracted, in a characteristic way, the intestine of the guinea pig, cat, and dog and the uterus of the guinea pig, rat and cat; it was also inactivated by serum and by chymotrypsin.

A comparison of the relative ability of various substances which release smooth-muscle stimulants from serum showed that for a particular substance the release was not the same in sera of different species; the results indicate a degree of species specificity of kallikrein and renin for their serum substrates. Rabbit and hen serum failed to release a smooth-muscle stimulant on dilution. Compound 48/80, egg white, and wasp venom did not release kallidin or bradykinin from serum. The hypothesis is advanced that dilution of serum releases kallidin through activation of kallikreinogen. These results are of importance from a practical standpoint, since under the conditions which exist in many biological assays, serum or plasma may be diluted in the procedures and so form a potent smooth-muscle stimulant. G. P.

Tiofenatin, Pharmacology of. S. Ya. Arbuzov. (*Farmakologiya i Toksikologiya*, 1956, **19**, No. 1, 16.) The pharmacological properties of Fenatin (β-phenyl*iso*propylamide of nicotinic acid) and Tiofenatin (β-phenyl*iso*propylamide of thionicotinic acid) are compared. In experiments on cats and rabbits, intravenous doses of Tiofenatin varying from 0·005 to 0·01 g./kg. produced a hypotensive effect much greater in intensity than that obtained with Fenatin. After bilateral vagotomy and denervation of both carotid sinuses this effect was not only maintained but somewhat enhanced and its duration was also increased. Tiofenatin is less toxic than Fenatin; the LD50 of Tiofenatin given subcutaneously to white mice was 2100 mg./kg., which compares with 1200 mg./kg. for Fenatin. Tiofenatin dilates the vessels of the isolated rabbit ear in concentrations of 1 : 100,000 to 1 : 10,000,000; the effect was also observed on the innervated isolated ear when Tiofenatin was given in doses of 0·005 to 0·01 g./kg. In various experiments Tiofenatin showed a powerful hypotensive effect in rabbits and in cats. It also increased the depth of respiration. E. H.

Tubocurarine, The Effect of, on the Neuromuscular Blocks Caused by Di-*iso*propylfluorophosphate (Dyflos) and Acetylcholine. J. A. B. Barstad. (*Arch. Int. Pharmacodyn.*, 1956, **107**, 4.) Acetylcholine (ACh) and dyflos cause reversible neuromuscular blockades in the isolated diaphragm-phrenic nerve preparation of the rat. In the early stages of block with ACh (2 to 4 minutes after addition of the drug) the block was reversed by tubocurarine. Where the ACh had been in contact with the muscle for 15 minutes or more the tubocurarine deepened the block. With moderate amounts of dyflos a similar pattern of reversal and potentiation by tubocurarine could be reproduced. However, where higher concentrations (ca 2 to 5 $\times$ 10^{-4}M) of dyflos were used, the neuromuscular blockade could at all stages of development be increased by tubocurarine. In interpreting these results, where the effect of tubocurarine was to reverse a partial block, this could most readily be explained in terms of a repolarizing effect of the alkaloid on an end-plate membrane depolarized by excess ACh. The additive effect in the later stages of blockade with ACh cannot be understood without simultaneous records of the electrical state of the end-plate membrane. With low concentrations of dyflos the events can be likened to those obtained with ACh, and can be explained by the anticholinesterase effect of the compound, but with higher concentrations another action has to be considered: this may have its site in the presynaptic nerve endings. G. P.

LETTERS TO THE EDITOR

Irradiation of Penicillin

SIR,—We have recently exposed sodium benzylpenicillin in the dry state and in aqueous solutions to gamma irradiation from a cobalt 60 source, and have assayed the materials by the method described by Royce, Bowler and Sykes[1]. Table I shows the results obtained. The controls consisted of solutions of the same batch of material as the irradiated samples, sealed in ampoules at the same time and kept under identical conditions. The loss of potency on irradiation is negligible.

TABLE I

Batch number	Radiation in rads × 10^6	Assay of original	Assay after irradiation	Control
		Solution of Penicillin		
6174	1·00	*15,200 units/ml.	15,140 units/ml.	15,180 units/ml.
6174	1·00	*19,300 "	15,800 "	16,000 "
6734	2·00	*22,500 "	18,400 "	19,200 "
		Dry Penicillin		
	1·00	—	1588 units/mg.	1600 units/mg.

* Mean of three samples

We have also exposed to gamma irradiation, soil cultures of spores known to be highly resistant to heat. After irradiation these spores failed to germinate in culture media. The amount of irradiation ($0{\cdot}5 \times 10^6$ rad) to which they were exposed was half the lowest dose applied to the penicillins. It thus appears probable that if gamma radiation were developed as a method of sterilising pharmaceutical products, solutions of penicillin would be sufficiently stable to withstand this process.

Work is proceeding with other labile substances.

For the assay of the penicillin, it was found necessary to allow four hours for the penicillinase reaction to be completed, a longer time than that used by Royce and his colleagues.

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REFERENCE

1. Royce Bowler and Sykes, *J. Pharm. Pharmacol.*, 1952, **4**, 904.

The Identity of Vasicinine from *Adhatoda vasica*

SIR,—One of us in the course of examining Indian drugs isolated a water soluble base "Vasicinine" from the leaves of *Adhatoda vasica* Nees (Acanthaceae), and certain derivatives were described[1]. Subsequently the compound was thought to be a pyrrolidine derivative[2]. Re-examination of "Vasicinine hydrobromide" has now shown that it is in fact identical with betaine hydrobromide: a comparison of derivatives is shown below:

	Vasicinine	*Betaine*
	m.p.	m.p.
Base	292° (decomp.)	293° (decomp.)[3]
Hydrobromide	232–233° (decomp.)	232–233° (decomp.)[4]
Picrate	179–180°	180–181°[5]

Bases and hydrobromides behaved identically when their melting points were determined simultaneously. Admixture of "Vasicinine" picrate with an authentic sample derived from betaine gave no depression in melting point.

Examination of the infra-red spectrum (potassium bromide discs) revealed the identity of peaks in the case of "Vasicinine" and betaine hydrobromides: viz. 1742, 1637, 1477, 1453, 1425, 1402, 1324, 1242, 1185, 1132, 993, 951, 929, 884, 833, 779 cm^{-1}.

Dr. J. F. Cavalla of Parke Davis and Co. Ltd. has independently likewise identified "Vasicinine" with betaine, and we are indebted to him for communicating this information to us.

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4. Stoltzenberg, *Z. physiol. Chem.*, 1914, **92**, 471.
5. Bebeschin, *ibid.*, 1911, **72**, 385.