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In conformity with an international agreement reached by the Editors of biological journals, a revision in the method of presentation of the summaries of research papers intended for publication in the *Journal of Pharmacy and Pharmacology* is drawn to the attention of authors. The form of summary in numbered paragraphs which previously appeared at the end of papers is suspended. The summary will now appear at the beginning of the text.

The summary should be brief but self-explanatory, giving results and conclusions described in the paper, and in the form of an abstract suitable for use as such by abstracting journals.

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REVIEW ARTICLE

HOST RESISTANCE TO INFECTION*

By George Brownlee, D.Sc., Ph.D.

Reader in Pharmacology, University of London, King's College

The application of chemotherapy to the control of infectious disease has had an unexpected result in revealing the contribution made by the resistance of the host to the desired result.

Most experience has been gained from a study of the pathogenesis and treatment of disease in the experimental animal. The reviewer has turned to infections with the tubercle bacillus as examples because he has had more experience with that parasite than with any other. But there is another reason; infections with the tubercle bacillus provide a model in which host resistance is not complicated by the presence of circulating protective antibodies.

Let us first saddle man with a definition. Thus a "host" must by definition supply the conditions favourable for reproduction of a bacterial "parasite." The use of the term "soil" by some tuberculosis workers refers to their belief that some unknown biochemical factors essential to the reproduction of tubercle bacilli may determine susceptibility, yet so far as can be traced only two factors have clearly been established, as growth conditions, to play a creative part in bacterial susceptibility. They are the availability of free oxygen and the temperature of the host.

The availability of free oxygen limits the pathogenic potentialities of all strictly aerobic bacteria. Tubercle bacilli are strictly aerobic. How different would be their pathogenic potentialities and how different the resultant patterns of disease were these parasites facultative rather than obligate aerobes. How much more dangerous to mammals would the tetanus bacillus be were it a facultative aerobe.

The reasons for the high pathogenicity of the bovine tubercle bacillus and the low pathogenicity of the human strain for the rabbit while both are pathogenic for the guinea-pig but have poor pathogenicity for dogs, rats, and mice, remain subjects for debate and speculation.

The presence of a genetically transmitted resistance to tuberculosis is recognised, notably in some strains of rabbits; and this knowledge may be subjected to a statistically significant experimental proof. A discussion of these experiments will be made later.

Host Resistance in Man

Those physicians who observe the tissue change seen with tuberculosis, recognise the presence of clinical disease as a manifestation of the absence of host resistance. Yet it was the advent of substances with the capacity to modify favourably the course of the disease which first enabled the

* Forming the basis of three lectures arranged by the Convention Culturelle Franco-Britannique, given in Angers, Tours and Rennes in May 1956.

significance of host resistance to be assessed. Those Gram-positive infections, a single attack (and recovery) of which conferred a solid lasting immunity, provided the first clue to the subsequent discovery of passive and active immunity. Diphtheria provides an excellent example.

It is difficult not to conclude that resistance of man to diphtheria is high, and unless swamped, or as the result of pharmacological damage with toxins, the body has little difficulty in digesting the parasite.

It is with the classical studies of Anderson with pneumococcal pneumonia that we are able to identify one facet of host resistance[1–4].

Epidemic uncomplicated (lobar) pneumonia in adults in Glasgow was caused by type I, II and IV organisms. Mortality before chemotherapy was about 30 per cent; with chemotherapy this was reduced to 8 per cent, the peak efficiency being reached with sulphathiazole; penicillin, aureomycin and other antibiotics did not modify this figure.

Endemic (broncho-) pneumonia was caused by types III, VII, XV and XXI, and, before chemotherapy, had a mortality of some 30 per cent; with the advent of chemotherapy the mortality was reduced only to two thirds of this figure. These organisms were recovered from the mouths of most adults[1–4].

Here then is evidence for a lack of host resistance in the endemic pneumonia group.

Host Resistance in Animals

We must now turn to work with experimental animals the better to see some possible sites of action of the mechanism of host resistance. By observing the incidence of infection in rabbits with high or intermediate native resistance to known numbers of tubercle bacilli Lurie and his colleagues[5–7] have been able to make a measure of host resistance.

With the bovine tubercle bacilli, each bacillus of maximum virulence which is ingested by an alveolar phagocyte gives rise to a tubercle, irrespective of the resistance of the rabbit. With the human type bacilli, 47 ± 10 organisms will generate a single tubercle in susceptible rabbits, whereas 684 ± 159 organisms of the same virulence are required to produce a single primary pulmonary focus in the genetically resistant rabbits, which are therefore about 16 times more resistant to disease caused by the human strain.

When pharmacological doses of cortisone are given to rabbits which inhale human type bacilli the number of primary tubercles is markedly increased. Also, the foci swarm with bacilli and there is little granulation tissue; in addition the draining lymph nodes contain few micro-organisms. The controls show typical tuberculous granulomata with well-developed caseation and marked lymphatic dissemination. Cortisone does not increase the number of foci generated in rabbits exposed to bovine bacilli of maximum virulence, for not more than one primary tubercle can arise from a single cell. The disturbed hormone balance produced by cortisone appears to deprive the phagocytes of their capacity to inhibit the growth of the bacilli within their cytoplasm though their phagocytosis is not impaired. In similar ways cortisone has been shown

to have a rapidly acting, deleterious effect on many bacterial, protozoal, viral and rickettsial infections in a variety of species[8]. The rapidity of the intervention by cortisone in acute infections suggests that its effect is not related to an interference with antibody mobilisation. Lurie's experiments also showed (1) there was a greater initial destruction of human type bacilli in the resistant animal; (2) the lag phase which precedes the logarithmic multiplication of inhaled human bacilli in the lungs of both resistant and susceptible animals is longer in the former; (3) the rate of multiplication during the growth phase is lower and (4) the acquired resistance developed is greater in the resistant than in the susceptible animal. This evidence was interpreted to mean that the inhibitory influence on the intracellular growth of the bacilli is of first importance in host resistance. The cortisone evidence was interpreted to mean that this property is under the influence of the hormone balance of the individual.

This knowledge of the part played by the phagocyte has received unexpected support by a chance discovery made by Hart and his colleagues[9]. It was observed that a commercial non-ionic surface-active agent Triton WR 1339 and similar large molecules synthesised for the purpose suppressed the development of experimental tuberculosis in the mouse. This observation introduced a new type of antituberculosis agent. Other workers[10] reported synergism with dihydrostreptomycin in the treatment of murine tuberculosis. Recently is was shown by Rees[11] that regression and healing of an established infection in the guinea-pig is possible.

Three series of these non-ionic macromolecules were synthesised differing in their polyoxyethylene chain length from 10 to 90 units. In these three homologous series, alterations in the lipophilic-hydrophilic balance in the individual products, brought about by varying the polyoxyethylene chain length, can influence the outcome of the tuberculosis infection. As the lipophilic to hydrophilic ratio decreases, activity passes from antituberculosis to inactive, and then to protuberculosis.

Yet none of the antituberculosis members of the different series inhibited the multiplication of tubercle bacilli *in vitro* even at high concentrations, while none of the protuberculosis enhanced growth. Moreover, no tuberculostatic substance was found in the blood or tissue fluids from animals heavily dosed with a therapeutically active agent. On this evidence, the authors presumed that the various effects were mediated more directly through the host. Mackaness[12] introduced tubercle bacilli *in vitro* into preparations of monocytes (macrophages) obtained from animals previously treated with Triton WR 1339. Although phagocytosis was not increased, the ingested bacilli were partially or completely destroyed. In the controls there was free intracellular multiplication.

Direct contact of the agent with monocytes was ineffective. It was recently shown that the surface-active agent entered monocytes. *in vivo* This was done using a dye, Victoria Blue B 150, which is made soluble by Triton. A suspension of the particulate dye (1μ in diameter) is rapidly phagocytosed by monocytes from normal and detergent treated animals.

Whereas the particles of dye ingested by monocytes from normal animals remain intact, those ingested by monocytes from the detergent-treated animals rapidly dissolved to form blue patches which then coloured the whole cell. From simple colour tests it was concluded that 0·1 per cent of detergent entered the cell and was uniformly and freely available within the cells.

The range from anti- to growth-promoting action seemed to mean that the physical properties of the detergents alter in some way the surface of the tubercle bacillus *in vivo* so that its suspectibility within the monocyte is increased or decreased.

Hart and Rees[13] have used to forward their argument the analogy which has been shown to exist between the behaviour to sudden cooling of human red cells previously exposed to these detergents, and the behaviour of tubercle bacilli exposed to detergents within the macrophages. Those agents that decrease the haemolysis of red cells in thermal shock are antituberculosis and those that increase it are protuberculosis; those that are inactive in one instance are inactive in the other.

Preliminary chemical analysis of the lipids of red cells after exposure to the detergents, was found by Lovelock[14] to be consistent with the fact that those detergents with few ethylene oxide units displaced cholesterol preferentially from the red cell membranes, while those with many ethylene oxide units displaced phospholipid preferentially. It is thought by Hart and Rees[13] that if the surface-active agents affect tubercle bacilli and red cells in a similar way then those with few ethylene oxide units per molecule would displace the more hydrophobic lipids of the tubercle bacillus and so make it more easily digested inside the phagocytes.

These model experiments of Hart and his colleagues have proved stimulating and no doubt direct observations of the effects of enzymes on both treated and untreated cells will follow. A stage nearer to the living animal would be reached should both red cells and tubercle bacilli previously treated with the "few ethylene oxide" detergent molecules prove to be more easily digested with pepsin than untreated similar cells.

There are, of course, other possible mechanisms by which the detergents could produce their effect, such as modification of enzymic activity of the monocyte or of the tubercle bacillus. However, whatever their precise mode of action, they demonstrably influence the operation of the cellular defences in tuberculosis infection. The immediate point of attack is apparently the same as that previously seen as a unique difference in genetically resistant or genetically susceptible rabbits by Lurie[7].

He showed that the host resistance could be overcome by virulent organisms—in this case bovine bacilli—and also observed from cortisone experiments that those tubercle bacilli which were not phagocytosed were multiplying vigorously—"swarming." Another factor known to influence this is cord factor or virulence factor.

Cord Factor

Virulent tubercle bacilli grow on or in artificial media in a characteristic manner; they form sheafs or "cords" consisting of bacilli in close parallel

arrangements. The phenomenon was described by Koch[15] and its significance reinvestigated by Middlebrook and others[16]. From these original observations and all that has been learned since, it seems that under the conditions in which tubercle bacilli are usually grown, this morphological pattern is characteristic of strains which multiply in susceptible hosts.

Let us see where these new observations may fit into the jigsaw puzzle. When strains which multiply in a host are cultured in an aqueous medium, cord factor, as Bloch[17,18] calls it, which is extremely water-insoluble, accumulates at the bacterial surface, and can be removed with organic solvents without killing the bacteria. It is produced only by cord-forming strains of the tubercle bacillus.

In a lipophilic environment, it is partly released into the surrounding medium. Bacteria which are specifically prevented from synthesising cord factor *in vitro*, or which are grown under conditions where they release the compound into the medium, are significantly less virulent than control suspensions of bacteria from the same strain consisting of equal numbers of viable tubercle bacilli[19].

Chemical Nature of Cord Factor

Noll, Asselineau and Lederer (quoted by Bloch and Noll[20]) have made a partial characterisation of cord factor. It is an ester of molecular weight 1580 containing α-mycolic acid. It is not identical with any of the known constitutents of the tubercle bacillus. On alkaline hydrolysis an ether-soluble part is split off which has been identified as α-mycolic acid. The water-soluble part is a non-reducing carbohydrate fraction containing one atom of nitrogen. Acid hydrolysis splits this fraction into a reducing sugar, indentified as glucose, and a nitrogen-fragment of unknown structure.

The well-documented relations between the presence of cord factor and bacterial virulence have been supplemented by experiments in which injections of cord factor were combined with controlled and standardised tuberculosis infections in animals of known susceptibility[20]. These results may be summarised.

1. A single injection of cord factor, by itself innocuous, aggravates the course of mild as well as severe tuberculosis infections when given between one and 72 hours before infection.

2. A similar injection given to mice with chronic but stationary tuberculosis causes a flare of rapidly progressive disease.

3. These effects are quantitative in nature and the amounts of cord factor required to produce the aggravation depends on the susceptibility of the animal. The minimum effective does for mice is 1 to 10 μg.

4. The enhancing effect is specific; staphylo-, pneumo- or streptococcal infections are not influenced. Likewise bacterial components related chemically to cord factor but lacking the characteristic toxicity of the compound show no influence on murine tuberculosis.

5. Cord factor interferes with the chemotherapeutic activity of isoniazid. Tuberculosis infections aggravated by the action of cord

factor do not respond to isoniazid as well as do comparable control infections. This effect is quantitative and observed only in the living animal.

When cord factor is injected intraperitoneally it is reabsorbed and deposited in the lungs and liver and some in the kidneys. The distribution varies with the solvent. Followed histologically[20], the main injury which is seen consists of small haemorrhages caused by increased permeability of capillary vessels. Necrosis appears after repeated injections. There is haemostasis in the capillaries of the area and transgression of cellular elements of the blood into the perivascular tissue. The nature of the tissue damage is unspecific, but it is of the kind which has long been observed in early tuberculosis lesions by Canetti[21] and more recently by Florey and his colleagues[22].

Bloch postulates that after penetrating an organ the tubercle bacilli release cord factor from their cellular surfaces into the surrounding tissue, thus causing the observed tissue reaction.

There are many obvious reasons why the bacilli would find the haemorrhagic, oedematous mentruum adequate for their multiplication and why phagocytosis would be less effective in such an environment. With fewer bacilli destroyed, multiplication would begin earlier. Thus cord factor would enable more bacteria to multiply in the host during the very early phase of infection and presumably in later stages whenever metastatic foci are formed and new tissues invaded. In this way cord factor is seen by Bloch to act as an "aggressin" (Keppie, Smith and Harris-Smith[23]), and so to play an important part in the virulence of the tubercle bacillus and consequently in the pathogenesis of tuberculosis.

Acquired Immunity

Antibody Responses

No discussion of host resistance, with tuberculosis as a model, is complete without reference to acquired immunity. It is usual to say that antibodies play no part in acquired resistance to tuberculosis. This view is supported by the inability to correlate antibody responses, however measured, and resistance in experimental animals; also the effects of serum or other tissue extracts from vaccinated subjects on the bacillus *in vitro* are variable, and again the transfer of passive protection from vaccinated donors to normal animals has failed.

Raffel[24] has reported a recent investigation which was unable to show any indication that antibodies or other humoral factors serve as defences in tuberculosis. The exercise was remarkable for the use of a variety of current serological methods, and the use of most of the major isolatable constituents of the bacillary cell as test antigens.

The serological tests included complement fixation, agglutination, agar diffusion, and haemagglutination and haemolysis[25]. The developing method[26] for detecting incomplete antibodies was also used. The antigens were cultured bacilli, freshly isolated bacilli from sputum or other fluids, synthetic medium filtrates from bacillary cultures, cytoplasm of bacilli, proteins, polysaccharide from wax[27], phosphatides, wax[28] and firmly bound lipid[29].

Sera were from guinea-pigs treated with B.C.G. or bacilli killed with heat, mechanically, cathode irradiation or ultra-violet irradiation; or with water soluble bacillary constituents, or tuberculoprotein, wax, wax fractions, polysaccharides and phosphatides alone and mixed with tuberculoprotein and with killed bacilli extracted with organic solvents.

Protection against tuberculosis follows vaccination with B.C.G., and in a degree the water-paraffin-dead organism emulsion[30]. In the above tests the titres of the sera obtained from the protected animals did not exceed the reactions of the sera from animals treated with the variety of components which did not protect.

Passive Serum Transfer

The conventional test by which acquired resistance is challenged is passive serum transfer. The fact that tuberculosis is a chronic disease is evidence of the inadequacy of acquired resistance[32] and forewarns of the special precautions needed to effect artificial transfer. Calmette[33] lists the early unsuccessful trials and Long[34] should be consulted for an up to date record. Raffel and Efford[24] made two exacting studies in which the effects of serum were investigated on the progress of a light and a severe infection in guinea-pigs. Serum was provided by a donor group of 235 guinea-pigs vaccinated during six months with B.C.G. and also by a second non-vaccinated group of equal numbers acting as controls. Careful design of the experiment, made it possible to inject subcutaneously freshly-drawn donor serum; the dose was adequate—equivalent to about 250 ml. daily for man. Raffel describes the results of both experiments to be unequivocal, the animals receiving immune serum deriving no benefit from the transfer. This failure should be contrasted with the successful demonstration in another chronic infection, malaria, in which Coggleshall and Kumm[35] demonstrated passive immunity with small amounts of low titre antibody.

Implanted Semipermeable Capsules

Immunity mechanisms have also been studied by implanting semi-permeable capsules in the body[36,37]. Usually a plastic capsule of about 1 ml. volume is used. A window at one end is sealed with a Gradacol membrane; an average pore diameter of 60 to 70 mμ does not pass tubercle bacilli or host cells yet is permeable to plasma proteins. Known numbers of tubercle bacilli are sealed in these capsules which are then placed in the peritoneal cavities of normal and immune guinea-pigs. At intervals up to three months capsules are removed from both kinds of animals and quantitatively sampled for growth of tubercle bacilli. No differences have been found[24].

Phagocytic Mechanisms of Resistance

The repeated failure to demonstrate the participation of humoral factors in acquired resistance directs attention to the action of phagocytes in vaccinated animals. Obervations in this field are complicated by difficulties introduced by the special methods used and by the life span of both

tubercle bacilli and phagocytes. Differing opinions which have been recently expressed will no doubt be resolved by the discovery of the common artifact.

Lurie[38,39] implanted phagocytes from vaccinated rabbits in the anterior ocular chamber of normal rabbits and observed them to inhibit bacillary growth. Suter[40] also found phagocytes from B.C.G. vaccinated guinea-pigs to inhibit growth of tubercle bacilli in tissue culture. But Mackaness[41,42] finds macrophages from vaccinated rabbits failing to inhibit the growth of virulent bacilli. Again Suter finds *normal* macrophages to inhibit avirulent tubercle bacilli but not attenuated strains like B.C.G. But Mackaness, although observing a prolonged lag in the growth of avirulent bacilli, finds an even greater inhibition of attenuated strains. Raffel[24] using macrophages from B.C.G. vaccinated guinea-pigs noted the same inhibitory effect observed by Suter and not seen by Mackaness. However, when Raffel used the avirulent strain H37 Ra with normal macrophages he found no bacillary inhibition. This disagrees with both Suter and Mackaness. With B.C.G. Raffel found growth with normal macrophages which agrees with Suter's work and disagrees with Mackaness.

At the present time the situation is confused and it may be as Raffel[24] suggests that the development of a method for the direct observation of bacilli within living macrophages may resolve some of the inconsistencies.

Acquired Immunity—Summary

The evidence up to date is that acquired immunity in tuberculosis is not activated by circulating antibodies or other elements of the body fluids. This observation is based on serological tests, passive transfer experiments, and the use of semipermeable containers seeded with tubercle bacilli and implanted in the peritoneal cavities of immune animals.

In short, the required proof, successful elsewhere, is absent. Whether a cellular immunity to *M. tuberculosis* exists is still a matter for research. Current studies of infected macrophages by the method of tissue culture and the intraperitoneally implanted semipermeable capsules have not yet supplied proof, but the advances are promising.

No antibacterial activity of plasma, splenic juice, or disrupted macrophage substance of immunised animals could be demonstrated.

Finally, although it seems probable that immunity to *M. tuberculosis* is not dependent upon an antibody-leucocyte relation, the nature of the mechanism is still unsolved.

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RESEARCH PAPERS

ARYLOXYPROPANE DERIVATIVES

PART III. SOME ARYLOXYPROPANOLUREAS

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AN attempt to prepare 2-hydroxy-3-*o*-toloxypropylurea (I; Ar = R; X = NH·CO·NH_2) by heating 2-hydroxy-3-*o*-toloxypropyl chloride (I; Ar = R; X = Cl) with 4 molar equivalents of urea at 180° to 190° for 1 hour[1] led to the formation of 5-*o*-toloxymethyloxazolid-2-one (II) in *ca.* 30 per cent yield. This was improved by using 1:2-epoxy-3-*o*-toloxypropane in place of the chloride and was raised to *ca.* 60 per cent by heating the epoxide with urethane at 195° for 1 hour in the presence of a catalytic quantity of potassium hydroxide. The oxazolidone (II) was additionally prepared[2] by heating the chloride (I; Ar = R; X = Cl) with potassium cyanate in aqueous ethanolic solution. Its structure followed from its formation from 2-hydroxy-3-*o*-toloxypropylamine (I; Ar = R; X = NH_2) and phosgene in benzene solution.

The required urea (I; Ar = R; X = NH·CO·NH_2) was finally obtained by heating the amine (·HCl) (I; Ar = R; X = NH_2) with potassium cyanate in aqueous solution. On reaction with ethyl sodiomalonate in boiling ethanol it passed into *N*(2-hydroxy-3-*o*-toloxypropyl)-barbituric acid. Reaction of the amine (·HCl) (I; Ar = R; X = NH_2) with potassium thiocyanate furnished 2-hydroxy-3-*o*-toloxypropylthiourea (I; Ar = R; X = ·NH·CS·NH_2).

When 3-*o*-toloxypropanediol (mephenesin) (I; Ar = R; X = OH) was heated with urea[3] at 190° for 5 hours the product consisted of unchanged material admixed with mephenesin carbonate, also prepared in nearly quantitative yield by condensing mephenesin with methyl or ethyl carbonate on the steam bath in the presence of sodium ethoxide as catalyst.

Fischer and Krämer[4] have described the condensation of 2-hydroxy-3-phenoxypropyl chloride with ethyl sodiomalonate to give a product hydrolysed to the lactone (III; Ar = Ph; R = H). We now find that this reaction proceeds more readily employing 1:2-epoxy-3-phenoxypropane in place of the propyl chloride, when 3-ethoxycarbonyl-2-oxo-5-phenoxymethyltetrahydrofuran (III; Ar = Ph; R = Et) is readily obtained in good yield. 1:2-Epoxy-3-*o*-toloxypropane likewise yields the *o*-toloxy analogue (III; Ar = *o*-tolyl; R = Et), directly converted into 5(2′-hydroxy-3′-*o*-toloxypropyl)-barbituric acid by reaction with urea. Ethyl sodioacetamidomalonate may also be employed in these condensations to give with 1:2-epoxy- or 1-chloro-2-hydroxy-3-*o*-toloxypropane an intermediate ester hydrolysed by hydrochloric acid to 3-amino-2-oxo-5-*o*-toloxymethyltetrahydrofuran (IV). Reaction of the last compound with

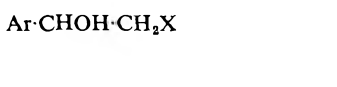
potassium cyanate gives 5(2′-hydroxy-3′-*o*-toloxypropyl) hydantoin (V).

By condensing *p*-ureidophenol with 2:3-epoxypropyl chloride in boiling ethanol-sodium ethoxide, Speckam[5] obtained a product formulated as 1:2-epoxy-3-(*p*-ureidophenoxy) propane (VI; Ar = R′). We now find that this material is actually 1:3-bis (*p*-ureidophenoxy)-2-hydroxypropane (R′·CHOH·R′), the authentic epoxide (VI; Ar = R′) being readily obtained by condensing *p*-ureidophenol with a large excess (6 moles) of 2:3-epoxypropyl chloride in aqueous alkaline solution. Decrease in the proportion of epoxy chloride employed results in the predominant formation of the bis-compound R′·CHOH·R′. The epoxypropane (VI; Ar = R′) was characterised by condensation with succinimide and phthalimide by methods previously described[6], and by reaction with piperidine and piperazine to give 2-hydroxy-1-piperidino-3-(*p*-ureidophenoxy)-propane (I; Ar = R′; X = NC_5H_{10}) and 1:4-bis (2-hydroxy-3-*p*-ureidophenoxypropyl)-piperazine (VII), respectively.

2-Hydroxy-3-*p*-ureidophenoxypropylurea (I; Ar = R′; X = ·NH·CO·NH_2) was prepared from 3-*p*-acetamido-1:2-epoxypropane (VI; Ar = R″). This was converted into N-(3-*p*-acetamidophenoxy-2-hydroxypropyl)-succinimide (I; Ar = R″; X = –$NC_4H_4O_2$) by reaction with succinimide in ethanolic solution containing a trace of pyridine as catalyst, from which 3-(*p*-aminophenoxy)-2-hydroxypropylamine (dihydrochloride) was obtained by hydrolysis with hydrochloric acid. Condensation with potassium cyanate in aqueous solution gave the urea (I; Ar = R′; X = ·NH·CO·NH_2). 3-*p*-Ureidophenoxypropane-1:2-diol (I; Ar = R′; X = OH)[7] was prepared (i) from the known 3-*p*-acetamidophenoxypropane-1:2-diol by acid hydrolysis, followed by condensation with potassium cyanate and (ii) from *p*-ureidophenol by reaction with 1:2-epoxypropan-3-ol. 1-*p*-Ureidophenoxy-4-oxahexane-2:6-diol (VIII) was similarly obtained by reaction between *p*-ureidophenol and 1-chloro-4-oxahexane-2:6-diol or 1:2-epoxy-4-oxahexan-6-ol (cf. ref. 8), or alternatively from *p*-acetamidophenol by the appropriate reaction sequence.

3-*o*-Ureidophenoxypropan-1:2-diol (I; Ar = R‴; X = OH), 2-hydroxy-3-*o*-ureidophenoxypropylurea (I; Ar = R‴; X = ·NH·CO·NH_2) and 1:3-bis (*o*-ureidophenoxy) propan-2-ol (R‴·CHOH·R‴) were similarly obtained from 3-*o*-acetamidophenoxy-1:2-epoxypropane.

Biological study of the foregoing compounds by Dr. A. David and his colleagues is in progress.



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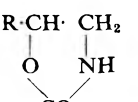

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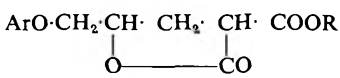

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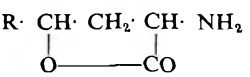

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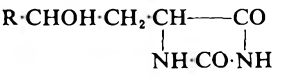

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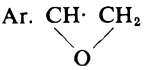

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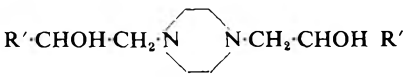

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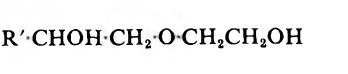

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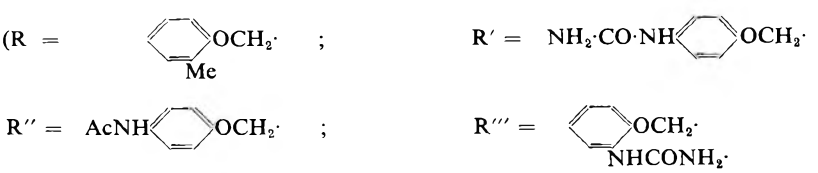

Experimental

5-o-*Toloxymethyl-oxazolid*-2-*one* (II)

(*a*) A mixture of 2-hydroxy-3-*o*-toloxypropyl chloride (20 g.) and urea (24 g.) was heated at 180° to 190° for 1 hour with occasional shaking. After cooling, the residue was suspended in water and extracted with chloroform. Concentration of the chloroform followed by dilution with light petroleum (b.p. 60° to 80°) yielded the product (6·5 g.), which was purified by crystallisation from ethyl acetate, forming colourless prisms, m.p. 128° to 129°. Found: C, 63·9; H, 6·1; N, 7·1. $C_{11}H_{13}O_3N$ requires C, 63·8; H, 6·3; N, 6·8 per cent.

(*b*) 1:2-Epoxy-3-*o*-toloxypropane (16·4 g.) and urea (7·2 g.) were heated at 175° to 180° and the product (II) (8·1 g.) isolated as in (*a*). The yield was not improved by using up to 4 moles of urea.

(*c*) To a solution of 1:2-epoxy-3-*o*-toloxypropane (16·4 g.) in ethanol (50 ml.) was added powdered sodium cyanate (7·8 g.) followed by concentrated hydrochloric acid (8·6 ml.). After heating under reflux for 4 hours, the mixture was diluted and the product (5·5 g.) isolated by extraction with chloroform.

(*d*) A mixture of 1:2-epoxy-3-*o*-toloxypropane (65·6 g.) and urethane (42·8 g.) was melted and potassium hydroxide (0·5 g.) in methanol (5 ml.) added as catalyst. The mixture was heated at 190° to 195° for one hour and the ethanol which distilled off was collected (23 ml.). The product (49 g.), isolated as in (*a*), had m.p. and mixed m.p. 127° to 128°. Pyridine was less satisfactory than potassium hydroxide as catalyst.

(*e*) A suspension of 2-hydroxy-3-*o*-toloxypropylamine hydrochloride (21·8 g.) in dry benzene (100 ml.) was treated with a solution of phosgene (20 g.) in dry benzene (250 ml.). The mixture was heated on the steam bath for 16 hours and the solvent removed at reduced pressure. The residue was dissolved in water and extracted with chloroform. Concentration of the chloroform extract followed by crystallisation from ethyl acetate-light petroleum (b.p. 60° to 80°) yielded the product (II) (7·5 g.), m.p. 126° to 128°, not depressed on admixture with a sample prepared by method (*a*).

