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REVIEW ARTICLE

DISCOVERIES IN THERAPEUTICS*

J. H. GADDUM, M.A., Sc.D., F.R.S.

THE attitude of the medical profession towards drugs has undergone a remarkable change during the last 50 years. At the end of the 19th century it had become clear that a large proportion of the traditional remedies had no real effect on the course of disease. Sir Henry Dale[1] (1943) has given a striking picture of his disappointment as a medical student when he realised the position. Quinine was effective against malaria, ipecacuanha against dysentery and mercury against syphilis. Digitalis, morphine, cocaine, bromides, salicylates, and general anæsthetics had their uses but their effects were palliative rather than curative and although there were 857 drugs in the British Pharmacopœia most of the others seemed to be comparatively useless.

Great changes have occurred since then. A large number of powerful new drugs have been introduced and a large number of old drugs have been transformed into useful therapeutic tools. The physician of to-day can do very much more for his patients than the physician of 50 years ago and if progress continues at the present rate the physician of to-morrow will be able to do much more still. From the point of view of the patient this kind of progress is of supreme importance. The object of this review is to analyse the methods by which new remedies are discovered, in the hope that progress in therapeutics may be accelerated.

A complete account of the methods by which all modern remedies were discovered would be a vast task, and it has been necessary for me to prepare a select list of important remedies for discussion. The list was originally based on the index of the "Textbook of Medical Treatment" by Dunlop, Davidson and McNee,[2] but has been modified. It includes about 200 drugs all of which have some claim to be separate and effective remedies. This list is, of course, an arbitrary one, and can be criticised in detail, but it probably contains most of the really potent drugs introduced before 1950.

A study of this list confirms the impression that therapeutics is advancing to-day more rapidly than it has ever advanced before. The number of remedies in the select list introduced in the 50 years between 1900 and 1950 is equal to the number introduced before that time.

It may perhaps seem surprising that so large a proportion of the drugs in use to-day were already known in 1900. Quite a number of really effective remedies were first used at a very early date, but for long ages their value was obscured by the fact that they were buried in long lists of other remedies, most of which were quite useless. The modern pharmacopœia is a much more select and critical document than its predecessors. Many useless drugs have been cast out, but many other old drugs have been made into useful remedies in the light of modern knowledge.

* Based on two University of London lectures delivered at The School of Pharmacy. February 4 and 5, 1954.

Some of the remedies still in use to-day were discovered before the dawn of recorded time and the real history of the beginnings of pharmacology will never be written. We shall never know who it was that first discovered the interesting pharmacological effects of alcohol. Some of the virtues of milk and honey and salt and sulphur were known from the earliest times.

The earliest recorded pharmacological experiments were carried out in 2700 B.C. by the emperor Shen Lung of China, who tasted all the drugs in the pharmacopœia of his day and classified them accordingly. Ma Huang was classified as a medium drug and when its active principle was isolated nearly 4000 years later it was called ephedrine. It had been used for hundreds of years in China as a stimulant of the circulation, as a diaphoretic, as an antipyretic and a sedative in coughs. Modern observations confirm that it is effective in most of these conditions. Ephedrine was isolated in 1887 and for the next 30 years it provided a happy hunting ground for the chemists. Its effect on the pupil was likened to that of the sympathetic in 1892 before adrenaline was discovered. In 1917 its pharmacology was investigated again by two Japanese workers who showed that it had sympathomimetic effects like its chemical relative adrenaline. Chen and Schmidt isolated ephedrine and showed that it acted like adrenaline before they discovered that these facts were already known. However, their paper, published in 1924, had a very important effect because it was written in English. It is interesting that fundamental pharmacological facts about ephedrine were discovered three separate and independent times before its use became general.

Two other drugs which we owe to ancient China are tea and rhubarb, both of which were known to Shen Lung.

The Ebers papyrus was written in Egypt about the year 1550 B.C. It is a textbook of therapeutics based partly on the experience of the author himself and partly on the writings of earlier authors[3,4]. It contains many interesting and true observations particularly with regard to purgatives; it has always been easy to make clinical observations on the action of purgatives.

The papyrus recommends castor oil, figs, manna, senna and colocynth as purgatives, and plants containing tannin as astringents, pomegranate for round worms and liver for night blindness. This last remedy, which presumably owed its action to vitamin A, is distinguished by a special note that it is really excellent. Copper, antimony, hyoscyamus and iron are also mentioned. Over a dozen of the drugs on my list are included, but many of the drugs mentioned in this papyrus have not been identified and the Egyptians may perhaps have known of even more of our modern remedies than these calculations suggest.

The Greeks inherited many drugs from Egypt but added a large number of their own. Theophrastus, who was born in 380 B.C., was a pupil of Aristotle and his book on materia medica probably represents Aristotle's information on the subject. It included opium, mustard plasters and tar for the skin, filix mas for tapeworms, and aloes as a purgative.

Dioscorides wrote a comprehensive treatise on materia medica in the first century A.D. which contains references to many other drugs used

to-day, including santonin for worms, alkaline diuretics for œdema, gentian, chalk, mercury and lead salts and clay poultices—which may be regarded as the origin of the kaolin poultice. The fact that goitre depends on the water supply has been known for at least 2000 years, but the first clear reference to the use of a remedy rich in iodine in the treatment of goitre is in the writings of Roger of Salerno who recommended burnt sponge for this purpose in 1170 A.D.

The schools of medicine which flourished in Mohammedan cities in Persia, in Mesopotamia and in Spain in the 10th and 11th centuries, and which used the Arabic language, preserved the ancient medical lore of Egypt and Greece for posterity, and introduced four of the drugs in my select list. They get the credit for introducing camphor from Malay and China. Colchicum had been described by Dioscorides as a poison; the Arabic writers recommended it for gout. They also introduced silver and gold into medicine, though not for the same diseases as Dunlop, Davidson and McNee. They used silver for auricular fibrillation. In later years, it was given to lunatics because it was associated in men's minds with the moon, which was well known to control the actions of lunatics. The local use of silver dates from the 16th century, when it was used as a hair dye. It was first used as a caustic in the 17th century and its antiseptic action was discovered by Neisser and Behring in the 1880's. The Arabs also used potable gold as an elixir of life.

Between the years 1300 and 1850 progress in therapeutics was still fairly slow. The discovery of America led to the introduction of cinchona for malaria, ipecacuanha for dysentery, chenopodium for worms, lobelia for asthma and quassia as a bitter. During this period Dutch sailors discovered the value of lemons in the treatment of scurvy, and magnesium sulphate was first described as the active purgative in the spring water which had become a fashionable drink at Epsom.

Digitalis was an old folk remedy grown by Gerard and Parkinson and Salmon and other herbalists and used for scrofula (that is tuberculous glands) and for local application to sores and for epilepsy. Its real value was established by Dr. Withering of Birmingham in a book published in 1785[5]. He described his results with foxglove on 100 cases and noted its effect on the heart and as a diuretic. He met with a lot of opposition, but did succeed in establishing digitalis as a valuable remedy.

Oil of eucalyptus was first used in 1790 by the early settlers in Australia as a substitute for oil of peppermint in the treatment of wind and colic.

Salicylates were first obtained from willow bark (Salix), and willow bark was introduced into medicine in the first place as a substitute for cinchona bark which previously had become known as a substitute for the bark used in preparing balsam of Peru. Salicylic acid was first synthesised commercially from phenol in 1874 and within two years it was established as an antiseptic, antipyretic and antirheumatic, and salicylates came into general use.

The history of ergot is complicated but interesting[6]. Its use by midwives was described in 1582, but it was not introduced into official medicine until 1808. In the early years of the 20th century extracts of ergot were

found to contain a large number of active drugs. They were the first natural source of histamine, acetylcholine, and a number of other interesting substances which are widely distributed in nature, but they also contain specific alkaloids which are found nowhere else. In 1932 ergotoxine had been on the market for 25 years, and ergotamine for about 10 years. These specific alkaloids were generally thought to be the real active principles, but, in spite of repeated efforts by the pharmacologists, it had been so far impossible to persuade the clinicians to produce good evidence of their real value. The official British extract of ergot contained no ergotoxine and no ergotamine; some people said it was nevertheless effective, but opinion was divided. The discovery of the real active principle was due to the application of a pharmacological technique to a human patient. Chassar Moir (1932) recorded the human uterine contractions after childbirth and showed that ergotoxine and ergotamine were both active in stimulating the uterus, but he also found that liquid extracts containing neither of these substances were even more effective and quicker in action. This work provided a test for the most important active substance, which was soon isolated by chemists in 3 different countries and became known as ergometrine.

TABLE I

DRUGS INTRODUCED BEFORE 1850

PREHISTORIC—Water, alcohol, milk, salt, turpentine.
EARLY CHINA—Caffeine, rhubarb, ephedrine.
EGYPT, 1550 B.C.—Glucose (honey), olive oil, bran poultice, castor oil, senna, tannin (for diarrhœa), liver for night blindness, bone marrow, atropine (hyoscyamus), iron for anaemia, copper, antimony, zinc.
THEOPHRASTUS, 300 B.C.—Filix mas for worms, opium, aloes, mustard plaster, tar.
DIOSCORIDES, 78 A.D.—Santonin, clay poultice, gentian, alkaline diuretics, chalk, mercury, lead, lanoline.
12TH CENTURY—Burnt sponge for goitre, colchicum for gout, camphor, gold, silver, berberine.
16TH CENTURY—Lemons for scurvy, croton oil, chaulmoogra for leprosy.
17TH CENTURY—Blood transfusion, Epsom salt, nux vomica, cinchona, ipecac, chenopodium.
18TH CENTURY—Digitalis, willow, vaccination, oxygen, charcoal, menthol, quassia, eucalyptus.
1800-1850—Ergot, cod liver oil, lobelia, nitrous oxide, ether, chloroform.

The most important pharmacological event in the first half of the 19th century was the introduction of general anæsthesia[7]. The anæsthetic action of nitrous oxide was discovered by Humphrey Davy. He published his results in 1800 when he was 22 years old[8]. His book is a model for all pharmacologists to copy. He described the chemical properties of nitrous oxide and its effects on a wide variety of animals. He showed that pure nitrous oxide caused death and that for anæsthesia it was necessary to mix oxygen with it. He repeatedly anæsthetised himself with it and recommended that it should be used in surgical operations. In 1844 Horace Wells, a dentist of Hartford, Connecticutt, used the gas for the extraction of teeth, but nitrous oxide was not finally introduced till 1863.

Meanwhile, the intoxicating effects of ether were fairly well known. They had been compared with those of nitrous oxide in 1818 in a note attributed to Michael Faraday. Ether was first used as an operation in America. The chief credit for its introduction is due to Morton of Boston who gave a successful demonstration on October 16, 1846, and within a few months ether was in general use all over the world. Chloroform was first used as a general anæsthetic by Sir James Simpson in

Edinburgh on November 16, 1847—so that all these important general anæsthetics were first used in medicine within a few years of one another. Chloroform, like ether, was generally adopted quite rapidly.

It was a little earlier than this that there appeared the first signs of the scientific upheaval which has revolutionised therapeutics. During this time the fundamental facts of physics and chemistry and physiology were

TABLE II

DRUGS INTRODUCED SINCE 1850

British Commonwealth

1850	Permanganates, bromides.
1860	Eserine, apomorphine, phenol, amyl nitrite.
1870	Nitroglycerine.
1890	Typhoid vaccine, thyroid, strophanthus, hydrogen peroxide.
1900	Pituitary extract, carbon dioxide.
1910	Pollen, gum acacia, acriflavine, eusol, chloramine-T.
1920	Insulin, parathyroid, peptone, ammonium chloride, cyclopropane.
1930	Alum toxoid, typhoid serum, Russell viper venom, magnesium trisilicate, diamidines, stilboestrol, mandelic acid.
1940	Antibiotics, cyanocobalamin, dyflos, dimercaprol, mutagens, mephenesin, methonium drugs.

U.S.A.

1880	Liquid paraffin.
1900	Adrenaline.
1910	Dysentery serum, lactose, carotene.
1920	Scarlet fever antitoxin, liver, vitamin E, nicotinic acid, carbon tetrachloride, tryparsamide, iodophthalein, hexylresorcinol.
1930	Progesterone, helium, riboflavin, neostigmine, phenytoin, amphetamine, dinitrophenol.
1940	Other antibiotics, dicoumarol, hyaluronidase, folic acid, cyanocobalamin, antituberculosis drugs, nalorphine, antithyroid drugs, dibenamine, chlormethine, dimethylcarbamazine.

German speaking countries

1860	Trichloroacetic acid, chloral hydrate.
1880	Cocaine, ichthyol, creosote, bee venom, formalin, betanaphthol, paraldehyde, iodoform, phenazone, phenacetin, methylene blue, gentian violet.
1890	Diphtheria antitoxin, tetanus antitoxin, urea, X-rays, aspirin, amidopyrin, hexamine, orthocaine.
1900	Kaolin, barbiturates, procaine, cinchophen, arsphenamine.
1910	Phenylhydrazine, phenolphthalein.
1920	Gonadotrophin, suramin, pamaquin, chiniofon, mersalyl, leptazol, nikethamide, bromethol.
1930	Œstrin therapy, sulphonamides, mepacrine, carbachol, dihydrotachysterol.
1940	Dihydroergotamine, isoprenaline, methadone, leucanthone, caramiphen, *p*-aminosalicylic acid, lysergic acid diethylamide

The rest of the world

1850	Chytsarobin, curare.
1870	Thymol.
1880	Rabies vaccine.
1890	Cholera vaccine, anthrax serum, vitamin B, thallium.
1900	Whooping cough vaccine, radium, trypan blue.
1910	Benzyl benzoate.
1920	Bismuth, thiosulphate, acetarsol.
1930	Vitamin K, vitamin P, antiadrenalines.
1940	Antihistamines, synthetic curares, disulfiram.

established. The whole technique of scientific experiment and scientific logic was built up into a powerful weapon which has been used to devise new methods of preserving human life, as well as new methods of destroying it.

This has led to a great increase in the rate of discovery of new remedies. During the 17th and 18th centuries the drugs on my list were being introduced at the rate of about 5 per century, or one drug every 20 years. In the 19th century the rate of discovery rose rapidly and between 1890 and 1900, 16 of these remedies were introduced or 1.6 remedies per year. Since then the rate has risen still further to a maximum of 2.7 remedies per

year, but the rise has not been a steady one. The recent data are divided into 4 groups, according to whether the remedy was first introduced in the British Empire or in the U.S.A. or in German-speaking countries or in the rest of the world. This has involved some arbitrary decisions, and the assumption has been made that, with a few obvious exceptions, the nationality of authors was the same as that of the country in which their results were published. All the German-speaking countries are classified together because it would be difficult to disentangle them, but most of the remedies thus classed together were actually discovered in Germany.

The first thing to be seen in this list is that the British have not been doing so badly after all. Since 1910 the British have introduced 24 of these remedies, the Americans 29, and the German-speaking countries 22.

The Americans introduced the general anæsthetics ether and nitrous oxide in the 1840's, but after that they get credit for nothing more until 1900 except liquid paraffin. The Americans did not really start introducing new remedies until the first world war was over, but since then they have shot ahead of all other nations.

In the years 1850–1880, 12 of these remedies were introduced. Permanganates were introduced by Condy in 1857 and bromides were first used in the treatment of epilepsy at about the same time.

Curare had been studied for half a century before Claude Bernard[9] showed in 1856 that it acted by stopping the transmission of impulses from motor nerves to voluntary muscles. Quite recently anæsthetists have started using curare to increase muscular relaxation during operations. It is interesting to enquire why over 80 years elapsed before this application of Claude Bernard's work became a practical proposition. Curare was first used clinically in 1859 and during the next 40 years it was given to patients in France suffering from rabies, tetanus, epilepsy and chorea. Interest in the subject then appears to have lapsed, probably because the drug was difficult to get, variable in potency, and liable to contain toxic impurities. In 1930–1935 Ranyard West[10] did much to attract attention to curare and tested its use in various clinical conditions. He helped to arouse the interest of Dr. Harold King[11] who isolated the alkaloid, which is known as tubocurarine, and established its chemical structure. In 1940 Bennett[12] injected curare to control the convulsions of patients undergoing treatment with convulsant drugs, using an extract known as intocostrin and this was used by Griffith and Johnson[13] as an aid to muscular relaxation during anæsthesia. The pure alkaloid tubocurarine is also effective and has the advantage that its composition is certain to be constant. Recent progress on curare has been due to the skill of the pharmacologists and chemists, but it is doubtful if they would have worked in this field if they had not been encouraged by the enthusiasm of the clinicians.

The ordeal beans used in the trials of witches at Calabar on the coast of Nigeria were first made known in Europe in 1840. Most of the fundamental work on this poison was done in Edinburgh where Professor Christison ate the beans, and survived because he took an emetic[14]. His assistant, Thomas Fraser, analysed their effect with great care[15], and isolated their active principle, which he called eserine. He demonstrated its

constrictor effect on the pupil and first used it for this effect. This substance has played a very important part in the development of physiological knowledge, since it was found by Loewi and Navratil[16] in 1926 to protect acetylcholine from the enzyme which destroys it. Without eserine our knowledge of the neuromuscular transmission of nervous impulses would be much less complete than it is to-day. The chemical structure of eserine was established by the Stedmans who synthesised many substances with similar actions. This work led to the introduction of neostigmine in 1931, but the latter substance was actually first prepared and used in America and this drug has been allotted to the U.S.A. The most interesting use of neostigmine is in the treatment of myasthenia gravis in which it temporarily cures the impaired conduction at the nerve ending. This discovery depended upon Claude Bernard's work on curare. It was shown by Pal[17] that the paralysing action of curare was antagonised by eserine. Clinical observations on myasthenia during the early part of this century led to the conclusion that the symptoms of this disease were similar to the effects of curare. These facts were the logical basis of the discovery in 1934 of the effect of eserine on myasthenia by Dr. Mary Walker[18].

In the 1880's therapeutics developed in two important directions. There was a sudden great output of synthetic drugs in Germany and immunology was established by the work of Louis Pasteur[19] on rabies. New chemical compounds were made with the deliberate object of discovering new remedies and were selected from among a host of allied substances, by experiments on man, or more usually on other animals. Organic chemistry was beginning to stretch itself, and to realise that a new technique for the fixing of methyl groups to a particular site in a molecule could be applied without much trouble to the fixing of dozens of alternative groups to the same site. Chemists were pleased to find that substances produced in this way could be handed over to pharmacologists, and that the pharmacologists would say that some were inactive and some were too toxic and that out of each series there was usually one that was better than all the others and worth trying on patients. Phenacetin is an example of a drug discovered in this way. It was chosen from a series of homologues containing groups of different sizes. The higher members of the series were inactive and the lower members were too toxic. The choice was made by pharmacological experiments and it has stood the test of time. A number of allied substances were made and marketed, but phenacetin has been the most successful.

Rabies vaccine was introduced by Pasteur in 1880. Vaccination against smallpox had been practised for centuries, and its value had been established as a scientific fact by Edward Jenner in 1798. Pasteur's discovery of the value of rabies vaccine was due to the accident that while he was away on a holiday, preparations of the virus of chicken cholera which he had left in his laboratory lost their virulence, so that the chickens into which he injected them did not get chicken cholera, but did become immune to this disease. This showed Pasteur that it was possible to confer immunity without exposing the patient to the danger of the disease. It needed great

courage to apply this knowledge to the treatment of rabies in man, but the success of Pasteur's experiments led directly to much other fruitful work in immunology.

In the 1890's the synthetic drug industry was flourishing in Germany but nowhere else. In these years great advances were also made in immunology. Behring and Kitasato announced the discovery of an effective tetanus antitoxin prepared by immunising animals with the exotoxin liberated into culture media by the tetanus bacillus, and a week later the same authors announced that diphtheria antitoxin could be made by the same method. Effective vaccines were made by Almroth Wright for typhoid, and by Haffkine for cholera. All these discoveries have played an important part in the world.

The same period saw the first successful use of hormones in therapy. Dr. George Murray[20] of Newcastle, made a glycerol extract of sheep's thyroid which he injected into a patient with myxœdema with good results. This was a logical thing to do, since it had just been proved that myxœdema was due to lack of thyroid. But it was also a brave thing to do and was the beginning of an important branch of therapeutics.

Another discovery of this period was the use of strophanthus in heart disease. This was the outcome of careful researches in Edinburgh by Sir Thomas Fraser[21] on the pharmacology of poisoned arrows.

The first 20 years of the present century were not particularly fruitful. The pressor effects of extracts of the adrenal medulla and of the pituitary posterior lobe were discovered by Oliver and Schafer[22] about 1895 in University College, London, but the credit for adrenaline goes to the U.S.A. because it was there that it was first isolated, and it was there that it was first used therapeutically. Germany still led the world in the synthetic drug industry. Only two synthetic drugs in the list were introduced outside Germany in the first ten years of the century. Phenolphthalein was used as an indicator of *p*H for 30 years before its purgative action was accidentally discovered[23]. Trypan blue was first used as a wool dye. Its chemotherapeutic action was discovered in France[24]. In these same years the Germans introduced the barbiturates as hypnotics[25], procaine as a local anæsthetic[26], cinchophen for gout[27], and arsphenamine (or salvarsan) for syphilis[28]—four big achievements.

The work of Ehrlich which led to the introduction of arsphenamine established the importance of the experimental study of the treatment of infections. Ehrlich investigated the effects of a long series of compounds on mice infected with various protozoa and so selected drugs which were active without being too toxic. He used the word chemotherapy to describe his work, and this word now means the study of the effects of drugs on infected animals, whether the infective agent is a protozoon or a bacterium or a worm.

The period of the first world war was not very fruitful. Judging by my list the war stimulated the British more than it stimulated anyone else. Their war effort included the first use of gum acacia in the treatment of shock[29], and of acriflavine[30], eusol and chloramine-T[31], all of which are effective disinfectants for wounds.

The most dramatic achievement of the 1920's was the introduction of insulin in 1923. This was the culmination of a long period of patient work on the physiology of the pancreas[32]. It was already known that diabetes mellitus was due to deficiency of a hormone liberated by the pancreas. This hormone was first called insuline in 1909 and many people had made extracts of the gland and injected them into animals. There had even been clinical trials in which patients had been treated with pancreatic extracts. These trials were abandoned because the extracts caused toxic effects, and it is quite likely that those toxic effects were really due to an overdose of insulin, methods of biological standardisation being very crude in those days. But it is not enough in therapeutics to have a good idea, even if you try it out on a few patients. The success that came in 1923 was due to the fact that Banting and Best were carried forward by faith that was undaunted by the failures of others, and overcame all difficulties until insulin was a practical proposition. They were greatly helped by the fact that during the period since the time of the earlier experiments a practical method of estimating the blood sugar had been devised. This gave them a quick proof that their extracts were active and a ready means of measuring their activity.

The most remarkable feature of the 1920's taken as a whole was that the U.S.A. suddenly took the lead in the production of new remedies. Only 5 of the remedies in the list have been credited to America in *all* the years before 1920, but during the next 10 years the Americans introduced 8 more of these remedies. Three of these were the result of work in laboratories on bacteriology, physiology and biochemistry. The use of liver in the treatment of pernicious anæmia was discovered by direct clinical trial. This was inspired by theoretical work on anæmia, but it was really an accident that this inspiration led to the right result. Whipple had shown that liver was effective in the treatment of microcytic anæmia in rats. Minot[33] was inspired by the knowledge of this fact to try giving liver to a patient with macrocytic pernicious anæmia, and it worked. The effect on rats was due to copper and the effect on pernicious anæmia was due to a complex organic compound called cyanocobalamin. Liver was tried because it contained copper, and it was a very fortunate coincidence that it happened also to contain cyanocobalamin.

During this period the Americans also produced a respectable number of synthetic remedies though not so many as the Germans, who still held the lead in this field. About half the synthetic drugs in my list for the 1920's are examples of the results of research in chemotherapy. They were discovered by infecting animals with diseases and then comparing the therapeutic effect of large numbers of chemicals. The introduction of carbon tetrachloride was the result of experiments on dogs infected with worms[34]. The introduction of suramin was the result of experiments on mice infected with trypanosomes. The introduction of pamaquin was the result of experiments on canaries infected with malaria[35]. When the Germans could not get quinine in the Kaiser's war they started looking for a substitute. Methylene blue was the only synthetic drug that was known to be effective at that time. They set out to improve on methylene

blue. They attached a side chain to it and by a prolonged series of trials and errors they gradually improved their side chain, testing dozens of drugs in the process. When they had found the perfect side chain, they tried changing the original nucleus to which the side chain was attached, so that when they had finished there was left no vestige of the original methylene blue from which they had started. Some years later they made an even better antimalarial (called atebrin or mepacrine), by attaching the same side chain to another nucleus.

In recent years many thousands of substances have been tested for antimalarial activity using infected chickens and ducklings, and many active compounds have been discovered. The discovery of proguanil was based on ingenious chemical arguments about the shapes of molecules.[36]

During the 1930's Germany lost the lead in therapeutics, although her output was still considerable. It was she who first discovered the wonderful properties of the sulphonamides and she has got all the credit for this achievement, but it is doubtful whether this is just. The activity of sulphanilamide itself was discovered in France, and the first convincing clinical trials and the first derivative more active than sulphanilamide were discovered in Britain and many of the later developments occurred in the U.S.A. In any case, even if Germany is to get all the credit for the sulphonamides her achievement in these 10 years did not obviously exceed either that of the British, or that of the Americans. The combined output of the English-speaking countries was now clearly greater than that of the German-speaking countries.

