REVIEW ARTICLE

PHARMACOLOGY OF LYSERGIC ACID DIETHYLAMIDE AND SOME OF ITS RELATED COMPOUNDS

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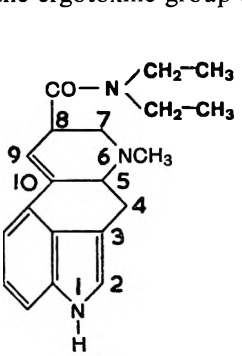
THE mental disturbances and autonomic effects induced by lysergic acid diethylamide (LSD, I) in man have initiated increased interest among workers in neurophysiology, neuropharmacology, biochemistry and psychiatry in the old problem of the mode of action of psychotomimetic agents. Since the first paper appeared[1] in 1947 the literature has become so extensive that it seems advisable to review the present state of LSD studies and to assess the contribution of the drug to the recent advances made in the study of psychogenic agents. The scope of this review is limited to the main experimental results of the LSD problem.

(+)-Lysergic acid diethylamide (LSD) is one of the many partially synthetic derivatives of (+)-lysergic acid[2,3]. This acid is the link between natural, dihydrogenated and partially synthetic alkaloids of ergot. The natural and dihydrogenated alkaloids of ergot possess central actions which are non toxic. In 1923 the inhibition of the depressor reflexes was described[4]; in 1934 we showed that ergotamine potentiates the action of phenobarbitone without itself possessing a hypnotic effect[5]; and in 1945 we demonstrated the central actions of the hydrogenated alkaloids of the ergotoxine group[6].



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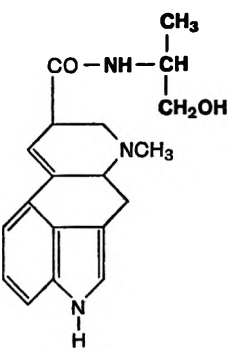

(II)

LSD is closely related to ergometrine (ergonovine, II) and was investigated pharmacologically in 1938. It proved to have a pronounced action upon the uterus of the rabbit both *in vitro* and *in vivo*, but its activity was 1·5 times weaker *in vivo* than ergometrine and its toxicity was higher. After intravenous injection of 0·1–0·2 mg./kg. in the anaesthetised rabbit we observed an unusual excitation combined with muscular cramps. Subcutaneous injection of 0·2 mg./kg. of LSD in the cat and dog induced motor effects like ataxia and spastic paresis and a change of the behaviour in a catatonic pattern. From these results it was not to be expected that

LSD would offer any clinical advantages, and therefore no further importance was attached to it.

As the drug was not considered for clinical trial, its psychogenic action in man went undetected until 1943, when Dr. Hoffman[7], the chemist engaged in its preparation, inadvertently ingested a minute quantity. The effect produced led him to repeat the experiment by taking orally 250 μg. which reproduced the same effects as before. This mental action of LSD was confirmed on myself and my staff and colleagues. We found the effective oral dose in normal subjects to be as small as 0·5 to 1·0 μg./kg. This unexpectedly potent activity in man is characteristic of LSD, since 3000–5000 times as much mescaline, a similarly acting substance, is required to produce similar psychic changes.

The first systematic psychiatric analysis of LSD was carried out[1] in our laboratory and in the Department of Psychiatry of the University Hospital in Zurich. This basic study of LSD on normal and schizophrenic subjects evoked great interest among neuropharmacologists, neurophysiologists and psychiatrists. Stoll's findings were subsequently confirmed and enlarged by numerous workers. I do not propose to discuss the specific psychiatric aspects of the LSD problem, but only the neurophysiology and pharmacology of this fascinating compound. However, the key of understanding of "psychosis" might lie, to quote Cholden[8], "in the reproduction of an experimental, predicable, controllable, reproducible state—an artificial psychosis". The interest of LSD lies primarily in the way it produces its psychic effects. We still do not know the drug's mechanism of action, but attempts at its elucidation have helped towards a fuller understanding of the normal and pathological functioning of the brain. Co-ordinated biochemical, neurophysiological and pharmacological investigations using psychological and psychiatric concepts are the only effective means of exploiting the potentialities of LSD and similarly active agents.

General Pharmacology

Toxicology

The intravenous acute LD50 of LSD varies according to species: 46 mg./kg. for the mouse, 16·5 mg./kg. for the rat and 0·3 mg./kg. for the rabbit. The corresponding relative toxicity[9,10] is: mouse 1, rat 2·8 and rabbit 150. None of the natural or hydrogenated ergot alkaloids exhibits so high an activity in acute toxicity experiments. Acute LSD poisoning is not specific; both autonomic and somatic symptoms are observed. These are mydriasis, piloerection, salivation, vomiting, increased reflex activity, ataxia and spastic paresis; death results from respiratory failure.

In chronic toxicity experiments, rats tolerated 2·5 mg./kg. intravenously daily for 30 days. The animals show increased reflex response, mydriasis, piloerection and slowing of growth. Since the maximum tolerated single intravenous dose in the rat is about 3·2 mg./kg., there seems to be no cumulative effect. Also animals pretreated chronically with LSD require the same LD100 as untreated animals; therefore no tolerance appears to develop.

Absorption, Distribution and Excretion

The tartrate of LSD is readily soluble in water and well absorbed when given orally. The fate of LSD in the body has been investigated in two ways. The first, a method in which the highly sensitive antagonistic action of LSD to 5-hydroxytryptamine (5-HT)[11] is used to determine LSD in the blood and tissue extracts[12], and the second, a method which measures the radioactivity in the same substrates after giving LSD-^{14}C [13–15].

LSD is neither bound nor destroyed in the blood of the rat. In homogenates of liver and muscle its activity decreases by nearly 50 per cent in a few minutes with little further reduction during the next 17 hours[12]. In brain homogenates the activity of LSD was reduced to 42 per cent after 10 minutes and to 20 per cent after 17 hours. These investigations do not reveal whether the loss of activity is due to the binding of LSD by a tissue constituent or to biochemical transformation into an inactive metabolite. That the decrease of activity at both 3° and 38° is similar does not support the idea of a metabolic transformation in the homogenates.

The distribution of LSD in the blood and in different organs has been investigated by the first method in mice[12] after intravenous injection and by the second method in mice and rats[13–15] after intravenous or intraperitoneal injection. The results of the two methods are in good agreement. The 50 per cent disappearance time from blood was 7 to 10 minutes as measured by the radioactivity method, but 35 minutes when assayed by the 5-HT antagonism method. After two hours, only traces could be found in the blood and organs. The maximum level of LSD in most organs was reached after 10 to 15 minutes, but in the liver after 30 minutes. The amounts in the different organs in decreasing order was: gut (inclusive of contents), liver, kidney, adrenals, lung, spleen, pancreas, large intestine, heart, muscle, skin, brain. The concentration in the brain was always lower than that in the blood. After 12 hours, 70 per cent of the radioactivity was found by Boyd and colleagues[13,14] to be in the intestinal contents. Only 7 to 8 per cent of the total radioactivity was excreted within 12 hours, half of this in the expired air and half in urine and faeces. In experiments with a bile fistula[15] 70 per cent of the total radioactivity was found in the bile. Thus we can say that LSD passes quickly from the blood, that it rapidly reaches a maximum in the organs and brain, that it is found in all organs, and is excreted quickly in the bile. Much of the LSD probably undergoes chemical change because the excreted compounds or metabolites are water-soluble in contrast to LSD. Paper chromatography[15] has revealed that the bile contains three different radioactive compounds, which have not yet been defined chemically or biologically because no reference substances are available.

Recently, Axelrod and colleagues[16] investigated the fate of LSD in the cat by spectrofluorophotometer, an instrument sensitive to as little as 0·001 μg./ml. of LSD. These authors found that 90 minutes after intravenous injection of 0·2 mg./kg., LSD was present, decreasing in amount in the following order of fluids and organs: bile, plasma, lung, liver, kidney, brain, intestine, spleen, cerebrospinal fluid, muscle and fat. The 50 per cent disappearance time in blood is 130 minutes for the cat

and 100 minutes for the monkey. These findings are in part agreement with our results. Axelrod and his colleagues confirm the overall distribution of LSD in the organism and also that its excretion is mainly through the bile. But they find that LSD is strongly bound (65 to 90 per cent) by a protein constituent of the blood of the cat, whereas Lanz and colleagues[12] could recover the total activity of LSD added to blood when assayed using 5-HT, showing that if LSD is bound in the rat's blood it still remains active. Stoll and colleagues[15] recovered from the bile of rats with a bile fistula 2 hours after the injection, 70 per cent of the total radioactivity, but found that the greater part of the LSD had undergone a biochemical transformation showing chromatographically three different metabolites, while the American authors report that the compound excreted into the bile is unaltered LSD. Axelrod and colleagues also show for the first time that LSD passes into the cerebrospinal fluid in measurable amounts. Unexpectedly, they find more LSD in the lungs than in the liver. They also report that guinea pig liver tissue is the only tissue that metabolises LSD *in vitro*. A careful study suggests that LSD is transformed *in vitro* into 2-oxyLSD by an "enzyme system, present in microsomes only, that requires oxygen and a reduced triphosphopyridine nucleotide generating system". The new compound does not possess the psychogenic action of LSD. So far two of the metabolites found in the bile by Stoll and colleagues do not correspond with 2-oxyLSD, but the question whether the third metabolite, which does not give the van Urk reaction, characteristic of lysergic acid and its derivatives, is identical with 2-oxyLSD, remains unanswered.

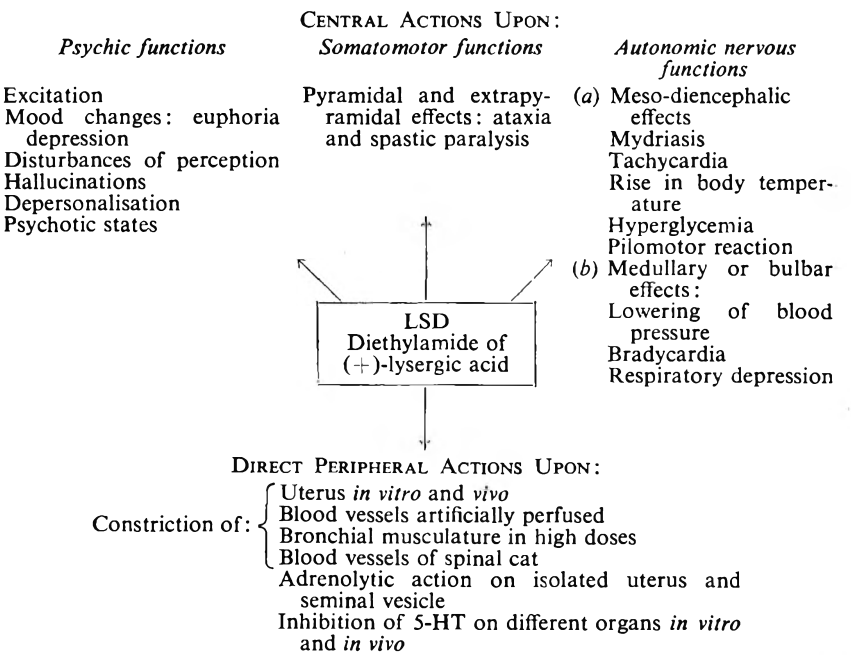

FIG. 1. PHARMACOLOGICAL PROPERTIES OF LSD (DIETHYLAMIDE OF (+)-LYSERGIC ACID

Pharmacological Data

In Figure 1 the complexity of the actions of LSD is shown (after Rothlin and Cerletti[10]). We distinguish two basically different kinds of action: the direct peripheral actions and those upon the central nervous system.

Direct peripheral actions. LSD has a contracting effect *in vitro* and *in vivo* on the uterus of the rabbit which is 1·5 times weaker than that of ergometrine. The drug causes vasoconstriction in the perfused blood vessels of the rat's kidney[9,10] and rabbit's ear[17], and also in the spinal cat[9,10]. In the intact animal the depressant action on the vasomotor centre predominates, and results in a fall in blood pressure. The adreno-sympatholytic action characteristic of ergotamine, ergotoxine and other natural and hydrogenated ergot alkaloids with the tripeptide group is present but relatively weak.

LSD selectively antagonises 5-HT, named enteramine by Erspamer[18] and serotonin by Page[19]. This antagonism was discovered by Gaddum[20] in the isolated uterus of the rat, and can also be demonstrated on the smooth muscle of other organs, for example the gut of the guinea pig[20], blood vessels[21,22], and also blood vessels[23] and bronchial musculature *in vivo*[24,25].

LSD elicits an expansion of the chromatophores of the female guppy (*Poecilia reticulatus*). Long pretreatment with 5-HT can inhibit this effect[26]. LSD produces a maximum increase in the amplitude of the heart of *Venus mercenaria* without altering its sensitivity to acetylcholine[27]. The drug also mimics the action of 5-HT on the heart of this mollusc (5-HT seems to be a normal transmitter in this organ) with the difference that the action of 5-HT can be easily washed out whereas 30 minutes' washing of the heart treated with LSD produces if anything a further increase in amplitude.

Effects on the central nervous system. The central effects elicited by LSD are more numerous and interesting than the direct peripheral actions. They can be subdivided in three groups: autonomic, somatomotor and psychic effects.

Autonomic effects. Some of these are sympathetic and others parasympathetic in nature. Mydriasis is induced in various species, mouse, rat, rabbit, cat[28], and also in man[29–31]. LSD increases the blood sugar, and the heat-regulating centre exhibits a very high sensitivity to the drug[28] which provokes increase in body temperature in the cat, dog and rabbit[32], while in the rat only very high doses have the same effect, and smaller doses decrease the temperature. Piloerection is induced in various animals. All these autonomic responses to LSD are sympathetic and are of central origin since pretreatment with either ganglion-blocking agents as, for example, hexamethonium, or sympathicolytics like hydergine (the hydrogenated ergot alkaloids of the ergotoxine group) inhibits them[28].

LSD also evokes parasympathetic activity producing salivation and lacrimation, especially in the dog[9,10], with nausea and vomiting in higher doses.

Blood pressure is not affected significantly by small doses of LSD. However, 50 to 100 μg./kg. in anaesthetised cats causes bradycardia,

resulting from central vagus stimulation, and a fall in blood pressure, the latter action being due to a depressant action upon the vasomotor centre, since in the spinal cat LSD increases blood pressure and bradycardia is absent[9,10].

In man, the action of LSD on blood pressure[29,30], cerebral circulation and metabolism was thoroughly investigated in normal and schizophrenic subjects[31]. When the psychic effects reached a maximum 40 minutes after intravenous injection of 100 to 120 μg. of LSD, blood pressure was slightly increased, the internal jugular venous pressure was unchanged in normal subjects and slightly but significantly raised in schizophrenic patients. Cerebral blood flow, vascular resistance, oxygen consumption, glucose utilisation, arterio-venous glucose and oxygen differences and respiratory quotient showed no significant change despite the obvious occurrence of the characteristic psychological and mental effects of LSD. Mydriasis was also present.

Respiration[9,10] is affected by doses of 10 to 50 μg./kg., which produce stimulation or inhibition. Higher doses produce only inhibition, and death is due to respiratory paralysis. Similar respiratory changes were also observed by other authors[33,34].

From this multiplicity of autonomic centrally induced effects the most significant action is the response of the temperature regulation centre in the rabbit to LSD. Doses as small as 0·5 to 1·0 μg./kg. raise the temperature. This dose is comparable to the dose of LSD inducing psychic changes in man. These two responses show that LSD has not only an outstanding activity in the psychic sphere but also in the field of autonomic functions but this does not imply that the psychic and autonomic functions are related. The responses in man and in rabbit are accompanied by parallel effects in both in that tachyphylaxis develops and tolerance occurs, reproducible effects being obtained only after several days interval between experiments.

Somatomotor effects. These are both pyramidal and extrapyramidal in nature. The symptoms in cats and dogs are ataxia and show especially spastic paresis. High doses are necessary to produce these motor changes when compared with the minute amounts of LSD inducing mental disturbances in man and changes in temperature regulation in rabbits.

Psychic effects. The characteristic psychic changes induced in man by LSD administered by mouth are: alteration of the mood either towards euphoria or depression, alteration of the behaviour, hallucinations, mostly optical, distortion of the body image, a sense of depersonalisation, and "psychotic" states. Whether the psychic alterations correspond to a real psychotic state, with a pattern which can be regarded as a "model psychosis", as many psychiatrists define it, can only be discussed when agreement is reached on the definitions of psychosis. With Hoch and colleagues[35] we believe that the psychological disturbances produced by LSD and similarly active agents are chiefly linked to the category of functional psychoses not causing gross impairment of memory, orientation and awareness. This is especially so for the acute action of LSD in a single dose.

Characteristic of the psychological LSD response is the individual reaction, the actual psychic induction and the environment during the assay. A distinctive feature in the production of the psychic and autonomic changes by LSD in man is the long period of latency after oral administration. While the autonomic effects are seen in about 20 minutes after administration, the psychic effects do not appear until 40–60 minutes. The peak of the mental changes is reached after 2–3 hours, and the mean duration is 8–12 hours. A comparative study of the onset, peak and duration of the actions when the drug was given by different routes was made by Hoch[36] with the following results. The mean doses were: 120 μg. orally and intramuscularly, 100 μg. intravenously, and 20–60 μg. intraspinally. The onset of autonomic and psychic effects was slightly faster by the intramuscular route than orally, only several minutes after intravenous and practically instantaneous after intraspinal injection. The peak of the psychic symptoms is reached in 1 hour and lasts 9–10 hours after intravenous and intraspinal administration. No qualitative but only quantitative differences could be observed for the different routes. These were particularly in the onset of symptoms, less so in the duration of action. The overall pattern of psychic changes induced by LSD resembles that produced by mescaline in much higher doses.

The lengthy period before onset of the psychic changes after oral administration gave rise to the hypothesis[37] that LSD, active at 0·5 to 1·0 μg./kg., does not induce the mental changes in man by direct action on the brain but by a metabolite produced by a primary disturbance of the metabolism of another organ, probably the liver. The results of administering LSD by different routes, especially intravenously and intraspinally where onset of action is instantaneous, makes this unlikely.

Neuropharmacological and Neurophysiological Data

The problem of the central actions of LSD has been approached by the newer neuropharmacological and neurophysiological techniques. When LSD was administered with the Feldberg-Sherwood[38] canula into the lateral ventricle of the brain, Gaddum and Vogt[39] found that doses up to 200 μg. induced only minor diffuse autonomic symptoms, but 800 μg. aroused the unanaesthetised cats to a display of "sham rage" beginning three minutes after the LSD and lasting 45 minutes. Subcutaneous injection of 3 mg. produced a similar pattern lasting for 2 hours. It is astonishing that such high doses are needed to produce apparent symptoms after intraventricular administration compared with the minute doses active orally in man or intravenously in the rabbit. Intraventricular administration is a promising pharmacological approach, but no better localisation of the point of attack of a drug is obtained in this way than by systemic administration.

The electrophysiological studies with LSD relate to spontaneous cortical activity and to effects on transmission in specific synaptic systems. Marrazzi and Hart[40,41] found that electrical submaximal stimulation

applied to an optical cortex in the cat slightly anaesthetised with thiopentone sodium evokes a cortical potential transcallosal response at a symmetrical point in the contralateral cortex. After ipsilateral intracarotid administration of 8 μg./kg. of LSD the amplitude of the postsynaptic response is apparently reduced. The same effect is obtained by mescaline, 5-HT, bufotenine, adrenaline, noradrenaline and adrenochrome: thus this effect does not seem specific. Rovetta's[42] investigations on the cortical response to photic stimulation in anaesthetised cats by local administration of LSD in concentrations as high as 9 per cent as well as by intravenous injection of 40 μg./kg. have been without clear results. Purpura[43,44] found in unanaesthetised cats immobilised with suxamethonium, facilitation of the primary cortical responses in the auditory and visual system by small doses of LSD; higher doses, 40 to 60 μg./kg., intravenously, depressed the auditory response while facilitation for visual stimuli remained. These effects could not be reproduced by thiopentone anaesthetised cats. With 4 μg./kg. intravenously, Purpura observed activation of the recovery cycle consisting of a shortened initial recovery phase and a prolonged phase of supernormality. Evarts[45] who has recently made an excellent review on the electrophysiological studies, investigated the synaptic transmission in the visual system of the cat in pentobarbitone anaesthesia. Intracarotid injection of 30 μg./kg. of LSD caused a mean decreased of 80 per cent in the amplitude of the geniculate postsynaptic response to a single stimulus of the optic nerve, while transmission within the retina and between geniculate radiation fibres and cortical cells was resistant to LSD. Block of geniculate transmission by LSD was associated with absence of responsiveness to visual stimuli (behaviour blindness) while the cats respond briskly to auditive stimulation. The same effects were obtained with monkeys.

On the basis of recent investigations by Grundfest and Purpura[46] on the general problem of dendritic activity, Purpura suggests that LSD elicits differential actions on axodendritic and axosomatic synapses, that is, LSD inhibits the axodendritic and facilitates the axosomatic transmission. Dendrites are synaptically excitable only by the physiological transmitters and drugs, and not by electrical stimulation. In several studies it was found that the electrographic picture produced by stimulation of the reticular formation and the action of LSD are similar (arousal effect in the EEG). Purpura suggests in the light of the new experimental data that this common picture does not imply a direct action of LSD on the reticular formation but a diffuse effect on cortical as well as on subcortical synapses whose activation will facilitate or inhibit transmission. The quality of the effect depends on whether this activation causes depolarisation or hyperpolarisation of the synaptic membrane. From this concept, the central actions of LSD and similarly active drugs seem to be diffuse rather than localised.

Cook and Weidley[48] investigated the behaviour effects of LSD using the "conditioned avoidance-escape response" (CR) in trained rats. LSD significantly blocked the conditioned response after subcutaneous injection of 1·5 mg./kg., but not the unconditioned response. The purpose

of this study was to antagonise the CR-blocking action of various agents with doses of 0·1 to 0·5 mg./kg. of LSD that had no measurable action themselves on the CR. These doses of LSD antagonised the CR-blocking action of chlorpromazine, morphine, reserpine and 5-HT. The latter depressed only the spontaneous locomotor activity in rats and mice, and there was no stimulant action. Mescaline failed to block the CR and did not antagonise the CR-blocking action of chlorpromazine, morphine, reserpine and 5-HT.

Apter and Pfeiffer[49] believe that cats are capable of having hallucinations when given nontoxic doses (0·10 mg./kg.) of LSD intraperitoneally and 150 mg./kg. of mescaline. Both compounds provoke spontaneous action potentials in both retina and optic nerve, appearing first in the retina and later in the optic nerve. Large spikes are recorded from the visual cortex in cats after LSD. These spikes are higher and larger than in the retinal potentials and they disappear after severing the optic nerve. From these findings the authors conclude that the spontaneous visual-system activity induced by LSD begins in the retina, travels through the visual pathways with facilitation at each synapse[43] with higher spikes from each nucleus and maximal activity in the cortex. The activity in the cortex depends on the intact connection with the retina. Thus the origin of optic hallucination by LSD is located in the retina.

Grenell[50] suggests that LSD acts directly on the cerebral cortex of the anaesthetised cat when administered intravenously or by local cortical perfusion (2·5 and 2 μg./ml. of LSD) by microcannulation into a pial vessel. The drug inhibits the cortical response to direct electrical stimulation. The most striking effect of LSD under these experimental conditions was in producing "dendritic spikes". This is in agreement with the assumption of Purpura[44]. But LSD can act more directly on the cortical response, because the inhibition of this response was also observed after interruption of the ascending reticular activating system.

Keller and Umbreit[51] observed that LSD induced a special locomotor behaviour in the guppy (*Lebistes reticulatus*). Previous or simultaneous exposure to 5-HT caused no observable effect on the typical LSD-response. No antagonistic action was seen with other indole derivatives and tryptophan, but the latter compounds prolonged the LSD induced behaviour for as long as a week after the drug, and in some cases for months. Fishes with this abnormal LSD-plus-indole-behaviour were completely restored to normal behaviour by reserpine. The action of 5-HT under the same conditions does not appear to have been studied. Changes of behaviour have been reported by Rothlin and Cerletti[9,10] who described an alertness pattern, and by Woolley[52] who compared the behaviour of LSD-treated mice with that of normal mice when placed on a tilting glass plate. He suggested that the behaviour of the LSD-treated mice was due to hallucinations. Any attempt to antagonise the LSD-induced behaviour by 5-HT failed.