Reaction of Mephenesin with Urea

A mixture of mephenesin (45·5 g.) and urea (30 g.) was heated at 180° to 190° for 5 hours, and then poured into water. The suspension was extracted with chloroform and the extract washed with water. The chloroform was removed and the residue distilled *in vacuo* to yield a mixture of unchanged mephenesin (12·4 g., m.p. 71°), mephenesin carbonate (8·5 g., m.p. 94° to 96°) and 5-*o*-toloxymethyloxazolid-2-one (16 g., m.p. 127° to 129°).

5-*Phenoxymethyloxazolid*-2-*one*, prepared from 1:2-epoxy-3-phenoxypropane and urea as for (II), had m.p. 125° to 127°, after crystallisation from a mixture of chloroform and light petroleum (b.p. 60° to 80°). Found: C, 62·2; H, 5·2; N, 7·0. $C_{10}H_{11}O_3N$ requires C, 62·2; H, 5·7; N, 7·3 per cent.

5-o-*Chlorophenoxymethyloxazolid*-2-*one*, prepared by interaction of 2-hydroxy-3-*o*-chlorophenoxypropyl chloride and sodium cyanate in aqueous ethanol, separated from ethyl acetate in colourless prisms of m.p. 151°. Found: C, 53·1; H, 4·4; N, 5·8. $C_{10}H_{10}O_3NCl$ requires C, 52·7; H, 4·4; N, 6·2 per cent.

5-o-*Toloxymethyldioxol*-2-*one*. ("Mephenesin carbonate")

A mixture of mephenesin (182 g.) and ethyl carbonate (118 g.) was warmed until homogeneous and a solution of sodium (0·5 g.) in ethanol (10 ml.) added. The mixture was heated on the steam-bath for 30 minutes, ethanol being allowed to distil off freely. The residue solidified on cooling and was crystallised from ethanol-light petroleum (b.p. 60° to 80°) or from benzene. Yield 90 per cent of a product m.p. 96°. Found: C, 63·5; H, 5·9. Calculated for $C_{11}H_{12}O_4$; C, 63·4; H, 5·8 per cent.

5-p-*Chlorophenoxymethyldioxol*-2-*one* ("chlorphenesin carbonate"), prepared from chlorphenesin and ethyl carbonate, separated from ethanol in shining needles of m.p. 96° to 97°. Found: C, 52·8; H, 4·2; Cl, 15·8. $C_{10}H_9O_4Cl$ requires C, 52·5; H, 4·0; Cl, 15·5 per cent.

2-*Hydroxy*-3-o-*toloxypropyl urea* (I; Ar = R; X = $NH{\cdot}CO{\cdot}NH_2$)

To a solution of 2-hydroxy-3-*o*-toloxypropylamine hydrochloride (30 g.) in water (100 ml.) was added a solution of sodium cyanate (12 g.) in water (30 ml.) and the mixture warmed for a few minutes. The urea separated on cooling. It was crystallised from ethyl acetate forming small shining plates, m.p. 131° to 132°. Found: C, 59·2; H, 6·9; N, 12·6. $C_{11}H_{16}O_3N_2$ requires C, 58·9; H, 7·2; N, 12·5 per cent.

2-*Hydroxy*-3-o-*toloxypropyl thiourea* (I; Ar = R; X = NH_2) was prepared as above, but using potassium thiocyanate in place of sodium cyanate. It crystallised from ethanol-ether in fine white needles, m.p. 120° to 122°. Found: C, 54·6; H, 6·5; S, 12·8. $C_{11}H_{16}O_2N_2S$ requires C, 54·9; H, 6·7; S, 13·3 per cent.

N-(2-*Hydroxy*-3-o-*toloxy*)-*propyl barbituric acid*

A solution of 2-hydroxy-3-*o*-toloxypropylurea (28·5 g.) in absolute ethanol (120 ml.) was added to a solution of ethyl sodiomalonate prepared from ethyl malonate (20·4 g.), and sodium (2·93 g.) in absolute ethanol (150 ml.). The mixture was refluxed for 15 hours, cooled to 50° and water (50 ml.) added to dissolve the bulk of the solid. The solution was then acidified with concentrated hydrochloric acid (12 ml.) and filtered immediately. The product (26 g.) separated rapidly, it had m.pt. 170° to 172°. The m.p. was not raised by crystallisation from a large volume of water. Found: C, 57·3; H, 5·6; N, 9·9. $C_{14}H_{16}O_5N_2$ requires C, 57·5; H, 5·5; N, 9·6 per cent.

3-*Methoxycarbonyl*-2-*oxo*-5-*phenoxymethyltetrahydrofuran* (II, Ar = Ph; R = Me).

1 : 2-Epoxy-3-phenoxypropane (30 g.) was added in one portion to a warm solution of sodiomalonic ester, prepared from ethyl malonate (32 g.) and sodium (4·6 g.) in absolute methanol (100 ml.). The moderately exothermic reaction was completed by heating on the steam bath for 1 hour. Most of the methanol was then boiled off and the residue was treated with 50 per cent aqueous acetic acid (25 ml.), diluted with water and the oil extracted with chloroform. The chloroform was removed at reduced pressure. A portion of the residue was distilled *in vacuo* and had b.p. 190°/1·0 mm. Slight decomposition occurred. Found: C, 62·3; H, 5·5. $C_{13}H_{14}O_5$ requires C, 62·4; H, 5·6 per cent.

5-(2′-*Hydroxy*-3′-o-*toloxy*)-*propyl barbituric acid*

1 : 2-Epoxy-3-*o*-toloxypropane (16·4 g.) was added to a solution of sodiomalonic ester, prepared from ethyl malonate (16 g.) and sodium (2·3 g.) in dry methanol (80 ml.). The mixture was refluxed for 1 hour and a solution of urea (6 g.) in dry methanol (50 ml.) was then added. Refluxing was continued for 10 hours. The solid which separated on cooling was collected, suspended in hot water (75 ml.) and acidified with concentrated hydrochloric acid (9 ml.). The product which separated (27·5 g.) was crystallised from water and then from ethanol and had m.p. 200°. Found: C, 57·7; H, 5·4; N, 9·9. $C_{14}H_{16}O_5N_2$ requires C, 57·5; H, 5·5; N, 9·6 per cent.

3-*Amino*-2-*oxo*-5-o-*toloxymethyltetrahydrofuran* (IV)

2-Hydroxy-3-*o*-toloxypropyl chloride (50 g.) was added to a solution of methyl sodio-acetamidomalonate, prepared from ethyl acetamidomalonate (54·3 g.) and sodium (5·8 g.) in anhydrous methanol (300 ml.), and the mixture heated under reflux for 5 hours. The precipitated sodium chloride was filtered off and washed with a little methanol. The combined filtrate and washings was concentrated and the residual oil hydrolysed by heating under reflux with concentrated hydrochloric acid (60 ml.) for 3 hours. The acid was removed at reduced pressure and the residue crystallised from methanol-ether. The crude crystalline solid was crystallised further from methanol-ethyl acetate yielding the product (9·8 g.) m.p.

228° to 230° (decomp.), which separated from methanol in small shining plates, m.p. 230° to 232° (decomp.).

Found: C, 56·0; H, 6·2; N, 5·3; Cl, 13·9. $C_{12}H_{16}O_3NCl$ requires C, 55·9; H, 6·3; N, 5·4; Cl, 13·8 per cent. Glycine methyl ester hydrochloride (14 g.) m.p. 176° to 178° (decomp.) was isolated from the mother liquors.

A similar result was obtained when 1:2-epoxy-3-*o*-toloxypropane was used in place of 2-hydroxy-3-*o*-toloxypropyl chloride in the reaction.

5-(2′-*Hydroxy*-3′-o-*toloxy*)-*propyl hydantoin* (V)

The preceding amine hydrochloride (4·4 g.) was dissolved in water (50 ml.) and treated with an aqueous solution of sodium cyanate (2 g.). The solid product was collected, washed with water and purified by crystallisation from ethyl acetate, separating in needles m.pt. 136° to 138°, solidifying rapidly and remelting at 210°. Found: C, 58·8; H, 6·0; N, 10·3. $C_{13}H_{16}O_4N_2$ requires C, 59·1; H, 6·1; N, 10·6 per cent.

1:2-*Epoxy*-3-(p-*ureidophenoxy*)-*propane* (VI, Ar = R′)

To a solution of *p*-ureidophenol (30·4 g.) in water (420 ml.) containing sodium hydroxide (8 g.), 2:3-epoxypropyl chloride (epichlorohydrin) (110 g. = 6 mole equivs) was added in one portion with stirring. The mixture was stirred vigorously at room temperature for 6 hours. The solid was collected, washed with water and dried. Yield 41·5 g., m.p. 149° to 151°. Crystallisation from ethanol-light petroleum (b.p. 60° to 80°) raised the m.p. to 152° to 153°. Found: C, 58·2; H, 6·0; N, 13·2. $C_{10}H_{12}O_3N_2$ requires C, 57·7; H, 5·8; N, 13·5 per cent.

1:3-*bis*-(p-*Ureidophenoxy*)-2-*hydroxypropane*

To a stirred solution of *p*-ureidophenol (30·4 g.) in water (250 ml.) containing potassium hydroxide (11·2 g.) was added 2:3-epoxy-propyl chloride (9·3 g.) and the mixture stirred at room-temperature for 8 hours. The solid was collected, washed well with water and purified by crystallisation from aqueous ethylene glycol. Yield 26 g., m.p. 234° to 235° (decomp.). Found: C, 55·9; H, 5·9; N, 14·7. $C_{17}H_{20}O_5N_4$ requires C, 56·6; H, 5·6; N, 15·6 per cent.

2-*Hydroxy*-1-*succinimido*-3-(p-*ureidophenoxy*)-*propane*

A mixture of 1:2-epoxy-3-(*p*-ureidophenoxy)-propane (10·4 g.) and succinimide (5 g.) was dissolved in hot ethanol (150 ml.), pyridine (5 drops) was added as catalyst and the mixture heated for 5 hours with concentration to *ca.* 100 ml. The product which separated on cooling was crystallised from water, forming white needles of m.p. 202° to 203°. Found: C, 54·5; H, 5·8. $C_{14}H_{17}O_5N_3$ requires C, 54·7; H, 5·6 per cent.

2-*Hydroxy*-1-*phthalimido*-3-(p-*ureidophenoxy*)-*propane*

A mixture of 1:2-epoxy-3-(*p*-ureidophenoxy)propane (5·2 g.) and phthalimide (3·7 g.) was dissolved in ethanol (40 ml.), pyridine (2 drops) was added as catalyst, and the solution heated for 10 hours. The product

which separated after slight concentration and cooling was crystallised from acetic acid and had m.p. 199° to 200°. Found: C, 60·3; H, 4·7; N, 11·8. $C_{18}H_{17}O_5N_3$ requires C, 60·8; H, 4·8; N, 11·8 per cent.

2-*Hydroxy*-1-*piperidino*-3-(p-*ureidophenoxy*)-*propane* (I, Ar = R′; X = $N{\cdot}C_5H_{10}$)

To a solution of 1:2-epoxy-3-(*p*-ureidophenoxy)-propane (10·4 g.) in ethanol (40 ml.) was added piperidine (4·3 g.). The solution was refluxed for 4 hours and then treated at once with a slight excess of hydrochloric acid gas. The hydrochloride (10·3 g.) was purified by crystallisation from methanol-ethyl acetate and had m.p. 198° to 199°. Found: C, 54·3; H, 7·5; N, 12·6; Cl, 10·7. $C_{15}H_{24}O_3N_3Cl$ requires C, 54·6; H, 7·3; N, 12·7; Cl, 10·8 per cent.

1:4-*bis*-(2-*Hydroxy*-3-p-*ureidophenoxy*)-*propyl piperazine* (VII)

A solution of 1:2-epoxy-3-(*p*-ureidophenoxy)-propane (8·2 g.) in ethanol (150 ml.) was treated with piperazine hexahydrate (3·9 g.) and the mixture heated on the steam bath for 1 hour. The product (9 g., m.p. 204° to 208°) was purified by dissolving in dilute hydrochloric acid and precipitating with dilute sodium carbonate solution. It then had m.p. 206° to 208°. Found: C, 56·4, 56·0; H, 7·1, 6·7; N, 16·7. $C_{24}H_{34}O_6N_6$; $\frac{1}{2}H_2O$, requires C, 56·3; H, 6·9; N, 16·4 per cent.

3-(p-*Aminophenoxy*)-2-*hydroxypropylamine dihydrochloride*

N-(3-*p*-Acetamidophenoxy-2-hydroxy)-propylsuccinimide[6] (20 g.) was heated under reflux with concentrated hydrochloric acid (50 ml.) for 6 hours. The mixture was evaporated to dryness at reduced pressure, the residue dissolved in water (50 ml.) and extracted with three portions of ethyl acetate to remove succinic acid. After concentration at reduced pressure again the solid residue (14·6 g.) was crystallised from aqueous ethanol and had m.p. 256° to 260° (decomp.). Found: N, 10·7; Cl, 27·2. $C_9H_{16}O_2N_2Cl_2$ requires N, 11·0; Cl, 27·8 per cent.

2-*Hydroxy*-3-(p-*ureidophenoxy*)-*propylurea* (I; Ar = R′; X = $NH{\cdot}CO{\cdot}NH_2$)

The foregoing dihydrochloride (2·55 g.) was dissolved in water (7 ml.) and treated with a solution of sodium cyanate (1·63 g.) in water (15 ml.). The product (2·5 g.) separated on standing and crystallised from water in nodules, m.p. 180° to 182°. Found: C, 48·9; H, 5·9; N, 21·4. $C_{11}H_{16}O_4N_4$ requires C, 49·2; H, 6·0; N, 20·9 per cent.

3-p-*Acetamidophenoxypropane*-1:2-*diol* was prepared by condensation of *p*-acetamidophenol with 2:3-dihydroxypropyl chloride (glycerol α-chlorohydrin) in aqueous alkaline solution or with glycidol in alcoholic solution using pyridine as catalyst. It crystallised from ethyl acetate containing a few drops of methanol and had m.p. 136° to 138°. Found: C, 58·4; H, 6·7; N, 6·2. Calculated for $C_{11}H_{15}O_4N$: C, 58·7; H, 6·7; N, 6·2 per cent.

3-(p-*Aminophenoxy*)-*propane*-1 : 2-*diol hydrochloride*

Hydrolysis of the foregoing acetamido-compound (5 g.) in concentrated hydrochloric acid (30 ml.) for 1 hour at reflux temperature yielded the amine hydrochloride, which separated from ethanol-ether in white needles, m.p. 166° to 168°. Found: C, 48·6; H, 6·5; N, 6·0; Cl, 15·8. Calculated for $C_9H_{14}O_3NCl$: C, 49·2; H, 6·4; N, 6·4; Cl, 16·1 per cent.

3-p-*Ureidophenoxypropane*-1 : 2-*diol* (I; Ar = R′; X = OH)[7]

A solution of the foregoing amine hydrochloride (20 g.) in water (100 ml.) was treated with a solution of sodium cyanate (7·1 g.) in water (20 ml.). The product which separated after a few hours standing was crystallised from ethanol-ether and had m.p. 156° to 157°. Found: C, 52·8; H, 6·2; N, 12·2. Calculated for $C_{10}H_{14}O_4N_2$: C, 53·1; H, 6·2; N, 12·4 per cent.

The same compound was obtained when *p*-ureidophenol (15·2 g.) dissolved in water (50 ml.) containing sodium hydroxide (4 g.) was treated with 2 : 3-dihydroxypropyl chloride (13·2 g.) and the solution stirred at room temperature for 2 hours. It was also formed by condensation of *p*-ureidophenol with glycidol in concentrated alcoholic solution in the presence of a basic catalyst.

1-p-*Acetamidophenoxy*-4-*oxahexan*-2 : 6-*diol*

(*a*) A mixture of *p*-acetamidophenol (20 g.) and 1 : 2-epoxy-4-oxahexan-6-ol[8] (15·6 g.) in the minimum of hot ethanol was treated with pyridine (3 drops) and the mixture heated on the steam bath for 3 hours with concentration.

The gummy residue crystallised on boiling with ethyl acetate containing a few drops of ethanol and had m.p. 116° to 117°. Found: N, 5·1. $C_{13}H_{19}O_5N$ requires N, 5·2 per cent.

(*b*) To a solution of *p*-acetamidophenol (15·1 g.) in water (85 ml.) containing sodium hydroxide (4 g.) was added 1-chloro-4-oxahexane-2 : 6-diol (15·6 g.) and the mixture heated with stirring for 1 hour. After concentration at reduced pressure the residue was purified as in (*a*).

1-p-*Aminophenoxy*-4-*oxahexane*-2 : 6-*diol*

The foregoing compound (8 g.) was hydrolysed by heating with concentrated hydrochloric acid (30 ml.) for 2 hours. The amine hydrochloride, isolated in the usual manner, was purified by crystallisation from ethanol-ether and had m.p. 151° to 152°. Found: C, 49·7; H, 6·9; N, 5·4. $C_{11}H_{18}O_4NCl$ requires C, 50·1; H, 6·9; N, 5·3 per cent.

1-p-*Ureidophenoxy*-4-*oxahexane*-2 : 6-*diol* (VIII)

To a solution of the foregoing hydrochloride (4·5 g.) in water (20 ml.) was added a solution of sodium cyanate (1·1 g.) in water (10 ml.). The product which separated on standing was crystallised from ethanol-ether and had m.p. 169° to 171°. Found: C, 53·3; H, 6·7; N, 10·0. $C_{12}H_{18}O_5N_2$ requires C, 53·3; H, 6·7; N, 10·4 per cent.

3-o-*Acetamidophenoxy-propane*-1 : 2-*diol* was prepared by condensation of *o*-acetamidophenol with 2 : 3-dihydroxypropyl chloride in aqueous alkaline solution by the standard method. The product was crystallised from ethyl acetate-ether, and had m.p. 146° to 147°. Found: C, 58·7; H, 6·8; N, 6·3. $C_{11}H_{15}O_4N$ requires C, 58·7; H, 6·7; N, 6·2 per cent.

3-o-*Aminophenoxypropane*-1 : 2-*diol* was prepared by hydrolysis of the corresponding acetamido-compound with concentrated hydrochloric acid. It crystallised from ethanol-ether in white needles, m.p. 170°. Found: C, 49·1; H, 6·3; N, 6·1. Calculated for $C_9H_{14}O_3NCl$; C, 49·2; H, 6·4; N, 6·4 per cent. Treatment of this compound with sodium cyanate in aqueous solution yielded 3-o-*ureidophenoxypropane*-1 : 2-*diol* of m.p. 95°, after crystallisation from ethanol-ether. Found: N, 12·1. Calculated for $C_{10}H_{14}O_4N_2$: N, 12·4 per cent.

2 : 3-*Epoxy*-1-(o-*acetamidophenoxy*)-*propane* was prepared by condensation of *o*-acetamidophenol with excess (5 mole equivs) of 2 : 3-epoxy-propyl chloride in aqueous alkaline solution. It crystallised from light petroleum (b.p. 80° to 100°) in white fluffy needles, m.p. 105°. Found: C, 64·0; H, 6·0; N, 7·0. $C_{11}H_{13}O_3N$ requires C, 63·8; H, 6·3; N, 6·8 per cent.

Condensation of this epoxide with succinimide in ethanol with pyridine as catalyst yielded 1-o-*acetamidophenoxy*-2-*hydroxy*-3-*succinimidopropane* which separated from ethyl acetate-light petroleum (b.p. 60° to 80°) in white needles, m.p. 112° to 114°. Found: C, 59·1; H, 5·8; N, 9·2. $C_{15}H_{18}O_5N_2$ requires C, 58·8; H, 5·9; N, 9·2 per cent. Hydrolysis of the foregoing succinimido-compound in the usual manner with concentrated hydrochloric acid yielded the dihydrochloride of 1-o-*aminophenoxy*-2-*hydroxy-propylamine*, which crystallised from ethyl acetate containing a little methanol, in white needles, m.p. 232° (decomp.). Found: C, 42·4; H, 6·1. $C_9H_{16}O_2N_2Cl_2$ requires C, 42·4; H, 6·4 per cent.

Treatment of this dihydrochloride in aqueous solution with sodium cyanate yielded 2-*hydroxy*-1-o-*ureidophenoxypropyl urea*, which had m.p. 174° after crystallisation from alcohol-ether. Found: C, 48·6; H, 6·0; N, 20·6. $C_{11}H_{16}O_4N_4$ requires C, 49·2; H, 6·0; N, 20·9 per cent.

1 : 3-bis-(o-*Acetamidophenoxy*)-2-*hydroxy propane*

Condensation of *o*-acetamidophenol (2 moles) with 2 : 3-epoxypropyl chloride (1 mole) in aqueous alkaline solution yielded the product which separated from aqueous ethanol as the monohydrate, m.p. 124° to 126°. Found: C, 60·2; H, 6·4; N, 7·8. $C_{19}H_{24}O_6N_2$ requires C, 60·6; H, 6·4; N, 7·4 per cent. The anhydrous compound was obtained on drying at 95° for some hours or by crystallisation from ethylene dichloride-light petroleum (b.p. 80° to 100°) and had m.p. 165° to 166°. Found: N, 7·9. Calculated for $C_{19}H_{22}O_5N_2$: N, 7·8 per cent.

Hydrolysis of the preceding compound with concentrated hydrochloric acid yielded 1 : 3-*bis*-(o-*aminophenoxy*)-2-*hydroxy propane dihydrochloride*, which separated from methanol-ether in white needles, m.p. 280° to 282° (decomp.). Found: C,51·7; H, 5·9; N, 7·8. $C_{15}H_{20}O_3N_2Cl_2$ requires C, 51·8; H, 5·8; N, 8·1 per cent.

Treatment of the dihydrochloride with sodium cyanate in aqueous solution yielded 1 : 3-*bis*-(p-*ureidophenoxy*)-2-*hydroxy propane* as a microcrystalline powder, m.p. 174° to 184° after repeated crystallisation from aqueous methanol. Found: C, 56·7; H, 5·7; N, 15·9. $C_{17}H_{20}O_5N_4$ requires C, 56·6; H, 5·6; N, 15·9 per cent.

Summary

1. Attempts to prepare an aryloxypropanolurea by condensing 3-aryloxy-2-hydroxypropyl chloride or 3-aryloxy-1 : 2-epoxypropane with urea led to the formation of the corresponding 5-aryloxymethyloxazolid-2-one. The required ureas were ultimately obtained in excellent yield by reaction between the 3-aryloxy-2-hydroxypropylamine and an alkali metal cyanate.

2. Some 3-ureidoaryloxypropane-1 : 2-diols and 2-hydroxy-3-ureidopropylamines were also prepared.

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DERIVATIVES OF ACETAMIDE AND BENZAMIDE AS HYPNOTICS

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It has been known since 1910[1] that certain dialkyl-arylacetamides possess sedative, hypnotic and antipyretic properties, and Lumière and Perrin[2] later described the hypnotic activity of phenylacetamides in dogs. Trialkylacetamides have been reported to possess spasmolytic[3] and hypnotic[4] properties, but none of the compounds described appears to have found its way into clinical use.

Our interest in 3-methylpentynol, and in hypnotics in general, led us to reinvestigate and extend earlier work in this connection, in the hope of discovering a compound with hypnotic properties intermediate between those of methylpentynol and the barbiturates, but without the disadvantages of the latter.

Although a number of active compounds were found, none was sufficiently outstanding to justify its introduction to clinical use. Nevertheless, in addition to the preparation of a number of new compounds, we have attempted a correlation of their hypnotic activity and chemical constitution within the group of acetamide derivatives.

Benzamide[5] has also been reported to possess hypnotic properties and a number of derivatives have been examined, but without discovering any which was of practical interest.

Narcotic Effect

The criterion of narcotic activity adopted was the abolition of the righting reflex in mice. Screening tests were carried out at four geometric dose levels, 1·14, 0·77, 0·52 and 0·35 g./kg. on groups of five female mice (Schofield strain), with methylpentynol as the reference compound in all cases, and observations were made at half an hour, 1 hour, and then hourly intervals up to 6 hours after oral administration of the appropriate dose in suspension in 5 per cent acacia solution. The doses chosen were such that on the top dose all the animals treated with methylpentynol invariably showed abolition of the righting reflex, whereas on the lowest dose only rarely did more than one animal show a positive response. Only in those in which the narcotic activity of the compound was similar or greater than that of methylpentynol, was a more detailed examination made using a larger number of animals, except when it appeared necessary to assist correlation of narcotic activity with

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chemical constitution. An estimate of the median narcotic dose (ND50) was made as described by Marshall and Vallance[6], when required.

Toxicity

Determinations of acute oral toxicity (LD50) were made only when narcotic activity indicated possible clinical value. Indications of gross toxicity were obtained in many cases during narcotic screening tests. Appropriate compounds were administered orally to female mice (Schofield strain) under the conditions specified by Marshall and Vallance[6] and the LD50 estimates were made as described by Bliss[7].

Preparation of chemical compounds

Aliphatic Derivatives of Acetamide

The alkyl acetamides were prepared by the action of alkyl halides on acetonitrile in the presence of sodamide in liquid ammonia[8], and subsequent hydrolysis of the nitrile with 85 per cent sulphuric acid. In the case of the ethyl and *n*-propyl derivatives, mixtures of the di- and tri-alkyl acetonitriles (with a trace of the monoalkyl acetonitrile) were obtained and were separated by fractionation, but *n*-butyl-bromide gave only the corresponding tri-alkyl derivative. Tri-chloroacetamide, *N*-ethyl and *NN*-diethyltrichloroacetamide were all prepared from trichloroacetyl chloride by reaction with ammonia or the appropriate amine. Siccatamide B was prepared from the acid, via the acid chloride.

Derivatives of Phenylacetamide

All the compounds of the phenylacetamide series have been prepared from the corresponding nitrile by hydrolysis. Nitriles with an α-hydrogen atom or an α-methyl group can be hydrolysed by 90 per cent (w/w) sulphuric acid, but sulphonation occurred with *o*- and *p*-tolyl-dimethyl-acetonitrile, and a modified procedure was adopted in these cases. The higher homologues are found to be very resistant to hydrolysis and require heating with 10 per cent potassium hydroxide in amyl alcohol for up to 48 hours. Unfortunately, under these conditions a varying amount of non-volatile material, possibly a polymer, is formed, which may increase the difficulty of purifying the amide. In the case of diallyl-phenylacetamide so much of the material was formed that none of the desired product could be isolated.

Symmetrical di-alkyl phenylacetonitriles were prepared by the reaction of the theoretical quantity of sodamide with phenylacetonitrile in ether and the subsequent addition of the appropriate alkyl halide. Unalkylated and monoalkylated nitriles were separated by reaction with acrylonitrile to give high-boiling fractions, which were easily separated by distillation.

In all the derivatives prepared containing two different alkyl groups, one of these was either methyl or ethyl. Bulk quantities of phenyl-ethyl-acetonitrile and phenyl-methyl-aceto-nitrile were prepared by the alkylation of phenyl cyanacetic ester followed by hydrolysis and decarboxylation. The second alkyl group was then introduced by the action of the alkyl halide on the sodio-derivative in ether. This reaction failed with

hydratroponitrile and *tert.*-butyl chloride. The following are typical examples:

Phenyl-αα-diethylacetonitrile. 31·5 g (0·81 mole) of sodamide and 750 ml. of dry benzene were heated to boiling point and, after removal of the source of heat, 90 g. (0·77 mole) of phenylacetonitrile was added at a rate sufficient to maintain gentle boiling. After refluxing for a further half-hour the dark red solution was cooled and 90 g. (0·825 mole) of ethyl bromide was added dropwise during about 2 hours and the whole left to stand overnight. After addition of a further 31·5 g. of sodamide the mixture was refluxed for 12 hours and then reacted as above with a further 90 g. of ethyl bromide. After cooling to room temperature, 200 ml. of water was added and the whole was extracted with benzene and once with ether. The combined extracts were washed with 2N-hydrochloric acid, 2N-sodium carbonate and water, and dried over anhydrous magnesium sulphate. Removal of the solvent left 70 g. of a crude product which contained unchanged material together with mono- and diethyl derivatives. 65 g. (1·23 moles) of acrylonitrile was added together with a few drops of sodium ethoxide in ethanol. The temperature was kept below 30° by cooling and, when the reaction had subsided a further small amount of catalyst was added until no further substantial rise in temperature occurred. After standing overnight 30 ml. of water was added, the base was neutralised with acetic acid, and the mixture was extracted with ethylene dichloride. The extracts were washed with water and dried over anhydrous magnesium sulphate. After removal of the solvent the residue was fractionally distilled and 55 g. of phenyl-αα-diethylacetonitrile, b.p. 137 to 139°/21 mm., was obtained. The high-boiling residue consisted of condensation products of acrylontrile with the other nitriles originally present.