The discovery of the chemotherapeutic activity of diamidine compounds, such as propamidine, provides an interesting example of the curious ways that new discoveries are sometimes made. Many workers had studied the survival of trypanosomes outside the body. It was found that they used up oxygen rapidly and burned glucose rapidly, and if they were deprived of glucose they became less active and underwent various other changes. It was clear that any drug which deprived the trypansomes of glucose or which prevented them using glucose, would be bad for them. In 1938 two Hungarian scientists Jansco and Jansco[37] came to the conclusion that the drug suramin did actually act in this way and decided to try whether other drugs would do so too. Synthalin causes a fall of blood sugar by poisoning the liver and might be expected to deprive the trypanosomes of their main food. This led them to try the effect of synthalin on mice infected with trypanosomes. The experiments were successful and Jancso and Jancso claimed that this was the first time that it had been possible to discover a compound with chemotherapeutic activity by theoretical reasoning instead of by pure accident. This, they said, was impressive evidence of the importance of the investigation of the mode of action of drugs.

Professor Warrington Yorke[38] of Liverpool was, however, sceptical. It seemed to him unlikely that the hypoglycæmia would kill the trypanosomes before it killed the mouse and he decided to re-examine the phenomenon. He soon found that synthalin killed trypanosomes *in vitro* in concentrations as low as 1 in 200 millions, and of course synthalin does not

cause hypoglycæmia *in vitro*, so that the hypoglycæmia was not the cause of the effect. This conclusion was confirmed by experiments in which insulin caused hypoglycæmia without harming trypanosomes. It is still possible that synthalin acts by interfering with the glucose metabolism of the trypanosomes, but there is now no evidence that this is so. These experiments showed that the drug acted directly on trypanosomes in very low concentrations, and King and Ewins then synthesised a number of similar compounds, several of which proved very effective on trypanosomes, and other microbes.

In spite of the war there was no falling off in the rate of discovery of new remedies between 1940 and 1950. The war itself was in fact a potent stimulus to certain kinds of research and quite a number of new remedies came more or less directly from the work undertaken in connection with chemical warfare. The substance which is known as mustine or chlormethine ($CH_3.N(CH_2CH_2Cl)_2$) was first studied because it acts like mustard gas and it was thought that it might be used in chemical warfare. Its toxic effects were studied in the hope of finding an antidote and it was found that it caused the leucocytes to disappear from the blood. After the war it was used to produce just this effect in leukæmia when there are too many leucocytes in the blood. Drugs of this group were also found to cause mutations by acting on the germ cells in the gonads so that the next generation was abnormal. These were the first drugs known to produce such an effect and they were christened mutagens[39]. Their interest is, of course, academic; doctors don't ever want to produce abnormalities in the next generation. The substance dibenamine also contains a chlorethylamine group and was discovered by investigators who had been working on chemical warfare agents. Its place in therapeutics is not yet established but it is interesting because it causes paralysis of the sympathetic system which lasts longer than that due to any drugs that were known before.

The substance dimercaprol was discovered in the search for an antidote to the arsenical vesicant known as lewisite[40]. It was known that substances containing SH groups combined with arsenic but the combination was more easily reversible than the combination between arsenic and the tissues. After much study it was concluded that arsenic must combine with two neighbouring SH groups in the tissues and it was found that antidotes with two neighbouring SH groups were able to compete on more equal terms with the tissues. The substance dimercaprol was thus found to be a potent antidote for arsenic and for various other toxic elements such as mercury, cadmium, zinc and gold. One reason why it is not more used than it is, is that arsenic poisoning has become rare owing to the use of antibiotics instead or organic arsenicals in the treatment of syphilis.

The anticholinesterases[14] have perhaps aroused more interest than other drugs which have been studied in connection with chemical warfare. These include some of the most poisonous substances known; they have been much used to kill vermin. Men have been poisoned accidentally in peace time and might be poisoned intentionally in war time. More is known about the biochemical explanation of their action than of that of any other class of drug and there is still clearly much to discover. These

drugs are a striking example of the fact that new pharmacological ideas may stimulate other branches of science.

These examples suffice to show that the researches which were undertaken in wartime in connection with chemical warfare have borne various kinds of fruit. Let me now turn to a group of discoveries which have been based on investigations of the ways in which drugs may antagonise one another. One of the earliest examples to be studied was the antagonism between carbon monoxide and oxygen which compete with one another for hæmoglobin. Carbon monoxide forms a much more stable combination than oxygen and a small amount of carbon monoxide can prevent large amounts of oxygen from combining with the hæmoglobin in the blood so that the animal dies. A similar state of affairs was studied by biochemists in many experiments where two substrates were competing for the same enzyme. The importance of this idea of competition was also emphasised by various pharmacological studies of antagonism in the early part of this century[41]. To take one example, Pohl[42] discovered that the insertion of an allyl group instead of a methyl group in codeine produced an antagonist for morphine and believed that this was because the new compound combined with the same groups in the tissue as the morphine but did not produce the same effect. In 1931 Stedman[43] used this theory to explain the action of eserine on cholinesterase. Chemical groups in this enzyme (which are known as receptors) normally combine with acetylcholine and hydrolyse it rapidly. Eserine is an ester like acetylcholine and it may combine with the same receptors so that the acetylcholine is excluded from the receptors and preserved from hydrolysis. It thus became apparent that competition is liable to occur between any two drugs which are closely related to one another and this fact was used for the discovery of new antagonists. It led to the discovery of drugs which antagonise adrenaline and drugs which antagonise histamine. These antihistamines were discovered in Paris during the exciting years between 1937 and 1944 and they were soon found to have many uses, so that they are now almost as popular as aspirin.

In 1940 this theory of competitive inhibition was used to explain the action of sulphonamides. It was found that their action could be inhibited by very small amounts of *p*-aminobenzoic acid and it was suggested by Woods and Fildes that this acid was a growth factor for the organisms the utilisation of which was inhibited by the sulpha drugs. This theory is now generally accepted; *p*-aminobenzoic acid is normally used by the microbes to make folic acid and many of the chemical details of how this occurs and how the sulpha drugs interfere with it are known.

This work with the sulpha drugs did much to popularise the theory of competitive inhibition and led to the discovery of various antivitamins. For example, aminopterin has the same chemical structure as folic acid except that one OH group is replaced by NH_2. It competes with folic acid and prevents the formation of new cells in the body. It has not been found effective in the treatment of cancer, but it is a very powerful and interesting poison and has been used in the treatment of leukæmia because it inhibits the formation of new leucocytes.

The substance dicoumarol was isolated in 1939 as the active substance in a disease of cattle caused by the eating of spoiled clover hay. It interferes with the clotting of the blood. Its resemblance in chemical structure to vitamin K immediately suggested that these two substances were competitive antagonists and this is still thought to be the true explanation of their action[44].

In more recent times substances allied chemically to thyroxine have been found to be antagonists of thyroxine[45].

From the practical point of view the most important recent discoveries have been in the field of chemotherapy. The great potency of diaminodiphenylsulphone (dapsone, B.P.C.) was first discovered by Buttle[46] although the application of its derivatives to tuberculosis was an American discovery. A complex of dapsone was the first drug to be effective in tuberculosis but was quickly replaced by better drugs. However, it forms the essential part of the molecule of solapsone B.P.C., probably the first sulphone to be used to treat leprosy[53]. This drug has completely transformed the position of lepers, who can now be rescued from a life of misery as outcasts, and restored to their families. They say the only difficulty in this programme depends on the fact that it all seems too good to be true and that the lepers' relations can only with difficulty be persuaded that it is safe to welcome the lepers home again.

It is unnecessary to say much about antibiotics. Their properties had been widely studied for many years before the advent of penicillin. In 1929 Fleming followed up a fortunate accident and showed that a mould formed a substance which inhibited bacteria and that this substance was not toxic. In 1940 Florey and Chain and their colleagues made the exciting discovery that this substance had a chemotherapeutic action when injected into infected mice. It was really this observation which showed that it was worth spending much time and money on penicillin and it was the success of penicillin which created a new industry devoted to antibiotics. Active substances have been found in bacteria, and in actinomycetes and other fungi. Many of these organisms were obtained from soil, but penicillin was found in a bacteriological laboratory and the streptomycin-producing organism was found in a chicken's throat. In recent years new antibiotics have appeared in rapid succession and there are now few if any visible microbes simpler than spirochætes which cannot be inhibited by antibiotics. This is a great practical achievement which we owe very largely to the Americans. It can scarcely be said that each new substance is a fundamentally new discovery, but a large proportion of the credit for the revolution in therapy which antibiotics have caused must go to those who were responsible for the practical developments.

Dramatic advances have been made in recent years in the chemotherapy of tuberculosis[47]. Derivatives of dapsone gave promising results in animals but were disappointing when tested on man. The antibiotic streptomycin gave dramatically good results in animals and in human tubercular meningitis and elaborate clinical trials were arranged to test its value in pulmonary tuberculosis. The methods which were devised in connection with these trials have had a very important effect since they

have made it possible for the first time to assess new treatments for tuberculosis in a few months, and various new drugs have been assessed in this way. *p*-Aminosalicylic acid was discovered as the result of fundamental work on the metabolism of the tubercle bacillus. Thiosemicarbazones were discovered as the result of a systematic search based on the fact that sulphathiodiazoles have some curative effect on tuberculous animals[47]. Isoniazid was discovered in much the same way, but the most important fact which has been established by these trials is that when two remedies are used together the organisms are often less liable to develop resistance to either of them. This discovery may revolutionise the treatment not only of tuberculosis, but also of other diseases in which the development of resistant strains is a bar to progress. We have even been told lately that tumours may become resistant to drugs. It is possible that the secret of the cure of cancer may lie in the combination of two or more drugs in this way.

There have, of course, been many other important advances in chemotherapy during recent years. Thousands of new drugs have been tried in the treatment of malaria and some of the new remedies for this very important disease are better than the old remedies. Synthetic drugs have been found to relieve those afflicted with the distressing tropical and subtropical diseases due to bilharzia[48] and filaria[49]. All these things are advances in chemotherapy and they make an impressive list.

It is uncertain whether work on repellants is chemotherapy or not, but the methods of investigation are similar to those used in the study of chemotherapy[50]. In wartime it became especially important to prevent insects from biting men and women, since their bites were not only unpleasant, but liable to transmit dangerous diseases. It was known that if oil of citronella was applied to the skin insects were repelled for a while, but this effect did not last long enough. Systematic researches were therefore undertaken in the Orlando Institute in America in which human arms were smeared with hundreds of compounds and then exposed to healthy and hungry mosquitoes. The time to the first bite was measured and so it was found that dimethylphthalate had a prolonged effect and this substance is now widely used to repel mosquitoes and midges and allied creatures.

The most interesting discoveries are the unexpected ones, and in spite of the fact that research is much more organised to-day than it was once, unexpected discoveries are still made. In 1948 Hald, Jacobsen and Larsen[51] were interested in the substance tetraethyl thiuram disulphide because it combines with copper and they hoped to kill intestinal parasites by depriving them of copper. Experiments on animals had given promising results and the substance did not appear to be very toxic, but they found that when the substance, which is now known as disulfiram or antabuse, had been taken, alcohol always caused flushing, headaches, nausea and vomiting so that those who were normally too fond of alcohol lost their desire for it. Disulfiram now plays a useful part in the treatment of chronic alcoholism but Hald and Jacobsen were not content with this and continued their experiments until they found out how it worked. They noticed a smell of acetaldehyde and this led them to the biochemical

explanation of their discovery. Ethyl alcohol is normally oxidised in the body first to acetaldehyde and then to acetic acid. Disulfiram inhibits the second reaction and so causes the accumulation of acetaldehyde in the body and it is this which causes the symptoms. The moral of this tale is that even such things as unexplained headaches may be turned into important discoveries when someone has the energy and skill to seize the opportunity.

Another example of an unexpected discovery is the work of Hofmann and Stoll on lysergic acid diethylamide[52]. Hofmann was working on the chemistry of ergot derivatives in Basel and one day he felt very queer indeed. The whole world looked unusual and he had difficulty in getting home. Next day he felt better and wondered to himself whether perhaps he had been poisoned by the lysergic acid diethylamide which he had been making. In order to test the theory he took 0.25 mg. by the mouth and the effects were worse than ever. This is now recognised as one of the most active known drugs. A dose of 0.03 to 0.05 mg. by the mouth causes a condition resembling schizophrenia but lasting only for a few hours. The victim feels as if his own body does not belong to him and is intensely aware of the world around him which looks like a picture by a surrealist. Anyone who can get this stuff can now tell what it feels like to be mad. In the end this discovery may throw light on the action of the brain. In the meantime it presents a fascinating problem but has no obvious practical applications.

What are the general impressions produced by this rapid review of the origins of some of the drugs of today?

The most important fact is that, in the words of Starling: "Every discovery however important and apparently epoch-making, is but the natural and inevitable outcome of a vast mass of work, involving many failures, by a host of different observers." It is true that many discoveries have been accidents, but these accidents would not have occurred to anyone who was not engaged on a systematic search for new knowledge, and without all the technique and apparatus of modern science they would usually have passed unheeded by the world at large. Jansco and Jansco would never have discovered the effects of synthalin on trypanosomes if they had not been engaged on systematic pharmacological experiments. They had a lucky accident, but it was only because they were expert in the technique of chemotherapy that they were able to prove a new fact and give it to the world. This fact might, however, quite easily have been lost in the limbo of forgotten achievements buried in the back numbers of scientific journals, if Yorke and King and Ewins had not confirmed it and developed it until they had discovered a whole group of new remedies.

It is of course true that chance has played an important part in the discovery of new synthetic remedies. It seems likely to play a less important part in the future. New types of drug do seem to have been discovered recently in the light of pure reason, but we must be warned by the example of Jansco and Jansco who gave too much credit to the intellect in discussing the origins of their own discovery. It will probably always be more important to try a thing out, than to argue about it.

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RESEARCH PAPERS

A COMPARISON OF THE BRONCHODILATOR ACTIVITIES OF ADRENALINE AND NORADRENALINE: A PROPOSED PROCEDURE FOR THE BIOLOGICAL ASSAY OF ADRENALINE SOLUTIONS CONTAINING NORADRENALINE*

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IT is well known that noradrenaline possesses a weaker bronchodilator activity than adrenaline. The exact ratio of the relative activities of these two amines, however, has not been established. For example, using the aerosol method Luduena, Ananenko, Siegmund and Miller[1] found the ratio to be 1 : 3 while Chen, Portman, Russell and Ensor[2], found it to be 1 :60. On the isolated perfused lung of the guinea-pig Cameron and Tainter[3] and Lands, Luduena, Grant and Ananenko[4], found the ratio to be approximately 1 : 7 ,while Luduena *et al*[1] found the value to be 1 : 17. On the isolated tracheal chain of the guinea-pig McDougal and West[5] found the ratio to be 1 : 5. While such discrepancies might be due to differences in experimental conditions, it is of interest to note that the results obtained in 4 experiments performed by Hawkins and Schild[6] on human bronchial chains were also markedly different. Since these 4 experiments were presumably carried out under identical conditions, the existence of certain inherent differences in the response of the bronchial muscle to these two amines in different animals seems apparent. However, this was not determinable on account of the experimental design used. An attempt was therefore made to find out (1) whether the bronchodilator activities of adrenaline and similar agents could be studied by a more exact method and (2) whether significant differences in the comparative activities of adrenaline and noradrenaline could be demonstrated in different animals.

It is also known that adrenaline solutions obtained from natural sources not infrequently contain 10 to 20 per cent. of noradrenaline[7,8]. Such solutions will be less effective as bronchodilator agents than solutions of pure adrenaline although equally potent in vasopressor activity, if such solutions have been assayed only for their pressor effect; noradrenaline has been shown to have a vasopressor activity equal to or greater than that of adrenaline[9]. In order to ascertain, therefore, that an adrenaline solution will have a bronchodilator and a vasopressor activity equivalent to those of a standard solution of pure adrenaline it would seem desirable to assay that solution not only for its vasopressor but also for its bronchodilator activity. Further experiments were therefore performed to determine the feasibility of the method described herein for assaying the

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bronchodilator activity of adrenaline solutions containing 10 to 20 per cent. of noradrenaline; methods suitable for assaying the vasopressor activity have been worked out and adopted by a number of pharmacopœias.

METHOD

For these experiments the tracheal chain of the guinea-pig prepared according to the method of Castillo and de Beer[10] was employed. The contraction and relaxation of the circular muscle of the tracheal preparation were recorded on a kymograph by means of a light lever. Either Locke's or van Dyke-Hasting's solution was used in the tissue bath. After the chain had been suspended in the bath for $\frac{1}{2}$ to 1 hour, the relaxant actions of *l*-adrenaline and *l*-noradrenaline or mixtures of these two amines were compared, either under normal tension or following the addition of one of the following spasmogens: acetylcholine chloride, histamine acid phosphate or barium chloride. When a spasmogen was used, it was added to the bath and allowed to act for 5 minutes. The relaxant agent was then added and allowed to act also for 5 minutes before the bath was washed out. When the sympathomimetic amines were tested under normal tension, they were added to the bath, without any other drug, and allowed to act for 5 minutes. A suitable amount of acetylcholine was then added to the bath to hasten the recovery of the tension of the tracheal muscle. After the acetylcholine had remained in the bath for 5 minutes, the bath was washed out. A recovery period of 10 minutes was found to be adequate for both types of experiments. Thus, each dose required about 20 minutes. A total of about 18 doses was tested in each experiment.

The assay was usually begun after 2 orientating doses. For most experiments the design described by Noel[11] for the assay of adrenaline was used. Following this design 4 sets of tests were carried out in each experiment. Each set consisted of 4 tests, one on each of the 2 dose levels of the standard and the unknown, given in a randomised order.

RESULTS

A comparison of the bronchodilator activity of adrenaline and noradrenaline

Table I shows the results obtained from experiments in which Locke's solution was used as the nutrient fluid. The first column indicates the spasmogens used in the various groups of experiments. In the experiments of the last group the sympathomimetic amines were tested on tracheal chains under normal tension. The bronchodilator activity of noradrenaline in terms of adrenaline in per cent. is shown in the second column. It may be noted that it varied from 4·84 to 13·52 per cent. and the geometric mean potency of the whole series was 8·54 per cent. The weighted mean and its corresponding confidence limits for each group of experiments are also listed in the table. It was found that there were significant differences in potency within each group of assays, although the differences in the weighted means of the different groups were not significant at $P = 0{\cdot}05$. Table II shows the results obtained from similar experiments in which van Dyke-Hasting's solution was used instead of

Locke's solution. The geometric mean bronchodilator activity of noradrenaline in terms of adrenaline was 6·27 per cent. This value was found to be significantly different from that of the estimates obtained from experiments performed in Locke's solution. Moreover unlike those experiments significant differences in the relative activity were found not only within the different groups but also between the weighted mean of the barium chloride group and the means of the other groups.

TABLE I

THE BRONCHODILATOR ACTIVITY OF NORADRENALINE IN TERMS OF ADRENALINE.

(1) LOCKE'S SOLUTION

Spasmogen	Potency per cent.	Standard error per cent.	Test for parallelism*
1. Acetylcholine	11·58	0·38	2·20
	11·87	0·67	0·27
	6·76	0·28	0·63
	13·69	0·46	0·10
	7·00	0·36	0·17
	10·32 (7·73–13·78)†	0·43‡	
2. Histamine	10·24	3·39	0·17
	11·19	0·65	2·18
	5·68	0·41	0·06
	8·57 (5·21–13·03)	1·48	
3. Barium	7·57	0·47	0·73
	6·97	0·48	0·10
	4·84	0·42	0·09
	6·68 (5·24–8·52)	0·46	
4. None	13·52	2·39	0·09
	7·82	0·53	4·09
	7·09	0·81	0·40
	8·07 (6·22–10·48)	1·24	

* Test for parallelism of dose-response lines. The critical value (at P = 0·05) is 2·26 for the experiments listed in this and the following tables.
† Weighted mean and confidence limits (P = 0·05).
‡ Mean of standard errors.

It was also observed that in the experiments in which barium chloride was used as the spasmogen and van Dyke-Hasting's solution as the nutrient fluid the average standard error was the smallest. This would indicate that these assays were somewhat more precise than those carried out under other conditions. Furthermore, in these assays, the bronchodilator activity of noradrenaline in terms of adrenaline was the weakest. This would indicate that a better differentiation of these two amines would be observed under these conditions. For these reasons it was considered that barium chloride was the most suitable spasmogen and van Dyke-Hasting's solution was superior to Locke's for this type of experiment. All the following experiments were therefore conducted under these conditions.

A determination of the accuracy of the method.

In view of the fact that reproducible results were not obtained when the activities of 2 different substances were compared even in experiments carried out under identical conditions (as shown in Tables I and II), it

TABLE II

THE BRONCHODILATOR ACTIVITY OF NORADRENALINE IN TERMS OF ADRENALINE.

(2) VAN DYKE-HASTING'S SOLUTION

Spasmogen	Potency per cent.	Standard error per cent.	Test for parallelism
1. Acetylcholine	7·21	0·41	0·32
	7·28	0·48	1·73
	7·58	0·16	1·95
	9·48	1·13	0·55
	5·73	0·46	1·41
	7·45 (6·92–8·02)*	0·53†	
2. Histamine	8·92	0·67	1·19
	4·67	0·27	0·74
	5·32	0·33	0·09
	5·70 (3·94–8·25)	0·42	
3. Barium..	5·72	0·23	—
	5·90	0·46	2·27
	3·49	0·14	5·13
	3·30	0·37	0·88
	5·20	0·30	0·62
	4·69	0·39	0·52
	4·97 (3·77–5·69)	0·32	
4. None	8·37	0·88	1·38
	5·90	0·24	0·98
	8·64	0·88	0·76
	7·54	0·50	0·46
	6·31 (4·68–8·50)	0·63	

* Weighted mean and confidence limits (P = 0·05).
† Mean of standard errors.

was thought of interest to test solutions of various strengths of the same substance to check the accuracy of the results obtainable with this method. Table III shows the results of these tests. Adrenaline solutions were used as the standard and unknown in 5 and noradrenaline as the standard and unknown in 4 experiments as shown in the first column. In the second column are listed the strengths of the "unknown" solutions in terms of the standard. The differences between the true potency and the potency found, listed as actual errors, varied from 0·0 to 3·9 per cent. and were all smaller than the respective standard errors. The significant differences in the results obtained in the experiments as listed in Tables I and II are thus

TABLE III

RESULTS OF ASSAYS OF ADRENALINE AND NORADRENALINE SOLUTIONS OF KNOWN CONCENTRATION

Solution	True potency per cent.	Potency found per cent.	Standard error per cent.	Test for parallelism	Actual error per cent.	Corrected error per cent.
Adrenaline	100	102·4	6·21	1·25	2·4	2·4
"	100	96·1	4·16	—	3·9	3·9
"	100	100·0	3·98	—	0·0	0·0
"	79·4	77·7	6·46	1·35	1·7	2·1
"	79·4	77·9	4·65	1·31	1·5	1·9
Noradrenaline	100	99·5	3·30	0·29	0·5	0·5
"	100	102·0	6·08	1·31	2·0	2·0
"	79·4	81·7	4·08	0·11	2·3	2·9
"	79·4	78·0	4·67	1·01	1·4	1·8
					Average	1·9

the consequence of an inherent difference in the response of tracheal chains to these 2 amines in different guinea-pigs.

Assays on adrenaline solutions containing 10 *to* 20 *per cent. of noradrenaline.*

Since precise and accurate results were obtainable when solutions of the same amine were compared, it was considered likely that assays on adrenaline solutions containing only 10 or 20 per cent. of noradrenaline against pure adrenaline would also yield reproducible results. Table IV shows the results of a number of such assays. The results were found to be homogeneous in each of these two series of unknowns. Furthermore these experiments evidently show that adrenaline solutions containing 10 or 20 per cent. of noradrenaline have definitely weaker bronchodilator activities than solutions of pure adrenaline.

TABLE IV

RESULTS OF ASSAYS OF SOLUTIONS CONTAINING VARIOUS AMOUNTS OF ADRENALINE AND NORADRENALINE IN TERMS OF PURE ADRENALINE

Contents of "unknown"		Potency found per cent.	Standard error per cent.	Test for parallelism
Adrenaline per cent.	Noradrenaline per cent.			
90	10	92·9	7·66	0·50
90	10	92·4	8·96	0·68
90	10	79·7	8·09	0·58
90	10	89·3	6·54	—
90	10	88·5	4·26	—
90	10	91·3	2·06	—
80	20	79·8	7·12	—
80	20	70·9	11·25	1·14
80	20	76·5	3·30	1·58
80	20	92·9	5·24	3·82
80	20	84·3	2·39	—
80	20	93·7	9·52	—

The weighted means and confidence limits ($P = 0{\cdot}05$) for the first 6 and the last 6 experiments are 90·4 (87·1 to 93·7 per cent.) and 81·9 per cent. (78·4 to 85·5 per cent.).

It is to be noted that, in addition to Noel's design as cited above, the design proposed by Vos[12] for the assay of ergometrine was used in some of these experiments. The latter design has been adopted by U.S. Pharmacopeia XIV for the assay of adrenaline and posterior pituitary extract and also used by the authors for the assay of coronary dilator drugs as reported recently[13]. In the experiments in which this design was used the parallelism of dose-response curves was not tested. Apart from this point, it appears from this limited amount of data that these two designs are both suitable for this type of experiment.

TABLE V

THE BRONCHODILATOR ACTIVITY OF AMINOPHYLLINE IN TERMS OF ADRENALINE

Potency per cent.	Standard error per cent.	Test for parallelism
0·056	0·012	0·09
0·042	0·003	1·02
0·082	0·011	0·12
0·059	0·006	1·01
0·057	0·003	3·48

A comparison of the bronchodilator activity of aminophylline and adrenaline.

Another series of experiments were conducted under the aforementioned experimental conditions. The results are listed in Table V. The weighted mean potency in this series of experiments was 0·054 per cent. and the confidence limits ($P = 0.05$) were 0·050—0·059 per cent.

