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Chairman: A. D. POWELL

CHAIRMAN'S ADDRESS

Fifty Years of Pharmaceutical Progress

In choosing the subject of my address at this eight-seventh Conference I have been influenced by several considerations, not the least of which is that eighty-six addresses have been given previously, covering almost every aspect of pharmacy and its allied subjects, and that therefore my choice must to some extent impinge upon ground already covered by my predecessors in this chair. I was influenced also by the thought that this year of 1950 marks the completion of a half-century of progress in the sciences of chemistry, physics and medicine, and also in the fields of social welfare and legislation, which has without doubt affected pharmacy in its various branches to an extent greater than had been experienced in any like period in the history of our profession. My other reason was a personal one. Although I cannot claim a full fifty years of active association with pharmacy, the period I have chosen for review, which from a pharmaceutical standpoint may be dated by the British Pharmacopœias of 1898 and 1948, was in its earliest years when I first took up a test-tube in the pursuit of chemistry in its application to pharmaceutical analysis. For these reasons I have taken as the title of my address to you to-day "Fifty Years of Pharmaceutical Progress."

I shall of necessity include under this title developments in fields other than pharmacy. A new concept in the world of pure science, or a new line of approach in medical research, or a new application of scientific principles to engineering, may at the time of their birth seem far removed from the realm of pharmacy. They may, nevertheless, either singly or in combination, prove to have a considerable influence on the production or the very nature of the drugs which the pharmacist may in course of time be called upon to know about and handle in the exercise of his vocation.

I will illustrate this relationship with one example; there are numerous others which could provide a fruitful source of interest and instruction to anyone who takes the time and trouble to delve into their history. In the last pharmacopœia one of the new preparations is injection of aneurine

hydrochloride. The busy pharmacist who supplies an ampoule of this preparation and the equally busy doctor who administers the drug to his patient have little time to consider the processes which resulted in its existence. Yet this small ampoule embodies in miniature a characteristic story of the combination of creative ideas, patient research, scientific knowledge and technical skill resulting in a valuable addition to the list of medicaments.

In my example, the history begins with Sir Gowland Hopkins's inspired forecast of the existence of accessory food factors. Research in the field of human deficiency diseases confirmed the idea by the discovery of the presence of a curative substance in rice polishings and later by the isolation from this source of an impure active substance which in minute doses was effective against beri-beri. Organic chemical science solved the constitution of the vitamin and then elaborated its synthesis and the technical chemist was then able to produce the drug we now know as aneurine hydrochloride. The story does not however end here. The drug was now available to the medical profession, but its solutions were found to be unstable unless sufficiently acid. Acidity in excess being undesirable in a solution given by injection, it became necessary to control the acidity between limits ensuring stability on the one hand and tolerance by the patient on the other. It was equally necessary to control the dosage of drug in the ampoule. Progress in physical chemistry supplied the necessary knowledge for these controls. The modern conception of acidity in relation to hydrogen ion activity had resulted in the evolution of electrometric instruments which could measure the hydrogen ion potential with great accuracy and the now familiar method of *p*H adjustment supplied the answer to control of acidity. For dosage control, physical methods were also available, in this case by the newly developed methods of fluorimetry. Finally, the skill of the glass technologist was called into play in the provision of the container, the glass of which had to withstand long contact with the solution of the drug without yielding alkali which would upset its stability.

This interplay of new conceptions and discoveries in the various sciences, of which the above instance is an example, has been responsible for the evolution of classes of compounds of entirely different character from the older drugs, remarkable in general for specific action and very great potency. The vitamins and hormones are examples. In addition, developments in organic chemistry have resulted in an enormous output of synthetic compounds, many of which have been shown by pharmacological testing to be capable of use as curative agents in place of naturally occurring drugs.

The medical scientist has consequently had placed at his disposal new therapeutic agents of proved activity with which to conduct his own researches into the causes and treatment of human disease. The branch of medicine known as chemotherapy, to which I shall refer again later, and which has marked a significant change in medical practice, has

developed largely as a result of the availability of these new compounds. The extent of the changes resulting from this progress in chemical and medical science and from the newer aspects of medical treatment may be illustrated by some comparison of medicine and pharmacy as practised to-day with the conditions which applied at the beginning of the century.

The drugs and galenical preparations used by the pharmacist, in particular those included in the pharmacopœia, are an indication of current medical practice in the treatment of human ailments, and consequently of medical thought and outlook. A comparison of the official drugs and preparations of the pharmacopœias of 1898 and 1948 enables one to appreciate how far methods of treatment have changed from the alleviative and palliative treatment of symptoms to the direct attack on the causal agent responsible for the condition. In the earlier pharmacopœia drugs were mainly natural products of vegetable or mineral origin and there were only a dozen or so of organic compounds prepared by synthetic means. Substances of animal origin, with the exception of those included only for their physical virtues in the compounding of preparations, as for example fats, waxes, colouring and sweetening agents, accounted for still less a number. Cantharides, ox bile, cod liver oil (its vitamin content unknown), two enzymes, pepsin and pancreatin, and one hormone, thyroid, constituted the total in this class. Relics of former medical methods of treatment persisted in the presence of musk and leeches.

Galenical preparations were, on the whole, similar to those which are in common use to-day, although, with the exception of a small group which contained the more potent alkaloids and a few solutions of easily assayed inorganic drugs, they were not required to comply with any standard. Notable differences in the character of preparations were the large number of plaster masses, now entirely eliminated from the pharmacopœia, and an extensive list of pill masses which, with a few exceptions, have also lost their official status. Other changes in methods of administration of drugs are shown by the complete absence of compressed tablets—the only official tablet being the trinitrin tablet in the familiar chocolate base—and by the paucity of preparations intended for use by injection, of which four only were included under the title of hypodermic injections.

By way of contrast, the present pharmacopœia contains a host of drugs and preparations indicative of the new methods of treatment, including the acridine and other medicinal dyes, anti-malarial drugs such as mepacrine and pamaquin, a group of drugs derived from barbituric acid, numerous of the "sulpha" drugs derived from sulphanilamide, as well as biological preparations such as insulin, liver extracts, preparations of the pituitary gland, heparin, sera and vaccines, and the œstrogenic hormones. Most of these were quite unknown at the beginning of the century. The presence of seventy-five injections of these and other drugs is significant evidence of the changed trends in methods of administration.

I will now turn from this general aspect to a consideration of some

of the developments in different fields affecting pharmacy which have played their part in bringing about the changes I have indicated.

Developments in Chemistry

In considering this aspect of my subject, I will first deal with the growth of synthetic organic chemistry in its relation to the elaboration of large numbers of substances of therapeutic value, and continue with an account of the more recent development of the chemotherapeutic agents.

During the first twenty or thirty years of the century the principles of medical treatment maintained their traditional character. Drugs were usually administered for the purpose of alleviating the patient's condition. The prevailing attitude was summed up by Sir Henry Dale in a recent reminiscence. He said " One of the clear impressions left by my student days in hospital, at the beginning of the present century, is that of surprise and disappointment at the lack of conviction, and even of interest, which our teachers showed in medical treatment, in contrast to the care and enthusiasm which they devoted to diagnosis.... Treatment would so often be prescribed with no better hope than to make the patient easier by alleviation of his symptoms, leaving Nature to deal, if possible, with the cause of the trouble." He went on to say, in relation to the paucity of specific therapeutic agents then prevailing, " It is clearer to me in retrospect that it was at the time that there were very few remedies then which were even expected to deal with the cause of disease."

This being the general outlook of the times, it is not surprising that the ideas and energies of organic chemists were directed principally towards the evolution of new compounds which, by reason of their molecular structure in relation to the known constitution of naturally occurring active principles of vegetable drugs, might be expected to exert similar activity. A new branch of science, that of practical pharmacology, was called into service as a necessary complement to chemistry in order that the trial of the thousands of derivatives so prepared might reveal which had the desired action to the best degree, and whether such action was accompanied or not by toxic or other undesirable activity.

Although the main goal of this research was the discovery of new drugs exhibiting similar action to naturally occurring compounds of proved activity, and generally to improve upon Nature, the knowledge which accumulated of the effect of certain groupings in the molecular structure was of a great value when, at a later date, chemists turned their attention to the synthesis of drugs of specific activity against particular classes of organisms; and thus assisted in the advance of chemotherapy. This period in the evolution of pharmaceutical chemistry has been productive of many notable achievements, some arising from the elucidation of the constitution of complex natural substances, others from the successful building up by synthesis of the counterpart of the natural drug or of a new series of derivatives which have proved to have inherent medicinal virtues. Aspirin, one of our commonest and most widely used organic drugs, represents an outstanding example of a purely synthetic substance which

by reason of its effectiveness has become almost a household word. It may surprise some of you to hear that it was only introduced to medicine at the beginning of the century and did not become an official drug until 1914. Other achievements of this period were the synthesis of local anæsthetics such as orthocaine and procaine, the introduction of the barbiturate group of hypnotics, and the development of the acridine dyestuffs.

Effects of the First Great War

Synthetic chemistry continued to occupy the centre of the picture until well after the first Great War of 1914-1918. The war had profound influences in accelerating progress in all branches of chemical and medical science. One of its permanent effects was the establishment on a firm basis of the fine chemical industry in this country and in the United States of America, resulting in a great impetus to research and requiring the education of a large body of trained chemists for all its branches. Another was the extraordinary growth of the application of preventive treatment in medicine arising from the necessity of maintaining millions of armed men in a state of fighting fitness in spite of wounds and of living under conditions conducive to the rapid spread of disease. The end of the war also left millions of human beings in the blockaded countries in a state of serious ill-health as a result of continued malnutrition, and the study of their condition pointed the way to a fuller understanding of nutritional needs and to the essential importance of the vitamins in the treatment of deficiency diseases. The stage was now set for the great advance in chemotherapy which was to follow and which has run parallel with progress in synthetic organic chemistry from that time onwards.

Chemotherapy

I must now retrace my steps in order to give a brief review of this important aspect of the perpetual struggle between mankind and the diseases which afflict him.

The German chemist, Ehrlich, following on the discovery that malaria, sleeping sickness and certain other tropical diseases were due to parasites capable of transmission from one host to another by such agencies as the bite of an insect, began a search for chemical agents which would have a toxic action against these parasites without unduly affecting their host; he thus introduced a new conception into the treatment of disease to which he gave the title of chemotherapy and his ideal chemotherapeutic agent was defined as a substance which would possess a maximum affinity for the parasite and a minimal affinity for the organs of the affected subject.

His first attempts had been in the direction of finding an alternative to quinine as an anti-malarial agent. Methylene blue and various other dyestuffs were tried, but with no real success until much later, when others who followed Ehrlich produced the active drug suramin, which although

colourless, had been derived from a study of the dyes trypan red and afridol violet. Suramin found its main use as a trypanocide, and Ehrlich's successors continued the search for anti-malarial drugs to a successful conclusion when they produced plasmoquine (pamaquin) and atebrin (mepracrine). The later discovery of paludrine (proguanil) is a purely British contribution in this field of research.

Ehrlich, subsequently to his earlier investigations, turned his attention to the organic arsenicals as agents against spirochætal infections, and his discovery of salvarsan not only revolutionised the treatment of syphilis, but established chemotherapy as a new factor in medical treatment. Until 1935 its applications were confined to the treatment of diseases of protozoal origin. In that year Domagk announced the anti-bacterial properties of prontosil, a new drug obtained by the condensation of *m*-phenylenediamine with sulphanilamide. This discovery opened the field of chemotherapy to include the more familiar diseases of Western civilisation, many of which are due to bacterial infection. When it became recognised that the activity of prontosil was due to the sulphonamido- group in its molecule and that sulphanilamide was in itself an active antibacterial, development in this field was rapid. It has resulted in the synthesis and trial of many hundreds of derivatives of sulphanilamide, of which a few have proved to possess outstanding activity. Most of these compounds exert a preferential activity against particular organisms; some are more liable to produce toxic effects, or may have undesirable side-reactions; consequently these factors have to be taken into account when deciding on the drug of choice for the treatment of different conditions

Another group of anti-parasitic agents, the amidines, has been the object of much research during the last fifteen years. In one respect they have an interesting history. The first compound of this class, synthalin, was introduced into medicine for the oral treatment of diabetes, following on observations that a simpler substance, guanidine, caused a fall in the blood sugar content when administered to healthy animals. This led to an investigation of its possible value against the parasitic trypanosomes by interference with their metabolism. Although the effectiveness of the amidines as anti-parasitics was proved by these researches, it was not for the expected reason but rather because they were found to exert direct trypanocidal activity. Further developments in this field are still to be expected and as chemotherapeutic agents the amidines may become of great importance in the treatment of many tropical diseases, notably sleeping sickness.

The final chapter in the review of chemotherapeutic development is still in process of being written and I can only refer briefly to the discovery of the antibiotics. Once again the urgent necessities of a great war gave an impetus to scientific advancement, on this occasion culminating in the isolation of penicillin and its commercial manufacture in huge quantities within a remarkably short time. This is a recent story and has been told elsewhere. Discoveries of other antibiotic drugs have followed, and will

probably continue as a result of the intensive research which the opening of this new field has engendered, with incalculable possibilities in the progress of medical treatment.

Quality Control and Standardisation

The changes which have occurred in the variety and nature of drugs, arising from the advances in other fields which I have just summarised, have been accompanied by a great expansion in the control of their purity and strength which has extended to the whole of the substances and preparations used in pharmacy. As some classes of the newer preparations are not amenable to assay by chemical methods, this expansion has not been confined to pharmaceutical chemical analysis, but has embraced new methods of testing based on the sciences of bacteriology and pharmacology.

As I have said earlier, the requirements of the older pharmacopœias demanded little in the way of standardisation. The three revisions which have appeared during the last fifty years have each marked stages in the progressive control of quality of official drugs and preparations. Progress along similar lines has brought the non-official medicaments into a like position. The British Pharmaceutical Codex, having first come into being as a book of reference and information, published by direction of the Pharmaceutical Society for the benefit of prescribers and dispensers, has, in the course of successive revisions, extended its scope and has become a book of standards covering most non-pharmacopœial drugs and galenicals which are in common use. Its requirements have the effect of law in many parts of the British Commonwealth and, although not having expressed legal status in this country, its standards are generally given considerable weight in the pharmaceutical industry. The book has therefore become complementary to the pharmacopœia in the control of quality of the materia medica of to-day.

Its production represents a piece of pharmaceutical progress which our profession may regard with pride as one of their contributions to the general advance in pharmaceutical knowledge, as it has been fostered by our own Society and brought to its present state of importance as a result of the collaborative work of successive men and women, most of whom have had a pharmaceutical training

In addition to the main body of standards the Therapeutic Substances Act, by its regulations, imposes stringent standards for all preparations to which the Act applies.