Phenyl-αα-diethylacetamide (*Compound* 437). 65 g. (0·376 mole) of the above nitrile was added to a hot solution of 30 g. (0·535 mole) of potassium hydroxide in 300 ml. of technical amyl alcohol, and the mixture refluxed for 24 hours, cooled, and poured into an equal volume of water. After ether extraction the organic layer was washed free from alkali with water, and dried over anhydrous magnesium sulphate. After removal of the solvent the residue gave on fractional distillation, a forerun of 19·2 g. (largely unchanged nitrile) and phenyl-αα-diethylacetamide; yield 33·9 g. (70 per cent on nitrile hydrolysed), m.p. 51 to 52° ex cyclohexane, *lit.* 53°. Found: C, 75·40; H, 8·82; N, 7·15. $C_{12}H_{17}ON$ requires C, 75·35; H, 8·96; N, 7·32 per cent.

From the alkaline liquors 9·7 g. (19·4 per cent on reacted nitrile) of phenyl-αα-diethylacetic acid was obtained on acidification.

Hydratroponitrile. $C_6H_5{\cdot}CH(CH_3)CN$ 23 g. of sodium were dissolved in 350 ml. of dry ethanol and cooled to about 70°, and 189 g. (1 mole) of ethylphenylcyanacetate were added, followed by 145 g. (1·01 mole) of methyl iodide dropwise to maintain gentle boiling. Refluxing was continued for 2 hours, 10 ml. of water was added, most of the ethanol was distilled off, and the residue was poured into water and extracted with ether. The ether extract was washed with water, dried over

anhydrous magnesium sulphate and the solvent was removed. Fractional distillation of the residue yielded 96 g. (73·6 per cent) of hydratroponitrile, b.p. 108°/10 mm. (*cf.* Newman and Closson[10], Goerner and Workman[9]). Hydratroponitrile was alkylated to give the unsymmetrical phenyl-αα-dialkylacetonitriles, as described for phenyl-αα-diethylacetonitrile above.

α-Phenyl-αγ-dimethylvaleramide. $C_6H_5{\cdot}C(R,R'){\cdot}CONH_2$·(R = Me; R′ = *iso*-Bu) 9·27 g. (0·05 mole) of α-phenyl-αγ-dimethylvaleronitrile was mixed with 35 g. of 90 per cent (w/w) sulphuric acid, heated to 100° and concentrated sulphuric acid added dropwise until all the oil dissolved. Heating was continued for a further 15 hours, and the mixture cooled and poured into water. The product was extracted with ether, washed with aqueous sodium carbonate and water, and dried (magnesium sulphate). After removal of the ether, the residual oil was fractionated. Yield, 6·2 g. (61·5 per cent), b.p. 182°/8 mm. Found: C, 76·05; H, 9·13; N, 6·70. $C_{13}H_{19}ON$ requires C, 76·05; H, 9·33; N, 6·82 per cent. The product was a viscous liquid. The above method gave better results than the alkaline hydrolysis described for phenyl-αα-diethylacetonitrile.

Diphenylacetamide. Diphenylacetonitrile (4 g.) prepared by Shapiro's method[11] was hydrolysed by Anschutz and Romig's method[12]. Yield, 2·2 g. (50 per cent), m.p. 167 to 168° (*lit.* 166°).

Nuclear substituted phenylacetamides. *o*- and *p*-Methyl benzyl chlorides were prepared from the respective xylenes, and *o*-, *m*-, and *p*-chlorobenzyl chlorides were prepared from the corresponding chlorotoluenes, by an adaptation of the method of Kharasch and Brown[13]. The corresponding acetonitriles were then prepared by known methods, and the subsequent alkylation was carried out as described for phenyl-αα-diethylacetonitrile. The hydrolysis of the chloro-compounds was carried out by the method described above, but *o*-tolyl-dimethylacetonitrile was attacked vigorously by 90 per cent sulphuric acid with the formation of water soluble products, presumably sulphonic acids. This nitrile was hydrolysed by refluxing with 20 per cent potassium hydroxide in ethanol for 7 hours. The *p*-isomer was hydrolysed by warming with 75 per cent (w/w) sulphuric acid for 25 minutes.

Derivatives of Benzylacetamide

Haller and Bauer[14] found that benzylacetamide derivatives with both the α-hydrogen atoms replaced by alkyl groups may be prepared by the action of sodamide on the corresponding ω-di-alkyl benzylacetophenones in xylene. The latter were obtained by benzylation of ketones prepared by the Friedel-Crafts reaction with acid chlorides and benzene. The splitting reaction did not occur in the expected way with the di-propyl and the methyl propyl derivatives. The preparation of mono-alkyl benzyl acetophenones by the catalytic reduction of the analogous chalcones failed because, with all the catalysts tried, the carbonyl group was reduced rather than the double bond.

α-*Benzyl*-iso*butyrophenone*. This compound was prepared by Haller and Bauer's method[14]. Yield, 15 per cent, b.p. 176 to 177°/7 mm. (*lit.* 180 to 185°/11 mm.).

αα-*Dimethyl-hydrocinnamic amide*. (Benzyldimethylacetamide). This compound was prepared by Haller and Bauer's method[14]. Yield, 33·6 per cent, m.p. 65° ex light petroleum (*lit.* 62 to 63°).

TABLE I

NEW CHEMICAL COMPOUNDS

Nitriles	B.p.	Yield per cent (ex corresponding phenylaceto-nitrile)
Phenyl-αα-methyl*iso*propylacetonitrile ..	123°/15 mm.	59·5
Phenyl-αα-ethyl-*n*-propylacetonitrile ..	122–130°/12 mm.	73·0
Phenyl-αα-di-*n*-propylacetonitrile	98°/1·3 mm.	41·0
o-Chlorophenyl-αα-dimethylacetonitrile ..	80–81°/0·2 mm.	12·0
m-Chlorophenyl-αα-dimethylacetonitrile	134–135°/20 mm.	22·5
p-Chlorophenyl-αα-dimethylacetonitrile ..	122–127°/7 mm.	by-product
p-Chlorophenyl-αα-diethylacetonitrile ..	149–150°/10 mm. (m.p. 38·5°)	32·4

Amides	Yield per cent (ex Nitriles)	M.p.	Found per cent	Calc. per cent
Phenyl-αα-methyl-*n*-propylacetamide ($C_{12}H_{17}NO$)		b.p. 127°/0·09 mm.	C 75·1 H 9·06 N 7·35	75·35 8·96 7·32
Phenyl-αα-methyl*iso*propylacetamide		53–54° (b.p. 132°/0·5 mm.)	C 75·8 H 8·82 N 7·15	
Phenyl-αα-methyl*sec.*-butylacetamide ($C_{13}H_{19}NO$)	<30	b.p. 136°/0·5 mm.	C 76·11 H 9·61 N 6·29	76·05 9·33 6·82
Phenyl-αα-methyl*iso*butylacetamide ..	61	b.p. 182°/8 mm.	C 76·05 H 9·13 N 6·7	
Phenyl-αα-ethyl-*n*-propylacetamide ($C_{13}H_{19}NO$)		54–56° (b.p. 136°/0·5 mm.)	C 75·99 H 9·15 N 6·6	76·05 9·33 6·82
Phenyl-αα-ethylallylacetamide ($C_{13}H_{17}NO$)	<10	b.p. 142–144°/0·5 mm.	C 77·03 H 8·57 N 6·9	76·81 8·43 6·89
o-Chlorophenyl-αα-dimethylacetamide ($C_{10}H_{12}ONCl$)	55	84–85°	C 61·2 H 6·09 N 7·25	60·7 6·07 7·1
m-Chlorophenyl-αα-dimethyl-acetamide	37	121·5–123°	C 61·0 H 6·5 N 7·1	
p-Chlorophenyl-αα-dimethylacetamide	<20	123–124°	C 60·9 H 6·38 N 6·8	
p-Chlorophenyl-αα-diethylacetamide ($C_{12}H_{16}ONCl$)	<20	107–108°	C 63·7 H 7·1 N 6·2	63·9 7·15 6·2
*Phenyl-αα-diethyl-N-allylacetamide ($C_{16}H_{21}ON$)	46 (exchloride)	74°	C 77·9 H 9·0 N 5·7	77·8 9·15 6·1
*α-t-Butyl-αγγ-trimethylvaleramide (Siccatamide B) ($C_{12}H_{25}ON$) ..		131°	C 72·9 H 12·72 N 6·8	72·3 12·64 7·1

*These compounds were prepared via the acid chloride with allylamine and ammonia respectively.

TABLE II

BIOLOGICAL RESULTS

No.	Compound	Narcotic activity relative to methylpentynol	Toxicity relative to methylpentynol
Aliphatic Amides			
460	Acetamide	Inactive at 1·14 g./kg.	
420	Trichloroacetamide	Inactive	
422	*N*-ethyl-trichloroacetamide	Less active	
430	*NN*-Diethyl-trichloroacetamide	Inactive	
431	Diethylacetamide	Similar	More toxic
432	Triethylacetamide	Similar	Much more toxic
439	Di-*n*-propylacetamice	Similar	More toxic
443	Tri-*n*-propylacetamide	Less active	
456	Tri-*iso*-propylacetamide	Inactive	
446	Tri-*n*-butylacetamide	Inactive	
536	α-*t*-Butyl-αγγ-trimethylvaleramide (Siccatic acid B amide)	Less active	Similar
Derivatives of Phenylacetamide			
445	Hydratropamide	Less active	Less toxic
429	Phenyl-αα-dimethylacetamide	Less active	
452	Phenyl-α-ethylacetamide	Similar	Similar
457	Phenyl-αα-methyl-ethylacetamide	Similar	Much more toxic
497	Phenyl-αα-methyl-*n*-propylacetamide	Less active	Similar
459	Phenyl-αα-methyl-*iso*-propylacetamide	Similar	More toxic
437	Phenyl-αα-diethylacetamide	ca. twice as active	More toxic
509	Phenyl-α-*n*-butylacetamide	Inactive	
471	Phenyl-αα-methyl-*n*-butylacetamide	Inactive	
508	Phenyl-αα-methyl-*iso*-butylacetamide	Less active	
462	Phenyl-αα-methyl-*sec.*-butylacetamide	Less active	
455	Phenyl-αα-ethyl-*n*-propylacetamide	Similar	Less toxic
461	Phenyl-αα-ethyl-*iso*-propylacetamide	Less active	Similar
469	Phenyl-α-*n*-amylacetamide	Less active	More toxic
444	Phenyl-αα-di-*n*-propylacetamide	Less active	Less toxic
447	Diphenylacetamide	Inactive	
454	Phenyl-αα-allylacetamide	Less active	
492	Phenyl-αα-ethylallylacetamide	Less active	Less toxic
420	*p*-Tolyldimethylacetamide	Inactive	
563	*o*-Tolyldimethylacetamide	Similar	Much more toxic
518	*p*-Chlorophenyldimethylacetamide	Similar (c.f. 429)	Less toxic
643	*m*-Chlorophenyldimethylacetamide	Similar	Less toxic
644	*o*-Chlorophenyldimethylacetamide	Appreciably greater	Much more toxic
529	*p*-Chlorophenyldiethylacetamide	Similar (c.f. 437)	Similar
540	*N*-Allylphenyldiethylacetamide	Inactive	Convulsant
Derivatives of Benzylacetamide			
510	Benzyl-αα-dimethylacetamide	Less active	Similar
517	Benzyl-αα-methylethylacetamide	Inactive	
Derivatives of Benzamide			
458	Benzamide	Less active	Less toxic
467	*N*-Methyl-benzamide	Less active	Similar
466	*N*-Ethyl-benzamide	Less active	Similar
468	*NN*-Diethyl-benzamide	Less active	Similar
500	*p*-Toluamide	Inactive	
499	*m*-Toluamide	Less active	Similar
498	*p*-Nitrobenzamide	Inactive	Less toxic
463	Salicylamide	Less active	
464	*o*-Methoxy-benzamice	Less active	Similar
465	*N*-Acetyl-salicylamide	Less active	Less toxic
472	Phthalimide	Inactive	
513	Phthalic diamide	Inactive	
Derivatives of Hydroxyacetamide			
438	α-Hydroxy-*iso*-butyramide	Inactive	
451	Atrolactinamide	Similar	

The only other compound prepared satisfactorily by this method was α-methyl-α-ethyl-hydrocinnamic amide (benzylmethyl-ethylacetamide), an oil b.p. 183 to 184°/6 mm.

Derivatives of Benzamide

Benzamide and its *N*-alkyl derivatives were prepared from benzoyl chloride, and *p*-nitrobenzamide was also prepared from the acid chloride. *m*- and *p*-Toluamide were prepared from the corresponding nitriles by

hydrolysis with 90 vol. hydrogen peroxide in warm dilute alkali[15]. The nitriles were obtained by a Sandmeyer reaction from the amines. Salicylamide, *o*-methoxy benzamide and *N*-acetyl salicylamide were also prepared by known methods.

α-Hydroxyacetamides

α-Hydroxy-isobutyramide. This compound (m.p. 93 to 94°) was obtained in low yield by refluxing a solution of the ethyl ester in ammonia (0·880) for 65 hours, followed by evaporation and crystallisation from acetone. Ciamician and Silber[16] gave m.p. 96°.

Atrolactinamide. This compound (m.p. 99 to 100°) was obtained from acetophenone cyanhydrin by Staudinger and Ruzicka's method[17].

New Chemical Compounds

So far as we can ascertain, the compounds in Table I have not been described hitherto in the literature.

Our findings with regard to *p*-tolyl-dimethylacetamide, prepared by hydrolysis of the corresponding nitrile, agree with those of Lambert and others[18] who report m.p. 143 to 144° for the compound obtained by catalytic reduction (Raney nickel) of the hydroxamic acid, whereas Rupe and Burgin[19] give m.p. 119°, and Wallach[20] gives m.p. 123 to 124°. Our synthesis from *p*-tolyl-acetonitrile gave a product m.p. 142°. Found: C, 74·3; H, 8·36; N, 8·0; Calc. for $C_{11}H_{15}ON$: C, 74·53; H, 8·5; N, 7·9 per cent. The intermediate *p*-tolyl-dimethylacetonitrile had b.p. 72 to 73°/0·3 mm. (Lambert and others[18] give 122 to 123°/12 mm. and Wallach[20] gives 247 to 248°).

Discussion

The criterion of activity was a narcotic effect greater than that of methylpentynol, and a better therapeutic index. Di- and triethylacetamides are the only compounds in the aliphatic group that are more active than methylpentynol, and they are much more toxic. (Table II).

The most interesting compounds belong to the αα-disubstituted phenylacetamide group, of which the most active are phenyl-αα-diethylacetamide (compound 437), phenyl-αα-ethyl-*n*-propylacetamide (455) and *o*-chlorophenyl-αα-dimethylacetamide (644). Compound 437 seemed most worthy of further investigation, since it appeared to be twice as active as methylpentynol. The results are shown in Table III. The acute oral toxicity (LD50) was found to be 489 mg./kg. (limits 422 to 567 mg./kg., cf. Bliss[7]), compared with 760 to 860 mg./kg. for methylpentynol[6], Table IV.

Optimal activity within the phenylacetamide group occurs when the α-carbon atom is substituted by two alkyl radicals. The αα-diethy-compound (437) and the α-ethyl-α-*n*-propyl compounds are the most active, whilst the isomeric α-methyl-α-butylacetamides are all less active. Maximal activity seems to reside in compounds containing *n*-alkyl groups with the total number of carbon atoms 4 or 5.

Phenyldimethylacetamide (429) and *p*-tolyldimethylacetamide (420) are inactive, whereas the corresponding *o*-tolyl compound (563) is more

TABLE III

COMPARATIVE NARCOTIC ACTIVITY OF PHENYLDIETHYLACETAMIDE AND METHYLPENTYNOL

Experiment	Compound	No. of mice	Route	Dose g./kg.	Time after injection (hours) ½ N* D†	1 N D	2 N D	3 N D	4 N D	5 N D	6 N D
I	Methylpentynol	5	Oral	1·14	5	5	5	3 2	0 5	0 5	0 5
				0·77	5	5	5	5	5	5	5
				0·52	5	5	5	4	4	4	3
	Phenyldiethyl-acetamide (437)	5	Oral	1·14	1 4	0 5	0 5	0 5	0 5	0 5	0 5
				0·77	3 2	3 2	2 3	1 4	1 4	1 4	1 4
				0·52	5	5	5	5	5	5	5
				0·35	5	5	5	5	5	5	5
II	Methylpentynol	5	Oral	1·14	5	5	5	5	3 2	2 3	1 4
				0·77	5	5	5	5	5	5	5
				0·523	4	5	5	2	3	1	1
				0·348	1	1	1	0	1	0	0
	Phenyldiethyl-acetamide (437)	5	Oral	0·523	5	5	5	4 1	3 2	3 2	2 3
				0·348	5	5	5	5	5	3 1	3 1
				0·233	3	5	3	1	2	2	1
				0·155	2	2	1	0	1	0	0

* N = Narcotised. † D = Dead.

active than methylpentynol. It was also shown that *o*-chlorophenyldimethylacetamide (644) was more active than the *m*- and *p*-isomerides (Table V), and all the isomerides were more active than compound 429. Nevertheless, similar substitution in the phenyl group of compound 437 resulted in a reduction in activity.

It would therefore appear that the level of narcotic effect may be largely a question of molecular weight, apart from the demonstrated influence of the orientation of the phenyl-substituents, and it might be of interest to test this by the preparation of compounds with other phenyl-substituents.

TABLE IV

ACUTE ORAL TOXICITY OF PHENYLDIETHYLACETAMIDE (437)

Dose mg./kg.	No. of mice	Deaths (5 days)
1200	18	18
800	18	15
533	18	10
356	18	3
237	18	2
158	14	0

LD50 = 489 mg./kg.
Fiducial limits = 422–567 mg./kg. (cf. Bliss[7]).
The acute oral toxicity of methylpentynol was found to be 760–860 mg./kg.[6]

TABLE V

COMPARATIVE NARCOTIC ACTIVITY OF THE ISOMERIC CHLOROPHENYLDIMETHYLACETAMIDES

Compound	No. of mice	Route	Dose g./kg.	Time after injection (hours) ½ N* D†	1 N D	2 N D	3 N D	4 N D	5 N D	6 N D
518 *p*-Chlorophenyldimethylacetamide	10	Oral	0·506	0	1	4	6	6	9	8 2
			0·338	0	0	1	2	4	4	5
643 *m*-Chlorophenyldimethylacetamide	10	Oral	0·506	1	4	9	9	9	9	9
			0·338	0	1	4	4	4	4	3
644 *o*-Chlorophenyldimethylacetamide	10	Oral	0·506	8	8	9	10	10	4 6	1 8
			0·338	6	6	8	8	8	5 4	4 5
Methylpentynol	10	Oral	0·506	2	6	7	8	7	8	8 1
			0·338	0	1	1	1	1	0	0

*N = Narcotised. †D = Dead.

Summary

1. A series of derivatives of acetamide and benzamide has been synthesised and examined for narcotic activity, in comparison with methylpentynol.

2. Of the alkylacetamides only di- and triethylacetamides are more active and they are also more toxic.

3. The most interesting compounds belong to the group of aryldialkylacetamides. Phenyldiethylacetamide (compound 437) is approximately twice as active as methylpentynol, but also more toxic.

4. *ortho*-Substitution in the phenyl radical considerably enhances the narcotic activity compared with that of the *m*- and *p*-isomerides, but increases the acute toxicity at the same time.

5. A *p*-chlorine substituent increases the activity of phenyl-dimethylacetamide (429), but depresses that of compound 437.

6. Variation in narcotic activity, apart from the demonstrated effect of orientation of the phenyl-substituents, may possibly be correlated to some extent with molecular weight.

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THE REACTION BETWEEN DIGITOXIN AND 3:5-DINITROBENZOIC ACID*

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3:5-DINITROBENZOIC acid was first used as a reagent by Bollinger[1] and by Benedict and Behre[2] for the determination of creatinine. Since, under certain conditions, the presence of digitoxin could be detected in a similar manner to that of creatinine, Kedde[3] investigated the use of 3:5-dinitrobenzoic acid for the estimation of digitoxin and of other cardiotonic glycosides. Langejan[4] and Rowson[5] have also used this reagent in a similar manner to that of Kedde. Pratt[6] used a quaternary base in place of sodium hydroxide in this reaction.

The reagent in ethanolic solution is mixed with a solution of the glycoside in dilute ethanol, standard solution of sodium hydroxide in fixed amount is then added and the intensity of the colour, which develops after a short time, is measured at a wavelength ranging between 5350 and 5500 Å. The final concentration of 3:5-dinitrobenzoic acid is usually 0·4 per cent, that of sodium hydroxide is 0·4 per cent (= 0·1N) and that of ethanol is 52 to 58 per cent. The majority of workers[3-5] have used the same concentrations of reagents. In general it has been recognised that, in order to obtain good results, it is necessary to control both the temperature of the reaction and the time allowed for colour development, also colour change in the blank has been noted. Precise details of these matters, however, are infrequently presented.

We have considered it desirable to study the optimum concentrations and conditions for carrying out this reaction. We have examined the influence of the solvent, the concentration of 3:5-dinitrobenzoic acid, the concentration of sodium hydroxide, and the amount of water in the reaction mixture. The reaction was always carried out as follows: digitoxin 0·4 mg. was dissolved in 4 ml. of solvent either with or without the addition of water, then 5 ml. of solution of 3:5-dinitrobenzoic acid in the same, undiluted solvent was added. After standing, 1 ml. of standard sodium hydroxide solution was added, the time at which the addition was made being noted. The extinction of the reaction mixture was measured at 5400 Å with a spectrum band width of 40 Å in a Bleeker (Holland) spectrophotometer.

Solvent

We have used methanol, ethanol and *n*-propanol, undiluted, as solvents for the digitoxin and also with which to prepare both 1 and 2 per cent solutions of 3:5-dinitrobenzoic acid; 1, 1·5 and 4N sodium hydroxide

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solutions were employed. Extinctions were measured one minute after adding the sodium hydroxide and then at half-minute intervals. Results are shown in Figure 1.

From these results it was concluded that *n*-propanol (curve 4) was unsuitable as a solvent, for the maximum extinction in it is low, N sodium hydroxide is adequate and an increase in its concentration greatly reduces the extinction values. Moreover, this solvent does not readily mix with water and the addition of more than 1 ml. of an aqueous solution is difficult.

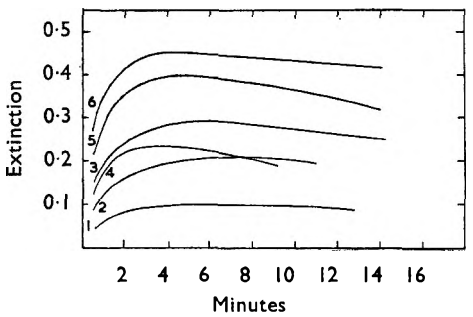

FIG. 1. Influence of solvent on colour development.

Curve 1: methanol, 2 per cent 3:5-dinitrobenzoic acid, 1·5N sodium hydroxide.

Curve 2: methanol, 1 per cent 3:5-dinitrobenzoic acid, 4N sodium hydroxide.

Curve 3: methanol, 2 per cent 3:5-dinitrobenzoic acid, 4N sodium hydroxide.

Curve 4: *n*-propanol, 1 per cent 3:5-dinitrobenzoic acid, 1N sodium hydroxide.

Curve 5: ethanol, 1 per cent 3:5-dinitrobenzoic acid, 1·5N sodium hydroxide.

Curve 6: ethanol, 2 per cent 3:5-dinitrobenzoic acid, 1·5N sodium hydroxide.

Using methanol as solvent the maximum *E* values were much lower than with ethanol; also 4N sodium hydroxide was necessary for optimum reaction (curve 3) and when only 1·5N alkali was used (curve 1) the colour was much less intense.

Ethanol as solvent gave the highest *E* values and for this reason it was used as solvent in the subsequent work.

Concentration of 3:5-Dinitrobenzoic Acid

From Figure 1 it is noted that the concentration of 3:5-dinitrobenzoic acid influences both the maximum extinction value and also the time required for its development. For this reason we have employed this reagent in concentrations of 0·5, 0·75, 1·0, 1·5, 2·0, 2·5, 3·0 and 4·0 per cent in ethanol. At the same time we have varied the normality of the sodium hydroxide solution between 1·0, 1·5 and 2·0N. The curves for maximum extinction values only are shown in Figure 2.

It will be seen that there is a marked increase in maximum values of *E* with increasing concentrations of 3:5-dinitrobenzoic acid reagent up to a 2 per cent reagent. Above that concentration an increase in strength of reagent resulted in maximum *E* values remaining almost the same or decreasing (curves 1 and 4). Since the changes in *E* values are relatively great for small changes in lower concentrations of 3:5-dinitrobenzoic acid, it is desirable to employ as reagent a 2 per cent solution of this acid, giving a final concentration of 1 per cent in the reaction mixture. The influence of 3:5-dinitrobenzoic acid on the time of reaction is not great as shown in Table II.

TABLE I

INFLUENCE OF SODIUM HYDROXIDE CONCENTRATION ON EXTINCTION AND ON RATE OF REACTION

1 per cent 3:5-Dinitrobenzoic acid			2 per cent 3:5-Dinitrobenzoic acid		
	Extinction			Extinction	
Sodium hydroxide N	Maximum	After (minutes)	Sodium hydroxide N	Maximum	After (minutes)
0·5	0·14[6]	7	0·5	0·11	7
1·0	0·32	7	1·0	0·40	7
1·5	0·39[6]	4·5	1·5	0·45	4
2·0	0·42	3	2·0	0·46	2·5
2·5	0·42	2	2·5	0·46[5]	2
3·0	0·42	1·5	3·0	0·46	1·5

Normality of Sodium Hydroxide

From Figure 1, curves 1 and 3, it may be seen that in a methanolic reaction mixture an increase in normality of sodium hydroxide resulted in a marked increase in *E* values. Figure 2 shows similar results and, up to a concentration of 2 per cent of 3:5-dinitrobenzoic acid reagent, the normality of the sodium hydroxide is of considerable significance. This is so when either ethanol (curves 1–3) or diluted ethanol (curves 4–6) are used as solvents. Strengths of 1N or lower must be avoided since small variations produce marked changes in *E* values; such changes in *E* values are much smaller when 1·5 or 2N sodium hydroxide solutions are used.

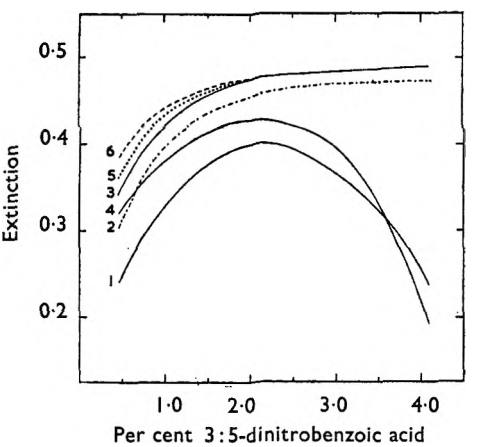

FIG. 2. Influence of concentrations of 3:5-dinitrobenzoic acid, sodium hydroxide and ethanol on maximum extinction.

Curve 1: ethanol 96 per cent, 1N sodium hydroxide.

Curve 2: ethanol 96 per cent, 1·5N sodium hydroxide.

Curve 3: ethanol 96 per cent, 2N sodium hydroxide.

Curve 4: ethanol 17·5 per cent, 1N sodium hydroxide.

Curve 5: ethanol 17·5 per cent, 1·5N sodium hydroxide.

Curve 6: ethanol 17·5 per cent, 2N sodium hydroxide.

Table I records the maximum *E* values and the times taken for their development when using either 1 or 2 per cent 3:5-dinitrobenzoic acid and varying the strength of sodium hydroxide between 0·5 and 3N; ethanol being the solvent for digitoxin. It was concluded that there is an optimum concentration of sodium hydroxide and above 2N the time of colour development is too short for convenience.

In Table II we have recorded, for different concentrations of 3:5-dinitrobenzoic acid and for different normalities of sodium hydroxide,

the times required for development of maximum E values (column A) and the time during which they remain constant (column B); ethanol and diluted ethanol were used as solvents. It will be seen that an increase in normality of sodium hydroxide not only speeds up the development of maximum E values but also reduces the time over which this maximum is maintained.

TABLE II

INFLUENCE OF CONCENTRATIONS OF SODIUM HYDROXIDE AND OF 3 : 5-DINITROBENZOIC ACID ON RATE OF REACTION

Minutes

Solvent	Sodium hydroxide	Concentration of 3 : 5-dinitrobenzoic acid reagent per cent															
		0·5		0·75		1·0		1·5		2·0		2·5		3·0		4·0	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Ethanol 96 per cent	1·0N	8	5	8	2	7	2	7	2	7	2	7	2	7	2	5	1
	1·5N	6	3	5	2	4·5	1·5	4	1	4	1	4	1	4	1	4	1
	2·0N	4·5	1·5	4	1	3	1	3	1	3	1	2·5	1	2·5	1·5	2	1
Ethanol 17·5 per cent	1·0N	11	6	9	5	9	5	9	5	8	5	8	5	8	5	8	5
	1·5N	7	4	6	4	6	3	6	3	6	3	6	3	6	3	6	2
	2·0N	6	3	4·5	2·5	4	2	4	1	4	1	4	1	4	1	4	1

A = Time for development of maximum extinction
B = Time during which maximum extinction is a constant

Influence of Water

When the ethanol concentration of the reaction mixture is reduced, it influences the E values. Thus in Figure 2 we see that E values are greater when using 17·5 per cent ethanol than when using 96 per cent ethanol (curves 1 and 4, 2 and 5, 3 and 6). This effect is more pronounced when the concentration of 3 : 5-dinitrobenzoic acid is low and it is less pronounced when using this reagent in a 2 per cent solution; using the more dilute ethanol the rate of reaction is also slowed down and the maximum extinction is maintained for a longer time also, especially when using the weaker solution of 3 : 5-dinitrobenzoic acid.