Discussion

It is of interest to note that precise as well as accurate results were obtainable with the method described herein. The precision and accuracy are evidenced by the small standard errors and actual errors observed in these experiments. Moreover, it was found in a number of experiments, the results of which have not been cited here, that the regression of response on dose was linear. These, as well as the fact that the dose-response lines of the standard and the unknown were parallel in most experiments, may be taken as indications that this method is suitable for the assay of bronchodilator drugs.

In spite of the accuracy of this method, assays on the comparative bronchodilator activity of unlike substances did not always yield reproducible results, even in experiments carried out under identical conditions. The estimate obtained in a single experiment is thus not dependable. However, the weighted means of the different groups of assays reported in this paper on noradrenaline in terms of adrenaline (using different spasmogens), with the exception of that of one group, were not significantly different from each other. These weighted means are thus probably fairly reliable. It also appears from these results that relative bronchodilator activities of noradrenaline and adrenaline do not depend, to any great extent, upon the spasmogens used.

Significant differences in the relative activities of these two sympathomimetic amines, such as those found in these experiments, have also been noted in other tissues. Thus according to Euler[14] the vasopressor activity of noradrenaline was 1 to 5 times as potent as that of adrenaline in the cat. While it is not altogether clear as to the mechanism responsible for the differences in the comparative bronchodilator activities of these two amines as observed in different animals, it may be of interest to note that thyroxine has been found to potentiate the action of adrenaline but not that of noradrenaline[15,16]. The differences of the comparative activities thus could be the result of a difference in the function of the thyroid in the different animals.

Although the results obtained in these experiments have shown that significantly different responses to these two amines exist in different guinea-pigs, the extent of variation is far too small to account for the discrepancy in the results obtained with the aerosol method as cited above. Differences in experimental conditions and factors such as differences in the rate of absorption, elimination, etc. in different guinea-pigs may also be responsible.

The geometric means of the bronchodilator activity of noradrenaline in terms of adrenaline, in the absence of a spasmogen, were 9·08 per cent.

and 7·53 per cent. for experiments performed in Locke's and van Dyke-Hasting's solution respectively. The geometric mean of the four experiments performed by Hawkins and Schild[6] on human bronchial chains was 4·4 per cent. It is to be noted that the racemic noradrenaline was used in their experiments. Since the *d*-isomer has been found to have only 1/20 to 1/60 the activity of the *l*-isomer[1], their estimate would thus be approximately 8·5 per cent. when computed in terms of *l*-noradrenaline. Furthermore the mean potency of aminophylline in terms of adrenaline has been found by Hawkins and Schild[6] to be 0·053 per cent., which compares favourably to 0·054 per cent. as observed in the experiments reported in this paper. These facts show that the results obtained in the tracheal chain of the guinea-pig are in close agreement with those obtained in the human bronchial chains.

While the true potency of the "unknown" solution used in the experiments as listed in Table IV is not known, the sum of the potencies of its two individual ingredients may be taken as its theoretical potency, assuming that there is no interference in activity when the two amines are present in the same bath. Thus as the bronchodilator activity of noradrenaline in terms of adrenaline under these experimental conditions was found to be approximately 5·0 per cent. (Table II), the potencies of the two "unknown" solutions (containing 90 per cent. of adrenaline + 10 per cent. of noradrenaline and 80 per cent. of adrenaline + 20 per cent. of noradrenaline) would be approximately 90·5 per cent. (90 per cent. + 0·5 per cent.) and 81·0 per cent. (80 per cent. + 1·0 per cent.) respectively. The results obtained in these experiments (90·4 per cent. and 81·9 per cent. respectively) are thus in fairly good agreement with the theoretical values. It may be concluded therefore that following the procedure described herein the tracheal chain of guinea-pigs is suitable for the standardisation of adrenaline solutions for their bronchodilator effect, while their vasopressor effect may be assayed by the blood pressure method.

It is to be noted that while this work was in progress a paper by West[17] appeared, suggesting, for reasons similar to those stated at the beginning of the present paper, that pharmacopœial adrenaline solutions should contain not more than 10 per cent. of noradrenaline; the determination of noradrenaline content is to be made in conjunction with a determination of either (*a*) the concentration of adrenaline (as by a chemical or a chromatographic method) or (*b*) the combined concentration of adrenaline and noradrenaline (as by a blood pressure method). Such a restriction on the noradrenaline content of adrenaline solutions will undoubtedly have its merits in the standardisation of these solutions for their bronchodilator and vasopressor effects. However, it should be pointed out that, in view of the fact that a variation in these concentrations (usually to the extent of 10 per cent.) is also permissible, adrenaline solutions standardised according to these criteria may possess a much weaker bronchodilator or a much stronger vasopressor activity than is indicated by their noradrenaline content. For example, adrenaline solutions containing slightly less than 10 per cent. of noradrenaline and having a combined concentration of

adrenaline and noradrenaline nearly 10 per cent. lower than the concentration of a solution of pure adrenaline, will possess a bronchodilator effect much weaker than that of the reference standard. In other words, their bronchodilator activity will be equivalent to that of solutions containing about 20 per cent. of noradrenaline but having a combined concentration of adrenaline and noradrenaline equal to that of the standard. On the other hand, adrenaline solutions containing slightly less than 10 per cent. of noradrenaline and having a concentration of adrenaline nearly 10 per cent. higher than that of a standard will possess a vasopressor effect much stronger than that of the standard. It is evident, therefore, that in order to ascertain that adrenaline solutions will possess a bronchodilator as well as a vasopressor activity equivalent to that of a standard preparation, the determination of these two activities as proposed in this paper would be more satisfactory than the determination of noradrenaline content.

Moreover, it should also be pointed out that most chemical and chromatographic methods are not entirely satisfactory for the standardisation of commercial adrenaline solutions in which different preservatives are invariably present. For example, the chemical method described by Auerbach[18] for the determination of noradrenaline is suitable only for adrenaline preparations in the powdered form; the chemical method described by Euler and Hamberg[19] yielded rather unreliable results as shown by West[17]; the chromatographic method usually has to be supplemented with a biological method in order to obtain accurate results[17,20]. Thus technically the method proposed in the present paper would also appear to be more satisfactory.

While the data pertaining to the bronchodilator and vasopressor activities *per se*, as obtained with the tracheal chain and the blood pressure methods, are adequate for the standardisation of adrenaline solutions, the noradrenaline content of these solutions may be computed from these data when this is desired. This computation may be made readily by using the formula given by Gaddum and Lembeck[21].

Summary

1. For the study of the comparative bronchodilator activities of adrenaline, noradrenaline and aminophylline a method has been described using the tracheal chain of guinea-pigs. The method has been found to yield data suitable for proper statistical treatment. The results from assays on different solutions of the same substance have been found to be accurate as well as precise. On the other hand, the ratios of the bronchodilator activities of different substances, while being precise, vary significantly in different experiments; the possible mechanism responsible for such differences have been discussed. The mean of the estimates from a group of assays, however, appears to give a fairly reliable result.

2. The mean bronchodilator activity of noradrenaline in terms of adrenaline has been found to be from 5·0 to 10·3 per cent. in the different groups of these experiments, and that of aminophylline has been found to be 0·054 per cent.

3. The method described herein, used in conjunction with the blood pressure method, seems to be suitable for the assay of adrenaline solutions containing 10 or 20 per cent. of noradrenaline.

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THE RELATIONSHIP BETWEEN THE INFRA-RED ABSORPTION SPECTRA OF SOME 5:5′-SUBSTITUTED BARBITURIC ACIDS AND THEIR PHARMACOLOGICAL ACTIVITY

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As barbiturates are used so extensively in clinical practice as depressants of the central nervous system, it is important to provide as much information as is possible about the relationship between their physicochemical and pharmacological properties. Barbiturates are classified pharmacologically on the basis of the duration of depression of the central nervous system which they produce and, therefore, representative members of the series from the long, moderate, short and ultra-short duration of action groups were selected for study.

This paper records the frequencies of the peaks of the absorption bands in the infra-red region of the spectrum associated with the carbonyl groups in each of the 14 barbiturate molecules considered.

It was thought that the various alkyl and aryl groups substituted in the 5:5′-position and the introduction of a sulphur atom in place of the oxygen atom in the 2-position might modify the double-bond single-bond resonance, $C{=}O$, $\overset{+}{C}{-}\overset{\cdot\cdot}{\underset{\cdot\cdot}{O}}\overset{-}{:}$, of the carbonyl bonds in the barbiturate nucleus to an extent which would cause changes in the frequencies of the infra-red carbonyl bands. Although the band shifts observed were small there appears to be some connection between the pharmacological action and the absorption spectra in that the shorter the duration of the action of the drug the lower were the frequencies of the carbonyl bands. The physicochemical implications arising from the analysis of the absorption bands and their relation to the carbonyl bonds are also considered below.

Method

The infra-red absorption spectra of the barbiturates were recorded in the frequency range from 1650 to 1850 wave numbers (wavelength range from 6·1 to 5·4 microns) on a rock-salt prism spectrometer. Solution strengths of about 1/100 molar in diethyl ether were found to give suitable spectra in path lengths of about 1 mm. The choice of solvent proved difficult since it is important to avoid any appreciable shift of the absorption bands, which may arise from molecular association in strongly polar solvents, if the bands are to be used to characterise the potentialities of the isolated molecule. The barbiturates studied were unfortunately

insoluble in normal non-polar solvents such as carbon tetrachloride, carbon disulphide, hydrocarbons, etc., with the exception of amylobarbitone, which was sufficiently soluble in carbon tetrachloride for a spectrum to be obtained. In order to decide whether chloroform, in which all the derivatives are very soluble, would be a sufficiently inactive solvent to use, the infra-red spectrum of amylobarbitone obtained using chloroform as a solvent was compared with that obtained in carbon tetrachloride. It was found that the absorption peaks in chloroform were very much broadened and displaced towards lower frequencies and it was therefore reluctantly concluded that there was too much solute-solvent association for chloroform to be a useful solvent. Various

TABLE I

THE FREQUENCIES OF THE PEAKS OF THE INFRA-RED ABSORPTION BANDS AND THE DURATION OF PHARMACOLOGICAL ACTION OF SOME BARBITURATES. THE SHORTER THE DURATION OF CENTRAL NERVOUS DEPRESSION THE LOWER ARE THE FREQUENCIES OF THE THREE BANDS.

Duration of action on C.N.S.		Barbiturate compound	Substituted groups R	Substituted groups R′	Peak of infra-red absorption band (wave numbers) *a*	*b*	*c*	*Duration of action in rabbits of 60 per cent. of LD50 intraperitoneally hours	minutes
Long	1	PHENOBARBITONE	Ethyl-	phenyl-	1712	1746	1754	22	—
	2	BARBITONE	Ethyl-	ethyl-	1712	1739	1756	18	10
	3	RUTANOL	Methyl-	phenyl-	1717	1745	1754	—	—
	4	METHYL PHENOBARBITONE	Ethyl-	phenyl-methyl-	1695	1729	1756	—	—
Moderate	5	ALLOBARBITONE	Allyl-	allyl-	1712	1742	1755	—	—
	6	PROBARBITAL	Ethyl-	*iso*propyl-	1710·5	1741	1762	8	30
	7	APROBARBITAL	Allyl-	*iso*propyl-	1710	1740	1756	6	0
	8	AMYLOBARBITONE	Ethyl-	*iso*amyl-	1710	1742	1764	3	54
	9	SANDOPTAL	Allyl-	*iso*butyl-	1708	1741	1755	—	—
Short	10	PENTOBARBITONE	Ethyl-	1-methylbutyl-	1711	1741	1755	2	48
	11	CYCLOBARBITONE	Ethyl-	*cyclo*hexenyl-	1709	1739	1753	2	32
Ultra-short	12	HEXOBARBITONE	Methyl-	*cyclo*hexenyl-1-methyl-	1701	1722	1754	—	†42
	13	THIALBARBITONE	Allyl-	*cyclo*hexenyl-2-thio-	1708	1737		—	—
	14	THIOPENTONE	Ethyl-	1-methylbutyl-2-thio-	1708	1743		—	†28

* After Fitch and Tatum[1], Werner, Pratt and Tatum[2].
† By intravenous administration.

other solvents such as some of the higher alcohols and the chlorinated hydrocarbons were tried and found unsatisfactory. Diethyl ether was finally chosen as all the derivatives with one exception were soluble in it and the spectrum of amylobarbitone in diethyl ether was virtually the same as in carbon tetrachloride as was also true of several other non-barbiturate compounds containing the carbonyl group which were soluble both in ether and carbon tetrachloride. Anæsthetic grade ether, freed from water by standing it over anhydrous sodium sulphate, was employed and this was found to be completely transparent in thicknesses of 1 mm., over the spectral range investigated. The average spectral band width over the range of observations was about 2 wave numbers and the frequencies were determined by reference to the well-defined atmospheric bands of water vapour which fell within the same range.

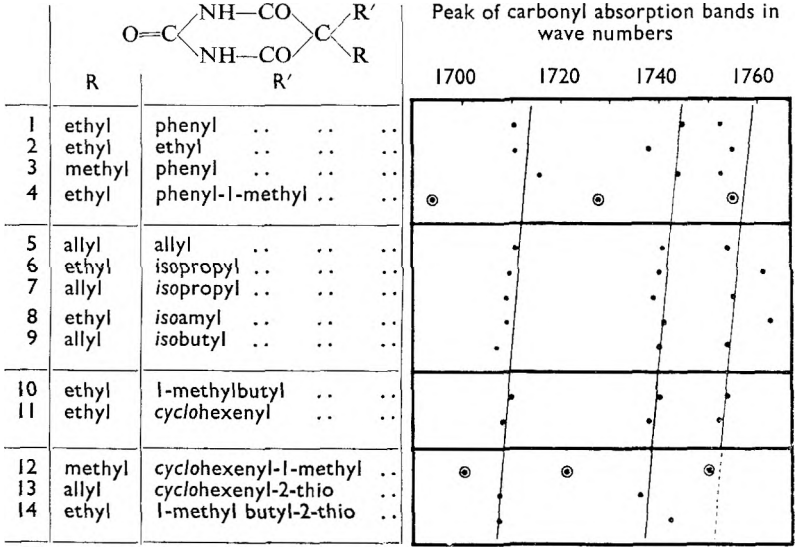
Results

The frequencies of the peaks of the infra-red absorption bands (*a*, *b* and *c*) associated with the carbonyl groups in the barbiturates studied are recorded in Table I together with the duration of the depression of the central nervous system in rabbits produced by the intraperitoneal administration of 60 per cent. of the LD50 of certain of the barbiturate sodium salts (Fitch and Tatum[1], Werner Pratt and Tatum[2]). Throughout this paper the order in which the barbiturates are tabulated commences with those of the longest duration of action and terminates with those with the shortest actions (Tatum[3]).

Discussion

Relationship between infra-red absorption bands and pharmacological action

When the frequencies of the peaks of the absorption bands associated with the carbonyl groups are plotted for each of the barbiturates arranged in order of pharmacological activity (Fig. 1), a degree of correlation appears. In general the shorter the duration of activity, the lower are the frequencies of the 3 absorption bands. This might be associated with a slight increase in water solubility of the substance which would follow from the increased resonance of the carbonyl to $\overset{+}{C}—\overset{-}{O}$ structures indicated by such shifts. However, these changes are only minor ones and exceptions must be expected where other factors depending more



	R	R′
1	ethyl	phenyl
2	ethyl	ethyl
3	methyl	phenyl
4	ethyl	phenyl-1-methyl
5	allyl	allyl
6	ethyl	isopropyl
7	allyl	*iso*propyl
8	ethyl	*iso*amyl
9	allyl	*iso*butyl
10	ethyl	1-methylbutyl
11	ethyl	*cyclo*hexenyl
12	methyl	*cyclo*hexenyl-1-methyl
13	allyl	*cyclo*hexenyl-2-thio
14	ethyl	1-methyl butyl-2-thio

FIG. 1. The frequencies of the peaks of the infra-red absorption bands are plotted for fourteen barbiturates arranged in order of decreasing duration of depression of the central nervous system as in Table I. The peak frequencies for those barbiturates having a methyl group substituted at the 1-position are indicated by the symbol ⊙. In these barbiturates, the 2 lower of the 3 bands are displaced towards the low-frequency end of the spectrum.

upon the properties of the substituent groups themselves are involved. There are 2 types of exception to this rule. The first concerns methylphenobarbitone and hexobarbitone, each of which possesses a methyl group attached to the C atom at the 1-position. This appears to be associated with a displacement of the 2 lower of the 3 absorption bands towards the low frequency end of the spectrum. The second exception is that of the thiobarbiturates which have a C=S linkage in place of the carbonyl group in the 2-position. The physico-chemical implications of these 2 exceptions are discussed in the next section.

If the actual duration of action is plotted against the peak frequencies of the (*a*) absorption bands (Table I), the trend for the short duration of action drugs to have a lower frequency of absorption in the infra-red than the longer duration barbiturates is seen to be broken only by point 10, that in the case of pentobarbitone (Fig. 2).

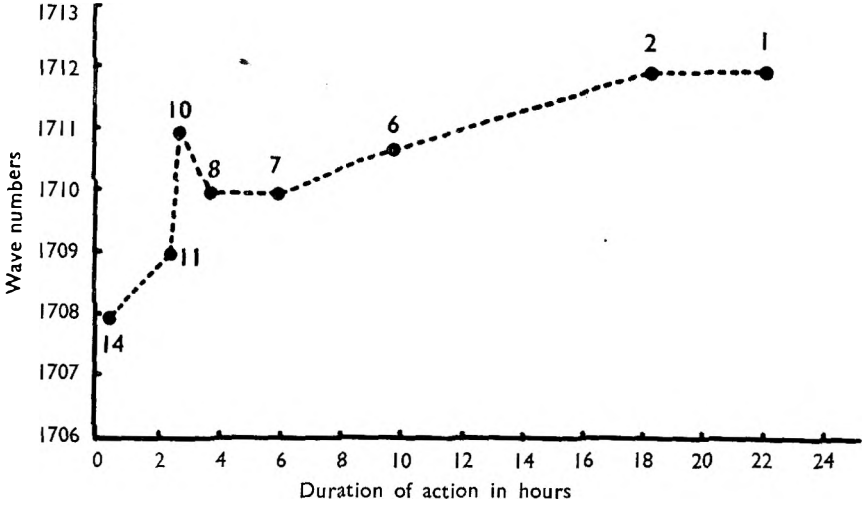


FIG. 2. This graph shows the relation between the duration of action of some barbiturates and the peak of the lowest (*a*) of the infra-red absorption bands associated with the carbonyl groups in the molecule. It is compiled from the data in Table I, and the number beside each point refers to the order of the barbiturate in Table I.

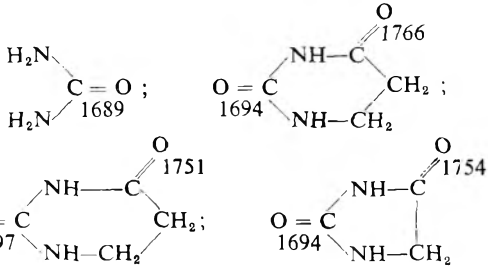
As the difference in the frequencies of the carbonyl absorption bands between members of the series is so small, the infra-red absorption spectra cannot be used to identify the individual members of the barbiturate series of drugs by consideration of the carbonyl frequencies alone. They can, of course, be identified by using the whole spectral range (Umberger and Adams[4]). Solvent difficulties may be avoided with the pressed potassium bromide disc technique. This, however, would not give the spectra characteristic of unassociated molecules which are required in order to explain differences in their chemical activity attributable to changes in the barbiturate nucleus.

In Figure 2, there are only 3 wave numbers between phenobarbitone with a duration of action of 22 hours and cyclobarbitone which acts for

2 hours 32 minutes and this indicates that extensions in the duration of pharmacological action are not to be sought in major changes in the chemical nature of the nucleus. The small frequency shifts that occur *can, however, probably* be linked with small changes in water solubility or lipin solubility which may be one important factor in the duration of activity of the drug.

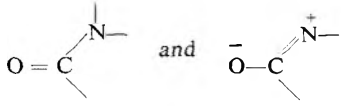
Analysis of absorption bands and their relation to the carbonyl bonds

In Figure 1, there appear to be 3 strong absorption bands in the wave number region 1700 to 1770 with each barbiturate of the long, medium and short duration of action groups. The first of these bands occurs around 1710, the second around 1740 to 1745 and the third around 1755 wave numbers. A comparison of the band positions with those of a number of structurally related molecules such as those given below (from Randall, Fowler, Fuson and Dangl[5]) leads to the conclusion

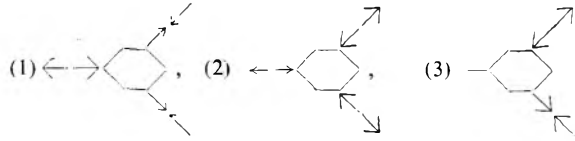


that the 1710 frequency is associated with the carbonyl in the 2-position while that in the 1755 cm.$^{-1}$ region is associated with vibrations of the carbonyl in the 4- or 6-positions. The band in the 1740 position is probably associated with a vibration which is an in-phase combination of the vibrations in the carbonyl bonds in the 2- and 4-, 6-positions.

In order to understand the vibrations of the system it should first be stated that according to modern resonance theory (Pauling, Corey and Branson[6]) the resonance in the barbiturate ring between forms containing



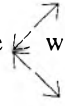
is such as to make coplanar all atoms except those in the R and R′ groups. The three carbonyl bonds act as three coupled oscillators giving rise to the following mode of vibrations which are clearly obtained by



coupling the oscillators "in phase" and "out of phase." Vibration (1) is to be associated mainly with vibrations in the carbonyl bond in the 2-position as this is expected to have the lowest bond frequency because

it has two neighbouring nitrogen atoms from which it can draw charge enabling it to have more single bond polar ($\overset{+}{C}—\overset{-}{O}$) character. In such a system the lower frequency bond largely controls the frequency and the motion is mainly in this bond. The higher frequency bonds move in phase with relatively small displacements. At higher frequencies when the vibration does correspond to motion mainly in the 4 and 6 bonds as in vibration 2, the vibration in the 2 carbonyl bond because of its lower value must follow it in opposite phase and will produce a vibration of somewhat lower frequency than that which would be associated for example, with a molecule with 4 and 6 carbonyl bonds but none in the 2-position. Thus vibration 2 is to be associated with the bands in the 1740 cm.$^{-1}$ region. It should be noted that the changing dipole associated with vibrations (1) and (2) are along the OX direction which in this case is the axis of symmetry and the motions mix with one another on this account. On the other hand, vibration (3) has its change perpendicular to the axis and therefore cannot involve motion in the 2 bond. Thus it can attain the highest frequency without modification by the lower value of the 2 bond. It is therefore to be identified with the highest frequency group around 1755 cm.$^{-1}$.

In the two substances with a methyl group attached to the nitrogen in the 1-position (viz., methylphenobarbitone and hexobarbitone) bands are obtained at lower frequencies than for any of the other compounds. In particular, it is the 2 lowest frequency bands whose frequencies are reduced, while the highest frequency band remains largely unaffected. This is consistent with the above analysis which indicates mixing with the 2 carbonyl bond for vibrations (1) and (2) but not for (3). The effect of substituting methyl groups is largely to permit greater charge transfer from the 1.N atom to the 2 and 4 carbonyl bonds thus enabling these bonds to assume greater single bond polar character. Similar frequency reductions are obtained in replacing hydrogen atoms by alkyl groups in simpler cases, e.g., the carbonyl bond in urea at 1689 cm.$^{-1}$ is shifted to 1618 cm.$^{-1}$ in *sym*-diethyl urea.

Finally we have to consider the two derivatives with C=S bonds in the 2-position. This bond has a much lower frequency (*ca.* 860 cm.$^{-1}$) than that of a carbonyl and thus does not interact appreciably with them and we are left with two carbonyl vibrations one in phase  with dipole change along the axis of symmetry and one  in which the change is perpendicular to this axis. Another important factor is that, since the C=S bond has much less electronegativity than has the C=O bond there is practically no drain of negative charge from the 1 and 3 nitrogen atoms to the 2-position in these substances and this charge is therefore released to go to the carbonyl bonds in the 4 and 6-positions. The result is that the bands corresponding to vibrations of these bonds occur

at lower frequencies than do the corresponding bands in sulphur-free barbiturates. This also indicates that the 4:6-carbonyl bonds in the thiobarbiturate compounds are more strongly polar than the same bonds in non-sulphur barbiturates containing molecules.

The correlation of the pharmacological activity with the condition of the carbonyl bond as judged from Figure 1, would indicate that the greater the double bond character of the carbonyl groups, the higher the bond frequencies, the lower the polarity, the longer the duration of activity. The differences between the different drugs are, however, small and the activity appears to depend on other factors in addition. It does not seem that the various groups R and R′ can exert much more than a steric effect. Of probably more importance is their capacity to increase the fat solubility, an effect which would be expected also to increase with increase in double bond character (reduction in polarity). This latter effect may well be the important factor which controls the duration of activity by permitting longer retention of the intact barbiturate ring in the body. The infra-red evidence presented here, though intrinsically interesting, and indicating features which should result in somewhat greater fat and lower water-solubility of the active nucleus for the longer acting drugs, does not suggest any other major factors which might affect the duration of their activity.

Summary

1. The infra-red spectra of a number of barbiturates classified according to the duration of their activity in depressing the central nervous system have been recorded in the range of the carbonyl frequencies.

2. An analysis of the bands obtained has been made and their relation to the bonds derived.

3. It is found that the longer acting drugs tend to have a band system at slightly higher frequencies.

4. This indicates slightly lower polarities and greater fat-solubility, which is suggested as being a possible contributory cause to the duration of their activity.