The maintenance of the present-day standards has imposed a considerable responsibility on the pharmaceutical manufacturing industry. Whereas at the beginning of the century a small analytical laboratory with one or two chemists sufficed for the testing of the products of a wholesale drug house, routine quality control now requires the provision of extensive laboratories and skilled staffs whose function is to assay the products of the manufacturing departments, to prove the absence of undesirable impurities which might have a toxic or deleterious action, and

to ensure that all injectable preparations are sterile. To fulfil this function, workers trained in analytical and physical chemistry, practical pharmacology and bacteriology are needed in considerable numbers. New methods and techniques have become necessary and the analyst relies not only on chemical reactions but on spectrophotometry, fluorimetry, polarography and other electrometric techniques in order to deal with the problems of his profession.

I have so far drawn your attention mainly to the effects of scientific progress in various fields indirectly affecting pharmacy, and it is perhaps desirable, lest undue emphasis may have given a distorted picture, to attempt to put them in their proper perspective as seen against the practice of medicine and pharmacy as a whole. They constitute additions to, and not replacements, of older practices. The mass of knowledge which has accumulated gradually and progressively since the study of drugs and their action began to be recorded many centuries ago is still in process of accretion, and for most complaints of non-specific origin treatment of symptoms remains the common practice and will probably continue to be for some time to come. Old beliefs in the value of individual drugs may die for lack of supporting evidence. Drugs may disappear from the pharmacopœias as they become outmoded and their places are taken by others of more certain action, but the well-established and proved remedies remain as the principal media of medical treatment. A famous physician—I believe it was Lord Horder—put the matter in the right light in a pithily worded warning that one should not dismiss the accumulated experience of three thousand years on the evidence of the latest galvanometer reading.

There is therefore no likelihood that the practice of pharmacy will undergo any revolutionary change in character as a result of the changes I have mentioned; on the contrary, they may in some degree allow him to exercise his skill and training in new directions.

Legislation and Pharmacy

The factors which have most directly affected pharmacy and the pharmacist during the last fifty years have resulted from the mass of new legislation, concerned either with social welfare or with the direct control of pharmacy and the sale of medicines.

At the beginning of the century the practice of pharmacy was governed by the Pharmacy Act of 1868, with certain amendments, although some of the provisions of the original Act of 1852 were still in force.

The chemist and druggist of those days—the title "pharmacist" at that time was reserved to those possessing the Society's higher qualification—was at liberty, if he so wished, to keep his premises open until late hours and expected equally long hours of service from his assistants. He was not required to keep a special guard on those habit-forming compounds which have since been classified as Dangerous Drugs, although he had to be careful to whom he sold arsenic and a few other poisons. Neither was he forbidden to recommend and sell remedies purporting to

cure venereal diseases or certain other complaints which have since been the subject of restrictive enactments.

In order to qualify for his title, he had to undergo an apprenticeship which gave him a good grounding in the practice and art of dispensing and also to fit himself by study of the sciences related to pharmacy. The examination he had to pass at the end of this training was a severe test of his practical skill and care in the exercise of his vocation, but did not demand anything like the theoretical knowledge of the sciences of chemistry, physics, botany, and physiology—either pure or applied—which is required nowadays.

The relationship between the individual qualified chemist and druggist and his chartered body was a loose one. If he had the wider interests of his profession at heart he would apply for membership of the Pharmaceutical Society and might continue his studies and sit for the "Major" diploma. On the other hand, once qualified and registered as a chemist and druggist, the individual owed nothing but a moral loyalty to the Society and was not required to become a member of it. Subject to his duties as an ordinary citizen, he was free to carry on his business much as he pleased. If, as is possible in all professions, he turned out to be a "black sheep" there was no professional statutory body empowered to investigate his conduct and either call him to order or expel him from the profession.

It is a tribute to pharmacists as a class and to the traditions and training of pharmacy at that time, that, in spite of this lack of professional control, pharmacy maintained a general standard deserving of the high respect which it received from the public which it served.

Beginning with the Poisons and Pharmacy Act of 1908, which incidentally gave the right to the chemist and druggist to use the title of pharmacist, new legislation affecting all branches of pharmacy was to come with great regularity. An outstanding landmark was the passing of the first National Health Insurance Act of 1911, which, with its successor of 1936 and the recent National Health Service Act, has considerably altered the relationship between the pharmacist and the community. The Shops Act of 1912, although not solely concerned with pharmacy, regulated the hours of work in the retail pharmacy and the duties of the pharmacist to his employees in this respect.

The Venereal Diseases Act of 1917 prevented the recommendation and sale to the public of remedies for these diseases; later legislation extended the prohibition to other conditions for which unqualified treatment is undesirable. The vice of drug addiction which began to assume serious proportions after the 1914-1918 war was responsible for the control of a class of habit-forming drugs which came to be known as Dangerous Drugs after the passing of the first Dangerous Drugs Act of 1920. The growth of chemotherapy introduced into pharmacy the class of potent drugs which came under stringent control under the provisions of the Therapeutic Substances Act of 1925. The Pharmacy and Poisons Act

of 1933 directly affected the pharmacist and the practice of pharmacy. This Act has made every pharmacist a member of his professional Society, liable to the payment of an annual retention fee, and answerable to the Statutory Committee of the Society with respect to his professional conduct. Briefly, this Act has replaced the loose relationship which obtained between the individual and the chartered body at the beginning of the century by a constitution of pharmacy on the lines of the other closed professions.

Pharmacy in the Future

The question remains: Where does pharmacy now stand in relation to this changed state in the surrounding fields of science? In the eyes of the community the conception of the pharmacist as a trained exponent of the science and art of the compounding of medicines, and as a confidential guide, still remains. The esteem in which he is held will, in the future, depend in a large degree on the maintenance of this reputation. There is no finality in the advance of scientific knowledge. Research will continue to produce results—new drugs, improved preparation of galenicals, new methods of treatment, and new methods of analysis and control. Pharmacy cannot stand still in a world of change. The chief danger which confronts it is that of failure to make progress on parallel lines to advancements in these other branches.

Pharmaceutical education and training, while giving due emphasis to the importance of the main function of pharmacy, and providing some knowledge of those parts of chemistry and other sciences which lie on its borderland, should also be directed towards a widening of general knowledge and culture. This cannot be gained from a mere storage of facts, but only by study of the history, development, and principles of these sciences. The pharmacist of the future will need this wider outlook if pharmacy is to hold its own. Though some forms of research are the province of the chemist, physicist, or biologist, there remain other fields in which chemists trained in the pharmaceutical applications of these sciences can find ample scope for utilising their particular skill and knowledge. This Conference has, from its inception, provided the arena in which such matters may be ventilated and discussed. Its continued value to the community will depend upon the willingness and zeal of the present and future generations of pharmacists and others engaged in pharmaceutical pursuits, to devote themselves to this branch of investigation and research. The proceedings of the science sessions of previous Conferences provide a long record of the endeavours of such men in the past and represent a tradition which should be prized by their successors in the future. It is a tradition worthy of upholding and deserving of the best efforts our profession can make to maintain and strengthen.

THE PREPARATION OF COMPRESSED TABLETS

PART III.—A STUDY OF THE VALUE OF POTATO STARCH AND ALGINIC ACID AS DISINTEGRATING AGENTS

BY H. BERRY AND C. W. RIDOUT

From the Pharmaceutics Research Laboratory, School of Pharmacy, University of London

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WHEN a drug intended for internal administration is, for convenience, presented as a compressed tablet, it should be axiomatic that the process of tableting should not alter either the therapeutic action of the drug or the time in which this action is produced. In other words the same effect should be produced in the same time when a tablet is swallowed as when the drug is taken in powder or solution form. Any deviation from these criteria is a measure of the pharmaceutical skill used in formulating and producing the tablets. The disintegration time of tablets intended to be swallowed whole is therefore of great importance, and the British Pharmacopœia 1948 has specified a test in which this time should not exceed 15 minutes except in the cases of tablets of barbitone and phenobarbitone, when the time is extended to 30 minutes.

The disintegration time is a function of (a) the formulation, (b) the degree of compression, (c) the speed of compression and (d) the type of coating used, if any.

The degree of compression is important, particularly to the manufacturer, for a tablet must be produced which will be hard enough to withstand abrasion during handling, transport and storage. Often, however, increase in the degree of compression causes an increase in the disintegration time, and a compromise has to be adopted. This compromise whereby the hardness is balanced against an optimum time of disintegration is referred to, in this paper, as the "*optimum compression.*"

This investigation is concerned with (a) ascertaining the effects of variation in degree of compression on disintegration time, the other factors being constant, i.e., formulation, speed of compression and absence of coating; (b) studying the relative values of potato starch and alginic acid as disintegrating agents when the other factors are constant, i.e., compression ratio, speed of compression and absence of coating.

It was hoped that, as a result of these investigations, it would be possible to recommend that the disintegration time for all uncoated tablets intended to be swallowed whole should be 15 minutes.

In previous papers[1,2] in this series it has been shown that 5-gr. phenacetin tablets were one of the types of compressed tablets that may

give extended and erratic disintegration times, and it was decided to use these tablets as a basis for study.

Experimental

The machine used for the compression of the granules was a single punch, hand operated, Manesty machine. This machine is fitted with an oscillating hopper which caused some segregation of granule size, and in order to avoid this the hopper was removed and the granules were hand-fed into the die. By using this method it was found that variations in weights of the tablets were decreased, as also were the variations in the disintegration times of any single batch of tablets.

Times of disintegration were measured by the method of the British Pharmacopœia, but in order to give more accurate values 12 tablets were used and a mean of these values taken. In order to standardise the experiments as much as possible a very large batch of granules was made by hand and stored in an air-tight container. From this batch, granules were taken as required, lubricant and disintegrating agent added and tablets compressed. Batches of approximately 40 to 50 tablets were prepared for each compression, and each batch was investigated by weighing and measuring 20 tablets selected at random from each batch.

Since the tablets were all prepared from the same batch of granules, it was decided that the degree of compression could conveniently be represented by a "compression ratio." $\frac{\text{Weighting.}}{\text{"l" cm.}}$ where "l" is the thickness along the axis of rotation (Fig. 1).

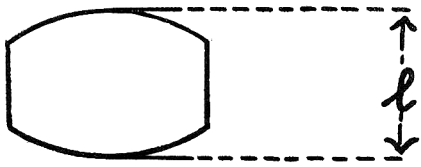

Fig. 1. "Compression ratio"—measurement of "l."

It is realised that the value of this expression "compression ratio" will vary with the curvature of the punches—using a die of constant size—and it is, consequently, a function of the particular punch used and it is not intended that this ratio should be used generally as a means of standardisation of compression. The moistening agent employed in preparing the granules was a 50 per cent. w/w solution of sucrose in water. The lubricant was 1 per cent. w/w of stearic acid.

The Investigation of the Effects of Variation in Degree of Compression on Disintegration Time

Table I shows the results of the average "compression ratio" measurements and disintegration times for 4 batches of tablets containing 15 per cent. of potato starch as a disintegrating agent, and it can be seen that there is a large increase in disintegration times on either side of the value 0·69. In order to determine whether this rise was accidental or

TABLE I

RESULTS OF DISINTEGRATION TESTS AND "COMPRESSION RATIOS" FOR THE FIRST BATCH OF TABLETS USING 15 PER CENT. OF POTATO STARCH

Average "compression ratio"	0·57	0·65	0·69	0·71
Average disintegration time	14′ 45″	6′ 5″	4′ 0″	7′ 10″

′=minutes. ″=seconds.

TABLE II

RESULTS OF DISINTEGRATION TESTS AND "COMPRESSION RATIOS" FOR THE SECOND BATCH OF TABLETS USING 15 PER CENT. OF POTATO STARCH

Average "compression ratio" ...	0·63	0·64	0·65	0·70	0·72	0·75
Average disintegration time ...	7′ 55″	8′ 20″	7′ 20″	2′ 10″	19′ 5″	25′ 10″

′=minutes. ″=seconds.

whether any significance could be attached to it, further batches of tablets were compressed, care being taken to produce compressions on either side of this value of 0·69. The results from this test are recorded in Table II. These results, combined with the previous results, give the graph shown in Figure 2.

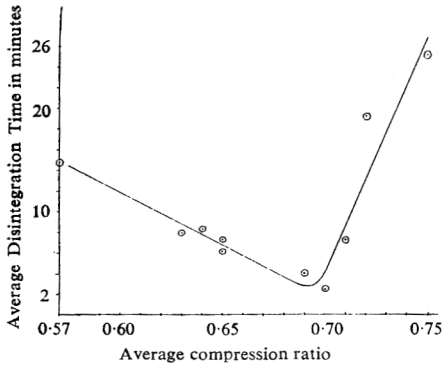

FIG. 2. Relation between Disintegration Time and "Compression Ratio" for Phenacetin Tablets using 15 per cent. of Potato Starch.

This graph shows that, using 15 per cent. of potato starch as a disintegrating agent, there is a critical compression which will give a minimum time of disintegration. A study of the manner in which the tablets disintegrate may lead to an understanding of the reasons for this critical compression. It was found that the tablets disintegrated from the outside but in a somewhat erratic manner. Fairly large pieces of the tablet would break off and these pieces, in turn, would then disintegrate. This would continue until all the tablet had broken up into small pieces. It is generally assumed that the disintegration of the tablets relies upon the swelling of the starch grains. Under a very light compression the grains can swell, but owing to large intergranular spaces they can do so to a considerable extent before they begin to exert pressure on the surrounding granules. Consequently disintegration time is long. At the critical compression, as soon as the grains swell they exert pressure on the surrounding granules and the tablets disintegrate rapidly. With a heavy compression, time is required for the water to seep through the outer layers of the tablets before the starch grains can start to swell and commence to disintegrate the tablet.

Actually, the critical pressure has very little importance in practice, since the tablet is far too friable and the "optimum compression" lies somewhere to the right of the dip in the curve.

Alginic Acid as a Disintegrating Agent

Alginic acid is a polymerised mannuronic acid extracted from certain types of seaweed. It is insoluble in water, but has the property of swelling with water. The acid used in the following experiments was a commercial sample supplied by Albright and Wilson, Ltd., of edible grade HS/LD and had the following characteristics:—

Viscosity. A 1 per cent. dry weight solution of the sodium salt had a viscosity of 6·3 c.s. at 20°C.

Mesh size. 100.

Moisture content. 14 to 16 per cent.