In all the variations of the process of estimation described above we have plotted the absorption curve of the colour produced and the maximum extinction was always obtained at a wavelength of 5400 Å.

From the foregoing it was concluded that the following conditions should be observed in the reaction: the 3 : 5-dinitrobenzoic acid reagent should be of 2 per cent concentration (giving a 1 per cent concentration in the final reaction mixture); the sodium hydroxide solution should be 1·5N (0·15N = 0·6 per cent in the final reaction mixture); the digitoxin may be dissolved in either ethanol or diluted ethanol since the concentration of ethanol has almost no influence on results under these conditions. Using this reaction mixture and dissolving the digitoxin in ethanol for reason of convenience, we have investigated the influence of temperature upon this reaction, the stability of the reagent, the variations in the colour of the

blank during the assay and also the possible influence of different concentrations of digitoxin.

Influence of Temperature

Solutions were maintained in a thermostat for 30 minutes at temperatures of 15°, 20°, 25° and 30°; the reaction mixture when prepared was allowed to stand for one minute at the same temperature and the extinction was determined immediately. Results are given in Table III. It is seen that *E* values decrease with increase in temperature, especially at 25 and 30° a change of 10 per cent being recorded. The rate of reaction is less influenced. It is recommended that this reaction be carried out at 20° and that higher temperature be avoided.

TABLE III

INFLUENCE OF TEMPERATURE ON EXTINCTION

Temperature degrees	Extinction	
	Time of development minutes	Maximum
15	4	0·44[3]
20	4	0·44
25	4	0·42[6]
30	3	0·40[6]

Stability of Reagent

Since a divergence of opinion exists as to the necessity of preparing a fresh solution of 3:5-dinitrobenzoic acid, we have investigated the problem. Samples of 0·4 mg. digitoxin were estimated on successive days with the same 2 per cent solution of 3:5-dinitrobenzoic acid in ethanol, the reagent being stored away from light. From Table IV it will be seen that the reagent may be stored for a number of days without exerting any significant variation in the results.

TABLE IV

INFLUENCE OF AGE OF REAGENT ON EXTINCTION

Age in days	0	2	3	4	5	6	7	11	14	18	21
Extinction	0·44[3]	0·44	0·43[7]	0·44	0·44	0·44[5]	0·44[3]	0·45[5]	0·46[9]	0·46[7]	0·46

Variations in the Blank

During these researches it was observed that the colour of the blank tended to darken, for in each assay a freshly prepared blank has been used. The changes in colour of a blank consisting of 5 ml. of reagent, 4 ml. ethanol and 1 ml. of 1·5N sodium hydroxide were measured against a mixture of 9 ml. ethanol and 1 ml. of 1·5N sodium hydroxide, readings being taken every 10 minutes at 5400 Å. From Table V it is seen that the colour of the blank does deepen after some time, thus if one blank were used for successive estimations the results would be too low. Hence the preparation of a fresh blank for each estimation is necessary.

TABLE V

CHANGE IN COLOUR OF BLANK WITH TIME

Time in minutes	1	10	20	30	40	50	60	70	75
Extinction	0·04	0·04[6]	0·05[2]	0·06[1]	0·07	0·07[7]	0·08[3]	0·08[9]	0·09[2]

Influence of different Concentrations of Digitoxin

When the reaction was carried out using 0·2, 0·4, 0·6 and 0·8 mg. digitoxin the time for development of maximum colour remained the same and was four minutes from the addition of the sodium hydroxide. Absorption curves for each quantity of digitoxin are plotted in Figure 3 from which it is seen that maximum E values are always obtained at 5400 Å. The reaction obeys the Beer-Lambert law and E(1 per cent, 1 cm.) = 111 ± 1·5.

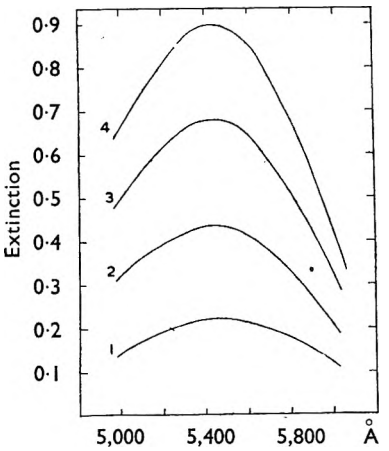

FIG. 3. Absorption curves for digitoxin.

Curve 1: 0·2 mg.; 2: 0·4 mg.; 3: 0·6 mg.; 4: 0·8 mg. digitoxin.

SUMMARY AND CONCLUSIONS

Digitoxin may be estimated by means of 3:5-dinitrobenzoic acid under the following conditions:

1. Digitoxin, up to 0·8 mg., dissolved in 4 ml. ethanol, is mixed with 5 ml. of a 2 per cent solution of 3:5-dinitrobenzoic acid in ethanol, 1 ml. of 1·5N sodium hydroxide is then added and the colour intensity measured in a 1 cm. cell at a wavelength of 5400 Å exactly four minutes after the addition of the alkali.

2. The reaction obeys the Beer-Lambert law: E(1 per cent, 1 cm.) = 111 ± 1·5.

3. The reaction should be carried out at a temperature of 20°, a reduction to 15° exerts no influence, above 25° a diminution in E values results.

4. A fresh blank should be made for each estimation.

5. A diminution in ethanol content of reaction mixture exerts only slight influence on maximum extinction or on rate of reaction.

6. Solutions of 3:5-dinitrobenzoic acid in ethanol may be stored for several days without deterioration.

We wish to thank UCLAF (Paris) for the supply of digitoxin used in these researches. Thanks are also due to the Netherlands Organisation for Pure Research (Z.W.O.) by whose assistance this work was carried out.

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EXCRETION AND STORAGE OF ^{131}I LABELLED IODO ANALOGUE OF CHLOROTRIANISENE*

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INTRODUCTION

IN recent years the long-acting synthetic pro-oestrogen†, chlorotrianisene (tri-*p*-anisylchloroethylene, TACE) has been used successfully for anti-androgenic therapy of prostatic carcinoma[1], for suppression of postpartum lactation and for vasomotor symptoms of menopause[2]. Thompson and Werner[3] demonstrated that after administration of TACE to laboratory animals the oestrogenic activity from the faeces was greater than that administered whereas urine had only negligible activity. They attributed the long duration of oestrogenic activity of TACE to fat storage as revealed by oestrogen bioassay in fat depots of animals administered the compound. Greenblatt and Brown[5] and Thompson and Werner[6] observed considerable and prolonged oestrogenic activity in fat depots of women administered the pro-oestrogen.

The present study was undertaken to gain further information about the metabolism of TACE and other halotrianisenes. The iodo analogue iodotrianisene (hereafter abbreviated as TAIE) was chosen for this purpose because of the ease with which it could be labelled with a radioactive element. Since both compounds, and the corresponding bromo analogue all possess comparable potency and duration of oestrogenic response[7], labelled TAIE may be reasonably assumed to represent this group of halotrianisenes in metabolic studies. Its use offers the additional advantage over conventional bioassay methods in that non-oestrogenic as well as oestrogenic metabolites may be detected. This paper describes the preparation of this radioactive compound and its storage and excretion by animals.

EXPERIMENTAL

Materials and Methods

TAIE was recrystallised from ethanol to remove traces of dark coloured impurities. The purified material melted 117·5–119°, and had absorption maxima at 250 and 292 mμ. At 250 mμ the molar extinction was $2{\cdot}62 \times 10^4$.

Preliminary experiments showed that TAIE could be labelled with ^{131}I by an exchange with $Na^{131}I$. The optimum pH for exchange between iodide and organic iodine compounds is reported to be about 5[8,9]; this pH was also satisfactory for the present reaction. TAIE (20 mg.) was

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†The terms oestrogen and pro-oestrogen are used in the sense defined by Emmens.[4]

dissolved in 10 ml. of warm methanol and the solution was acidified with about 3 drops of 10 per cent glacial acetic acid in methanol. To this solution was added 0·5 ml. of an aqueous carrier-free solution of sodium iodide ^{131}I containing about 5 mC. of radioactivity. The solution was refluxed gently on a heating mantle in a hood. Maximum exchange took place within 12 hours, but only about 70 per cent of the theoretical ratio of iodine incorporation was obtained. Possibly volatisation of the ^{131}I in the form of iodine, or its adsorption on the glassware contributed to the loss. After 24 hours, water was added to the point of turbidity, the solution clarified by briefly reheating and it was then placed in the cold for several hours. The resultant suspension was diluted to about 30 ml. with water and centrifuged. The precipitant was recrystallised from dilute methanol and dried *in vacuo*. The product (18 mg.) had an activity of $9{\cdot}5 \times 10^6$ c.p.m./mg. as measured with the counter used. It had an infra-red spectrum identical to that of the unlabelled TAIE. The radioactive preparation was administered as a 1 per cent corn oil solution.

For comparative excretion studies, the diiodo derivative of the true oestrogen, hexoestrol, was employed. This was prepared and made radioactive in the following manner. To 500 ml. of hot 6 N hydrochloric acid were added 5 g. of hexoestrol in 225 ml. of ethanol. The hot solution was treated with 10 ml. of iodine monochloride in 50 ml. of 6 N hydrochloric acid. After several mintues on the steam bath a heavy crystalline precipitate formed. The solution was heated for an additional 15 minutes, then was placed in the cold overnight. The tan crystals were filtered by suction, washed with water and dried. The product, composed of a mixture of various iodohexoestrols, was dissolved in 100 ml. of ethanol and hot water was added to the point of turbidity. After adding sufficient sodium sulphite to remove the colour, the solution was briefly heated and then allowed to cool slowly, finally in the cold. The crystals, after filtering and drying *in vacuo*, were recrystallised from hot Skellysolve E, giving 4·7 g. of a white product, m.p. 143–144°, which on elementary analysis gave C, 41·5; H, 4·38 and I, 52·3; calculated for $C_{18}H_{20}O_2I_2$, C, 41·4; H, 3·83 and I, 48·6. It has not been determined whether this compound is the 3:5- or 3:3′-diiodo derivative. The oestrogenic potency was found by Dr. Sheldon Segal of the Department of Urology to be approximately 1/20th that of hexoestrol. The exchange between iodide ^{131}I and diiodohexoestrol was carried out in the same manner as used for the preparation of TAIE-^{131}I. No comparable studies were made on the kinetics of this exchange although the extent of incorporation of ^{131}I into diiodohexoestrol was found to be of the same magnitude as that with TAIE.

For localisation studies, the animals were killed at a designated period after the injection. Weighed samples of tissue were digested in 10 volumes of 10 per cent caustic soda at 100°. In the case of the fatty tissues, addition of Bloors solution was necessary to dissolve the tissues completely. Triplicate 1 ml. aliquots were dried in cup planchets, and were counted to a 95 per cent level of confidence with an end window

geiger counter for all but the least active of the tissue samples. Certain of the determinations were made with a scintillation counter. Both methods of counting were standardised with ^{131}I samples from the National Bureau of Standards. For the excretion studies, the animals were placed in metabolism cages in which urine and faeces were readily separated. The urine was evaporated directly in planchets for counting. The faeces were suspended in water, and disintegrated in a blendor. Aliquots were dried in planchets. As a precautions against iodine loss during drying, 0·5 ml. of 2 per cent sodium sulphite was added to urine and faeces samples before drying. Triplicate values for the activity in the excreta were averaged and the values are expressed as the fraction of total administered activity. Because of the uncertainty about the chemical stability of the radioactive iodine on TAIE, a dilute ethanolic solution of TAIE and urine samples from animals administered TAIE were treated with carrier sodium iodide and silver nitrate so as to precipitate any iodine ^{131}I present. No radioactivity could be detected in the silver precipitate from the TAIE solution. Although the activity was too low to measure accurately, the 12 hour and 5 day urine samples contained only about 0·9 and 1·3 per cent respectively of their total radioactivity in the form of inorganic iodide, indicating that liberation of ^{131}I from TAIE was relatively minor.

Results and discussion

Preliminary experiments on tissue localisation of TAIE indicated that the subcutaneous route of administration was preferable; by intraperitoneal injection a high concentration of radioactivity was found at injection site and oral administration was undesirable since we wished to study the concentration of TAIE and its metabolites in the gastro-intestinal tract.

Four days after 70 mg. of TAIE-^{131}I in 2·5 ml. of corn oil (equivalent to $3{\cdot}9 \times 10^6$ c.p.m.) was injected subcutaneously into the leg of a 7 kg. male dog, the animal was killed and the radioactivity in the tissues was measured. As shown in Table I, the thyroid contained the highest localisation of radioactivity; intraperitoneal fat and adrenals were the only other tissues which had counts per minute appreciably above the background. It appears likely that the ^{131}I which was concentrated in the thyroid is the result of deiodination of TAIE and subsequent incorporation of the radio-iodide into thyroid metabolites. The radioactivity in the fat depots is consistent with the view that this type of pro-oestrogen is readily stored in body lipids by virtue of its lipid solubility. The concentration of radioactivity in the adrenals might be

TABLE I

Distribution of ^{131}I in tissues of a dog administered TAIE-^{131}I 4 days previously

Tissue*	Per cent administered ^{131}I/g. wet tissue
Thyroid	0·583
Fat, intraper.	0·050
Adrenals	0·009
Bone marrow	0·003
Spleen	0·001
Testis	0·001

*Other tissues examined and found to contain less than 0·001 per cent of administered ^{131}I were: liver, kidney, lung, skeletal muscle, brain, blood, pancreas and prostate.

explained on their high lipid content. The remaining tissues studied contained so little radioactivity that their sequential order is probably not significant.

To verify these findings with a larger number of animals, rats were used for subsequent studies. Adult male animals (with one exception) were used because of our interest in possible localisation of this material in the male sex organs. The animals were administered 2 mg. of TAIE-^{131}I in 0·2 ml. corn oil and a comparison group was given 0·2 ml. of a solution containing 15 μC. of ^{131}I iodide in 0·02 mg. of potassium

TABLE II

RECOVERY OF ^{131}I IN RAT TISSUES AFTER ADMINISTRATION OF TAIE-^{131}I OR Na^{131}I

Compound	TAIE-^{131}I		Na^{131}I	
Hours after administration	4	48	4*	48*
No. animals	2	4	3	3
Thyroid (total organ)	0·016	0·480	16·170	6·700
Stomach	0·120	0·098	1·180	0·016
Intestine	0·257	0·086	0·276	0·014
Mesenteric fat	0·022	0·064	0·097	0·009
Testicular fat	0·005	0·088	0·097	0·016
Adrenals (total organ)	0·004	0·001	0·017	0·001
Liver	0·035	0·030	0·110	0·047
Kidney	0·022	0·012	0·203	0·020
Lung	0·021	0·024	0·173	0·010
Pancreas	0·025	0·024	0·100	0·023
Testis	0·015	0·014	0·167	0·014
Skeletal muscle	0·007	0·007	0·053	0·006
Brain	0·003	0·001	0·026	0·001

Data expressed as per cent of administered ^{131}I/g. wet tissue (except for thyroid and adrenals) from mean values of the group.

*All tissues in this group were counted directly with a scintillation type counter.

iodide subcutaneously. The animals were killed after 4 hours or 48 hours and the distribution of ^{131}I in the tissues measured thus is summarised in Table II.

Slow absorption from the site of injection undoubtedly accounted for the relatively low ^{131}I content of tissues of rats given TAIE four hours previously as compared with the tissue radioactivity of comparable animals given radioactive iodide; even after two days a highly radioactive fluid pocket could be found at the site of TAIE injection. The variable rate of absorption of TAIE makes the absolute magnitude of the recovered tissue ^{131}I of little significance. On the other hand, the relative concentration in the different tissues is illuminating.

In the TAIE administered animals, the highest content of ^{131}I after 4 hours was found in intestine, stomach and liver; no appreciable concentration was observed in fat at this time. The content of ^{131}I in the thyroid was low, indicating that little deiodination of TAIE had occurred. This is marked contrast to the distribution in rats administered ^{131}I iodide in which the organs having most radioactivity were thyroid and stomach, particularly the pyloric portion, probably due to parietal secretion, as suggested by Goldsmith and others[10] from similar observations in humans.

After 48 hours, the tissue distribution of ^{131}I in TAIE treated animals was greatly different. The tissues having the highest content of ^{131}I were thyroid, stomach, fat and intestine. The content in the thyroid and in the pyloric portion of the stomach is taken as evidence that release of inorganic iodide from TAIE had become appreciable after two days. The intestinal content of ^{131}I probably reflects the continued excretion of TAIE metabolites as well as thyroid metabolites. The content of ^{131}I in fatty depots became appreciable after two days. Although the presence of radioactivity does not necessarily connote the presence of the administered compound, comparison of the distribution of tissue ^{131}I after administration of TAIE-^{131}I and Na^{131}I clearly shows that despite the activity in the thyroid, deiodination of TAIE was relatively small. Thus after 48 hours the thyroid had taken up 0·48 and 6·7 per cent, respectively, of the total TAIE-^{131}I and Na^{131}I administered; at the same time, mesenteric fat contained 0·064 and 0·009 per cent of the administered ^{131}I after giving TAIE-^{131}I and Na^{131}I respectively. Although these data must be interpreted with some reservations, especially regarding the amount of radioactivity remaining at the injection site, they appear to be consistent with the conclusions of Thompson and Werner[3] that extensive storage of TACE in depot fat accounts for its long duration of oestrogenic activity. Other tissues appeared to have no appreciable content of ^{131}I either after 4 or 48 hours. The chemical and metabolic instability of ^{131}I tagged compounds is well known. Most organic iodine compounds appear to participate in an exchange with Na^{131}I in hot ethanolic solution; however, at pH 7 at 37° in aqueous solution, the exchange appears to be sufficiently slow to justify metabolic studies of iodinated compounds.

Because of the well known differences in duration of oestrogenic effect between TACE and the true oestrogens, it was of interest to compare the rate and route of excretion of TAIE and iodohexoestrol as indicated by radioactivity in urine and faeces of rats which had been administered these compounds labelled with ^{131}I. TAIE (1 mg. $= 8 \times 10^6$ c.p.m.) was given intraperitonially in 1 ml. of ethanol; diiodohexoestrol (0·5 mg. $= 1{\cdot}2 \times 10^4$ c.p.m.) was given similarly in 1 ml. of propylene glycol to adult male rats weighing approximately 200 g. The intraperitoneal route was selected because rapid absorption was desired and tissue localisation was not of prime interest in this portion of the study.

In Figure 1 is illustrated excretion of radioactivity in faeces and urine over an eight-day interval. It will be noted that diiodohexoestrol and/or its metabolites were predominantly excreted in the faeces, probably via the biliary pathway. On the other hand, the excretion of TAIE and its metabolites was approximately equal in both urine and faeces.

Thompson and Werner[3] have shown that compared with hexoestrol, TACE possesses a long duration of oestrogenic activity, accompanied by a low rate of excretion of biologically active material. In agreement with these authors, the present data show that the TAIE or its metabolites, compared with hexoestrol, are slowly cleared from the body. Indeed, only about 37 per cent of the total administered radioactivity

was recovered from faeces and urine after eight days whereas 85 per cent of the hexoestrol radioactivity was recovered after a similar interval.

A comparison of the amount of radioactivity in faeces and urine shows that with iodohexoestrol the faecal route is predominant, in agreement with Thompson and Werner. With TAIE, however, the activity was found to be equally distributed between urine and faeces. This is in striking contrast to the finding of these authors that oestrogenically active material was present only in the faeces of animals administered TACE. When larger doses of TAIE were given subcutaneously the faecal route became more important but the urine still contained considerable activity. For example, eight days after a 10-mg. subcutaneous dose, 4·8 per cent of the total dose was recovered in the faeces whereas only 0·3 per cent was found in the urine. This further suggests that there is a limit to the renal clearance of TAIE metabolites, and that mobilisation and excretion from the subcutaneous route is slow. The discrepancy between these results and those of Thompson and Werner must therefore be due to the urinary excretion of a non-oestrogenic metabolite of the halotrianisene. In a preliminary attempt to determine whether this might be due to urinary non-oestrogenic glucuronic acid conjugate of an oestrogenic TACE or TAIE metabolite, 48-hour urine samples of adult male rats administered 1 mg. doses of TACE were bioassayed for oestrogenic activity after incubation with β-glucuronidase (200 units/ml. urine) but failed to show any oestrogenic activity.

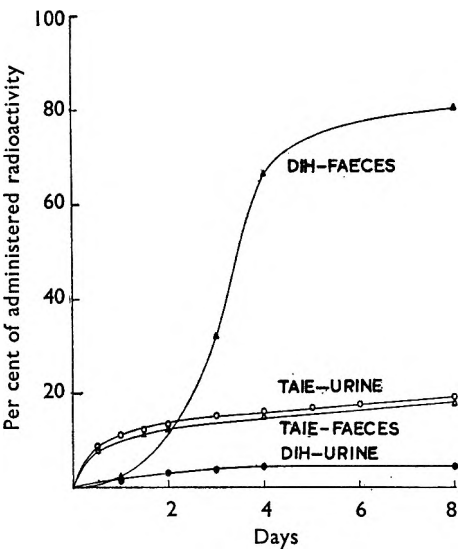

FIG. 1. Comparison of excretion of radioactivity in faeces and urine after administration of TAIE and diiodohexoestrol (DIH) to rats.

SUMMARY

1. The pro-oestrogen, iodotrianisene (TAIE) was labelled with ^{131}I by exchange between unlabelled TAIE and ^{131}I iodide.

2. Appreciable deposition of ^{131}I in depot fat was observed several days after subcutaneous administration of TAIE-^{131}I to a dog and to rats. The concentration of ^{131}I in the thyroid and stomach of rats, two days after the subcutaneous administration of TAIE-^{131}I, indicated that some release of the ^{131}I from TAIE-^{131}I had occurred. This was shown to be of small magnitude by comparative studies with rats given $Na^{131}I$. Other than in the above-mentioned tissues and in the gastrointestinal

tract, there was no significant localisation of radioactivity following TAIE-^{131}I administration.

3. Excretion of radioactivity in both faeces and urine was slow following administration of TAIE-^{131}I to rats and only traces of the radioactivity were excreted in the form of inorganic iodide. On the other hand, the excretion of diiodohexoestrol labelled with ^{131}I was rapid and occurred predominantly by the way of the faeces.

We are indebted to Mr. C. R. Thompson of Wm. S. Merrill and Co. for generous supplies of TAIE.

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THE DETERMINATION OF ISONIAZID IN PHARMACEUTICAL PREPARATIONS CONTAINING SODIUM *p*-AMINOSALICYLATE

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With the increased use of combined therapy in the treatment of tuberculosis there are now a number of preparations containing small quantities of isoniazid in combination with other antituberculosis drugs of the aminosalicylic acid type. The difficulty of determining isoniazid in the presence of aminosalicylic acid is obvious when reviewing the methods at present available. Most of the macromethods deal with pure product and the majority, including the B.P.C. method[1] are based on the oxidation of the hydrazine group by various oxidants[1–11]. Under these conditions aminosalicylic acid is also oxidised.

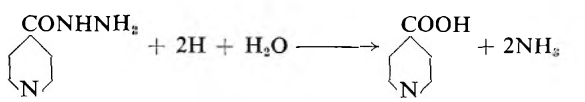
An attempt to separate isoniazid from calcium aminosalicylate was first made by Biffoli[12] who precipitated the free aminosalicylic acid with concentrated hydrochloric acid and titrated the filtrate with potassium iodate in the presence of chloroform or carbon tetrachloride. However, when the quantity of isoniazid is small, the separation is not complete, since either quantities of the isoniazid are retained by the voluminous precipitate of aminosalicylic acid hydrochloride or, on prolonged washing with acid, small amounts of the aminosalicylic acid hydrochloride are dissolved and high results obtained. Strickland and Hentel[13] used a gasometric micromethod, oxidizing hydrazine derivatives to nitrogen with sodium iodate in alkaline solution in the presence of aminosalicylic acid. This method has been tested by the authors as a macrodetermination in the presence of fifty times as much aminosalicylic acid and found to give satisfactory results, although subject to the usual errors of a gasometric estimation. Various other methods have been described for the estimation of isoniazid, including non-aqueous titrations, polarographic and colorimetric procedures, but these have not been investigated by the authors since the methods were either not sufficiently accurate or required the use of special equipment and, in many cases, the presence of aminosalicylic acid would have caused interference.

It would appear, therefore, that the determination of isoniazid in the *presence of aminosalicylic acid and its derivatives must be carried out* either by separation of the isoniazid and assaying it by one of the known methods, e.g., the B.P.C. assay procedure, or by direct estimation of the isoniazid, using a method which is not affected by large quantities of aminosalicylic acid. Separation from isoniazid by the use of ion exchange resins was considered, but preliminary experiments were unsuccessful and, since the high aminosalicylic acid content of commercial preparations would necessitate frequent regeneration of the resin, this line of investigation was not pursued. Attention was, therefore, concentrated on producing a simple method of assay that would be unaffected by large

quantities of aminosalicylic acid. In particular, the possibility of developing a method based on the reduction of the hydrazine group was investigated, since the aromatic amino group in aminosalicylic acid would not be affected.

In discussing the properties of hydrazine, Ephraim[14] states that it is decomposed in alkaline solution to give ammonia, nitrogen and water in varying proportions, depending on the catalyst used. Suzuki[15] distinguishes between hydroxylamine and hydrazine by reducing both to ammonia with ferrous sulphate in a glucose solution, the first reaction occurring in the presence of sodium carbonate and the second requiring sodium hydroxide. The possibility of estimating isoniazid by reducing the hydrazine group quantitatively to ammonia, followed by distillation into standard acid was, therefore, considered since, under these conditions, aminosalicylic acid and its derivatives would be stable and should not produce interference. It is interesting to note that Sanchez[16] describes a colorimetric assay in which isoniazid is reduced to an aldehyde with zinc powder and hydrochloric acid, the aldehyde being subsequently reacted with phenylhydrazine.

To obtain a quantitative reduction of isoniazid to ammonia according to the reaction



various reducing agents, including ferrous sulphate, powdered zinc, Devardas alloy, powdered tin, and zinc-iron, tin-copper, and iron-copper couples were tested in alkaline solution, but all gave less than 75 per cent of the theoretical yield of ammonia. Encouraging results were obtained with a zinc-copper couple in potassium hydroxide solution, recoveries being about 95 to 100 per cent and subsequent investigations were, therefore, restricted to the use of this reducing agent. It was found that, in the absence of sodium aminosalicylate, recoveries were occasionally above the theoretical. These anomalous results were due to traces of what appeared to be γ-picoline produced during the reaction, since the distillate, which had the odour characteristic of pyridine bases, gave a positive reaction with 1-chloro-2:4-dinitrobenzene[17,18]. In the presence of sodium aminosalicylate, however, the results were dependent only upon the zinc-copper ratio and the alkalinity of the solution. In addition, large quantities of zinc salts inhibit the reaction and the following optimum conditions have been found for the reduction of isoniazid in the presence of aminosalicylic acid:

1. The zinc-copper couple prepared from zinc powder and copper sulphate has to be washed thoroughly to remove all the zinc sulphate.
2. The ratio of zinc powder to copper sulphate should be about 4:1.
3. The quantity of zinc powder used should be between 4–10 g.
4. The normality of the potassium hydroxide solution should be between 0·1 and 0·5N.

5. The volume of the mixture at the beginning of the reaction should be 400 to 500 ml. and should contain about 5 g. sodium aminosalicylate.

The following procedure is recommended:

Reagents. Zinc powder, 25 per cent w/v aqueous copper sulphate solution, 2·5 per cent w/v aqueous potassium hydroxide solution (approximately 0·4N), 0·1N sulphuric acid and 0·05N sodium hydroxide.

TABLE I

THE ESTIMATION OF ISONIAZID IN THE PRESENCE OF 5 G. QUANTITIES OF SODIUM AMINOSALICYLATE

Weight of isoniazid taken (mg.)	Titre ml. 0·1N H_2SO_4	Isoniazid recovered	
		mg.	per cent
25	3·53	24·23	96·92 } 96·2
	3·49	23·88	95·52 }
50	7·43	50·94	101·88 } 101·4
	7·36	50·44	100·88 }
75	10·93	74·92	99·88 } 100·0
	10·96	75·13	100·16 }
100	14·56	99·78	99·78 } 99·9
	14·62	100·10	100·10 }

Procedure. About 10 g. of zinc powder, 25 ml. water and 10 ml. of 25 per cent copper sulphate solution are shaken in a 1-litre round flask until the supernatant liquid is nearly colourless. The liquid is decanted and the residue washed three times with 25 to 50 ml. portions of water, decanting each washing. About 5 g., accurately weighed, of the sodium aminosalicylate-isoniazid sample, containing 50 to 150 mg. of isoniazid, is placed in the flask, together with 400 ml. of 2·5 per cent potassium hydroxide solution. The mixture is distilled into 25 ml. of 0·1N sulphuric acid until 100 to 150 ml. has been collected (15 to 30 minutes). The distillate is back titrated with 0·05N sodium hydroxide solution, using methyl red screened with methylene blue as an indicator.