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ON THE METABOLISM OF SOME AROMATIC NITRO-COMPOUNDS BY DIFFERENT SPECIES OF ANIMAL

PART I. SOME FACTORS INFLUENCING THE ELIMINATION OF 4:6 DINITRO-*o*-CRESOL FROM THE BLOOD OF THE RAT

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INTRODUCTION

KING and Harvey[1] have shown that the rat, the rabbit and man eliminate dinitro-*o*-cresol at widely differing rates. This observation suggested that further work should be carried out to investigate, first, the influence of various factors, for example, age, weight, sex and environmental temperature on the elimination rate from the blood of some commonly used laboratory animal, and secondly, the elimination of this and analogous nitro-compounds by different species of animal.

The present communication reports the results of the first of these investigations, namely, the effect of some common variables on the elimination of dinitro-*o*-cresol by the rat.

EXPERIMENTAL

The methods employed were essentially similar to those described by King and Harvey[1]. Hooded rats of the same strain were used throughout. Statistical analysis for variance, and the method employed for the determination of regression lines (b) were essentially as described by Emmens.[2]

RESULTS

These are given in Tables I and II and in Figure 1. Table I summarises the effects of various common factors on the slope value b. This shows that the decay of dinitro-*o*-cresol from the blood is exponential and that the range of 17 values is $-0{\cdot}010$ to $-0{\cdot}022$. Table II compares values of b following tail bleeding and cardiopuncture (after ether anæsthesia) methods of obtaining blood, and demonstrates that the former method results in values of b that are about 30 per cent. smaller than the latter.

Figure 1 is a diagrammatic representation of the results in terms of the extreme limits of time necessary to eliminate dinitro-*o*-cresol almost completely from the blood of the rat when the initial blood level is 50 to 60 μg./g.

DISCUSSION

The overall range of elimination rates derived from 17 experiments is $-0{\cdot}010$ to $-0{\cdot}022$, or 120 per cent. variation. This is equivalent to saying that an initial blood level of about 60 μg./g. will be eliminated almost completely from the blood in 82 to 182 hours (Fig. 1).

However, this range includes at least 3 abnormal variables, namely,

TABLE I

EFFECT OF VARIOUS FACTORS ON THE ELIMINATION OF DINITRO-*o*-CRESOL FROM THE BLOOD OF RATS

Serial number	Description of factor	Weight g.	Number of animals	Dose mg./kg.	Mode of administration	$b \pm$ SE b (a)	Per cent. difference of b between pairs. (Based on lower value)	Remarks
	(A) Sex and Size							
1	Female	150–200	4	9 × 20	Oral	− 0·010 ± 0·0004		
2	Female	,,	5	1 × 30	,,	− 0·011 ± 0·0006	− 7	All doses given at normal temperature, i.e., 18° to 20° C and relative humidity 50 to 60 per cent.
3	Male	,,	5	1 × 20	Intraperitoneal inj.	− 0·017 ± 0·001	− 53	
4	Female	,,	6	1 × 20	,, ,,	− 0·013 ± 0·0003	− 30	
5	Large (♂ + ♀)	350–400	4	1 × 20	,, ,,	− 0·017 ± 0·0008	− 12	
6	Small (♂ + ♀)	40–50	16	1 × 20	,, ,,	− 0·019 ± 0·0008		
	(B) Mode of Administration and Environmental conditions							
7	Female	150–200	6	2 mg./cu.m/5 hrs.	Aerosol	− 0·010 ± 0·0009	− 0	25° to 30° C and increasing relative humidity
8	Male	,,	6	1 × 5	Intraperitoneal inj.	− 0·010 ± 0·0005	− 7	37° C and relative humidity 50 per cent.
9	Male	,,	6	1 × 10	,, ,,	− 0·011 ± 0·0005	− 40	Pretreated at 37° C and relative humidity 50 per cent. for 7 days before administration of dinitro-*o*-cresol
10	Male	,,	6	1 × 10	,, ,,	− 0·014 ± 0·0007		
11 (a)	Mixed (♂ + ♀)	,,	6	1 × 15	,, ,,	− 0·011 ± 0·003	− 36	Given 7 days rest before oral administration
(b)		,,		1 × 15	Oral	− 0·015 ± 0·005	− 15	64
12 (a)	Mixed (♂ + ♀)	,,	6	1 × 15	,,	− 0·013 ± 0·0005	− 38	Given 7 days rest before intraperitoneal injection
(b)		,,		1 × 15	Intraperitoneal inj.	− 0·018 ± 0·0007		
	(C) Metabolic Depressant—Methylthiouracil							
	Mixed (♂ + ♀)	150–200	60	1 × 15	Intraperitoneal inj.			Divided into 3 groups (serials 13, 14, 15) of 20 each.
13			20		,, ,,	− 0·018 ± 0·0009	22	1 × 15 mg./kg. of methyl thiouracil by intraperitoneal injection
14			20		,, ,,	− 0·022 ± 0·0009	30	6 3 × 15 (1 daily) thiouracil by intraperitoneal injection
15			20			− 0·017 ± 0·0008		Control—No methylthiouracil

NOTE.—Serials 6, 13, 14 and 15 bled by cardiopuncture—the remainder by tail.

high environmental temperatures and relative humidities and the effect of a metabolic depressant. Comparison of some smaller sub-groups of values indicates that the differences are less. For example, the values of *b* for series 1, 2, 11b and 12a following oral administration are − 0·01, − 0·011, − 0·015, − 0·013 (mean − 0·0124 ± 0·001), and for serials 3, 4, 5, 11a and 12b following intraperitoneal injection are − 0·017, − 0·013, − 0·017, − 0·011 and − 0·018 (mean − 0·015 ± 0·003).

TABLE II

EFFECT OF VARIATION OF MODE OF OBTAINING BLOOD ON THE ELIMINATION OF DINITRO-*o*-CRESOL BY THE RAT

32 hooded male rats; 83 to 117 g., mean 102 g. ± 1·9, given a single dose of 15 mg./kg. by intraperitoneal injection. They were then divided into 2 groups; (A) of 6 for tail bleeding, and the remaining 28 (B) for cardiopuncture under ether anæthesia. These animals were killed after the blood samples were obtained.

GROUP A—TAIL BLEEDING

Individual Analysis

Rat No.	$b \pm SE\,b$	
1	− 0·0143 ± 0·00063	
2	− 0·0179 ± 0·00049	
3	− 0·0167 ± 0·00098	Mean slope b =
4	− 0·0145 ± 0·00191	− 0·0158 ± 0·00064
5	− 0·0171 ± 0·00147	
6	− 0·0145 ± 0·00131	

Block Analysis

$\bar{x} = 37 \quad \bar{y} = 0{\cdot}92895$

	S. squares	D.F.	Mean square	F.
Between Times	3·92648	3	1·30883	262
Linear Regression	3·91544	1	3·91544	783
Departures	0·01104	2	0·00552	1
Within Times	0·10085	20	0·005	

From this $b \pm SE\,b = -0{\cdot}0158 \pm 0{\cdot}00064$

GROUP B—CARDIOPUNCTURE

Block Analysis

$\bar{x} = 37, \quad \bar{y} = 0{\cdot}08920958$

	S. squares	D.F.	Mean square	F.
Between Times	7·14692	3	2·3823	45
Linear Regression	7·00081	1	7·00081	134
Departures	0·14611	2	0·07306	1·4
Within Times	1·04110	20	0·05206	

From this $b \pm SE = -0{\cdot}021 \pm 0{\cdot}0018$

From this observation it can be calculated that:

b_B is 31 per cent. greater (faster) than b_A

$\sqrt{SE\,b_A + SE\,b_B} = 0{\cdot}0018$, or

the difference between b_A and b_B is significant.

Two general conclusions can be made from these observations. First, that variations such as sex, magnitude and frequency of the dose of dinitro-*o*-cresol do not have any very marked effects on its elimination rate. Secondly, that any of the values of *b* in the smaller ranges or even in the complete range will permit at least a semi-quantitative comparison with values obtained under similar conditions for other species of animal, e.g., the rabbit (King and Harvey[1]).

The methods employed in administering dinitro-*o*-cresol and in collecting the blood gave different results. Up to the present no theory can be advanced to explain why *b* values calculated from concentrations

in blood samples obtained by cardiopuncture following intraperitoneal injection are significantly greater (elimination rate more rapid) than those obtained by oral administration or by inhalation.

Certain practical aspects of these experiments have a bearing on the design of toxicological assays. Obviously there is considerable value in determining some index of the elimination rate of a toxic material, and it is essential to design experiments that will embody as many natural conditions of environment and of exposure as possible. Clearly it is better to administer a substance *via* the alimentary canal or through the lungs and to obtain blood by tail bleeding than to adopt the less natural

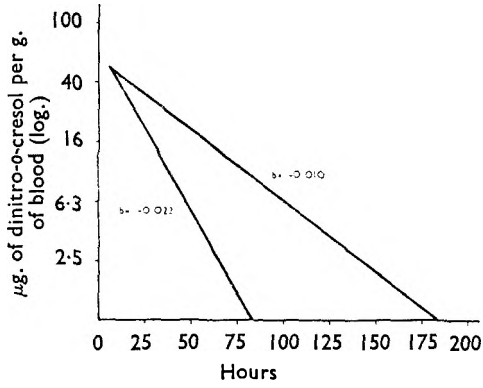

FIG. 1. Elimination of dinitro-*o*-cresol by the rat, range values of *b*.

procedures already referred to. Of necessity cardiopuncture may have to be employed, for example, if growing rats or small animals (for example, mice) are used, but such a procedure will involve a greater waste of animal life. By use of a few (for example, 6) animals for some preliminary experiments, considerable information can be obtained on elimination rates from the blood. There is no doubt that the results obtained will give some guide in assessing any possible accumulation of the substance in the animal following repeated exposures.

Several facts emerge from this study which may have some practical applications in maintaining safe conditions for spray operators and others handling dinitro-*o*-cresol. First, it is clear that a sudden increase in the environmental temperature and in the relative humidity is unlikely to cause any marked changes in the elimination rate. In fact the actual rates of elimination of 2 groups of rats given 5 and 10 mg./kg. dinitro-*o*-cresol are identical ($-0{\cdot}01$). This value is numerically the lowest of the range, and therefore represents the slowest rate of elimination. Secondly, acclimatisation to these two environmental factors results in a somewhat faster elimination rate and one that is more comparable with that obtained under normal temperatures and humidities. The exacerbating effect of high environmental temperatures on the action of dinitro-*o*-cresol is well known (Bidstrup and Payne[1]; Parker, Barnes and Denz[1];

King and Harvey[5]), but it has been demonstrated (King and Harvey[5]) that heat does not alter significantly the highest blood concentration in intoxicated animals.

These observations add further emphasis for the need for maintaining the strictest safety measures among men handling dinitro-*o*-cresol, especially in hot weather. Not only does heat cause an increase in toxicity but it fails to assist its elimination from the blood. Thirdly, methylthiouracil has been suggested as a therapeutic agent in reducing high metabolic rates caused by excessive doses of dinitro-*o*-cresol (Siedek and Hoffman Credner[6]). The present studies indicate that the elimination rate from man is unlikely to be altered greatly by the employment of methylthiouracil. In other words it is at all times essential to encourage the natural elimination from man by removing him from exposure once early toxic symptoms appear (Bidstrup, Bonnell and Harvey[7]), and by carrying out the recognised treatment, including bed-rest in cool conditions, on persons who are seriously poisoned (Pollard and Filbee[8]).

Summary

1. Elimination rates of dinitro-*o*-cresol from the blood of hooded rats have been determined under normal and abnormal (high environmental temperature, metabolic depressant) conditions. The effects of varying the methods of administration and of obtaining the blood have also been studied. Seventeen values of b ranged from $-0{\cdot}010$ to $-0{\cdot}022$.

2. Elimination rates are faster when dinitro-*o*-cresol is administered by intraperitoneal injection than by mouth or by inhalation of an ærosol, and when blood is obtained by cardiopuncture rather than by tail bleeding.

3. Sudden application of high environmental temperatures to animals poisoned by dinitro-*o*-cresol caused a slight slowing of the elimination rate. Acclimatisation for 7 days under the same conditions resulted in a faster elimination rate.

Our thanks are due to Miss Jean Peal for her constant and reliable assistance, and to Miss Audrey Mackrill and Miss June Welsher for help with the animals.

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CHRONIC TOXICITY OF BREAD ADDITIVES TO RATS

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Currently there is much interest in the use of "chemical" additives in the food industry. Probably a considerable proportion of this interest stems from the report of Sir Edward Mellanby in 1946[1] that running fits had been induced in dogs by including in their diets flour that had been bleached and improved by treatment with nitrogen trichloride. The offending agent was finally isolated and found to be a derivative of methionine, methionine sulphoximine.[2] As a result of these discoveries several countries, Canada included, switched to the use of chlorine dioxide as a flour improving and bleaching agent. More recently, a great many compounds have been investigated in an effort to establish whether they might be added to food with a reasonable surety that no harm would result. At the time this investigation was planned a great deal of interest was being shown in the additives used in bread manufacture. Of the many compounds intentionally added to bread, chlorine dioxide, sodium propionate, the antioxidants *n*-propyl gallate and butylated hydroxyanisole, and the emulsifier polyoxyethylene (8) monostearate* were selected for study. The number of compounds which could be tested in a chronic toxicity trial was limited by the facilities available and it was considered that these 4 additives were most deserving of study.

Most investigations of this nature have been made by adding such compounds singly, in abnormally large amounts, to otherwise normal experimental diets. This may result in unequal concentrations of proteins, vitamins, minerals, and calories among the diets and the difficulty of providing adequate controls under these conditions is obvious. It appeared that a sounder method of checking for chronic toxicity would be to incorporate the additives in bread at abnormally high levels and, maintaining the levels of the nutritive elements as close to those of the control diets as possible, to give this bread as a major part of a good experimental diet. It was decided to reverse the usual procedure and to check the toxicity of the additives in combination rather than singly since the toxicity of the finished bread was of major concern.

In view of the popular "100-fold margin of safety"[3] or "margin of uncertainty" as it is rather aptly termed by Oser[4], it was decided to incorporate the additives under test in bread at 50 times the normal concentration and to give the finished bread as 75 per cent. of the experimental diet. It was considered that giving this amount would correspond to over 100 times the usual rate of intake for man.

The seven diets used are shown in Table I. The emulsifier, polyoxyethylene (8) monostearate, and the mould inhibitor, sodium propionate,

* The use of this material in bread had not been prohibited in Canada at the time these investigations were started.

were taken as approximately equivalent, on a caloric basis, to carbohydrate and additions of these ingredients were made at the expense of glucose. On this basis all 6 bread diets were approximately isocalorific and were as near alike in all respects, except for the additives under test, as could be achieved.

Diet I contained bread made to an essentially normal formula with the exception that the glucose content was elevated to permit substitution of high levels of emulsifier and mould inhibitor in other diets. Diet II contained all 4 test ingredients at 50 times the level used in diet I. With the flour and antioxidants this was accomplished conveniently with little weight change in the diet ingredients. Diets III, IV, V and VI were similar to diet II with the exception that one ingredient in each, emulsifier, propionate, antioxidants, or chlorine dioxide, respectively, was reduced to the control concentration. Diet VII consisted of a commercial cubed diet* ground to a consistency similar to that of the other diets. The basal diet used as 25 per cent. of diets I to VI supplied adequate supplementary amounts of all the required vitamins as well as high quality protein, bulk, minerals, and fat.

Technical difficulties were encountered in the preparation of the various breads, which are referred to here by the same numbers as the corresponding diets. Those mixes which contained the high concentration of propionate showed very poor yeast action. Increasing the quantity of yeast had little beneficial effect. To overcome this difficulty to some extent the dry propionate was incorporated only into the surface of the dough so that fair yeast action in the bulk of the material was obtained. With this modification of the mixing scheme it was possible to produce breads which had been proofed approximately equivalent lengths of time. As might be expected, loaf volume and texture on numbers II, III, V and VI were poor. To a lesser extent bread I was inferior to normal quality because of the inhibitory effect of the high glucose content on yeast action. Bread IV was excessively friable and open textured.

After baking, the loaves were wrapped and stored in the refrigerator for short periods before being thinly sliced. The slices were then placed on edge in a special drier and dried in a forced draft hot air oven at 70° C. The drying process usually took 3 to 5 hours, and, for those loaves high in propionate, was accompanied, as was the baking, by a strong odour of propionate. Subsequently the dried bread was ground in a hammer mill and stored in sealed metal containers in the refrigerator (4° C.) until required for use. This dried, ground bread was rarely kept for more than 1 month. Diets were mixed at intervals of no greater than 2 weeks and were kept in closed cans in the refrigerator except during feeding periods.

A preliminary feeding trial of 3 weeks' duration indicated that there was little difference in the acceptability of the several diets to weanling rats. None of the ingredients was acutely toxic at the concentrations under test. Accordingly, a large scale experiment was started. A group of 182 male albino rats of Wistar strain ranging in age from 27 to 41 days

* Master Fox Cubes, Toronto Elevators, Toronto.

TABLE I

DIET COMPOSITIONS

Part A. 75 per cent. of the diet	Diet						
Ingredient	I	II	III	IV	V	VI	VII
Commercial bread flour[1]	70·5	—	—	—	—	70·5	G
Bread flour 50 × ClO_2[1]	—	70·5	70·5	70·5	70·5	—	R
Commercial lard[2]	1·8	—	—	—	1·8	—	O
Lard 50 × Antioxidants[2]	—	1·8	1·8	1·8	—	1·8	U
Sodium propionate[3]	0·1	5·0	5·0	0·1	5·0	5·0	N
Polyoxyethylene (8) monostearate[4]	0·3	15·0	0·3	15·0	15·0	15·0	D
Cerelose (glucose)	20·4	0·8	15·5	5·7	0·8	0·8	
Salt (NaCl)	1·8	as I	as I	as I	as I	as I	M
Skim milk powder	2·2	"	"	"	"	"	A
Malt flour	0·5	"	"	"	"	"	S
Yeast food[5]	0·2	"	"	"	"	"	T
Wytase[6]	0·9	"	"	"	"	"	E
Yeast	1·3	"	"	"	"	"	R
	100·0	100·0	100·0	100·0	100·0	100·0	

Part B. 25 per cent. of the diet	Diet						
Ingredient	I	II	III	IV	V	VI	
Casein	60·0	as I	as I	as I	as I	as I	F
Alphacel[7]	9·4	"	"	"	"	"	O
Corn oil	12·8	"	"	"	"	"	X
Mineral mix (a)	12·0	"	"	"	"	"	C
Vitamins and excipient (b)	5·8	"	"	"	"	"	U
	100·0	100·0	100·0	100·0	100·0	100·0	B
							E
							S

(a)—Minerals—the 12·0 g. of mineral mix present in 100 g. of basal diet were made up as follows—

KCl	1·580	$FePO_4$	0·195
KH_2PO_4	4·100	$KAl(SO_4)\cdot 12H_2O$	0·001
$Ca_3(PO_4)_2$	1·970	$CuSO_4\cdot 5H_2O$	0·080
$MgSO_4$	1·190	NaF	0·076
$MnSO_4\cdot H_2O$	0·027	KI	0·001

(b)—Vitamins—the 5·8 g. of vitamins and excipient in 100 g. of basal diet were made up as follows—

Thiamin hydrochloride	0·0020		
Riboflavin	0·0040	Menadione	0·0004
Calcium pantothenate	0·0080	E concentrate*	0·0560
Pyridoxine	0·0020	A and D concentrate†	0·1600
Nicotinamide	0·0080	Corn oil	4·2830
l-Inositol	0·0800		
Choline chloride	0·4000		
Folic acid	0·0008		
p-Aminobenzoic acid	0·0040		
Liver fraction‡	0·8000		

* Vitamin E concentrate, 350 mg. of *dα*-tocopheryl acetate equivalent per g.
† Navitol, 65000 A, 13000 D per g.
‡ Wilson's Liver fraction L.

NOTES

1. Chlorine dioxide.—Canadian number one patent flour was treated with 0·3 g. of chlorine dioxide per barrel (196 lb.) to yield commercial bread flour and with 15·0 g. of chlorine dioxide per barrel to yield high ClO_2 flour.

2. Antioxidants.—Tenox II, a commercial preparation containing 20 per cent. butylated hydroxyanisole, 6 per cent. *n*-propylgallate, 4 per cent. citric acid and 70 per cent. propylene glycol was used at the rates of 0·05 per cent. in commercial lard and 2·5 per cent. in high antioxidant lard.

3. Propionate.—The commercial sodium propionate sold by Du Pont de Nemours and Co., under the trade name Mycoban was used.

4. Polyoxyethylene monostearate.—The material sold by Atlas Powder Co. as polyoxyethylene (8) monostearate under the trade name Myrj 45 was used.

5. Yeast food.—The commercial material sold under the name RKD by Standard Brands was used.

6. Wytase.—This material is sold by J. R. Short Canadian Mills Limited, and is used commercially to improve whiteness.

7. Alphacel is a commercial cellulose supplying bulk without nutritive value.

and in weight from 41 to 91 g. was divided into 7 sub-groups by arranging the rats in order of decreasing weight and assigning consecutive lots of 7 rats by random selection to the sub-groups. The 182 female rats

aged 25 to 41 days and weighing 36 to 86 g. were grouped in a similar manner. The oldest rats of each sex had been maintained on diet VII from weaning at 22 days of age until placed on test. The 14 groups of rats were housed in cages with wire screen floors. Each cage held 1 group (26 rats). Food and water were allowed *ad libitum*. For the first 3 weeks, feed consumption and body weight were recorded twice weekly; thereafter the measurements were made once a week. Cages were moved in a regular rotation on the racks so that all spent approximately equal intervals at the 4 different levels above the floor. Mortality was recorded and the probable cause of death (autopsy) was noted for those rats which died and were found before autolysis was too far advanced.

At 13 and 26 weeks, 3 rats of each sex from each diet were chosen by random selection and killed. Tissue samples from the bladder, small intestine, spleen, stomach, pancreas, adrenal, kidney, liver, heart, lung, thyroid, brain, and either testicle or ovary were preserved in formalin-saline fixative and reserved for histopathological examination. At the 52nd week all surviving animals were killed and the heart, liver, spleen and both kidneys were weighed. In addition, the usual tissue samples were reserved for histopathological examination. Gross abnormalities which were observed were recorded during this work. It should be noted that the rats were group fed and that the food consumption figures include wastage. Every effort was made to keep wastage at a minimum and there is no reason to believe, on the basis of observations made, that any particular group wasted more food than other groups.

RESULTS

The mean body weights, at weekly intervals, of the rats in the 14 groups are presented graphically in Figures 1A (males) and 1B (females). The mean body weights of the survivors on each diet at 52 weeks and the standard error of the means are shown for males, females, and the sexes combined in Table II.

TABLE II

FINAL WEIGHT OF SURVIVORS AT 52 WEEKS

Weight in grams ± standard error

	Sexes combined		Males		Females	
Diet	Mean wt.	No.	Mean wt.	No.	Mean wt.	No.
I	248 ± 8·8	16	284 ± 9·8	6	226 ± 5·9	10
II	262 ± 10·8	17	288 ± 17·9	8	238 ± 6·9	9
III	266 ± 6·7	18	290 ± 7·5	8	251 ± 6·7	10
IV	251 ± 12·5	18	303 ± 25·7	6	226 ± 5·7	12
V	260 ± 9·4	22	295 ± 10·8	10	231 ± 7·5	12
VI	278 ± 16·7	14	333 ± 12·8	7	224 ± 6·8	7
VII	244 ± 7·8	19	266 ± 11·7	8	229 ± 7·7	11

The commercial diet (VII) was approximately equivalent to the bread diets in supporting the growth of female rats. Female rats on diet III surpassed in growth those on diet VII toward the end of the experiment. For male rats, with their more rapid growth, diet VII was inferior to

bread diet I in the earlier weeks of the experiment. At 10 weeks the mean weight of male rats on diet I was significantly greater than that of male rats on diet VII. At the end of the 52nd week, this difference had disappeared and the mean body weight of male rats on diet VII was not significantly lower than that of male rats on diet I or on all 6 bread diets combined.

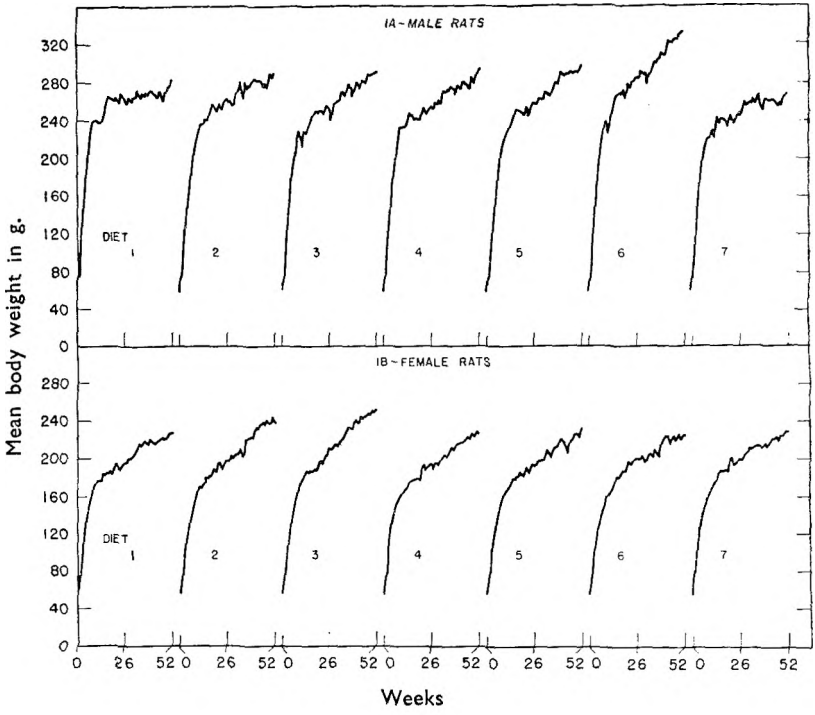

FIG. 1.