The cells of oligodendroglia of the brain are characterised by a slow pulsating rhythmical movement in tissue culture. Benitez and colleagues[53] and Woolley[52] observed that LSD, 5 μg./ml., first causes relaxation and

vacuolisation of these cells of foetal human brain, and secondly causes contraction. When LSD is given with 5-HT the first effects of relaxation and vacuolisation are antagonised by 5-HT but the contraction is increased. Thus there are both antagonistic and additive effects between the two agents. The meaning of these cerebral actions of LSD and 5-HT is not evident, but there is an analogy with the actions of the two agents on isolated smooth muscle.

The results of several authors working on the spontaneous cortical activity (EEG) produced by LSD in animals under various experimental conditions and in man are in good agreement. Delay and colleagues[55] found in rabbits a decrease in the amplitude of spontaneous activity and no block of the LSD response to activation. Bradley and colleagues[56], in conscious unrestrained cats, recorded after oral LSD an EEG-pattern consisting of diffuse, fast activity with low amplitude. The same results have been described by Schwarz and others[57] using the intraventricular route. In pentobarbitone-induced narcosis Bradley and colleagues[56] and Evarts and colleagues[58,59] found the "barbiturate spindles" abolished. Himwich[60] and Rinaldi and Himwich[61] concluded that one of the central effects of LSD is the stimulation of the mesodiencephalic activating system (arousal response) but in contrast to these findings Killam and Killam[62] recorded an increase of the threshold for EEG-arousal to sciatic nerve and reticular formation stimulation.

In man, the LSD effect on the EEG of normal volunteers consisted in favouring or increasing the alpha-rhythm[63–65].

Biochemical Data

Hoagland[66] discusses the biochemical changes induced by LSD *in vivo* and suggests that these might be related to the phosphate metabolism. A marked reduction in urinary phosphate excretion was found in drug-free schizophrenic men as well as in LSD-treated normal men (dose 0·5 to 1·0 μg./kg. orally). ACTH in schizophrenic and LSD-treated subjects enhanced phosphate excretion[67]. The data is believed to be consistent with the view that some unknown metabolite in schizophrenic and LSD-treated normal subjects facilitates the conjugation of phosphates with some organic substances in the cells or the decrease of the phosphate turnover rate.

Bain[68] recently reviewed the *in vitro* biochemical work on LSD and similarly acting compounds like mescaline and adrenochrome. All these agents have been studied primarily in relation to the metabolism of carbohydrates. The most interesting finding by Mayer-Gross and colleagues[69] is that the homogenate of guinea pig brain in the presence of 4×10^{-9} M of LSD stimulates the glucose oxidation by some 30 per cent and inhibits the utilisation of hexose monophosphate by 40 per cent. This was not confirmed by Lewis and McIlwain[70] who did not find stimulation, but 40 per cent inhibition of glucose oxidation with brain slices, electrically stimulated, of the guinea pig and by applying concentrations up to 5×10^{-9}M of LSD. Unstimulated slices did not show any effect.

Under the same conditions, lactate production was inhibited by 40 per cent. Clark and others[71], and Bain[68], quoting Bain and Hurwitz and Bain and Morrison, confirmed the results of Lewis and McIlwain. Bain[68] found the mitochondria of liver and brain of the rat insensitive to LSD in uptake of oxygen and in associated phosphorylation in concentrations up to 10^{-4}M of LSD. Attempts to reverse the inhibition of glucose oxidation by LSD in electrically stimulated brain slices with 5-HT were without success.

Rudolph and Olson[72] investigating the metabolism of glucose-1-^{14}C and -6-^{14}C with prostate slices of dogs could not detect differences in the incorporation of ^{14}C into CO_2 and lactate in the tissue between normal and hypoglycemic animals. The total radioactivity in CO_2 from glucose-1-^{14}C was considerably higher than from glucose-6-^{14}C. With 5×10^{-4} M of LSD in the system the labelled carbon in CO_2 was markedly increased with glucose-6-^{14}C but not with glucose-1-^{14}C. The results were identical in both normal and hypoglycemic tissues.

Poloni and Maffezzoni[73] observed an increase of acetylcholine in the brain of guinea pigs after administration of LSD. Hence Thompson and others[74] investigated the action of LSD on cholinesterase and found a 50 per cent inhibition of the activity of the serum cholinesterase of human plasma and brain with 5×10^{-6} M of LSD. Only 10 per cent inhibition of true cholinesterase occurred with LSD-concentrations up to 5×10^{-5} M. Zehnder and colleagues[75] have confirmed these findings and found that 2-brom-lysergic acid diethylamide acts similarly. Of the natural ergot alkaloids only ergometrine was active, having one-fifth the activity of LSD. Inhibition of 50 per cent of the serum cholinesterase was also found by Fried and Antopol[76] in the same concentration (5×10^{-6} M) of LSD, and with 2×10^{-3} M of 5-HT the same effect was seen. When the non-enzymatic acetylcholine hydrolysis in the presence of the drug was subtracted from the total manometric values, a marked enhancement occurred with concentrations of 6×10^{-9} M of LSD (50 per cent) and 5×10^{-6} M of 5-HT (31 per cent). The antagonistic effect of the two drugs was not studied.

The inhibiting effect of LSD on serum cholinesterase may explain the accumulation of acetylcholine observed by Poloni and Maffezzoni in animals treated with LSD, but the primary question of whether this biochemical effect on the serum cholinesterase *in vitro* is correlated with the central activity of LSD on autonomic and mental functions has to be considered carefully (Thompson). Indeed the active concentration of LSD of 5×10^{-6} M in these *in vitro* experiments on the serum cholinesterase are in contrast with the doses of 0·5 to 1·0 μg./kg. which provoke the mental changes in man. Allowing a uniform distribution of the human dose, the concentration is at least 1000 times lower (10^{-9}). The sensitivity of true cholinesterase toward LSD is more than 10 times less and yet this enzyme is presumably more directly correlated with the nervous functions than is pseudoesterase. When discussing the sensitivity of these enzymes to LSD and other compounds it is wise to take into account the differences of the milieu *in vitro* and *in vivo*.

Compounds Related to LSD

The characteristic activity of LSD on mental functions in man prompted the synthesis of related compounds. Stoll and Hofmann[2] provided us with a series of substances for pharmacological testing obtained by partial synthesis with (+)-lysergic acid as a common link. These were screened by different tests. Table I gives the data on the antagonistic actions to 5-HT of the most interesting compounds on the isolated uterus of the rat[10]. This antagonism has the advantage of being specific, reproducible and a highly sensitive test for LSD. For a reliable analysis of the specificity of the 5-HT antagonism the following criteria must be considered: (1) the extent of the latency period for the maximum effect, (2) the reversibility of the antagonistic action and (3) the absence of the inhibiting action toward adrenaline and noradrenaline, acetylcholine and histamine at concentrations that cause significant 5-HT inhibition[20,77]. These criteria have not been considered for compounds of groups 4 and 5 in Table I (with the exception of LSD), because their activity was weak and transient.

TABLE I

INHIBITION OF 5-HT BY VARIOUS AMIDES OF LYSERGIC ACID (from ref. 10)

1. *LSD and its isomers*	
(+)-Lysergic acid diethylamide (LSD)	Very active (standard)
(−)-Lysergic acid diethylamide (+)- and (−)-*iso*lysergic acid diethylamide	Practically inactive, i.e., more than 100 × weaker
2. *Derivatives of LSD obtained by saturation of the double bond between C(9) C(10)*	
Dihydro-(+)-lysergic acid diethylamide	1·6× weaker
Lumi-(+)-lysergic acid diethylamide	Practically inactive
3. *Derivatives of LSD with substitution in the indole nucleus of (+)-lysergic acid*	
(+)-1-Methyl-LSD	3·5× stronger
(+)-1-Acetyl-LSD	2·0× stronger
(+)-2-Brom-LSD	1·5× stronger
(+)-2-Iodo-LSD	2·0× weaker
(+)-1-Oxy-methyl-LSD	1·5× weaker
4. *Monosubstituted amides of (+)-lysergic acid*	
Monomethylamide of (+)-lysergic acid	15·5× weaker
Monoethylamide of (+)-lysergic acid	8·5× weaker
Mono*iso*propylamide of (+)-lysergic acid	5·0× weaker
Monopropylamide of (+)-lysergic acid	2·5× weaker
Monobutylamide of (+)-lysergic acid	1·5× weaker
5. *Disubstituted amides of (+)-lysergic acid*	
Dimethylamide of (+)-lysergic acid	5·0× weaker
Diethylamide of (+)-lysergic acid = LSD	= standard
Di*iso*propylamide of (+)-lysergic acid	4·0× weaker
Dibutylamide of (+)-lysergic acid	3·0× weaker

Group 1. It is surprising that of the diethylamides of the four known isomers of (+)-lysergic acid only the (+)-lysergic acid diethylamide (LSD) is a strong 5-HT antagonist. The other three isomers are more than 100 times weaker than LSD. None of these three isomers had a competitive effect toward LSD in the 5-HT antagonism and in other tests.

Group 2. These are compounds in which the double bond between C(9) and C(10), the most unstable of the five double bonds in the molecule of lysergic acid, is saturated either by two atoms of hydrogen giving the dihydro-(+)-lysergic acid diethylamide, or by the addition of H_2O at the same positions giving the so-called lumi (+)-lysergic acid diethylamide. While the first compound possesses an activity only 1·6 times weaker than LSD, the latter is inactive. This agrees with the fact that lumi-ergotamine also has no activity on the uterus *in vitro* and *in vivo* and no specific adrenolytic action.

Group 3. This group is the most interesting, and comprises derivatives with substitution in the nucleus of (+)-lysergic acid. (+)-1-Methyl-LSD, (+)-1-acetyl-LSD and (+)-2-brom-LSD are stronger antagonists of 5-HT than LSD, the methyl 3·4 times[81], the 1-acetyl- twice, and the 2-brom-compound 1·5 times as active. These compounds have not only this high sensitivity to 5-HT but also other effects which will be discussed later. The (+)-2-iodo-LSD and the (+)-1-oxymethyl-LSD are respectively twice and 1·5 times weaker 5-HT antagonists than LSD.

Group 4. Monosubstitution at the amide nitrogen of the (+)-lysergic acid amides gives rise to a series of homologues mono-methyl, mono-ethyl, mono-*iso*propyl, mono-propyl and mono-butyl compounds. The anti-5-HT activity of all compounds is stronger than that of (+)-lysergic acid amide but weaker than that of LSD. Following the sequence of the homologous series the anti-5-HT activity increases gradually from 15·5, 8·5, 5, 2·5 to 1·5 times lower than LSD. Hence the activity increases with the length of the side chain.

Group 5. Disubstitution at the amide nitrogen of the (+)-lysergic acid amide. In this final group, dimethyl, diethyl, di*iso*propyl and dibutyl amides of the (+)-lysergic acid were investigated and found to have a 5 to 3 times weaker anti-5-HT activity than LSD which belongs to this group.

The most important compounds of all these related substances are without doubt the three derivatives of LSD with substitution in the indole nucleus of the lysergic acid: 1-methyl, 1-acetyl, and 2-brom-LSD which possess a stronger anti-5-HT activity than LSD on the isolated uterus of the rat. It is known that differences do exist in the anti-5-HT effect with respect of intensity in different functions, and that agents with apparent anti-5-HT effect *in vitro* might be inactive *in vivo*[78,79]. A comparison of the 5-HT inhibiting action of LSD, 1-acetyl-LSD and 2-brom-LSD on various functions *in vitro* and *in vivo* shows that 2-brom-LSD is significantly stronger on the isolated uterus of the rat and in the artificially perfused renal vessels of the rat and the cat, and that 1-acetyl-LSD is twice as active as LSD[80]. *In vivo* the three compounds show about the same activity to 5-HT on the peripheral vessels in the anaesthetised cat, on the bronchial musculature of the guinea pig, and in the inhibition of the potentiation to barbiturates by 5-HT in mice. In contrast to some indole derivatives active only *in vitro*[78], these compounds also have a pronounced anti-5-HT activity *in vivo*[80]. There are difficulties in the quantitative evaluation of this antagonism in some functions. A disturbing factor is the above-mentioned tachyphylaxis.

While 1-acetyl-LSD induces similar psychic changes in man as does LSD, the 2-brom-LSD is inactive in this respect, as appear to be all other compounds in Table I with the exception of the monoethylamide of (+)-lysergic acid (LAE). The preliminary assays, in volunteers, of the other compounds in doses up to 10 times those of LSD which elicit autonomic effects, produced no apparent psychotogenic activity. 1-Methyl-LSD was not examined[81].

Of the derivatives of LSD and related compounds described, only the monoethyl amide of (+)-lysergic acid (LAE), the diethylamide of (+)-lysergic acid (LSD) and the 1-acetyl-diethylamide of (+)-lysergic acid induce apparent psychotic changes in man, and it is probable that 1-methyl (+)-lysergic acid diethylamide behaves similarly. The psychic effects induced by these three compounds are similar, but there are great quantitative differences. While LSD and acetyl-LSD are active in doses of 0·5 to 1·0 μg./kg., LAE required 8 to 10 times this amount[9,10].

From the chemical point of view it is noteworthy that only the monoethyl (LAE) and diethyl amide (LSD) compounds possess the psychogenic action, the mono and di-methyl, propyl and butyl derivatives do not produce psychic alterations. The latter compounds can elicit autonomic effects in animal and man in doses at which LAE is inactive. Thus 50 μg. of the dimethylamide of (+)-lysergic acid produces autonomic effects for which 500 μg. of LAE are necessary. But in this dose LAE elicits pronounced psychic changes. So the production of autonomic and psychic effects are probably not linked. It seems evident that the ethyl group or groups on the amide nitrogen of (+)-lysergic acid derivatives are of primary importance for the production of psychogenic action.

The presumed causal relationship between the psychic action of LSD and the inhibition of 5-HT by LSD is much more complicated than it appeared when the first arguments were presented by Gaddum[82] and Woolley[83]. The facts are that LSD was found to be a specific and highly active inhibitor of 5-HT on the isolated uterus of the rat and isolated blood vessels. Soon afterwards, 5-HT was detected in the brain by Page[84] and by Gaddum[85] and was admitted as a new neurohumoral transmitter playing an important role in cerebral functions. From this was derived the fascinating hypothesis that the inhibiting action of LSD towards 5-HT may be the key to the explanation of the cerebral action of LSD (Gaddum and Woolley).

That many substances antagonistic to 5-HT *in vitro* may have no such effect *in vivo* has already been mentioned. Of the numerous derivatives of LSD investigated by us, 2-brom-LSD was found to be as potent an inhibitor of 5-HT as LSD *in vitro* and *in vivo*[80]. The surprising fact is that 2-brom-LSD despite its pronounced anti-5-HT activity is without any effect on the psyche. Doses up to 650 μg. were administered to 19 volunteers in our laboratory, and only autonomic effects were observed. Hirsch and others[86] administered 5 to 7 μg./kg. in volunteers, Waldenström (personal communication) 1500 μg., Snow and others[87] up to 7500 μg. and Page (personal communication) 20,000 μg. of 2-brom-LSD in carcinoid patients without provoking significant changes in the psyche. The largest dose of 2-brom-LSD given to man is at least 200 times the usual active psychogenic dose of LSD.

The next step towards elucidating the relations of the psychic and 5-HT inhibiting action of LSD was a comparison of the pharmacological actions of LSD and 2-brom-LSD on different functions[9,10]. Table II summarises these results.

TABLE II

COMPARISON OF THE PHARMACOLOGICAL PROPERTIES OF LSD AND 2-BROM-LSD

Functions	LSD	2-brom-LSD
Uterus and vagina in anaesthetised rabbit	Contraction approximately 1·5× weaker than ergometrine	No contraction, in higher doses inhibition of spontaneous rhythm and relaxation
Adrenolytic action (seminal vesicle of guinea pig)	Approximately 50× weaker than ergotamine	Approximately 10× as strong as LSD
Blood vessels: perfused kidney of the rat and the rabbit ear	Constriction	No constriction
Blood pressure in anaesthetised cat	Decrease	No specific action
Heart rate in anaesthetised cat	Bradycardia	No effect
Eye, pupil	Mydriasis	No effect
Body temperature: rabbit, cat, dog " " rat	Increase in all doses Decrease, increase in toxic doses	Decrease in high doses Decrease in all doses
Blood sugar in normal rabbits	Increase	No effect
Behaviour in normal mice	Excitation	Sedation
Amphetamine-excitation in mice	Potentiation	Inhibition
Effect on walzing mice	Inhibition on walzing due to excitation	Inhibition due to sedation
Effect on EEG	Activation	No activation
Psychic action in man	Very pronounced	Absent
5-HT constriction on rat uterus 5-HT constriction on perfused blood vessels	Inhibition Inhibition	Inhibition) 1·5× as strong Inhibition) as LSD
5-HT constriction on bronchial musculature *in vivo*	Inhibition	Inhibition
5-HT-potentiating action to hypnotics in mice	Inhibition	Inhibition
Reserpine-potentiating action to hypnotics in mice	Inhibition	No inhibition
Effect on chromatophores of *Poecilia reticularis*	Spreading	Spreading 2·5× as strong as LSD
Effect on cholinesterase of brain slices *in vitro*	Inhibition	Inhibition

LSD produces mainly a sympathetic pattern of symptoms: mydriasis, increase of temperature and of the blood sugar, piloerection, behavioural alertness, activation in the EEG and the typical psychic changes in man. None of these effects is produced by 2-brom-LSD. Instead of sympathetic stimulation and behavioural alertness, 2-brom-LSD induces general sedation, inhibition of the stimulatory effects of amphetamine and no activation of the EEG-pattern. Both compounds have a strong anti-5-HT effect *in vitro* and *in vivo* and inhibit the potentiating action of 5-HT to barbiturates in mice. Yet 2-brom-LSD is without any psychotogenic effect. Sedation and the inhibition of the potentiation effect of 5-HT on barbiturates by 2-brom-LSD are of central origin. Therefore it cannot be argued that 2-brom-LSD is unable to produce mental changes because it does not penetrate into the brain. Moreover, 2-brom-LSD could be detected in the brain in the same way as LSD. Brain extracts of mice treated with LSD and 2-brom-LSD exerted the same anti-5-HT activity

on the rat uterus. We therefore concluded[80] that on the basis of these results with LSD and 2-brom-LSD it is difficult to admit a correlation of the psychic effects induced by LSD and its anti-5-HT property, since 2-brom-LSD possesses the same anti-5-HT activity *in vitro* and *in vivo*, but it lacks the psychotogenic action.

The problem of interference of 5-HT and LSD in cerebral functions has been studied under various conditions. It became particularly attractive when the fundamental studies of Brodie and his associates[89] on the mode of action of reserpine provided evidence that the effects of this alkaloid are correlated with the metabolism of 5-HT. The actual concept of these authors[90] is as follows: reserpine primarily impairs the 5-HT binding sites in the brain but also those of the blood platelets and of the intestine. The consequence of this is the release of 5-HT in free form from the depots in the brain and elsewhere. Free 5-HT is rather an unstable substance and metabolised[91] easily by the action of monoamine-oxidase. But 5-HT is continuously synthesised and it persists free in low concentration in the brain. This free 5-HT has been considered to provoke the actions attributed to reserpine. 5-HT (serotonin) acts as the transmitter substance for the parasympathetic and noradrenaline for the sympathetic centres. LSD is supposed to block the "serotonergic" parasympathetic brain centres and by doing so unmasks the action of the opposing sympathetic system by causing an imbalance between the two antagonistic systems. This is a clever and attractive hypothesis, but, as the authors say, probably too simple to explain the differential functions of autonomic, somatic and psychic nature. Recently Carlsson and others[92] and Shore and Brodie[93] gave evidence that reserpine causes a long-lasting depletion of noradrenaline as well as of serotonin from brain and other organs. These results suggest a similar mechanism of action of reserpine on the catechol amines as on serotonin. Carlsson supposes that lack, rather than excess in free form, of serotonin and noradrenaline has to be considered as the cause of some pharmacodynamic effects of reserpine. These new results do not fit Brodie's theory based mainly upon the 5-HT releasing action of reserpine, since this agent provokes a long-lasting depletion of 5-HT and noradrenaline in the brain as well as in other organs.

In conclusion some experimental findings are presented that do not agree with the theory that the actions of LSD, and particularly its psychogenic effects, are caused by the relationship of LSD to 5-HT.

As well as the parallel and divergent effects of LSD and 2-brom-LSD previously noted, there exists the inhibition by LSD of the potentiating effect of reserpine to barbiturates, while 2-brom-LSD unexpectedly has no action. Therefore we suggest that reserpine does not potentiate by releasing 5-HT but by another mechanism. If reserpine acts by releasing 5-HT it is difficult to understand why 2-brom-LSD inhibits administered 5-HT as effectively as LSD and not the free 5-HT released by reserpine.

LSD causes a state of excitation with "sham rage" and antagonises the depressant action of 5-HT in unanaesthetised cats, when both drugs are given intraventricularly. LSD also antagonises the depressant action of

reserpine, but only temporarily, for the action of reserpine is of longer duration than that of LSD. When administered together there is no inhibiting influence by 5-HT on the alerting response of LSD. Therefore LSD antagonises the action of 5-HT but not vice versa.

Ergometrine is a strong anti-5-HT compound. It produces excitation and sham rage when given intraventricularly, and antagonises the depressant effect of 5-HT like LSD. Yet ergometrine has no psychogenic properties at all.

Mescaline induces similar psychic changes in man as LSD, but no relation with 5-HT is known.

Amphetamine produces similar autonomic and somatic functions as LSD, it induces the arousal pattern, reverses the depressant action of 5-HT and potentiates the effect of barbiturates, but does not provoke the psychic effects of LSD.

Morphine causes excitatory action and a sympathetic pattern similar to LSD and amphetamine but does not have the psychogenic property of LSD. Morphine and amphetamine have no anti-5-HT effects.

These experimental results are evidence that for the inhibition of the central effects of 5-HT and reserpine the anti-5-HT activity is not obligatory.

The effects of LSD and similar agents are not the result of circulatory changes (vasoconstriction or vasodilatation) nor metabolic factors as for example glucose and oxygen consumption. The neuropharmacological and electrophysiological findings, the alerting response due to the influence on the lower reticular formation activating system, the inhibition of the transcallosal synaptic response, and the postsynaptic geniculate synapse, the axodendritic inhibition and axosomatic facilitation are most valuable and stimulating. But present experimental results do not explain the profound mode of action of these agents in the brain, nor do they give coherent understanding of the production of psychic changes peculiar to psychotomimetic drugs. There is as yet no answer to the main question of whether these interesting experimental results have any relation with the psychic functions and their alterations by the psychotomimetic agents in man. While we have learned much about psychotomimetic drugs as a result of the advances in neurophysiology and neuropharmacology, the psychogenic activity of LSD and similarly acting agents still awaits a plausible explanation.

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RESEARCH PAPERS

THE SYNTHESIS OF CERTAIN BIS*ISO*QUINOLINE DERIVATIVES STRUCTURALLY RELATED TO EMETINE

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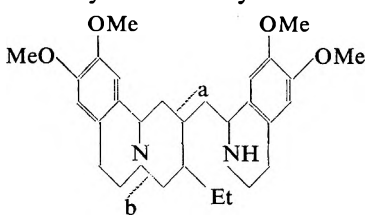
α ω-Bis(3 : 4-dihydro-6 : 7-dimethoxy-1-*iso*quinolyl)-propane,-butane, and -2- methylpropane have been prepared, the latter for the first time and the structures confirmed by comparing the ultra-violet light spectrophotometric measurements with those for ethyl γ-(3 : 4-dihydro-6 : 7-dimethoxy-1-*iso*-quinolyl) butyrate. The prepared compounds have been hydrogenated to give the corresponding tetrahydro*iso*quinoline derivatives and the structures confirmed by spectrophotometric measurements and by comparison of the pKa values with those of the corresponding dihydrocompounds. The tetrahydro*iso*quinoline derivatives are of comparable basic strength with emetine. Biological results are not yet available. An alternative route to the necessary amides required as intermediates for the above has been found to yield amides free from the unwanted cyclic imides, but the yields are not as good as by the other route. The use of a potassium bisulphate sodium sulphate mixture for ring-closing the amides has been found to yield cyclic imides instead of the required *iso*quinoline derivatives.

Until the structure of emetine was successfully established by Robinson[1] and confirmed by the work of Battersby and Openshaw[2] the preparation of synthetic analogues had been based on a structure proposed by Brindley and Pyman[3].