An attempt was made to incorporate the alginic acid into the bulk granules prepared previously, but this was not found to be successful since the acid was a fine free-flowing powder, had no "clinging" properties and, on placing in a hopper, separation of the powder and granules resulted. Fortunately, however, alginic acid can be treated with water or an aqueous solution, dried and still retain its full swelling properties. Consequently, it was decided to mix the phenacetin with the alginic acid and then proceed to granulate so that the alginic acid was incorporated within the granules. The same type of moistening agent was used as before, and batches of tablets were made incorporating 3, 6 and 10 per cent. of alginic acid. Tables III, IV and V give the disintegrating times for tablets of various "compression ratios" containing the above percentages of alginic acid.

TABLE III

Results of disintegration tests and "compression ratios" for tablets using 3 per cent. of alginic acid

Average "compression ratio"	0·56	0·61	0·68
Average disintegration time	2′ 20″	4′ 35″	25′ 25″

′=minutes. ″=seconds.

TABLE IV

Results of disintegration tests and "compression ratios" for tablets using 6 per cent. of alginic acid

Average "compression ratio"	0·56	0·58	0·62	0·65	0·75
Average disintegration time	1′ 20″	3′ 10″	1′ 0″	4′ 5″	45′ 15″

′=minutes. ″=seconds.

Figure 3 shows the graph of "compression ratio" plotted against disintegration times for these results.

A comparison of Figures 2 and 3 shows that the mechanism whereby alginic acid, added before granulation, acts as a disintegrating agent is

TABLE V

RESULTS OF DISINTEGRATION TESTS AND "COMPRESSION RATIOS" FOR TABLETS USING 10 PER CENT. OF ALGINIC ACID

Batch A.						
Average "compression ratio"	0·57	0·58	0·60	0·64	0·71
Average disintegration time	0′ 50″	1′ 15″	1′ 0″	2′ 15″	15′ 30″
Batch B.						
Average "compression ratio"	0·56	0·57	0·60	—	—
Average disintegration time	0′ 30″	0′ 35″	0′ 30″	—	—
Batch C.						
Average "compression ratio"	0·57	0·60	0·64	0·71	—
Average disintegration time	0′ 45″	1′ 0″	1′ 45″	14′ 15″	—

′=minutes. ″=seconds.

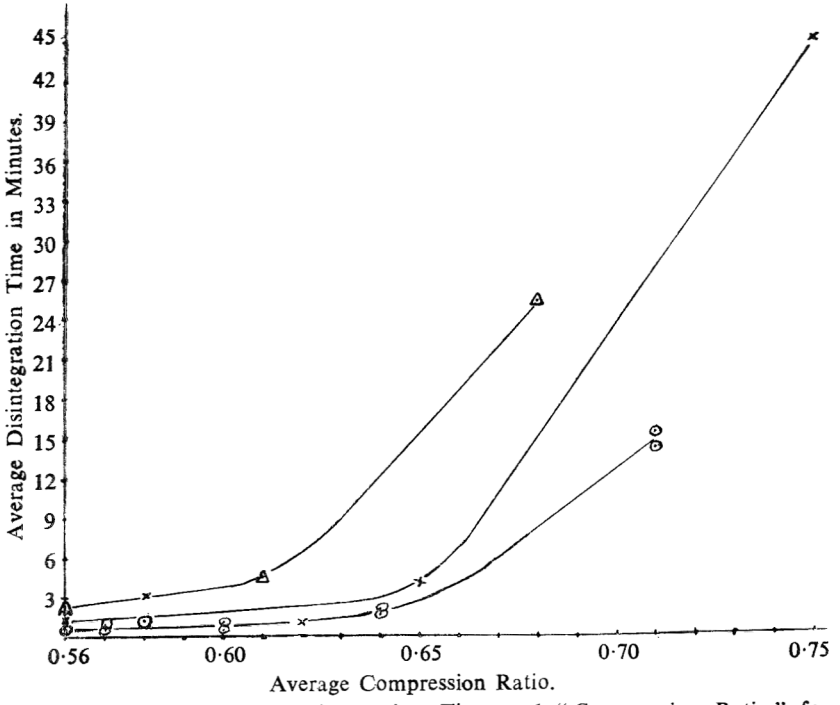

FIG. 3. Relation between Disintegration Time and "Compression Ratio" for Phenacetin Tablets using 3 per cent. (upper curve), 6 per cent. (middle curve), and 10 per cent. (lower curve) of Alginic Acid.

different from starch added after granulation. Once again, observation of the tablet during disintegration gives a clue as to the method involved. As before, disintegration begins from the outside of the tablet, but instead of large pieces breaking off, small fragments, smaller than the original granules, fall away and this continues until the whole tablet

has broken up. This appears to be due to the fact that the alginic acid is now an integral part of each granule, and as it swells, when immersed in water, it breaks up the original granules. Also, it is possible that the wet granules, when made by means of an aqueous solution contain enlarged alginic acid particles. On drying, these grains of alginic acid would decrease in size, leaving air spaces in the granules. Although it is likely that compression would decrease the size of these intragranular air spaces, yet their existence must present a narrow capillary through which water may be drawn into each granule and so aid the rupture of the granule and of the tablet itself.

Comparison of Potato Starch and Alginic Acid as Disintegrating Agents

Experiments were carried out on various B.P. tablets to compare the values of potato starch and alginic acid as disintegrating agents. As far as possible the same compression was used for each batch of tablets, irrespective of the disintegrating agent. The results of the disintegration tests are shown in Table VI.

TABLE VI

Results of disintegration tests on various Tablets using potato starch or alginic acid

		Disintegrating agent	Average "compression ratio"	Average disintegration time
Barbitone	gr. 5...	Potato starch 10 per cent.	0·78	greater than 60′ 0″
		Potato starch 15 ,, ...	0·78	5′ 0″
		Alginic acid 10 ,, ...	0·76	5′ 10″
Digitalis	gr. 1...	Potato starch 10 ,, ...	0·30	16′ 10″
		Alginic acid 10 ,, ...	0·28	9′ 20″
Phenobarbitone	gr. 1...	Potato starch 10 ,, ...	0·28	greater than 75′ 0″
		Potato starch 15 ,, ...	0·28	about 75′ 0″
		Alginic acid 10 ,, ...	0·27	8′ 0″
Thyroid	gr. 1...	Potato starch 10 ,, ...	0·27	6′ 45″
		Alginic acid 10 ,, ...	0·27	4′ 30″

′=minutes. ″=seconds.

Time did not permit hardness tests such as recommended by Smith[3,4] to be performed on these tablets, but it appears that more force is required to break, between the fingers, a tablet using alginic acid as a disintegrating agent than it does to break a similar tablet, having the same "compression ratio," but using starch as a disintegrating agent.

Ageing of Tablets

The batches of tablets shown in Table VI were subjected to disintegration tests after a period of 3 months. The results are shown in Table VII. No change was observed in the "compression ratio" for any of the tablets.

TABLE VII

RESULTS OF DISINTEGRATION TESTS ON VARIOUS TABLETS AFTER 3 MONTHS' STORAGE

	Disintegrating agent	Average disintegration time	
		At time of preparation	After 3 months
Barbitone gr. 5	Alginic acid	5′ 10″	7′ 25″
	Potato starch	5′ 0″	6′ 45″
Digitalis gr. 1	Alginic acid	9′ 20″	11′ 40″
	Potato starch	16′ 10″	16′ 25″
Phenobarbitone gr. 1	Alginic acid	8′ 0″	10′ 20″
	Potato starch	about 75′ 0″	—
Thyroid gr. 1	Alginic acid	4′ 30″	4′ 30″
	Potato starch	6′ 45″	10′ 35″

′ = minutes. ″ = seconds.

The results of the few experiments so far carried out and the reports of other workers[5] indicate that the storage of tablets causes an increase in their disintegration times, and controlled experiments have been set up to determine the cause of this effect over long periods of time.

CONCLUSIONS

(1) In the experiments so far carried out 10 per cent. of alginic acid gave a more rapid disintegration than the corresponding percentage of potato starch.

(2) In the manufacture of phenobarbitone tablets, 10 per cent. of alginic acid gave a much better disintegration time than 15 per cent. of potato starch, and in the case of barbitone tablets the time of disintegration was approximately the same.

(3) From the results obtained it seems possible to recommend that the maximum disintegration time for all uncoated tablets which are to be swallowed whole, and which are in the British Pharmacopœia, be reduced to 15 minutes.

(4) The fact that alginic acid can be granulated with the medicaments in a tablet has the following advantages:—

(a) The addition of a very fine powder, such as starch to the granules before tableting, means that there is a considerable risk of separation of powder and granules during the transfer of the bulk material to the tablet machine and also, whilst the material is in the hopper, due to the vibration of the hopper or of the machine itself. This separation of fine powder will cause variation in weight of the tablets and also variation in the amount of active ingredient in each tablet. There is also the difficulty—by no means inconsiderable—of ensuring an even distribution of a large quantity of starch in a bulk of granules.

(b) Since the alginic acid in these experiments is an integral part of the granules, when the tablet breaks up it will do so to give material which is smaller than the original granule. The active constituents thus

being presented in a finer form will be more quickly absorbed and give a more rapid therapeutic action.

(c) The process of tableting will be simplified.

(5) In tablets prepared of barbitone, digitalis and phenobarbitone, using alginic acid as a disintegrating agent, some increase in disintegration time was noticed after 3 months' storage, but even so, all the tablets disintegrated within 15 minutes.

(6) Thyroid tablets showed no increase in disintegration time after storage for 3 months when alginic acid was used as a disintegrating agent, but showed a considerable increase in disintegration time when potato starch was used.

SUMMARY

(1) An investigation has been carried out to ascertain the effects of varying degrees of compression upon phenacetin tablets using either potato starch or alginic acid as disintegrating agents.

(2) The value of potato starch and alginic acid as disintegrating agents have been compared at the same compression, and alginic acid has been shown to have an advantage in that it can be incorporated within the granules, thus giving finer disintegration.

(3) There is a tendency for disintegration time to increase with storage and further investigations of this phenomenon are necessary.

(4) It is suggested that the maximum disintegration time for all uncoated tablets which are in the British Pharmacopœia and which are to be swallowed whole, be reduced to 15 minutes.

We wish to thank Albright and Wilson, Ltd., for supplying the alginic acid.

REFERENCES

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2. Berry and Smith, *ibid.*, 1944, **17,** 248.
3. Smith, *Pharm. J.*, 1949, **163,** 477.
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DISCUSSION

An abstract of the paper was read by Mr. Ridout.

THE CHAIRMAN (Mr. A. D. Powell) said that the subject of the paper had for some years assumed growing importance, with the control and standardisation of tablets in the British Pharmacopœia.

MR. N. ALLPORT (London) thought the idea of using alginic acid ought to appeal to everyone concerned with tablet manufacture. Did the mixture containing alginic acid run easily in the machines on a manufacturing scale, especially in rotary machines? Generally, hand machines gave results which could not be obtained on a manufacturing scale. Was it an economic proposition to use 10 per cent. of alginic acid instead of 15 per cent. of potato starch?

MR. A. NUTTER SMITH (Nottingham) said that one of the main difficulties with awkward drugs, such as phenobarbitone and phenacetin, was that these substances in a supposedly No. 60 powder contained a good deal which would not go through a No. 40 sieve. For these two substances it was necessary to have a minimum of an 80-powder or, preferably, a 100-powder to get a good product. There was a better chance of working moisture into these finer powders; coarse powders became sticky before sufficient moisture had been absorbed. For moistening he preferred a 10 per cent. solution of soluble starch to cane sugar (50 per cent.) or acacia (5 or 10 per cent.). He found it confusing that the authors had not stated the sieves used in the granulation. This was important as, with phenacetin, it was possible to get the results the authors had obtained by bad technique. He would use a No. 16 or 20 sieve. In the case of phenacetin tablets, he could not agree with the authors' conclusions. One reason was that there were many ways of incorporating starch as a disintegrant. The authors' experiments were inadequate. On the Continent, starch was often used in conjunction with pectin or agar, and he wished that the authors had tried alginic acid with starch. He asked what fillers were used to keep the compression ratio constant when 3 per cent. and 6 per cent. alginic acid were used. He imagined that the alginic acid was much lighter than starch. The disintegration time of 75 minutes for phenobarbitone tablets was surprising; a freshly-made tablet should not take that time to disintegrate if it were properly made. Three months was not a long enough period for the keeping tests; it was necessary to keep tablets over a period of years. He thought it would be wrong to alter the B.P. as a result of laboratory experiments, the results of which had not been proved on a manufacturing scale.

MR. A. W. BULL (Nottingham) referred to the authors' statement that the disintegration rate was affected by the speed of compression, and to their claim to have kept the latter constant. He suggested that this was very difficult to do with a hand-operated machine, and thought that a mechanically-operated one should have been used. Results were reported as average disintegration times; he would like to know what variation from the mean occurred within each batch of 40 to 50 tablets. How far was this factor responsible for the apparently large differences shown in Tables I and II in the disintegration rates for tablets of similar compression ratios? Table VII showed that the rate of disintegration of certain tablets made with alginic acid had increased more rapidly than when starch was used. Had the authors carried on their observations for more than three months, and was this increase significant? Was there any variation of disintegration rate due to humidity and storage?

MR. D. SMITH (Bexleyheath) asked whether the degree of whiteness was affected by the presence of alginic acid. He believed it caused a certain " off-whiteness " which might be a deterrent to its use if this were so in all cases.

DR. E. F. HERSANT (Dagenham) said that the physical form of the ingredients had a considerable influence on disintegration time. The authors had combined Tables I and II to produce one graph, but if two graphs were drawn they did not coincide. He thought it was always difficult to say exactly when a tablet had disintegrated. If the results were going to be quoted in seconds, it must be borne in mind that the B.P. did not prescribe any particular mesh size to take as a criterion of complete disintegration. The result might be very misleading if different authors took different mesh sizes as criteria of disintegration.

MR. R. W. GILLHAM (Leeds) asked whether they had standardised the moisture content of the starch before using it in the tablets as this would affect the disintegrating properties of the starch. Had the authors used maize starch, and did it cause any difference in the rates of disintegration? Could they give any information on the keeping properties of phenacetin tablets? One sample that he had recently handled had a disintegration time well beyond the B.P. limit after storage, although when originally purchased they disintegrated within 15 minutes. Some observations of the effect of alginic acid on the keeping properties of phenacetin tablets would be very interesting.