The final weights recorded in Table II were subjected to an analysis of variance based on the statistical methods of Snedecor[5] for disproportionate groups with significant interaction. The results for the commercial diet (VII) were excluded from these and subsequent calculations since the rats on this diet were included only to illustrate the usual growth, mortality, and pathology of animals from the laboratory colony. The results of the statistical analysis shown in Table III indicate that although sex had a significant effect on final weight, as was to be expected, diet was without significant influence.

The significant interaction apparently arose from the fact that male rats on the 4 diets high in polyoxyethylene (8) monostearate had a higher mean weight than those on the 2 diets low in this ingredient and female rats on the 4 diets high in chlorine dioxide had a higher mean weight than those on the 2 diets low in chlorine dioxide. These differences however were below the 5 per cent. point level of significance when the

"t" test was applied to the appropriate means. Since the effects of diet were not significant it may be concluded that none of the additives tested, in the amounts and manner used, had any deleterious effect on the growth of male or female rats during one year.

TABLE III

ANALYSIS OF VARIANCE OF FINAL WEIGHT DATA

Main effect	D.F.	Mean square	F
Sex	1	109908	47**
Diet	5	951	<1
Diet × sex ..	5	2346	2·56*
Error	93	919	

* significant at P = 0·05 ** significant at P = 0·01

Cumulative food consumption per rat per day over the 52-week period is shown in Figure 2A. It is readily observed that the commercial diet was eaten to a greater extent than the other diets, particularly in the case of the male rats, where significantly larger amounts of the diet VII were consumed. The cumulative food efficiency data, shown in Figure 2B, were plotted in a log relationship with time and straight lines were fitted

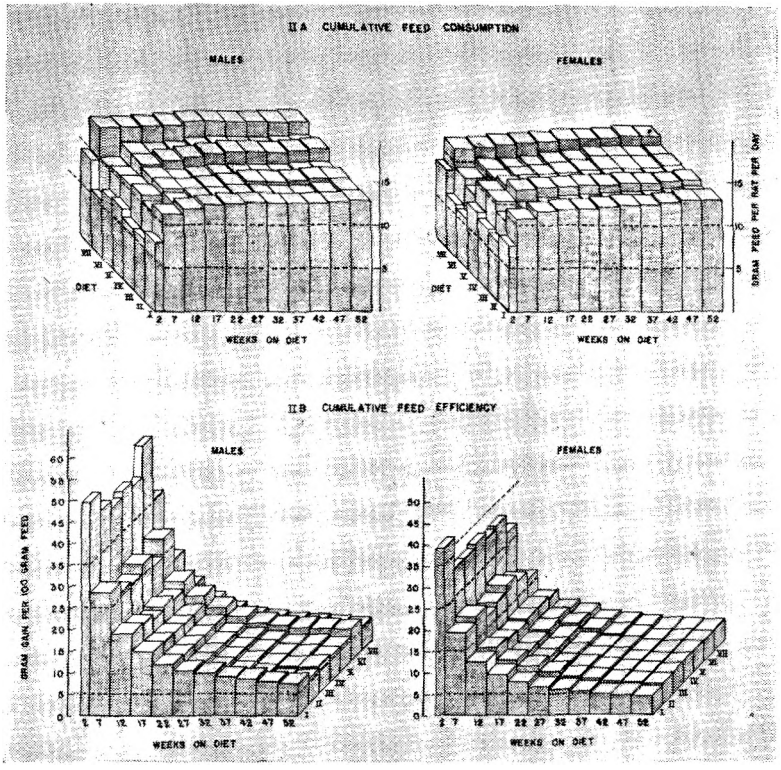

FIG. 2.

TABLE IV

A. HISTOPATHOLOGICAL FINDINGS

	Sex													
	Male							Female						
Diet	I	II	III	IV	V	VI	VII	I	II	III	IV	V	VI	VII
Number of rats examined*	12	14	14	12	16	13	14	16	15	16	18	18	13	17
Normal rats	9	7	10	9	11	8	10	13	7	8	10	14	9	14
Brain liquefaction necrosis (embolus)	—	—	—	—	—	—	—	—	—	—	—	1	—	—
Brain inflammation (cerebromeningitis)	—	—	—	—	—	1	—	—	—	—	—	—	—	—
Lungs—respiratory infection	1	2	2	1	1	1	2	2	—	3	1	—	2	2
Heart—fatty degeneration	—	—	—	—	—	—	1	—	—	—	—	—	—	—
Liver—cloudy swelling, fatty degeneration	—	1	—	—	—	1	1	—	2	—	—	—	—	—
Liver—excessive deposits of haemosiderin	—	—	—	—	—	—	—	—	—	—	2	—	—	—
Spleen—excessive deposits of haemosiderin	—	—	—	—	—	—	—	—	—	—	2	—	—	—
Stomach—inflammation of submucosa	—	—	—	—	1	—	—	—	—	—	—	—	—	—
Intestine—erosion	—	—	—	—	—	—	—	1	—	1	2	—	—	—
Kidney—degeneration of tubules	—	—	—	—	1	—	—	—	1	—	2	1	2	—
Kidney—glomerulonephritis	—	—	—	—	—	—	—	—	2	2	—	—	—	—
Kidney—hydronephrosis	—	—	—	—	—	—	—	—	1	1	—	1	—	—
Adrenal—cloudy swelling, necrosis in zona fasiculata	—	—	—	—	1	1	—	—	1	—	—	1	—	—
Bladder—parasite Trichosomoides crassicauda	2	5	2	3	3	1	1	—	1	3	—	1	2	2
Testicle—degeneration	—	—	1	—	—	—	—	—	—	—	—	—	—	—

* 3 rats were killed at 13 weeks, 3 at 26 weeks, and the remainder at 52 weeks.

B. MORTALITY DATA

Died on test	14	12	12	14	10	13	12	10	11	10	8	8	13	9
Undiagnosed	5	4	9	7	6	9	8	7	9	5	4	7	8	4
Respiratory infection	8	6	3	7	4	3	3	1	2	5	4	1	2	5
Middle ear disease	1	2	—	—	—	1	1	2	—	—	—	—	1	—
Tumor—fibroadenoma	—	—	—	—	—	—	—	—	—	—	—	—	2	—

to the points by the method of least squares. These lines were not significantly different for female rats but for the males the food efficiency on diet VII was significantly less than that on diet I.

The sections from the tissues of the 208 rats were stained with *hæmatoxylin and eosin and examined microscopically.* The group to which the various rats belonged was unknown to the pathologist (H.C.G.) at the time the tissues were examined. The histopathological findings are presented in Table IVA and the results have been summarised by the pathologist as follows:

"The tubular degeneration and perhaps the glomerulonephritis seen mainly in the kidneys of female rats are the only histopathological findings considered to be significant. The tubular degeneration is, in all probability, due to a toxic substance. The lack of coexisting glomerular change is remarkable as the glomerulus is usually affected before the tubules. It may be that there is a tubular specificity of the injurious agent or that there is an inherent weakness of the tubular epithelium in these particular rats. The former supposition is more probable. The hydronephrosis in each case was unilateral. Dilatation of the kidney pelvis may be caused by concretions, parasites, or tumors in the urinary bladder or it may be congenital. Concretions were not found in any of the bladders, there were no tumors present in the bladders of any of the animals examined, and there was no evidence of congenital atresia of the ureters. While parasites were not observed in the bladders of rats suffering from hydronephrosis, these could have been missed in histological

cross section (4 microns) and the known incidence of such parasites is sufficient to attribute the hydronephrosis to bladder parasites."

The significant kidney changes in female rats were found on diets II, III, IV and V. While the incidence is low, it should be noted that these were the rats receiving flour treated with the high concentration of chlorine dioxide. Tubular damage from propyl gallate has been demonstrated by Orten, Kuyper and Smith[6], and this may have been a contributing factor. Again, the incidence is so low that the distribution may be fortuitous.

The overall mortality data are tabulated in Table IVB. It will be noted that a considerable number of deaths occurred for which no explanation is given. These deaths occurred at night and by the time the bodies

TABLE V

EFFECT OF DIET ON THE WEIGHT OF VARIOUS ORGANS

Mean tissue weights in mg. per g. of rat

Diet	Number	Liver	Right kidney	Left kidney	Heart	Spleen
A.	Males					
I	6	32·7	3·56	3·55	3·96	2·82
II	8	31·0	3·66	3·83	3·81	2·51
III	8	32·5	3·72	3·71	3·65	2·71
IV	6	37·4	3·38	3·31	3·22	2·16
V	10	34·0	3·60	3·51	3·42	2·64
VI	7	34·2	3·47	3·35	3·38	2·59
VII	8	34·1	3·41	3·19	3·84	2·67
Over-all standard deviation		4·6	0·43	0·42	0·40	0·38
B.	Females					
I	10	35·0	4·10	4·07	4·48	3·47
II	9	39·5	4·95	5·06	4·29	3·31
III	10	41·5	4·82	4·81	4·70	4·01
IV	12	37·3	4·28	4·24	4·10	4·03
V	12	38·0	5·19	5·02	4·68	3·65
VI	7	35·6	4·46	4·61	4·47	3·43
VII	11	38·4	4·02	4·06	4·08	3·59
Over-all standard deviation		4·5	0·81	0·67	0·42	0·85

TABLE VI

ANALYSES OF DATA ON TISSUE WEIGHTS

Main effect	D.F.	Liver		Right kidney		Left kidney		Heart		Spleen	
		m.s.	F.	m.s.	F.	m.s.	F.	m.s.	F.	m.s.	F.
Sex	1	3088	15*	15932	49**	16934	61**	11508	46**	25473	33**
Diet	5	212	1·0	618	2·1	714	2·6	299	1·2	361	<1·0
Sex × diet ..	5	203	1·4	324	<1·0	276	2·1	248	3·4**	765	1·5
Error	93	147		397		133		73		493	

* significant at P = 0·05 ** significant at P = 0·01

were retrieved in the morning autolysis was too far advanced to allow conclusive autopsy findings. It may be stated with a considerable degree of confidence that most of these deaths were due to respiratory infection. A χ^2 analysis of the data revealed no significant influence of diet on mortality.

The mean weights of the livers, kidneys, hearts, and spleens of the rats which survived to the time of sacrifice at 52 weeks are listed in Table V.

For statistical analysis the data in Table V were transformed into percentages of the mean weight for each tissue. The resulting data were analysed by Snedecor's methods for disproportionate subclass numbers. The results of these analyses are presented in Table VI.

As might be expected, sex had a significant influence on the weight of the tissues examined. Since the sex × diet interaction was significant in one instance and in 4 of 5 instances the mean square for interaction was of appreciable magnitude these interactions were used in the calculation of the F for diet and sex. In no instance did diet have a significant influence on tissue weight.

Discussion

Propionate.

Very few experimental data on the toxicity of sodium propionate are available. In a 3-week trial Harshbarger[7] fed concentrations of 1 and 3 per cent. sodium or calcium propionate in the diets of rats. No effect on weight gains was observed. Heseltine[8] reported that sodium propionate had a weak antihistamine action about 1/7·5 that of diphenhydramine. Daily oral doses of 6 g. to men led to the production of a fairly alkaline urine but no appreciable diuresis, catharsis, or other effects.

These findings are confirmed and extended by the present data which show that at concentrations of 5 per cent. in bread ingredients before baking, a finished product is obtained which exerts no discernible toxicity when fed as 3/4 of the diet of rats for 1 year; this in spite of the added stress of the presence of 3 other additives at high concentration in the diet.

Antioxidants.

n-Propyl gallate is a fat-soluble ester of the widely-occurring natural antioxidant gallic acid. It has been fairly intensively investigated by Orten *et al.*[6], who found that concentrations of 1·17 per cent. in the diet of male rats for 6 months caused growth depression, a decrease in hæmoglobin levels, and some damage to the kidney tubules. Guinea-pigs receiving 0·011 per cent. in the diet for 1 year and dogs receiving similar amounts for 14 months showed no ill-effects. Lehman, Fitzhugh, Nelson and Woodard[9] found this antioxidant even less toxic and, in rats, detected no growth inhibition at 1 per cent. of the diet of males or females. At 5 per cent. of the diet growth depression occurred in male rats, there was some increase in mortality over the 2-year feeding period, and there was evidence of patchy hyperplasia of the proventriculus. Allen and De Eds[10] and Van Sluis[11] reported very low toxicity for lauryl gallate given to rats.

Wilder and Kraybill[12] reported that butylated hydroxyanisole (the commercial material is usually a mixture of the 2- and 3-tertiary butyl esters) when given to rats for periods up to 21 months at concentrations up to 0·06 per cent. of the ration caused no toxic effects. At 0·12 per cent. palatability of the ration was lowered but no pathology was detected. Evidence accumulated by the U.S. Food and Drug scientists[13] indicated that this antioxidant was safe when used in the usual concentrations.

The amounts of the several ingredients of the antioxidant mixture fed in the various diets is shown in Table VII.

In the amounts in which they occurred there is no reason to suspect any direct toxic action from the propylene glycol or the citric acid. The high level of butylated hydroxyanisole was about 1/10 that found to cause no harmful effects and the high level of *n*-propyl gallate was about 1/50 of the maximum non-harmful amount. Even the combination of these 2 failed to exert any significant influence on the health of the rats.

TABLE VII

ANTIOXIDANT AND PROPYLENE GLYCOL CONTENT OF DIETS I–VI

	Diets I and V	Diets II, III, IV and VI
	mg./kg.	mg./kg.
Butylated hydroxyanisole ..	1·35	67·50
n-Propyl gallate	0·405	20·25
Citric acid	0·27	13·50
Propylene glycol carrier ..	4·725	236·25

Polyoxyethylene (8) *Monostearate.*

The literature on the toxicity of polyoxyethylene (8) monostearate is rather considerable. The chief conclusion reached after a careful scrutiny of these data is that, under certain conditions, polyoxyethylene (8) monostearate in fairly large amounts in the diet may cause harmful effects[14,15,16,17]. Whether or not these certain conditions are of such a nature as to lend practical significance to the observed toxicity is a matter of argument. The literature is too voluminous to review in detail here. Suffice to say that loss in weight, diarrhœa, bladder stones, etc., have been observed in rats fed diets containing 5 per cent. or more of this emulsifier. Other investigators have not demonstrated any harmful effects at concentrations up to 5 per cent. in the diet[18]. To quote Frazer[19] "Grossly excessive amounts of polyoxyethylene monostearate—some workers have used as much as 15 per cent. of the diet—may produce some effects. Some of the results of these experiments are not convincing and in any case they have little relevance to the normal dietary use of these materials."

Culver, Wilcox, Jones and Rose[20] have shown that in humans receiving polyoxyethylene (40) monostearate the polyoxyethylene was recovered almost completely in the urine and fæces, that this portion of the molecule was recovered free of combined fatty acid, and that there was no evidence of storage.

In the experiments reported here, polyoxyethylene (8) monostearate occurred as 0·3 or 15·0 per cent. of the breads which made up 75 per cent. of the diet. Under these conditions and in spite of 2 or 3 additional dietary stresses, no toxic effect of this material was observed.

Chlorine Dioxide.

The toxicity of chlorine dioxide-treated flour has been the subject of several investigations. Arnold[21] gave dogs, as 80 per cent. of their diet, flour treated with 5 g. of chlorine dioxide per 100 lb. for periods up to 28 days without detecting any ill-effect. Newell, Gershoff, Suckle,

Gilson, Erikson and Elvehjem[22], carried out similar test giving 60 per cent. flour in the ration with a maximum chlorine dioxide treatment of 4 g. per 100 lb. and continuing the trials for 13 weeks. The results of these studies on dogs were negative. Similar studies on rabbits (6 weeks) monkeys (5·5 months) and rats (5 weeks) also gave negative results. A group of 13 humans receiving 55 g. of chlorine dioxide-treated wheat gluten (20 g. of ClO_2 per 100 lb. of gluten) daily for 6 weeks showed no detectable injury. Nakamura and Morriss[23] gave to dogs flour treated with high concentrations of chlorine dioxide for prolonged periods without seeing any evidence of canine hysteria. With the exception of the last experiment most of the studies were of short duration and no long-term studies on rats have been reported.

In the present investigation it has been shown that rats receiving a diet containing 75 per cent. of bread made from flour treated with 15 g. of chlorine dioxide per barrel (196 lb.), 50 times the normal rate of treatment, for 1 year from weaning age exhibited no signs of toxic effect as evidenced by growth, mortality, gross observation, organ weights or histopathological examination of the tissues.

Summary

1. Bread containing 50 times the normal concentration of chlorine dioxide, propyl gallate and butylated hydroxyanisole, polyoxyethylene (8) monostearate, or sodium propionate as 75 per cent. of the diet for 1 year did not harmfully affect growth or mortality of rats.

2. The high concentrations of polyoxyethylene (8) monostearate, sodium propionate, antioxidants and chlorine dioxide had no detectable effect on organ weights or on histopathology of the tissues.

3. No evidence was obtained that the simultaneous presence of high concentrations of more than one potential toxicant in the diet led to any synergistic action.

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THE ESTIMATION OF THE COMPONENT CARDIAC GLYCOSIDES IN DIGITALIS PLANT SAMPLES

PART II. THE ESTIMATION OF THE DESGLUCO-GLYCOSIDES AND SOME OBSERVATIONS ON THE PRODUCTION OF ULTRA-VIOLET FLUORESCENCE WITH TRICHLORACETIC ACID

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IN a previous communication[1] a method was described for the separation of the naturally occurring lanatosides of *Digitalis lanata*. Using paper chromatography with a solvent mixture of 84 parts of ethyl acetate and 16 parts of benzene, saturated with water and containing 0·5 to 3 per cent. of ethanol, a satisfactory separation of the 3 lanatosides was obtained but these conditions were unsuitable for the desglucoglycosides which travel too near the solvent front and merge too closely together for a convenient separation. In view of the importance of the desgluco-glycosides as the first breakdown products in the naturally occurring enzymatic hydrolysis and the therapeutic and commercial status of digoxin and digitoxin a comprehensive study was made of possible methods for achieving more complete separation.

The present paper describes a method permitting reliable and reproducible separation and estimation of digoxin, digitoxin and gitoxin in the presence of the original lanatosides and other glycosides present in crude plant extracts.

In the previous paper it was mentioned that a mixture of chloroform, benzene and water in the proportions 65:15:50 and containing 5 per cent. of methanol will separate digitoxin from gitoxin and digoxin but that this procedure does not allow the separation of gitoxin and digoxin from each other.

As the result of trying a variety of solvents (chloroform, carbon tetrachloride, benzene, *cyclo*hexane, ethyl acetate, acetone, *n*-butanol, ethanol and methanol) in different mixtures it was found that a mixture of chloroform, benzene, ethyl acetate and water in the proportions of 60:20:20:50 by volume was suitable for the estimation of digitoxin using the ascending method of irrigation. Digitoxin gives an R_F value of 0·7 under these conditions whereas no other glycoside travels very near and the estimation of this material can readily be performed in this way. Digoxin and gitoxin however give R_F values of 0·25 and 0·15 respectively which are too similar for an unambiguous separation unless advantage is taken of the different behaviour of these two substances when treated with trichloracetic acid mixtures as described later in this paper. But in this case only one of these two glycosides can be determined at a time. By using the descending method of irrigation and

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extending the time of running the chromatogram to 18 to 22 hours the same or a slightly modified mixture of solvents is very suitable for the separation of the closely related isomers digoxin and gitoxin, which differ only in the position of the hydroxyl group in positions 12 and 16 respectively. Under these conditions the digitoxin and all the glycosides travelling in front of it are washed away with the effluent solvent. Digoxin then becomes well separated in front of gitoxin. There is no marked tailing on the developed chromatogram provided that the quantities applied are less than 5 μg. and this should preferably be in the range 0·5 to 3 μg. The digoxin spot is usually an elongated elipsoid with an axis ratio 1 : 2 to 3. The working details and solvent modifications are given in the experimental account below.

The trichloracetic acid reagent used to render the glycoside spots visible under ultra-violet irradiation was usually that described by Svendsen and Jensen[2]. It was found however during an extensive use of this elegant and sensitive reagent that some seemingly unexplained difficulties may be experienced which were at first very difficult to understand and necessitated a complete study of this reaction. It was found that the reagent prepared from the same stock of chemicals behaves differently toward different glycosides depending on the age of the solution and the conditions under which it has been stored. It has been found possible to produce an artificial ageing of the reagent by shaking with air, exposure to light, or by the addition of hydrogen peroxide[3].

Since this work was done Jensen[4] published his experiences with the use of trichloracetic acid and he recommends the use of ethanol as a solvent for the acid, claiming that the reagent so prepared is more stable. He proposes also the use of a trichloracetic acid solution with chloramine in aqueous ethanol for the differentiation of the purpurea glycosides. From the table given by this author it can be seen that purpurea glycosides of the B series react with "stabilised" trichloracetic acid solution with a blue fluorescence and give a negative test with the glycosides of the A series. Further, the chloramine-containing reagent gives a yellow fluorescence with the A series, and the B series again fluoresce blue. Our observations generally confirm those of Jensen but extend these findings to the C series of glycosides.

The reagents used in this work comprised (a) a freshly prepared 25 per cent. solution of trichloracetic acid in chloroform containing 1 per cent. of ethanol, (b) the same solution with the addition of 1 or 2 drops of 100 volume hydrogen peroxide to 10 ml. of the reagent. The hydrogen peroxide can be replaced by 1 to 3 per cent. of benzoyl peroxide. The reagent (a) was also "aged" by storage in a half-filled conical flask at room temperature exposed to daylight. For some purposes a stronger reagent was prepared containing 4 drops of hydrogen peroxide in 10 ml of solution. The results of experiments with the reagents are given in Table I. It will be seen that digoxin is quite invisible when the fresh reagent is used but fluoresces with a bright blue colour with the "aged" solution with an additional intensity with the stronger reagent mentioned

above. With the latter solution lanatoside B and gitoxin appear as greenish spots which are easily distinguished from the intense blue colour of digoxin even when these spots occur in proximity upon a chromatogram. The absence of fluorescence of digoxin with a freshly prepared solution has been used routinely by Ferry and Murphy[3] to test this drug for freedom from gitoxin.

When lanatoside A occurs on chromatograms from plant materials it usually shows a faint greenish fluorescence whether sprayed with fresh or aged trichloracetic acid reagent. This is probably due to trace impurities in the chromatogram which are sufficient to change the colour reaction of this glycoside; digitoxin, however, separated from crude plant extracts behaves as indicated in Table I and does not show this aberrant colour.

TABLE I

Glycosides	Freshly prepared reagent	"Aged" reagent (or with hydrogen peroxide)
Lanatoside A ..	no fluorescence	yellow
Digitoxin	" "	yellow/orange
Lanatoside B	bright blue	greyish blue
Gitoxin	" "	greenish blue
Lanatoside C ..	bright grey-blue	blue
Digoxin	no fluorescence	blue

Jensen[4] in discussing the changes which take place on keeping the trichloroacetic acid solution in chloroform assumes that the difference of action of a freshly prepared and old reagent is due to "free" chlorine which is present in an "aged" solution. To obtain similar results this author recommends the addition of chloramine as a source of free chlorine. Since no molecular chlorine can be generated from chloramine in the absence of hydrochloric acid by a simple reaction, we assume that Jensen understood by free chlorine a source of active chlorine atoms and since similar results can be obtained by adding hydrogen peroxide, benzoyl peroxide, or shaking with air we would like tentatively to point out that all these reagents and procedures have in common processes which are assumed to involve free radicals in their reaction mechanism. This is also the prevailing view with regard to the decomposition of chloroform and its instability on keeping. It seems to us therefore not improbable that changes in the colour of the fluorescence reaction proposed by Svendsen and Jensen are influenced by processes involving a homolytic type of reaction mechanism; a point which merits further experimental investigation.

Experimental

Solvents: The chloroform and ethyl acetate, B.P. grade, used for the chromatography were washed with a 10 per cent. potassium carbonate then with water, dried over anhydrous potassium carbonate and distilled through a short column rejecting the first 5 to 10 per cent. as a fore-run and 5 per cent. as the residue.

The benzene was a commercial nitrating grade reagent, benzene

crystallisable, which was distilled through an 18-in. Vigreux type column; 15 per cent. was rejected as fore-run and 5 per cent. left as residue.

The trichloracetic acid was of A.R. grade.

Apparatus: The tank for the upward method was a square glass jar 6 in. × 8 in. × 14 in. high. For the downward chromatography a round jar 6½ in. in diameter and 15 in. high was used. The tanks were covered by glass plates carrying a rubber stopper through which a bent glass rod could be moved allowing the paper to be dipped into the solvent without opening the container and disturbing the phase equilibrium.

Chromatography: Whatman filter paper No. 1 was used throughout. The "spotted" paper is placed in the tank for 12 hours to equilibrate with the solvent-saturated gas phase and then irrigated for 2½ to 4 hours for the upward method (solvent mixture I) or 18 to 22 hours for the downward method (solvent mixture I); 24 to 28 hours solvent mixture II, 16 to 20 hours solvent mixture III. After removing from the tank the paper was dried by hanging in an airy room for 1 to 2 hours and sprayed with a 25 per cent. trichloracetic solution either freshly prepared or "aged" according to the problem under investigation as explained earlier. Satisfactory results were obtained at temperature 22° to 24° C. Solvent mixtures: I chloroform, 60, ethyl acetate 20, benzene 20, water 50.

II chloroform 40, ethyl acetate 30, benzene 30, water 50.

III chloroform 50, benzene 40, water 50; to the separated organic layer 10 to 15 parts of methanol are added.

The developed fluorescing chromatograms can be photographed as described previously[1] for a permanent record.