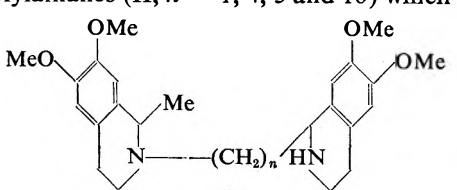
Emetine (I) has been synthesised by Preobrazhenskii[4] and many



(I)

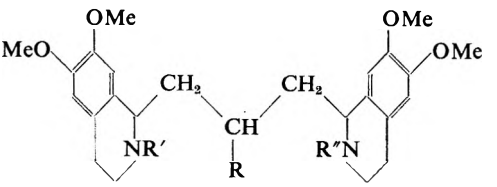
synthetic analogues, based on this structure have been prepared[5–8], some of which show marked activity against *Entamoeba histolytica.*

Osbond[7] first prepared a series of α-tetrahydro-*iso*quinolino-ω-tetrahydro-1-*iso*quinolylalkanes (II, n = 1, 4, 5 and 10) which may be regarded



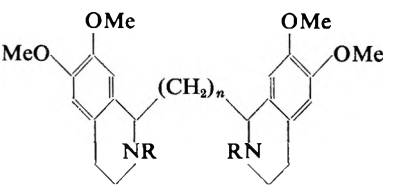
(II)

as being derived theoretically from emetine by rupture at the dotted line "a" (I). *In vitro* activity against *E. histolytica* was shown by a concentration of $1:10^4$ whereas, under the same conditions emetine was active at $1:10^6$. A second series prepared by Osbond[8] were the bistetrahydro*iso*quinolylalkanes (III), R = Et, Pr, R′, R″ = H or Me), recalling to



(III)

mind the straight-chain *iso*quinolylalkanes (IV) prepared by Child and Pyman[9] in 1929.



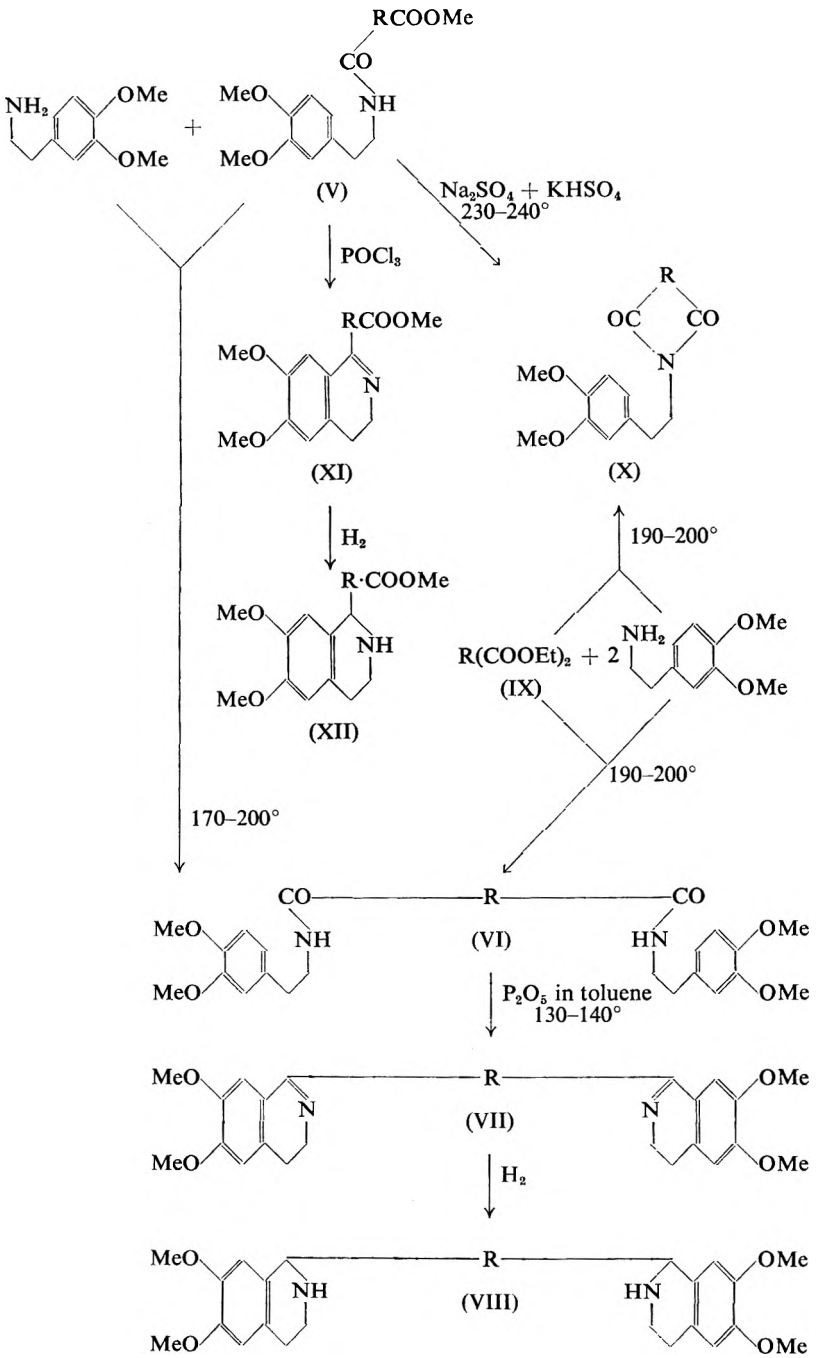
(IV, n = 4, 5, 8; R = H, Me)

In Osbond's second series the activity (*in vitro*) depended very much on the natures of the groups R, R′ and R″ and in those compounds where R′ and R″ were different no activity was observed at concentrations as low as $1:10^4$. In the structurally symmetrical compounds activity was observed when R′ = R″ = H and R = Et. Two "Racemates" of this structure were isolated (one of these would, of course, be a "meso" form and not a racemate). "Racemate A" was inactive but "Racemate B" was active at a concentration of $1:10^3$. When R′ = R″ = H but R = propyl instead of ethyl the activity increased and both "racemates" were active at concentrations as low as $1:10^4$.

The same sort of activity was obtained when R′ = R″ = Me and R = Et, but if R = Pr and R′ and R″ = Me the activity disappears. All these compounds may be theoretically derived from emetine by rupturing the molecule I at "b".

In view of the above results it seemed desirable to prepare further compounds of this nature and as methylation of the two *N* atoms appears not to enhance amoebecidal activity it was decided to keep R′, and R″ = H and to concentrate on varying R in the structure III. The nearest approach to emetine would be obtained by making R = CH(Me)(Et) but it was decided to commence with R = Me followed by R = $CH(Me)_2$ and also to prepare certain of the "straight chain" (R = H) compounds prepared by Child and Pyman (IV, n = 3, 4, R = H) in order to make a comparative study of the physico-chemical data.

The compounds were prepared according to the scheme shown overleaf.



R = $(CH_2)_3$; $(CH_2)_4$, *iso*Pr; $(CH_2{-}CH{-}CH_2)$ *iso*Pr.

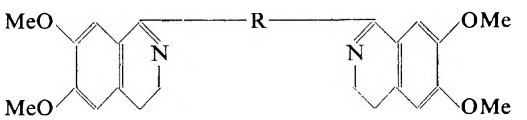
The rather longer route to the diamides (VI) by way of the amido esters (V) was investigated as the route followed by Child and Pyman[9] and Osbond[8] yielded the cyclic imides (X) as by-products. However, although the former route yielded diamide free from imide the increase in the yield was not sufficiently great to justify the more lengthy synthesis of the amide esters (V) against that of the simple esters (IX) (five stages instead of one).

Child and Pyman[9] ring-closed the diamides of the type VI (R = $(CH_2)_n$ where $n = 4, 5, 8$) by means of phosphorus oxychloride to yield the corresponding bis*iso*quinolines (VII) but were unable to isolate any product with the corresponding succinamide (VI, R = $-(CH_2)_2-$) and obtained anomalous results with the glutaramide (R = $-(CH_2)_3-$). After we failed to obtain ring-closure with the same reagent or with polyphosphoric acid or hydrofluoric acid, we applied the reagent consisting of a mixture of potassium hydrogen sulphate (4 parts) and anhydrous sodium sulphate (1 part) at 230–240°, first used by Baddar and Gindy[10].

TABLE I

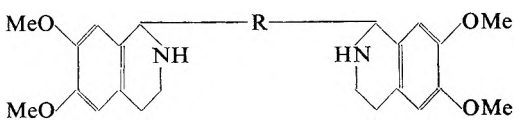
ULTRA-VIOLET LIGHT ABSORPTION OF DIHYDRO- AND TETRAHYDRO-*ISO*QUINOLINES

1. Dihydro-*iso*quinolines



Reference No.	R =	λ max in mμ	ε	Log ε
XI	$-(CH_2)_8COOEt$ (base) (one *iso*quinoline ring)	244 303	15,080 8,412	4·1784 3·9249
VIIa	$-CH_2{\cdot}CH(Me)CH_2-$ (Dihydrochloride)	243 304	28,210 16,390	4·4504 4,2146
VIIb	$-(CH_2)_4-$ (base)	243 302	27,960 16,530	4·4465 4·2183
VIIc	$-(CH_2)_8-$ (base)	243 304	29,890 17,140	4·4755 4·2340

2. Tetrahydro-*iso*quinolines



Reference No.	R =	λ max in mμ	ε	Log ε
XII	$-(CH_2)_3COOEt$ (hydrochloride) (one *iso*quinoline ring)	230 282	7,598 3,864	3·881 3·587
VIIIa	$-CH_2{\cdot}CH(Me)CH_2-$ (hydrochloride)	230 282	13,740 6,237	4·138 3·795
VIIIb	$-(CH_2)_4-$ (base)	228 282	15,040 9,137	4·177 3·819
VIIIc	$-(CH_2)_3-$ (dihydrochloride)	229 282	12,540 5,282	4·098 3·722

The desired bis*iso*quinolines were not obtained; no one appears yet to have prepared *iso*quinolylethane, VII, (R = $-(CH_2)_2-$). The compounds actually obtained were the corresponding imides (X, R = $-(CH_2)_2-$, $-(CH_2)_3-$, etc.). Finally, in all cases (except that of the succinamide) the desired bis*iso*quinolylalkanes were obtained by following Osbond's method using P_2O_5 in toluene.

The tetrahydro*iso*quinolines (VIII) were prepared from the corresponding dihydro-derivatives (VII) by hydrogenation at room temperature and pressure in the presence of Adams' platinum oxide catalyst.

1 : 3-Bis(3 : 4 : -dimethoxy-1 : 2 : 3 : 4-tetrahydro-1-*iso*quinolyl)-β-methyl propane (III, R = CH_3) which could not be obtained solid was characterised as its hydrochloride and by comparing its ultra-violet light absorption curve with that of known compounds, (III, R = H, and VIII, R = $(CH_2)_4$), and with that of ethyl-γ-(6 : 7-dimethoxy-1 : 2 : 3 : 4-tetrahydro-1-*iso*-quinolyl)butyrate. Further evidence that hydrogenation had occurred as required was supplied by a study of the pKa values of the bisdihydro and the bistetrahydro-compounds respectively (see Table II). The corresponding *iso*propyl compound (III, R = $CH(Me)_2$) was not obtained, analysis and absorption data showing that the dihydro compound (VII, R = CH_2–CH(*iso*Pr)CH_2) had not been obtained in a pure condition. A dipicrate with a satisfactory analysis for the required compound was obtained pure after several recrystallisations, but only in small yield.

TABLE II

EFFECT OF HYDROGENATION ON THE BASIC STRENGTHS OF CERTAIN DIHYDRO*iso*QUINOLINES

(For Formulae of Compounds see Table I)

Before hydrogenation		After hydrogenation	
Reference No. of compound	pKa	Reference No. of compound	pKa
XI	7·4	XII	8·3
VIIa	6·8	VIIIa	8·2
VIIc	6·8	VIIIc	8·2

Dissociation constants for emetine—pKa_1 = 8·2, pKa_2 = 7·4

The tetrahydro compounds are comparable in basic strength with emetine itself, probably one of the prerequisites for amoebicidal activity. Those prepared for the first time (VIII, R = $CH_2CH(Me)CH_2$, and XII, R = $(CH_2)_3COOEt$) have been submitted for biological testing.

EXPERIMENTAL

(The micro analyses were made by Mr. G. Crouch of these laboratories.) Melting points are uncorrected.

γ-Carbethoxybutyryl Chloride

This was obtained in 84 per cent yield by the method of Clark[11] b.p. 75–76°/1·4 mm. p-*Toluidide* m.p. 98–99°. Found: C, 67·7; H, 7·6; N, 5·6 per cent. $C_{14}H_{19}O_3N$ requires C, 67·4; H, 7·6; N, 5·6 per cent.

Ethyl N-(β-3 : 4-*dimethoxphenethyl*) *Glutaramate* (V, R = $(CH_2)_3$)

A stirred solution of homoveratrylamine (β-3 : 4-dimethoxyphenethylamine) (18·1 g.) in dry ether (150 ml.), cooled in ice-salt mixture was treated dropwise with a solution of γ-carbethoxybutyryl chloride (8·9 g., 0·5 equiv.) in dry ether (50 ml.), over a period of one hour. The stirring

was continued for a further two hours, the mixture left overnight, and then refluxed for a further two hours. Water (100 ml.) was added, and the product worked up as described by Battersby, Openshaw and Wood for ethyl *N*-(3:4-dimethoxyphenethyl) malonamate. Yield 12·9 g. (80 per cent). Recrystallised from dry ether with cooling in acetone and CO_2 to give fine, colourless needles (18·8 g.) m.p. 47–48°. Found: C, 63·5; H, 7·7; N, 4·4 per cent. $C_{17}H_{25}O_5N$ requires C, 63·1; H, 7·8; N, 4·3 per cent.

N-(*β*-3:4-*Dimethoxyphenethyl*) *Glutarimide* (X, R = $(CH_2)_3$)

Ethyl *N*-(3:4-dimethoxyphenethyl) glutaramate (1·61 g.) was added to a melted mixture of potassium hydrogen sulphate (5 g.) and anhydrous sodium sulphate (2 g.) and the mixture heated at 230–240° (bath temperature) for 45 minutes. The cooled mass was extracted with hot water, and after cooling the deposited imide was collected after skimming off a black tar from the surface of the solution. Yield 0·48 g. (35 per cent). Recrystallised from benzene and light petroleum (40 to 60°) as white needles, m.p. 113 to 114°. Found: C, 64·5; H, 6·8; N, 5·1 per cent. $C_{15}H_{19}O_4N$ requires C, 64·9; H, 6·9; N, 5·1 per cent.

Ethyl N-(*β*-3:4-*dimethoxyphenethyl*) *Adipamate* (V, R = $(CH_2)_4$)

This was prepared as described above for ethyl *N*-(3:4-dimethoxyphenethyl) glutaramate, using δ-carbethoxyvaleryl chloride (9·6 g.) prepared by the method of Blaise and Koehler[13]. Yield of adipamate 11·2 g. (69 per cent) as colourless needles, m.p. 46 to 47, 5° on crystallisation from ether. Found: C, 64·3; H, 8·3; N, 4·2 per cent. $C_{18}H_{27}O_5N$ requires C, 64·1; H, 8·1; N, 4·2 per cent.

Methyl N-(*β*-3:4-*dimethoxyphenethyl*) *Succinamate*

This was prepared in the same way as ethyl *N*-(3:4-dimethoxyphenethyl) glutaramate, using 36·2 g. of homoveratrylamine and *β*-carbmethoxypropionyl chloride (15·05 g., 0·5 equiv.), prepared by the method of Cason[14]. Yield 25 g. (85 per cent) yielding colourless needles, m.p. 66 to 67·5°, from benzene and light petroleum (40 to 60°). Found: C, 61·5; H, 7·1; N, 4·6 per cent. $C_{15}H_{21}O_5N$ requires C, 61·0; H, 7·1; N, 4·7 per cent.

N-(*β*-3:4-*Dimethoxyphenethyl*) *Succinimide*

Methyl *N*-(3:4-dimethoxyphenethyl) succinamate (2 g.) was heated at 230 to 240° with a mixture of potassium hydrogen sulphate (8 g.) and anhydrous sodium sulphate (2 g.) as described for *N*-*β*-(3:4-dimethoxyphenethyl) glutarimide. Yield 0·4 g. (22 per cent). Crystallised from alcohol in colourless needles m.p. 128-129°. Found: C, 63·6; H, 6·3; N, 5·3; MeO, 23·6 per cent. $C_{14}H_{17}O_4N$ requires C, 63·8; H, 6·5; N, 5·3; MeO, 23·6 per cent.

*γ-Carbethoxy-β-*iso*propylbutyryl Chloride*

γ-Carbethoxy-*β-iso*propylbutyric acid (5·05 g.) was heated with freshly-distilled thionyl chloride (11·9 g.) at 50 to 60° for 3½ hours. After the

excess of thionyl chloride had been removed *in vacuo* with the aid of dry benzene, the residue was distilled. Yield 7·1 g. (68 per cent) of colourless liquid, b.p. 75 to 76°/0·05 mm.

Ethyl N-(*β*-3:4-*dimethoxyphenethyl*)-*β*-iso*propyl Glutaramate* (V, R = $CH_2.CH(iso\text{Pr})CH_2$)

This was prepared from homoveratrylamine (12·5 g.) and *γ*-carbethoxy-*β*-*iso*propylbutyrylchloride (7·4 g., 0·5 equiv.) as described for ethyl *N*-(3:4-dimethoxyphenethyl) glutaramate. Yield 7·4 g. (60 per cent) of pale yellow gum, b.p. 275 to 280°/0·0005 mm. (decomp.). The product was purified by passing in a solution in benzene (dry) (0·3 g. in 25 ml.) through columns of activated alumina (2 × 10 cm.), developing with dry benzene (150 ml.) and eluting with ethanol benzene (1:20, 1400 ml.). Removal of the solvent from the eluate under reduced pressure yielded a colourless gum (0·252 g.). Found: C, 64·9; H, 8·4; N, 3·6 per cent. $C_{20}H_{31}O_5N$ requires C, 65·7; H, 8·6; N, 3·8 per cent.

NN-*Bis*(*β*-3:4-*dimethoxyphenethyl*) *Glutaramide* (VII, R = $(CH_2)_3$)

(*a*) Ethyl *N*-(*β*-3:4-dimethoxyphenethyl) glutaramate (3·23 g.) was heated with veratrylamine (1·81 g.) for 4 hours at 170 to 180° (oil bath). On cooling, a white solid mass, 2·9 g. (63 per cent) was obtained, which gave white needles, m.p. 130 to 131° from ethanol. The yield was increased to 73 per cent on repeating the experiment in a sealed tube (Carius furnace). Found: C, 65, 4; H, 7·6; N, 6·2 per cent. $C_{25}H_{34}O_6N_2$ requires C, 65·5; H, 7·5; N, 6·1 per cent.

(*b*) The same compound was prepared from homoveratrylamine (3·62 g.) and diethyl glutarate (1·9 g.) by following Child and Pyman's method[9]. Yield 2·6 g. (75 per cent), m.p. 130 to 131°. Mixed m.p. with previous sample, 129 to 130° with softening at 127°.

NN-*Bis*-(*β*-3:4-*dimethoxyphenethyl*) *Adipamide* (VI, R = $(CH_2)_4$)

(*a*) From ethyl *N*-(3:4-dimethoxyphenethyl) adipamate (3·3 g.) and homoveratrylamine (1·81 g.) by heating at 190° to 200° as described above. Yield 2·8 g. (60 per cent) raised to 65 per cent in a sealed tube (Carius furnace), m.p. 169 to 170°.

(*b*) By Child and Pyman's method (see above) by heating homoveratrylamine (3·6 g.) and diethyl adipate (1·9 g.) at 190 to 200° for four hours. Yield 2·45 g. (74 per cent) m.p. 169 to 170°. Found: C, 66·2; H, 7·7; N, 5·9 per cent. $C_{26}H_{36}O_6N_2$ requires C, 66·1; H, 7·7; N, 5·9 per cent. Mixed m.p. with product (*a*), 169 to 170°.

NN-*Bis*(*β*-3:4-*dimethoxyphenethyl*) *Succinamide*

(*a*) From ethyl *N*-(3:4-dimethoxyphenethyl) succinamate (2·95 g.) and homoveratrylamine (1·81 g.) by heating for 4 hours at 170 to 180° (oil bath) as described above for the corresponding adipamide and glutaramide. Yield 2·35 g. (53 per cent) raised to 60 per cent when heating was conducted in a sealed tube. Colourless needles from benzene and light petroleum. m.p. 173 to 174°.

(*b*) By Child and Pyman's method, used above, by heating homoveratrylamine (8·6 g.) and diethyl succinate (4·1 g.) for four hours at 170 to 180°. The crude product (6·5 g.) was separated by boiling water into an insoluble portion (3·9 g., m.p. 168 to 170°) and a soluble fraction (2·2 g., m.p. 124 to 128°). The former yielded *NN*-bis-(β-3:4-dimethoxyphenethyl) succinamide, m.p. 173 to 174° from benzene and petroleum and the latter, after removal of the water under reduced pressure, gave *N*-(β-3:4-dimethoxyphenethyl) succinimide, m.p. 128 to 129° after repeated recrystallisation from ethanol. The melting points of both diamide and imide were undepressed when admixed with authentic samples. Found: C, 64·6; H, 7·5; N, 6·3 per cent. $C_{24}H_{32}O_6N_2$ requires C, 64·8; H, 7·3; N, 6·3 per cent.

NN-*Bis*-(β-3:4-*dimethoxyphenethyl*)β-*methyl Glutaramide* (VI, R = $CH_2CH(CH_3)CH_2$)

Diethyl β-methyl glutarate (10·1 g.) and homoveratrylamine (18·1 g.) were heated for 4 hours at 200 to 210° (flask fitted with condenser). After cooling, benzene (50 ml.) was added and the whole set aside for 17 hours. The solid (9 g.) was washed with cold ethanol. A second crop (1·5 g.) was obtained by concentrating the benzene and ethanol mother liquors. Total yield 10·5 g. (45 per cent). Needles, m.p. 157 to 158°, from ethanol. Found: C, 65·8; H, 7·6; N, 5·9 per cent. $C_{26}H_{36}O_6N_2$ requires C, 66·1; H, 7·7; N, 5·9 per cent.

The mother liquors from this experiment (and two other batches) were evaporated to quarter bulk and left in a refrigerator for one week. The crystals (3·1 g.) which had separated yielded small needles, m.p. 119 to 120° after four recrystallisations from ethanol. This compound was shown to be N-(β-3:4-*dimethoxyphenethyl*)β-*methyl glutarimide* (see below). Mixed m.p. with authentic sample 118 to 120°. Found: C, 65·4; H, 7·0; N, 5·1 per cent. $C_{16}H_{21}O_4N$ requires: C, 65·9; H, 7·2; N, 4·8 per cent.

NN-*Bis*(β-3:4-*dimethoxyphenethyl*) β-iso*Propyl Glutaramide* (VI, R = $CH_2.CH(iso\text{Pr})\text{–}CH_2$)

(*a*) Homoveratrylamine (18·1 g.) and diethyl β-*iso*propylglutarate (11·5 g.) were heated at 190 to 200° for 4 hours, and the product worked up as described above for the corresponding β-methylglutaramide. Yield 8 g. (33 per cent). Small needles, m.p. 156 to 157°, from ethanol. Found: C, 67·2; H, 7·8; N, 5·5 per cent. $C_{28}H_{40}O_6N_2$ requires C, 67·2; H, 8·1; N, 5·6 per cent.

The mother liquors, on concentrating to quarter bulk and leaving in the refrigerator for three days yielded N-(β-3:4-*dimethoxyphenethyl*) β-iso-*propyl glutarimide*; 2·6 g. (17 per cent), m.p. (after four recrystallisations from aqueous ethanol) 119 to 120°. Found: C, 67·7; H, 7·9; N, 4·4 per cent. $C_{18}H_{25}O_4N$ requires C, 67·9; H, 7·9; 4·4 per cent.

(*b*) Ethyl*N*-(β-3:4-dimethoxyphenethyl)β-*iso*propyl glutaramate (3·6 g.) and homoveratrylamine (1·8 g.) were heated together at 190 to 200° for 4 hours. The solid obtained on cooling, 2·1 g. (42 per cent) yielded, on recrystallisation from ethanol, small needles, m.p. 156 to 157°, undepressed when admixed with a sample obtained by method (a).