Each ml. of 0·1N sulphuric acid used is equivalent to 0·006858 g. of isoniazid.

TABLE II

THE ESTIMATION OF ISONIAZID IN SODIUM AMINOSALICYLATE/ISONIAZID CACHETS

Weight of sodium aminosalicylate per cachet (g.)	Weight of isoniazid per cachet (mg.)	
	Stated	Found
1·50	33	33·13 } 32·8
		32·50 }
1·25	25	25·12 } 25·4
		25·63 }
1·50	50	49·28 } 50·1
		50·84 }
1·25	25	24·86 } 24·9
		24·86 }
1·50	33	32·66 } 32·9
		33·12 }

The above method was used with varying quantities of isoniazid in the presence of 5 g. of sodium aminosalicylate; the results obtained are given in Table I.

The greater error in the lower concentrations of isoniazid is probably due to the small titre difference. In general, when reasonable quantities of isoniazid are employed, a satisfactory accuracy is obtained.

Six commercial samples of isoniazid/sodium aminosalicylate cachets, containing various quantities of isoniazid, were examined and the isoniazid contents obtained are given in Table II.

Summary

A rapid and accurate method for the determination of isoniazid in the presence of sodium *p*-aminosalicylate has been developed. The hydrazine group of the isoniazid is quantitatively reduced by a zinc-copper couple in potassium hydroxide solution to give ammonia, which is titrated after distillation into standard acid.

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STRUCTURE-ACTIVITY RELATIONS IN TWO NEW SERIES OF ANTIFOLIC ACIDS

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ANTIFOLIC acids† are clinically useful in acute leukaemia (aminopterin, A-methopterin) and in malaria (proguanil, pyrimethamine); but aminopterin and its relatives possess the disadvantages of producing severe side-effects and of evoking drug-resistance[1], which latter can also be a problem with the antifolic antimalarials[2]. It therefore seemed useful to search for new types of antifolic acids, which might allow the development of additional therapeutically useful substances, not cross-resistant with existing types. Taking the 2:4-diaminopteridines known to possess very high antifolic acid activity in various bacterial systems[3–6] as a starting point, two series of compounds—the aryl-azopyrimidines and 8-azapurines —were prepared and investigated. Since both series were readily varied chemically, they allowed structure-activity relations to be explored in some detail. In the account that follows the chemical and biological investigations are described in separate sections and the results then brought together in a discussion of structure-activity relations.

This work was started in the Autumn of 1953, and some of the compounds were described[7] at the International Chemical Congress in 1955, where conversation with Dr. E. J. Modest revealed that somewhat later than ourselves he had prepared and begun to examine some similar compounds. Arrangements were made with Dr. Modest for simultaneous publication and a paper by himself and his colleagues[8] appears alongside our own.

CHEMICAL SECTION‡

INTRODUCTION

Antagonists of folic acid in bacterial systems have been found by various workers amongst the following simple derivatives of 2:4-diaminopteridine; 6:7-diaryl- (I, $R' = R'' =$ aryl)[3,4], 7-amino-6-aryl- (I, $R' =$ aryl, $R'' = NH_2$)[9] and 6:7-dialkyl- (I, $R' = R'' =$ alkyl)[5,6]. As a further simplification of structure we first made 2:4-diamino-5-4′-chlorophenylazo-6-dimethylaminopyrimidine (II, R = Cl) (No. 24) since this would exist in the *trans* form[10] (as shown) and would therefore show some spacial similarity to a 2:4-diamino-6-chlorophenylpteridine. The chlorine

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† The term antifolic acid is used to refer to an antagonist of any vitamin of the folic acid group.

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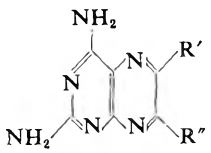
substituent in the benzene ring was selected in the hope that it would have a favourable effect because of the marked enhancement of antifolic activity in the 4:6-diamino-1-aryl-1:2-dihydro-5-triazines by the introduction of halogen substituents in position 3′ or 4′ or 3′ and 4′ of the benzene ring[11]. The discovery of antifolic activity in the azo-pyrimidine (II, R = Cl) encouraged further exploration using a diethanolamino- and morpholino-, instead of the dimethylamino- substituent but when it was found that much greater activity arose from using an unsubstituted amino group the series III was explored in detail. In the pyrimidine ring, variations involved the hydroxy, amino, thio, chloro and 4′-chlorophenylthio substituents and in the benzene ring chloro, bromo, nitro, methoxy and ethoxy substituents were used. In place of the benzene ring naphthalene and quinoline were introduced. Since amino-aryl-azopyrimidines (III) are, in general, easily oxidised to 8-azapurines[12] (IV) it was thought that this conversion might occur *in vivo* and that the 8-azapurines might therefore be active. Many active compounds were found in this series but the evidence, given later in this paper in the discussion of structure-activity relations, is against the hypothesis that the aryl-azopyrimidines owe their activity to this conversion. The 8-aryl-8-azapurines were all made by a general method[12] from the aryl-azopyrimidines (III) which were usually synthesised by the method[12] of coupling the appropriate pyrimidine and aryl diazonium salt. The azo compounds were oxidised by copper sulphate in boiling pyridine, the yields being greatly improved by passing oxygen through the reaction mixture. Azo compounds containing a tertiary amino group in the 4- (or 6-) position of the pyrimidine, e.g., II, were made by reacting the appropriate chloro-aryl-azopyrimidine (X) and secondary amine.

The dimethylamino derivatives (II, R = Cl and NO_2) were also formed by reacting the chloro-pyrimidines (X, R′ = Cl and NO_2, R″ = Cl) and guanidine in a mixture of ethanol and dimethylformamide, respectively under hot and cold conditions. Presumably the reaction of dimethylformamide and guanidine liberates dimethylamine. From X, (R′ = NO_2, R″ = Cl) in this reaction there is also formed 2:4-diamino-6-ethoxy-5(4′-nitrophenylazo)pyrimidine (X, R′ = NO_2, R″ = OEt) but none of the corresponding ethoxy derivative could be isolated from the chlorophenyl analogue (X, R′ = Cl, R″ = Cl). From X, (R′ = Cl, R″ = Cl) and hot 2-ethoxyethanol in the presence of guanidine the corresponding 2-ethoxyethoxy derivative (X, R′ = Cl, R″ = OCH_2CH_2OEt) was analogously formed, the guanidine being a sufficiently strong base to take the place of the sodium alkoxide usually used in this type of reaction. When 4:6-diamino-2-thiopyrimidine was coupled with *p*-chlorobenzene diazonium chloride the desired 4:6-diamino-5(4′-chlorophenylazo)-2-thiopyrimidine (XI, R = SH) was formed in small yield, the major product (XI, R = SC_6H_4Cl (*p*)) having been formed by coupling with two mols of the diazonium chloride. Previously[13] 2-thiouracil and 4-methyl-2-thiouracil have been coupled with this diazonium chloride but yielded only the product derived from two mols of the reagent; in our case the 4:6-diamino groups therefore appear to direct the coupling to the 5-position more strongly.

In order to observe the effect of changing the position of the aryl substituent in the 8-azapurine series some 9-aryl derivatives were made.

9-Phenyl-2-amino-8-azapurine (VII, R′ = H, R″ = Ph) was synthesised from 2-amino-4-chloro-5-nitropyrimidine by condensation with aniline to form 2-amino-4-anilino-5-nitropyrimidine, reduction, and ring-closure of the 4 : 5-diamine by treatment with nitrous acid. Similarly were prepared the *p*-chlorophenyl analogue (VII, R′ = H, R″ = $C_6H_4Cl(p)$) and the 6-methyl derivative (VII, R′ = CH_3, R″ = Ph).



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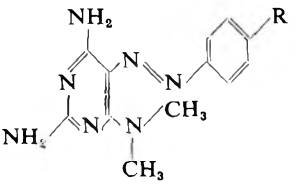

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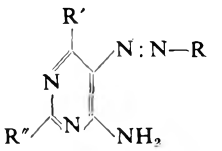

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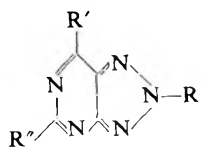

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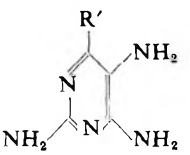

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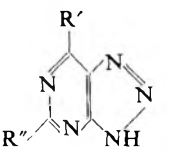

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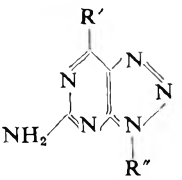

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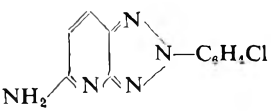

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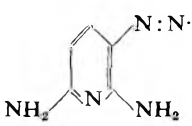

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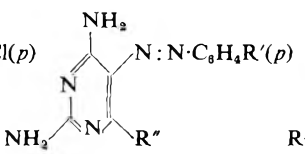

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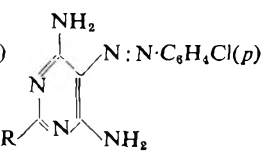

(XI)

From structural analogy, the two pyridine-containing structures VIII and IX were potentially interesting especially since the benzeneazo analogue of IX (2-benzeneazo-2 : 6-diaminopyridine, "Pyridium") is a powerful bactericide. These compounds (Nos. 66 and 60 respectively), which were synthesised by methods exactly analogous to those used for the azopyrimidines and 8-aryl-8-azapurines, were inactive.

6-Thio-8-aryl-8-azapurines were made by thiation of the 6-hydroxy analogues with phosphorus pentasulphide in boiling pyridine solution. 2 : 6-Diamino-8-azapurine (VI, R′ = R″ = NH_2) (No. 65) made according

to Cavalieri and others[14], and 8-azapurine (No. 64), made by ring-closure of 4:5-diaminopyrimidine, were synthesised to ascertain the effect on activity of removing successively the aryl substituent and the amino groups from the diamino-8-azapurines. Both were inactive.

2:4:5-Triamino-6-hydroxypyrimidine (No. 68) and 2:4:5:6-tetraminopyrimidine (No. 67) were made, by Traube's method, for antifolic tests as possible *in vivo* reduction products of the diamino-hydroxy and triamino-arylazopyrimidines respectively. Since both types of azopyrimidine are active, the fact that V ($R' = OH$)[15] and V ($R' = NH_2$)[16] are respectively inactive and active, argues against the hypothesis that they might be the active forms.

New compounds are indicated in the experimental section by italics and, in the Tables, by the absence of references to their preparation.

Experimental

Melting points were determined using an electrically heated copper block and are uncorrected unless otherwise stated. Samples for analysis were dried at 100° in a high vacuum unless otherwise stated. Analyses were by Drs. Weiler and Strauss (Oxford) and by Mr. P. R. W. Baker (Beckenham).

2:4-*Diamino*-6-*chloro*-5(4′-*nitrophenylazo*)-*pyrimidine* (CB. 2297, No. 19)

2:4-Diamino-6-chloropyrimidine (1·44 g.) in dilute acetic acid (3N, 75 ml.) was cooled in ice-water and stirred and then treated with a solution of *p*-nitrobenzenediazonium chloride (from *p*-nitraniline (1·38 g.)), freed from nitrous acid by treatment with excess of sulphamic acid. A slight change in colour was noted. After stirring the solution for 5 minutes, it was treated with crystalline sodium acetate to bring the pH to 6–7 and stirring was continued for 5 hours, during which time a thick orange-red precipitate was obtained. This was collected, washed with water copiously, dried and recrystallised from aqueous 2-ethoxyethanol to yield the *azo-compound* as scarlet needles, m.p. 298 to 299° (decomp.) (Found: C, 38·7; H, 3·3; N, 31·4; Cl, 11·3. $C_{10}H_8O_2N_7Cl.1H_2O$ requires C, 38·5; H, 3·2; N, 31·5; Cl, 11·4. Found on a sample dried at 110°; C, 41·15; H, 2·9; loss 5·8. $C_{10}H_8O_2N_7Cl$ requires C, 40·9; H, 2·7; loss, 5·8 per cent).

2:4-*Diamino*-6-*chloro*-5(4′-*chlorophenylazo*)*pyrimidine* (CB. 2298, No. 20)

2:4-Diamino-6-chloropyrimidine (1·44 g.) in dilute acetic acid (3N, 75 ml.) was treated with *p*-chlorobenzenediazonium chloride (from *p*-chloroaniline (1·275 g.)) and the product isolated as above. Recrystallised from aqueous 2-ethoxyethanol, the *azo-compound* formed fine yellow needles m.p. 271 to 272° (decomp.). (Found: C, 42·1; H, 3·1; N, 29·3; Cl, 25·0. $C_{10}H_8N_6Cl_2$ requires C, 42·4; H, 2·8; N, 29·7; Cl, 25·1 per cent).

2:4-*Diamino*-6-*dimethylamino*-5(4′-*nitrophenylazo*)-*pyrimidine* (CB. 2269, No. 21)

Method 1. 2:4-Diamino-6-chloro-5(4′-nitrophenylazo)-pyrimidine (1 g.) in dry 2-ethoxyethanol (40 ml.) was treated with an ethanolic solution

of dimethylamine (33 per cent, 3 ml.) and the mixture was heated under reflux at 100° when the azo compound passed into solution. After one hour, water was added to turbidity and the solution filtered. On cooling, the solution deposited *dark maroon crystals* with a green lustre (0·87 g.), which after recrystallisation from aqueous 2-ethoxyethanol had m.p. 252 to 255°, undepressed on admixture with a sample prepared according to Method 2.

Method 2. 2:4-Diamino-6-chloro-5(4′-nitrophenylazo)pyrimidine (1 g.) was dissolved in warm dimethylformamide (20 ml.) and treated with a solution of guanidine (prepared from guanidine hydrochloride (0·95 g.) and sodium (0·23 g.) dissolved in ethanol (20 ml.), with removal of the precipitated sodium chloride). There was an immediate precipitation of crimson solid, which was allowed to stand overnight. The precipitate (1 g.) was then collected and fractionally crystallised from aqueous 2-ethoxyethanol to yield:—

(i) 2:4-*Diamino*-6-*dimethylamino*-5(4′-*nitrophenylazo*)*pyrimidine*. This formed the less soluble fraction and recrystallised from aqueous 2-ethoxyethanol as deep maroon leaflets with a strong metallic green lustre, m.p. 252 to 255°. (Found: C, 48·0; H, 4·6; N, 37·45; $C_{12}H_{14}O_2N_8$ requires C, 47·7; H, 4·6; N, 37·1 per cent). This was sparingly soluble in dilute hydrochloric acid and on examination in ultra-violet light, the solution had a light blue fluorescence.

(ii) 2:4-*Diamino*-6-*ethoxy*-5(4′-*nitrophenylazo*)*pyrimidine*. This was precipitated on dilution of the mother liquors from which (i) had separated. It recrystallised from aqueous 2-ethoxyethanol as deep orange needles, m.p. 214 to 215°. (Found: C, 47·7; H, 4·6; N, 32·0. $C_{12}H_{13}O_3N_7$ requires C, 47·5; H, 4·3; N, 32·35 per cent). It yielded a sparingly soluble yellow hydrochloride with dilute hydrochloric acid.

2:4-*Diamino*-6-*morpholino*-5(4′-*nitrophenylazo*)*pyrimidine* (CB. 2270, No. 22)

2:4-Diamino-6-chloro-5(4′-nitrophenylazo)pyrimidine (1 g.) was treated with dry morpholine (10 ml.) when the mixture became warm. The reaction was completed by heating under reflux for 10 minutes, and on cooling the mixture deposited red crystals. These were collected, washed with cold ethanol and recrystallised from aqueous 2-ethoxyethanol to yield the *morpholino* compound as deep red prisms, m.p. 275° (decomp.). (Found: C, 49·2; H, 4·60; N, 32·5. $C_{14}H_{16}O_3N_8$ requires C, 48·9; H, 4·65; N, 32·55 per cent). The compound had a pronounced golden-green metallic lustre.

2:4-*Diamino*-6-*diethanolamino*-5(4′-*nitrophenylazo*)*pyrimidine* (CB. 2271, No. 23)

This was prepared similarly from the azo compound (1 g.) and dry diethanolamine (5 ml.). The *diethanolamino* compound, obtained by dilution of the reaction mixture with water, crystallised from very dilute ethanol as deep maroon plates, with a metallic lustre, m.p. 211 to 212°

(decomp.). (Found: C, 46·2; H, 5·1; N, 30·7. $C_{14}H_{18}O_4N_8$ requires C, 46·4; H, 5·0; N, 30·9 per cent). It was very soluble in ethanol.

2:4-*Diamino*-5(4′-*chlorophenylazo*)-6-*dimethylamino pyrimidine* (CB. 2266, No. 24)

Method 1. 2:4-Diamino-6-chloro-5(4′-chlorophenylazo) pyrimidine (1 g.) in dry 2-ethoxyethanol (40 ml.) was heated under reflux for one hour with an ethanolic solution of dimethylamine (33 per cent, 3 ml.). The clear solution was then filtered and concentrated, and on dilution then yielded a yellow precipitate. This on recrystallisation from aqueous 2-ethoxyethanol yielded the *dimethylamino* compound (0·6 g.) as long lustrous yellow needles, m.p. 210° (corr.), undepressed on admixture with the product from Method 2.

Method 2. 2:4-Diamino-6-chloro-5(4′-chlorophenylazo)pyrimidine (0·5 g.) was dissolved in dimethylformamide (10 ml.) and treated with a solution of guanidine in ethanol (from guanidine hydrochloride (0·675 g.) and a solution of sodium (0·16 g.) in ethanol (5 ml.), with removal of sodium chloride). The mixture was heated under reflux at 60 to 70° for 4 hours and then the solvent was removed under reduced pressure. Addition of water yielded a yellow solid which was crystallised from aqueous 2-ethoxyethanol to yield the 6-*dimethylamino* compound as yellow needles, m.p. 210° (corr.) or lustrous golden leaflets, m.p. 187 to 188° depending on the rate of cooling. (Found: C, 49·3; H, 4·9; N, 33·75; Cl, 12·3. $C_{12}H_{14}N_7Cl$ requires C, 49·4; H, 4·8; N, 33·6; Cl, 12·2 per cent).

Careful search failed to reveal a second component (cf. above).

2:4-*Diamino*-5(4′-*chlorophenylazo*-6(*β-ethoxyethoxy*))*pyrimidine*

2:4-Diamino-6-chloro-5(4′-chlorophenylazo)pyrimidine (0·5 g.) was added to a solution of guanidine (from guanidine hydrochloride (0·675 g.) and sodium (0·16 g.) dissolved in 2-ethoxyethanol (20 ml.) with removal of the sodium chloride) and the mixture heated under reflux at 80° for three hours. Concentration of the filtered solution under reduced pressure at 100° and dilution of the residue with water yielded a yellow solid which on recrystallisation from aqueous 2-ethoxy ethanol (90 per cent) afforded the *azopyrimidine* as yellow lustrous silky needles, m.p. 161 to 162°. (Found: C, 50·1; H, 5·0; N, 25·1; Cl, 10·6. $C_{14}H_{17}O_2N_6Cl$ requires C, 49·95; H, 5·05; N, 25·0; Cl, 10·55 per cent).

2:4-*Diamino*-5(4′-*chlorophenylazo*)-6-*morpholinopyrimidine* (CB. 2279, No. 25)

The chlorophenylazo-6-chloropyrimidine (0·3 g.) and morpholine (2 ml.) were heated together under reflux for one hour, cooled and diluted with water to yield a yellow precipitate. This, on crystallisation from aqueous 2-ethoxyethanol, afforded the *morpholino pyrimidine* as golden-yellow lustrous plates, m.p. 221 to 222°. (Found: C, 50·7; H, 4·7; N, 28·9; Cl, 10·5. $C_{14}H_{16}ON_7Cl$ requires C, 50·4; H, 4·8; N, 29·4; Cl, 10·65 per cent).

2:4-*Diamino*-5(4'-*chlorophenylazo*)-6-*diethanolaminopyrimidine* (CB. 2280, No. 26)

The chlorophenylazo-6-chloropyrimidine (1·0 g.) and diethanolamine (5 ml.) were heated together at 140° for 2 hours, cooled and diluted with water. The resulting oily gum crystallised on being allowed to stand overnight and the solid, after crystallisation from very dilute ethanol, yielded the *diethanolamino* compound as orange brown prisms, m.p. 160 to 161°. (Found: C, 48·15; H, 5·12; N, 28·25; Cl, 10·4. $C_{14}H_{18}O_2N_7Cl$ requires C, 47·8; H, 5·12; N, 27·9; Cl, 10·1 per cent).

2:4:6-*Triamino*-5-*arylazopyrimidines*

General Method. 2:4:6-Triaminopyrimidine (5·0 g., 1/25 mol.) was dissolved in water (160 ml.) containing crystalline sodium acetate (16 g.) and the mixture stirred mechanically at 0° during the addition of a filtered solution of the required diazotised amine. The latter solution was generally prepared by dissolving the arylamine (1/25 mol.) in a mixture of concentrated hydrochloric acid (12 ml.) and water (20 ml.), cooling to 0° and adding a solution of sodium nitrite (3·04 g.) in water (20 ml.); after 2–3 minutes, an excess of solid sulphamic acid was added and the solution filtered rapidly through a chilled funnel under suction. Modification of the procedure was necessary only if the amine was too weakly basic to be soluble in dilute hydrochloric acid. An extreme example was 2:4:6-tribromoaniline, which was diazotised as follows. The amine (6·6 g., 1/50 mol.) was dissolved in hot glacial acetic acid (79 ml.) and crystallised on cooling. The suspension of small crystals was added to a solution of sodium nitrite (1·54 g.) in cold concentrated sulphuric acid (10 ml.) and gave a pale yellow solution. This coupled normally on addition to a solution of triaminopyrimidine (2·5 g.) in water (500 ml.) and the product was collected after adjustment of the pH of the solution to 6–7 by the addition of sodium acetate crystals.

Occasionally, despite the presence of sodium acetate in the solution before addition of the diazonium salt, the azo compound was precipitated as the hydrochloride. This could be converted to the base by boiling with aqueous pyridine.

The azo compounds so obtained are listed in Table I.

Other 4-*amino*-5-*arylazopyrimidines*

2:4-*Diamino*-5(4'-*chlorophenylazo*)-6-*hydroxypyrimidine* (CB. 2288, No. 27)

2:4-Diamino-6-hydroxypyrimidine hydrochloride (6·5 g.) was suspended in water (360 ml.) stirred with sodium acetate (42 g.) and filtered. A solution of *p*-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)) was slowly added to the ice-cold filtrate with stirring and the orange-yellow precipitate was collected after two hours. It was crystallised from aqueous pyridine when a pyridine salt was obtained, which was decomposed by stirring with hot ethanol to liberate the *azo-compound* as a yellow microcrystalline powder, m.p. 314 to 315° (decomp.). (Found:

TABLE I

ARYL-AZOPYRIMIDINES (III, $R' = R'' = NH_2$)

CB. No.	R	Formula	Colour and crystal form	M.p. °	Solvent‡	Analysis									
						Found					Required				
						C	H	N	Cl	Br	C	H	N	Cl	Br
*2295 No. 1	C_6H_5-	$C_{10}H_{11}N_7$	Yellow leaflets	262–263	CW	52·5	4·6	42·4	—	—	52·4	4·8	42·8	—	—
2310 No. 2	$2-ClC_6H_4-$	$C_{10}H_{10}N_7Cl$	Yellow needles	290–291	PW	45·7	3·9	36·6	13·6	—	45·5	3·8	37·2	13·5	—
2309 No. 3	$3-ClC_6H_4-$	,,	Yellow needles	264–265	PW	45·7	3·7	37·0	13·6	—	,,	,,	,,	,,	—
2277 No. 4	$4-ClC_6H_4-$	,,	Golden-yellow flat needles	262	CW	45·9	4·2	36·7	13·3	—	,,	,,	,,	,,	—
2328 No. 5	$2-BrC_6H_4-$	$C_{10}H_{10}N_7Br$	Golden-yellow crystals	298–299	CW	39·5	3·3	31·9	—	26·0	39·0	3·25	31·8	—	26·0
2311 No. 6	$4-BrC_6H_4-$	,,	Yellow prisms	261–262	CW	39·0	3·3	31·7	—	25·7	,,	,,	,,	—	,,
2326 No. 7	$2:3-Cl_2C_6H_3-$	$C_{10}H_9N_7Cl_2$	Orange-yellow leaflets	316–317	CW	40·6	2·9	32·7	23·9	—	40·3	3·0	32·9	23·8	—
2307 No. 8	$2:4-Cl_2C_6H_3-$	,,	Long yellow needles	304–305 (decomp.)	PW	40·3	3·0	32·5	24·1	—	,,	,,	,,	,,	—
2322 No. 9	$2:5-Cl_2C_6H_3-$	,,	Yellow needles	319–320	PW	40·4	3·0	32·7	23·6	—	,,	,,	,,	,,	—
2283 No. 10	$3:4-Cl_2C_6H_3-$	,,	Yellow leaflets	269–270	PW	40·4	3·0	32·6	23·7	—	,,	,,	,,	,,	—
2349 No. 11	$2:4-Br_2C_6H_3-$	$C_{10}H_9N_7Br_2$	Orange-yellow leaflets	328–329	PW	31·3	2·5	25·3	—	41·3	31·0	2·3	25·3	—	41·4
2347 No. 12	$2:4:6-Br_3C_6H_2-$	$C_{10}H_8N_7Br_3$	Orange-yellow needles	304–305 (decomp.)	CW	25·8	2·0	20·6	—	51·4	25·75	1·7	21·05	—	51·5
2299 No. 13	$4-MeOC_6H_4-$	$C_{11}H_{13}ON_7$	Orange-yellow prisms	231–232	CW	50·8	5·4	37·7	—	—	51·0	5·0	37·8	—	—
2323 No. 14	$4-EtOC_6H_4-$	$C_{12}H_{15}ON_7$	Yellow needles	280–282	CW	53·1	5·3	35·7	—	—	52·8	5·5	35·9	—	—
†2296 No. 15	$4-NO_2C_6H_4-$	$C_{10}H_{10}O_2N_8$	Red needles	352–353	ACOH	44·1	3·5	40·7			43·8	3·65	40·9	—	—
2330 No. 16	1-naphthyl	$C_{14}H_{13}N_7$	Golden-brown plates	264–265 (decomp.)	CW	60·6	4·6	35·3	—	—	60·2	4·7	35·15	—	—
2315 No. 17	2-naphthyl	,,	Yellow needles	298 (decomp.)	B	59·9	4·4	34·9	—	—	,,	,,	,,	—	—
2331 No. 18	3-quinolyl	$C_{13}H_{12}N_8$	Orange leaflets, blue lustre	315–316 (decomp.)	PW	56·0	4·5	39·9	—	—	55·7	4·3	40·0	—	—

* First prepared by Hartzel and Benson(*J. Amer. chem. Soc.*, 1954, 76, 2263) who give m.p. 235° (decomp.).
† Yellow acetate, initially isolated, is decomposed either on standing or on washing with ethanol and ether.
‡ Solvents: **B**, *n*-butanol; CW, aqueous 2-ethoxyethanol; PW, aqueous pyridine; ACOH, glacial acetic acid.

C, 45·5; H, 3·5; N, 31·5; Cl, 13·2. $C_{10}H_9ON_6Cl$ requires C, 45·4; H, 3·4; N, 31·73; Cl, 13·4 per cent).

2:4-*Diamino*-5(4′-*bromophenylazo*)-6-*hydroxypyrimidine* (CB. 2336, No. 28)

This was obtained in a similar manner when *p*-bromobenzene-diazonium chloride was used. The *azo-compound* was purified by successive crystallisations from aqueous pyridine and aqueous 2-ethoxyethanol and formed yellow crystals. (Found: C, 38·9; H, 3·5; N, 27·3; Br, 26·0. $C_{10}H_9ON_6Br$ requires C, 38·9; H, 3·0; N, 27·2; Br, 25·9 per cent).

4-*Amino*-5(4′-*chlorophenylazo*)-2:6-*dihydroxypyrimidine* (CB. 2313, No. 29)

4-Amino-2:6-dihydroxypyrimidine (5·1 g.) was suspended in water and sufficient 2N sodium hydroxide was added dropwise to yield a clear solution. This solution was then cooled in ice, stirred and treated with a filtered solution of *p*-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)). Glacial acetic acid was added dropwise to the mixture to adjust the pH to 5–6 and after the mixture had remained at 0° in the ice-chest overnight, the product was collected. It was purified by crystallisation from hot 90 per cent formic acid by the addition of water and the resulting yellow solid was dissolved in hot 6N ammonium hydroxide, the solution filtered and the product reprecipitated by neutralisation using glacial acetic acetic acid. The *azo-compound* formed a fine yellow powder which was thoroughly washed by means of water, ethanol and ether successively. (Found: C, 45·15; H, 2·9; N, 26·2; Cl, 13·2; $C_{10}H_8O_2N_5Cl$ requires C, 45·2; H, 3·0; N, 26·4; Cl, 13·4 per cent).