Note: Based on a very comprehensive experience covering the determination of the lanatosides by the previously described process some further details may be useful to ensure satisfactory results. The maintenance and the achievement of a complete saturation of the paper sheet before irrigation is of vital importance for a good separation and to avoid tailing. Satisfactory equilibration is more readily obtained in medium sized tanks by hanging papers, dipping into the aqueous layer on the bottom, down the side walls. Good results can also be obtained in a larger tank, 10 in. × 10 in. × 18 in., but it is better in this case to extend the equilibration time to 24 or 36 hours before irrigation. Much poorer results were obtained when the working temperature dropped below 22° C. or was raised over 28° C. The temperature during the irrigation period (2½ to 4 hrs.) should be kept constant to ± 1° and uniform throughout the tank. By adhering to these precautions, reliable and reproducible results were obtained in screening tests with a variety of plant samples and in storage tests performed in these laboratories by other workers[5]. In the case of the desgluco-glycosides separation described in this paper, the conditions of equilibration are less critical and a period of 8 to 12 hours is found to be sufficient in most instances.

Discussion

The procedures described above were applied to fractions collected from chromatographic columns and to follow up fermentation studies of

the transformation of the lanatosides in the leaf material to the desgluco derivatives, also for analysis of different plant samples of *Digitalis lanata*. In all these cases it is important to compare the corresponding unknown spots with known purified compounds and not to rely on the previously determined R_F values even if the experiments are performed under exactly identical conditions. This point was elaborated in our previous communication and was also stressed by Schindler and Reichstein[6] and Jensen[4]. This is especially the case when working with crude extracts of plant material which contain a great variety of glycoside materials. In addition to the known lanatosides, their desgluco-derivatives and aglycones there may also be intermediate compounds such as acetyl desgluco-glycosides described by Stoll and Kreis[7], which we have not had available for comparison. There may also be other glycosides as yet unidentified and in such instances it is helpful if on a certain chromatogram only a limited number of spots are present, as is obtained by using different solvent mixtures for different groups of glycosides, or by extending the time of irrigation in the downward chromatography thus removing the faster travelling components into the effluent solvent. It may be of interest to mention that in all the analysed samples of *Digitalis lanata* we have noticed a very intense spot placed between the lanatosides C and B which we could not identify with any of the glycosides at our disposal. This same compound was also present in the mixtures of the crude lanatosides which were prepared according to the method described by Stoll and Kreis, and as far as it was possible to judge from the colour intensity the concentration of this substance was very considerable.

In some cases it may be a disadvantage compared with the chromatography on formamide-soaked paper that in the case of the desgluco-glycosides only rather small quantities of the order of 0·5 to 5 μg. of a single component can be applied for separation; this is not the case with the lanatosides where higher concentrations (1 to 15 μg.) with the A and C component give the best results. On the other hand sheets of paper up to 8 to 9 in. in width with up to 8 spots on the same starting line can be run at the same time thus making possible the comparison of a greater number of samples under identical conditions. Furthermore in applying the method to the semiquantitative estimation of certain glycosides it is possible to place a larger number of the known test substances at different levels of concentration and so lower the limit of error involved in the method of comparison of colour intensities.

In view of the great complexity of the mixtures of glycosides often present and at the same time, of the great similarity of the physical and chemical properties, it was often found by the formamide method, that two components travel at the same rate and cannot be sufficiently separated. This is also the case with some substances with the method used in our chromatograms and a more limited number of compounds have been compared by this method than in the case of formamide-soaked paper, and therefore it may prove advantageous to confirm the identity and homogeneity of a glycoside on several different systems since

there is then less likelihood that two compounds having similar or identical fastness on one system will behave in a completely parallel manner on another system of chromatography.

We should like to point out that the use of the term "lanatoside" is more in accord with the nomenclature of the cardiac glycosides than is the older term "digilanid" and the former has been used in this paper in place of the latter synonym used previously.

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Correction.

MORPHINE DERIVATIVES WITH ANTIANALGESIC ACTION

By A. F. GREEN, G. K. RUFFELL and E. WALTON

This Journal, 1954, **6**, 390

Page 393, line 3. For R′ read R.

Page 395, line 5. For R′ read R″ and for R″ read R′.

Page 396, line 30. For R′ read R″ and for R″ read R′.

line 32. For R^1 read R, and for R^2 read R′.

PHENOL AS THE PRESERVATIVE IN INSULIN INJECTIONS

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The purpose of this paper is to describe a laboratory investigation into the use of phenol as the added bacteriostatic in insulin injections, an investigation which arose partly because other accepted agents, such as chlorocresol and phenylmercuric nitrate, are considered unsuitable either for physiological or for chemical reasons, and partly because The United States Pharmacopœia XIV recommends phenol or cresol in the different injections in concentrations ranging between 0·1 and 0·28 per cent. Insulin preparations of American origin have been found to contain phenol as the bacteriostatic, whereas certain commercial insulins of non-American origin have contained either cresol or *o*-cresol. In view of these facts it was considered desirable to study in detail the merits of phenol as the preservative, especially as there is also published evidence[1,2,3] showing that the rate of loss of phenol through rubber closures on storage is smaller than with other bacteriostatics. The particular preparations to be considered are the B.P. injections of insulin, globin insulin and protamine zinc insulin. The first two are in acid solution, *p*H about 3, and the last is neutral, *pH* 6·9 to 7·3.

The British Pharmacopœia, 1953, requires all injections dispensed in multi-dose containers to contain "sufficient of a bacteriostatic to prevent the growth of micro-organisms," but it further states "A bacteriostatic need not be added if the medicament itself has sufficient bacteriostatic power." The classical example of a suitable bacteriostatic is 0·5 per cent. of phenol; but no indication is given how activity is to be measured, or how comparisons with other substances are to be made. Although described as bacteriostatic, a solution containing 0·5 per cent. of phenol possesses considerable bactericidal properties which vary according to the nature of other constituents of the solutions, its temperature and the type of infecting organism, and these must be taken into account in assessing the comparative antibacterial properties of any other solution.

It is well known that acid solutions themselves are inhibitory to bacterial growth and at sufficiently low *p*H values are lethal; the activities of bacteriostatics are also affected by *p*H value. It seems proper, therefore, in any comparative work to use 0·5 per cent. of phenol in neutral solution as the standard, and to attribute any advantage gained by acidity or from any constituent of the solution to the natural bacteriostatic properties of the preparation. Anti-fungal properties would need separate consideration since moulds are much less sensitive to acid conditions than are bacteria. We have evidence, for example, of the growth of *Cladosporium* in insulin solution in the presence of 0·17 per cent. of cresol after several months in cool storage.

Bacteriostatic and Bactericidal Tests with Phenol

To test the influence of acidity on the bacteriostatic properties of phenol solution, serial dilutions were made in 0·5 per cent. peptone water and adjusted to the required *p*H values with 0·02M phosphate-citrate buffer. These solutions were inoculated with (*a*) mixed 24-hour cultures of *Staphylococcus aureus* (including the F.D.A. strain), (*b*) mixed 24-hour cultures of *Bacterium coli* (N.C.T.C. 86 and 5934), and (*c*) a spore suspension of *Bacillus subtilis*, and the concentrations just preventing growth in 5 to 7 days at 37° C. and at 22° C. recorded. Similar solutions were also inoculated with mixed mould cultures, including *Penicillium*, *Aspergillus* and *Cladosporium* species, and tested at 22° C. only. Numerous replicates were made at different times and Table I gives the maximum concentrations in the whole series which just prevented growth under the conditions quoted. At *p*H 4 or less, the acidity alone was sufficient to prevent bacterial growth. There were no significant differences between tests at 37° C. and 22° C., and addition of 2 per cent. of glycerol did not affect the results.

TABLE I

Bacteriostatic concentrations of phenol in peptone solution

Test organism	Percentage concentration just preventing growth at			
	*p*H 3	*p*H 4	*p*H 5	*p*H 7·5
Staph. aureus			0·1	0·2
Bact. coli			0·15	0·2
B. subtilis (spores)			0·2	0·25
Mould spores	0·25	0·25	0·2	0·2

The bactericidal properties of phenol solutions at different *p*H values were measured in comparison with 0·5 per cent. of phenol in neutral solution (*p*H 7·5). Serial dilutions in either saline solution or nutrient broth were adjusted to the required *p*H values with 0.02M phosphate-citrate buffer and inoculated heavily with mixed cultures of *Staph. aureus* and *Bact. coli* as used in the bacteriostatic tests to give initial counts of the order of 20×10^6 per ml. After short intervals, standard samples were removed and the surviving bacteria counted (by plating 0·1 ml. amounts in nutrient agar). The results from the several groups of tests made were somewhat variable, and typical ones are shown in the composite Table II. They show that phenol solutions have a substantial and fairly rapid bactericidal activity, and that a 0·2 per cent. solution at *p*H 3 is at least equivalent in activity to a standard 0·5 per cent. solution at *p*H 7·5. At *p*H 3·5, a 0·2 per cent. solution is less effective and is somewhat inferior to the standard.

Tests were also made to ascertain the survival of *B. subtilis* spores under the same conditions. Whereas they remained viable in 0·5 per cent. phenol at *p*H 7·5 for at least two weeks, there was an appreciable death rate with as little as 0·1 per cent. of phenol at *p*H 3 and 4 within 8 days.

Tests with Insulin Injections

Insulin. Insulin solution is adjusted to *p*H approximately 3, and contains only crystalline insulin and added glycerol. The total solids

TABLE II

BACTERICIDAL PROPERTIES OF PHENOL SOLUTIONS

Test Organism	Tests in	Surviving organisms in phenol solutions (per cent.) after (hr.)	at *p*H 3				at *p*H 3·5				at *p*H 4			at *p*H 7·5
			0·4	0·3	0·2	0·1	0·4	0·3	0·2	0·1	0·4	0·3	0·2	0·5
Bact. coli	Saline *solution*	½					0 : +	0 : +	+ + : + + +	+ + +	+	+ : + + + +	+ + +	+ + +
		1					0	0 : +	+ : + +	+ + +	0 : +	+ : + + + +	: + + +	0 : +
		2					0	0	0 : +	+ : + + +	0	0 : +	+ + : + + +	0
	Nutrient broth	½	+	+ : + +	+ +	+ + +	0 : +	0 : +	+ : + + +	+ + +				+ + +
		1	0	0	0	+ : + +	0	0	+	+ + +				0
		2	0	0	0	0	0	0	0 : +	+ : + +				0
Staph. aureus	Saline solution	½					0 : + +	+ : + +	+ +	+ + +	+ +	+ + : + +	+ + +	+ + +
		1					0	0 : +	0 : + +	+ + +	0 : +	+	+ + : + + +	+ + : + + +
		2					0	0	0 : + +	+ : + + +	0	0	0 : + +	0 : +
	Nutrient broth	½	+	+	+ +	+ +	0 : +	0 : +	0 : + +	+ + +				+ : + + +
		1	0	0 : +	0	+ : + +	0	0	0 : +	+ : + +				0 : + +
		2	0	0	0	0	0	0	0	+ : + +				0

Initial inoculum equivalent to approx. 2×10^6/0·1 ml.

O = no survivors in 0·1 ml.

\+ = few survivors only, less than 100.

++ and +++ = increasingly large numbers of survivors.

Two values indicate responses in different tests.

and organic matter present is very small, hence the bactericidal and bacteriostatic levels of phenol under these conditions will not be different from those found in the foregoing tests with phenol in broth or in buffer solution.

Globin Zinc Insulin. This injection has a *p*H value of about 3. To confirm that phenol exerts the same activity in it as in other solutions at similar *p*H values, a standard preparation of globin zinc insulin was made at *p*H 3·15 without any added bacteriostatic. To portions of this were added different amounts of phenol and these were inoculated with mixed 24-hour cultures of *Staph. aureus*, *Bact. coli*, *Pseudomonas pyocyanea* and *Proteus vulgaris*, and the survivors counted after short intervals. Results are given in Table III. They confirm clearly the earlier findings at low *p*H values and show that 0·2 per cent. of phenol is satisfactory for globin zinc insulin.

TABLE III

BACTERICIDAL PROPERTIES OF PHENOL IN GLOBIN ZINC INSULIN

Test organism	Period of Test (hr.)	Surviving organisms from Globin insulin + phenol (per cent.)					0·5 per cent. of phenol in saline solution
		0·3	0·25	0·2	0·15	Nil	
Staph. aureus	1	0	++	+++	+++	+++	++
	2	0	0	+	+	+++	0
	6	0	0	0	0	+++	0
	24	0	0	0	0	0	0
Bact. coli ..	1	0	0	+++	+++	+++	+
	2	0	0	0	0	+++	0
	6	0	0	0	0	+++	0
	24	0	0	0	0	0	0
Ps. pyocyanea ..	1	0	0	0	++	+++	0
	2	0	0	0	+	++	0
	6	0	0	0	0	+	0
	24	0	0	0	0	0	0
Proteus vulgaris	1	+	+	++	+++	+++	+
	2	0	0	+	++	+++	0
	6	0	0	0	+	+++	0
	24	0	0	0	0	+++	0

Initial inocula equivalent to 3–4·5 × 10^6 orgs./0·1 ml.
O = no surviving organisms in 0·1 ml.
+ = few survivors only, less than 100.
++ and +++ = increasingly large numbers of survivors.

Protamine Zinc Insulin. This injection is prepared at *p*H about 7. It is evident, therefore, that if a concentration of less than 0·5 per cent. of phenol is to be of any value, one or more of the constituents must contribute to the antibacterial properties of the final preparation. There is already published evidence[4,5] that protamine (salmine) is itself antimicrobial. To check this, a series of tests with phenol and protamine were made, using protamine at a concentration of 0·5 mg./ml., the normal amount used in the injection. Graded amounts of phenol were added to the protamine solution in 0·5 per cent. peptone water buffered to *p*H 7, and these were inoculated with 24-hour cultures of the test organism as before. For comparison, a similar series of dilutions of phenol without protamine were included. Surviving organisms were counted at intervals up to 24-hours with results as recorded in Table IV. The

TABLE IV

BACTERICIDAL PROPERTIES OF PHENOL AND PROTAMINE IN PEPTONE SOLUTIONS (*pH* 7)

Test Organism	Period of test (hr.)	Surviving organisms from												
		Protamine (0·5 mg./ml.) with phenol (per cent.)						Phenol alone (per cent.)						
		0·4	0·3	0·25	0·2	0·15	0	0·5	0·4	0·3	0·25	0·2	0·15	0
Staph. aureus	1	+ +	+ + +	+ + +	+ +	+ +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	2	+ +	+ + +	+ + +	+ +	+ +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	6	0	+ +	+	0	0	+	0	+	+ +	+ +	+ +	+ + +	+ + +
	24	0	0	0	0	0	0	0	0	+	G	G	G	G
Bact. coli	1	0	0	+	+ +	+ +	+ +	+ +	+ + +	+ + +	+ + +	+	+ + +	+ + +
	2	0	0	0	+ +	+	+ +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
	6	0	0	0	0	0	0	0	0	+ +	+ + +	+ + +	+	+ +
	24	0	0	0	0	0	0	0	0	+	+ +	+ +	G	G
Ps. pyocyanea	1	0	0	0	+	+ +	+ +	+ + +	+ + +	+ + +				
	2	0	0	0	0	0	+ +	+	+ + +	+ + +				
	6	0	0	0	0	0	0	0	+ +	+ +				
	24	0	0	0	0	0	0	0	0	+ +				

Initial inocula—*Staph. aureus* 20×10^8/0·1 ml.
Bact. coli 30×10^8/0·1 ml.
Ps. pyocyanea 100×10^8/0·1 ml.

O = no survivors in 0·1 ml.
+ = few survivors only, less than 100.
+ + and + + + = increasingly larger numbers of survivors.
G = visible growth.

strong lethal action of the protamine solution is clearly shown, which action is enhanced by adding even 0·15 per cent. of phenol. Two tests with *Ps. pyocyanea* showed the protamine solution to be still lethal at half the concentration normally used, but at a lower level; it was without action against bacterial spores.

To confirm these observations, protamine zinc insulin preparations, made to the normal formula but containing either phenol or cresol as preservative, were examined. They were inoculated with the same test organisms, and the survivors counted. Table V gives the composite results of 2, or in some cases 3, such tests, and shows complete killing of the test organisms in 6 hours and often in 2 hours. The killing rate with 0·2 per cent. of phenol was almost equal to that with 0·2 per cent. of cresol; this rate can be considered satisfactory for the purpose required.

TABLE V

BACTERICIDAL PROPERTIES OF PROTAMINE ZINC INSULIN

Test organism	Period of test (hr.)	Surviving organisms from protamine zinc insulin with				
		Phenol per cent.			Cresol 0·2 per cent.	Nil (control)
		0·3	0·25	0·2		
Staph. aureus ..	1	+:++	+:+++	++:++	+:++	+++
	2	+	+	+:+	+	+++
	6	0	0	0	0	+++
	24	0	0	0	0	++:+++
Bact. coli ..	1	+	+	+:++	+	+++
	2	0	0	0	0:+	++
	6	0	0	0	0	++
	24	0	0	0	0	++
Ps. pyocyanea ..	1		+	+	0:+	+++
	2		0	0	0	++
	6		0	0	0	++
	24		0	0	0	++
Proteus vulgaris	1		+:++	++:+++	0	+++
	2		0:+	+	0	+++
	6		0	0	0	+++
	24		0	0	0	+++

Initial inocula—*Staph. aureus* 0·9–3 × 10^6/0·1 ml.
Bact. coli 2–4 × 10^6/0·1 ml.
Ps. pyocyanea 5 × 10^6/0·1 ml.
Proteus vulgaris 1·6 × 10^6/0·1 ml.

O = no survivors in 0·1 ml.
\+ = few survivors only, less than 100.
++ and +++ = increasingly larger numbers of survivors.

SUMMARY

1. Acid conditions enhance the antibacterial properties of phenol such that a 0·2 per cent. solution at *p*H 3 behaves similarly to a 0·5 per cent. solution at neutral *p*H values; it does not behave in this way to moulds.

2. 0·2 per cent. of phenol is, therefore, a suitable bacteriostatic for insulin and globin insulin injections at *p*H 3.

3. Because of the antibacterial properties of protamine, 0·2 per cent. of phenol is also a suitable bacteriostatic to add to protamine zinc insulin injection.

This work was carried out at the request of the Technical Committee of the British Insulin Manufacturers.

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POLYVINYLPYRROLIDONE AS A DRUG RETARDANT

PART III. EFFECT ON SODIUM *p*-AMINOSALICYLATE

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CLINICAL evidence has been accumulated by Weiss, Seven and Eisenberg[1], demonstrating the improved blood levels of *p*-aminosalicylate when the drug is administered intravenously with polyvinylpyrrolidone. While *p*-aminosalicylate is usually given orally, gastrointestinal intolerances sometimes indicate its intravenous use. In such instances the reported prolonged retention of effective blood concentrations and the increase in maximum blood levels of the drug when given with polyvinylpyrrolidone suggest that this is a distinct advance in therapeutics.

As part of a broader study of the drug retardent action of polyvinylpyrrolidone[2,3], the combination of *p*-aminosalicylate with polyvinylpyrrolidone was investigated in rabbits and rats. This investigation was particularly desirable in view of the earlier report of Bertolani and Farinetti[4] which indicated that the intravenous injection of polyvinylpyrrolidone had little or no effect on the blood and urine concentrations of *p*-aminosalicylate given orally or intravenously.

EXPERIMENTAL METHODS AND RESULTS

Male and female rabbits used in these experiments were of mixed breeding and weighed 1·6 to 2·8 kg. Samples of free-flowing blood were taken from the ear vein just before and at intervals after the injection of *p*-aminosalicylate solutions. Measured aliquots of the blood, usually 0·2 ml., were added immediately to sufficient distilled water to give a total volume of 7·0 ml. Protein was removed by adding 3·0 ml. of 20 per cent. trichloracetic acid solution and filtering, after at least 5 minutes, through Whatman No. 42 filter paper. *p*-Aminosalicylate determinations on 5·0 ml. or smaller aliquots of the filtrate were carried out by the method of Klyne and Newhouse[5] using *p*-dimethylaminobenzaldehyde reagent (2 per cent. solution in 95 per cent. ethanol). If aliquots smaller than 5·0 ml. were used they were diluted to 5·0 ml. by the addition of 6 per cent. trichloracetic acid solution. After the addition of 1·0 ml. of citrate buffer (39·4 g. of citric acid dissolved in 100 ml. of 2 N sodium hydroxide and diluted to 250 ml. with distilled water) and 2·0 ml. of *p*-dimethylaminobenzaldehyde reagent, the percentage transmission of the coloured solutions was read at 420 mμ in the Evelyn colorimeter. The blank consisted of 5·0 ml. of 6 per cent. trichloracetic acid solution, 1·0 ml. of citrate buffer, and 2·0 ml. of *p*-dimethylaminobenzaldehyde reagent.

In the experiments in which rats were used, the analytical procedure was the same. Blood samples were obtained from the cut neck veins

of the rat when killed. Aliquots (0·3 ml.) were added at once to 6·7 ml. of distilled water without the use of an anticoagulant. The rats used were all male albinos and all within the weight range 130 to 180 g.

In some of the earlier experiments the *p*-aminosalicylate solutions for injection were prepared by suspending *p*-aminosalycylic acid in distilled water and adjusting the *p*H to approximately 6 with 2 N sodium hydroxide solution before diluting to the final volume. In the later experiments sodium *p*-aminosalicylate was used. Usually the salicylate derivative was made up at 5 times the required concentration and diluted with 4 volumes of polyvinylpyrrolidone solution to the required strength.

Rabbits were dosed in groups of 5, one rabbit receiving each of the 10 per cent. sodium *p*-aminosalicylate solutions containing 0, 5, 10, 20, or 40 per cent. polyvinylpyrrolidone. These solutions were injected at the rate of 2·0 ml./kg. of body weight. Blood samples were taken for analysis at 0 minutes and 20, 40, 60, 90, 120 and 180 minutes after injection. The analytical values on blood samples obtained after injection of the drugs were corrected by subtraction of the appropriate 0 time level. The results of 5 such experiments involving 25 rabbits are shown in Table I.

TABLE I

EFFECT OF POLYVINYLPYRROLIDONE ON THE BLOOD LEVELS AND RETENTION OF *p*-AMINOSALICYLATE IN RABBITS

Polyvinyl-pyrrolidone per cent.	Blood level of *p*-aminosalicylic acid μg./ml. of blood ± standard error					
	Minutes after injection (intravenous)					
	20	40	60	90	120	180
0	384 ± 9	236 ± 31	112 ± 14	49 ± 12	14 ± 5	trace*
5	378 ± 11	237 ± 33	87 ± 9	36 ± 9	10 ± 2	"
10	403 ± 21	225 ± 39	95 ± 24	43 ± 15	14 ± 3	"
20	402 ± 32	190 ± 27	62 ± 16	27 ± 7	7 ± 2	"
40	217 ± 39	119 ± 13	40 ± 14	24 ± 6	13 ± 4	"

* The corrected values at 180 minutes usually (22 cases) were less than 50 per cent. of the control level and are considered to be of very doubtful validity.

These data were subjected to appropriate application of the "t" test and it was found that the blood level of *p*-aminosalicylate at 20, 40, or 60 minutes after injection of the drug in 40 per cent. polyvinylpyrrolidone was significantly lower than the control. By 90 minutes after injection this difference was lost. After 120 minutes the blood level of *p*-aminosalicylate was very low and in 16 of 25 rabbits was zero at 180 minutes.

Most of the clinical investigations employed 12·5 to 25 per cent. polyvinylpyrrolidone. Since the clinical data of Weiss *et al.*[1] were obtained using 3·5 per cent. of polyvinylpyrrolidone, additional tests on rabbits were made to determine whether or not concentrations of polyvinylpyrrolidone less than 5 per cent. might be effective. The results with 3·5 per cent. of polyvinylpyrrolidone were essentially the same as those obtained with 0 or 5 per cent. of the retardant.

On the chance that the plasdone* brand of polyvinylpyrrolidone used in most of the experiments reported here might fail as a retardant because

* Plasdone was supplied by General Aniline and Film Corporation. K (Fikentscher) (1 per cent. solution) 30 ± 2.

of its chemical or physical properties, experiments using subtosan† brand of polyvinylpyrrolidone were conducted. The results, regardless of concentration over the range 2·6 to 40 per cent., were indistinguishable from those obtained with plasdone.

In the experiments with rats the *p*-aminosalicylate (10 per cent.) in aqueous polyvinylpyrrolidone solutions (0 to 40 per cent.) were injected intraperitoneally at the rate of 5·0 ml./kg. The injections were made at carefully timed intervals and the rats were killed 25, 50, 75, 125, or 200 minutes later. In each of two identical experiments 5 rats on each dose were killed at each of the 5 intervals. The results of the chemical determinations were subjected to an analysis of variance which indicated that there was no significant difference between the results of the two experiments.

The analytical data for the pooled experiments are presented in Table II.

TABLE II

EFFECT OF POLYVINYLPYRROLIDONE ON THE BLOOD LEVELS AND RETENTION OF *p*-AMINOSALICYLATE IN RATS

Polyvinyl-pyrrolidone per cent.	Blood level of *p*-aminosalicylic acid μg./ml. of blood ± standard error				
	Minutes after injection (intraperitoneal)				
	25	50	75	125	200
0	857 ± 29	693 ± 32	437 ± 42	196 ± 20	33 ± 6
5	726 ± 72	722 ± 53	426 ± 31	168 ± 25	45 ± 17
10	753 ± 35	672 ± 15	444 ± 23	149 ± 17	36 ± 4
20	678 ± 18	643 ± 18	439 ± 18	217 ± 26	57 ± 7
40	498 ± 26	518 ± 12	396 ± 42	250 ± 22	104 ± 8

Appropriate application of the "t" test to the means of the combined experiments indicated that the presence of 10, 20, or 40 per cent. of polyvinylpyrrolidone in the injected material significantly decreased the blood level of *p*-aminosalicylate measured 25 minutes after injection. At 50 minutes after injection this effect was significant only in the 40 per cent. polyvinylpyrrolidone group and at 75 minutes there were no significant differences among the mean blood levels for all 5 groups. In the presence of 40 per cent. polyvinylpyrrolidone 125 minutes after injection the blood *p*-aminosalicylate concentration was significantly greater than that in the presence of 5 or 10 per cent., but not in the presence of 0 or 20 per cent. of retardant. At the end of 200 minutes the blood level of *p*-aminosalicylate was significantly elevated over the control in the presence of 20 or 40 per cent. polyvinylpyrrolidone.