DR. F. HARTLEY (London) pointed out that, therapeutically, tablets might not necessarily be the most effective form of administering a substance orally. With fairly rapidly metabolised materials the therapeutic effect might depend on the intensity of pharmacological action. It was not certain that the same effect would be produced in the same time when a tablet was swallowed as when the drug was taken in solution form. The tablet might disintegrate in a reasonable time, say, 5 to 15 minutes, but other physicochemical factors, such as the rate of solution of the substance, were involved. For example, a substance soluble to the extent of 1 per cent. and metabolised on reaching the bloodstream in an hour or an hour and a half might well be best administered as a solution in a suitable solvent which would form a colloidal solution on mixing with the gastric juice. This might enable more rapid absorption into the blood stream. If the substance dissolved only slowly, and was metabolised quickly, no matter how quickly the tablet disintegrated, the concentration in the blood would never be sufficient to produce the maximum therapeutic effect. Conversely, it might not matter whether the tablet disintegrated in 15 or 30 minutes, so long as the substance was not metabolised in less than, say, one to one and a half hours.

MR. W. R. HOWARD (London) asked if the authors had considered the possible effects of their lubricants on the disintegration results. His experience had been that the lubricant, particularly if it were of a fatty nature, was apt to waterproof the tablet. Had that effect come into play to produce the increased disintegration times, which they had reported as compression was increased? Might it not have been better to try compressing in the absence of any lubricant, perhaps making use of some device such as a taper die to ease ejection and to prevent shatter-

ing of the specimen which, of course, would vitiate any disintegration determination carried out subsequently? Alginic acid was a most useful admixture in granulations already containing starch. Only small proportions were necessary.

MR. C. W. RIDOUT, in reply, admitted that they had not used a rotary punch machine, but he could see no reason why the process should not work just as well as on a single punch hand-operated machine. When the alginic acid was used internally there was less dust than when starch was used externally as a disintegrant, especially if 10 to 15 per cent. of the latter was used. Even if some starch was added before and some after granulation, there was a fair amount of fine powder which could separate. With alginic acid this did not occur, and the only fine powder to separate was the lubricant. A No. 22 sieve had been used and it was possible to feed the die from the hopper, but they had obtained more consistent results by hand-feeding. They had not used a mixture of alginic acid and starch as the paper set out to give a comparison of the two used separately. The disintegration time for phenobarbitone tablets was surprising, but the compressions used were not comparable with those used for a manufacturer's tablets. In all cases the tablets were much harder than normal as their main concern was to keep the compression ratio constant for all the tablets made.

He agreed that three months was insufficient for the keeping tests. The only reason for using three months was that the paper had to be ready by a certain date. Figures for 6 months' storage were now available, and in all cases showed some slight increase in disintegration time which was generally less than the increase after 3 months. It seemed to be falling off to a constant level. Hand-feeding the granules into the hopper avoided one difficulty about the speed of compression. Great care was taken to ensure that the handle was turned at a constant rate. The variation from the mean of the average disintegration times (Tables I and II) had been measured and the results submitted to the Student's "t" test, and the probability that the widely varying figures all came from the same group was very small.

Ten per cent. of alginic acid caused some discoloration, but it was doubtful if this could easily be noticed, unless the tablets were compared with others prepared with starch. Maize starch had been used, but not very successfully. Data gathered on the keeping properties of phenacetin tablets had unfortunately been lost. The effect of the lubricant had not been investigated, and throughout the work they had used 1 per cent. of stearic acid. The compression had never been hard enough to form a solid coating of lubricant, and prevent the water from seeping in; in these experiments he did not think that the lubricant had any effect on the disintegration times. If used in large proportions certain lubricants would have such an effect.

THE DISINTEGRATION OF COMPRESSED TABLETS

The Effect of Age and Certain Associated Factors

By H. Burlinson and C. Pickering

From the Laboratories of Thomas Kerfoot and Co., Ltd.

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It is the purpose of this paper to examine some of the factors which influence the disintegration of a tablet, and then assess the possible effect of ageing upon the disintegration time of tablets after storage. In the past, reference has been made to the fact that tablets may show variation in their time of disintegration when stored over prolonged periods[1,2,3]. This has been summarised by Berry and Nutter Smith[2] who stated that ". . . Some tablets tend to harden on keeping, though this is by no means universal . . . . It is necessary, of course, to know the disintegration times when the tablets were first made before definite conclusions can be drawn as to the progressive effect of age."

The breakdown in water of a tablet consisting entirely of soluble substances is one of solution rather than disintegration. An examination was therefore made of tablets consisting of drugs which are either insoluble or slowly soluble in water. Disintegration is usually brought about by the use of one of a variety of starches, and all experimental tablets contained starch as a disintegrant.

Preliminary experiments showed that a study must be made of other factors which may have an adverse effect upon the initial disintegration of a tablet, and therefore obscure the effect due to age. The following factors are those primarily concerned in the initial rate at which a tablet disintegrates:—

(1) The variety of starch used.
(2) The amount of starch contained in the tablet.
(3) The method of granulation.
(4) The nature of the moistening agents.
(5) The pressure used during tableting.

As a result of this work, a formula was devised for use in preparing a series of tablets which, when freshly made, would disintegrate rapidly. Tablets thus produced were kept over a period of four years, and determinations made of the rate of disintegration after certain intervals of time.

Before discussing the experiments it is important to define the nature of the tests used in the physical examination of the various batches of tablets.

Experimental

Disintegration Test. The British Pharmacopœial apparatus was used and the time taken when complete disintegration was observed in all 5 tubes. These times were recorded to the nearest half minute.

Friability Value. Hardness in tablets is a term which is difficult to define. A number of tests have been suggested from time to time, but the true test is that the tablet should reach the consumer in as good a condition as when it was made. It must, therefore, be hard enough to be handled in bulk quantities without "rubbing" and to withstand the hazards of packaging and transit tests. It is desirable to have some mechanical means of measuring this resistance to wear, other than the time-honoured method of the snap produced when a tablet is broken between fingers and thumb.

If a weighed number of tablets are vigorously agitated by controlled mechanical means for a fixed period of time, the percentage of powder produced by this treatment indicates the resistance to wear of the tablet and may be called the Friability Value.

In practice, 20 tablets were weighed into a two ounce, screw-capped, wide mouthed, round bottle, and placed in a laboratory shaking machine, similar to the one which has been fully described and illustrated in a recent paper by Nutter Smith[4]. After shaking for 5 minutes, the powder produced was separated from the tablets by means of a sieve (16 mesh. 26 S.W.G.). The rubbed tablets were weighed and the difference, expressed as a percentage of the original weight, was called the Friability Value. Findings are given to the nearest whole number.

It is important that there should be no tendency towards "capping" in the batch, otherwise an unduly high value will be obtained.

Moisture Content. The moisture contents of the tablets were determined by drying to constant weight at 100°C., except in the cases of aspirin, cinchophen, sulphonal, guaiacol carbonate, guaiacum and sulphur and mercury with chalk, which were dried in a vacuum desiccator over concentrated sulphuric acid. Determinations were made upon freshly produced tablets, and after storage for four years.

Granulation Processes. In preparing batches of tablets, use was made of the three recognised methods of granulation. These may be described briefly as follows:—

Moist Granulation Process. This may be divided into two methods, A and B, depending upon whether all the starch is included when the mixed powders are moistened, or added as a powder to the dried granules.

Dry Granulation Process. If the drug is insoluble, it is reduced to a suitable particle size and with it is mixed an amount of dried starch. The mixture is then suitable for compression.

Granulation by Preliminary Compression. The starch and other ingredients, in fine powder, are well mixed and roughly compressed into large tablets. These are broken down into granules of suitable size before final tableting.

Granules obtained by the above methods may require the addition of a lubricant before compression.

An Examination of Common Varieties of Starch. The use of starch as a disintegrating agent lies in its property of rapidly absorbing water, thereby swelling and creating a pressure inside the tablet, which

causes it to disintegrate. The following starches may be used:—maranta, potato, sago, tapioca, maize and rice. In order to determine their comparative values, 6 batches of tablets of phenobarbitone were made, differing only in the fact that each contained 15 per cent. of a different variety of starch. The moist granulation process A was used and care taken to ensure that there was no alteration in pressure whilst making the tablets. Similarly, 6 batches of aspirin tablets were made by the dry granulation process using 9 per cent. of starch. The disintegration times for the tablets and the moisture contents of the starches are shown in Table I.

TABLE I

DISINTEGRATION TIMES OF TABLETS MADE WITH DIFFERENT STARCHES

	Moisture content	Phenobarbitone gr. 1	Aspirin gr. 5
	per cent.		
Potato starch	13·1	< ¼′	< ¼′
Sago ,, ...	14·9	< ½′	< ½′
Maranta ,, ...	15·4	< ½′	< ½′
Tapioca ,, ...	12·5	< ½′	< ½′
Maize ,, ...	8·5	< ½′	< ½′
Rice ,, ...	12·1	< 1′	< ½′

Reference to this table indicates that, using the amounts of starch shown above for the purpose of disintegration, there is no significant difference in any of the six varieties, despite considerable variation in the size of the starch grains. It was decided to use maize starch as the disintegrating agent in all future experiments.

The Amount of Starch Contained in the Tablet. Whilst 15 per cent. and 9 per cent. of maize starch were used in the above experiment, and proved satisfactory, it is desirable to investigate the effect of starch when used as in disintegrant in larger or smaller proportions.

Batches of tablets of phenobarbitone, phenobarbitone and theobromine, and sulphathiazole were made by the moist granulation process A and tablets of aspirin by the dry granulation process. Varying amounts of maize starch from 0 per cent. to 20 per cent. were used in each case and lactose was used to replace starch in the varying proportions.

It is seen that 10 per cent. of maize starch is adequate to ensure disintegration within the pharmacopœial limits for the four different tablets examined. To give a margin of safety, 15 per cent. of starch is to be used for all future experimental batches made by moist granulation process A.

The Method of Granulation. In this experiment, tablets were made by three of the processes previously described:—Moist granulation process A; moist granulation process B; granulation by preliminary compression.

A constant amount of disintegrant was added for three different methods of granulation in order to ascertain whether this results in a noticeable variation in the disintegration times of the tablets.

TABLE II

EFFECT OF PERCENTAGE OF STARCH ON DISINTEGRATION TIMES

Maize starch per cent.	Phenobarbitone gr. 1	Phenobarbitone and theobromine B.P.C.	Sulphathiazole 0·5 g.	Aspirin gr. 5
0	> 60′	> 60′	> 60′	> 60′
1	> 60′	45′	5′	> 60′
2	> 60′	40′	2′	> 60′
5	40′	1′	½′	1′
10	5′	< ½′	< ½′	< ½′
20	< ½′	< ½′	< ½′	< ½′

TABLE IIIA

DISINTEGRATION TIMES OF TABLETS MADE BY DIFFERENT PROCESSES

	Moist granulation process A.	Moist granulation process B.	Preliminary compression
Carbromal, gr. 5	1′	1′	< ½′
Phenobarbitone, gr. 1	½′	1′	< ½′
Sulphathiazole, 0·5 g.	1′	< ½′	1′
Calcium lactate, gr. 5	6′	9′	1′

The result of this experiment shows there is little difference in the relative disintegration times of tablets made by the three methods of granulation, and all are well within the Pharmacopœial limits. It is *interesting to note that the act of moistening the starch in granulation* process A has not adversely affected its function as a disintegrant. It was further observed that tablets made by this method were less friable than when starch was added as a dry powder. Friability values were therefore determined, using the same batches of tablets as for Table IIIA, with the following results:—

TABLE IIIB

FRIABILITY VALUES OF TABLETS MADE BY DIFFERENT PROCESSES

	Moist granulation process A.	Moist granulation process B.	Preliminary compression
Carbromal, gr. 5	2	39	49
Phenobarbitone, gr. 1	3	14	12
Sulphathiazole, 0·5 g.	2	4	28
Calcium lactate, gr. 5	4	9	13

This shows that tablets made by moist granulation process A have considerably lower friability values than those made by the other two methods, although all appeared to be equally hard at the time of manufacture when tested between the fingers and thumb. This is of particular

importance when resistance to wear has to be considered in relation to the handling and packing of tablets.

It is suggested that a satisfactory tablet should have a friability value not exceeding 8.

The Nature of the Moistening Agent. A number of suitable moistening agents are mentioned in the British Pharmacopœia. Of these, starch mucilage (10 per cent. w/v) and acacia mucilages (50 per cent. w/v and 25 per cent. w/v) are commonly used, and were employed in the granulation of similar batches of a number of different drugs.

As before, the moist granulation process A was used and the formula included 15 per cent. of maize starch. The disintegration times of the tablets produced from these batches of granules are shown in Table IV.

TABLE IV

EFFECT OF MOISTENING AGENT USED ON DISINTEGRATION TIMES

	Starch mucilage 10 per cent. w/v	Mucilage of Acacia	
		50 per cent. w/v	25 per cent. w/v
Guaiacum and sulphur	1′	> 60′	> 60′
Phenobarbitone, gr. 1	1′	9′	6′
Calcium lactate, gr. 5	6′	28′	15′
Carbromal, gr. 5	1′	36′	11′
Sulphathiazole, 0·5 g.	1′	> 60′	27′
Phenacetin compound, B.P.C.	1′	35′	15′

The most striking result of this experiment was the retarded disintegration of tablets when mucilages of acacia of the strengths indicated had been employed, despite the inclusion in the formulae of an adequate amount of disintegrant. They contrast unfavourably with similar tablets of approximately equal hardness made with starch mucilage as the moistening agent.

The Pressure Used During Tableting. Provided that disintegration is not seriously affected, the harder the tablet the better. Two batches of tablets were made from portions of the same batch of granule. In the first case, moderate pressure was used to produce a tablet of normal hardness; in the second case, excessive pressure was used to produce a tablet which was extremely hard, and could only be broken in the fingers with difficulty. The results of the disintegration times and friability values for 4 different tablets are given in Table V.

It was interesting to observe in the series of tablets examined that a tablet could be produced which still had a satisfactory disintegration time despite the use of pressure much in excess of that which would normally be used in tablet practice.

The Effect of Age Upon the Tablet. The following conclusions were made from the results obtained with these preliminary tests.

TABLE V

EFFECT OF EXCESSIVE PRESSURE ON DISINTEGRATION TIMES AND FRIABILITY VALUES

	Moderate pressure		Excessive pressure	
	Disintegration Time	Friability Value	Disintegration Time	Friability Value
Phenobarbitone, gr. 1	½′	3	4′	2
Carbromal, g. 5	1′	2	3′	1
Sulphanilamide, 0·5 g. ...	½′	2	¾′	2
Phenobarbitone and theobromine B.P.C.	¼′	4	1′	3

The inclusion of 15 per cent. of maize starch was sufficient to give satisfactory disintegration when tablets were made by any of the methods described. Mucilage of acacia should be used with caution, since it can result in the production of a tablet with retarded disintegration.