1 : 3-*Bis*-(3 : 4-*dihydro*-6 : 7-*dimethoxy*-1-iso*quinolyl*) *propane* (VII, R = $(CH_2)_3$) (see Osbond[8]).

To a solution of *NN*-(β-3 : 4-dimethoxyphenethyl) glutaramide (2·29 g.) in anhydrous boiling toluene (30 ml.), phosphorus pentoxide (7 g.) was added all at once with continuous stirring. More phosphorus pentoxide (7 g.) was added after half an hour. After refluxing for a total of three hours the product was cooled and the supernatant liquid decanted. The residue was decomposed with water and after extraction with ether (2 × 20 ml.) the aqueous solution was cautiously basified with ammonium hydroxide solution. The yellow oil which separated was extracted with ether (5 × 50 ml.) and the ethereal solution dried and evaporated. The crude product was crystallised from ethanol. Yield 1·14 g. (54 per cent) of white felted needles, m.p., 161 to 163° (Osbond quotes 159 to 161°). Found: C, 70·8; H, 7·1; N, 6·5 per cent. $C_{25}H_{30}O_4N_2$ requires C, 71·1; H, 7·2; N, 6·6 per cent. Titration equivalent, 204; $C_{25}H_{30}O_4N_2$ requires 211. pKa = 6·8 (approx.).

1 : 4-*Bis*-(β-3 : 4-*dihydro*-6 : 7-*dimethoxy*-1-iso*quinolyl*) *butane* (VII, R = $(CH_2)_4$)

(*a*) This compound was obtained in the same way as the corresponding propane homologue using *NN*-bis-(β-3 : 4-dimethoxyphenethyl) adipamide (2·36 g.) in anhydrous toluene (35 ml.) and phosphorus pentoxide (2 × 8 g.). The crude product, 1·6 g. (72 per cent) crystallised from dry ethyl acetate in hard needles, m.p. 172 to 173°. Found: C, 72·2; H, 7·5; N, 6·0 per cent. $C_{26}H_{32}O_4N_2$ requires C, 71·5; H, 7·4; N, 6·4 per cent. Titration equivalent 216, $C_{26}H_{32}O_4H_2$ requires 218. pKa = 7·5 (approx.).

(*b*) *Using phosphorus oxychloride.* *NN*-Bis-(β-3 : 4-dimethoxyphenethyl) adipamide (2·36 g.), dry toluene (30 ml.) and freshly-distilled phosphorus oxychloride (7 ml.) were refluxed for one hour. After cooling, and decanting the toluene, the residue was washed with light petroleum (40 to 60°), dried and dissolved in ethanol (50 ml.), and basified with solution of sodium hydroxide. The free base was precipitated on the addition of water. Yield 1·8 g. (82 per cent).

1 : 3-*Bis*-(3 : 4-*dihydro*-6 : 7-*dimethoxy*-1-iso*quinolyl*)β-*methyl propane* (VII, R = *iso*Pr)

This was obtained in the same way as the corresponding *n*-propane homologue, using *NN*-bis-(β-3 : 4-dimethoxyphenethyl)-β-methylglutaramide (4·72 g.), dry toluene (100 ml.) and phosphorus pentoxide (2 × 15 g.) at a bath temperature of 110 to 120°. Yield 3·0 g. (69 per cent). Crystals from ethyl acetate, m.p. 172 to 173°. Found: C, 71·0; H, 7·5; N, 6·1 per cent. $C_{26}H_{32}O_4N_2$ requires C, 71·5; H, 7·4; N, 6·4 per cent. Titration equivalent 213. $C_{26}H_{32}O_4N_2$ requires 218. pKa = 6·8 (approx.).

Dipicrate m.p. 172 to 173·5° after drying for 4 hours at 100° over P_2O_5 *in vacuo*. Found: C, 57·2; H, 4·3; N, 12·8 per cent. $C_{38}H_{38}O_{18}N_8$ requires C, 57·0; H, 4·3; N, 12·5 per cent.

Dihydrochloride from hydrochloric acid gas and the base in dry benzene. Recrystallised from methanol and ether m.p. 175 to 177° (decomp.).

Found: C, 58·0; H, 6·6; N, 5·2; Cl, 14·0 per cent. $C_{26}H_{34}O_4N_2Cl_2$ requires C, 61·5; H, 6·7; N, 5·5; Cl, 13·9 per cent.

Dimethiodide m.p. 190 to 192° dried over P_2O_5 *in vacuo* at 100°. Found: C, 45·1; H, 5·4; N, 3·7; I, 36·0 per cent. $C_{28}H_{38}O_4N_2I_2$ requires C, 46·6; H, 5·3; N, 3·8; I, 35·2 per cent.

Chlorplatinate Found: Pt, 24·0 per cent. $C_{26}H_{34}O_4N_2PtCl_6$ requires Pt, 23·0 per cent.

1 : 3-*Bis*(3 : 4-*dihydro*-6 : 7-*dimethoxy*-1-iso*quinolyl*)-2-iso*propylpropane* (VII R = $-CH_2{\cdot}CH(iso\text{Pr})CH_2-$)

This was obtained in the same way as the corresponding *n*-propane homologue, using *NN*-bis-(β-3 : 4-dimethoxyphenethyl)-β-*iso*propylglutaramide (5 g.) dry toluene (50 ml.) and phosphorus pentoxide (2 × 15 g.) at a bath temperature of 110 to 115°. Yield 3·6 g. of brown gum which was finally crystallised from ether/light petroleum (40 to 60°) (charcoal). Repeated crystallisations gave colourless prisms m.p. 78 to 80°. Found C, 70·0; H, 7·9; N, 5·6 per cent. $C_{28}H_{36}O_4N_2$ requires C, 72·4; H, 7·8; N, 6·0 per cent. Better analytical results could not be obtained and the ultra-violet light absorption spectrum did not conform with those of homologous compounds. (On repeating the above experiment at a temperature of 120 to 130° for four hours the only isolable product proved to be *N*-(β-3 : 4-dimethoxyphenethyl)-β-*iso*propylglutarimide, m.p. 119 to 120° (see above). Mixed m.p. with authentic sample 118 to 120°).

Dipicrate. The substance of m.p. 78 to 80° yielded after repeated recrystallisations a dipicrate, m.p. 194·5 to 196°. Found C, 52·6; H, 4·5; N, 12·0 per cent. $C_{40}H_{42}O_{18}N_8$ requires C, 52·1; H, 4·6; N, 12·1 per cent.

Dimethiodide, *dihydrochloride*, and *platinichloride* of the base were all obtained but none yielded satisfactory analytical results.

Ethyl-γ-(3 : 4-*dihydro*-6 : 7-*dimethoxy*-1-iso-*quinolyl*) *Butyrate* (XI, R = $(CH_2)_3$)

Ethyl *N*-(β-3 : 4-dimethoxyphenethyl) glutaramate (3·23 g.) anhydrous toluene (35 ml.) and freshly-distilled phosphorus oxychloride (10 ml.) were heated at 120 to 130° (oil bath) for 45 minutes. After cooling the mixture was diluted with light petroleum (40 to 60°, *c.* 50 ml.). After 2 hours the solvent was decanted and the brown gum dissolved in water (30 ml.) by slightly warming. After the removal of non-basic matter with ether (2 × 10 ml.) the aqueous solution was basified with dilute solution of ammonia and extracted with ether (5 × 20 ml.) and ethyl acetate (2 × 15 ml.) and the combined extracts dried over sodium sulphate (anhydrous). On evaporation of the solvent 2·3 g. (75 per cent) of yellow gum was obtained. This yielded long buff-coloured prisms from petroleum (80 to 100°). Found: C, 66·8; H, 7·6; N, 4·6 per cent $C_{17}H_{23}O_4N$ requires C, 66·9; H, 7·6; N, 4·6 per cent. Titration equivalent 301, pKa 7·4 (approx.) $C_{17}H_{23}O_4N$ requires 305. *Picrate*, m.p. 172 to 173·5°. Found: C, 51·9; H, 5·2; N, 10·7 per cent. $C_{23}H_{26}O_{11}N_4$ requires C, 51·7; H, 4·9; N, 10·5 per cent. *Hydrochloride*, m.p. 124 to

125°. Found: C, 59·6: H, 6·9; N, 4·0; Cl, 10·1 per cent. $C_{27}H_{24}O_4NCl$ requires C, 59·9; H, 7·1; N, 4·1; Cl, 10·4 per cent.

1 : 3-*Bis*-(6 : 7-*dimethoxy*-1 : 2 : 3 : 4-*tetrahydro*-1-iso*quinolyl*) *propane* (VIII, R = $(CH_2)_3$)

The corresponding dihydro compound (1 g.) dissolved in glacial acetic acid (15 ml.) was treated with Adams' platinum catalyst (0·05 g.) and hydrogenated at room temperature and pressure. Uptake of hydrogen after 2 hours 118 ml.; theory requires 106 ml. After filtration, the solvent was removed *in vacuo*. The residual oil (1 g.) failed to crystallise (*cf.* Osbond[7]).

Dipicrate from ethanol (72 per cent of theory). Yellow needles, m.p. 211 to 213° (decomp.). Found: C, 49·9; H, 4·9; N, 10·4 per cent. $C_{37}H_{40}O_{18}N_8$ requires C, 50·2; H, 4·6; N, 12·6 per cent.

Dihydrochloride, needles from methanol, m.p. 265 to 268° (decomp.). Equivalent weight (by titration) 251; $C_{25}H_{36}O_4N_2Cl_2$ requires 249. pKa = 8·2 (approx.).

1 : 4-*Bis*(6 : 7-*dimethoxy*-1 : 2 : 3 : 4-*tetrahydro*-1-iso*quinolyl*) *butane* (VIII, R = $(CH_2)_4$)

The corresponding bis dihydro compound (1 g.) in methanol (25 ml.) with Adams' catalyst (0·075 g.) was hydrogenated as described for the corresponding propane. Uptake of hydrogen after $2\frac{1}{2}$ hours = 112 ml.; theory requires 103 ml. The oil remaining after the removal of solvent finally yielded needles m.p. 127 to 128° from ethyl acetate. (Child and Pyman[9] quote 126 to 127°). Found: C, 70·4; H, 8·1; N, 6·3 per cent. $C_{26}H_{36}O_4N_2$ requires C, 70·9; H, 8·2; N, 6·4 per cent.

1 : 3-*Bis*(3 : 4-*dimethoxy*-1 : 2 : 3 : 4 : *tetrahydro*-1-iso*quinolyl*)β-*methylpropane Dihydrochloride* (VIII, R = *iso*Pr)

This was prepared in the same way, and using the same quantities for 1 : 3-bis-(6 : 7-dimethoxy-1 : 2 : 3 : 4 : tetrahydro-1-*iso*quinolyl) propane. Uptake of hydrogen after 3 hours 115 ml.; theory requires 103 ml. The residual oil failed to crystallise after removal of the acetic acid. It was taken up in dry benzene and converted into the hydrochloride. Yield after three recrystallisations from methanol and ether 0·6 g., 50 per cent of theory. m.p. 278 to 280° (decomp.). Found: C, 59·7; H, 7·2; N, 5·1; Cl, 14·2 per cent. $C_{26}H_{38}O_4N_2Cl_2$ requires C, 60·8; H, 7·5; N, 5·4; Cl, 13·8 per cent. Equivalent weight by titration = 249. Theory requires 256·7. pKa = 8·2 approx..

1 : 3-*Bis*(3 : 4-*dimethoxy*-1 : 2 : 3 : 4-*tetrahydro*-1-iso*quinolyl*)β-*methylpropane Dihydrobromide*

This was prepared from the corresponding bisdihydro-β-methylpropane using tin and hydrochloric acid as described by Osbond[7] for the *n*-propane derivative. Yield of dihydrobromide, 0·78 g., from 1 g. of bisdihydro-compound. Prisms from ether and methanol m.p. 276 to 278° (decomp.). Found: C, 49·9; H, 6·2; N, 4·5; Br, 26·3 per cent. $C_{26}H_{38}O_4H_2Br_2$ requires C, 51·8; H, 6·3; N, 4·7; Br, 26·5 per cent.

Measurement of Ultra-violet Absorption Spectra

Solutions contained about 2 mg. accurately weighed, in 100 ml. of 0·1N HCl. Absorption was measured between the wavelengths of 220 and 330 mμ in 1 cm. cells with a Hilger Uvispek and a hydrogen arc.

Calculation of the approximate pKa Values

A suitable quantity of free base or hydrochloride was dissolved in 100 ml. of a mixture of 3 parts of ethanol, 97 per cent, and 2 parts of distilled water and titrated potentiometrically with 0·1 N HCl, or 0·1 N NaOH. A Morton D.C. amplifier pH meter was employed, with glass and saturated calomel electrodes. The approximate pKa values were calculated from measurements of the pH of the solutions at half neutralisations.

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ASSESSMENT OF DIRECT CHOLINESTERASE INHIBITORY ACTIVITY BY PUPILLARY MIOSIS

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Factors affecting the pupillary response to ocular administration of a direct inhibitor of cholinesterase have been investigated. The miotic response is dependent on the volume and concentration of solution and on the direct inhibitory activity of the compound. Measurements of miotic activity of twelve anticholinesterase materials show good agreement with *in vitro* biochemical measurements of their direct inhibitory activity. A rapid and simple method is suggested which reveals ten-fold differences of direct inhibitory activity, and detects activity equivalent to 0·01 μg. of TEPP.

The measurement of direct cholinesterase inhibitory activity by *in vitro* methods, although accurate, demands special biochemical laboratory facilities, and may be adversely influenced by the presence of organic solvents present in commercial preparations. A more rapid semi-quantitative method of assessment would frequently be of value, for example, in the preliminary screening of organic phosphorus insecticides, for detecting the presence of direct inhibitors as impurities in samples of indirect inhibitors (e.g., schradan, parathion or dimefox), and for examining commercial preparations.

Among the known "direct inhibitory" responses in mammals, pupillary constriction (miosis) is perhaps the most definite. It has the advantage of being readily seen and measurable. A method based on this response has already been described for confirming the potency of di*iso*propyl phosphorofluoridate (dyflos), after storage, using the miotic effect upon the rabbit pupil, and comparing the stored material with dyflos standards of known potency[1]. In the rabbit the rate of pupillary constriction depends on the concentration of dyflos administered[2]. Studies on ocular effects accompanying miosis have been made[2,3], and an abrupt dose-response relation has been observed in man[3].

Some organic phosphorus compounds, the indirect inhibitors, become highly potent anticholinesterases only after "conversion" to direct inhibitors within the mammalian body, or by insect or plant tissues[4]. The only mammalian tissue known to produce this conversion to a significant extent is the liver, so that a purely local cholinergic miosis should in theory be produced only by a direct inhibitor. Also, miosis should occur only above a limiting concentration proportional to the direct inhibitory activity of a compound, subject to some modification by its physical properties.

Materials and Methods

The present investigation involved, firstly, a study of some of the factors affecting the production of miosis by a known direct inhibitor,

and thus the development of a standardised technique; tetraethyl pyrophosphate (TEPP) was selected for this purpose. Secondly, the miotic effects of serial dilutions of several organic phosphorus compounds and commercial insecticidal preparations were assessed, and the results compared with the corresponding *in vitro* I 50 concentrations. This is defined as the molar concentration of substance producing 50 per cent inhibition of rat brain cholinesterase on incubation at 37° for 30 minutes.

Young albino guinea pigs were used. The standard inhibitor was a "pure" (96 per cent) sample of TEPP supplied by Messrs. Albright and Wilson Ltd. The solvents were water, propylene glycol, and tetrahydrofurfuryl alcohol containing 10 per cent v/v added water; none of these alone caused miosis, although propylene glycol and tetrahydrofurfuryl alcohol caused very slight transient irritation. The needles used for ocular administration were standard No. 16 hypodermic (0·55 mm. diameter, 24 s.w.g.).

Ocular administration was by allowing slowly-formed drops to fall from the vertically-held needle and syringe on to the centre of the cornea, from a height of 3 or 4 mm. The lower lid was then pulled upwards and outwards and released, to trap the drop and promote even spreading. Periodic observations of pupillary diameter were then made for a suitable period, with special emphasis on the speed of onset and of attainment of maximum response. In most tests, the other pupil was used as a control; alternatively the pupils of a similar but untreated animal were used. Between and during pupillary inspection, all animals were kept under comparable diffuse lighting conditions, sufficiently bright for adequate observation, without causing enough reflex pupillary constriction to mask a partial contraction.

The guinea pig and rabbit pupils are circular, or almost so, at all stages of constriction. However, it was not found possible to standardise lighting or operational conditions sufficiently to measure the pupillary diameter accurately and reproducibly. An arbitrary division of response was adopted, based on visual estimation of the degree of maximal contraction, and the time required to reach this maximum.

It was noted that the presence of an incomplete contraction could often be confirmed by exposing the treated and control pupils to bright light for a few seconds. The partially contracted pupil re-expanded less fully and less rapidly than did the normal pupil.

Factors Affecting Response

Dose-response Relations

Two dosage variables had to be considered, the volume and the concentration of solution administered. There was found to be a definite increase in miotic response with increasing volume of solution administered at constant concentration. Hence the dose volume was standardised at one drop of volume 8 to 10 μl. The effect of ten-fold concentration changes was then found to be marked and reproducible. A small amount of individual variation was expected, and could be minimised by using two or more animals at each dose level. On no occasion has a gross

discrepancy been noted, provided the correct technique was used; statistical calculations were not possible.

No difference in response has been detected between the sexes in young guinea pigs. However, fully adult (500 g.) guinea pigs gave a lesser response than young (200 g.) animals. The rabbit eye was found to be about one-tenth as sensitive as the guinea pig eye.

Effect of Solvent

Substitution of propylene gylcol for water as diluent for 1×10^{-5} v/v TEPP produced a slightly more rapid onset of contraction, but no difference in final effect.

Tetrahydrofurfuryl alcohol containing 10 per cent v/v added water, as solvent for 1×10^{-5} v/v TEPP, was not detectably different in effect from propylene glycol; the undiluted alcohol caused slight irritation.

Possibility of False Positives

Some samples of commercial materials which were obviously irritant also caused a transient miosis, occuring within a minute or less, partial in degree, and returning to normal within 5 to 10 minutes. In some animals such a response caused a "sympathetic" miosis in the untreated eye, presumably due to a pain reflex. All such transient effects were accompanied by signs of corneal damage.

The introduction into the eye of a corrosive substance, such as 4 per cent aqueous sodium hydroxide, produced a rapid transient contraction of this type, even if the cornea had been locally anaesthetised with 2 per cent butacaine sulphate.

Miosis due to direct cholinesterase inhibitors never occurred within less than two minutes of application, even after 100 per cent TEPP. This was presumably due to the time taken for diffusion to the site of action, and for acetylcholine accumulation to occur. Also, recovery was delayed, and contraction of the untreated eye was never detected. There was no difficulty in distinguishing between a response produced by a direct inhibitor of cholinesterase and one produced by corneal irritation. No other cause of a "false positive" result has been seen.

Comparison of Miotic and *In Vitro* Results

Serial dilutions of a number of anticholinesterase substances were next examined by the standardised technique. These substances are listed in Table I.

TABLE I

Anticholinesterase substances examined by the standard technique

Schradan	Octamethyl pyrophosphoramide
Dimefox	Bis(dimethylamido)phosphorofluoridate
DDVP	Dimethyl 2:2-dichlorovinyl phosphate
Parathion (technical)	Diethyl *p*-nitrophenylthionophosphate
Tabun	Ethyldimethylamidophosphorocyanidate
Sarin	*O-iso*Propyl methylphosphonofluoridate
"AC 528" (technical 100 per cent)	2:3-Dioxylenebis(*OO*-diethylphosphorodithioate)
"FAC 20" (commercial 20 per cent)	*OO*-Diethyl *iso*propylcarbamoylmethyl dithiophosphate
Methyldemeton (thiolo- and thiono-isomers)	*OO*-Dimethyl (2-ethylthioethyl) thiophosphate
Demeton (thiolo-isomer)	*OO*-Diethyl(2-ethylthioethyl) thiophosphate

The results obtained, and a comparison with those of corresponding *in vitro* biochemical tests, are summarised in Table II.

TABLE II

COMPARISON OF MIOTIC AND *in vitro* DIRECT INHIBITORY POWERS OF VARIOUS ANTICHOLINESTERASE ORGANIC PHOSPHORUS MATERIALS

Substance(*a*)	Miotic limit (*b*) (Active ingredient)	Molar I 50	Miotic limit / Volume I 50
TEPP	10^{-7}	10^{-8}	100
Schradan	10^{0}	10^{-2}	
Dimefox	10^{0}	10^{-1}	
DDVP	10^{-4}	10^{-6}	1,000
Parathion (technical)	10^{-2}	10^{-3}	100
Tabun	10^{-6}	10^{-9}	100
Sarin	10^{-9}	10^{-10}	100
"AC 528" (technical 100 per cent)	10^{-4}	10^{-5}	100
"FAC 20" (commercial 20 per cent)	10^{-1}	10^{-3}	1,000
Methyldemeton ("Metasystox"):			
thiolo-isomer	10^{-4}	10^{-5}	100
thiono-isomer	10^{-3}	10^{-4}	100
Demeton ("Systox") (thiolo-isomer)	10^{-4}	10^{-6}	1,000

(*a*) Dissolved in propylene glycol
(*b*) Highest non-miotic volume concentration

Pure undiluted schradan and dimefox were found to produce no miosis, and fatal doses of these two materials could be absorbed by the eye without miotic response.

DISCUSSION

It would be expected that the magnitude of miotic response due to a direct cholinesterase inhibitor would be dependent on the amount of the substance reaching the effector end organs of the sphincter pupillae muscle, and hence on the amount of inhibitor administered.

The experimental findings are in agreement with this supposition. The magnitude of miotic response in the young albino guinea pig was dependent on both the volume and the concentration of solution of inhibitor administered. The results proved to be reproducible, and variation between individual animals was small. This test served to detect direct inhibitory activity equivalent to 0·01 μg. of TEPP.

The substitution of propylene glycol or tetrahydrofurfuryl alcohol for water as solvent for TEPP affected only the speed of onset of miosis. This is consistent with the supposition that change of solvent affects the rate of transport and release of inhibitor to the site of action, but not the amount carried. Malathion (*OO*-dimethyl-(1 : 2-dicarbethoxy)-ethyl dithiophosphate), a water-insoluble substance, gave a similar final response whether as aqueous emulsion or a propylene glycol solution.

The twelve substances selected for comparison of miotic and *in vitro* inhibitory activity showed a wide variation in structure and physical properties. In spite of this, the lowest concentration at which miosis could be detected showed a consistent relation to the I 50 concentration. The molar I 50 concentrations obtained by *in vitro* assay can be converted to approximate volume dilutions by multiplying by the molecular weight and dividing by 1000 times the density, giving a factor of about 0·1 for the substances tested, whose molecular weights are about 150 to 300.

Hence, Table II shows that, for most materials, the highest non-miotic concentration was about 100 times the I 50 concentration. This ratio of 100 was found with all but three of the substances tested, DDVP, FAC 20 and the thiolo-isomer of demeton, for which the ratio was 1000. Thus, graphs plotted from the logarithms of the corresponding pairs of concentrations showed a good linear relation. Observations made during these tests indicated that the main effect of differences in physical properties between compounds may be a slight effect upon the rate of development of miosis.

The possibility exists that miosis due to rapidly reversible direct inhibitors may be of detectably shorter duration than with "irreversible" inhibitors. It was observed that the duration of miosis produced by 1×10^{-3} v/v DDVP ($2\frac{1}{2}$ to 3 hours) was much shorter than that of similar miosis produced by 1×10^{-5} v/v TEPP (4 to 20 hours). Also, a rather sharp break-off of miotic effect with dilution was observed with DDVP. This is consistent with the known reversibility of DDVP inhibition[5].

The following method is now used for the estimation of miotic activity.

A series of ten-fold dilutions of the substance is made up in a non-irritant water miscible solvent. A standard volume of each dilution is then administered, usually one drop from a No. 16 needle, or 10 μl. from a micrometer syringe. One eye in a young albino guinea pig is used for each dilution. Comparisons with the control eye are made at regular intervals for one hour under lighting conditions sufficiently bright for adequate observation without producing appreciable reflex pupillary constriction. The results are then interpreted in relation to those obtained with a standard direct inhibitor, such as TEPP. Precision may be improved, by testing two or more eyes with each dilution. A reflex pupillary constriction due to corneal pain or irritation may be distinguished from a true cholinergic miosis by its more rapid and transient nature, often with sympathetic miosis in the other eye.