Since the depressed early blood levels might have been caused by excessive amounts of polyvinylpyrrolidone interfering with the analytical method for *p*-aminosalicylic acid, this point was investigated. Amounts of polyvinylpyrrolidone corresponding to as much as 12·5 mg./ml. of blood were added to known amounts of *p*-aminosalicylic acid and the analyses performed. There was no detectable influence of polyvinylpyrrolidone on the results obtained.

It seemed possible that the more highly concentrated solutions of

† Subtosan was supplied by Poulenc, Ltd. K (Fikentscher) value (1 per cent. solution) 33.

polyvinylpyrrolidone injected intravenously might cause a temporary hæmodilution thereby leading to low blood levels of the salicylate. In an attempt to test this possibility, serial hæmoglobin determinations were run on 6 rabbits given 2·0 ml./kg. of 10 per cent. *p*-aminosalicylate in water or in 40 per cent. polyvinylpyrrolidone solutions. The hæmoglobin determinations were made on 0·05 ml. aliquots of blood by a slight modification of Rimington's pyridine hæmochromogen method.[6] The results indicated that the hæmoglobin levels increased slightly during the first 20 to 30 minutes after injection of the drug and then returned to the original level whether the carrier solution was water or an aqueous solution of polyvinylpyrrolidone. This finding is not compatible with a hæmodilution which would have to be rather considerable to explain the 40 per cent. initial depression of the blood *p*-aminosalicylate level.

With intraperitoneal injections in rats it is likely that the high viscosity of the more concentrated polyvinylpyrrolidone solutions mechanically hindered absorption of *p*-aminosalicylate into the blood stream. This could account for the initial low blood levels and more prolonged retention of *p*-aminosalicylate in the blood stream.

On the basis of these experiments in rabbits and rats it is not possible to account for the findings of Weiss *et al.*[1] that 3·5 per cent. polyvinylpyrrolidone in the *p*-aminosalicylate solutions for intravenous injection in man doubled both the maximum blood level and the duration of measurable blood levels of the antituberculosis drug. The results reported here would suggest that polyvinylpyrrolidone was without effect except at relatively high concentration and in such instances lowered the maximum blood concentration of *p*-aminosalicylate.

Summary

1. Polyvinylpyrrolidone at 40 per cent. concentration in the injected material effectively lowered the maximum blood level of *p*-aminosalicylate when the drugs were given together intravenously to rabbits or intraperitoneally to rats.

2. The duration of retention in the blood stream of significant amounts of the intravenously injected *p*-aminosalicylate was not influenced by polyvinylpyrrolidone.

3. When the combined drugs were given intraperitoneally appreciable blood levels persisted longer in the presence of 20 or 40 per cent. of polyvinylpyrrolidone.

The authors wish to acknowledge the able assistance of Mr. R. Slinger in the early phases of this investigation.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Achilleine, Alkaloid of *Achillea millefolium* L. F. M. Miller and L. M. Chow. (*J. Amer. chem. Soc.*, 1954, **76**, 1353.) From the yarrow plant, *Achillea millefolium* L., a crystalline alkaloid, achilleine, $C_{14}H_{26}N_2O_6$, m.pt. 247 to 248° C., $[\alpha]_D$–14·3° (water, *c* 10) was isolated. It is weakly basic, gives positive, but non-reproducible, results in the *N*-methyl determination, and contains no methoxyl group. It is very soluble in water, slightly soluble in ethanol, but almost completely insoluble in less polar solvents. On the basis of physical data and chemical evidence, achilleine is tentatively formulated as a glyco-alkaloid containing a pyrrolidine or piperidine nucleus bearing an *N*-methyl group and a carboxamide function. It was found to reduce the clotting time of blood in rabbits as determined by the Sabraze method. A. H. B.

***Rauwolfia serpentina*, A Preliminary Chemical Examination of.** W. L. Holt and C. H. Costello. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 144.) Extracts were prepared by percolation with a mixture of 10 parts of acetone and 90 of ethanol, 2 per cent. of strong solution of ammonia being added for the preliminary maceration, since this increased the potency of the extracts. Of 9 samples tested, Dehra Dun and Himalayan materials generally showed the greatest hypotensive action, Coastal Plain and Bihar being about half and Malabar 1/5th as potent. Coastal Plain and Malabar varieties were adulterated with another species of *Rauwolfia*. The total alkaloidal content as determined by the B.P.C. 1949 method did not run parallel with hypotensive activity, because of variations in the proportions of the different alkaloids present. The best solvents for extraction of total alkaloids and hypotensive principles were found to be chloroform, acetone, and ethylene dichloride. A method of extraction of the alkaloids and separating them into main groups is described. G. B.

***Rauwolfia Serpentina* Benth. Isolation of δ-Yohimbine and a New Related Alkaloid from.** F. L. Weisenborn, M. Moore and P. A. Diassi. (*Chem. Ind.*, 1954, 375.) In addition to reserpine, δ-yohimbine (previously encountered only in *Coryanthe yohimbe*) and a hitherto undescribed related alkaloid were isolated from *Rauwolfia serpentina* Benth. After crystallisation of reserpine from a fraction containing the weakly basic constituents of the root, the mother liquors were chromatographed on acid-washed alumina. Elution with 1:9 ether-benzene gave a new alkaloid $C_{22}H_{26}O_4N_2$, m.pt 240 to 241° C. The alkaloid δ-yohimbine, was obtained by further elution of the column with 1:1 ether-benzene, and was shown to be identical with py-tetrahydroserpentine (mixed m.pts., ultra-violet and infra-red spectra). A. H. B.

ANALYTICAL

Gitoxin, Fluorimetric Determination of. J. F. A. Fruytier and J. A. C. van Pinxteren. (*Pharm. Weekbl.*, 1954, **89**, 99.) The method of Jensen (*Acta pharm. tox., Kbh.*, 1952, **8**, 101) for the fluorimetric determination of gitoxin uses as reagent a mixture of hydrochloric acid and glycerol. The method can

not however be applied satisfactorily to a mixture of digitalis glycosides as the material is not sufficiently soluble in the solvent. More satisfactory results are obtained by using a mixture of 5 volumes of hydrochloric acid (38 per cent.), 5 of glycerol and 1 of ethanol. The fluorescence is measured after 1 hour. Digitoxin does not interfere with the determination. By this method it was possible to demonstrate the presence, in commercial digitoxin, of quantities up to 15 per cent. of gitoxin. For determination in digitalis, the drug was first treated by the method of Langejan and van Pinxteren (*Pharm. Weekbl.*, 1953, **88**, 529). Of the mixture of glycosides and genins obtained in this way from 1 g. of leaf, dissolved in 50 ml. of ethanol (24 per cent.), 5 ml. was evaporated cautiously and the residue dissolved in 20 ml. of ethanol (96 per cent.): 1 ml. of this solution was mixed with 10 ml. of hydrochloric acid-glycerol mixture, shaken vigorously for 1 hour, and then compared with a standard gitoxin solution treated in the same way. Three successive determinations showed a content of 0·17, 0·16 and 0·16 per cent. of gitoxin (aglycone + glycoside). The total content of substances giving a colour with dinitrobenzoic acid was 0·24 per cent., calculated as digitoxin. Thus a large proportion of the glycosides belong to the gitoxin group. G. M.

Magnesium, Calcium, Zinc, Cadmium, Titanium and Zirconium, Spectrophotometric Titrations with Ethylenediamine tetra-acetic Acid. P. B. Sweetser and C. E. Bricker. (*Analyt. Chem.*, 1954, **26**, 195.) In an effort to extend the versatility of ethylenediamine tetra-acetic acid (versenate) as a volumetric reagent, the ultra-violet region of the spectrum was studied for use in the spectrophotometric determination of the end-point. A procedure is described for the determination of calcium, magnesium, cadmium and zinc in ammonia-ammonium chloride solutions; calcium in the presence of magnesium and cadmium in the presence of zinc. A method was developed for the determination of zirconium involving the addition of excess of versenate and back-titration of the excess with ferric iron solution. Versenate solutions as dilute as 0·001 M give a very sharp end-point even when the total volume of the solution being titrated exceeds 100 ml. A. H. B.

Morphine, Colorimetric Method for Quantitative Determination of. L. Szabolcs. (*Hungarian J. Chem.*, 1953, **59**, 67, *Through Hungarian Technical Abstracts.*) The reaction of morphine with potassium iodate in acid media serves as a basis for the colorimetric determination of morphine. After rendering the reaction mixture alkaline a stable colour was formed. The amount of morphine salt present can be measured photometrically since a linear relation exists between the colour intensity and concentration. Experimental procedure: to 4 ml. of the solution to be analysed (containing 1 to 4·5 mg. of morphine/ml.) one drop of a 10 per cent. solution of hydrochloric acid and 0·2 ml. of a potassium iodate solution (saturated at room temperature) was added. After 1 minute the colour intensity reaches its maximum, and then 0·2 ml. of a 10 per cent. solution of sodium hydroxide is added. Stand the solution for about 20 minutes, and make up to 25 ml. The colorimetric determination is made using a Pulfrich photometer with an S 50 filter. The extinction of 1 mg. morphine hydrochloride is 0·190 using a 30 mm. cuvette. Apomorphine, pyramidon and acetylsalicylic acid interfere with the determination. They can be eliminated by preparing a stock solution in the presence of bismuth subnitrate and subsequently filtering. J. R. F.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Castle's Intrinsic Factor, Isolation of. A. L. Latner, R. J. Merrills and L. C. D. P. Raine. (*Lancet*, 1954, **266**, 497.) The authors claim to have isolated the intrinsic factor of Castle from the hog gastric mucosa in a satisfactory pure state. It is mucoprotein in nature. The material was tested for activity in man by determining its effect on the fæcal excretion of radio-active vitamin B_{12} given orally. Details of the isolation are to be published shortly. From its behaviour in the ultracentrifuge it is shown to contain 5 per cent. of protein. The remaining 95 per cent. appeared to be homogenous and to have a molecular weight below 20,000. It is similar to a material previously obtained from human gastric juice by paper strip electrophoresis. G. F. S.

Glycyrrhetinic Acid, Metabolism of, in Human Subjects. V. M. v. Katwijk and L. G. Huis In't Veld. (*Nature. Lond.*, 1954, **173**, 733.) Using a method by which the urinary excretion of the acid, in amounts as small as 0·01 mg. per 24 hours, could be determined, none could be detected in the urine of a patient with Addison's disease who was treated with 2·28 g. of the acid orally every 24 hours. The urine of a patient with a jejunal ulcer who received 2·5 g. for 24 hours orally also failed to show a trace of this compound. The detection of possible catabolites of the acid was then considered, and it was found that after administration, extraction of the urine with butanol at 15° C. yielded a substance which gave a red colour with 71 per cent. sulphuric acid in methanol solution. Glycyrrhetinic acid itself does not give a colour. Of 20 samples of urine from patients not being treated with the acid 1 gave a positive result, and of 30 samples collected during treatment with the acid all gave a positive result. The red solution has an absorption maximum lying between 555 and 560 mμ as measured with a Beckman spectrophotometer. Work on the optimum condition for colour development and the isolation and identification of this possible catabolite is in progress. J. R. F.

***Mycobacterium tuberculosis*, Chromatographic Isolation of Polysaccharides from.** B. Siegel, G. A. Candela and R. M. Howard. (*J. Amer. chem. Soc.*, 1954, **76**, 1311.) An electrolytic extraction of the polysaccharides from the tubercle bacillus was devised. By this process, additional polysaccharides not present in the autolysate, were extracted. The polysaccharides were isolated by adsorption on silica gel and then elution with the following sequence of increasing polar solvents: hexane, chloroform, methanol and water. 12 different polysaccharide fractions were isolated from the autolysate from a human strain of *Mycobacterium tuberculosis* and 21 from the electrolytic extraction of the cells. Of these polysaccharide fractions, 19 had the ability to bind antibodies *in vitro*. All the 21 fractions gave typical polysaccharide absorption curves in the ultraviolet. A. H. B.

BIOCHEMICAL ANALYSIS

Chloramphenicol, Comparison of a Microbiological and a Chromatographic Assay Method for. T. Higuchi, A. D. Marcus and C. D. Bias. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 135.) Preparations of chloramphenicol were assayed by chromatographic separation of the antibiotic followed by measurement of the ultra-violet absorption of the chloroform-ethyl acetate solution at

278 mμ. Good recoveries were achieved and the results were in agreement with those of microbiological assays based on the inhibition of the oxygen uptake of *Escherichia coli*. The assay was carried out by dissolving a sample in chloroform containing 10 per cent. of ethyl acetate. 5 ml. was placed on a silicic acid column with water as the internal phase. Chloroform was passed through the column until about 40 ml. of eluate containing impurities less polar than chloramphenicol had been obtained. The solvent was changed to chloroform containing 10 per cent. of ethyl acetate and this fraction, containing chloramphenicol was collected for spectrophotometric analysis. This method is applicable to samples containing a fraction of a mg. of chloramphenicol, but needs modification before it can be used for quantities of a few μg. G. B.

Lead in Blood, Determination of, with Dithizone. M. Mokranjac and S. Radmić. (*Acta Pharm. Jug.*, 1953, **3**, 253.) The determination of small quantities of lead in blood using dithizone is inconvenient because of the presence of iron and the formation of a yellow colour which masks that of the normal colour of lead dithizonate. The following method is suggested. Place 10 ml. of blood in a 150-ml. beaker to which 10 ml. of concentrated nitric acid and 0·25 ml. of concentrated sulphuric acid are added. Heat carefully until nearly all the nitric acid has been driven off. Cool a little, and add 5 ml. of nitric acid and repeat the heating. Then add a further 5 ml. of the acid and heat until sulphur trioxide is formed. Cool, add 1 ml. of 30 per cent. hydrogen peroxide, heat carefully until the peroxide has evaporated, and repeat the addition and evaporation of peroxide 3 or 4 times. A whitish residue is left which is then heated to remove all the sulphur trioxide. Cool, add 1 ml. of a 1 : 1 mixture of hydrochloric and nitric acid, and heat until almost dry. Add 5 ml. of 50 per cent. hydrochloric acid, and to the cooled solution in a separator introduce 10 ml. of ether. Shake well to allow the iron to pass into the ether, separate and then evaporate the solution to dryness. Add 2 ml. of water, heat to boiling, and after the addition of 5 ml. of a 20 per cent. citric acid solution, cool, and introduce 2 to 3 drops of thymol blue (in 0·25 per cent. ethanol) and ammonia to *p*H 9·2 (colour changing to blue). Then add 3 ml. of a 10 per cent. solution of potassium cyanide and 10 ml. of a solution containing: 15 ml. of a 10 per cent. potassium cyanide solution, 5 ml. of concentrated ammonia, 15 ml. of a 20 per cent. citric acid solution, and water to 100 ml. Shake 5 ml. of dithizone solution (5 mg. per cent. in carbon tetrachloride) with the solution in a separator for 5 minutes. Run off the carbon tetrachloride solution into another separator and repeat the extraction using 3 ml. of dithizone solution each time until its green colour changes. Remove excess dithizone from the mixed solutions with 1 in 300 ammonia. Filter into a 10 or 25 ml. graduated flask, and make up to the mark with carbon tetrachloride. This solution is now ready for spectrophotometric or electrophotometric determination. It is necessary to make a calibration curve for the instrument used. The error calculated after many analyses varied between 1·1 and 4·3 per cent. J. R. F.

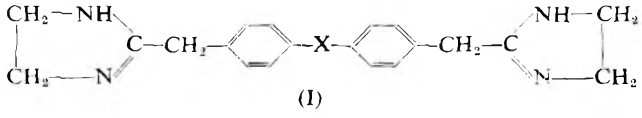
Penicillin in Feeding Stuffs, Assay of. S. A. Price and K. A. Boucher. (*Analyst*, 1954, **79**, 150.) A paper disc plate method, developed from the procedure of Esposito and Williams (*Proc. Soc. Exp. Biol. N.Y.*, 1952, **81**, 660), is described for the assay of penicillin in feeding stuffs, using *Bacillus subtilis* as the test organism. The standard solution is prepared by dilution in methanol of a buffered aqueous solution of sodium benzylpenicillin, final dilutions for assay

being made in an unfortified extract of the feeding stuff under test. Feeding stuffs were extracted with methanol and the solutions were taken up on paper discs, the dried discs being applied to the medium in a randomised Latin square assay design. With the method as described, the limits of error (P = 0·95) were of the order of 90 to 111 per cent. in an 8 × 8 assay of two samples with 8 replicates per dose, or 85 to 117 per cent. if four samples were used and the number of replicates reduced to 4. Both sodium benzylpenicillin and procaine benzylpenicillin were found to be unstable when dissolved in aqueous methanol, unless phosphate was present. R. E. S.

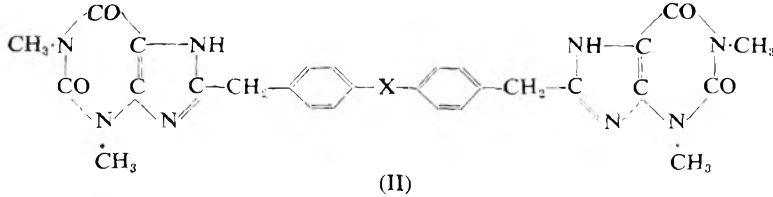
"Strepogenin," Determination of, with *Lactobacillus bifidus*. P. Roine, H. Gyllenberg and V. Salakivi. (*Acta. chem. scand.*, 1954, **8**, 161). An assay process for strepogenin based on the fact that it is an essential growth factor for certain strains of *Lactobacillus bifidus* is described. Its effect on this organism is specific at least in that asparagine and glutamine have no activity. The use of this test organism has the following further advantages: the blank values are always very small, the growth response is pronounced, and the basal medium is very simple. Figures for the strepogenin-activities of 13 different preparations are given, using peptonised milk as the strepogenin standard. A. H. B.

CHEMOTHERAPY

Theophylline Derivatives, Bifunctional, and Corresponding Iminazolines. G. P. Hager and C. Kaiser. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 148.) A series of iminazoline derivatives of type I were prepared (X = –O–, $-CH_2.CH_2-$, $-O(CH_2)_3O-$, and $-O(CH_2)_5O-$) by fusion of the corresponding dinitriles with ethylenediamine *p*-toluenesulphonate. Another bifunctional analogue of tolazoline, 1 : 5-bis(2-methylene-3-iminazoline) naphthalene, which is also related to naphazoline, was prepared, and appeared to be the most active compound.



(I)



(II)

These substances lowered the blood pressure of dogs, but only when administered in doses which inhibited respiration. Compounds of type II were obtained by fusion of the dicarboxylic acid with 1 : 3-dimethyl-5 : 6-diaminouracil and cyclisation with sodium hydroxide, or by refluxing with phosphorus oxychloride. These bifunctional analogues of 8-benzyltheophylline produced an effect on blood pressure similar to that of the parent substance but had the disadvantage of limited solubility in water. G. B.

PHARMACY

GALENICAL PHARMACY

Bacitracin, Stability of Solutions of. V. Würtzen. (*Dansk Tidsskr. Farm.*, 1954, **28**, 34.) Bacitracin, although a polypeptide containing cysteine, does not give any reaction with nitroprusside, with or without the addition of cyanide. Old solutions however react with nitroprusside in presence of cyanide, indicating the presence of a disulphide group. The deterioration of solutions appears to be a process of oxidation initiated by light. Neither nordihydroguaiaretic acid, ascorbic acid nor hydroquinone were effective in preventing the loss of strength of solutions of bacitracin when exposed to diffused daylight. By replacement of the air by nitrogen, a solution may be kept in diffused light for 3 months with a loss in activity not greater than 10 per cent. A certain amount of antioxidant action was shown by *p*-phenylenediamine and *p*-aminophenol. G. M.

Cyanocobalamin and Ascorbic Acid, Stability of, in Liquid Formulations. A. Bartilucci and N. E. Foss. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 159.) The effects of *p*H and of various added chemicals were studied in solutions containing 10 μg. of cyanocobalamin and/or 50 mg. of ascorbic acid per ml., containing 0·05 per cent. of propyl 4-hydroxybenzoate as preservative. The maximum stability of ascorbic acid and ascorbic acid/cyanocobalamin solutions occurred at *p*H 6·0 to 7·0, but solutions of cyanocobalamin alone were most stable at *p*H 4·5 to 5·0. Tetrasodium ethylenediamine tetra-acetate had a stabilising effect on ascorbic acid solutions, and slightly improved the keeping properties of ascorbic acid/cyanocobalamin solutions stored at 40° C. This substance was harmful to cyanocobalamin solutions, unless acidified. Ascorbic acid appeared to be most stable in solutions containing high concentrations of propylene glycol with water, glycerol or D-sorbitol solution. In mixed ascorbic acid/cyanocobalamin solutions, a vehicle consisting of equal quantities of propylene glycol and glycerol gave maximum stability, there being no loss of cyanocobalamin and about 10 per cent. loss of ascorbic acid after 6 months' storage at room temperature. It appears that the products of the decomposition of ascorbic acid play an important part in the destruction of cyanocobalamin. G. B.

NOTES AND FORMULÆ

Nitrofurantoin (Euradantin). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1954, **154**, 339.) Nitrofurantoin is *N*-(5-nitro-2-furfurylidene)-1-aminohydantoin and occurs as a yellow bitter powder with a slight odour; it decomposes at 258° to 262° C., and is very slightly soluble in ethanol and almost insoluble in ether and water. It dissolves in sodium hydroxide solution to give an orange-red colour. A 0·0005 per cent. solution prepared with dimethylformamide and water exhibits ultra-violet absorption maxima at about 266 and 368 mμ ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ about 753) and a minimum at about 305 mμ; the ratio of the absorption at 368 mμ to that at 266 mμ is 1·26 to 1·46. When dried at 105° for 5 hours, nitrofurantoin loses not more than 1·0 per cent. of its weight; sulphated ash, not more than 0·05 per cent. It contains 95·0 to 105·0 per cent. of nitrofurantoin and is assayed by measuring the absorption of a 0·0005 per cent. solution at 368 mμ. Nitrofurantoin is useful for the treatment of bacterial infections of the urinary tract. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Adenosine 5-Monophosphate, Treatment of Calcific Tendinitis with. A. M. Susinno and R. E. Verdon. (*J. Amer. med. Ass.*, 1954, **154**, 239.) A total of 36 patients with chronic calcific tendinitis of the shoulder were treated with intramuscular injections of 20 mg. of adenosine 5-monophosphate given daily or on alternate days in 1 ml. of gelatin solution. Thirteen controls were given 1 ml. of either saline solution or gelatin solution. Results were evaluated on loss of pain and significant improvement in abduction, rotation or elevation of the arm. 31 of the 36 patients gave satisfactory responses with 3 to 14 injections (average 9 injections). There were 6 recurrences in this group; four were given a further course of treatment, with similar results, while in the other two the recrudescence was so mild that further treatment was considered unnecessary. Of the controls, one responded to saline solution. 10 of the others did not respond after an average of 16 injections but responded promptly to adenosine 5-monophosphate. No severe reaction of any kind was encountered with intramuscular administration of the drug. A number of patients reported diuresis, flushing, or a slightly painful site of administration. Almost half the cases exhibited an apparent "flare-up" of symptoms and disability in the fifth to tenth day of treatment, followed in a day or two with a notable overall improvement. An additional 17 patients were similarly treated and 13 of these gave satisfactory results. G. R. K.

Adrenaline and Noradrenaline, Comparative Effects of, in the Dog. R. Ahlquist, J. Taylor, C. Rawson and V. Sydow. (*J. Pharmacol.*, 1954, **110**, 352.) In this paper relative potencies of adrenaline and noradrenaline are determined and the results analysed statistically. The test preparations used are the arterial blood pressure, heart rate, renal and femoral blood flow, splenic contraction and intestinal activity of dogs, anæsthetised with sodium pentobarbitone.

Noradrenaline was found to be significantly more effective than adrenaline as a pressor agent and in producing vagal bradycardia whereas adrenaline was found to be significantly more effective in producing splenic contraction, intestinal relaxation and vasoconstriction in the femoral and renal vascular beds. M. M.

Adrenaline and Noradrenaline in Separate Adrenal Medullary Cells. N. Hillarp and B. Hökfelt. (*Acta. physiol. scand.*, 1953, **30**, 55). A method is described for the cytological differentiation of noradrenaline from adrenaline in the adrenal medulla. Adrenal glands of the rat, guinea-pig, rabbit, cat, dog, cow, sheep, horse and domestic fowl are treated with 2·5 per cent. potassium iodate in acetate buffer at *p*H 6, when the noradrenaline in the gland is converted into insoluble brownish-black granules. Under these conditions adrenaline does not form an insoluble pigment. Both amines can be demonstrated by treatment with 2·5 per cent. potassium bichromate at *p*H 5·6 when adrenaline and noradrenaline give brown pigments. No method has yet been found for the selective cytological demonstration of adrenaline. By these methods it is shown that 5 to 15 per cent. of the medullary cells in the rat, 20 to 50 per cent. in the cat, 15 to 30 per cent. in the cow and 10 to 40 per cent. in the dog, sheep and horse show an intense noradrenaline reaction, while in the guinea-pig and rabbit there is no noradrenaline pigmentation. These figures agree well with biological and chemical estimates of the noradrenaline content of the glands. Noradrenaline is therefore apparently selectively stored in certain specific cells of the adrenal

medulla. This cytological selectivity suggests that noradrenaline is not only a precursor of adrenaline but also an independent hormone. M. M.