The use of the moist granulation process A has been shown to give satisfactory tablets with a low friability value and able to withstand the application of excessive pressure without causing serious increase in the rate of disintegration.

As a result of this information, 13 different batches of tablets were made by the moist granulation process A, each containing 15 per cent. of maize starch as the disintegrant, and using starch mucilage (10 per cent. w/v) as moistening agent. A small proportion of magnesium stearate was used for the purpose of lubricating the dry granules. A batch of aspirin tablets was also made by the dry granulation process employing aspirin crystals mixed with 9 per cent. of dried maize starch.

The tablets were kept in partially filled glass jars and stored on a shelf at room temperature. They were examined over a period of 4 years for disintegration rates, and determinations were also made of the friability values and moisture contents of the tablets at the beginning and end of this period. It was thought that if any significant increase in the time of disintegration was observed as a result of ageing, this might be related to a corresponding change in the moisture content of the tablet.

Table VI shows the results of these experiments.

The following observations can be made from an examination of *Table VI*.

Batches of different tablets show variations in their initial rates of disintegration, although all comply with the requirements of the British Pharmacopœia. It is interesting to note that calcium lactate, which is the most soluble of the drugs examined, takes the longest time to disintegrate when in tablet form. The remainder of the series of tablets show only slight differences in the rates of disintegration at the beginning and conclusion of the period under review, and where increases do occur they have no practical significance.

TABLE VI

THE EFFECT OF STORAGE ON DISINTEGRATION TIMES, FRIABILITY VALUES AND MOISTURE CONTENT

Tablet	Disintegration Times									Friability Values		Moisture Content per cent.	
			Months			Years							
	Initial	1 Week	1	3	6	1	2	3	4	Initial	4 Years	Initial	4 Years
Barbitone, gr. 5	2	2	1	1	1½	1½	1½	2	2½	1	1	2·1	1·4
Calcium lactate, gr. 5	6	6	6	6	6	6	6	6	6	4	4	25·0	25·0
Carbromal, gr. 5	1	1	1	1	1	1	1	2	2	2	1	3·3	3·0
Cinchophen, gr. 5	1	1	1	1	1	1	1	1	1	5	6	1·5	1·7
Guaicol carbonate, gr. 5	2	2	2	2	2	2	2	3	3	6	3	2·3	2·5
Guaiacum and sulphur	1	1	1	1	1	1	1	1	1½	3	2	1·4	2·0
Mercury with chalk, gr. 1	3	4	4	4	4	4	4	4	4	12	8	1·9	1·4
Phenacetin compound	1	1	1	1	1	1	1	1	1	4	2	1·4	1·8
Phenobarbitone, gr. 1	½	½	1	1	1	1	1	1	1	3	2	3·0	3·5
Phenobarbitone and theobromine ...	½	½	½	½	½	½	½	½	½	4	4	1·6	1·5
Sulphanilamide, 0·5 g	½	½	½	½	½	½	½	½	½	2	3	1·6	1·3
Sulphathiazole, 0·5 g	1	1	1	1	1	1	1	1	2	2	1	3·7	1·0
Sulphonal, gr. 5	½	½	½	½	½	½	½	½	1	4	5	3·5	2·6
Aspirin, gr. 5	½	½	½	½	½	½	½	½	½	5	5	1·2	0·9

In general, the friability values have decreased and the tablets may be said to have " hardened " a little after storage.

The moisture contents of the tablets show both increases and decreases, but they do not bear any relation to the friability values or disintegration times.

Summary

1. Factors affecting the initial time of disintegration of tablets made from insoluble drugs have been discussed.

2. Experiments have been made to examine the influence which each of these factors may have upon disintegration.

3. An experimental formula has been devised in which factors adversely affecting initial disintegration have been eliminated.

4. Thirteen batches of different tablets have been made using this formula and the effect of age upon the time of disintegration examined over a period of 4 years.

5. Results of these experiments show that there is no significant increase in the time of disintegration after prolonged periods of storage.

Our thanks are due to the Directors of Thomas Kerfoot and Co., Ltd., for permission to publish this work.

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DISCUSSION

An abstract of the paper was read by Mr. Burlinson.

MR. A. NUTTER SMITH (Nottingham) referred to the friability test used by the authors. With this test, it was necessary to standardise all the conditions to obtain reliable results. The true hardness of certain tablets, such as aspirin and mercury with chalk, was not apparent at first. All those who had worked on the subject were convinced that there was no direct relationship between hardness and disintegration. Starch was a very good lubricant, but it must be dry. It might contain as much as 20 per cent. of moisture but he had found from 8·5 to 9 per cent. to give the best results, if the starch were added last. The method of storage for ageing tests was important, as moisture could be readily absorbed by the tablets. The authors' method of partially-filled glass jars gave the worst possible conditions. In the keeping tests, many of the friability values were low. This may have been due to the absorption of water. However, he had found it difficult to link up friability values and moisture content.

DR. G. E. FOSTER (Dartford) asked how the hardness had been measured.

DR. NORMAN EVERS (Ware) asked whether the authors had investigated the effect of elevated temperatures, which no doubt caused an increase in disintegration time. He wondered whether the question of disintegration had been over-emphasised; there might well be cases where it would be a great advantage to have a tablet which would disintegrate slowly.

MR. S. G. STEVENSON (Birkdale) pointed out that disintegration was a function of the permeability of a tablet to water, and it was necessary for the moisture to penetrate right through the tablet. If alginic acid were used would not the gel hold the water and prevent it from reaching the centre of the tablet?

PROF. H. BERRY (London) stated that with alginic acid as disintegrant the granules themselves split and in the disintegration test a powder was obtained and not granules.

MR. H. BURLINSON, replying to the discussion, said that he agreed with Mr. Nutter Smith's remarks about the effect of physical form, especially with phenobarbitone which was difficult to work with in some forms and easier in others. It was suggested that mercury with chalk tablets should be crushed before use. The figures given in the paper showed them to be considerably softer than the other tablets when first made. They had used maize starch not because it had a low moisture content, but because it was widely used and was reliable. The drying of the starch was particularly important when it was added to the dry granules but not in the wet granulation process. For the storage conditions they had tried to reproduce the conditions in an average pharmacy. They did not use any elevated temperatures in the test. The degree of hardness of the tablets was not measured but they did not alter the pressure and took care to produce a uniform granule in the sieving process. The machine was a single punch machine working at 60 r.p.m. Disintegration was related to permeability and the latter to the way in which the disintegrant was used. He thought that the type of disintegration obtained by the use of alginic acid could be obtained by incorporating the starch prior to granulation.

THE REMOVAL OF BACTERIA FROM OILS BY FILTRATION

By G. SYKES and A. ROYCE

From the Microbiology Division, Standards Department, Boots Pure Drug Co., Ltd., Nottingham

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ATTEMPTS to influence the rate of diffusion of drugs given by injection have directed attention to the importance of the vehicle and as a result of this an oily basis is frequently used for injections of thermolabile drugs. The sterilisation of these injections requires some consideration in view of opinions expressed that the method of sterile filtration so generally applicable to aqueous solutions is unsatisfactory with oils. Indeed, the Pharmacopœia of the United States (XIII, pp. 696-697) states: "Liquid petrolatum and other oils are not to be sterilised by this method as they may increase the permeability of the filter to bacteria." It then goes on to explain that the process of filtration is not simply a matter of relative sizes of pores of the filter and of particles to be filtered, but involves many other factors.

As early as 1926, Holman[1] showed that by pre-treatment with a mixture of petroleum and paraffin oil, candle filters could be rendered much more permeable to the passage of certain bacteria than before treatment or even after the removal of the oil. He did not give the porosity of his candles, but it would appear that they were not of sufficiently fine porosity to yield sterile filtrates of aqueous solutions. The observation on increased permeabilities was later extended by Kramer[2], by Varney and Bronfenbrenner[3], and by others, to vehicles other than oils. The generally accepted explanation of this phenomenon is that fine-pore filters depend for their efficiency not only on mechanical sieving but also on the relative electrical charges on the filter and on the particles in the liquid being filtered. Thus, coating a filter with oil may be considered to insulate its charge and thereby reduce its efficiency.

In contrast, Hurni[4] believed that the only method of sterilising oil, other than by dry heat, was filtration through a Berkefeld candle under reduced pressure at a temperature of 80° to 90°C. He used bacterial spores as the test organisms and presumably employed the elevated temperature to assist in the rate of filtration. He found that these same organisms suspended in oil would pass through a Seitz-type filter pad under the comparatively high filtration pressure of $1\frac{1}{2}$ atmospheres, and concluded that Seitz and other asbestos-type filters are useless for the sterilisation of oils. He gave no information on the efficiency of Seitz filters under reduced pressure. Berry[5] considered that the pressure at which a filtration was carried out materially affected the efficiency of the filter, thus the contrast in response of the candle filter and filter pad reported by Hurni may have been due to the different conditions under which his experiments were carried out.

Berry[6] also stated in a paper on bacterial filtration that a Seitz filter depended for its efficiency on the swelling of the fibres of the pad and that where such swelling, caused by inbibition of the solvent being filtered, did not take place, the effectiveness of the filter was seriously impaired. Davies and Fishburn[7], on the other hand, describing their filter pad technique for sterility tests, stated that oils could be tested satisfactorily by this method, thus implying the complete retention by the filter pad of any organisms present in the oils.

In the past few years we have successfully handled some hundreds of batches of oily solutions. Each batch has been filtered under reduced pressure at normal temperatures through sterilised and dry Ford Sterimats (S.B. grade), and tested for sterility by approved methods at both the bulk and final container stages. No batch has yet been failed and only very occasionally has a repeat test been found necessary. Our success may have been due to the comparative freedom of oils from bacterial contamination (as suggested by Hurni[4]), and to the recognised difficulties in detecting organisms in small numbers in water-immiscible liquids, nevertheless, it gives substantial support to the practical value of bacterial filters in the sterilisation of oils. In view of the statements quoted above, it seemed desirable to re-investigate this whole matter, and the following paper describes some of the results of this work.

Practical Considerations.—Coulthard and Chantrill[8] and Coulthard and Croshaw[9], in private communications, have shown that hæmolytic streptococci protected with blood, and also tubercle bacilli, will remain infective in oils for periods up to several weeks, and it is well known that dry bacterial spores will also survive for prolonged periods in oils. We considered it desirable, however, to carry out our experiments with very small, unprotected, vegetative organisms, and chose, therefore, for most of the work *Bacterium prodigiosum* either on nutrient agar slopes or in broth culture. A number of preliminary tests showed that these relatively unprotected cells became non-viable in the space of a few hours when suspended in the oils commonly used as vehicles for injections, and so adequate controls were included in every group of experiments to ascertain the rate of survival during the filtration period. In some tests a soil-dust-spore mixture was substituted. Both candle filters and asbestos pad filters were tested under the different conditions outlined below.

Experimental Technique

Liquid paraffin, arachis oil and ethyl oleate, all of B.P. quality, were used in the experiments, and 18 to 24-hour cultures on agar slopes or in broth of *Bact. prodigiosum* provided the source of this test organism. It was not found possible to prepare a completely dry suspension in oil of sufficiently high viability for the purpose required, and so the following method was adopted. Small agar slope cultures were sludged with a few ml. of the oil, taking care not to break the surface of the agar, and this suspension was diluted with a large volume of sterile oil—usually 1 to 2 l.—giving a final concentrated bacterial suspension of very low moisture

content. That these organisms were virtually unprotected receives considerable support when their very high death-rate is compared with the comparative stability of blood-protected organisms such as those used by Coulthard and Chantrill[8].

Counts were made on the oil suspensions by preparing tenfold serial dilutions in the same oil (using a fresh sterile pipette at each stage) and testing each dilution for the presence of viable bacteria. This was done by a method similar to that given in the U.S.P., XIII, p. 691:—1 ml. of the sample was inoculated into 50 ml. of sterile nutrient broth in a screw-capped bottle and the mixture shaken vigorously. It was incubated at 37°C., shaking daily, and at the end of the incubation period of 5 days subcultures were made to confirm the presence or absence of viable bacteria. Counts were made in this manner just before the commencement of the filtration period and immediately on completion of the filtration, usually some 6 to 8 hours later, the latter sample being tested simultaneously with the filtrate. This precaution was taken to confirm that viable organisms were still present in the oil and to counteract as far as possible any false-negative results due to spontaneous death of bacteria in the filtrate. All the counts recorded indicate the weakest dilution showing a positive response on subculture, for example, a recorded bacterial count of 1,000 per ml. indicates growth in the 1 in 1,000 dilution but not in the 1 in 10,000 dilution.

All filtrates were examined by a similar shaken broth technique except that much larger samples, up to 50 ml. (in 5 ml. portions) were employed with correspondingly larger volumes of broth.

In experiments not using *Bact. prodigiosum*, 5 g. of a dry soil-dust-spore mixture was shaken with 1 l. of the oil, and roughly filtered to remove coarse particles. The oily suspension was then used in the manner described above.

Candle Filters.—It is our experience, based on work we hope to publish in the near future, that candles of an estimated maximum pore size of about 2·4 microns, corresponding to a "bubble-pressure test" in water of about 18 lb. per sq. in, must be used to obtain a sterile filtrate from a nutrient solution over a sustained filtration period. We, therefore, chose for this series of experiments Doulton and Berkefeld candles of bubble pressure to 18 to 22 lb./sq. in. We also included a few tests with candles of greater pore size with bubble pressures of approximately 10 lb./sq. in. The bacterial suspensions in oils were prepared as already described and the filtrations were carried out under reduced pressures. The results of the experiments are given in Table I.

It is obvious that the rate of filtration of the relatively viscous oils through candles of such porosity is very low, too low in fact to be of practical value on the large scale. The results do show, however, that candles which are reliable in giving sterile aqueous filtrates will sterilise even highly contaminated oils. A candle of greater pore size, and, therefore, not suitable for sterile filtration of aqueous solutions, was also found

unsuitable for removing small organisms such as *Bact. prodigiosum* but was apparently effective in removing the larger sized bacterial spores.