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OBSERVATIONS ON THE EFFECT OF SULPHUR DIOXIDE IN BLACKCURRANT SYRUP ON THE DEVELOPMENT OF ANEURINE DEFICIENCY IN RATS

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Sulphur dioxide, 350 p.p.m., administered orally in blackcurrant syrup, once daily, in a dose of 0·5 ml./150 g. body weight, failed to influence the rate of growth of aneurine deficient rats.

Two facts concerning the toxicity of sulphite are already well known. Firstly, clear evidence has been presented that sulphite treatment of raisins[1], potatoes and cabbage[2] greatly reduces their aneurine content. Secondly, Williams, Waterman, Keresztesy and Buchman[3] demonstrated that the products of interaction between sulphite ions and aneurine molecules had no vitamin B_1 activity. Further evidence of the destruction of the aneurine in a solid diet by sulphite mixed with that diet was provided by Fitzhugh, Knudsen and Nelson[4]. These workers fed young rats with a diet which contained insufficient aneurine to maintain normal growth, and found that the addition of 0·05 per cent of sodium bisulphite, 307 p.p.m. as SO_2, further decreased their growth.

By contrast, no investigation of the effect of the oral administration of sulphite in a fluid medium on the development of aneurine deficiency in animals has been reported. Such an investigation appeared necessary because of the widespread use of sulphur dioxide in a concentration of 350 p.p.m. for the preservation of fruit juices. The experiments reported below constitute a preliminary examination of this problem. A blackcurrant syrup, to which 350 p.p.m. of sulphur dioxide had been added, has been administered orally, once daily to young rats which were fed a solid diet partially deficient in aneurine. The effect of this treatment on the rate of development of aneurine deficiency has been observed.

Methods

Hooded, inbred, female rats, weighing 85 to 95 g., were divided by weight into three comparable groups. They occupied individual cages with raised wide-meshed wire floors, thus ensuring that their droppings passed out of reach without delay. The atmosphere was well ventillated and thermostatically maintained within a temperature range of 65 to 70° F. Water and the prescribed diets were continually within reach of the animals, which were weighed twice a week.

Experimental diets were derived from a basic diet called D1 which consisted of dextrinised starch, 79 parts, casein, 19 parts, and Steinbock's salt mixture, 4 parts. The basic diet D1 was autoclaved to provide the experimental diets D2 to D4. Diet D2 was autoclaved for 2 hours, D3 for two periods of 2 hours, and D4 for 8 hours. Brewers' yeast 25 parts,

autoclaved for 6 hours, was added to these diets shown in Table I. In addition to the prescribed diet each rat received 0·5 ml. of cod-liver oil weekly by pipette.

The syrups were given by mouth in the early evening shortly before the rats became active and started eating. The control syrup contained sucrose 33 g., glucose 22 g., and fructose 16·5 g./100 ml. of water brought to pH 3·5 with N hydrochloric acid. It contained no sulphur dioxide. The fruit syrup was an extract of blackcurrant, sweetened with sucrose, and to which 350 p.p.m. of SO_2 were added. It had a pH of 3·5 and a composition as follows, sucrose 33·2 g., glucose 21·9 g., fructose 16·6 g., potassium 166 mg., sodium 1·4 mg., ascorbic acid 71 mg., and citric acid 1·7 g./100 ml. Syrups were administered by stomach tube, once a day, in a dose of 0·5 ml./150 g. rat.

Results

The results are summarised in Table I. In the first experiment parallel growth took place in the three groups of rats until the end of the sixth week. Growth continued in control group A throughout the seventh and eighth weeks, but was greatly reduced in groups B and C during the seventh week, and was arrested in these two groups during the eighth week. The rats of groups B and C developed anorexia, muscle weakness, and bradycardia during the eighth week. These symptoms clearly indicated aneurine deficiency in Diet D2. There was, moreover, significant difference in the eighth week between the mean weights of the control animals (group A) and the syrup treated animals fed the reduced aneurine diet (groups B and C); t calculated was 2·49, $P = < 0·05$. Since the rats

TABLE I

THE EFFECT OF A BLACKCURRANT SYRUP, CONTAINING SO_2 350 P.P.M., ADMINISTERED ORALLY, ONCE DAILY, IN A DOSAGE OF 0·5 ML./150 G. RAT, ON THE GROWTH OF RATS MAINTAINED ON DIETS DEFICIENT IN, BUT NOT FREE OF, ANEURINE

Expt.	Group	No. Rats	Mean weights in g. ± S.D. Intervals from onset of special diet and treatment					Diet	Treatment
			End of 4th week	End of 5th week	End of 6th week	End of 7th week	End of 8th week		
I	A	6	135 ± 3·8	146 ± 4·0	153 ± 6·1	162 ± 5·7	168 ± 5·8	D1 + yeast	Nil
	B	6	138 ± 5·2	150 ± 5·9	156 ± 5·6	158 ± 5·9	156 ± 6·3	D2 + yeast	Simple syrup
	C	5	134 ± 4·5	142 ± 4·5	151 ± 5·1	154 ± 6·2	154 ± 5·7	D2 + yeast	SO_2 syrup
			End of 4 weeks	End of 5 weeks	End of 6 weeks	End of 7 weeks	End of 8 weeks		
II	A	5	125 ± 5·2	134 ± 5·4	145 ± 5·6	155 ± 5·8	163 ± 5·5	D1 + yeast	Nil
	B	5	133 ± 6·2	143 ± 6·4	150 ± 6·3	155 ± 6·6	148 ± 6·0	D3 + yeast	Simple syrup
	C	5	130 ± 5·0	142 ± 5·2	153 ± 5·5	156 ± 5·6	151 ± 5·4	D3 + yeast	SO_2 syrup
			End of 3 weeks	End of 4 weeks	End of 5 weeks	End of 6 weeks	End of 7 weeks		
III	A	6	121 ± 5·0	132 ± 5·4	137 ± 5·4	141 ± 6·2	143 ± 6·5	D2 no yeast	Nil
	B	6	123 ± 7·2	130 ± 6·8	132 ± 7·0	134 ± 7·0	132 ± 6·9	D2 no yeast	Simple syrup
	C	6	129 ± 6·4	137 ± 6·5	141 ± 6·8	144 ± 6·8	144 ± 6·6	D2 no yeast	SO_2 syrup
			End of 4 weeks	End of 5 weeks	End of 6 weeks				
IV	A	6	125 ± 4·8	133 ± 5·1	142 ± 5·3			D1 + yeast	Simple syrup
	B	6	126 ± 5·2	129 ± 6·5	126 ± 6·1			D4 + yeast	Simple syrup

in groups B and C did not differ in their weight gains during the experiment, or in the severity of their symptoms during the eighth week, no toxicity could be ascribed to the fruit syrup containing 350 p.p.m. SO_2 administered to group C.

The diet fed to groups B and C in the second experiment had been increased in aneurine deficiency by further autoclaveing. Rats receiving this diet D3 showed reduced growth in the seventh week, lost weight and developed anorexia, muscle weakness, and bradycardia in the eighth week. Control rats fed diet D1 grew throughout the experiment. At the end of the experiment there was a significant difference between the mean weight per rat in Group A, and the corresponding mean weight for groups C and B; the value of t calculated was 2·61; $P = < 0{\cdot}05$. Again no deleterious effects from the sulphite in the blackcurrant syrup appeared; groups B and C grew, then failed to grow, in parallel.

The third experiment differed from those preceding in two respects: no autoclaved yeast was added to the diet, and all rats received diet D2. Normal growth was maintained in each of the three groups until the end of the fourth week of the experiment, but growth decreased in the fifth week and had ceased by the end of the seventh. Autoclaved yeast was added to the diet for the first three days of the eighth week; a mean weight increase of 2·2 g. $\pm$ 0·8 g. (S.D.) was observed on the 4th day. Autoclaved yeast was replaced by natural brewers' yeast on the 4th day for a period of three days. This produced a further mean weight increase of 6·9 $\pm$ 1·2 g. (S.D.) on the 7th day of yeast treatment. It may be concluded that a deficiency both of aneurine and of the B2 group vitamins had been induced in this experiment. Daily oral treatment with either simple syrup, or blackcurrant syrup containing 350 p.p.m. of SO_2 failed to influence the course of this deficiency.

In the fourth experiment the growth of rats (group A) receiving control diet D1 was compared with that of rats (group B) fed diet D4 which had been autoclaved for eight hours. Autoclaved yeast was added to both D1 and D4, and both groups of rats were treated with the control syrup. Growth proceeded normally in group A throughout the experiment. Failure of growth was observed in group B during the fifth week of the experiment, and the majority of rats in the group lost weight during the sixth week. A significant difference between the mean weights of animals in groups A and B became demonstrable in the sixth week; the value of t calculated was 4·55, $P = < 0{\cdot}01$. The experiment was planned to last for eight weeks, and was continued for this period. One animal in group B died in the seventh week; two further animals in this group died in the eighth. All animals in group B developed anorexia, extreme muscle weakness, and bradycardia, indicative of aneurine deficiency.

Discussion

Rats fed a basic diet which had been autoclaved for 8 hours in experiment IV showed more rapid failure of growth and more severe symptoms of aneurine deficiency than did rats which received the basic diet autoclaved for 2 and 4 hours in experiments I and II. It may therefore be

concluded that the diets used in experiments I, II, and III contained aneurine, but in amounts insufficient to supply the needs of the animals.

Once daily oral administration of a fruit syrup containing 350 p.p.m. of sulphur dioxide in a dose of 0·5 ml./150 g. rat proved no more toxic than a simple control syrup which contained no sulphur dioxide.

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A SIMPLE METHOD FOR THE PRODUCTION OF HIGH TITRE PENICILLINASE

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The culture filtrates of certain penicillinase-constitutive mutant strains of *B. cereus*, grown overnight in casein hydrolysate and citrate, can serve, without further treatment, as a source of stable, high-titre penicillinase suitable for inactivation of penicillin in sterility tests, blood cultures, etc.

Many laboratories in hospitals, research institutes and pharmaceutical establishments need supplies of penicillinase from time to time, largely in order to permit valid sterility tests on penicillin preparations or for bacteriological investigations on body fluids of patients under treatment with penicillin. The enzyme is produced by a wide range of bacteria[1,2] in extremely variable quantities. In the genus *Bacillus*, which gives the highest yields, it is usually adaptive, and penicillin must be added to the culture for optimal production. Under many conditions the enzyme is unstable and penicillinase activity may be lost rapidly. For most purposes the amount required is so modest that high titres and stable preparations, though desirable, may not be essential. However, the recent isolation[3,4] of penicillinase-constitutive mutant strains of *B. cereus*, forming very large quantities of the enzyme extracellularly in casein hydrolysate without the need for stimulation with penicillin, has provided a means by which a stable, high titre preparation can be assured in any laboratory, at minimal cost in time and effort.

Technique

Strains, B. cereus 5/B (NCTC9946[3]) or *B. cereus* 569/H (NCTC9945[4]), both penicillinase-constitutive mutant strains. Strain 569/H produces higher titres than strain 5/B, but is genetically, perhaps rather less stable, and for most purposes strain 5/B is probably preferable.

Medium. Casein hydrolysate, Difco, technical grade 10 g.; KH_2PO_4, 2·72 g.; sodium citrate, 5·88 g. dissolved in 200 ml. of water; concentrated NaOH solution added to give pH 7·2, and water to 1 l. It is sterilised by heating in an autoclave. A portion (100 ml.) of a solution containing 0·41 g. $MgSO_4.7H_2O$ and 1 ml. of a 0·16 per cent solution of $(NH_4)_2$ $Fe(SO_4)_2$, 6 H_2O is autoclaved and added to the solution separately.

Production. Using an inoculum of washed spores[6], or vegetative cells from a nutrient agar slope, the culture is left standing at any convenient temperature (between 18° and 37°) until growth becomes visible whereupon it is shaken in a conical flask aerobically at 35° to 37° for approximately 16 hours overnight. Efficient aeration is essential for maximal enzyme formation. The cells are separated by centrifugation, and the supernatant fluid retained. If sterilisation is not required, the

culture supernatant can be preserved by the addition of a few crystals of oxine (8-hydroxyquinoline). For many purposes this may be undesirable, when the supernatant can be sterilised by filtration through an Oxoid membrane filter (Oxo Ltd.). This may involve 20 per cent loss in activity, through adsorption to the membrane. Sintered glass or Seitz filters may adsorb most of the enzyme, particularly with preparations obtained from cultures grown in casein hydrolysate, and are therefore unsatisfactory. The sterile filtrate should contain from 2000 to 10,000 units of enzyme per ml. This compares with penicillinase activities of 15, 25, 190, 380 and 1300 units/ml. respectively found in unconcentrated preparations dispensed by five leading pharmaceutical firms trading in this country. The unit of penicillinase, previously defined[7], is that amount which will hydrolyse one micromole of benzylpenicillin per hour at 30° and pH 7·0 under conditions where the enzyme is saturated with substrate: that is, for practical purposes, at a penicillin concentration above 1000 units/ml. One-tenth ml. of the filtrate, as prepared, should therefore be able to destroy between 1,000,000 and 5,000,000 units of benzylpenicillin per hour under these conditions.

This activity corresponds to a production of from 6 to 30 μg. of pure penicillinase per ml. of culture. This single enzyme may, in fact, constitute up to 30 per cent of the total extracellular protein formed by the cells. It can, if necessary, be isolated from the culture medium, and finally crystallised, by a procedure already described[4,5]. For many purposes, however, the culture supernatant has sufficient activity and is quite satisfactory, without further treatment.

The sterilised filtrate should be kept in the refrigerator where its activity is maintained at least for several weeks. Alternatively, it can be freeze-dried and sealed in ampoules, where it can be preserved without loss of activity, certainly for many months.

Loss of activity in solution is due largely (*a*) to hydrolysis of the enzyme by a proteinase which may also be present in the culture medium and (*b*) to adsorption on solid surfaces, particularly glass, for which penicillinase has an unusually high affinity[4]. However, the incorporation of citrate in the culture medium removes the Ca^{++} which is essential both for formation and function of bacterial proteinase[7,8]; this factor is not therefore one which, in this method, will cause any serious decrease in activity. Loss of activity due to adsorption on glass is liable to occur if the enzyme preparation is given opportunity for fresh contact with glass surfaces such as may occur on pipetting or transfer from one vessel to another particularly if it is diluted in water or aqueous buffer solution. This loss can be completely prevented by the incorporation of 1 per cent gelatin in the solution. This, however, may not always be desirable and might have to be omitted in which case a certain amount of loss on dilution may be inevitable. This will not be serious if the technique recommended here is adopted because the initial penicillinase titres are so high that the proportional loss of activity will be low.

The fact that the Michaelis affinity constant of this enzyme for benzylpenicillin is approximately 20 units/ml.[9] should be borne in mind when

calculating the quantity of enzyme needed for *complete* destruction of a given amount of penicillin in a given time. As soon as the penicillin concentration falls much below 1000 units/ml. the enzyme will cease to be saturated with its substrate and the rate of hydrolysis will begin to decrease appreciably. It is, therefore, suggested that, for this purpose, at least 10 times the amount of enzyme theoretically needed under conditions where the enzyme is always saturated with substrate, be in fact added in order to ensure that the reaction is complete in the time required.

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THE STEPWISE ALKOXYLATION OF PHENYL PHOSPHORODICHLORIDATE

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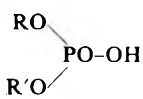
The reaction of phenylphosphorodichloridate (P.P.D.C.) with various alcohols in the presence of a tertiary base has been studied. With equimolar proportions of the P.P.D.C. and the requisite alcohol in light petroleum, the following compounds have been obtained: phenyl *n*-undecyl, phenyl *n*-butyl and phenyl methyl hydrogen phosphates. When two molar proportions of the alcohol in acetonitrile are added to P.P.D.C., phenyl bis-*n*-undecyl, phenyl bis-*n*-butyl and phenyl bis-*n*-amyl phosphates may be isolated. By hydrogenolysis (of the phenyl group diundecyl, dibutyl, and dimethyl hydrogen phosphates result.

The occurrence of phosphodiester linkages in biologically important compounds such as the phosphatides and the nucleic acids has stimulated research on mixed phosphoric esters. Methods of synthesising compounds of structure I have been devised, based upon the reactions of



(I)

hydroxylic compounds with phosphoric acid or phosphorus pentoxide[1,2], phosphorohalidates[3–9] or alkyl metaphosphates[29]. Alternatively, the reaction of metallic phosphates with halogen compounds has been employed and the synthesis of certain phosphatides by this method has been claimed[10–14]. Although the identity of many earlier products is in doubt, recent applications of this route have been more definitive[15].

Apart from this, the synthesis of phosphatides has been based almost entirely upon the stepwise replacement of the chlorine atoms of phosphoryl chloride or its derivatives in the presence of tertiary base[16–18]. Monoalkoxylation of phosphoryl chloride is difficult to achieve, and the most successful synthetic application of this type[19] has involved the stepwise alkoxylation of phenyl phosphorodichloridate (II). For example, a number of lecithins (VI, R = $CH_2OCOR''{\cdot}CHOCOR''{\cdot}CH_2$–, R' = $CH_2CH_2{}^{+}NMe_3$) have been produced in this manner[19].

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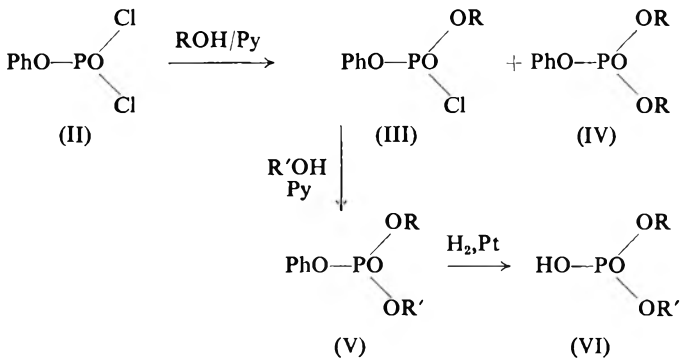
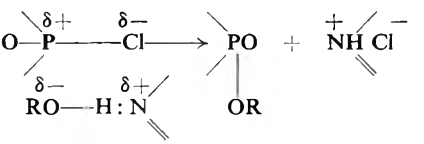


Whilst in the case of glycerides with acyl chains C_{18} and C_{16} (stearoyl and palmitoyl) the amount of unwanted tertiary ester IV remained small[20], repetition by us of published details using glycerides with smaller acyl chains (C_{10} and C_{12})[30] led to much greater amounts of tertiary ester being formed. In addition we were unable to isolate any lecithin.

For this reason the present work was undertaken to study the relative rates of the two alkoxylation steps (II → III and III → V) and to delineate, if possible, conditions which would permit their definite separation.

The reaction of an aliphatic alcohol with phenyl phosphorodichloridate and pyridine was followed by determination of the chloride ion liberated according to the equation



Since the reaction takes place initially by way of a dipole-dipole interaction[21] the rate at which it proceeds should be small, and hence more amenable to investigation, in a non-polar solvent.

Initially the case of *n*-undecanol and phenyl phosphorodichloridate was considered. When molar proportions of these and pyridine were allowed to react in light petroleum at 5°, formation of chloride ion was fairly rapid, and by the end of 60 minutes was equivalent to the complete removal of one chlorine of phenyl phosphorodichloridate (Fig. 1, Expt. 1). If two molar proportions of *n*-undecanol were used, this same amount of chloride ion was formed almost immediately. Thereafter, the rate of reaction decreased markedly (Fig. 1, Expt. 2). It thus appeared that in light petroleum at low temperature and using only the theoretical quantity of the alcohol, alkoxylation could largely be limited to substitution of one chlorine only.

Accordingly the isolation of an intermediate phosphorochloridate was attempted. Although this proved impracticable for the pure halides, hydrolysis of these with alkali yielded the free acids, and reaction with an amine, the amides: alkyl phenyl phosphates (VII), and alkyl phenyl

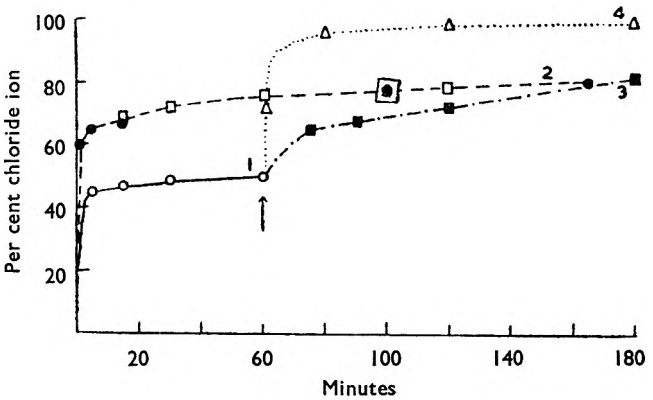


Fig. 1. Reaction of alcohols with P.P.D.C. in the presence of pyridine. At arrow, pentanol added in expt. 3 and 4.

○——○ *n*-Undecanol 1, P.P.D.C. 1 mol. eq.

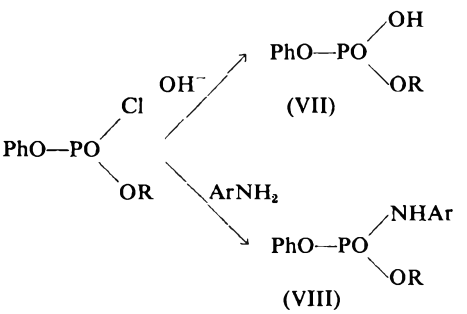
●——● *n*-Undecanol 2, P.P.D.C. 1 mol. eq., direct method.

□——□ Ditto, indirect method.

■——■ *n*-Undecanol, 1, P.P.D.C., 1, followed after 1 hour by *n*-pentanol 1 mol. equiv. in petroleum.

△——△ Ditto, *n*-pentanol in acetonitrile.

phosphoroamidates (VIII) respectively. In this way compounds 1 to 6 in Table I were prepared. Yields of the derivatives VII and VIII with *n*-undecanol and *n*-butanol provide confirmatory evidence that the main reaction in this stage is as stated above.

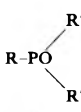


In light petroleum the replacement of the second chlorine was extremely slow. Thus, when one molar equivalent of a second alcohol (*n*-pentanol) was added in light petroleum to crude phenyl undecyl phosphorochloridate in the presence of pyridine, the amount of chloride liberated was only 60 per cent of theory even after $2\frac{1}{2}$ hours (Fig. 1, Expt. 3). Reactions of this type, as has been shown by Dostrovsky and Halmann[22,23] are bimolecular nucleophilic substitutions, and as such are accelerated in polar solvents. When crude phenyl *n*-undecyl phosphorochloridate was allowed to react as above with *n*-pentanol, in acetonitrile instead of light petroleum, elimination of a second equivalent of chloride ion was much more rapid, being essentially complete in about 60 minutes (Fig. 1, Expt. 4).

Tertiary esters (V; R = R′ = alkyl) were therefore readily prepared by allowing phenyl phosphorodichloridate to react with two molar equivalents of an alcohol and base in acetonitrile. Compounds 7 to 9 (Table I) were prepared in this manner. Hydrogenolysis of these dialkyl phenyl phosphates gave the free acids (VI; R = R′ = alkyl) in good yield as indicated (compounds 10 and 11).

TABLE I

ESTER DERIVATIVES OF PHOSPHORIC ACID



Ph = C_6H_5 Bu = n-C_4H_9

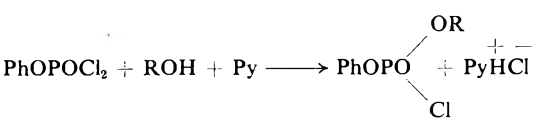
Un = n-$C_{11}H_{23}$ Am = n-C_5H_{11}

Me = CH_3

No.	R	R″	R′	cpd. isolated	m.p.	yield* per cent
1	PhO	UnO	PhNH	amide	51–51·5°	59
2	PhO	UnO	HO	Ba salt	—	66
3	PhO	BuO	β-$C_{10}H_7NH$	amide	93–93·5°	59
4	PhO	BuO	HO	Ag salt	—	59
5	PhO	MeO	PhNH	amide	77–77·5°	32
6	PhO	MeO	HO	Ba salt	—	43
7	PhO	UnO	UnO	ester	—	73
8	PhO	BuO	BuO	ester	b·p·125°/0·1	58
9	PhO	AmO	AmO	ester	b·p·135°/0·05	48
10	HO	UnO	UnO	free acid	53·5–54	72
11	HO	BuO	BuO	Ba salt	—	54
12	HO	MeO	MeO	Pb salt	141–142°	15

* Based upon P.P.D.C.