2:4-*Diamino*-5(4′-*chlorophenylazo*)*pyrimidine* (CB. 2329, No. 30)

This was prepared as described by Brown[17]. It formed yellow needles from aqueous 2-ethoxyethanol, m.p. 282 to 283° (decomp.). Brown (*loc. cit.*) gives m.p. 271 to 272°.

4-*Amino*-5(4′-*chlorophenylazo*)-6-*hydroxypyrimidine* (CB. 2304, No. 31)

A solution of 4-chlorobenzenzenediazonium chloride (from *p*-chloroaniline (10·2 g.)) was added slowly with stirring to a filtered, ice-cold solution of 4-amino-6-hydroxypyrimidine (8·82 g.) in water (850 ml.). The pH of the reaction mixture was adjusted to 5–6 by the addition of crystalline sodium acetate and the product collected after a period. Recrystallisation from aqueous 2-ethoxyethanol afforded the *azo-compound* as orange needles, m.p. 300°. (Found: C, 48·6; H, 3·3; N, 27·8; Cl, 14·1. $C_{10}H_8ON_5Cl$ requires C, 48·1; H, 3·2; N, 28·1; Cl, 14·2 per cent).

4:6-*Diamino*-5(4′-*chlorophenylazo*)-2-*thiopyrimidine* (CB. 2292, No. 32) and 4:6-*Diamino*-5(4′-*chlorophenylazo*)-2(4′-*chlorophenylthio*) *pyrimidine* (CB. 2290, No. 33)

A solution of 4-chlorobenzenediazonium chloride (from *p*-chloroaniline (5·1 g.)) was added slowly to an ice-cold, stirred solution of 4:6-diamino-2-thiopyrimidine (5·68 g.) in dilute hydrochloric acid (0·1N, 500 ml.).

On adjusting the pH to 5–6 by the addition of crystalline sodium acetate, a slow precipitation of yellow solid was observed. This was completed after the mixture had stood overnight at 0°. The product was extracted with boiling ethanol and the hot filtrate diluted with water to yield 4:6-*diamino*-5(4′-*chlorophenylazo*)-2-(4′-*chlorophenylthio*) *pyrimidine* as yellow feathery needles from aqueous ethanol, m.p. 211 to 212°. (Found: C, 49·3; H, 3·0; N, 21·35; S, 8·35; Cl, 17·6. $C_{16}H_{12}N_6SCl_2$ requires C, 49·1; H, 3·1; N, 21·5; S, 8·2; Cl, 18·2 per cent).

The residue from the ethanol extraction was crystallised several times from aqueous pyridine and afforded 4:6-diamino-5 (4′-chlorophenylazo)-2-thiopyrimidine as yellow needles, m.p. 278 to 279° (decomp.). Found: C, 43·2; H, 3·1; N, 29·7; S, 11·3; Cl, 13·0. $C_{10}H_9N_6SCl$ requires C, 42·8; H, 3·2; N, 30·0; S, 11·4; Cl, 12·7 per cent).

4:6-*Diamino*-5(4′-*chlorophenylazo*)*pyrimidine* (CB. 2287, No. 34)·

4:6-Diamino pyrimidine (4·4 g.) was dissolved in dilute hydrochloric acid (500 ml. 0·04N) and coupled by treating it with a filtered solution of 4-chlorobenzene diazonium chloride (from *p*-chloroaniline (5·1 g.)) in the usual manner. The addition of solid sodium acetate to the reaction mixture afforded a precipitate which was collected and recrystallised from aqueous 2-ethoxyethanol when the azo compound was obtained as flat yellow needles, m.p. 299 to 300° (decomp.). (Found: C, 48·6; H, 3·5; N, 34·0; Cl, 14·25. Calc. for $C_{10}H_9N_6Cl$: C, 48·3; H, 3·6; N, 33·85; Cl, 14·3 per cent). Lythgoe, Todd and Topham[18] give m.p. 301 to 302° (decomp.).

The preparation of 2:6-diamino-3(4′-chlorophenylazo)pyridine is given at the end of the experimental section.

8-*Aryl*-8-*azapurines*

With the exception of the few members noted below, which include a 1-deaza-8-azapurine (No. 60), the compounds of this series were all prepared in the following general manner.

General Method of preparation of 8-*aryl-azapurines*

The corresponding azopyrimidine (4 g.) was dissolved in a mixture of pyridine (100 ml.) and water (100 ml.) containing copper sulphate (10 g.). When the azo compound was insoluble in this mixture either a larger proportion of pyridine was used, e.g., 200 ml. pyridine to 100 ml. of water, or else a larger volume of mixed solvents in 1:1 proportion. The mixture was placed in a three-necked flask equipped with reflux condenser and a gas inlet tube reaching to the bottom of the flask, and heated to boiling under reflux. A slow stream of oxygen from a cylinder was then passed until the reaction was complete, which was indicated when the solution changed from a greenish-yellow hue to a rich royal blue colour. The hot solution was then poured into water (2 litres) and allowed to stand overnight to ensure complete precipitation. The 8-azapurine was then collected at the pump, washed with copious quantities of water and then with ethanol and finally ether. In nearly every case the yield was quantitative.

TABLE II

8-ARYL-8-AZAPURINES (IV)

CB No.	R	R′	R″	Formula	M.p. °	Solvent§	Analysis											
							Found						Required					
							C	H	N	Cl	Br	S	C	H	N	Cl	Br	S
*†2314 No. 35	C_6H_5–	NH_2	NH_2	$C_{10}H_9N_7$	344–345 (decomp.)	20 F	—	—	—	—	—	—	—	—	—	—	—	—
2321 No. 36	2–ClC_6H_4–	NH_2	NH_2	$C_{10}H_8N_7Cl$	283–284	20 F	45·9	3·2	37·7	13·3	—	—	45·9	3·1	37·5	13·6	—	—
2308 No. 37	3–ClC_6H_4–	NH_2	NH_2	,,	350 (decomp.)	20 F	46·2	3·0	37·2	13·8	—	—	,,	,,	,,	,,	—	—
2278 No. 38	4–ClC_6H_4–	NH_2	NH_2	,,	>360	20 F	46·3	2·9	37·3	13·4	—	—	,,	,,	,,	,,	—	—
2332 No. 39	2–BrC_6H_4–	NH_2	NH_2	$C_{10}H_8N_7Br$	271–272	10 F	37·65	2·8	27·5	—	23·2	—	37·5	2·8	27·85	—	22·7	—
2319 No. 40	4–BrC_6H_4–	NH_2	NH_2	$C_{10}H_8N_7Br + \frac{1}{2}HCO_2H$	>360	20 F	38·1	3·0	29·8	—	24·5	—	38·3	2·7	29·8	—	24·3	—
2333 No. 41	2:3–$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2 + \frac{1}{2}HCO_2H$	287–288	20 F	39·35	2·5	30·6	22·7	—	—	39·5	2·5	30·7	22·3	—	—
2318 No. 42	2:4–$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2$	320–321	20 F	40·1	2·4	32·5	23·85	—	—	40·55	2·4	33·1	24·0	—	—
2324 No. 43	2:5–$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2$	312–313	20 F	40·7	2·5	32·45	23·8	—	—	40·55	2·4	33·1	24·0	—	—
2282 No. 44	3:4–$Cl_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Cl_2$	>360	20 F	40·0	2·5	32·9	24·2	—	—	40·55	2·4	33·1	24·0	—	—
2364 No. 45	2:4–$Br_2C_6H_3$–	NH_2	NH_2	$C_{10}H_7N_7Br_2 + HCO_2H$	279–281 (decomp.)	20 F	30·6	2·5	22·5	—	37·0	—	30·65	2·1	22·75	—	37·15	—
2350 No. 46	2:4:6–$Br_3C_6H_2$	NH_2	NH_2	$C_{10}H_6N_7Br_3$	286–287	AW	26·2	1·3	20·9	—	51·3	—	25·9	1·3	21·1	—	51·7	—
2327 No. 47	4–$EtO–C_6H_4$–	NH_2	NH_2	$C_{12}H_{13}ON_7 + \frac{1}{4}HCO_2H$	310–311	20 F	51·7	4·4	34·5	—	—	—	52·0	4·8	34·7	—	—	—
2335 No. 48	1–$C_{10}H_7$–	NH_2	NH_2	$C_{14}H_{11}N_7$	290–291	20 F	60·4	3·7	35·2	—	—	—	60·7	3·9	35·4	—	—	—
‡2289 No. 49	4–ClC_6H_4–	OH	NH_2	$C_{10}H_7ON_6Cl$	>360	80 F	45·7	2·7	31·9	13·2	—	—	45·7	2·7	32·2	13·5	—	—
2337 No. 50	4–BrC_6H_4–	OH	NH_2	$C_{10}H_7ON_6Br$	>360	80 F	39·0	2·2	27·3	—	26·6	—	39·1	2·3	27·4	—	26·1	—
2285 No. 51	4–ClC_6H_4–	NMe_2	NH_2	$C_{12}H_{12}N_7Cl.HCl$	287–288	2N HCl	44·5	3·8	29·9	21·6	—	—	44·2	3·9	30·05	21·8	—	—
2293 No. 52	4–$NO_2C_6H_4$–	NMe_2	NH_2	$C_{12}H_{12}O_2N_8$	316–318	DMF	48·1	3·9	37·0	—	—	—	48·0	4·0	37·3	—	—	—
2291 No. 53	4–ClC_6H_4–	NH_2	4–ClC_6H_3S	$C_{16}H_{10}N_6SCl_2$	325–326 (decomp.)	80 F	49·3	2·7	21·25	17·9	—	8·1	49·4	2·6	21·6	18·3	—	8·2
2312 No. 54	4–ClC_6H_4–	NH_2	H	$C_{10}H_7N_6Cl$	367 (decomp.)	ACOH	49·1	3·1	34·0	14·1	—	—	48·7	2·8	34·1	14·4	—	—
2305 No. 55	4–ClC_6H_4–	OH	H	$C_{10}H_6ON_5Cl$	339–340	CW	48·7	2·5	28·5	14·8	—	—	48·5	2·4	28·3	14·3	—	—

* Parker and Webb, U.S.P. 2,543,333; *Chem. Abstr.*, 1951, **45**, 7605.
† Hartzel and Benson (*J. Amer. chem. Soc.*, 1954, **76**, 2263) give m.p. >300°.
‡ No preparation recorded in literature; compound mentioned in Stock and others (*Cancer Research, Suppl.* 1, p. 104 as compound No. 1189 (m.p. 375°), obtained from Calco Division, American Cyanamid Co.
§ Solvents: F, Aqueous formic acid, percentage content indicated; DMF, Dimethylformamide; ACOH, Glacial acetic acid; CW, Aqueous 2-ethoxyethanol; AW, Aqueous ethanol.

Table II lists the compounds prepared in this way. All were colourless solids, generally of microcrystalline form. The majority could only be crystallised from aqueous formic acid and occasionally retained formic acid tenaciously after prolonged drying at 100°. In every case the acid content was confirmed by drying at a higher temperature and recording the required loss, the product then analysed for carbon and hydrogen with satisfactory results. It is not possible to show this in the Table.

8-(4′-*Chlorophenyl*)-6-*thio*-8-*azapurine* (CB. 2320, No. 56)

8-(4′-Chlorophenyl)6-hydroxy-8-azapurine (3 g.) in dry pyridine (50 ml.) was treated with phosphorus pentasulphide (3 g.) and the mixture was heated under reflux for one and a half hours, all material dissolving during the initial half hour to yield a dark-brown solution. The reaction mixture was allowed to cool somewhat and was then smoothly decomposed by adding it to hot water (100 ml.). The product (2·87 g.) was collected next day and washed thoroughly with water and then with ethanol and ether. Two crystallisations from aqueous pyridine (charcoal) afforded the *thio-compound* as pale golden lustrous needles, m.p. 357 to 358° (decomp.). (Found: C, 46·0; H, 2·5; N, 26·3; S, 12·1; Cl, 13·4. $C_{10}H_6N_5SCl$ requires C, 45·5; H, 2·3; N, 26·6; S, 12·15; Cl, 13·5 per cent).

2-*Amino*-8(4′-*Chlorophenyl*)-6-*thio*-8-*azapurine* (CB. 2294, No. 57)

A mixture of 2-amino-8(4′-chlorophenyl)-6-hydroxy-8-azapurine (1 g.) and phosphorus pentasulphide (1 g.) in dry pyridine (50 ml.) was heated under reflux for two hours. A further portion of phosphorus pentasulphide (2 g.) and pyridine (50 ml.) was then added and heating continued. All material had dissolved after a further hour and after a total time of reflux of 4 hours, the mixture was decomposed by pouring into hot water (200 ml.). The primrose-yellow precipitate (Crude 0·91 g.) which was formed was collected next day and crystallised from pyridine (charcoal) to yield the *thio compound* as a yellow powder, m.p. 362 to 363° (decomp.). (Found: C, 45·3; H, 3·0; N, 28·9; S, 10·4; Cl, 12·1. $C_{10}H_7N_6SCl$–1/4 C_5H_5N requires C, 45·3; H, 2·8; N, 29·3; S, 10·7; Cl, 11·9 per cent).

2-*Amino*-6-*hydroxy*-8(3′-*quinolyl*)-8-*azapurine* (CB. 2338, No. 58)

2:4:6-Triamino-5(3′-quinolylazo) pyrimidine (12·0 g.) in pyridine (600 ml.) and water (100 ml.) was treated with a solution of copper sulphate pentahydrate (40 g.) in hot water (100 ml.) and the clear solution was heated in a three-necked flask fitted with reflux condenser while a slow stream of oxygen was passed through the liquid. A creamish-yellow precipitate soon began to form, and after two hours the colour of the supernatant solution assumed a clear royal blue. Whereupon the mixture was poured into water (4 l.). The product was collected next day, and purified by solution in hot 2N hydrochloric acid, treatment with charcoal and reprecipitation with 2N ammonium hydroxide several times. The product formed a *pale cream solid*, m.p. $>$ 360°. Found: C, 56·0; H, 3·2; N, 35·2; $C_{13}H_9ON_7$ requires C, 56·0; H, 3·2; N, 35·15 per cent).

Besides being soluble in dilute acid, the compound was soluble in hot dilute sodium hydroxide, revealing the presence of a hydroxy group.

2:6-*Diamino*-8-(3′-*quinolyl*)-8-*azapurine* (CB. 2381, No. 59)

The ring closure of triamino-5(3′-quinolylazo)pyrimidine was carried out exactly as described above. The crude product was recrystallised from propylene glycol (charcoal) several times, washed thoroughly by trituration with ethanol and finally ether and yielded a *pale cream solid*, m.p. $> 360°$, which however, was now not soluble in hot dilute sodium hydroxide. To obtain satisfactory analytical figures, it was necessary to dry the sample to constant weight at 140°. (Found: C, 56·3; H, 4·0; N, 39·9; $C_{13}H_{10}N_8$ requires C, 56·2; H, 3·6; N, 40·3 per cent).

5-*Amino*-2(4′-*chlorophenyl*)-*triazolo*(4′:5′-2:3)*pyridine* (CB. 2363, No. 60)

2:6-Diamino-3(4′-chlorophenylazo)pyridine (5 g.) was dissolved in a mixture of pyridine (100 ml.) and water (100 ml.) containing copper sulphate pentahydrate (10 g.) and was heated under reflux during the passage of oxygen. A rapid reaction occurred and a deep blue colour developed within 10 minutes. After one hour, the mixture was poured into water (2 l.) and the pale grey precipitate was collected. Crystallisation from aqueous 2-ethoxyethanol gave the *triazolopyridine* as long white felted needles, changing on standing to pale yellow prismatic needles. The change could be accelerated by warming; both forms melted at 258 to 259°. (Found: C, 54·1; H, 3·2; N, 28·65; Cl, 14·1. $C_{11}H_8N_5Cl$ requires C, 53·8; H, 3·3; N, 28·5; Cl, 14·5 per cent.) Treatment of this compound with hot acetic anhydride afforded the 5-*acetamido* derivative as colourless needles, m.p. 296 to 297° from *n*-butanol. (Found: C, 53·0; H, 3·5; N, 23·2; Cl, 12·2. $C_{13}H_{10}ON_5Cl.\frac{1}{2}H_2O$ requires C, 52·6; H, 3·5; N, 23·6; Cl, 12·0 per cent).

9-*Aryl*-8-*azapurines*

A. 2-*Amino*-9-*phenyl*-8-*azapurine*

(i) 2-*Amino*-4-*anilino*-5-*nitropyrimidine*. 4-Anilino-2-chloro-5-nitropyrimidine (3·0 g.)[19] was heated for four hours at 100° in a sealed tube with saturated ethanolic ammonia (25 ml.). The tube was cooled, opened and the lemon yellow product (2·91 g.) collected and crystallised from *n*-butanol to give the *aminonitropyrimidine* as lemon-yellow needles, m.p. 206 to 207°. (Found: C, 52·3; H, 3·9; N, 30·05. $C_{10}H_9O_2N_5$ requires C, 52·0; H, 3·9; N, 30·3 per cent).

(ii) 2:5-*Diamino*-4-*anilino pyrimidine*. The above aminonitropyrimidine (1·8 g.) was stirred into a solution of stannous chloride (7·6 g.) in concentrated hydrochloric acid (38 ml.) and the mixture was gently warmed on the steam bath. When all the yellow solid had been replaced by a white precipitate, the mixture was cooled and the solid collected and dissolved in water. The solution was treated with 40 per cent sodium hydroxide solution and the white precipitate collected using chloroform. Evaporation of the dried extract gave a pale yellow residue which was

crystallised from 2N hydrochloric acid (charcoal) to yield the *triamine dihydrochloride* as colourless needles, m.p. 249 to 250° (decomp.). (Found: 3, 44·0; H, 4·8; N, 25·9; Cl, 25·3. $C_{10}H_{11}N{\cdot}2HCl$ requires C, 43·8; H, 4·75; N, 25·6; Cl, 25·9 per cent). Treatment of an ethanolic solution of the dihydrochloride with an ethanolic solution of picric acid gave the *monopicrate* as yellow needles, m.p. 249 to 250° (decomp.) from aqueous ethanol. (Found: C, 44·4; H, 3·4; N, 25·6; $C_{10}H_{11}N_5{\cdot}C_6H_3O_7N_3$ requires C, 44·7; H, 3·3; N, 26·1 per cent).

(iii) 2-*Amino*-9-*phenyl*-8-*azapurine* (CB. 2355, No. 61). The above dihydrochloride (0·68 g.) was dissolved in water (40 ml.) containing a few drops of concentrated hydrochloric acid to suppress any hydrolysis and was stirred and cooled to 5°. A solution of sodium nitrite (0·24 g.) in water (1 ml.) was added when an immediate pale yellow precipitate was formed. After half an hour, the pH of the mixture was adjusted to 8 by the addition of ammonium hydroxide and the product was collected, and crystallised from aqueous methanol (charcoal). The *triazolopyrimidine* formed colourless rosettes of needles m.p. 167 to 168°. (Found: C, 56·6; H, 3·9; N, 39·7; $C_{10}H_8N_6$ requires C, 56·6; H, 3·8; N, 39·65 per cent).

B. 2-*Amino*-6-*methyl*-9-*phenyl*-8-*azapurine*

(i) 2-*Amino*-4-*anilino*-6-*methyl*-5-*nitropyrimidine*. 4-Anilino-2-chloro-6-methyl-5-nitropyrimidine (2·54 g.; Spickett[19], *loc. cit.*) was heated at 100° in a sealed tube with saturated methanolic ammonia solution (20 ml.) for 1½ hours. When cold, the tube was opened and the product (2·3 g.) was collected and crystallised from *n*-butanol to give the *amino* compound as yellow needles, m.p. 192 to 193°. (Found: C, 53·3; H, 4·3; N, 28·6. $C_{11}H_{11}O_2N_5$ requires C, 53·9; H, 4·5; N, 28·6 per cent).

(ii) 2:5-*Diamino*-4-*anilino*-6-*methylpyrimidine*. The nitro compound (3·0 g.) was reduced using a solution of stannous chloride (12 g.) in concentrated hydrochloric acid (60 ml.). The precipitated stannichloride was collected, decomposed with 40 per cent sodium hydroxide and the liberated base extracted with several portions of chloroform. Evaporation of the dried extracts gave the crude product as a crusty brownish solid (2·4 g.). This, on crystallisation from hot 2N hydrochloric acid (charcoal) gave colourless, lustrous needles, m.p. 239 to 240°, of the *dihydrochloride*. (Found: C, 45·8; H, 5·2; N, 24·45; Cl, 24·65. $C_{11}H_{13}N_5{\cdot}2HCl$ requires C, 45·9; H, 5·2; N, 24·3; Cl, 24·7 per cent). The *monopicrate* formed orange-yellow leaflets, m.p. 252 to 253° (decomp.) from dilute ethanol. (Found: C, 45·9; H, 3·8; N, 24·85. $C_{11}H_{13}N_5C_6H_3O_7N_3$ requires C, 45·95; H, 3·6; N, 25·25 per cent).

(iii) 2-*Amino*-6-*methyl*-9-*phenyl*-8-*azapurine* (CB. 2341, No. 62). 2,5-Diamino-4-anilino-6-methyl pyrimidine dihydrochloride (2·0 g.) in water (150 ml.) containing concentrated hydrochloric acid (4 drops) was cooled to 5° and treated with a solution of sodium nitrite (0·7 g.) in water (2 ml.) with stirring. A precipitate was formed immediately and after 15 minutes the pH was adjusted to 7–8 by the addition of ammonium hydroxide and the product collected. The *azapurine* formed colourless

needles, m.p. 188 to 189°, from aqueous methanol. (Found: C, 58·3; H, 4·6; N, 36·8. $C_{11}H_{10}N_6$ requires C, 58·4; H, 4·5; N, 37·1 per cent).

C. 2-Amino-9(4'-chlorophenyl)8-azapurine

(i) *2-Chloro-4-(4'-chloroanilino)-5-nitropyrimidine.* 2:4-Dichloro-5-nitropyrimidine (1·0 g.) in ethanol (25 ml.) was cooled to −10° in an ice-salt bath and treated all at once with a solution of *p*-chloroaniline (0·66 g.) in ethanol (12 ml.). After a few seconds the clear solution became a mass of yellow solid, which was immediately collected and recrystallised from ethanol, to yield *2-chloro-4(4'-chloroanilino)-5-nitropyrimidine* as yellow needles, m.p. 150 to 151°. (Found: C, 42·0; H, 2·6; N, 19·65; Cl, 24·5. $C_{10}H_6O_2N_4Cl_2$ requires C, 42·1; H, 2·1; N, 19·7; Cl, 24·9 per cent).

If the reaction was attempted on a larger scale, then a mixture was obtained with the di-*p*-chloroanilino compound. For comparison an authentic sample of this was prepared from the dichloronitropyrimidine (1 g.) in ethanol (15 ml.) and *p*-chloroaniline (2·64 g.) in ethanol (50 ml.), the pyrimidine being added to the amine this time. The mixture was then boiled under reflux to complete the reaction and the product, isolated by filtration, formed pale yellow needles, m.p. 216 to 217° from *n*-butanol. (Found: C, 51·3; H, 3·0; N, 18·8; Cl, 19·3. $C_{16}H_{11}O_2N_5Cl_2$ requires C, 51·0; H, 2·9; N, 18·6; Cl, 18·9 per cent).

2-Amino-4(4'-chloroanilino)-5-nitropyrimidine. 2-Chloro-4(4'-chloroanilino)-5-nitropyrimidine (2·0 g.) was heated at 100° for three hours in a sealed tube with saturated ethanolic ammonia solution (20 ml.). The product (1·9 g.), collected on cooling, crystallised from *n*-butanol as golden-yellow leaflets, m.p. 241°. (Found: C, 45·5; H, 3·45; N, 26·3; Cl, 13·6. $C_{10}H_8O_2N_5Cl$ requires C, 45·2; H, 3·0; N, 26·4; Cl, 13·4 per cent).

2:5-Diamino-4(4'-chloroanilino)pyrimidine. The above nitro compound (1·32 g.) was added to a solution of stannous chloride (5·0 g.) in concentrated hydrochloric acid (25 ml.) and the mixture warmed and ground. When reduction was complete, as shown by the replacement of the orange colour by a cream suspension of the stannichloride, the product was collected, decomposed by 40 per cent sodium hydroxide and the base collected by extraction with chloroform. The residue obtained by evaporation of the extract was crystallised from hot 2N hydrochloric acid (charcoal) to yield the *dihydrochloride monohydrate* as colourless needles, m.p. 286 to 287° (decomp.) (Found: C, 37·1; H, 4·5; N, 21·8; Cl, 32·7. $C_{10}H_{10}N_5Cl{\cdot}2HCl{\cdot}H_2O$ requires C, 36·75; H, 4·3; N, 21·45; Cl, 32·65 per cent). The *monopicrate* formed lemon-yellow rosettes of prisms, m.p. 256° (decomp.) from aqueous ethanol. (Found: C, 41·8; H, 3·1; N, 23·9; Cl, 7·9. $C_{10}H_{10}N_5Cl{\cdot}C_6H_3O_7N_3$ requires C, 41·3; H, 2·8; N, 24·1; Cl, 7·65 per cent).

2-Amino-9(4'-chlorophenyl)-8-azapurine (CB. 2356, No. 63)

2:5-Diammo-4(4'-chloroanilino)pyrimidine dihydrochloride (0·4 g.) was dissolved in water (80 ml.), concentrated hydrochloric acid (2 drops)

was added, and the mixture cooled to 5° in ice-water. A solution of sodium nitrite (0·12 g.) in water (1 ml.) was added and the mixture was well stirred. After adjustment of the pH to 8 by means of concentrated ammonium hydroxide, the precipitate was collected and crystallised from aqueous methanol to afford the triazolopyrimidine as rosettes of fine white needles, m.p. 236°. (Found: C, 48·7; H, 3·0; N, 34·2; Cl, 14·7. $C_{10}H_7N_6Cl$ requires C, 48·7; H, 2·8; N, 34·1; Cl, 14·4 per cent).

8-*Azapurine* (CB. 2354, No. 64)

4:5-Diaminopyrimidine (0·4 g.) in 95 per cent ethanol (10 ml.) was refluxed with amyl nitrite (5·5 ml., 10 mol.) for two hours, by which time the solution was nearly clear. It was cooled, filtered from a small amount of dark coloured matter which was discarded and the filtrate was evaporated to dryness to leave a brown solid. This was sublimed at 130 to 140°/1·5 mm. to yield a cream substance, smelling slightly of fatty acids. The m.p. varied with the rate of heating and standard conditions had to be adopted to obtain concordant results. The sample was placed in an electrically-heated copper block at 125° and the temperature raised at the rate of 2° per minute. The m.p. was then 170 to 171°.

The product was resublimed at 130 to 140°/1·5 mm. and the initial fraction (0·08 g.) m.p. 164 to 165° was rejected. Sublimation was continued and the second fraction (0·29 g.) had m.p. 175°. A small amount of yellowish substance was obtained subsequently and the remainder consisted of brown, involatile decomposition products. Repeated sublimation of the fraction, m.p. 175° only gave material of the same m.p. and an involatile residue. The final *sublimate* was a white solid, insoluble in ether, but very soluble in water to give a solution with a strongly acid reaction to litmus. On treatment with silver nitrate solution, this gave a heavy white precipitate of the silver salt; with sodium bicarbonate solution a vigorous effervescence is observed. (Found: C, 39·9; H, 2·6; N, 58·1; $C_4H_3N_5$ requires C, 39·7; H, 2·5; N, 57·8 per cent).

2:6-*Diamino*-3(4′-*chlorophenylazo*)*pyridine* (CB. 2357, No. 66)

2:6-Diaminopyridine (5·45 g.) in dilute hydrochloric acid solution (2N, 50 ml.) was cooled in ice, stirred and treated slowly with a solution of 4-chlorobenzenediazonium chloride (from *p*-chloroaniline (6·375 g.)). To the resulting dark-red solution was added crystalline sodium acetate to adjust the pH to 5–6 and the thick yellow slurry so obtained was diluted with water and filtered. The product on crystallisation from aqueous ethanol afforded the *azo compound* as yellow prisms, m.p. 184 to 185°. (Found: C, 53·45; H, 4·3; N, 27·9; Cl, 14·1. $C_{11}H_{10}N_5Cl$ requires C, 53·4; H, 4·0; N, 28·3; Cl, 14·3 per cent).

Biological Section*

Methods

Strains. Streptococcus faecalis (R) was used for experiments with pteroylglutamic acid (PGA) and *Leuconostoc citrovorum* (NCTC 7837)

* By H.O.J.C. and P.L.H.

with 5-formyltetrahydropteroylglutamic acid (folinic acid). Stock cultures of *Str. faecalis* were maintained in the liver-tryptone agar of Nymon and Gortner[20], and stock cultures of *Leuc. citrovorum* in brewer's yeast agar. Tests were carried out in a basal medium similar to that used by Collier and Phillips[6] for testing antagonists of folinic acid, which was based on the medium of Barton-Wright, Emery and Robinson[21]. This contained: acid hydrolysed casein (A. & H.), 6 g.; L-cystine, 100 mg.; DL-tryptophane, 120 mg.; glucose, 10 g.; sodium acetate (anhydrous), 10 g.; adenine HCl, 10 mg.; guanine HCl, 10 mg.; uracil, 10 mg.; xanthine, 10 mg.; aneurine, 100 μg.; riboflavine, 200 μg.; pyridoxine HCl, 100 μg.; calcium D-pantothenate, 500 μg.; D-biotin, 0·4 μg.; nicotinic acid, 100 μg.; NaCl, 5 g.; KH_2PO_4, 500 mg.; K_2HPO_4, 500 mg.; $MgSO_4 \cdot 7H_2O$, 200 mg.; $MnSO_4 \cdot 4H_2O$, 10 mg.; $FeCl_3$, 2 mg.; glass-distilled water, to 500 ml. The pH of this medium was adjusted to 7·0 and after steaming for 30 minutes the medium was filtered while hot.