Ford Sterimat Filters.—Single 14 cm. Ford Sterimats (S.B. and F.C. grades) were sterilised and used dry for these experiments. Filtrations were carried out at reduced pressures (Table II) and also at positive

TABLE I

FILTRATION OF OILS THROUGH CANDLES AT REDUCED PRESSURES

Type of candle	Test organism	Oil	Period of filtration (hours)	Volume filtered (litres)	Count/ml. of oil during experiment		Tests on filtrate	
					Start	Finish	Samples Tested	Number Sterile
18 to 22 lb. bubble pressure	*Bact. prodigiosum*	Arachis oil	6·5	1	1000	100	10 × 5 ml.	All
		Liquid paraffin	24	1	1000	100	,,	,,
		Ethyl oleate	6·5	2·5	100	10	,,	,,
18 to 22 lb bubble pressure	Soil-dust-spores	Arachis oil	6·5	0·5	1000	1000	10 × 5 ml.	All
		Liquid paraffin	24	1	1000	100	,,	,,
		Ethyl oleate	6·5	3	1000	100	,,	,,
10 lb. bubble pressure	*Bact. prodigiosum*	Arachis oil	6	1·5	1000	100	10 × 5 ml.	4 × 5 ml.
	Soil-dust-spores	,, ,,	6·5	3·5	1000	1000	,,	All

TABLE II

FILTRATION OF OILS THROUGH FORD STERIMATS AT REDUCED PRESSURES

Filter grade	Test organism	Oil	Period of filtration (hours)	Volume filtered (litres)	Count/ml. of oil during experiment		Tests on filtrate	
					Start	Finish	Samples tested	Number sterile
S.B.	*Bact. prodigiosum*	Arachis oil	4	1·5	100,000	1000	10 × 5 ml.	All
		Liquid paraffin	7	2	10,000	1000	,,	,,
S.B.	Soil-dust-spores	Arachis oil	7	1	1000	1000	10 × 5 ml.	All
		Liquid paraffin	7	1	1000	1000	,,	,,
S.B.	*Bact. prodigiosum* in broth culture*	Arachis oil	4	1	100,000	100,000	10 × 5 ml.	All
		Liquid paraffin	6·5	0·5	100,000	100,000	,,	,,
F.C. ...	*Bact. prodigiosum*	Arachis oil	0·5	1	100	100	3 × 5 ml.	2 × 5 ml.

* Prepared by shaking 1 per cent. of a broth culture of *Bact. prodigiosum* into a temporary emulsion in the oil.

pressures of 25 lb./sq. in. (Table III); the latter in an attempt to reproduce the finding of Hurni (*v.s.*). The rate of filtration was greater than with candle filters and, here again, all experiments using the sterilising grade of filter (S.B.) were completely successful, and there appeared

TABLE III

FILTRATION OF OILS THROUGH FORD STERIMATS UNDER POSITIVE PRESSURE

Test organism	Oil	Period of filtration (hours)	Volume filtered (litres)	Count/ml. of oil during experiment		Tests on filtrate	
				Start	Finish	Samples tested	Number sterile
Bact. prodigiosum ...	Arachis oil	4	2·5	10,000	10,000	10 × 5 ml.	All
	Liquid paraffin ...	3·5	3	1000	1000	,,	,,
	Ethyl oleate	2	10	100	10	,,	,,
Soil-dust-spores ...	Arachis oil	3·5	2·5	1000	1000	10 × 5 ml.	All
	Liquid paraffin ...	4	2·5	1000	100	,,	,,
	Ethyl oleate	2·75	18	1000	100	,,	,,
Bact. prodigiosum in broth culture*	Liquid paraffin ...	4	3	1000	1000	10 × 5 ml.	All
	Ethyl oleate	2	10	100	10	,,	,,

All filtrations using S.B. grade pads, and under an applied pressure of 25 lb./sq. in.
* Prepared by shaking 1 per cent. of a broth culture of *Bact. prodigiosum* into a temporary emulsion in the oil.

to be no difference between filtrations carried out under reduced pressure or at the positive pressure employed, although, of course, the rate of filtration was materially affected.

The F.C. grade filter, only intended for use as a clarifying filter, failed, as anticipated, to hold back bacteria in oily suspension.

ADDITIONAL TESTS

A small number of experiments, involving animal inoculations as well as *in vitro* tests, have been carried out by Coulthard, Chantrill and Royce[10] and the results will be reported in detail elsewhere. Briefly, 25 ml. amounts of heat-sterilised liquid paraffin were inoculated with *Streptococcus hæmolyticus* (*Richards strain*) by adding small amounts of blood taken from a mouse fatally infected with the organism. In one instance 2 drops of blood were added giving a theoretical count of 28,000 viable cells per ml. of oil, and in another 0·1 ml. of blood yielding theoretically 3,000 cells per ml. of oil. Part of the infected oil was filtered under reduced pressure through a 6 cm. Ford Sterimat (S.B.). Filtered and unfiltered portions of the oils were injected intraperitoneally into groups of about 15 mice. In each case, all the mice injected with the filtered oil survived the test period of 5 days, and all those injected with the unfiltered oil died within 48 hours. At the same time, counts were made on the oils as outlined in an earlier section but using 10 per cent. serum broth as the cultage medium. No bacteria were found in 0·6 ml. of the filtered oil, but the unfiltered oil had streptococci in the 1 in 1,000 dilution and not in the 1 in 10,000 dilution.

Breakdown Filtrations.—It has already been shown in the foregoing experiments that filters which are effective in removing bacteria from aqueous media are also effective in removing them from oily vehicles. In an attempt to find the conditions under which filters cease to be effec-

tive, as suggested by Holman[1] and others, a number of experiments have been carried out filtering oil-broth mixtures and also oil and aqueous suspensions alternately.

The oil-broth mixtures were made by shaking 10 ml. of an 18 to 24-hour broth culture of *Bact. prodigiosum* into a temporary emulsion in 1 l. of oil (giving a 1 per cent. water content). Filtrations were through Sterimats (S.B.) at either reduced pressures or under positive pressures, the oil suspension being shaken at intervals. Five such experiments were carried out, two with arachis oil, two with liquid paraffin and one with ethyl oleate. Although high initial counts of well over 100,000 viable cells per ml. of oil were found in some cases, the filtrates were all sterile, that is, no organisms were detected in 50 ml. of oil. Thus, the presence of small amounts of water admixed with the oil did not affect the efficiency of the filtration.

When 500 ml. of a diluted broth culture of *Bact. prodigiosum* (1 ml. of culture in 5 l. of saline solution) was first put through a Sterimat followed by 250 ml. of arachis oil and then a further 250 ml. of aqueous culture the whole of each broth fraction and the oil were found to be sterile. On the other hand, when a dry pad was saturated with oil and then an aqueous suspension of the test organism forced through it, the aqueous fraction was heavily contaminated. In one experiment the count was between 10 and 100 organisms/ml. and in another over 1,000 organisms/ml. (Table IV.)

In contrast, candle filters of 18 to 22 lb. "bubble pressure," which had previously been impregnated with arachis oil, liquid paraffin, or ethyl oleate, were completely successful in preventing the passage of bacteria in aqueous suspension even though the filtrations were continued for periods of 6 to 7 hours. (Table IV.)

TABLE IV

FILTRATION OF ALTERNATE OIL AND AQUEOUS SUSPENSIONS

Filter	Oil	Successive fractions filtered			Results
		1st	2nd	3rd	
Sterimat S.B.	Arachis oil	Saline + *Bact. prodigiosum*	Oil + soil-dust-spores	Saline + *Bact. prodigiosum*	All fractions sterile
" "	"	Oil	Saline + *Bact. prodigiosum*		Aqueous fraction contaminated
Candle 18 to 22 lb. bubble pressure	Arachis oil	Oil	Saline + *Bact. prodigiosum*		Aqueous fraction sterile
	Liquid paraffin	"	"		"
	Ethyl oleate	"	"		"
Candle 10 lb. bubble pressure	Arachis oil	Oil	Saline + *Bact. prodigiosum*		Aqueous fraction contaminated.

Discussion

The time factor is probably very important in sterile filtration and it may well be that filters, satisfactory when the period of filtration is brief, will break down when used over longer periods. This is, of course, particularly likely to occur with nutrient solutions. Asbestos pad filters of the type Ford Sterimat (S.B. grade) are quite satisfactory for periods of a few hours. In the case of candle filters, a grade giving a response of 18 lb./sq. in. in the " bubble pressure" test in water (equivalent to an estimated maximum pore size of 2·4 microns) may be necessary for filtrations sustained during several hours, whereas a grade of about 12 lb./sq. in. response may be satisfactory for short period filtrations. From the experiments reported here, using a variety of oily vehicles and of bacteria, it appears that these same standards might safely be applied to the sterile filtration of oils.

We have no evidence to show that filters of sufficiently good quality are normally less efficient in dealing with oily than with aqueous preparations. Neither have we any evidence to support the claim put forward by Hurni that pressure filtration through asbestos filters is unreliable. We have indicated, however, that coarser grade filters, the Sterimat F.C. grade or larger pore filter candles, which are not claimed to hold back all bacteria, do not yield sterile filtrates.

Filters have been caused to break down by somewhat artificial means, for example, by alternatively filtering oil and water. This applied to Sterimats only and not to candle filters as claimed by Holman. The difference was most probably due to Holman's use of a coarser grade candle, since he did not claim in the the first place to obtain a completely sterile filtrate even with an aqueous solution. This finding gives support to the electrostatic theory of filtration, but it also shows that the phenomenon is manifest over only a limited range of porosity and can be offset by the probably more reliable mechanical process of filtration with filters of sufficiently fine pore size. The fact that the efficiency of some filters can be reduced by coating with oil has little or no practical application with Seitz-type pads, which are used only once, but it may be of importance in dealing with candle filters. These should be washed free of oily vehicle before being re-used for aqueous solutions.

Summary

(1) Oils deliberately contaminated with *Bact. prodigiosum,* hæmolytic streptococci or bacterial spores have been successfully sterilised by filtration through Ford Sterimats or candle filters. Large scale batches of oily injections have also been handled with equal success over many years.

(2) It is important that the right grade of filter be used. Filters of grades suitable for the sterilisation of aqueous solutions are also suitable for handling oily vehicles.

(3) Sterimats have been caused to break down by first " insulating " the pads with oil and then filtering an aqueous suspension of organisms.

They are, however, satisfactory with oils containing only a small amount of water, or if a previously wetted pad is used.

(4) The breakdown effect has not been demonstrated with candles of sufficiently fine pore size.

It is a pleasure to record our thanks to Mr. C. Bowler for his able technical assistance. We are also indebted to Mr. C. E. Coulthard for his interest and criticisms, and to the Directors of Boots Pure Drug Co., Ltd., for permission to publish this work.

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DISCUSSION

An abstract of the paper was read by Mr. Sykes.

PROFESSOR H. BERRY (London) thought that there was difficulty in proving the results in work of this kind. The forcing of oil through a filter with a dispersed aqueous suspension of bacteria was not to be trusted, because of the difficulty that water has in passing through an oil-wetted filter. Normally oils were infected with air-dried organisms and it was therefore preferable to obtain a test organism which could be deprived of surface moisture and remain viable. Also the organism should be of the size of, say, *Bact. prodigiosum,* 0·75 mμ or it was no real test of the filter. He had tried to dry organisms with acetone but *Bact. prodigiosum, Bact. coli* and staphylococci died very rapidly. *B. subtilis,* particularly the spore form, would survive for long periods in acetone but was not a suitable organism with which to test the filter. He accepted the authors' results, but their negative character reduced their significance. He would like to know the size of the dust particles used and if the authors could confirm any difference in the behaviour of the Seitz pad when dry, wetted with water, and when wetted with oil. Had they observed a critical pore value for a filter candle which would pass either aqueous-wetted bacteria or bacteria wetted with oil ?

MISS V. W. BURRELL (Pinner) said that she had successfully used spores of *B. subtilis* and, for vegetative organisms, *Bact. coli* in similar experiments. Sterilisation had been effected by Ford sterimats of G.S. and S.B. grades. Suspensions of suitably dry organisms were obtained by taking a small portion of an agar slope culture and rubbing it down in a mortar. The experiments were all carried out under vacuum, and air was eventually allowed to pass in after being dried with sulphuric

acid. Out of well over a 100 heavily contaminated experimental batches, only two were found not to be sterile, possibly due to breakdown of the filter.

DR. K. BULLOCK (Manchester) asked for details of the authors' sludging process. When he had tried it he obtained an emulsion presumably of clumps of bacteria in a water phase in oil. It would not surprise him if they were filtered off by an oil-saturated filter. Did the authors check whether there was any clumping or whether the organisms were evenly distributed, and had they tested the oil for sterility before filtration as well as afterwards?

MR. R. LEVIN (Leeds) said that he had subcultured previously contaminated oils after incubating them for 6 weeks. Olive oil, with and without a fungstatic, showed growth, but samples of ethyl oleate without a fungistat did not. Had the authors any similar experiences and had they found similar properties for ethyl oleate?

MR. G. SYKES, replying to the discussion, said that the making of a bacterial suspension from an agar slope was the nearest they could get to the ideal. Time, porosity, thickness of the filter bed and the nature of the solution were all important factors in the filtration of bacteria. They had not tested production batches of oily preparations before filtration as the survival of bacteria in oils was very low and it was difficult to test oils for the presence of so few bacteria. It was possible that self-sterilisation occasionally occurred.

MR. A. ROYCE said that they, too, had found that certain organisms did not survive treatment with acetone. Their soil dust preparations were ground down in a mortar and were fairly fine. After initial trituration with oil, the mixture was filtered through gauze or thin cotton wool and no large particles of dust were visible in the final preparation. However, they could not guarantee that they had a suspension of single organisms only. A considerable number of organisms in an aqueous phase passed through an oil-saturated pad, but an oil filtrate from a water-wetted pad was sterile. Subsequent aqueous fractions filtered through the now oil-coated water-wetted pad gave filtrates which were also sterile. Work had not yet been completed on critical pore values, nor had G.S. grade pads been tested, and no attempts had been made to achieve perfect dryness in the system. The sludging technique consisted of adding a few drops of oil to an agar slope, mixing and then triturating the concentrate in a mortar with a large quantity of oil. Although a high dilution was used and there were no visible clumps they had not done any experiments to prove the absence of clumping. Ethyl oleate was markedly hostile to vegetative organisms, but the authors did not agree that it was self-sterilising.

SOME PHARMACEUTICAL ASPECTS OF VITAMIN B_{12}

By F. Hartley, P. Stross and R. E. Stuckey

From The British Drug Houses, Ltd., London, N.1

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Introduction

Vitamin B_{12} is an erythropoietic substance isolated almost simultaneously from liver by Rickes *et al.*[1] in U.S.A. and by Smith[2,3,4] in this country and subsequently by Ellis, Petrow and Snook[5]. It has since been obtained from the products of metabolism of *Streptomyces griseus*[6], *Streptomyces aureofaciens*[7] and other micro-organisms[6] and from the dung of animals[8].