While yields of the alkylphenyl secondary, and symmetrical dialkylphenyl tertiary esters, actually isolated (reactions II → III and III → IV respectively) were fair for preparative purposes, they fell short of those suggested by estimation of the chloride ion produced. The discrepancy seems unlikely to be due to experimental methods of isolation, and the reaction



appears to be complicated by a consecutive side reaction.

Attempts to synthesize mixed dialkylphenyl phosphates under the reaction conditions delineated above, were less successful When Experiment 4 (see p. 616) was repeated on a preparative scale, we were unable to isolate any single pure fraction using chromatographic methods employed successfully elsewhere in this paper, although some of the required *n*-amyl phenyl *n*-undecyl phosphate was undoubtedly formed Exchange of alkyl radicles in highly polar solvents such as acetronitrile would seem a likely competing side reaction: attack of any tertiary base used upon the phosphorochloridate has already been suggested[21] The nature of such side reactions however has not been investigated.

EXPERIMENTAL

Melting and boiling points are uncorrected. Microanalyses were performed by Mr. G. S. Crouch of this school, and by Drs. Weiler and Strauss, Oxford.

Materials

Phenyl phosphorodichloridate, prepared by the interaction of phenol and phosphoryl chloride[24,25], had b.p. 241 to 242°. Found: C, 34·3; H, 2·2; Cl, 33·6. Calc. for $C_6H_5O_2PCl_2$, C, 34·2; H, 2·4; Cl, 33·6 per cent.

n-*Undecanol* obtained by $LiAlH_4$ reduction of ethyl 9-undecenoate followed by hydrogenation of the double bond over palladium black, had b.p. 131°/14 mm., $n_D^{23°}$ 1·4392, saponification value zero.

Other materials, of reagent grade, were dried and redistilled.

Methods. Rate of Reaction of Phenyl Phosphorodichloridate (P.P.D.C.) with some Aliphatic Alcohols in the Presence of Pyridine

All reactions were conducted at 2 to 5°. The results of the undernoted experiments 1 to 4 are given graphically in Figure 1.

n-*Undecanol*

1. In each of five 50 ml. flasks a 2M solution of P.P.D.C. in light petroleum (b.p. 40 to 60°) (2·0 ml.) was rapidly mixed with a 2M solution of pyridine and *n*-undecanol (2·0 ml.). At intervals the contents of a flask were filtered, the precipitate washed rapidly with solvent and dissolved in water. Ionised chlorine in the aqueous solution was estimated gravimetrically as silver chloride. (Direct method.)

2. The experiment was repeated with twice the volume of alcohol-base solution. A duplicate experiment was made in which the reaction mixture was diluted with light petroleum (50 ml.), the chloride extracted with half-saturated ammonium nitrate solution and estimated gravimetrically. (Indirect method.) This procedure gave results which agreed well with those obtained by the direct method, and which were unaffected by the addition of acetonitrile (6·0 ml.).

n-*Undecanol and* n-*Pentanol*

Experiment 1 was prepared again and the reaction allowed to proceed for 1 hour. A 2M solution of pyridine and *n*-pentanol in light petroleum (b.p. 40 to 60°: 2·0 ml.) was added to the contents of each flask, and chloride determined in successive flasks at intervals by the direct method.

4. Experiment 3 was repeated with acetonitrile as solvent for the second stage: after one hour's reaction between *n*-undecanol, pyridine and P.P.D.C., light petroleum was removed *in vacuo* without heating. A molar solution of *n*-pentanol and pyridine in acetonitrile (4·0 ml.) was added, and chloride estimated in successive flasks at intervals by the indirect method.

n-*Undecanol and* 2-*Nitroethanol*

5. Experiment 4 was repeated with 2-nitroethanol in place of *n*-pentanol. After initial difficulties due probably to interaction of the

base with 2-nitroethanol[27], the same type of curve as in experiment 4 was obtained.

Derivatives of Alkyl Phenyl Phosphorochloridates

Phenyl n-*undecyl phosphorochloridate.* In a flask equipped with dropping funnel, sealed stirrer and guard tube was placed a solution of P.P.D.C. (2·11 g., 0·01 mole) in dry light petroleum (b.p. 60 to 80°; 20 ml.). The flask was immersed in an ice bath, and a solution in the same solvent (20 ml.) of *n*-undecanol (1·72 g., 0·01 mole) and dry pyridine (0·79 g., 0·01 mole) was added dropwise with stirring over a period of 15 min. The addition complete, stirring was continued at room temperature for 1 hr. The precipitated pyridine hydrochloride (1·16 g. calculated, 1·18 g.) was filtered off, and the filtrate concentrated at the pump, yielding crude *phenyl undecyl phosphorochloridate.* The n-*butyl* and *methyl* homologues were similarly prepared. An attempt to distil the methyl homologue under reduced pressure resulted in decomposition.

ON-*Diphenyl* O-n-*undecyl phosphoroamidate.* Crude phenyl undecyl phosphorochloridate (from 2·11 g. P.P.D.C.) was refluxed with aniline (1·86 g., 0·02 mole) in dry ethanol-free chloroform (40 ml.) for 4 hr. Solvent was removed at the pump, the residue dissolved in light petroleum (b.p. 60 to 80°) and the solution washed successively with N HCl, 1 per cent $NaHCO_3$ and water. After drying (Na_2SO_4) the solution was concentrated at the pump to yield a reddish-brown oil (3·64 g., 73 per cent) which was chromatographed on silica gel[30]. Isorefractive fractions were bulked, dissolved in dry ether, filtered (charcoal) and the ether evaporated. Colourless crystals of ON-*diphenyl* O-*undecyl phosphoroamidate* (2·41 g., 59 per cent) were obtained, m.p. 49 to 50°. For analysis the compound was twice recrystallised from aqueous ethanol; m.p. 51 to 51·5°. Found: C, 68·5; H, 8·5; N, 3·5; P, 7·7. $C_{23}H_{34}NO_3P$ requires: C, 68·5; H, 8·5; N, 3·5; P, 8·1 per cent. The following were prepared similarly:

O-n-*Butyl* N-2-*naphthyl* O-*n-phenyl phosphoroamidate*, isolated in 59 per cent yield as an oil which was crystallised from aqueous ethanol; m.p. 90 to 91°. For analysis the compound was recrystallised from ether-light petroleum (2:5); m.p. 93 to 93·5°. Found: C, 67·2; H, 6·5; N, 4·0; P, 8·5. $C_{20}H_{22}NO_3P$ requires: C, 67·6; H, 6·2; N, 3·9; P. 8·7 per cent.

O-*Methyl* ON-*diphenyl phosphoroamidate*, isolated in 36 per cent crude yield: m.p. 65 to 70°. The compound, twice recrystallised from ether-light petroleum (1:10), had m.p. 77 to 77·5°. Found: C, 60·5; H, 5·2; N, 5·1; P, 11·4. $C_{13}H_{14}O_3NP$ requires: C, 59·3; H, 5·4; N, 5·3; P, 11·8 per cent.

Phenyl n-*undecyl phosphate.* Crude phenyl *n*-undecyl phosphorochloridate (from 10·55 g. P.P.D.C.) was shaken with N NaOH (100 ml.) for 2 hr., the solution neutralised with N HCl, extracted with chloroform and treated with a slight excess of 10 per cent $BaCl_2$ solution. The precipitate, washed well with water and dried *in vacuo*, weighed 13·0 g. (66 per cent). For analysis the salt was twice recrystallised from methanol-acetone (2:1). Found: C, 51·9; H, 7·4; P, 8·7. $C_{34}H_{56}O_8P_2Ba$ requires: C, 51·6; H, 7·1; P, 7·8 per cent.

The barium salt (1·00 g.) was dissolved in boiling methanol and a slight excess of 0·1N HNO_3 added. The methanol was distilled off and the residue extracted with chloroform. The chloroform extract was divided into two parts, each of which was concentrated and dried to constant weight. Yield of *phenyl* n-*undecyl phosphate*, 0·81 g. (98 per cent), titration equivalent 332. $C_{17}H_{29}O_4P$ requires 328.

n-*Butyl phenyl phosphate.* Crude *n*-butyl phenyl phosphorochloridate (from 10·55 g. P.P.D.C.) was hydrolysed with N NaOH (100 ml.), the neutralised solution extracted with chloroform and acidified. The liberated acid was extracted into chloroform. Removal of the solvent left crude n-*butyl phenyl phosphate* (8·88 g., 77 per cent), titration equivalent 238; $C_{10}H_{15}O_4P$ requires 230.

The crude acid (8·0 g.) dissolved in acetonitrile (90 ml.) was shaken with silver oxide (3·2 g.) for 2 hours, the unreacted oxide filtered off and the filtrate diluted with ether (400 ml.).* After standing overnight at 0° the precipitate was collected, washed with ether and dried. Yield of *silver butyl phenyl phosphate*, 7·2 g. (59 per cent). For analysis the salt was thrice recrystallised from chloroform-benzene (1:4). Found: C, 35·8; H, 4·3; P, 9·2; Ag 32·3; equivalent weight (by titration with 0·1N KCl) 339. $C_{10}H_{14}O_4PAg$ requires: C, 35·6; H, 4·2; P, 9·2; Ag, 32·0 per cent; equivalent weight 337.

Extraction of an acidified aqueous solution of the silver salt (1·10 g.) with chloroform and removal of the solvent gave n-*butyl phenyl phosphate* (0·74 g., 99 per cent) as an oil. Titration equivalent 231; $C_{10}H_{15}O_4P$ requires 230.

Methyl phenyl phosphate. Crude methyl phenyl phosphorochloridate (from 10·55 g. P.P.D.C.) was hydrolysed as described for the butyl homologue. To the neutralised aqueous solution was added sodium chloride (30 g.) and 2N H_2SO_4 (25 ml.). Extraction with chloroform and removal of the solvent gave a residue (6·25 g.) which was dissolved in water. The solution was warmed with a slight excess of barium carbonate and filtered into a large excess of acetone. The precipitate was collected, washed with acetone and dried. Yield 4·4 g., (43 per cent). For analysis the compound was thrice recrystallised from methanol-ethanol (1:7). Found: C, 32·7; H, 3·2; P, 11·9. $C_{14}H_{16}O_8P_2Ba$ requires: C, 32·9; H, 3·2; P, 12·1 per cent. The free acid, isolated in a manner similar to that described for the butyl homologue, had a titration equivalent of 189. $C_7H_9O_4P$ requires 188.

Dialkyl Phenyl Phosphates and Dialkyl Phosphates

Phenyl di-n-undecyl phosphate. In a flask equipped with sealed stirrer, dropping funnel and guard tube was placed a solution of P.P.D.C. (7·03 g.) in dry acetonitrile (17 ml.). A solution of *n*-undecanol (11·46 g.) and dry pyridine (5·20 g.) in dry acetonitrile (10 ml.) was added dropwise over a period of 15 min. Stirring was continued for 3 hr. The solution was poured into 0·1N HCl (250 ml.) and the ester extracted into light

* The silver salt was protected from light as far as possible.

petroleum. The organic layer was washed with N HCl and dried over Na_2SO_4 in the presence of $NaHCO_3$. The dry, filtered solution was diluted with dry light petroleum to 100 ml., and 10 ml. chromatographed upon silica gel.[30] Isorefractive fractions totalled 1·17 g. (73 per cent). This material was again chromatographed for analysis. Found: C, 70·3; H, 10·9; P, 6·3. $C_{28}H_{51}O_4P$ requires: C, 69·7; H, 10·6; P, 6·4 per cent. $n_D^{17·5°}$ 1·4691; d_4^{22} 0·979. R_L found, 138; calculated, 140. The remainder of the crude product was worked up similarly.

Di-n-*undecyl phosphate.* Phenyl di-*n*-undecyl phosphate (4·84 g.) dissolved in ethanol (30 ml.) was hydrogenated at atmospheric pressure over Adams' platinum oxide (0·5 g.) until the theoretical volume of hydrogen had been absorbed. The catalyst was filtered off and the filtrate adjusted to 100 ml. Titration of two 10 ml. portions confirmed that hydrogenolysis was virtually complete. Treatment of a further 10 ml. portion with benzylamine (0·11 ml.) and evaporation of the solvent gave a gummy residue; the benzylamine salt, if formed, could not be crystallised.

The remaining 70 ml. was evaporated to small volume and set aside at room temperature. After two days a crystalline mass (2·80 g., 98 per cent) was obtained; this was purified through its sodium salt. Dissolved in ethanol, the crude acid was treated with the theoretical volume of boiling 0·1N NaOH, the solution cooled to about 50° and acetone added until a slight permanent turbidity was produced. After some hours at 0° the sodium salt was collected, washed with water, acetone and ether and dried at 100°. Yield, 2·05 g. (70 per cent.) For analysis the salt was twice recrystallised from ethanol-water-acetone (1:2:7). Found: C, 59·5; H, 10·9; P, 6·7. $C_{22}H_{46}O_4PNa,H_2O$ requires: C, 59·2; H, 10·8; P, 6·9 per cent. The water of crystallisation was not completely removed by drying at 100°/0·1 mm. for 24 hr.

The pure acid was recovered by dissolving the sodium salt (1·0 g.) in ethanol (10 ml.) and adding a slight excess of 0·1N HCl. The suspension was warmed until the precipitate started to melt, cooled a little, and ethanol added until a clear solution resulted. After some hours at room temperature the crystalline product was collected, washed with 25 per cent ethanol and dried *in vacuo*. Yield, 0·75 g. (79 per cent), m.p. 53·5 to 54°. Found: C, 65·6; H, 11·7; P, 7·5; titration equivalent, 407. $C_{22}H_{47}O_4P$ requires C, 65·0; H, 11·6 P, 7·6 per cent; titration equivalent 407.

Di-n-*butyl phenyl phosphate.* The crude ester, prepared from 31·6 g. P.P.D.C. as described for the undecyl homologue, was purified by fractional distillation. Two distillations gave 24·5 g. (58 per cent), b.p. 125 to 125·5°/0·1 mm., n_D^{22} 1·4740, d_4^{22} 1·079, R_L found, 75; calculated, 75. Found: C, 58·8; H, 8·2; P, 10·7. $C_{14}H_{23}O_4P$ requires: C, 58·7; H, 8·1; P, 10·8 per cent.

Di-n-*amyl phenyl phosphate* was similarly prepared in 48 per cent yield, b.p. 134 to 135°/0·05 mm., n_D^{22} 1·4715, d_4^{22}1·053, R_L calculated, 84; found, 84. Found: C, 61·0; H, 8·9; P, 9·7. $C_{16}H_{27}O_4P$ requires: C, 61·2; H, 8·6; P, 9·9 per cent.

*Di-*n*-butyl phosphate.* Di-*n*-butyl phenyl phosphate (14·3 g.) dissolved in ethanol (40 ml.) was hydrogenolysed as described for the undecyl homologue. The filtered solution was adjusted to 100 ml. and two 10 ml. aliquots titrated to confirm complete hydrogenolysis. The rest was treated with a solution of crystalline barium hydroxide (6·3 g.) in boiling water (10 ml.), then with excess acetone. The collected gelatinous precipitate was washed with acetone and dried. Yield, 10·3 g. (93 per cent). For analysis the salt was twice recrystallised from absolute ethanol-water (60:1). Found: C, 34·6; H, 6·5; P, 11·2. $C_{16}H_{36}O_8P_2Ba$ requires: C, 34·6; H, 6·5; P, 11·1 per cent.

The free acid was isolated in 84 per cent yield as described for butyl phenyl phosphate. Titration equivalent, 212; $C_8H_{19}O_4P$ requires 210.

Dimethyl phenyl phosphate. The crude ester, prepared as described for the butyl homologue from 21·1 g. P.P.D.C., gave after extensive fractionation 4·45 g. of a liquid, b.p. 92·5°/0·1 mm., the analytical figures for which corresponded poorly with those required for the ester. Found: C, 45·9; H, 5·7; P, 14·3. $C_8H_{11}O_4P$ requires: C, 47·5; H, 5·5; P, 15·3 per cent.

Dimethyl phosphate. The above compound (7·4 g.) was hydrogenated as described for the butyl homologue until uptake ceased. The filtered solution was diluted to 200 ml. with ethanol, refluxed gently for 10 min. with lead monoxide (4·1 g.) and filtered while hot. The cooled solution deposited lead dimethyl phosphate (6·7 g., 80 per cent), m.p. 141·5 to 142·5°. The m.p. was not raised by recrystallisation from ethanol. A previous claim to have obtained this compound, m.p. 155°, would seem to be in doubt[28]. Found: C, 10·7; H, 2·7; P, 13·2; Pb, 46·3. $C_4H_{12}O_8P_2Pb$ requires: C, 10·5; H, 2·7; P, 13·5; Pb, 45·3 per cent.

The free acid was obtained by passing H_2S through a warm ethanol solution of the lead salt, filtering and concentrating the filtrate. Yield of dimethyl phosphate 98 per cent. Titration equivalent 127; $C_2H_7O_4P$ requires 126.

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CHRONIC TOXICITY STUDIES ON FOOD COLOURS

PART III. OBSERVATIONS ON THE TOXICITY OF MALACHITE GREEN, NEW COCCINE AND NIGROSINE IN RATS

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New Coccine and Nigrosine, at levels of 0·03, 0·3 and 3·0 per cent in the diet, had no effect on mortality in male or female rats. In groups receiving Malachite Green at levels of 0·3 and 3·0 per cent in the diet there was 100 per cent mortality within the first week of the experiment. Female rats receiving Malachite Green at 0·03 per cent or Nigrosine at 3·0 per cent in the diet showed a significant decrease in growth rate. For the group on Malachite Green this was accompanied by a decrease in food consumption. In both male and female rats receiving Nigrosine at 3·0 per cent in the diet, there was an increase in the organ-body weight ratio for liver and kidneys. Histopathological changes attributed to the toxic effects of the colours were noted in kidneys and testes.

The work reported here is part of a continuing study on the chronic toxicity of food colours. Reports have been presented previously on the toxicity of Oil Yellow AB, Oil Yellow OB, Light Green SF Yellowish Orange SS and Oil Red XO[1,2]. Malachite Green, New Coccine, and Nigrosine are not included in the permitted list of food colours for Canada but are in use in some countries.

METHODS

The methods used were similar to those described in the first two papers of this series[1,2]. The colours were added to the normal laboratory diet in dry form to give concentrations of 0·03, 0·3 and 3·0 per cent for each dye. The rats, which were five to six weeks of age at the start of the experiment, were kept in individual cages and were given free access to food and water. Groups of ten males and ten females were assigned to each concentration level of each food colour. Ten rats of each sex were also used as control groups. Weight and food consumption were recorded weekly. Post-mortem examinations were made where possible on rats which died on test. At the termination of the experiment, the surviving rats were killed and post-mortem examinations made. Some of the organs were weighed and prepared for histological examination.

RESULTS AND DISCUSSION

Mortality

The mortality of control and test rats during the period of the experiment (64 weeks) is indicated in Table I. It seems logical to conclude that

TABLE I

CUMULATIVE NUMBER OF DEATHS

Treatment	Conc. of colour (per cent in diet)	No. rats on test	Time in weeks on test																
			1	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64
Males																			
Control		10	0	0	0	0	0	1	2	3	4	4	4	4	4	4	4	4	5
Malachite Green	0·03	10	1	1	1	2	2	3	4	4	4	5	5	5	6	6	6	6	7
	0·3	10	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	3·0	10	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
New Coccine	0·03	10	0	0	0	0	0	2	3	4	4	5	5	5	6	6	6	6	7
	0·3	10	1	1	1	2	2	2	2	2	2	4	4	4	4	4	4	6	6
	3·0	10	0	0	0	0	1	1	1	2	3	4	4	4	4	4	4	4	4
Nigrosine	0·03	10	0	0	0	0	1	1	2	2	2	2	2	2	2	3	3	4	5
	0·3	10	0	0	0	0	2	2	2	2	2	3	3	3	4	4	4	5	5
	3·0	10	0	0	0	0	0	0	0	0	0	0	2	2	3	3	3	6	6
Females																			
Control		10	0	0	0	0	0	0	1	1	1	1	1	1	1	3	3	3	3
Malachite Green	0·03	10	0	0	0	0	0	0	2	2	2	3	3	4	4	4	4	4	4
	0·3	10	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	3·0	10	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
New Coccine	0·03	10	0	1	2	2	2	2	2	2	2	3	3	3	4	4	4	6	6
	0·3	10	0	0	0	0	0	0	0	0	1	2	3	4	5	5	5	6	6
	3·0	10	0	0	0	0	0	0	1	1	1	1	2	2	2	2	2	3	3
Nigrosine	0·03	10	0	0	0	1	1	1	3	3	4	4	4	4	4	4	5	5	5
	0·3	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
	3·0	10	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	4	5

neither New Coccine nor Nigrosine, in the dose range used, had any effect on mortality. There was no correlation of mortality rate and concentration of dye for these two colours. All rats on the two higher levels of Malachite Green died within the first week of the experiment. Obviously these dosages were not suitable for a chronic toxicity study for this dye. There is some indication that Malachite Green at 0·03 per cent in the diet affected the mortality rate in the first half of the experiment, especially for male rats.

Growth, Food Consumption and Food Efficiency

Weight, food consumption and food efficiency data for the various test groups are presented in Tables II, III and IV. From the results

TABLE II

MEAN BODY WEIGHT OF RATS FED MALACHITE GREEN, NEW COCCINE, AND NIGROSINE

Treatment	Dosage (per cent of diet)	No. rats surviving No. rats on test	Mean body weight (g±S.E.)				
			Initial	16 weeks	32 weeks	48 weeks	64 weeks
Males							
Control		5/10	63±2	286±9	321±6	315±10	314±12
Malachite Green	0·03	3/10	60±3	259±12	323±12	336±12	346±11
New Coccine	0·03	3/10	64±2	270±9	317±18	327±20	352±36
	0·3	4/10	62±4	285±11	327±18	374±22*	354±12
	3·0	6/10	63±3	271±12	310±10	333±10	326±14
Nigrosine	0·03	5/10	61±4	263±10	307±15	324±15	294±13
	0·3	5/10	65±4	268±18	300±17	329±14	327±28
	3·0	4/10	65±3	290±5	346±10	358±9*	337±5*
Females							
Control		7/10	62±4	184±5	220±6	236±5	246±8
Malachite Green	0·03	6/10	59±2	169±2*	197±6*	214±6*	197±2*
New Coccine	0·03	4/10	61±3	177±6	202±9	243±5	254±12
	0·3	4/10	61±3	176±8	198±9	233±9	241±2
	3·0	7/10	60±3	167±3*	203±6	227±5	232±5*
Nigrosine	0·03	5/10	61±3	178±2	203±7	225±5	231±7
	0·3	9/10	59±1	175±2	207±2	228±5	233±5
	3·0	5/10	60±2	165±6*	194±8*	215±3*	227±3*

* Significant at P = 0·05 or less

in Table II the consistent effect on growth rate occurred only in female rats receiving 0·03 per cent Malachite Green and 3·0 per cent Nigrosine. These groups showed a significant decrease in growth rate throughout

the course of the experiment. For the group on Malachite Green this retardation was partly due to a decrease in food consumption as shown in Table III. There was no significant change in food consumption for the 3 per cent Nigrosine group. Table III also shows that female rats receiving 3·0 per cent New Coccine and 0·03 per cent Nigrosine had a lower mean food consumption throughout the experiment than did the

TABLE III

MEAN FOOD CONSUMPTION OF RATS FED MALACHITE GREEN, NEW COCCINE, AND NIGROSINE

Treatment	Dosage (per cent of diet)	Mean food consumption g./rat/day ± S.E.			
		16 weeks	32 weeks	48 weeks	64 weeks
Males					
Control		15·7 ± 0·4	16·4 ± 0·7	16·6 ± 0·3	16·9 ± 0·4
Malachite Green	0·03	15·2 ± 0·2	16·9 ± 0·5	17·1 ± 0·4	17·3 ± 0·7
New Coccine	0·03	16·0 ± 0·3	17·2 ± 0·7	17·5 ± 0·4	18·5 ± 0·4*
	0·3	15·7 ± 0·4	17·2 ± 0·4	17·9 ± 0·5*	18·1 ± 0·3*
	3·0	15·2 ± 0·4	15·3 ± 0·4	15·5 ± 0·4*	15·9 ± 0·4
Nigrosine	0·03	15·5 ± 0·4	16·9 ± 0·6	17·5 ± 0·6	16·9 ± 0·6
	0·3	15·4 ± 0·3	16·4 ± 0·6	17·9 ± 0·6	18·4 ± 0·6
	3·0	16·4 ± 0·4	17·8 ± 0·4	18·1 ± 0·5*	17·6 ± 0·3
Females					
Control		13·2 ± 0·2	14·1 ± 0·2	14·5 ± 0·3	14·9 ± 0·3
Malachite Green	0·03	11·8 ± 0·4*	12·4 ± 0·3*	13·0 ± 0·2*	13·2 ± 0·4*
New Coccine	0·03	12·7 ± 0·4	13·2 ± 0·3*	14·0 ± 0·4	13·9 ± 0·9
	0·3	13·1 ± 0·2	14·2 ± 0·4	15·9 ± 0·4*	16·0 ± 0·4
	3·0	11·3 ± 0·2*	12·5 ± 0·1*	13·4 ± 0·3*	13·7 ± 0·4*
Nigrosine	0·03	12·2 ± 0·4*	12·3 ± 0·4*	13·1 ± 0·2*	13·5 ± 0·3*
	0·3	13·0 ± 0·3	14·1 ± 0·2	14·8 ± 0·4	15·0 ± 0·4
	3·0	12·6 ± 0·4	13·6 ± 0·3	14·2 ± 0·4	14·0 ± 0·4

* Significant at P = 0·05 or less

control group. This did not produce any significant change in body weight except at 16 weeks for the rats on New Coccine. There were a few other instances where the food consumption differed significantly from the control values but only two of these were accompanied by corresponding changes in body weight.