Antifolic tests. The above medium, which was double-strength, was filled out in 5 ml. aliquots, in which solutions of test compounds were serially diluted. PGA was dissolved in a little potassium dihydrogen phosphate solution and made up to the required concentration in glass-distilled water. Five ml. quantities of PGA solution were added to aliquots of medium before autoclaving at 10 lb. for 10 min. Folinic acid was dissolved in sterile glass-distilled water and added with sterile precautions to double-strength medium after autoclaving. Inocula of bacteria were prepared from stock cultures by growing in liver tryptone broth for 20 hours at 37°. Cultures were centrifuged, washed twice with sterile saline and made up to correspond in opacity with Brown's No. 2 tube. This suspension was diluted 1 : 100 with saline and 0·02 ml. used as inoculum. After 60 to 64 hour incubation at 37°, growth was estimated by titration with 0·1N NaOH, using bromthymol blue as indicator. For reference, 4-amino-10-methylpteroylglutamic acid (A-methopterin) and 2 : 4-diamino-6 : 7-di*iso*propylpteridine (0/129) were used.

Experimental

Str. faecalis *and pteroylglutamic acid.* The technique of screening tests is illustrated in Figure 1. When *Str. faecalis* was cultivated in the presence of enough PGA to give good growth (2 ng./ml.), increasing concentrations of an inhibitor depressed growth to an increasing degree. When the level of PGA was raised to 20 ng./ml., higher concentrations of inhibitor were required to produce equal depression, indicating some degree of competitive antagonism.

The antifolic acid activities of all compounds described in the chemical section were examined by this method, using levels of 2 and 20 ng./ml. PGA. Compounds numbered 9, 12, 15, 19, 21, 22, 25, 29, 32–34, 49–58, 60–66, and 68 were inactive in saturated solution, while the remainder showed activity. The concentration of each active inhibitor required to depress growth by half at each level of PGA was determined in three or more independent experiments. The geometric means of these concentrations and their 95 per cent fiducial limits, together with the molar ratios

of inhibitor to PGA based on these mean values, and the ratios of the mean inhibitory concentrations at 20 and 2 ng./ml. PGA are expressed for aryl-azopyrimidines in Table III. Corresponding results for 8-azapurines, Compound 67, A-methopterin and pteridine 0/129 are given in Table IV. From these Tables it will be seen that the most active compounds among azopyrimidines were Nos. 5, 6, 11, 17 and 30 and among azapurines the considerably more potent Nos. 45 and 48. Compound 45 had the highest potency, which was about one-fiftieth that of A-methopterin in the same test.

Leuc. citrovorum *and folinic acid.* Replacing *Str. faecalis* by *Leuc. citrovorum*, some of the compounds antagonising PGA were screened at levels of 0·92, 4·6 or 23 ng./ml. folinic acid. Of the aryl-azopyrimidines tested, only compounds 1, 13 and 26 showed some degree of competitive antagonism, the remainder tested (Nos. 2, 3, 4, 6, 8, 10, 14, 16, 17, 20, 23, 24 and 27) being inactive. All the azapurines tested (Nos. 35, 37, 38, 40, 42 and 48) were inactive and so was Compound 67. A-methopterin had about one-hundredth and Compound 1 about one-fifth of their respective potencies with *Str. faecalis.*

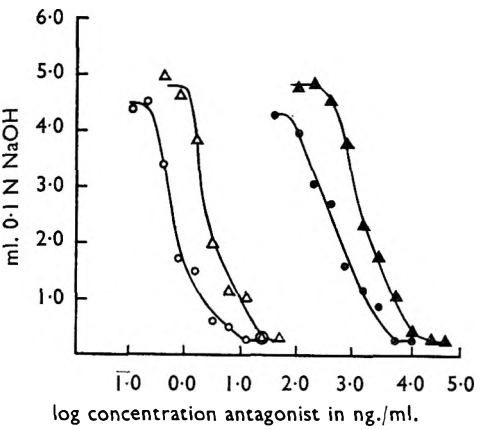

FIG. 1. Growth inhibition of *Str. faecalis* in the presence of pteroylglutamic acid (PGA).
● Compound 1 at 2 ng./ml. PGA
▲ ,, ,, ,, 20 ,, ,,
○ A-methopterin at 2 ng./ml. PGA
△ ,, ,, 20 ,, ,,

DISCUSSION

In 1914 Langley[22] showed that for any particular muscle of the frog, the ratio of curare (antagonist) to nicotine (agonist) necessary to prevent a response was constant and independent of the levels of concentration. In 1940, a similar constancy of ratio between sulphanilamide and aminobenzoic acid for *Str. pyogenes* was described by Woods[23], who ascribed it to competition between those substances for an enzyme. Since then, constancy of ratio between antagonist and agonist has commonly been used as a test of competition, particularly in bacteria. Schild[24], however has argued that "the mass action equation as developed by Gaddum[25] for a first order reaction requires a ninefold increase of antagonist corresponding to a five-fold increase of active drug. . . . Straight proportionality between drug and antagonist at low concentrations of the antagonist is presumptive evidence against the existence of a simple mass action relation". The above divergent viewpoints and the work of Timms[26] suggest that methods based simply on measuring ratios of antagonist to

growth-factor required for a given biological effect do not fully characterise competitive action. Such methods however can indicate some degree of competition.

It will be seen from Tables III and IV that for each compound a tenfold increase in PGA requires a twofold to eightfold increase in inhibitor to produce the same degree of inhibition. This indicates the presence of

TABLE III

INHIBITION OF GROWTH OF *Str. faecalis* BY VARIOUS ARYL-AZOPYRIMIDINES IN THE PRESENCE OF PTEROYLGLUTAMIC ACID (PGA). VALUES EXPRESSED AS GEOMETRIC MEANS AND THEIR 95 PER CENT FIDUCIAL LIMITS

Compound		μg./ml. to inhibit growth by half in presence of				Mean molar ratio inhibitor to PGA at		Ratio of means at 20 and 2 ng./ml. PGA
	CB Reference	2 ng./ml. PGA		20 ng./ml. PGA				
No.	No.	Mean	Limits	Mean	Limits	2 ng./ml.	20 ng./ml.	
1	2295	0·33	0·20–0·56	1·66	1·34–2·07	317·8	159·8	5·0
2	2310	0·25	0·09–0·67	1·13	0·39–3·26	209·2	94·55	4·5
3	2309	0·39	0·15–1·06	1·06	0·41–2·74	326·3	88·70	2·7
4	2277	0·21	0·08–0·59	1·08	0·45–2·61	175·7	90·37	5·1
5	2328	0·19	0·12–0·28	0·82	0·35–1·92	136·0	58·70	4·3
6	2311	0·17	0·12–0·23	0·81	0·60–1·09	121·7	57·98	4·8
7	2326	0·57	0·16–2·08	2·75	0·99–7·67	424·2	203·5	4·8
8	2307	0·41	0·14–1·17	0·71	0·50–1·00	303·4	52·53	1·7
10	2283	0·54	0·20–1·43	2·22	0·80–6·18	399·6	164·3	4·1
11	2349	0·27	0·11–0·65	0·54	0·25–1·19	153·8	30·76	2·0
13	2299	1·11	0·51–2·41	5·00	1·96–12·76	945·0	425·7	4·5
14	2323	3·67	1·30–10·35	12·88	4·60–36·08	2,964	1,040	3·5
16	2330	0·28	0·12–0·67	0·86	0·31–2·39	221·3	67·96	3·1
17	2315	0·16	0·06–0·45	0·43	0·21–0·90	129·4	33·98	2·7
18	2331	2·26	1·01–5·06	4·77	2·10–10·77	1,779	375·6	2·1
20	2298	12·28	7·25–20·81	—*	—	9,568	—	—
23	2271	22·41	10·95–45·84	7·60	28·45–49·68	13,650	2,290	1·7
24	2266	7·72	3·08–19·36	—*	—	5,840	—	—
26	2280	14·59	7·02–30·34	22·40	12·66–39·64	9,153	1,405	1·6
27	2288	0·49	0·38–0·64	1·10	0·46–2·63	408·5	91·70	2·3
28	2336	0·76	0·53–1·09	1·65	1·15–2·36	542·3	117·7	2·2
30	2329	0·13	0·05–0·39	0·54	0·27–1·09	115·3	47·90	4·2
31	2304	20·72	18·02–24·39	52·94	27·50–102·0	18,310	4,679	2·6

* Incomplete or no inhibition in saturated solution.

TABLE IV

INHIBITION OF GROWTH OF *Str. faecalis* BY VARIOUS 8-AZAPURINES AND OTHER COMPOUNDS IN THE PRESENCE OF PTEROYLGLUTAMIC ACID (PGA). VALUES EXPRESSED AS GEOMETRIC MEANS AND THEIR 95 PER CENT FIDUCIAL LIMITS

Compound		μg./ml. to inhibit growth by half in presence of				Mean molar ratio inhibitor to PGA at		Ratio of means at 20 and 2 ng./ml. PGA
	CB Reference	2 ng./ml. PGA		20 ng./ml. PGA				
No.	No.	Mean	Limits	Mean	Limits	2 ng./ml.	20 ng./ml.	
35	2314	1·16	0·62–2·15	5·43	4·24–6·93	1,126	527·5	4·7
36	2321	0·35	0·13–0·93	1·96	1·66–2·32	295·1	165·3	5·6
37	2308	0·62	0·26–1·52	4·68	1·75–12·51	522·9	394·6	7·6
38	2278	0·12	0·07–0·21	0·85	0·36–2·03	101·2	71·67	7·1
39	2332	0·46	0·26–0·84	1·83	1·53–2·18	331·5	131·9	4·0
40	2319	0·12	0·05–0·30	0·59	0·28–1·24	86·47	42·52	4·9
41	2333	0·22	0·09–0·58	0·73	0·34–1·58	163·9	54·38	3·3
42	2318	0·12	0·05–0·29	0·57	0·24–1·37	83·39	42·46	4·8
43	2324	0·19	0·12–0·28	1·20	0·44–3·28	141·5	89·39	6·3
44	2282	0·22	0·08–0·63	1·42	0·53–3·78	163·9	105·8	6·5
45	2364	0·04	0·02–0·10	0·11	0·04–0·30	22·90	6·30	2·8
46	2350	0·51	0·41–0·64	1·64	0·58–4·65	242·4	77·93	3·2
47	2327	0·98	0·37–2·63	2·71	0·98–7·45	797·4	220·5	2·8
48	2335	0·04	0·03–0·07	0·19	0·09–0·38	31·84	15·12	4·8
59	2381	0·10	0·04–0·24	0·42	0·22–0·81	79·32	33·32	4·2
67	2316	0·57	0·24–1·36	3·89	2·67–5·66	897·8	612·7	6·7
A-Methopterin		0·0005	0·0002–0·0012	0·0023	0·0013–0·0041	0·226	0·112	4·6
Pteridine 0/129		0·0026	0·0011–0·0060	0·0143	0·0071–0·0290	2·301	1·286	5·5

some degree of competitive antagonism of pteroylglutamic acid. A full test of competition depends on analysis of curves of log concentration of folic acid plotted against bacterial growth in the absence of and in the presence of various levels of inhibitor. Such curves have shown that Compound 1 competes with both PGA and folinic acid. These curves and others at present being obtained for further compounds will be published elsewhere.

Structure-Activity Relations

The fact that the 4-amino-5-aryl-azopyrimidines (III) can be readily oxidised, by chemical agents, to the 8-aryl-8-azapurines (IV) and that both series of compounds contain active substances suggested that the activity of the azo compounds might be due to this conversion by oxidation during bacterial metabolism. Detailed investigation however revealed a marked lack of the relation which, on this hypothesis, would have been expected between the activities of corresponding compounds in the two series. For example, for *Str. faecalis* and PGA, certain 8-azapurines (Nos. 49, 50 and 55) which are substituted with amino and hydroxy groups at the 2 and 4 positions (IV, $R'' = NH_2$, $R' = OH$) respectively, are inactive, whilst the corresponding azo compounds (Nos. 27, 28 and 31) are active. Again for *Leuc. citrovorum* and folinic acid the 8-azapurine No. 35 is inactive, but the corresponding azopyrimidine (No. 1) is active. On the other hand, for *Str. faecalis*, certain highly active 8-azapurines, e.g., Nos. 43 and 46, correspond to the inactive azopyrimidines, Nos. 9 and 12 respectively. A further discrepancy is revealed by comparison of the activities of three azopyrimidines (Nos. 2, 3 and 4), which differ only in the position of the chlorine atom in the benzene ring, and the corresponding 8-azapurines (Nos. 36, 37 and 38). The azo compounds are of comparable activity whilst the 8-azapurines showed more fluctuation.

An alternative hypothesis for the activity of the azopyrimidines was that they might be reductively split (a process known to occur *in vivo* with a variety of azo compounds[27–30]) to yield a 4:5-diaminopyrimidine (V) which might be the active form; but, although 2:4:5:6-tetraminopyrimidine (V, $R = NH_2$, No. 67), which might be derivable from many of the active compounds, was an antifolic acid, the inactivity of 2:4:5-triamino-6-hydroxypyrimidine (V, $R = OH$, No. 68), also derivable from the active azopyrimidines Nos. 27 and 31, rendered the hypothesis untenable. We conclude therefore that the antifolic activity found in the azopyrimidines and 8-azapurines is intrinsic for each series.

Simplification of the diamino-8-aryl-8-azapurines by removing the aryl substituent to produce 2:6-diamino-8-azapurine (No. 65) or by further removing the amino groups to give 8-azapurine itself (VI, No. 64) led to inactive compounds. The inactivity of No. 65 thus suggests that an aryl substituent may be necessary. Since, also, the three 9-aryl-8-azapurines prepared (VII, $R' = H$, $R'' = C_6H_5$, No. 61; $R' = CH_3$, $R'' = C_6H_5$, No. 62; $R' = H$, $R'' = C_6H_4Cl\,p$, No. 63) were all inactive, it may be that the particular position of the aryl substituent in the 8-aryl-8-azapurine structure relative to the pyrimidine ring is also important. As a flat

structure this substituent might be concerned in facilitating attachment to an enzyme. Modification of the two active structures by substitution of a pyrimidine by a pyridine ring as in VIII (No. 60) and IX (No. 66) led to inactivity.

In the aryl-azopyrimidines (III) activity is consistent with either one, two or three amino substituents in the pyrimidine ring, but for maximal effect three amino groups are generally desirable. The effects of substitution in the benzene ring with chlorine or bromine depend on the position and number of the substituent atoms. A single atom in the *para* position was beneficial, but dihalogen compounds were usually less active than the mono-substituted and the tribromo compound (No. 12) was inactive.

Of the twenty-five 8-aryl-8-azapurines tested, fifteen contained two amino groups in the pyrimidine ring and all were active. The remaining ten compounds contained one or no amino groups. Since none of these was active, we conclude that two amino groups are essential for activity in this series. In the azapurines, the effects of substitution in the benzene ring with chlorine or bromine differed in some ways from those in the azopyrimidine series. Substitution of a single halogen atom in any position increased activity, and the dihalogen compounds were generally more active than the monosubstituted. On the other hand, as in the azopyrimidine series, the *para* position in the monosubstituted derivatives was the most beneficial, and the tribromo derivative (No. 46) was much less active than the dibromo compound (No. 45).

The active compounds of the 8-azapurine series, some of which are more potent than any of the azopyrimidines tested, provide what appear to be the first examples of antifolic acid activity among 8-azapurines.

Summary

1. Thirty-four amino-5-aryl-azopyrimidines and thirty amino-8-aryl-8-azapurines and a few examples of three related structures were synthesised as potential antifolic acids because of a structural relation to pteridine.

2. Screened with *Str. faecalis* and pteroylglutamic acid (PGA), 23 aryl-azopyrimidines and fifteen 8-azapurines showed antifolic acid activity. This was greatest in Compound 45, which possessed about one-fiftieth the potency of A-methopterin in the same test.

3. With *Leuc. citrovorum* and folinic acid, 3 of 16 azopyrimidines and none of 6 azapurines tested showed antifolic acid activity. A-methopterin had about one-hundredth and Compound 1 about one-fifth of their respective potencies with *Str. faecalis*.

Consideration of the activities of the above compounds has enabled certain structure-activity relations to be deduced.

We are indebted to Professor F. C. Happold for the culture of *Str. faecalis* (R) and to the American Cyanamid Company for A-methopterin and the calcium salt of folinic acid ("Calcium leucovorin"). We also thank Mr. J. J. Grimshaw for statistical advice and analysis and Mr. J. Johnston and Miss J. A. Moore for technical assistance. The chemical part of this investigation was supported by grants from The British

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ANTIMETABOLITE ACTIVITY OF 5-ARYLAZOPYRIMIDINES

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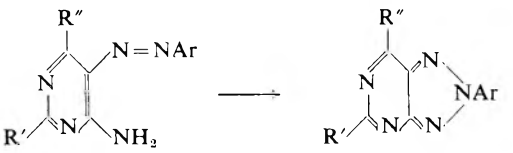
THE antimetabolite activity of a series of 5-arylazopyrimidines (I) has been observed in these laboratories. This type of compound, which is synthesised by the coupling of a diazotised arylamine with a 5-unsubstituted pyrimidine, has been described previously[1–4] but, although slight inhibition in certain bacterial systems was observed[2], no specific antimetabolite activity was reported. Compounds of structure I are converted on oxidation to 8-aryl-8-azapurines (II)[2–4], which proved to be essentially devoid of antimetabolite activity in our studies. This investigation began with the synthesis of new derivatives of structures I and II



(I) (II)

	R′	R″	Ar
a,	NH_2	SH	phenyl
b,	NH_2	OH	phenyl
c,	NH_2	OH	2′,6′-xylyl

containing the 6-mercaptopyrimido-moiety, common to the purine antagonists 6-mercaptopurine[5] and thioguanine[6], as potential antimetabolites and antitumor agents. During the course of these studies we learned of similar work at the Chester Beatty Research Institute in London on the antimetabolite activity of compounds of types I and II[7–9].

Certain compounds of structure I have now been found to display inhibitory activity in a previously described *Streptococcus faecalis* *8043-pteroylglutamic acid (PGA) bioassay system[10] and in a "maximum synthesis" system utilising *Escherichia coli* *6522[11], which in the former system is relieved by excess PGA and slightly by guanine, and in the latter system by adenine or guanine, but poorly by PGA (Table I). Inhibition in the *Str. faecalis* PGA system, which appears to be competitive over a narrow range, about 10 fold, of concentration, is also reversed by dihydro-PGA, 10-formyl-PGA, natural citrovorum factor (CF), or thymine. These compounds also are active in a previously described *Leuconostoc citrovorum* *8081-CF[12] bioassay system. The response of a guanineless mutant of *Aerobacter aerogenes* to guanine[13] is inhibited effectively by certain of the arylazopyrimidines.

Preliminary structure-activity correlation indicates that at least one

* American Type Culture Collection numbers.

TABLE I

50 PER CENT MINIMAL INHIBITING CONCENTRATIONS, μG./ML.

	Streptococcus faecalis					*Escherichia coli*		
Cpd.	Excess PGA 100 μg./ml.	PGA μg./ml. 0·1	0·01	0·001	Excess guanine, 100 μg./ml.	Medium alone	Excess PGA 100 μg./ml.	Excess purine* 50 μg./ml.
Ia	Complete reversal	34·0	2·8	2·8	2·2	9	20	70
Ib	,,	95·0	2·0	0·21	0·27	9	20	Complete reversal
Ic	,,	2·7	2·8	0·25	3·5	Inactive	Inactive	Inactive

* Adenine or guanine.

amino group *ortho* to the azo linkage is necessary for optimal activity of structure I. The aryl group (Ar) should be unsubstituted or contain electropositive substituents for maximum microbiological activity. Structure II, with few exceptions, has shown little activity.

Structure I is unusual in that it exhibits both anti-PGA and anti-purine activity. It is of interest that I exhibits this bivalent activity rather than II, which possesses the 8-azapurine nucleus of known antimetabolite properties[14,15]. Reduction of the azo linkage of I to the corresponding 5-aminopyrimidine results in a great decrease in microbiological activity.

Structural relationship of I to other similar antimetabolites[16–18] is evident. Further synthetic variations of both I and II are being made and studied, and further studies on the mechanism of action of these compounds and their activity in other biological systems are in progress[19].

SUMMARY

1. The discovery of both antipurine and antifolic activity is reported in certain 4-amino-5-arylazopyrimidines.

2. Details of the microbiological assays are indicated. Work on the 5-arylazopyrimidines and the chemically related 8-aryl-8-azapurines is being developed.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Alkaloids and Other Basic Drugs, Identification of, by Paper Partition Chromatography. L. R. Goldbaum and L. Kazyak. (*Analyt. Chem.*, 1956, **28**, 1289). A method is presented for the presumptive identification of μg. amounts of alkaloids and other basic drugs by means of the pattern of their R_F values or (better) the ratios of their movement in relation to a known substance (codeine being chosen) at 4 different pH values. Four chromatograms at each pH value are run simultaneously and an assumption as to identity is made by referring to tables recording the behaviour of known compounds (the data for 44 compounds, which are commonly encountered by toxicologists and pharmacologists are included). Confirmation can then be carried out by specific chemical tests if necessary after recovery of the compounds from the paper. D. B. C.

Benzalkonium Chloride, Colorimetric Determination of. K. Yoshimura and M. Morita. (*Pharm. Bull., Japan*, 1955, **3**, 432.) In this assay the benzalkonium chloride (100 to 300 μg./ml.) is precipitated with phosphomolybdic acid in strongly acid solution, the precipitate dissolved in warm acetone and the blue colour produced after the addition of stannous chloride is measured colorimetrically after 80 minutes at 25°. The time and temperature for maximum development are critical. The maximum extinction is at 730 mμ. A blank solution of the reagents is employed. The precipitation reaction is not specific for benzalkonium chloride but the advantage is that it can be determined in small amounts without any special reagent with an error less than 2 per cent. D. B. C.

Cinchona and Nux Vomica, New Assay of. E. Brochmann-Hanssen. (*J. Amer. pharm. Ass., Sci., Ed.*, 1956, **45**, 74.) The following assay for cinchona and nux vomica depends on extraction of the alkaloids with a cation exchange resin and purification with an anion exchange resin. 0·1 g. of finely powdered cinchona or nux vomica was placed in an extraction tube with 1 ml. of 2·5 N formic acid, 1 g. of activated cation exchange resin (Dowex 50-X2) of high porosity and 25 ml. of boiling water. After shaking for 30 minutes (15 for nux vomica), maintaining the temperature at 80–90°, the extracted drug was removed by back-washing. The resin was washed and the alkaloids eluted with 4 N ammonium hydroxide in methanol (70 per cent). The eluate was purified by passing it through a highly porous strongly basic anion exchange resin (Dowex 1-X1), and the alkaloids determined by ultra-violet spectrophotometry, using as blank, methanolic ammonium hydroxide which had been passed through Dowex 1-X1 resin. The method of extraction of cinchona described above was found to be more efficient than the usual extraction with solvents, giving rise to assay results 10 to 15 per cent higher. The results for nux vomica were also higher than by the official method, in which a loss of strychnine occurs during the oxidation of brucine with nitric acid and sodium nitrite. G. B.

CHEMISTRY—ANALYTICAL

New Indicator for the Titration of Calcium with EDTA. J. Patton and W. Reeder. (*Analyt. Chem.*, 1956, **28**, 1026.) A new indicator for calcium, 2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)-3-naphthoic acid, enables it to be accurately estimated in the presence of magnesium using standard disodium edetate. The colour change is from wine red to pure blue. Titration procedures are given for the estimation of calcium and magnesium in water, limestone, salt and boiler scale. The usual ammonia-ammonium chloride buffer is replaced by an odourless monoethanolamine-hydrochloric acid buffer containing complexed magnesium to render the end point sharper. The total calcium and magnesium is estimated by using eriochrome black T as indicator in the presence of the above buffer. Calcium is then estimated using the new indicator in a solution containing 4 ml. of 8 N potassium hydroxide in about 50 ml. In both cases about 30 mg. of each of potassium cyanide and hydroxylamine hydrochloride are added to prevent interference from zinc, copper, cobalt, nickel and manganese. Blank determinations are performed at every stage. The amount of magnesium is calculated from the difference between the two titrations. Interfering ions are discussed. In trial analyses, the maximum variation encountered was 2 p.p.m. as calcium carbonate and 1 p.p.m. as magnesium carbonate, and the results agreed with those obtained by other methods. D. B. C.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

***Digitalis purpurea*, Transformation of Unknown Glycosides into the Known Cardiac Glycosides.** K. B. Jensen. (*Acta pharm. tox., Kbh.*, 1956, **12**, 20.) The glycosides, A_1, A_2, A_3, A_4, A_5, B_2, B_3, B_4, B_5 and B_9 described in the preceding paper (*Acta pharm. tox., Kbh.*, 1956, **12**, 20), were isolated by paper chromatography as previously described. Appropriate cut-out filter strips were treated in M/1 phosphate buffer (pH 5·9) with the enzyme digipurpidase when only three of the glycosides, A, B_3 and A_2, were shown to be hydrolysable. With sodium bicarbonate, B_1 gave digitalinum verum, B_3 gave purpurea glycoside B, B_5 gave strospeside, and B_9 gave gitoxin. Acid hydrolysis was more complex but in every case gave either gitoxigenin or digitoxigenin. J. B. S.

ORGANIC CHEMISTRY

Steroids, Potent Oral Anabolic-Androgenic, Synthesis of. M. E. Herr, J. A. Hogg and R. H. Levin. (*J. Amer. chem. Soc.*, 1956, **78**, 500.) The preparation of three 11-oxygenated C-(19) steroids, 11β: 17β-dihydroxy-17-methyl-4-androsten-3-one 11β: 17β-dihydroxy-9α-fluoro-17-methyl-4-androsten-3-one and 17β-hydroxy-9α-fluoro-17-methyl-4-androstene-3: 11-dione is briefly described. Their oral anabolic activities in terms of 17-methyltestosterone activity (1·0) are 2·9, 20·0 and 22·0 respectively and their androgenic activities compared with the same standard are 0·9, 9·5 and 8·5. A. H. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

D-Glucosamine, Anaerobic Deamination of, by Bacterial and Brain Extracts. P. Faulkner and J. Quastel. (*Nature, Lond.*, 1956, **177**, 1216.) Experiments have been carried out to investigate the deamination of D-glucosamine. Suitably prepared extracts of *E. coli* and of brain tissue were used. Incubations were carried out in the Warburg manometric apparatus. Estimations of D-glucosamine, ammonia, hexosediphosphate, phosphates and sugar phosphates were

made. Deamination of D-glucosamine and *N*-acetylglucosamine takes place anaerobically in an extract of *E. coli*, the process being dependent on the presence of adenosine triphosphate and magnesium ions. Little or no breakdown of D-glucosamine takes place in the bacterial extract under aerobic conditions. Glucosamine is deaminated at a slower rate than *N*-acetylglucosamine, but when mixtures of the two are incubated with adenosine triphosphate a more than additive rate of ammonia production takes place. Thus it is likely that the substrate for D-glucosamine deamination is D-glucosamine-6-phosphate. A similar conclusion applies for *N*-acetylglucosamine. Dialysed *E. coli* extracts cause phosphorylation of glucose and D-glucosamine in the presence of adenosine triphosphate, the same hexokinase apparently being involved. The phosphorylation of *N*-acetylglucosamine requires a separate kinase. Glucosamine breakdown in an *E. coli* extract is inhibited by the presence of glucose. Adenosine triphosphate cannot be substituted by adenosine-5-phosphate for the anaerobic deamination of D-glucosamine in an *E. coli* extract. However, a mixture of acetyl phosphate and adenylic acid can replace the adenosine triphosphate. The D-glucosamine anaerobic deaminating system in *E. coli* is unaffected by the presence of either diphosphopyridine nucleotide or by L-glutamate or by pyridoxal phosphate. Electrophoresis on filter paper of a mixture of D-glucosamine, a trace of *N*-acetylglucosamine, magnesium ions, adenosine triphosphate and dialysed *E. coli* extract, after anaerobic deamination at 37° has taken place for one hour, shows the presence of D-glucosamine-6-phosphate and hexosediphosphate. It is possible that the hexosediphosphate formed is produced secondarily by phosphorylation of fructose-6-phosphate by excess adenosine triphosphate present. Thus the following reaction in an *E. coli* extract takes place:

$$\text{D-glucosamine} \xrightarrow{\text{ATP}} \text{D-glucosamine-6-phosphate} \rightarrow \text{fructose-6-phosphate} \xrightarrow[\text{ammonia}]{\text{ATP}} \text{hexosediphosphate}$$

Small quantities of *N*-acetylglucosamine stimulate the deamination of D-glucosamine. Such an effect cannot be produced by *N*-acetylmethionine, acetylcholine or *N*-acetylglycine. Thus it is suggested that the stimulatory or catalytic action of *N*-acetylglucosamine deamination may be due to an inhibitory effect on the hydrolysis of a phosphate ester formed during the course of the D-glucosamine breakdown. However the phosphate ester of *N*-acetylglucosamine might play a part in the catalytic reaction. D-Glucosamine is deaminated anaerobically by dialysed brain extract in the presence of adenosine triphosphate and magnesium ions. This process however, is not stimulated by the addition of *N*-acetylglucosamine. *N*-acetylglucosamine is not deaminated in brain extracts nor does it undergo phosphorylation in the presence of adenosine triphosphate. The presence of glucose inhibits anaerobic deamination of D-glucosamine in brain extracts, this probably being due to competition between glucose and glucosamine or between their respective esters. The evidence would indicate that D-glucosamine-6-phosphate is the substrate of the anaerobic deaminating enzyme in brain tissue as in *E. coli*, but that the factors in *E. coli*, operating with *N*-acetylglucosamine and which account for the effects of this substance in the bacterial deaminating system, are not present in the brain extract. M. M.