Vitamin B_{12} is obtainable as an intensely red crystalline substance which can be characterised by its absorption spectrum[5,9], by its ability to act as an essential growth factor in the culture of micro-organisms such as *Lactobacillus lactis* Dorner[10], *L. leichmannii*[11] and *Euglena gracilis*[12], and by its ability to serve as the "animal protein factor" in promoting the growth in chicks[13,8]. It is capable of inducing erythropoiesis in humans on injection in microgramme doses[14,15].

Vitamin B_{12} appears to be one member of a group of naturally occurring chemical substances whose relationship has not yet been completely elucidated. Woodruff and Foster[16] have isolated from fermentation broths and from liver extracts a substance termed vitamin B_{12a} which differs from vitamin B_{12} in its absorption spectrum and its lower biological activity. Vitamin B_{12a} was first described by Kaczka *et al.*[17], who obtained it on catalytic hydrogenation of crystalline vitamin B_{12}. Brockman *et al.*[18], however, found that under similar conditions vitamin B_{12} gave a different red crystalline substance which had properties identical with another member of the group named vitamin B_{12b}, which had previously been isolated by Pierce *et al.*[7], from cultures of *Streptomyces aureofaciens* and has since been extracted from liver[20]. A fourth member of the group[21] previously referred to as vitamin B_{12x} has recently been renamed vitamin B_{12c}.

Vitamin B_{12} alone, of the four members of the group, appears so far to have been studied intensively with a view to elucidation of its chemical structure, though recently a report has been published[21] on vitamin B_{12c} which appears to be more highly oxygenated than vitamin B_{12}. Vitamin B_{12} has a molecular weight of about 1400 and contains per molecule one atom of cobalt[9] and one atom of phosphorus[22], its analysis[9] indicating a formula in the range $C_{61\text{-}64}H_{86\text{-}92}N_{14}O_{13}PCo$. It is somewhat hygroscopic and is soluble in water to the extent of 1 per cent. or more. It is optically active having $[\alpha]_{5563}^{23°C.}$ approximating $-60°$, though accurate determinations were difficult due to the necessity of working with dilute solutions because of the intensely red colour of its aqueous solutions[9]. On fusion with alkali it forms products which react with *p*-dimethyl-

aminobenzaldehyde, indicative of the presence of certain cyclic 5-membered nitrogen-containing compounds such as pyrroles[9]. Hydrolysis with hydrochloric acid releases a ninhydrin-reacting fragment[5], phosphate[22], and a 5:6-dimethyl-benziminazole residue[23,24]. A *l*-D-ribofuranosido-5:6-dimethylbenziminazole has also been obtained[25] on degradation of vitamin B_{12}. Ammonia has been shown by Ellis and Petrow[26] to be liberated during acid or alkaline hydrolysis of vitamin B_{12}.

The widespread interest that has arisen in vitamin B_{12} both intrinsically and in the search for reliable methods for the quantitative determination of the amounts present in concentrates and other materials appears to be adequate justification for considering the determination of its stability a matter of importance. It is the purpose of this communication to report our experimental work on the stability of vitamin B_{12} in such a variety of conditions as would enable adequate appraisal of its pharmaceutical aspects to become possible. Any studies of the stability of a chemical substance, however, obviously first involve consideration of methods by means of which changes in the physical, chemical or biological properties of the substance may be detected.

Methods of Determination of Vitamin B_{12}

Physical. The physical property most widely used is the absorption spectrum of the aqueous solution. Ellis, Petrow and Snook[5] found that vitamin B_{12} exhibited a characteristic absorption spectrum with maxima at 278, 361 and 548 mμ, their data indicating values for $E_{1\text{ cm.}}^{1\text{ per cent.}}$ of 108 at 278 mμ, 183 at 361 mμ and 57 at 548 mμ. Brink *et al.*[9] subsequently reported $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 115 at 278 mμ, 204 at 361 mμ and 63 at 548 mμ, and recently the U.S.P. XIII Third Sheet Supplement has adopted the value of $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 207 at 361 mμ for the pure anhydrous vitamin B_{12}. We ourselves find values for $E_{1\text{ cm.}}^{1\text{ per cent.}}$ at 361 mμ slightly over 200 calculated with reference to the substance dried to constant weight at 90°C. *in vacuo*.

In our experience the determination of the absorption spectrum is of the greatest value in assessing the purity of vitamin B_{12}. Figure 1 shows the type of spectra obtained with impure samples, the curves being adjusted so that they relate to the same total amount of vitamin B_{12} in each case. Curve A represents the spectrum of the solution prepared from a concentrate containing about 0·6 per cent. of vitamin B_{12}; it shows no peak at 278, 361 or 548 mμ, since the presence of relatively highly absorbing impurities masks the characteristic vitamin B_{12} spectrum, leaving a slight shoulder only at *ca.* 520 to 550 mμ. A solid concentrate containing approximately 2 per cent. of vitamin B_{12} (Curve B) begins to show the peaks at 361 and 548 mμ, while for a solid concentrate containing 5 per cent. of vitamin B_{12} (Curve C) these peaks are more apparent and the divided peak can be seen in the visible region. It is necessary to have a much purer sample before the peak at 278 mμ can be realised, and even with samples having a purity of approximately 10 per cent. the value of $E_{\text{max.}}$ at 278 mμ is relatively high when compared with the

curve for pure vitamin B_{12}; the peaks at 361 and 548 mμ, however, approximate to those shown by pure vitamin B_{12}. Thus a suitable criterion exists for establishment of the purity of a sample of vitamin B_{12} in that the three peaks at 278, 361 and 548 mμ should be in a definite ratio and the Third U.S.P. XIII Sheet Supplement has taken advantage of this property in fixing a limiting range for the ratios

$$E^{1\text{ per cent.}}_{1\text{ cm.}}\ 361\ \text{mμ.}/E^{1\text{ per cent.}}_{1\text{ cm.}}\ 278\ \text{mμ.}\ \text{of}\ 1{\cdot}62\ \text{to}\ 1{\cdot}88,\ \text{and for}$$

$$E^{1\text{ per cent.}}_{1\text{ cm.}}\ 361\ \text{mμ.}/E^{1\text{ per cent.}}_{1\text{ cm.}}\ 548\ \text{mμ.}\ \text{of}\ 2{\cdot}83\ \text{to}\ 3{\cdot}45$$

This U.S.P. Supplement also fixes limits for wavelength values, allowing maxima within ± 1 mμ. at 278 and 361 mμ., and within ± 4 mμ. at 548 mμ., allowance being made in the latter case for the broad maximum showing in the visible region.

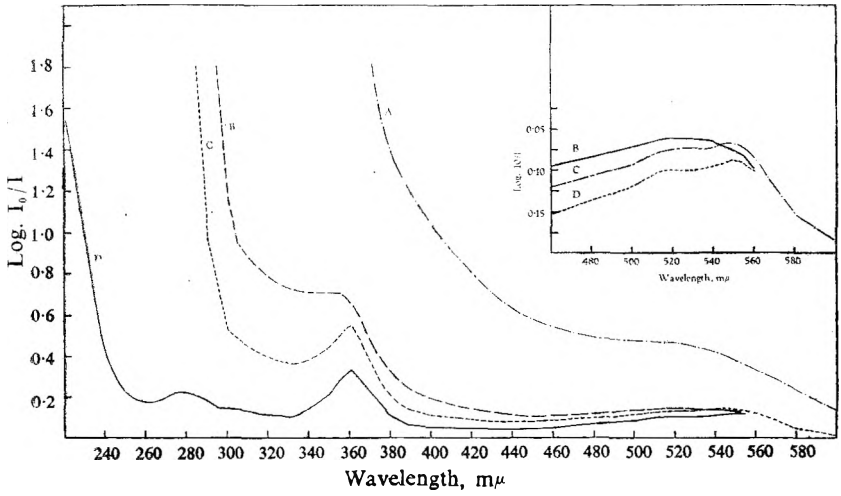

FIG. 1. Absorption spectra of vitamin B_{12} of varying purity. A, containing approximately 0·6 per cent.; B, containing approximately 2 per cent.; C, containing approximately 5 per cent.; D, pure vitamin B_{12}. The curves are adjusted so that they relate to the same total amount of vitamin B_{12} in each case.

Chemical. Although chemical methods of determination of vitamin B_{12} based upon the determination of cobalt are obviously possible, they are of more value in following purification from concentrates than in studies of stability as means are then required to differentiate between cobalt-containing decomposition fragments and unchanged vitamin B_{12}. Assays based on the microchemical determination of cobalt have proved useful in our laboratories in following enrichment during purification. The method used is based upon hydrolysis of the material and determination of the cobalt content of a butyl alcohol extract of the hydrolysate.

Fantes *et al.*[27] have based an assay on the fact, established by Ellis, Petrow and Snook[5], that the acidic fragment formed on acid hydrolysis of vitamin B_{12} can be esterified with methyl alcohol, although octyl alcohol was finally chosen as the esterifying agent. The concentration

of coloured ester formed was then determined photo-electrically. While in our hands the method has proved useful for examination of concentrates relatively rich in vitamin B_{12}, the high colour obtained with a blank determination precludes its use for estimating the vitamin B_{12} content of materials less rich in the vitamin. Furthermore it is insufficiently precise for stability studies.

Biological. As already mentioned, vitamin B_{12} possesses the ability to act as an essential growth factor for several micro-organisms and for chicks. Many microbiological assays have been devised and widely investigated. "Tube assays" have been described, for example, by Shorb and Briggs[28], Caswell[29], Lees and Emery[30], Shaw[31] and Hoffman, Jukes *et al.*[11], the preferred organisms being *L. lactis* Dorner or *L. leichmannii.* Most workers are, however, agreed that these assays are capricious in operation and the results obtained are found to vary from a large number of factors as emphasised by Shorb[28] and by Hartley[32]. The cup-plate method of assay, introduced by Cuthbertson[33], is the most useful for routine operation. In our hands this method based on the technique and medium described by Foster, Lally and Woodruff[34], and using *L. lactis* Dorner A.T.C.C. 10697, has proved to be the most capable of giving repeatable results, although the figures obtained show considerable variations between different laboratories and for some types of preparation, notably concentrates from fermentation broth, the method appears to yield erratic results.

Assays based upon measurement of the growth of chicks appear to show higher results than those obtained in corresponding assays against micro-organisms[19,35].

Stability of Vitamin B_{12}

It can readily be shown by spectrophotometric examinations that an aqueous solution of vitamin B_{12}, even when adjusted to a reaction in the range of greatest stability, namely, *p*H 4·0 to 7·0, suffers considerable change in its absorption spectrum within a few days on exposure to sunlight. On the other hand, in our experience, such solutions undergo no change, as shown by absorption spectra, on exposure during a week to diffuse daylight such as is encountered in a normal room or laboratory and may therefore be regarded as stable during the type of exposure occurring in normal handling for analysis, filling or packing.

The relatively low concentrations of vitamin B_{12} used for injection appeared to render it desirable to consider the stability of the pure material in solutions made isotonic with sodium chloride. It was found that no change occurred in the absorption spectra of solutions of pure vitamin B_{12} in concentrations of 10 to 20 microgrammes per ml. in injection of sodium chloride during 12 months' storage in sealed ampoules packed in cardboard boxes and maintained at normal room temperature.

Although it appears to be generally considered that vitamin B_{12} is stable in aqueous solutions at *p*H 4·0 to 7·0, decomposition occurring in

more acid solutions and more rapidly in alkaline solutions, it seemed to us desirable to examine more precisely the influence of *p*H of solution on the stability, especially at elevated temperatures such as would arise in sterilisation of solutions by autoclaving.

Solutions of vitamin B_{12} have therefore been examined spectrophotometrically and microbiologically for their stability under different conditions. The spectrophotometric assay has been based, in conformity with the Third Sheet Supplement to the U.S.P.XIII on the peak value at 361 mμ. The microbiological results were obtained by a technique based on that described by Foster, Lally and Woodruff[34] using *Lactobacillus lactis* Dorner A.T.C.C. 10697, the standard used being a carefully purified sample of crystalline vitamin B_{12} which was dissolved in water and standardised on the spectrophotometric result at 361 mμ.

Solutions buffered at *p*H 4·0 and *p*H 7·0 (measurements being made on a glass electrode) were first examined—both before and after autoclaving in sealed ampoules, at 115°C. for 30 minutes and after standing in the dark for 6 months. The absorption curves are shown in Figure 2, from which it will be seen that the curves at *p*H 4·0 and at *p*H 7·0 are

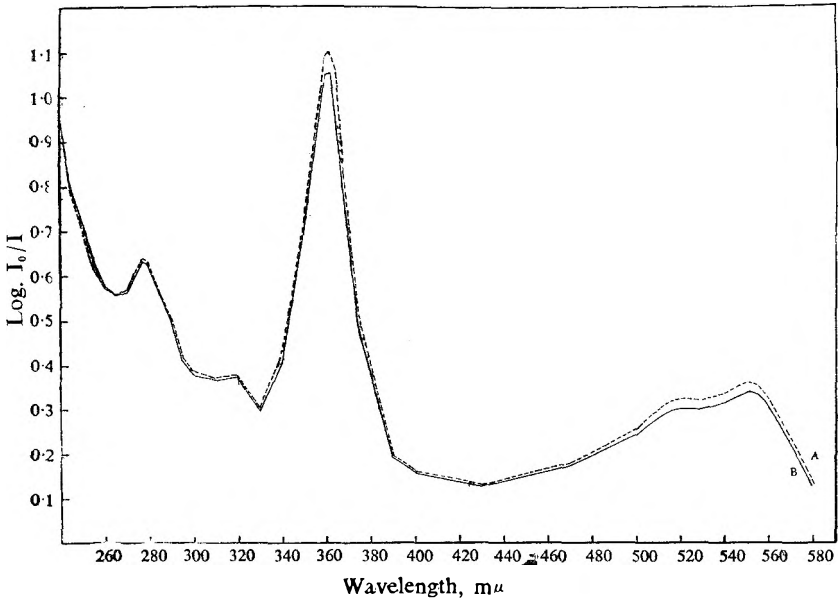

FIG. 2. Absorption spectra of vitamin B_{12}. A, at *p*H 4·0 and *p*H 7·0; B, at *p*H 4·0 and 7·0 after heating for 30 minutes at 115°C.

indistinguishable and that a slight spectrophotometric drop occurs on autoclaving.

Table I shows the comparison between spectrophotometric and microbiological results. Agreement between the two methods of assay in this instance is reasonably good, though such agreement is frequently only

obtainable by taking the mean of a relatively large number of replicate microbiological assays. The differences between the assays on the original solutions and on those of the solutions after standing for several months are, in our opinion, not significant. On the other hand, the microbiological results for the autoclaved solutions were obtained side by side with those for the original, and in our opinion the slight fall in activity (approximately 10 per cent. at both *p*H 4·0 and *p*H 7·0) is significant, confirmed as it is by a loss detected spectrophotometrically.