There was little effect of the colours on food efficiency (expressed as g. body weight gained per hundred g. of food consumed) as shown in Table IV. In only two cases of all the data tested was there a significant P value found and neither of these occurred in groups receiving the highest concentration of dye.

TABLE IV

MEAN FOOD EFFICIENCY DATA FOR RATS FED MALACHITE GREEN, NEW COCCINE AND NIGROSINE

Treatment	Dosage (per cent of diet)	Mean food efficiency g. gained/100 g. food consumed ± S.E.			
		16 weeks	32 weeks	48 weeks	64 weeks
Males					
Control		13·0±0·5	7·2±0·2	4·6±0·1	3·4±0·1
Malachite Green	0·03	12·0±0·6	7·3±0·2	4·8±0·3	3·7±0·4
New Coccine	0·03	11·8±0·4	6·6±0·3	4·4±0·3	3·5±0·5
	0·3	13·2±0·5	7·0±0·4	5·3±0·3	3·7±0·3
	3·0	12·5±0·5	7·2±0·3	5·2±0·3	3·7±0·2
Nigrosine	0·03	11·9±0·4	6·6±0·1*	4·5±0·2	3·3±0·2
	0·3	12·2±0·8	6·5±0·3	4·4±0·3	3·2±0·3
	3·0	12·6±0·2	7·2±0·1	4·9±0·1	3·5±0·3
Females					
Control		8·7±0·3	5·2±0·1	3·6±0·2	2·8±0·1
Malachite Green	0·03	8·3±0·4	5·1±0·3	3·7±0·1	2·4±0·2
New Coccine	0·03	8·3±0·2	4·8±0·2	3·8±0·2	3·0±0·1
	0·3	8·1±0·3	4·3±0·3*	3·2±0·1	2·5±0·2
	3·0	8·7±0·5	5·2±0·1	3·7±0·2	2·7±0·1
Nigrosine	0·03	8·8±0·2	5·1±0·2	3·7±0·2	2·7±0·2
	0·3	8·2±0·3	4·8±0·2	3·4±0·1	2·6±0·2
	3·0	7·8±0·5	4·5±0·4	3·4±0·2	2·8±0·2

* Significant at P = 0·05 or less

Organ Weights

Certain organs of the rats killed upon termination of the test were weighed. The mean weights (in mg./g. of weight) of these organs are given in Table V. Significant weight changes occurred most often in liver and kidneys but were also recorded for heart, spleen and testes. The effect on liver weight occurred with four groups and in each case was an increase over the corresponding control value. The increase was marked in both male and female rats on the diet containing 3 per cent of Nigrosine. It may be mentioned that no pathological changes in liver were recorded for any group. Of four significant changes in kidney weights, three were in groups receiving Nigrosine. One of these, on the low dose level, was a decrease in weight, whereas the other two, both at the 3 per cent level of Nigrosine, were increases over the control values.

Histopathology

A detailed examination was made of the haematoxylin-eosin stained paraffin sections of a number of organs including lung, heart, liver,

spleen, thyroid, pancreas, stomach, small intestine, kidney, urinary bladder, adrenal, testes, ovaries and thymus. A summary of the findings is given in Table VI.

The histopathological changes that were attributed to the toxic or adverse effects of the colours were those noted in the kidneys and testes.

TABLE V

ORGAN WEIGHTS OF RATS FED MALACHITE GREEN, NEW COCCINE AND NIGROSINE

Treatment	Dosage (per cent of diet)	No. rats sacrificed	Mean organ weight (mg./g. rat ± S.E.)					
			Heart	Liver	Spleen	Kidneys	Gonads	Adrenals
Males								
Control ..		5	4·5±0·6	31·1±1·3	2·3±0·1	8·2±0·2	8·1±0·3	0·11±0·02
Malachite Green ..	0·03	3	3·7±0·4	34·7±1·7	2·4±0·1	8·1±0·5	7·3±0·9	0·11±0·00
	0·03	3	3·7±0·1	31·1±1·1	2·1±0·1	7·6±0·3	7·9±0·5	0·11±0·01
New Coccine ..	0·3	4	3·9±0·2	29·6±1·3	2·1±0·2	7·1±0·7	7·0±0·5	0·11±0·01
	3·0	6	3·8±0·1	32·0±1·1	2·1±0·1	7·8±0·3	8·0±0·6	0·12±0·01
	0·03	5	3·8±0·1	28·1±0·7	2·5±0·2	7·1±0·3*	6·3±0·7*	0·12±0·02
Nigrosine	0·3	5	3·6±0·3	32·7±1·3	2·5±0·2	7·7±0·1	7·0±0·4	0·11±0·02
	3·0	4	4·2±0·4	40·1±0·8*	2·9±0·2*	9·5±0·3*	7·5±0·7	0·12±0·03
Females								
Control		7	4·6±0·2	34·0±1·0	3·3±0·2	8·1±0·3	0·44±0·02	0·27±0·04
Malachite Green ..	0·03	6	5·1±0·2	37·3±0·6*	3·4±0·4	8·5±0·4	0·46±0·05	0·26±0·04
	0·03	4	4·6±0·2	36·1±1·1	3·2±0·1	8·9±0·4	0·46±0·05	0·21±0·01
New Coccine ..	0·3	4	4·8±0·3	35·3±1·1	3·1±0·2	8·6±0·6	0·42±0·05	0·32±0·02
	3·0	7	5·3±0·2*	37·8±1·3*	3·2±0·2	10·4±0·4*	0·45±0·03	0·34±0·02
	0·03	5	4·6±0·2	36·6±2·4	3·6±0·4	9·1±0·5	0·45±0·00	0·26±0·03
Nigrosine	0·3	9	4·5±0·2	34·5±1·7	2·9±0·2	8·2±0·2	0·38±0·06	0·26±0·01
	3·0	5	4·7±0·1	44·8±2·2*	4·0±0·4	9·6±0·2*	0·49±0·02	0·27±0·01

* Significant at P = 0·05 or less.

The testicular changes were similar to those previously described[1]. The kidney changes in the 3 per cent Nigrosine group appeared more marked than in the other groups.

Haematology

Haemoglobin estimations were made on all surviving rats at the termination of the test. A slight modification of the pyridine-haemochromogen method of Rimington was used[3]. The only difference from control values occurred in the female rats on 0·03 per cent of Malachite Green, where an increased blood haemoglobin was recorded. The significance of this finding is not apparent.

TABLE VI

SUMMARY OF HISTOPATHOLOGICAL FINDINGS

Sex	MALE					FEMALE			MALE		FEMALE		MALE		FEMALE		Totals
Dosage (per cent of diet)	0·03 per cent					0·03 per cent			0·3 per cent		0·3 per cent		3·0 per cent		3·0 per cent		
Treatment	Control	Nigrosine	New Coccine	Malachite Green	Control	Nigrosine	New Coccine	Malachite Green	Nigrosine	New Coccine	Nigrosine	New Coccine	Nigrosine	New Coccine	Nigrosine	New Coccine	
Number of rats on test	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	160
Number of survivors ..	5	5	3	3	7	5	4	6	5	4	9	4	4	6	5	7	82
Number of rats examined	5	5	3	3	7	4	4	3	5	4	9	4	4	6	5	7	78
Bladder parasites ..	2	3	—	—	—	—	1	—	3	1	—	—	1	—	—	1	12
Hydronephrosis ..	—	—	—	—	—	—	4	—	—	—	3	1	—	—	—	2	10
Hydroureter	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1
Glomerulosis	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Hyaline casts kidney ..	2	—	2	2	2	—	—	—	1	—	1	1	4	—	4	1	20
Focal distal nephritis ..	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	1
Ephithelial crescents (glomerulus) ..	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1
Nephritis	—	—	—	—	—	—	—	—	—	1	—	—	2	—	2	—	5
Altered spermatogenesis	1	2	—	1	—	—	—	—	3	1	—	—	2	—	—	—	10
Ovarian cysts	—	—	—	—	—	—	—	—	—	—	3	—	—	1	—	—	4
Focal necrosis adrenal	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	1
Tracheitis	—	—	—	—	—	1	—	—	1	—	—	1	—	—	—	—	3
Ectopic myelopoiesis Spleen	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	1
T Uterine polypus ..	—	—	—	—	1	2	2	—	—	—	—	2	—	—	—	2	9
Fibro adenoma ..	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1	2
U Adenoma (coagulating gland ..	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
M Sertoli cell tumor ..	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
O Papilloma ureter ..	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1
R Chromaffinoma Adrenal	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Carvone, Determination of. D. H. E. Tattje. (*Pharm. Weekbl.*, 1956, **91**, 733.) This colorimetric method requires less time than the dinitrophenylhydrazine method and less material than the sulphite method: 4 ml. of a solution of carvone (at least 0·1 per cent) in aldehyde-free alcohol is mixed with 5 ml. of 2 per cent solution of ethyl 3:5-dinitrobenzoate in acetone-free methanol, followed by 1 ml. of 0·7N sodium hydroxide, and after 3·5 minutes the colour is measured in a 1 cm. cell at 537·5 mμ, using a blank of ethanol, reagent and sodium hydroxide. A fresh blank should be made for each estimation, and the reaction should be carried out at 20°. Between the concentrations of 0·05 and 0·4 per cent the calibration curve is a straight line—E (1 per cent, 1 cm.) = 2·55. The method is suitable for oil of caraway. G. M.

Chloramphenicol in Water-containing Preparations, Determination of the Hydrolytic Decomposition of. A. Brunzell. (*Svensk farm. Tidskr.*, 1957, **6**, 129.) Chloramphenicol yields on hydrolysis 1:3-dihydroxy-1-(*p*-nitrophenyl)propyl-2-amine, which may be detected or determined quantitatively by means of a colour reaction with sodium naphthoquinone sulphonate. To 1 to 5 ml. of a neutral solution equivalent to 0·2 to 2 mg. of hydrolysed chloramphenicol is added 10 ml. of buffer solution (Clark and Lubs), pH 8, and 5 ml. of naphthoquinone sulphonate solution. After 15 minutes, 2 ml. of 0·5N acetic acid and 2 ml. of ascorbic acid solution (10 per cent) are added to remove the excess of reagent, and the solution diluted to 25 ml. The light absorption is determined at 440 mμ against a reagent blank, 10 minutes after the addition of the acetic acid. The result is calculated from the datum that E(1 per cent, 1 cm.) = 162 for the amino alcohol, equivalent to 125 for decomposed chloramphenicol. The reagent is prepared freshly by dissolving 1 g. of sodium 1:2-naphthoquinone-4-sulphonate in 50 ml. of water and diluting to 100 ml. with ethanol. Propylene glycol, glycerol, lactose and undecomposed chloramphenicol do not interfere in the determination of the decomposition product. Solutions of chloramphenicol in water were found to lose about half their activity in 290 days at 20 to 22°. When buffered to pH 7·4 with borax and boric acid (as in oculoguttae chloramphenicoli Ph. Dan.) about 14 per cent of the activity was lost under these conditions. Propylene glycol solutions were relatively stable unless they contained much water. Aqueous solutions decomposed to the extent of about 3 per cent on heating at 100° for 15 minutes. G. B.

Iodine and Iodides, Control of Purity of. A. Berka and J. Zýka. (*Československ. Farm.*, 1957, **6**, 110.) Iodides and iodine are determined by titration against 0·01M *N*-bromosuccinimide; in solutions containing 15 per cent of HCl, the reagent oxidises these to iodine chloride. For the determination of iodine, 0·1 g. is dissolved in about 30 ml. of ethanol, 1 g. of NaCl is added and the solution is made up to 100 ml. Sufficient hydrochloric acid is added to a 10 ml. aliquot, which is then titrated against the reagent, the end point being determined

potentiometrically. Iodine chloride, sometimes present as an impurity in iodine, does not interfere. For iodides, a 5 to 10 mg. sample is dissolved in water, hydrochloric acid is added and the solution is titrated in the same way. Bromides and chlorides do not interfere. The method can be used for the determination of iodides in ointments. The reagent can also be used for the volumetric determination of arsenites and hydrazine and its derivatives, including isoniazid. E. H.

Methadone Hydrochloride, Ephedrine Hydrochloride and Hyoscine Hydrobromide, Separation of, by Paper Chromatography. F. Abaffy and S. Kveder. (*Acta pharm. Jug.*, 1956, **6**, 209.) Whatman No. 1 filter paper was used, with a mixture of butanol 10, glacial acetic acid 1 and water 4 as developing solvent. Ninhydrin was used for the detection of ephedrine and the modified Dragendorff reagent of Munier and Macheboeuf for the detection of methadone and hyoscine on the same paper. The R_F values obtained by the circular, ascending and descending techniques are given. G. B.

Morphine, Extraction of, from Poppy Capsules and its Recovery by Ion Exchange. C. L. Mehltretter and F. B. Weakley. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 193.) Dried poppy capsules were extracted with 2 quantities of *iso*butanol saturated with water, containing 0·23 per cent of ammonia. The morphine was removed from the extract by passing it through a bed of cation exchange resin, from which it was readily eluted with dilute aqueous alkali, and purified. Zeo-Karb H and Duolite C–10, sulphonated cation exchange resins containing few cross-links were employed, and Zeo-Karb H was found to have 55 per cent greater adsorptive capacity for morphine than Duolite C–10. The *iso*butanol solvent was recycled after the addition of a suitable quantity of ammonia. G. B.

Morphine Hydrochloride and the Main Non-phenolic Opium Alkaloids, Use of Ion Exchangers with. A. Jindra and J. Böswart. (*Československ. Farm.*, 1957, **6**, 77.) Morphine in both methanolic and aqueous solution is quantitatively retained by the anion exchangers Amberlite IRA-400, Lewatit MN and Wofatit L150 or L160, but it could be quantitatively eluted only from the first two; 0·5N acetic acid or N hydrochloric acid is used as eluent. On these resins morphine can be separated from codeine, narcotine, thebaine, papaverine and narceine. Of a number of cation exchangers tested, only Wofatit F proved to be suitable for the quantitative absorption and elution of morphine; a 3 to 5 per cent solution of ammonia is used as eluent. Strong alkalias attack the resin and with solutions of salts of strong bases the morphine is only partially recovered. E. H.

Morphine, Identification of, in the Presence of Chlorpromazine in the Urine. B. Košir and J. Košir. (*Acta pharm. Jug.*, 1956, **6**, 181.) A method is described in which morphine and chlorpromazine are extracted from urine, concentrated by evaporation and rendered slightly alkaline, by shaking with a 4:1 mixture of chloroform and ethanol. Chlorpromazine is removed by shaking the hydrochlorides of the extracted bases in hydrochloric acid solution with chloroform, in which the chlorpromazine salt is soluble, but the morphine salt is not. The presence of morphine in the extract can then be confirmed by

chromatographic analysis. The colour tests given below can be used for distinguishing between morphine and chlorpromazine.

Reagent	Morphine	Chlorpromazine
Ferric chloride	purple	red
Hydrochloric acid (concentrated)	no colour	red
Potassium ferricyanide-ferric chloride reagent (Ph.Jug.II)..	blue	purple

G. B.

Neovitamin A_1, Separation of, from All-*Trans* Vitamin A_1 by Chromatography. B. Barnholdt. (*Nature, Lond.*, 1956, **178**, 1401.) Good separation of the isomeric neovitamin A_1 and all-*trans* vitamin A_1 is obtained by chromatography on alumina prepared from fine-grained aluminium hydroxide. 1·5 mg. of a mixture was chromatographed on a column of alumina (75 cm. × 9 cm.), prepared from aluminium hydroxide, analytical grade (Baker's analysed), with a grain size distribution characterised in that 100 per cent passes sieve DIN 80, while 97 per cent passes sieve DIN 100 and 70 per cent passes sieve DIN 150 E. Aluminium hydroxide was heated to 800° for seven hours, cooled in a vacuum desiccator, and mixed with distilled water (6·5 g./100 g.) before use. Elution was with ether and light petroleum, the content of the former being gradually increased from 2–25 per cent by volume. The eluate was collected in 4 ml. fractions, the vitamin content of each determined by measurement of the ultra-violet absorption at 325 mμ. Complete separation was achieved. J. B. S.

Potassium Precipitants as Alkaloidal Reagents. L. Rosenthaler and F. Lüdy-Tenger. (*Pharm. Acta Helvet.*, 1957, **32**, 35.) By analogy of ammonium salts and organic bases with potassium, the authors have investigated the effect of nine of the best known potassium precipitants on 37 plant alkaloids and 8 synthetic bases, 1 per cent solutions being used. In 47·7 per cent of the 400 reactions tried there was precipitation, among which 28 per cent were crystalline. Some of these were characteristic enough to be used as auxiliary microchemical tests for identification purposes. For quantitative purposes however, only one reagent, viz., potassium tetraphenylboron appeared to be suitable. Some sodium precipitants were also tried, but rarely produced precipitate. D. B. C.

Protoveratrines A and B, Separation of, from Associated Alkaloids. J. Levine and H. Fischbach. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 191.) Chromatography on paper moistened with buffer solution, pH 3·5 was used to separate the alkaloids from commercial protoveratrines. A sharp separation of germitetrine, protoveratrine A and protoveratrine B was obtained using a mixture of ethylene chloride 15 ml., ethoxyethyl acetate 10 ml. and pyridine 1 ml. as the developing solvent, the R_F values of the alkaloids being 0·5, 0·7 and 0·25. The position of the alkaloids on the paper was detected with the aid of bromophenol blue. Column chromatography on Celite columns containing a mixture of McIlvaine's buffer solution (pH 3·0) and ethylene glycol was found suitable for the separation of protoveratrine A, protoveratrine B and other alkaloids by the use of a series of solvents of increasing solvent power. A suitable series consisted of (1) carbon tetrachloride 3, ethylene chloride 2, (2) ethylene chloride, (3) chloroform 2, ethylene chloride 1 and (4) chloroform. In this series protoveratrine was first eluted, followed by a fraction more hydrophilic than protoveratrine A and less than protoveratrine B. Protoveratrine B was obtained as a third fraction, and the final chloroform eluate contained alkaloids more hydrophilic than protoveratrine B. G. B.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

***Digitalis purpurea*, Effect of Fermentation on Glycosides of.** D. H. E. Tattje. (*Pharm. Weekbl.*, 1956, **91**, 937.) Digitalis leaves were examined after drying in the field, on screens and after fermentation in a heap at about 30°. In the case of the fermented samples the total aglycone content was significantly increased, and the gitoxigenin content very significantly. This phenomenon is the more striking since the content of aglycones combined with digitoxose is not increased. The explanation of this is not clear, but possibly during the fermentation a glycoside is formed which has no digitoxose in the molecule. The enzyme digipurpurase was not destroyed in the fermentation, and also the primary glycosides were not converted to secondary glycosides to any greater extent than with the other samples. G. M.

***Digitalis purpurea*, Inheritance of Glycosidal Composition in.** F. H. L. van Os and J. H. Stehouwer. (*Pharm. Weekbl.*, 1956, **91**, 942.) Strains of *Digitalis purpurea*, which were originally examined for botanical differences, were found to vary in the composition of their glycosides. These chemical characters can be fixed in the strains. Three types are described, with their characters and the composition of their glycosides. Distinction is made between the digipurpurin type, a stropeside type and a digitoxin type. This discovery is important since it shows that a determination of the total glycoside content gives no indication of physiological action. Most cultures consist of a mixture of strains, and the biological standard sample is also a mixture. In the case of samples from selected cultures or from certain natural regions it is necessary to establish this relation before it is possible to standardise them chemically. G. M.

Digitoxin, Estimation of, by Paper Chromatography. E. Fujiwara. (*Acta med. biol., Japan*, 1956, **4**, 137.) The apparatus consisted of a wire frame inserted through the lid of an hermetically sealed glass cylinder. On this frame two sheets of Whatman No. 1 paper (8 × 34 cm.) were hung; to each sheet four spots containing digitoxin in methanol were transferred. The sheets were suspended above the solvent at the bottom of the cylinder for 24 hours: the frame was then pushed down till the lower ends of the sheets were immersed in the solvent. By this means eight tests could be carried out under identical conditions. The solvent was a chloroform-water-methanol (10:10:2) mixture and the temperature used was 17°. After development the papers were dried, sprayed with a fresh mixture of 3 per cent aqueous chloramine-T and 25 per cent ethanolic trichloroacetic acid and heated at 120° for ten minutes. The digitoxin was identified by its yellow fluorescence in ultra-violet light. A "zone of flow" rather than a discrete spot was formed, and the author shows that the lengths of these zones, relative to the solvent front distance, are proportional to the concentrations of digitoxin. The results with solutions of pure digitoxin only are described in this paper. J. W. F.

Gitoside—A New Digitalis Glycoside. J. E. Murphy. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 170.) Gitoside was isolated from residues obtained in the processing of digoxin from *Digitalis lanata*, but has also been detected in other *Digitalis* species, including *D. purpurea*. The residues were treated with acetone, in which gitoside is relatively soluble, to remove most of the digoxin

The concentrate was placed on a Celite column containing formamide and water as the stationary phase and developed with chloroform:benzene (3:1). Further purification was achieved using formamide:water (2:1) as the stationary phase and ethylene chloride:benzene:acetone (10:10:2) as the developing solvent, and finally repeating with chloroform containing 10 per cent of heptane as the mobile phase. Hydrolysis of gitoside yielded one molecule of gitoxigenin and one molecule of digitoxose. It was noted that on hydrolysing glycosides containing digitoxose, part of the digitoxose formed another compound which still gave a reaction for 2-desoxy sugars when the paper chromatograms were sprayed with trichloroacetic acid in ethanol. G. B.

Flavonol Glycosides, Occurrence in Hips. D. Öiseth and A. Nordal. (*Pharm. Acta Helvet.*, 1957, **32**, 109.) Flavonol glycosides were isolated from the fruits of wild rose (mainly *R. canina*) by adsorption on Amberlite IRC-50, followed by separation by paper chromatography. Two of the glycosides were identified as *iso*quercitrin and campherol-3-glucoside respectively. The third glycoside gave on hydrolysis quercitron and glucose, and the fourth gave campherol, glucose and an unidentified component. The aglycones quercetin and campherol were found to be present also in leaves and buds of *R. canina*, and in the fruits of *R. afzeliana*, *R. cinnamomea*, *R. moyesi*, *R. rugosa* and *R. villosa*. G. M.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Bracken Poisoning of Cattle—Nature of the Poison. A. J. Thomas, I. A. Evans and W. C. Evans. (*Biochem. J.*, 1957, **65**, 5P.) Finely powdered, low temperature-dried bracken was exhaustively extracted with (*a*) acetone, (*b*) ether and (*c*) water. The extracts were concentrated under reduced pressure, and the dry residues fed to three yearling Welsh Black cattle, at the rate of 5 lb./day, mixed with bran; the rest of the diet was hay. Acetone, ether and water extracts, each equivalent to 5 lb. milled bracken, were given by rumen fistula to another three animals daily. No poisoning occurred with the ether and acetone extracts. Cattle receiving the residues developed clinical bracken poisoning within a month. Cattle receiving water extracts and residues gave equivocal results. The water extract led to a greater fluctuation in leucocytes and platelets than is usual with normal animals. J. B. S.