Anticholinesterases, Pharmacology of Some New. F. H. Shaw and G. A. Bentley. (*Aust, J. exp. Biol. Med. Sci.*, 1953, **31**, 573.) The anticholinesterase activity of some acridine, pyridine and thiazole derivatives was estimated manometrically. The modifications of the actions of acetylcholine on the isolated frog rectus abdominis, the Straub amphibian heart and isolated mammalian intestine and uterus by the compounds were also determined. There was some correlation of anticholinesterase activity and pharmacological effects in those derivatives showing a high degree of cholinesterase inhibition (e.g., the 2–, 3–, 4– and 5– aminoacridines), but this was not uniform throughout the range of test preparations and on the amphibian hearts especially some of the compounds had an atropine-like action. Also, others of the series having no anticholinesterase activity showed on some preparations a potentiation of the effects of acetylcholine. The significance of these results is discussed. G. P.

Carotid Body and Sinus, Effect of Drugs on. C. Heymans, A. L. Delaunois, L. Martini and P. Janssen. (*Arch. int. Pharmacodyn.*, 1953, **96**, 209). The effects of some cholinomimetic and cholinolytic drugs on the chemoreceptors of the carotid body and on the baroreceptors of the carotid sinus have been reinvestigated by the authors, since much of the work in this field has produced conflicting results. The technique adopted was that of Heymans *et al.* (Heymans *Actualités Pharmacologiques*, 5e serie, 1952, p. 111, Paris) and was conducted on dogs under morphine and chloralosane anæsthesia. Femoral arterial blood pressure, thoracic movements and respiratory volume were recorded. Injections of acetylcholine, lobeline and potassium cyanide were made through the thyroid artery into the common carotid circulation, all efferent branches of the carotid bifurcation except the occipital arteries supplying the carotid bodies being tied off. All other drugs were injected either intravenously or into the conjunctival space surrounding both carotid body and sinus areas. Positive significance to results was attributed only where changing one factor resulted in practically 100 per cent. change in response or where a dose-response relationship was clearly demonstrable. Local application of neostigmine to the carotid body and sinus induced moderate respiratory stimulation whereas eserine had no such action when applied similarly. Both drugs increased the sensitivity of the chemoreceptors to acetylcholine markedly, and sensitivity to lobeline slightly. Eserine also increased slightly the sensitivities of the chemoreceptors to potassium cyanide. Neither drug affected baroreceptor sensitivity to change in blood pressure. Intravenously, large doses of atropine did not affect responses of the chemoreceptors to acetylcholine, lobeline or potassium cyanide, but local application of similar doses to the carotid body, besides causing a rise in blood pressure blocked the action of these drugs on the chemoreceptors. Local application of small doses had no effect on either the chemoreceptor or baroreceptor sensitivity. Intravenous doses of tetraethylammonium, hexamethonium, methantheline, or pendiomide had no significant effect on chemoreceptor stimulation by acetylcholine, lobeline and potassium cyanide. Similarly local application of these ganglion-blocking agents did not modify either chemoreceptor response to the three stimulants or baroreceptor sensitivity to blood pressure variation. Some of the results are therefore at variance with those of other workers and do not support the suggestion that acetylcholine is the chemical mediator of carotid body chemoreceptor or carotid sinus barorec eptor activity G. P.

Demyelination. J. B. Cavanagh and R. H. S. Thompson. (*Brit. med. Bull.*, 1954, **10**, 47.) The series of changes involving the break-up and loss of the myelin sheath of the nerve fibre, either in peripheral nerves or in the central nervous system, constitutes one of the standard reactions to injury exhibited by nervous tissue, and occurs in a wide variety of different types of injury. From a survey of the various means by which demyelination can be brought about experimentally it appears that not only is it not yet possible to reproduce in laboratory animals the true counterpart of the lesions of disseminated sclerosis but that there is so far no clear idea as to the underlying biochemical mechanism in most of the experimental demyelinations. From the biochemical point of view four apparently dissimilar procedures can give rise to these lesions: (1) copper deficiency, (2) intoxication by cyanide, presumably acting by producing cerebral anoxia, (3) intoxication by certain of the anticholinesterases, and (4) deficiency of certain vitamins of the B complex, notably aneurine and cyanocobalamin. A further fact which has emerged from recent isotopic studies is that myelin is in a constant state of "turnover," with new formation balancing its disintegration, and it is possible that the complex set of synthetic reactions involved in this formation of myelin may become deranged. Indeed, it would seem more likely that the demyelination in anoxia or in aneurine deficiency would be due to a failure in endothermic synthetic processes. S. L. W.

Dextromethorphan Hydrobromide and Codeine, Quantitative Comparison of. L. J. Cass, W. S. Frederik and J. B. Andosca. (*Amer. J. Med. Sci.*, 1954, **227**, 291.) A statistical evaluation of the comparative antitussive activities of dextromethorphan hydrobromide, codeine and a placebo was conducted on 69 patients with varying degrees of cough. The dextromethorphan was given at three doses, 6, 12 and 18 mg., and codeine in a dose of 15 mg. The drugs were administered orally three times daily for seven days so that each patient had five drugs in 35 days. The drug sequence was allocated at random. Taking the placebo as being equivalent to zero dose of dextromethorphan, there was a good dose-response relationship for this drug. From this dose-response curve, 15 mg. dextromethorphan hydrobromide had an antitussive effect equal to that of 15 mg. codeine. There was, however, no significant statistical difference between 12 and 18 mg. of dextromethorphan hydrobromide or 15 mg. of codeine. On the other hand, the 6 mg. dose of dextromethorphan was significantly more effective than the placebo, and significantly less effective than 12 mg. dextromethorphan. The most suitable dose of the drug seems to lie between 10 and 15 mg. The incidence of side effects (nausea, vomiting, constipation and drowsiness) was much more marked with 15 mg. codeine than with any of the doses of dextromethorphan. G. P.

Histamine Analogues, Effect of on Cutaneous Pain. S. R. Rosenthal. (*Arch. int. Pharmacodyn.*, 1953, **96**, 220). The local anæsthetic activity of a series of 52 compounds related to histamine, including heterocyclic structures containing pyridine, pyridazine, pyrimidine, pyrazine, quinoxaline, quinoline, pyrazole, thiazole and imidazole nuclei, was determined by a guinea-pig weal method. A two per cent. solution of the drug was injected intradermally into the shaved skin of the guinea-pig to form a weal. The skin over the weal was stimulated electrically to obtain an axon reflex response and the duration of suppression of this reflex by the drug compared with one per cent. procaine solution injected similarly into an adjacent area. Twenty of the compounds showed some degree of local anæsthesia, 3:5 diphenyl 1-(β-aminoethyl) pyrazole, in particular, being much more effective than procaine. No strict correlation between local

anæsthetic activity and antihistamine activity was apparent. The pain-producing effect of some of the compounds was compared with that of histamine by intradermal injection in man, but only 2-methyl 4-(β-aminoethyl) imidazole approached the activity of histamine in this respect. The results further support the theory that histamine or a histamine-like substance may be the mediator for cutaneous pain. G. P.

Histamine, 5-Hydroxytryptamine and a Potent, Slow Contracting Substance, Presence of, in Wasp Venom. R. Jaques and M. Schachter. (*Brit. J. Pharmacol.*, 1954, **9**, 53.) Wasp venom contains three highly active smooth muscle stimulants—histamine, 5-hydroxytryptamine and a potent, slow-acting substance as yet unidentified. The presence of histamine was demonstrated chromatographically and by isolation using Code's method. These gave estimates for histamine content of 20 mg. and 16 mg., respectively, per g. of dried venom. 5-Hydroxytryptamine was identified chromatographically and the eluate from the corresponding chromatogram showed a content of 0·32 mg. per g. of dried venom. The third smooth muscle stimulant was demonstrated in presence of atropine and mepyramine on the guinea-pig ileum, after desensitisation of the ileum to tryptamine derivatives. This substance resembled bradykinin in that it withstood boiling at neutral—though not at high— *p*H, for long periods; it contracted the rat colon and was inactivated when the venom was extracted for histamine by Code's method. Also crude untreated wasp venom in a concentration of 3×10^{-7} will contract the guinea-pig ileum rendered unresponsive to histamine, acetylcholine and 5-hydroxytryptamine; bradykinin in similar conditions has a similar degree of activity. Crude wasp venom occasionally released histamine from the isolated perfused cat skin preparation. G. P.

Histamine—Effect of Intravenous Infusions of, on the Urinary Histamine and on Gastric Secretion in Man. H. M. Adam, W. I. Card, M. J. Riddell, M. Roberts and J. A. Strong. (*Brit. J. Pharmacol.*, 1954, **9**, 62.) The appearance of free histamine in the urine was compared with the rate of gastric acid secretion in three healthy male subjects after slow intravenous infusion of histamine acid phosphate. When the rate of infusion was sufficient to cause an increase in the acidity of the gastric juice there was a corresponding rise in urinary histamine. The threshold for the two effects varied from 7·4 to 13·0 ng./kg./min. With further increase in dose, the two secretions rose concomitantly. The proportion of free histamine excreted in the urine during the infusion was approximately one per cent. of the total dose and was independent of the rate of infusion. Free histamine also appeared in the gastric juice, where its concentration was similar to that in the urine, but more variable. Again the amount appearing was independent of the rate of infusion. Whether the histamine in the juice was derived from the plasma or from the gastric mucosa could not be determined from the results. The urinary free histamine would, however, seem from the evidence to be obtained from the plasma, although an increased plasma content could not be detected during the infusion, even when the dose was high. G. P.

4-Hydroxycoumarin, Metabolism of, in the Dog. S. Roseman, C. F. Huebner, R. Pankratz and K. P. Link. (*J. Amer. chem. Soc.*, 1954, **76**, 1650). The study of the fate of dicoumarol *in vivo* is difficult because only small continuous doses can be tolerated and therefore 4-hydroxycoumarin with about 1/20th the activity was chosen for study. When injected into the blood stream of the dog, approximately 75 per cent. appeared in the urine within 24 hours. The fate of the

remainder is unknown. No significant increase in the urinary output of the steam-distillable phenols or the ethereal sulphate fraction occurred and no salicylic acid was found in the urine. Approximately 50 per cent. of the 4-hydroxycoumarin injected appears in the urine in the free state and 25 per cent. as 4-hydroxycoumarin β-D-glucopyranosiduronic acid. Dilute acid hydrolysis of this latter compound formed *o*-hydroxyacetophenone. 4-Hydroxycoumarin underwent a similar degradation under similar conditions.

A. H. B.

Liver Residue, Protective Effect of, on Immature Male Rats Fed Toxic Doses of Acetylsalicylic Acid. B. H. Ershoff, H. B. McWilliams and E. W. Thurston. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 175.) Immature male rats, fed on a diet containing 0·5 per cent. of acetylsalicylic acid showed significant retardation of growth and reduction of the weight of the thymus, prostate and seminal vesicles, as compared with animals from whose diet the acetylsalicylic acid was omitted. These effects were largely counteracted by the administration of liver residue, the coagulated water-insoluble material remaining after the extraction of water-soluble matter. The factor protecting the animals against toxic doses of acetylsalicylic acid did not appear to be any known vitamin of the B group. It was not present in significant amounts in the purified casein which formed part of the diet of the rats. Possibly it is the same substance present in liver residue which has been observed to protect rats against the toxic effects of thyroid, thyroxine, mepacrine, cortisone etc. G. B.

Mercaptoethylamine; Failure to Protect against Mutagenic Effects of Radiation. W. D. Kaplan and M. F. Lyon. (*Science*, 1953, **118**, 777.) It has been shown that β-mercaptoethylamine gives protection against the lethal effects of radiation and produces a marked decrease in mortality when administered intraperitoneally to mice during the half hour preceding whole-body radiation. The object of these experiments, using *Drosophila melanogaster* and mice as test organisms was to determine whether this substance also protects against the mutagenic effects of radiation. The combined studies clearly indicate that mercaptoethylamine has no influence upon the genetic effects of radiation, nor does it protect the male germ cells against radiation death. These findings support those of other workers, suggesting that this substance exerts its protective action through the liver and that the primary effects of radiation on other organs are not prevented. S. L. W.

Nalorphine in the Prevention of Neonatal Asphyxia due to Maternal Sedation with Pethidine. S. J. Paterson and F. Prescott. (*Lancet*, 1954, **266**, 490.) Nalorphine, *n*-allylnormorphine, antagonises the pharmacological actions of morphine. It has been found effective in abolishing respiratory depression in the newborn infant due to the sedation of the mother with pethidine or 'omnopon'. After delivery the cord was not cut until the child had gasped and cried. If the child did not gasp within 10 secs. nalorphine 0·5 mg. in 2 ml. was injected into the umbilical vein. If the child did not cry within two minutes the dose was repeated. In the control series (262 babies), which did not receive nalorphine, 16 per cent. showed respiratory depression and resuscitation was necessary in 91 per cent. of these. In the test series (203 babies) 14 per cent. showed respiratory depression, and 80 per cent. of these gasped within half a minute of receiving an injection of nalorphine. The effects were striking in babies which were limp and asphyxiated before treatment. No ill effects from nalorphine were seen in any of the babies. It is suggested that nalorphine should permit the use of morphine, pethidine and related drugs nearer to the time of delivery. G. F. S.

Plasma Fractions in the Treatment of Injury. M. E. Mackay. (*Brit. med. Bull.*, 1953, **10**, 31.) In the treatment of injury by plasma proteins two groups of proteins are of major importance. These are fibrinogen and thrombin, which bring about coagulation of blood and prevent protein loss; and serum albumin which may be used in the treatment of trauma. The colloid osmotic pressure of serum albumin accounts for 80 per cent. of that of whole blood; 1 g. of albumin will hold 18 ml. of fluid in circulation. On this basis a standard dose of 25 g. of albumin, the considered equivalent of 500 ml. of citrated plasma, has been adopted by American workers. In traumatic shock doses of 25 g. of albumin may be given at 15 to 20 minute intervals. Blood should be given at the same time to correct anæmia, and saline if necessary for the dehydration. The place of albumin in hæmorrhagic shock is to tide the patient over while blood transfusion is arranged. Dramatic improvement has been obtained in patients with burns given albumin. Excessive doses of concentrated albumin may, however, cause pulmonary congestion and œdema, and it should not be given at or above a rate of 25 g./hour. Albumin has a low viscosity compared with plasma, and concentrated solutions are stable in aqueous media. A 25 per cent. w/v solution of albumin in 0·04 M acetyltryptophane may be heated for 10 hours at 60° C. thus destroying the causative agent of serum hepatitis. Fibrinogen and thrombin may be purified and concentrated, and these purified proteins, or materials manufactured from them, are used as hæmostatics. Fibrin clot, pressed into sheets and made plastic by heat or treatment with glycerol, is used in the repair of dural defects. Fibrin foam is made by whipping fibrinogen into a foam which is then converted to fibrin with thrombin, dried from the frozen state, and heated to 130° C. The sponge so formed is especially of value in neurosurgical operations; it is most useful in controlling capillary oozing and is efficacious in free venous bleeding, but less so in arterial bleeding. Mixtures of fibrinogen and thrombin have been used for sealing of small vessels in the cut surface of the lung and to reinforce pleural sutures over bronchial stumps. In skin grafting these mixtures are used as adhesives to hold the skin graft in place; vascularisation occurs in 3 days as compared with a month without the mixture, and there is less pigmentation than with pressure dressings. S. L. W.

Tuberculosis in Man, Chemotherapy of. N. D. D'Esopo. Report to the Council on Pharmacy and Chemistry. (*J. Amer. med. Ass.*, 1954, **154**, 52.) This report covers the 2-year period between January, 1951, and February, 1953; the number of persons reported on was 15,000, and the study was concerned almost exclusively with pulmonary tuberculosis. The use of streptomycin twice weekly and *p*-aminosalicylic acid daily is now considered the treatment of choice by most workers. The use of *p*-aminosalicylic acid alone is uncommon and undesirable. The use of dihydrostreptomycin has been largely discontinued, since experience has shown that it causes appreciably more deafness than the streptomycin. At present it is agreed that isoniazid should not be used alone in the treatment of pulmonary tuberculosis; the combined streptomycin and isoniazid regimen gives promise of being equally effective as streptomycin and *p*-aminosalicylic acid, but this has not yet been clearly established. Except for patients tolerating unusually high dosages, oxytetracycline would appear to be of low efficacy, but it is effective in delaying the emergence of resistance to streptomycin. Clinical trials of viomycin now in progress indicate that it may cause serious reactions and has only limited efficacy in pulmonary tuberculosis. Neomycin, mycomycin and erythromycin all appear to be ineffective.

(ABSTRACTS *continued on p.* 575).

BOOK REVIEWS

A TEXTBOOK OF FORENSIC PHARMACY. By Thomas Dewar. Third Edition. Pp. xvi + 287 (including Index). Edward Arnold (Publishers), Ltd., London. 1954. 18s.

The text and arrangement of this edition (November, 1953) follows almost exactly that of the second edition (1950) but embodies changes effected by at least 28 enactments made since 1950. This rate of forensic change is a measure of the importance of the new publication, and an illustration of the fact that *A Textbook of Forensic Pharmacy* deals exclusively with practical problems which are continually presenting themselves. It is essential, though often difficult, for practising pharmacists, teachers and students to keep accurate note of changes in regulations and conditions through the succession of acts, amendments and orders. Dr. Dewar's latest edition, therefore, comes as a most welcome landmark from which a fresh and accurate start can be made. Even so, the first marginal note to make is on page 1: that the Supplementary Charter stated sought by the Pharmaceutical Society was granted on December 31, 1953. Incidentally, the inclusion of more blank marginal space in any future editions would be most useful. Among the more noteworthy of the changes and innovations dealt with are those in the conditions governing the supply of medicinal opium, tincture of opium, and pethidine to certified midwives; then there is the inclusion of dimercaprol and aureomycin among the substances to which the Therapeutic Substances Act applies, the Regulations controlling chloramphenicol and isoniazid, and the charges payable by the patient under National Health Service Regulations for drugs or appliances. This fully documented and unique textbook is therefore an essential part of the reference library of every practising pharmacist and teacher; the author states that it is written primarily for students, yet the mass of necessary and intricate detail and the legal phraseology, however well arranged, may easily create a degree of confusion in the student mind unless that mind is tempered by practical pharmaceutical experience and comprehensive instruction.

E. W. SKYRME.

LEHRBUCH DER ORGANISCHEN CHEMIE, by Paul Karrer. Twelfth Edition. Pp. xix + 949 (including Index). George Thieme Verlag, Stuttgart, 1954. D.M. 59.70.

This very well-known textbook of organic chemistry, now in its twelfth edition, has been completely revised and extended to embrace many of the more recent advances of organic chemistry. The systematic treatment of the subject matter follows the familiar pattern of earlier editions, the main subdivisions being aliphatic, carbocyclic and heterocyclic chemistry; always with marked emphasis on substances of natural origin. As might be expected, the revision of an established textbook leaves much of the fundamental chemistry in its original presentation. Nevertheless, much new material has been included but, despite these additions, the book has been retained within a reasonable compass. It is fitting that this newer material finds its logical place in the systematic treatment of the subject; space has been given to a limited but adequate treatment of newer theoretical concepts, so that a nicely balanced presentation is achieved throughout. The account of modern ideas on the structure of the *cyclo*hexane ring is typical. The treatment is lucid, elementary, yet sufficient to enable those who may wish to pursue the subject further to do so without difficulty. The application of these ideas to the elucidation of the stereochemistry of the tropane

alkaloids provides an excellent example of the use of conformational analysis, though the absence of any mention of these studies in connection with the sterioids is indeed surprising. Notable additions include complete sections on fluorohydrocarbons, the tropolones, penicillin, the chemistry of acetylenic compounds and such newer synthetic reagents as lithium aluminium hydride. The treatment of reaction mechanism from the standpoint of fundamental electronic theory has been given much greater prominence throughout the book than was the case in earlier editions. There is no doubt that in this, as in many other ways, the utility of an already much valued textbook has been considerably enhanced.

J. B. STENLAKE.

METHODEN DER ORGANISCHEN CHEMIE (Houben-Weyl). Volume II. Analytische Methoden. Fourth Edition. Edited by Eugen Müller. Pp. xxiv + 1070 (including 252 illustrations) and Index. Georg Thieme Verlag, Stuttgart, 1953. D.M. 139.00.

The second volume of the new fourth edition of Houben-Weyl is devoted entirely to the application of analytical methods in organic chemistry. An extensive introductory section deals with methods of elementary analysis, both qualitative and quantitative. As in the rest of the book, the treatment is comprehensive, giving experimental details for all the more important analytical methods. Semi-micro, micro and macro methods of analysis are described and the section concludes with a short account of ultra-micro methods. By far the greater part of the book is devoted to a first class survey of analytical methods available for estimating organic functional groups. The treatment is systematic and includes all the more important types such as carbon-carbon, hydroxyl, carbon-oxygen functions and functional groups containing nitrogen and sulphur.

The remainder of the book which constitutes Part II consists of five sections, each devoted to some specialised aspect of organic analysis. General gasometric methods of analysis are described, including both chemical and physical methods, together with a number of more specialised techniques for particular classes of gaseous product. The study of melting and freezing points, boiling points and condensation temperatures forms the subject of yet another of these specialist sections. Thermal analysis and chromatographic analysis are each accorded an individual section of the book. The treatment is both theoretical and practical. The latter section is excellent and detailed, and provides a wealth of valuable information in all branches of chromatography. The concluding section is devoted to the analytical control of solvents and the analysis of solvent mixtures. Throughout the book the bibliography is extensive, seemingly complete and up to date. There are many excellent diagrams of both conventional and novel pieces of apparatus. This new volume would be a most valuable addition to any library.

J. B. STENLAKE.

(ABSTRACTS *continued from p.* 573).

At present there appears to be no place for the thiosemicarbazones in the treatment of pulmonary tuberculosis except possibly as a last resort in patients who have failed to respond to all other antituberculosis drugs. S. L. W.

War Gases, Physiological and Biochemical Effects of. H. Collumbine. (*Brit. med. Bull*, 1954, **10**, 18.) Whether gross tissue damage (as by the vesicants) or dysfunction (as by the lethal agents) is produced, it appears that some biochemical disturbances may be the fundamental "key" action of the war gases. Thus, the visible skin damage caused by mustard gas and the nitrogen mustards is accompanied by metabolic changes in the skin, for example, inhibition of glycolysis, which has been shown to be due to the inhibition of

(ABSTRACTS *continued on p.* 576.)

LETTER TO THE EDITOR

Alkaloids of *Duboisia leichhardtii*

SIR,—I have read with interest the paper[1] by Rosenblum and Taylor. It seems very likely that their "Base B" is in fact similar to the "Base D" that I described[2]. This was isolated in 0·06 per cent. yield from a specimen of the drug containing 4·1 per cent. of total alkaloids but differed from "Base B" in that it was optically inactive. Hence the authors' statement that I thought *d*-α-methylbutyryltropeine to be present is unfounded. I was able to prove that "Base D" consisted of tropine esters, but having less than 0·5 g. of the hydrobromide available, I did not succeed in identifying the acid produced on hydrolysis. It was evidently a mixture, and appeared to contain a valeric acid. By analogy with my earlier work on poroidine and *iso*poroidine[3] I suggested that *iso*valeric acid might be present, a suggestion that now seems to have been incorrect.

It is satisfactory that this problem now appears to have been solved, and I share the authors' interest in the isolation of a butyric ester alkaloid from a *Duboisia*, species of which have previously only yielded minor alkaloids that were esters of pentoic or pentenoic acids.

Stafford Allen & Sons Ltd., WM. MITCHELL.
London, N.1.
June 4, 1954.

REFERENCES

1. Rosenblum and Taylor, *J. Pharm. Pharmacol.*, 1954, **6**, 410.
2. Mitchell, *J. chem. Soc.*, 1944, 480.
3. Barger, Martin and Mitchell, *ibid.*, 1938, 1685.

(ABSTRACTS *continued from p.* 575.)

hexokinase. Hexokinase is an –SH enzyme and mustard gas attacks both the oxidised and the reduced forms. The mustard compounds can also produce other cellular changes. They can cause heritable mutations, can affect chromosome structure and can inhibit mitosis. The permeability of the local skin capillaries is altered within a few minutes, resulting in a loss of fluid and protein from the plasma so that local œdema occurs, the plasma volume and plasma protein content may fall and hæmoconcentration is produced. In addition serious damage is done to the hæmopoietic tissues, and these tissue changes are reflected by alterations in the cellular content of the blood. The whole metabolism of the bone marrow is depressed. Lewisite can also inhibit hexokinase, but there are differences in the gross pathology and the metabolic disturbances produced by lewisite and the mustard compounds, attributable to the presence of a tervalent arsenic atom in the lewisite molecule. Lewisite has a strong inhibitory action on the pyruvate oxidase system by combining with the SH groups in protein which are essential for the activity of this enzyme system. Dimercaprol, by forming stable ring compounds with lewisite, renders it relatively non-toxic and prevents it from inhibiting the pyruvate oxidase enzyme system. The lung irritants, such as phosgene, are, like the vesicants, able to produce profound shifts in body water merely by acting on the local exposed capillary vessels. The main toxic action of phosgene, however, is on the lung; the underlying mechanism of the effect on the lung capillaries is not known, but there is evidence that it may be enzymic in nature. The organophosphorus compounds produce their effects by inhibiting cholinesterase and so allowing acetylcholine to accumulate peripherally at cholinergic nerve-endings and possibly centrally in the brain and spinal cord, the body thus poisoning itself by its own production of acetylcholine.

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