BIOCHEMICAL ANALYSIS

Iron in Serum, Improved Determination of. P. Trinder. (*J. clin. Path.*, 1956, **9**, 170.) Heat 2 ml. of serum, 2·5 ml. of water and 1·5 ml. of 20 per cent

trichloroacetic acid for 10 minutes at 90 to 95°. Cool and centrifuge. To 4 ml. of the supernatant add 0·2 ml. of iron reagent, 0·6 ml. of 40 per cent sodium acetate and 0·4 ml. of 1 : 1 sulphuric acid. Read the optical density of the unknown in an absorptiometer using an Ilford 624 green filter, or in a spectrophotometer at 535 mμ. The iron content is obtained from a standard calibration curve. A blank is prepared by heating 3 ml. of water and 1 ml. of 20 per cent trichloroacetic acid. For the preparation of the iron reagent, add 0·5 ml. chlorsulphonic acid to 100 mg. of 4 : 7 diphenyl 1 : 10 phenanthroline, and boil for 30 seconds. Cool, add 10 ml. of water and heat at 100° until the precipitate is dissolved. Make up to 100 ml. with water and add 1 ml. of thioglycollic acid. The recovery of added ferric iron is quantitative and a moderate haemolysis does not affect the result. G. F. S.

Pyruvate in Plasma, Determination of. K. S. Henley, H. S. Wiggins and H. M. Pollard. (*J. Lab. clin. Med.*, 1956, **47**, 978.) An enzymatic method is described for the determination of pyruvate in plasma using lactic acid dehydrogenase and reduced diphosphopyridine nucletide (DPNH). Heat 1 ml. of plasma from heparinised blood in a boiling water bath for 15 minutes. Add 6 ml. of M/15 phosphate buffer (pH 7·41, prepared by mixing 80·8 ml. of M/15 anhydrous dibasic sodium phosphate solution with 19·2 ml. of M/15 monobasic sodium phosphate solution). Homogenise the mixture in a Potter-Elvehjem homogeniser with a Teflon pestle. Centrifuge and mix 5 ml. of the supernatant with 1 ml. of DPNH solution. Divide the fluid approximately equally between two matched cuvettes. To the first add 0·01 ml. of lactic acid dehydrogenase solution (0·01 ml. of enzyme to 0·5 ml. of 0·225 per cent saline), stir and allow reaction to go to completion (2 to 3 minutes). This is the blank and using it read the optical density of the second cuvette at 340 mμ. This density is an index of the DPNH oxidised and is equivalent to the amount of pyruvate in the solution. If C is the difference in optical density between contents of the two cuvettes and the micromolecular extinction coefficient of DPNH is 2·05 per 3 ml. at 340 mμ then

Micromols. pyruvate/100 ml. $= 100\ C \times 7/1 \times 6/5 \times 1/3 \times 2{\cdot}05 = 137\ C$
or mg. pyruvate/100 ml. $= 88/1000 \times 137\ C = 12{\cdot}1\ C$

The method has a standard error of 3·6 per cent and a normal plasma pyruvate level is $0{\cdot}69 \pm 0{\cdot}25$ mg. per 100 ml. G. F. S.

Pyruvic Acid in Blood, Determination of. S. Segal, A. E. Blair and J. B. Wyngaarden. (*J. Lab. clin. Med.*, 1956, **48**, 137.) An enzymatic spectrophotometric method is described for the estimation of pyruvic acid in whole blood. It is based on the measurement of the oxidation of reduced diphosphopyridine nucleotide (DPNH) by pyruvic acid in the presence of lactic dehydrogenase. Pipette 5 ml. of blood into a rubber stoppered centrifuge tube containing 5 ml. of 7 per cent perchloric acid. Centrifuge twice and place 2 ml. of the clear supernatant into a small beaker in ice. Adjust the pH to 3 or 4 with 5 N potassium hydroxide (about 0·2 ml.). Remove the precipitate of potassium chlorate by centrifuging. Place 1 ml. of the supernatant into a quartz cuvette and add 1 ml. of buffer (phosphate buffer 0·1 M pH 7·0 to 7·4), 0·1 to 0·2 μM. diphosphopyridine nucleotide (about 0·05 to 0·1 ml. of solution prepared by hydrosulphite reduction of DPN) and sufficient water so that after addition of the enzyme the total volume is 3 ml. Read the optical density (OD) at 340 mμ in a spectrophotometer against a water blank. It is initially 0·300 to 0·600 OD. Add lactic dehydrogenase (usually 3 to 5 μg. enzyme protein) such that the reaction, as followed by OD change, is complete in from 4 to 7

minutes. The change usually consists of − 0·40 to − 0·085 OD unit. An enzyme blank is run concomitantly with the substitution of 1 ml. of water for the filtrate. The concentration of pyruvate is calculated from

$$\frac{[(\Delta OD_{340\ m\mu}) - (\Delta OD_{340\ m\mu}\ \text{blank})]}{* 6{\cdot}22 \times 10^6\ \text{cm.}^2\ \text{mole}^{-1}} \times \text{mol. wt. pyruvate} \times \text{dilution} \times 100 = \text{mg./100 ml. of blood}$$

where * is the molar extinction coefficient of DPNH.

Recoveries of pyruvate added to whole blood were 97 to 104 per cent of the theoretical. The blood pyruvate level in fasting normal subjects ranged from 0·39 to 0·86 mg. per cent. Wide daily variations occurred. Results are reported in diseases in which elevated pyruvate levels occur, rheumatoid arthritis, liver disease and diabetes etc. G. F. S.

CHEMOTHERAPY

Antibacterial Activity of Dried Australian Plants. N. Atkinson. (*Austral. J. exp. Biol. med. Sci.*, 1956, **34**, 17.) This is an account of tests to detect the presence of antibiotic substances in flowering plants. The general methods used previously had entailed an extraction with water or other solvent and testing of the extracts by the cylinder or cup plate methods against several micro-organisms. The author found that these methods lacked sensitivity as was proved by the results of a new test—called the "direct plate test"—which was applied to 124 specimens of dried flowering plants collected in Australia. The test was carried out by seeding a tryptic digest agar with the test organism and pouring into Petri dishes. A small piece of the required plant part was placed on the agar surface, testing 4 or 5 specimens on each plate. Many of the plants were also powdered and a small heap of the powder placed on the agar. Widths of zones of inhibition were measured after incubation for 18 hours. The 3 test organisms used were specified strains of *Salmonella typhi*, *Staphylococcus aureus* and *Mycobacterium phlei*. Of the 124 plants studied 75 showed some inhibition by at least 1 plant part against at least 1 organism. Many of the reactions were very weak (zone width 0·5–1 mm.), but 30 plants showed fair to good zones of inhibition, best among which were the following which were active against all 3 test organisms: nut kernels of *Cycas circinalis* and *Cycas media* (Cycadaceae), root of *Plumbago zeylanica* (Plumbaginaceae), fruit of *Rhodomyrtus macrocarpa* (Myrtaceae), leaves and branchlets of *Flindersia xanthoxyla* (Rutaceae), and bark of *Lunasia amara* (Rutaceae). The most active family was Rutaceae and the least active was Leguminosae. All specimens were dried and some were about 3 years old. It is suggested that the antibiotics of these plants must be relatively stable substances and should be worth further investigation. B. A. W.

Antibacterial Substances Produced by Flowering Plants. N. Atkinson and H. E. Brice. (*Austral. J. exp. Biol. med. Sci.*, 1956, **33**, 547.) The essential oils from thirty-four Australian plants have been tested for antibacterial activity against *S. typhi*, *Staph. aureus* and *M. phlei* by the Tween agar and Tween broth dilution tests. Only a few of the oils had even a fair activity against *Staph. aureus* and *S. typhi*, but all the oils showed activity against *M. phlei.*, ranging from weak to very good. Oils from numerous members of the Myrtaceae were included, and the best overall activity was shown by oils from *Backhousia augustifolia*, *Backhousia citriodora*, *Leptospermum citratum*, *Leptospermum liversidgei*, *Melaleuca bracteata* and *Dacrydium franklini*. It is considered that Australian essential oils are not strong antibacterial agents. G. F. S.

PHARMACY

Antibacterial Agents in *Pseudomonas aeruginosa* Contaminated Ophthalmic Solutions. S. Riegelman, D. G. Vaughan, Jr. and M. Okumoto. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 93.) The solutions examined were phenylmercuric nitrate up to 0·01 per cent, benzalkonium chloride up to 0·02 per cent, chlorbutol up to 0·8 per cent, phenylethyl alcohol 0·5 and 2 per cent, and polymixin B sulphate 50 to 1000 units/ml. *In vitro* tests were carried out using various dilutions of the antiseptics in conjunction with solutions contaminated with *Ps. aeruginosa*, suitable inactivating agents being used to counteract the effect of the antiseptic during incubation. In addition, *in vivo* tests were carried out by injecting the solutions intracorneally into rabbit eyes. In the intracorneal tests infections were produced after one week's contact with concentrations of benzalkonium chloride and phenylmercuric nitrate shown to be bacteriostatic in the *in vitro* tests. It is concluded that "bacteriostatic" concentrations are not adequate for use in ophthalmic solutions. The best preservative for these preparations appeared to be polymixin B together with a bactericide effective against Gram-positive organisms. G. B.

Emulsions. The Effect of Two-stage High Pressure Homogenisation on the Stability of. J. D. Mullins and C. H. Becker. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 105.) Emulsions were prepared with 25 per cent of arachis oil, cod-liver oil, glyceryl ricinoleate or glyceryl trioleate, using acacia 2·5 per cent or a mixture of polysorbate 80 and sorbitan sesquioleate (Aracel C) as the emulsifying agent, and passing the product twice through a two-stage homogeniser with the first valve at 1500, 2000, 2500 or 3000 lb. wt./sq. in., and the second at 1000 lb. wt./sq. in. Samples of the emulsions were diluted with propylene glycol, placed in a haemocytometer cell and examined microscopically, using a globule size-frequency method of analysis. The specific interfacial area was found to increase directly with the homogenisation pressure, and during storage the emulsions prepared with non-ionic emulsifying agents showed a linear decrease in specific interfacial area. Emulsions prepared with acacia were characterised by a lower specific interfacial area and a greater proportion of relatively large oil globules. Deterioration in acacia emulsions was usually due to coalescence of globules to form larger ones, whereas in the polysorbate 80-sorbitan sesquioleate emulsions, deterioration took the form of separation of free oil rather than coalescence of globules. The viscosity of the emulsions was not affected by changes in the specific interfacial area or by the oil used, although it varied with different emulsifying agents. G. B.

Vitamin B_{12} in Solutions of Ascorbic Acid, Copper-promoted Decomposition of. A. J. Rosenberg. (*J. biol. Chem.*, 1956, **219**, 951.) The decomposition of vitamin B_{12} in solutions containing ascorbic or dehydroascorbic acid is considerably accelerated in the presence of cupric ions, although cupric ions are without effect on vitamin B_{12} alone. One cupric ion appears to be inactivated for each molecule of vitamin B_{12} destroyed. A study of the kinetics of the reaction indicates that the rate-determining step is a unimolecular transformation. It is suggested that a copper ion combines with 1 molecule of ascorbic acid to produce an intermediate product (X) which is then transformed into another product (Y) together with a cupric ion in an inactivated form. The product (Y) reacts with vitamin B_{12} yielding cobalamin-like intermediates which then undergo further decomposition. G. B.

PHARMACOLOGY AND THERAPEUTICS

Anticholinergic Drug (BL700B), Pharmacological Evaluation of. R. D. Judge, R. J. Bolt, B. L. Hirschowitz and H. M. Pollard. (*J. Lab. clin. Med.*, 1956, **47**, 950.) A new anticholinergic drug, αα-diphenyl-γ-dimethylaminobutyramide-ethobromide has been evaluated in humans. The compound was compared orally with atropine in males, all of whom were capable of a continuous secretion of acid gastric juice. Studies were made of the gastric juice obtained by stomach tube and gastric motility recorded from a balloon. Side effects were determined in 5 normal young men placed on a 12·5 mg. dose four times daily. Dosage response data indicated that 10 to 25 mg. orally reduced gastric acidity and secretory volume without significant side effects and 12·5 mg. orally was superior to 0·6 mg. of atropine sulphate orally. No tolerance was observed with 12·5 mg. four times daily for 7 days. G. F. S.

Ganglion-blocking Agents in Hypertension, Two New. S. Locket. (*Brit. med. J.*, 1956, **2**, 116.) This paper is a report on the use over a period of a year and a half of two new ganglion-blocking agents (356c54 and 139c55) in the treatment of 15 severe cases of hypertension. Chemically, 356c54 is *N'N'N"*-trimethyl-*N'*-(6-cyano-6:6-diphenylhexyl)ethylene-1-ammonium-2-morpholinium dichloride, and 139c55 (also called Presidal) is the 5-cyano-5:5-diphenylpentyl homologue. The compounds were given subcutaneously, using a solution containing 20 mg. of the dichloride of either drug per ml. With 356c54 the minimal effective therapeutic dose was 10 mg. and the maximum dose 40 mg.; with 139c55 the minimal dose was 5 mg. and the maximum 27·5 mg. With identical hypotension-producing doses the duration of hypotension, using 139c55, often exceeded 12 hours, and on occasion 24 hours, and was rarely less than 9 hours, while with 356c54 it was rarely less than 7 hours and might exceed 24 hours, though this latter duration occurred much less frequently than with 139c55. The maximum fall in blood pressure was reached 60 to 120 minutes after the onset and persisted for some time before a slow return was made to pre-treatment height. The drugs may also be administered intravenously or by mouth, but in the former case the duration of hypotensive action is much shorter than by subcutaneous injection, and with oral administration, even with doses 15 to 20 times the effective subcutaneous dose, the hypotensive response could not be accurately predicted. No hypertensive symptoms were made worse by the treatment, and where a change occurred it was for the better. Attacks of left ventricular failure stopped when treatment began, breathlessness was relieved and angina of effort ceased to occur. When electrocardiographic changes occurred they were towards normal, and heart size was reduced. In 7 patients the papilloedema which was present subsided. Headaches which were hypertensive in origin were immediately ameliorated, but recurred with inadequate treatment. Side-effects observed included a transient blurring of vision, within a few minutes of injection, dryness of the mouth (more frequent and severe with 356c54 than with 139c55), and mild constipation. Large doses of both drugs could produce postural syncope, and bradycardia was present in most cases. Nausea and vomiting did not occur, and the absence of marked effect upon the small-intestine propulsion was one of the most interesting and useful features of the drugs. The author concludes that the advantages of the compounds, particularly 139c55, in the treatment of hypertension, lie in the more gradual onset of activity, the greater duration of action, the slowing effect on the heart rate, and the relative lack of action on the small intestine. S. L. W.

Spiramycin, Acute Toxicity and Activity of. C. Cosar. (*Thérapie*, 1956, **11**, 324.) Spiramycin, an antibiotic obtained from cultures of *Streptomyces ambofaciens* is not very toxic to mice. By subcutaneous injection the LD50 is about 1·7 g./kg.; 5 g./kg. may be given by mouth. It is very active against peritoneal infections by pneumococci, streptococci and staphylococci, and is effective against strains of *Staphylococcus* which are resistant to other antibiotics such as penicillin and aureomycin. It is more effective against pneumococci than erythromycin or carbomycin. It is not active against *Trypanosoma congolense* and *T. brucei* but shows some activity against *Plasmodium berghei*.

G. B.

Spiramycin, Chronic Toxicity and Effects of Local Administration of. P. Dubost, R. Ducrot and M. Kolsky. (*Thérapie*, 1956, **11**, 329.) The administration of spiramycin to rats and dogs over a period of 4 weeks did not cause any serious untoward effects. Experiments were carried out in which a 10 per cent solution was instilled into the eyes of rabbits, a 2 per cent solution was injected subcutaneously into rats and a 1 per cent solution was injected intramuscularly into rabbits. In all these forms of local administration the drug was well tolerated. G. B.

Tremorine, Tremor produced by. G. M. Everett. (*Nature, Lond.*, 1956, **177**, 1238.) 1:4-Dipyrrolidino-2-butyne (Tremorine) in doses of 5–20 mg./kg. produces tremor, salivation, meiosis, slight muscular weakness and rigidity lasting several hours. The effects are similar in rats, mice, guinea-pigs, cats, dogs and monkeys. In monkeys the picture is very similar to Parkinsonism. The antagonism of these effects is brought about with various drugs, such as atropine and scopolamine, that are used for the treatment of Parkinsonism. In contrast to this, hypnotics, anticonvulsants and ganglionic blocking drugs are ineffective in doses below those causing marked depression. Banthine was found to control the parasympathetic effects of salivation and diarrhoea but had no effect on the tremor; thus demonstrating the distinct peripheral and central actions. Decerebrate animals develop tremor after the drug. It also causes a marked fall in body temperature. The chemical structure producing tremor is very specific within this chemical type. Twenty such analogues show no such action. Thus Tremorine may be a useful tool in the investigation of tremor and the search for more effective agents against Parkinsonism. M. M.

Triiodothyroacetic Acid (Triac): Effect on Blood-Cholesterol Levels. W. R. Trotter. (*Lancet*, 1956, **270**, 885.) Triiodothyroacetic acid (triac) was given to 3 patients with myxoedema and to 18 euthyroid patients. Its relative effects in lowering the blood-cholesterol level and in raising the B.M.R. were compared with those of thyroxine in 1 case of myxoedema; triac appeared to have a greater effect than thyroxine on the blood-cholesterol level. In a similar comparison with triiodothyronine in 2 other cases of myxoedema, the action of the two drugs appeared to be similar but triiodothyronine was about 75 times more effective than triac both in depression of the blood-cholesterol level and elevation of B.M.R. In euthyroid patients triac in a dose of 2–4 mg. daily lowered the blood-chloesterol level by about a quarter; triiodothyronine 0·08 mg. daily had a similar effect. In 3 patients whose dose of triac was increased gradually up to 3 or 4 mg. daily there was no obvious rise in the B.M.R. though the plasma-cholesterol level appeared to fall. Triac reduced the uptake of radioactive iodine in euthyroid patients, the dose required being about 25 times

greater than the triiodothyronine. It cannot be claimed at present that triac has any properties not shared by the other thyroid hormones or that it is likely to be particularly useful in lowering the blood-cholesterol level of euthyroid patients. S. L. W.

Viadril: A New Steroid Anaesthetic. L. H. Lerman. (*Brit. med. J.*, 1956, **2**, 129.) Viadril is a steroid, 21-hydroxypregnane-3 : 20-dione sodium succinate. It is a non-volatile crystalline solid, freely soluble in water, the solution having a pH of 7·8 to 10·2. Its value as an anaesthetic is that it telescopes the narcotic and relaxant functions and requires only minimal nitrous oxide and oxygen to maintain anaesthesia. The drug is given as a 2·5 per cent solution in normal saline and is injected into the giving-set tubing of a normal-saline drip, just below the inspection chamber. The intravenous injection of Viadril causes loss of consciousness, analgesia, absent corneal reflex, and muscular relaxation, particularly of the larynx. It has no demonstrable hormone effects. No electrocardiographic changes have been observed, no haemolysis, and no depression of respiration. Hypotension commonly occurs but blood pressure is soon restored by small doses of pressor drugs. Nitrous oxide has a marked synergism with Viadril. If necessary, relaxant drugs, pethidine, and the volatile anaesthetic agents can be given at the same time as Viadril, but much smaller doses are needed. Viadril induction is quite different from thiopentone induction. There is no yawning or sighing, no apprehension, and no unpleasant smell or taste. The patient falls asleep quite naturally and there is no stertor. He remains in an apparently normal sleep on return to the ward and can be roused in a short period. At the end of an hour or so he is fully awake, says he feels well and is in no pain, euphoria being a distinct feature of this agent. Vomiting is rare. The disadvantages of Viadril are the slowness of induction and the risk of thrombophlebitis in the injected arm. In the series of 19 cases presented in this paper the average time of operation was 26 minutes and the average dose of Viadril was 824 mg. All except 3 were premedicated with Omnopon and scopolamine. None received pethidine during the operation. Details of the 19 cases are tabulated and 3 case histories given to emphasize special points. S. L. W.

Viadril: An Intravenous Steroid Anaesthetic. A. G. Galley and M. Rooms. (*Lancet*, 1956, **270**, 990.) Viadril is a derivative of pregnanedione (21-hydroxypregnane-3 : 20-dione, sodium hemisuccinate) which has anaesthetic properties and is sufficiently soluble in water to be given intravenously. Animal experiments indicate that it has a much higher therapeutic index than thiopentone, that it is not carcinogenic and that it shows no signs of hormonal activity, such as salt retention. In contrast to all the ultra-short acting barbiturates, Viadril rapidly suppresses the pharyngeal and laryngeal reflexes, so that even under the lightest narcosis it is difficult to provoke vomiting or laryngospasm. Falls of blood pressure and a rise in pulse-rate are more frequent with Viadril than with thiobarbiturate anaesthesia, but the fall in blood pressure often rectifies itself or can readily be remedied by methylamphetamine. Vomiting is rare after Viadril anaesthesia, and post-operative fatigue is slight or absent, patients often experiencing a sense of well-being during recovery from operation. In concentrations greater than 0·5 per cent Viadril solutions give rise to venous thrombosis and it must therefore be given as an intravenous drip; this may limit the use of the drug for shorter operations, but for long operations where

the intravenous drip is inevitable it may well prove the anaesthetic of choice. A suitable solution may be readily prepared by dissolving the contents of five 500-mg. vials of Viadril in a 540-ml. bottle of intravenous saline; however rapidly it is run in, this solution is trouble-free and causes no venous sclerosis. With a drip-rate of 150 per minute the average patient becomes drowsy within 5 to 7 minutes and in 15 minutes he is deeply asleep, though he reacts to painful stimuli. The usual induction period of 20–25 minutes can be halved as follows: directly the patient falls asleep a mask is held within an inch or so of the face and pure nitrous oxide administered. The mask is gradually lowered until it touches the face, when 20 per cent oxygen is added to the mixture. After 2 minutes 30–50 mg. of pethidine is injected intravenously, followed by a suitable dose of a muscle relaxant. After another minute the mask can be removed, laryngoscopy performed, and an endotracheal tube passed; anaesthesia is maintained with nitrous oxide and oxygen. Doses of Viadril up to 2 g. do not appear to affect either the depth or rate of respiration. From experience gained in the use of Viadril in 100 operations the authors conclude that whether used alone or in place of thiopentone in the hypnotic-analgesic-muscle relaxant sequence, it offers not only ideal operating conditions but ensures a more comfortable recovery period than any other anaesthetic or anaesthetic combination in common use.

S. L. W.

BACTERIOLOGY

***Clostridium perfringens* Type A Toxin, Protection Against, by a Metal-chelating Compound.** M. Moskowitz, M. W. Deverell and R. McKinney. (*Science*, 1956, **123**, 1077.) The α-toxin of *Clostridium perfringens* (*C. welchii*) type A is a lecithinase enzyme known to be an important lethal factor in infections of this organism. The enzyme is activated by calcium and the authors sought to demonstrate whether a lethal outcome of experimental infections could be reduced by removal of Ca^{++} from the area of infection. Intracutaneous injection of lethal doses of organisms mixed with sodium oxalate or citrate into mice gave no decrease in mortality. The use of the metal-chelating agent ethylenediamine tetra-acetic acid (EDTA) as a calcium removing agent gave protection in some but not all cases. Further tests were made in which toxic culture filtrates were used in place of the organisms, when it was found that whereas 2 MLD doses of the toxin killed mice in 10 hours, a mixture of the same dose of toxin with 0·68 per cent solution of EDTA in borate buffer gave complete protection. This protection could not be reversed by addition of excess Ca^{++}, but when Zn^{++}, Co^{++}, or Mn^{++} (which also activate lecithinase) were added to the toxin-EDTA mixture, the toxicity was restored. Tests on the *in vitro* inhibition of lecithinase using the lecithovitellin test gave results similar to those obtained *in vivo*: EDTA inhibited the lecithovitellin reaction; excess of either Zn^{++}, Co^{++} or Mn^{++} reversed the inhibition but excess of Ca^{++} did not. It is suggested that Ca^{++} activates but is not essential for the activity of lecithinase, whereas Zn^{++}, Co^{++} and Mn^{++} are essential for activity, it appearing that these ions are preferentially chelated over Ca^{++}. Further experiments in which toxin and EDTA were separately injected on opposite sides of mice revealed that greater protection was afforded when toxin and EDTA were simultaneously injected, indicating that inhibition of the toxin at the site of injection is involved in the protection. The authors report that EDTA is non-toxic except in high doses which induce hypocalcaemia; but Ca-EDTA can be given in large quantities and has possible uses in gas gangrene therapy. B. A. W.

Dihydrostreptomycin and Anaerobiosis—Comparison with other Antibiotics and its Selectivity with regard to Obligate Anaerobes. G. M. Williamson and F. White. (*J. gen. Microbiol.*, 1956, **14**, 637.) The authors report on a comparison of the sensitivity to various antibiotics of a number of facultative anaerobic bacteria whilst growing under aerobic and anaerobic conditions. The organisms used were *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* type B, *Escherichia coli*, *Aerobacter aerogenes*, *Streptococcus pyogenes*, and *S. pneumoniae*. The antibiotics tested were dihydrostreptomycin, penicillin, chloramphenicol, erythromycin, carbomycin, chlortetracycline, oxytetracycline and tetracycline. The tests employed heated blood (10 per cent) agar (pH 7·8). Ditch plates were prepared, the ditches containing either 20 units per ml. penicillin or 20 μg. per ml. of one of the other antibiotics, and the inoculum consisted of one standard loopful of a suitable dilution of a fluid culture of the test organisms. Plates for anaerobic tests were incubated in a McIntosh and Fildes jar for 48 hours. Inhibition of growth was measured as linear distance of complete inhibition of growth extending from the edge of the ditch. It was found that dihydrostreptomycin was unique among the antibiotics tested in giving a greater inhibition of facultative anaerobes when growing aerobically than when growing anaerobically. Dihydrostreptomycin was found to be relatively inactive against *Clostridium welchii* and *C. novyi*, the inhibitory concentration being dependent on the inoculum size. Investigations into the possible use of dihydrostreptomycin as a selective agent for isolation of *C. welchii* revealed that use of the antibiotic (dissolved in cooked meat medium) was less efficient than was heating at 65° for 30 minutes. B. A. W.

Ethylene Oxide, Sporicidal Activity of. J. L. Friedl, L. F. Ortenzio and L. S. Stuart. (*J. Assoc. off. agric. Chem., Wash.*, 1956, **39**, 480.) A study of the sporicidal activity of ethylene oxide when tested against 5 aerobic and 5 anaerobic spore-forming bacterial species is described. The tests were carried out in accordance with the sporicide method of the Association of Official Agricultural Chemists, in which the test organisms are dried on surgical suture loop carriers at room temperature for 24 hours over $CaCl_2$. The contaminated carriers were placed in individual petri dishes with the lids suspended above the bases of the dishes in order to obtain contact between spores and gas. The dishes were stacked in a jar which could be evacuated and then filled with ethylene oxide. Exposure periods of 10, 20, 30 and 60 minutes and of 3, 6 and 18 hours were used. Subcultures were incubated for 7 days at either 37° or 55° depending on the organism. Simultaneous exposures were made on wet drained carriers and on dried carriers in the case of *Cl. sporogenes* and *B. subtilis*, when it was found that dried spores were much more resistant to ethylene oxide than were wet spores. Dried spores of all 10 test organisms were destroyed after an exposure of 18 hours. *B. subtilis* and *Cl. sporogenes* were able to survive a 6 hour exposure; *Cl. tetani*, *Cl. botulinum*, *Cl. lentoputrescens*, *B. stearothermophilus* and *B. coagulans* survived for 60 minutes; *B. anthracis* and *B. globigii* survived exposures of 30 minutes but were killed by an exposure of 60 minutes. Resistances of all the test organisms to constant boiling HCl at 20° were also tested and no direct relation found between these resistances and the resistances to ethylene oxide. The authors conclude that ethylene oxide can be used as a sterilising agent but that prolonged exposures are necessary to ensure destruction of spores of all commonly encountered bacteria. B. A. W.