Fluorocarbon, Purification of Poliovirus with. L. A. Manson, E. L. Rothstein and G. W. Rake. (*Science*, 1957, **125**, 546.) Poliomyelitis virus (type II, strain MEF-1) suspension was mixed with an equal volume of a solution of 1:2-difluorotetrachloroethane in *n*-heptane (s.g. 1·30), cooled to 4° and blended in a Servall Omnimixer at 14,500 r.p.m. After standing for 10 minutes to allow any aerosols to settle, the homogenate was centrifuged for 10 minutes at 1000 *g*, when three layers were obtained, (a) upper aqueous layer containing the virus, (b) lower fluorocarbon mixture, (*c*) protein accumulated at the interface. Repetition of this treatment permits the removal of up to 90 per cent of the protein. Homogenisation for much shorter periods (1 to 2 minutes) was found to be almost as effective as the longer blending time; increase in the proportion of the fluorocarbon mixture also increased the efficiency of the method. Assay of the residual infectious virus concentrations after successive fluorocarbon treatments shows that there is no significant decrease of titre after three such treatments. The purification procedure has been applied to

other viral suspensions, such as coliphage T5; poliovirus types 1 and 3; feline pneumonitis virus, and certain members of the adenovirus group. J. B. S.

BIOCHEMICAL ANALYSIS

Carbohydrates, Determination of, in Biological Material using the Thymol-Sulphuric Acid Reaction. M. R. Shetlar and Y. F. Masters. (*Analyt. Chem.*, 1957, **29**, 402.) An account is given of the different absorption curves given by nearly all sugars when they react with thymol in the presence of strong sulphuric acid. It was shown that while glycosidic linkages did not affect the reaction either qualitatively or quantitatively, the presence of protein had a slight effect on the absorption curves apparently due to the reaction of the sulphuric acid with the protein itself. The reaction could be used, however, to estimate carbohydrate bound to protein in serum, and was found to correlate closely with the method using tryptophan of Shetlar, Foster and Everett (*Proc. Soc. exp. Biol. N.Y.*, 1948, **67**, 125). Using a Beckman spectrophotometer, the complex was found to obey the Beer-Lambert law for samples between 10 and 100 μg. under the conditions described. For serum protein samples, 0·2 ml. of a one in five dilution sufficed. No limits of error are stated. D. B. C.

Iodide, Determination of, in Urine. H. L. Helwig, W. A. Reilly and J. N. Castle. (*J. Lab. clin. Med.*, 1957, **49**, 490.) A method is described for concentrating and analysing urinary iodide. To concentrate the urinary iodide add to 200 ml. of urine carrier free ^{131}I that has been reduced with sodium sulphite to give a suitable counting rate of 500 counts per ml. Accurately determine the actual counts/sec./ml. of urine. Acidify the urine to pH 5 with sulphuric acid and heat to 95°, filter to remove protein, cool and while stirring add 0·5 ml. of concentrated sulphuric acid until the urine is 0·1N with acid. Pour the cooled urine through a AgCl-cellulose column prepared in 1 cm. bore glass tubing 10 cm. long. [The column is prepared by adding in succession from the tip upwards a 1 cm. layer of glass wool, a 0·5 cm. layer of cellulose powder suspended in 0·1N sulphuric acid, a layer of AgCl-cellulose powder and a 1 cm. layer of glass wool. The AgCl-cellulose powder is prepared by adding 0·1 g. of silver nitrate to 1 g. of Whatman cellulose powder in 100 ml. distilled water. The suspension is acidified with sulphuric acid and a slight excess of sodium chloride is added while stirring. 33 ml. of the suspension is used in preparing the column and the column is washed with 100 ml. of 0·1N sulphuric acid to remove excess Cl and any free AgCl colloid.] Discard the effluent, wash the column with 200 ml. of 0·1N sulphuric acid and discard the washings. The iodide is removed as iodate from the column by adding 15 ml. of bromine saturated 0·05N sulphuric acid. To determine the urinary iodide heat the eluate from the column containing $Br_2{}^{127}{}^{131}I\ O_3$ in a water bath for one hour to remove bromine. Continue heating, add one drop of saturated potassium permanganate solution to oxidize any further unknown reducing substances present and after ten minutes add 1 to 2 drops of 10 per cent sodium nitrite to destroy oxides of manganese. After another ten minutes heating add solid urea to destroy traces of nitrous acid. Maintain the volume during boiling by the addition of water. Adjust the pH to 2·8 with sodium bicarbonate and add 1 ml. of citrate-phosphate buffer of pH 2·8 (16·83 ml. of 0·1N citric acid plus 3·17 ml. of 0·2M disodium phosphate). Place some of the solution in the cuvette of a spectrophotometer, adjust to zero optical density at 352 mμ. Add a drop of 50 per cent aqueous potassium iodide and measure the optical density of the iodine released. Determine the concentration of stable iodine from a standard curve relating optical

density to concentration of iodine as iodate. Pipette an aliquot of the reaction mixture in the cuvette and measure its radioactivity.

$$\text{Urinary I} - (\mu\text{g./ml.}) = \frac{\mu\text{g. I/ml. in cuvette}}{\frac{^{131}\text{I c./s./ml. in cuvette}}{^{131}\text{I c./s./ml in urine}}}$$

G. F. S.

Mercury, Determination of, in Urine. V. L. Miller and F. Swanberg Jr. (*Analyt. Chem.*, 1957, **29**, 391.) The method described is rapid and specific, and consists of catalytically oxidising the sample with hydrogen peroxide, reaction of the Hg^{++} with excess ditolyl mercury according to the equation: $Hg^{++} + R_2Hg = 2RHg^+$ (R = tolyl), extraction of the RHg^+ with chloroform and colorimetric determination of the mercury by the dithizone reaction. As little as 1 μg. of Hg/100 ml. of sample may be determined, the error being about ± 10 per cent. The procedure may also be applied to very dilute solutions of mercury. Silver and bismuth interfere by causing a precipitate during the oxidation procedure, which must be removed by filtration. There is no interference in the presence of 1000 μg. of cobalt, nickel, copper, zinc, cadmium, lead and manganese. D. B. C.

Morphine in Blood and Tissues, Determination of. J. C. Szerb, D. P. MacLeod, F. Moya and D. H. McCurdy. (*Arch. int. Pharmacodyn.*, 1957, **109**, 99.) A method is described to detect morphine in very low concentrations. It depends on the elimination of impurities by precipitation with benzene and the adsorption of the morphine on an ion exchange resin. The morphine is then determined colorimetrically with the aid of Folin-Ciocalteu's phenol reagent. The blue colour formed depends on the presence of free phenolic hydroxyl groups and is stable for over twenty-four hours. The lower limit of sensitivity of the method is three μg. of morphine free base per ml. of blood or gram of tissue and 0·6 μg./ml. of plasma. Recovery, after addition of 3 to 5 μg. of morphine to either blood, plasma or tissue homogenates gave an average of 80 per cent. Other morphine derivatives tested and recovered in proportions similar to morphine were: levorphanol, methyldihydromorphinan, nalorphine and levallorphan. Diacetylmorphine is hydrolysed during the procedure and may be estimated by the method. Normorphine, a metabolite of morphine, could only be recovered to the extent of 30 per cent since it is largely removed in the initial zinc sulphate-barium hydroxide precipitation.

G. P.

Urinary Indoles, Separation and Characterization of. R. Rodnight. (*Biochem. J.*, 1956, **64**, 621.) Examination of human urine using the ion exchange resin Zeo-Karb 227, followed by paper chromatography and tests on guinea pig ileum has shown the presence of two indoles. There was strong evidence that these were 5-hydroxytryptamine and tryptamine. *N*-Methyl-5-hydroxytryptamine or bufotenin was not detected. The excretion of "urinary 5-hydroxytryptamine" in twelve normal adults ranged from 45 to 120 μg. 24 hours and similar values were found for "urinary tryptamine" in six subjects. Addition of extra tryptophane to the diet nearly doubled the excretion of 5-hydroxytryptamine. Using the method described the recovery of 5-hydroxytryptamine, *N*-methyl-5-hydroxytryptamine, bufotenin and tryptamine added to urine was about 70 per cent. G. F. S.

CHEMOTHERAPY

Chlorophyll, Antibacterial Activity of. S. Mowbray. (*Brit. med. J.*, 1957, **1**, 268.) The water-soluble chlorophyll preparation used in this investigation was sodium potassium copper chlorophyllin. A fresh 10 per cent solution (the maximum possible concentration) was prepared daily in sterile distilled water and dilutions of this were used throughout. The sensitivity of various organisms to chlorophyll was determined using a plate method. The highest concentration of chlorophyll incorporated in solid media was 1/100. Chlorophyll sensitivity was also determined by making bacterial counts of organisms growing in fluid media. Some bacteriostatic action was shown against certain Gram-positive organisms, including *Staph. aureus*, *Str. pyogenes*, the pneumococcus, and the pathogenic clostridia. After a period of bacteriostasis bacterial multiplication proceeded in the presence of all concentrations of chlorophyll tested. After repeated culture in the presence of chlorophyll *Staph. aureus* becomes resistant to the temporary bacteriostatic effect. The possible potentiating effect of antibiotics on the antibacterial activity of chlorophyll was also investigated, using a routine plate assay method. The activity of penicillin, oxytetracycline, and streptomycin was shown to be markedly potentiated by the presence in the medium of subinhibitory concentrations of chlorophyll. S. L. W.

Di- and Triphenylmethane Dyes, Relation between Chemical Structure and Bacteriostatic Activity of. E. Fischer. (*Arzneimitt.-Forsch.*, 1957, **7**, 192.) This communication deals with basic di- and triphenylmethane dyes from their bacteriostatic aspect only, since many of these compounds behave quite differently when their bactericidal effects are compared. For an optimum bacteriostatic effect there must be three six-membered rings and at least two amino groups. These latter should be substituted by two ethyl radicals since it was found that methyl or phenyl substitution produced marked loss of activity. It is probable too, that propyl, butyl and *iso*butyl substitution causes loss of activity, but the author questions the purity of the products. Their activity was less than that of the methyl substitution products. D. B. C.

PHARMACY

Antacid Evaluation, An Approach to. R. H. Schleif. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 179.) An apparatus is described which facilitates the testing of antacids *in vitro*. To carry out an examination of an antacid preparation 50 ml. of simulated gastric juice (U.S. Pharmacopeia) and 50 ml. of water are placed in a beaker and warmed in a water bath to the appropriate temperature for the test (37°). The solution is stirred at a constant rate with a magnetic stirrer and the sample to be tested is released into the beaker by a timing mechanism. Throughout the experiment the reaction of the solution is recorded automatically. 50 seconds after the sample is released, the timing mechanism operates an automatic pipette which delivers simulated gastric juice at the rate of 4 ml./minute for 1 or 2 hours. It is claimed that the close control of experimental conditions which is achieved by the automatic operation of the equipment provides good reproducibility, and that the results are in agreement with that of another method involving continuous recording (that of Rossett and Flexner) which had previously been shown to yield results in agreement with those of *in vivo* methods. From the recordings the following figures are obtained: (1) speed of action, the time taken to reach pH 3 under the test conditions, (2) duration of action, the time taken to return to pH 2. G. B.

Atropine Solutions, Aged, Bioassay of. E. J. Huycke. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 160.) Commercial solutions of atropine sulphate and tartrate for injection which had been stored for some time under warehouse conditions (protected from light) were assayed for atropine. Dilutions of these solutions and of a standard atropine solution were injected intraperitoneally into groups of mice and the dilatation of their pupils determined under standard lighting conditions, after an interval of 15 to 18 minutes. The fiducial limits were estimated to be $\pm 15{\cdot}6$ per cent ($P = 0{\cdot}95$) using suitable dilutions of the solutions and 6 mice at each dose level. Tropine in quantities likely to be present did not affect the pupil. The method of instillation into the eyes of cats was less suitable owing to the difficulty of establishing narrow fiducial limits with small numbers of cats. Determinations were carried out by instilling the solutions into human eyes. The sensitivity depended upon the colour of the iris, but this difficulty could be overcome by instilling the standard solution into one eye and the solution under investigation into the other eye of the same subject. The experiments showed that atropine solutions slowly deteriorate, the amount of decomposition being approximately 35 per cent in 5 years. G. B.

B-Complex Vitamins and Ascorbic Acid in Aqueous Solutions. A. S. Gambier and E. P. G. Rahn. (*J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 134). In solutions of vitamin B-complex without vitamin B_{12}, the rate of decomposition was increased by making the solutions more acid, increasing the concentration of riboflavine or increasing the quantity of air in the vials. The rate of decomposition of riboflavine was increased by making the solution more alkaline, which also decreased the stability of pantothenates. Decomposition of aneurine was accompanied by the precipitation of thiochrome, and that of riboflavine by the precipitation of chloroflavine, when the aneurine concentration was 15 mg. or more/ml. and that of riboflavine 0·5 to 1 mg./ml. B-complex solutions were not usually stable for long periods of time, but it was possible to prepare "dosage forms" of 2 to 5 ml. of solution containing in each ml. aneurine hydrochloride 6 mg., riboflavine 0·5 mg., pyridoxine hydrochloride 1·1 mg., nicotinamide 26·25 mg. and sodium pantothenate 3·75 mg., and provided that ingredients of high purity were employed they could be stored for 18 months at room temperature without the development of turbidity. The addition of ascorbic acid to B-complex solutions displaced the oxidation/reduction equilibrium, preventing the precipitation of thiochrome, while increasing the tendency for chloroflavine to precipitate. The replacement of riboflavine by riboflavine 5-phosphate resulted in preparations which remained stable without precipitation during long periods at room temperature. In solutions of vitamin B-complex containing 0·5 to 1 mg. of cyanocobalamin per ml., a correlation was established between losses of aneurine and cyanocobalamin within the limits of pH 3·3 to 6·5. The optimum pH was that for aneurine solutions, pH 3·3. The stability of cyanocobalamin appeared to be reduced by decomposition products, particularly the thiazole moiety of aneurine. A solution containing aneurine hydrochloride 60 mg., nicotinamide 55 mg., and cyanocobalamin 1 mg./ml. at pH 4·5 lost 3·6 per cent of its cyanocobalamin content on storage for 12 months at room temperature. The best formulation containing vitamins B_1, B_6 and B_{12} was prepared from aneurine hydrochloride 60 mg., pyridoxine hydrochloride 27·5 mg., and cyanocobalamin 1 mg./ml. at pH 3·3. Decomposition tests at elevated temperatures were found to be unreliable owing to the thermolabile nature of the substances under investigation. G. B.

Rubber Closures, Extraction Tests for. E. J. Morrisey Jr. and W. L. Hartop Jr. (*Drug Standards*, 1957, **25**, 1.) A number of rubber caps varying from 2 to 20 according to the surface area, is placed with 200 ml. of distilled water in a 300 ml. flask, and autoclaved together with a similar flask containing only 200 ml. of distilled water, at 121° for 30 minutes. After allowing to cool, the liquid is decanted from both flasks and rejected. The flasks and rubber caps are rinsed and a further 200 ml. of distilled water added to each flask. After autoclaving at 121° for 2 hours the flasks are allowed to stand at room temperature for 3 to 5 hours. The extract is examined, using the contents of the other flask as a control. A high turbidity indicates the excessive use of dusting agents such as zinc stearate. Titration with 0·01N iodine may reveal the presence of reducing agents such as mercaptobenzothiazole, and a change in pH indicates the presence of acid or alkaline ingredients, usually alkaline substances used in accelerating rubber cure. The test is useful for the preliminary examination of rubber caps and rejection of unsuitable batches, and for obtaining evidence of uniformity of quality in a series of batches. G. B.

Solubilising Agents in the Preparation of Stable Calcium Gluconate Solution for Parenteral Use. D. C. Chakravarty and J. W. Jones. (*Drug Standards*, 1957, **25**, 4.) The stability of a simple 10 per cent solution of calcium gluconate was compared with that of a similar solution containing a small proportion of calcium saccharate (commonly used as a stabilising agent), and a number of solutions equivalent in calcium content, approximately half the calcium being in the form of another salt. The solutions were submitted to alternate freezing and thawing and daily shaking for 55 minutes in the presence of sand, which accelerated the rate of precipitation in the plain calcium gluconate solution, and the same solution containing a small amount of calcium saccharate or ethylenediaminetetra-acetic acid. Solutions in which about half the calcium was present as lactobionate, glucoheptonate or laevulinate withstood this treatment without the formation of a precipitate. Changes in pH and the presence of carbon dioxide had no effect on the stability of any of the solutions. G. B.

PHARMACOLOGY AND THERAPEUTICS

Aerosols in Chronic Bronchitis. K. N. V. Palmer. (*Lancet*, 1957, **272**, 611.) This is the report of a clinical study to assess the value of an aerosol solution in reducing the viscosity and aiding the expectoration of sputum in chronic bronchitis. The solution under investigation was Alevaire which contains 0·125 per cent of a detergent superinone (Triton WR 1339) in a sterile aqueous solution containing 2 per cent sodium bicarbonate and 5 per cent glycerol. As a control, a solution the same as Alevaire was employed except that it did not contain the detergent. Twenty-five patients with chronic bronchitis were studied over a period of 3 consecutive weeks; (1) a week with no treatment; (2) a week of daily inhalations of Alevaire; (3) a week of daily inhalations of the control solution. The solutions were given for an hour thrice daily, being nebulised at the rate of about 15 ml. an hour; the nebulisers were attached to a suitable air-compressor or oxygen supply. The results showed that aerosols of Alevaire and of the control solution were equally effective in bringing about considerable symptomatic improvement, the main effect being the greater ease of expectoration. A few patients showed objective improvement and changes in the viscosity of the sputum with either aerosol. A further

experiment with 2 patients, using on consecutive days Alevaire, the control solution, normal saline solution, or water, showed that the reduction in the viscosity of the sputum was about the same with all four aerosols. It would appear that the effect is due to the hydration of the viscid sputum, and it is suggested that a simple water aerosol given by an efficient nebuliser would be equally effective as the detergent aerosol. S. L. W.

Amphetamine Poisoning. R. Greenwood and R. S. Peachey. (*Brit. med. J.*, 1957, **1**, 742.) Three examples of acute amphetamine poisoning are described. One was due to excessive use of a benzedrine inhaler, a second to the swallowing of the contents of one and a half inhalers, and a third to the swallowing of 50 tablets of dexamphetamine. One case developed an acute psychosis, a common feature of severe amphetamine intoxication. All the patients recovered. Soluble phenobarbitone, 0·2 to 0·4 g., was given intramuscularly six-hourly; 2 of the patients were given in addition paraldehyde 10 ml. intramuscularly. These 3 cases bring the total number of cases of acute amphetamine poisoning reported in this country to 11, in 7 of which the source of the drug was an amphetamine-containing inhaler. S. L. W.

Antihaemophilic Factor; Clinical Trial in Haemophilia. R. A. Kekwick and P. Wolf. (*Lancet*, 1957, **272**, 647.) This paper describes the preparation of a concentrate of human antihaemophilic factor and its use in the treatment of 6 cases of haemophilia. The antihaemophilic activity of the concentrate was not less than 85 per cent of the fresh citrated normal human plasma used as the starting material, and the potency in terms of activity per mg. of protein was 20 to 25 times that of fresh plasma. The product is dissolved in a volume such that the resulting solution has 10 times the activity of normal plasma; the total protein content is about 2·2 g./100 ml. and the viscosity so low as to present no problem during administration. Such solutions can be held frozen at $-20°$ or dried from the frozen state with negligible loss in activity. Freeze-dried material has been stored at room temperature for 6 months with only slight loss in activity, and redissolves completely in 20 to 25 minutes when the appropriate volume of distilled water is added at room temperature. The dangers of circulatory overloading during treatment are largely avoided with this preparation since 100 ml. is equivalent in antihaemophilic activity to 1000 ml. of fresh plasma. The preparation was used with very satisfactory results in six cases, including single and multiple tooth extractions, haemorrhages into the stomach and joints, and radical surgery of the buttock and rectum, the dosage varying between 100 and 300 ml. given by transfusion. S. L. W.

Bracken Poisoning in Cattle—Therapeutic Treatment. W. C. Evans, I. A. Evans, C. M. Edwards and A. J. Thomas. (*Biochem. J.*, 1957, **65**, 6P). Bracken poisoning in Welsh Black bullocks was treated with subcutaneous injections of DL-batyl alcohol (D-α-octadecylglyceryl ether) in olive oil (1 g. in 10 ml.) on five consecutive days. Control animals receiving olive oil alone died within 3 days. Treated animals showed a leucocyte response, pyrexia abated and the animal recovered. In further experiments DL-batyl alcohol was solubilised with Tween 80 and administered by slow intravenous injection (25–50 ml. of 2 per cent solution) daily at the critical stage of poisoning. Treatment, coupled with wide-span antibiotic therapy, raised the leucocyte counts from 2000–2500/mm.^{3} to 4000/mm.^{3} and platelet counts from 60,000 and 65,000/mm.^{3} to 120,000 and 70,000/mm.^{3}, within four days. J. B. S.

Tranquillising Drugs in the Treatment of Allergic Conditions. B. C. Eisenberg. (*J. Amer. med. Ass.*, 1957, **163**, 934.) Clinical observations were made on three groups of allergic patients receiving tranquillising drugs. Patients were selected for study who exhibited undue anxiety, mental depression or tension, together with individuals whose symptoms failed to respond to the usual types of allergy treatment. The nature of the drugs used was unknown to the patients and they were given daily in the usual therapeutic dosage over a period of 6 weeks. Chlorpromazine was found of some benefit to 19/59 patients; meprobamate was of benefit in 32/83 patients; reserpine was of benefit in 11/52 patients. Mephenesin, used in lieu of control placebos in the meprobamate series, benefited about 10 per cent. None of the patients was "cured" but the sedative and relaxing effects appear to reduce the intensity of the allergic symptoms. Four of the patients in the chlorpromazine series had to discontinue the therapy because of weakness and sleepiness, and in the meprobamate series six patients had to discontinue therapy, one because of extreme drowsiness, one because of dizziness and headache, and four because of erythematous eruption with urticaria and angioneurotic oedema of the face and neck. S. L. W.

APPLIED BACTERIOLOGY

Haemolytic Material from Aerobic Sporing Bacilli. G. R. Williams. (*J. gen. Microbiol.*, 1957, **16**, 16.) In an investigation of the haemolytic activities of members of the genus *Bacillus* which do not produce lecithinase, 10 strongly haemolytic strains were identified. One strain of *Bacillus subtilis* was especially active and isolation of the haemolytic material produced by this strain was attempted. The culture filtrate was 4/5 saturated with ammonium sulphate. Purification of the resulting precipitate yielded a protein preparation only 6 to 7 times more active haemolytically per mg. of protein than the original culture filtrate. A more highly active preparation resulted from suspension of the ammonium sulphate precipitated material in acetone followed by acidification with 5N HCl. Inactive protein was filtered off and the acetone solution concentrated under reduced pressure and added to 5 volumes of water, the pH adjusted to 3·5 and the resulting precipitate dissolved in pH 8·0 buffer. This acidic material showed a maximum absorption at 275 mμ and was referred to as "275-acid". It gave a slow developing biuret reaction, a weak ninhydrin reaction and was found to have a N content of *c.* 1 per cent. It could be obtained from the haemolytic protein fraction at any stage of purification: as purification of the protein proceeded, the relative content of "275-acid" increased. Examination of culture filtrates of 7 other haemolytic strains of aerobic bacilli revealed that in only two of the strains could haemolytic activity be not attributed to "275-acid". The kinetics of haemolysis with "275-acid" were shown to resemble those of tyrocidine rather than those of most other haemolysins and the preparation was found to have very low activity compared with saponin. The author considers that "275-acid" is responsible for haemolysis, but it is so associated with protein in the culture filtrate as to modify its solubility properties. B. A. W.