TABLE I

STABILITY OF VITAMIN B_{12} AT *p*H 4·0 AND *p*H 7·0

	Spectrophotometric assay ex $E_{361m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution at *p*H 7·0	53·1	—	54·0	—
After standing for 6 months in the dark	52·1	—	52·9	—
After heating for 30 minutes at 115°C.	50·7	4·6	48·4	10·4
Original solution at *p*H 4·0	45·9	—	47·0	—
After standing for 6 months in the dark	46·9	—	45·9	—
After heating for 30 minutes at 115°C.	44·1	4·0	42·6	9·4

The stability results for vitamin B_{12} given in Table I appear to apply only to solutions of the vitamin having an order of purity of about 90 to 95 per cent. Solutions prepared from less pure samples of crystalline material or from solid concentrates containing the vitamin appear to be much less stable, the degree of instability varying with the nature of the impurities present. Table II shows results obtained at *p*H 5·0 with a solution prepared from a sample of vitamin B_{12} approximately 70 per cent. pure. In this case the loss on autoclaving is greater than with pure

TABLE II

STABILITY OF A SAMPLE OF IMPURE VITAMIN B_{12} (CALCULATED EX $E^{1\text{ per cent.}}_{1\text{ cm.}}$ 361 Mμ, APPROXIMATELY 70 PER CENT. PURE)

	Spectrophotometric assay ex $E_{361m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution at *p*H 5·0	20·9	—	20·9	—
After heating for 30 minutes at 115°C.	18·1	13·4	11·3	45·9
After heating for a further 30 minutes at 115°C.	16·0	23·4	7·8	62·7

vitamin B_{12}, as shown by the spectrophotometric results, and considerably greater, amounting to about 46 per cent. after 30 minutes' autoclaving, as shown by the microbiological results.

In alkaline solutions vitamin B_{12} suffers rapid decomposition on heating and is much less stable than in slightly acid solutions. Figures 3 and 4 and Tables III and IV show the results obtained at *p*H 9·0 and 12·0. The nature of the decomposition occurring in alkaline solution evidently differs from that in acid solution, in that with loss of activity there occurs loss of the pink colour of the solution.

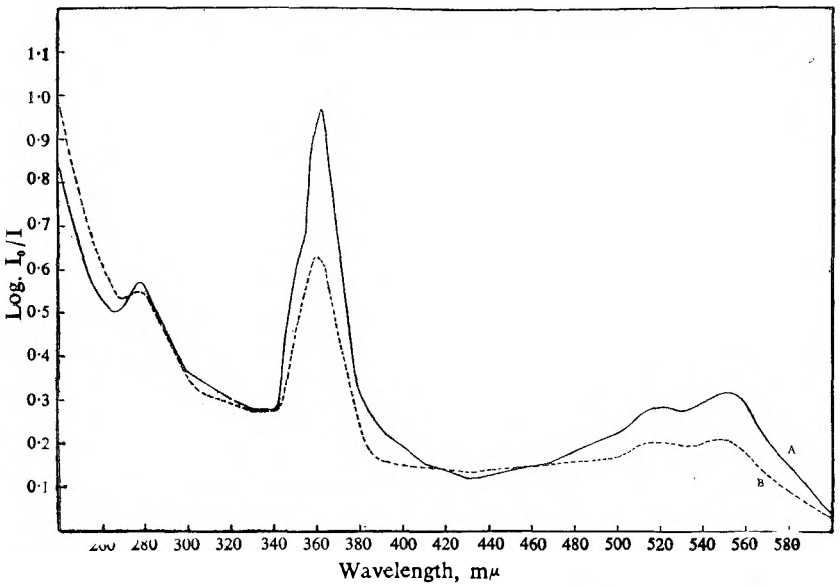

FIG. 3. Absorption spectra of vitamin B_{12}. A, at *p*H 9·0; B, at *p*H 9·0 after heating for 30 minutes at 115°C.

TABLE III

STABILITY OF VITAMIN B_{12} AT *p*H 9·0

	Spectrophotometric assay ex $E_{361m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution	46·9	—	48·8	—
After standing for 7 days in the dark...	46·9	—	48·8	—
After heating for 30 minutes at 115°C.	30·4	35·2	0·4	99·2

At *p*H 2·0, vitamin B_{12} was less stable than at *p*H 4·0, although the stability is relatively much greater than in an alkaline medium. Figure 5 and Table V show the stability results obtained at *p*H 2·0. Losses found

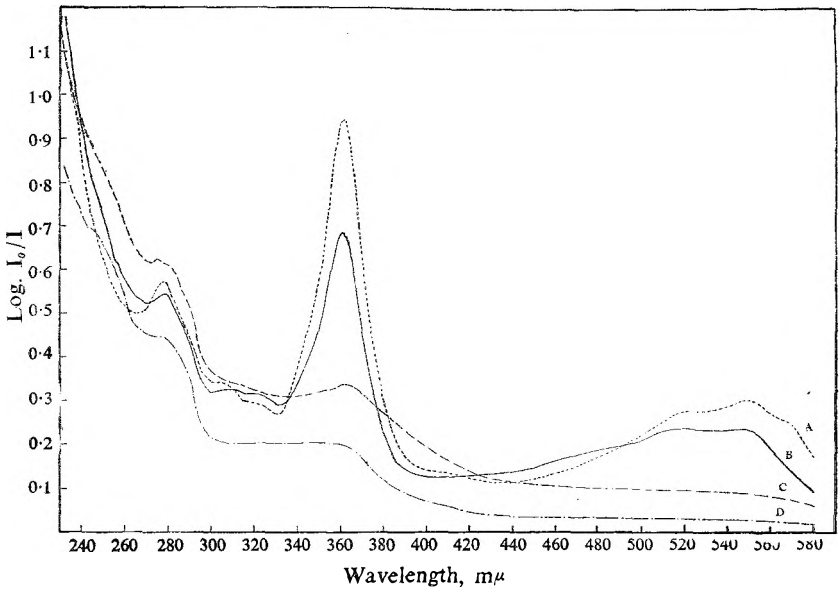

FIG. 4. Absorption spectra of vitamin B_{12}. A, at *p*H 12·0 determined immediately after adjustment; B, at *p*H 12·0, after standing in the dark for 24 hours; C, at *p*H 12·0, after heating for 30 minutes at 115°C.; D, at *p*H 12·0, after heating for 60 minutes at 115°C.

TABLE IV

STABILITY OF VITAMIN B_{12} AT *p*H 12·0

	Spectrophotometric assay ex E_{361} mμ		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution	45·7	—	—	—
After standing in the dark for 24 hours	33·1	27·6	2·6	—
After heating for 30 minutes at 115°C.	16·2	64·5	No response	100·0
After heating for 60 minutes at 115°C.	9·7	78·8	No response	100·0

microbiologically were again appreciably greater than spectrophotometric losses. The spectrum obtained after prolonged autoclaving is characteristic of the acid decomposition products obtained; among them being substituted benziminazoles[23,24].

Sterilisation of vitamin B_{12} solutions. In view of the significant, though relatively slight, decomposition of vitamin B_{12} occurring on autoclaving even in the *p*H range 4·0 to 7·0 and in view of the relatively larger loss under these conditions shown microbiologically, it is our opinion that the use of heat should be avoided in the sterilisation of solutions

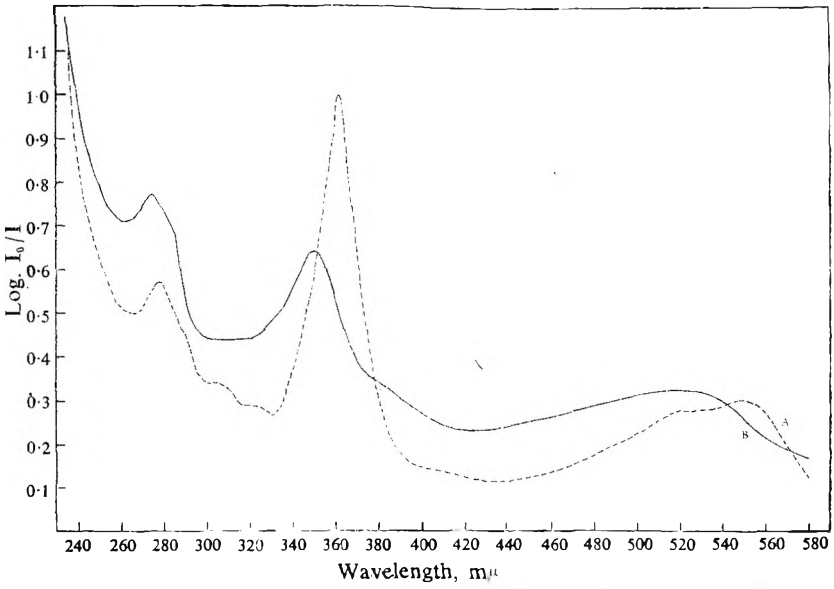

FIG. 5. Absorption spectra of vitamin B_{12}. A, at *p*H 2·0; B, at *p*H 2·0 after heating for 6 hours at 115°C.

TABLE V

STABILITY OF VITAMIN B_{12} AT *p*H 2·0

	Spectrophotometric assay ex $E_{361m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution	48·3	—	47·4	—
After standing for several days in the dark	48·3	—	47·4	—
After heating for 30 minutes at 115°C.	39·9	17·4	24·3	48·7
After heating for 6 hours at 115°C. ...	30·9*	36·0	No response	100·0

* Maximum at 360 mμ.

of vitamin B_{12} for injection. Although it is recognised that the microbiological assay of vitamin B_{12} is notoriously unreliable, on the basis of many results we consider the loss on autoclaving to be significant.

Stability of vitamin B_{12} in the presence of phenolic bacteriostatics. In view of the conclusion reached that vitamin B_{12} solutions should not be sterilised by autoclaving and the possibility of use in multi-dose containers, the stability of vitamin B_{12} solution in the presence of phenols was accordingly studied. Spectrophotometrically, although the peak at 278 mμ could not be realised owing to the presence of the phenol, there was no change after standing for 2 months in the presence of 0·5 per

cent. of phenol. Table VI shows the microbiological results, which also indicated that there was no loss of potency. Similar results were obtained in the presence of 0·3 per cent. of cresol B.P. Chlorocresol 0·2 per cent. has also been found satisfactory.

TABLE VI

MICROBIOLOGICAL RESULTS OBTAINED FOR VITAMIN B_{12} SOLUTIONS IN THE PRESENCE OF 0·5 PER CENT OF PHENOL

Time	Vitamin B_{12} μg./ml.	Average μg./ml.
1 week ...	13·9	—
	12·8	—
	12·0	12·9
2 weeks...	12·3	—
	12·8	—
	12·2	12·4
6 weeks...	12·2	—
	12·8	—
	12·2	12·4

In the light of the above results we consider that sterilisation of solutions of vitamin B_{12} for injection should be effected by filtration. In cases where the presence of a bacteriostatic is required, 0·5 per cent. of phenol or 0·3 per cent. of cresol B.P. may be used. The *p*H of the final solution should preferably be between 4·5 and 6·5.

Stability to other substances. In addition to its instability in relatively acid or alkaline solution, vitamin B_{12} has been shown to be unstable to hydrogen peroxide[27], sodium bisulphite[36], cysteine hydrochloride, hydroquinone[37] and thioglycollic acid[38]. In general, therefore, in pharmaceutical practice it should be regarded as incompatible with oxidising or reducing agents. For example, vitamin B_{12} is not stable in the presence of ascorbic acid, a solution containing 10 microgrammes of vitamin B_{12} and 1 mg. ascorbic acid per ml. becoming colourless within 24 hours and being then inactive to *L. lactis* Dorner. This result is in conformity with those reported by Gakenheimer and Feller[39].

SUMMARY AND CONCLUSIONS

1. The absorption spectrum is a convenient method of establishing the purity of vitamin B_{12} the maxima at 278, 361 and 548 mμ exhibiting definite ratios for which limits can be fixed. Under these conditions the concentration of vitamin B_{12} in solutions containing substantially pure material can be inferred from determination of the absorption value at 361 mμ.

2. While aqueous solutions of vitamin B_{12} undergo variation in absorption spectra within a few days on exposure to sunlight, such changes do not occur during exposure to the diffuse daylight of a normal room for one week. They can, therefore, be regarded as stable during normal handling for analysis, filling or packing.

3. Aqueous solutions of vitamin B_{12} undergo decomposition, especially on heating, at *p*H 2·0 and more rapidly at *p*H 9·0 and *p*H 12·0.

4. Aqueous solutions of vitamin B_{12} are stable at *p*H 4·0 and 7·0 at normal temperatures, but undergo slight but significant decomposition during autoclaving at 115°C. for 30 minutes. Less pure samples of vitamin B_{12} suffer greater loss on autoclaving in solution. It is, therefore, recommended that the use of heat should be avoided in the sterilisation of solutions of vitamin B_{12} for injection, and that the *p*H of the final solution should preferably be between 4·5 and 6·5.

5. No evidence was found, either spectrophotometrically or microbiologically, of deterioration of pure vitamin B_{12} on storage for 2 months in 0·5 per cent. phenol solution or 0·3 per cent. cresol solution. It is, therefore, recommended that solutions of vitamin B_{12} for injection should be sterilised by filtration, a suitable bacteriostatic being added if necessary.

6. Vitamin B_{12} should be regarded as unstable in relatively acid or alkaline solutions and in the presence of oxidising or reducing substances.

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DISCUSSION

An abstract of the paper was read by Dr. Hartley.

The CHAIRMAN (Mr. A. D. Powell) said that the paper indicated the rapidity with which discoveries were being made; when the abstract was written only vitamins B_{12a}, B_{12b} and B_{12c} were known, but Dr. Hartley now referred to a vitamin B_{12d}. The paper illustrated the great use which could be made of absorption spectrometry, and the authors were to be congratulated on the practical data on the preparation of stable solutions of this vitamin.

DR. G. E. FOSTER (Dartford) asked if the authors had done any work on the adsorption of vitamin B_{12} on the filter during sterilisation.

DR. W. F. J. CUTHBERTSON (Greenford) asked if any significant *p*H changes took place during Seitz filtration and, if so, to what extent, and what steps had been taken to cope with them. Had any stability experiments been done at elevated temperatures corresponding to tropical conditions?

PROFESSOR H. BERRY (London) asked if any decomposition measurements had been made at 100°C. As the vitamin was compatible with 0·2 per cent. of chlorocresol it should lend itself to sterilisation by heating with a bactericide. Did the authors suggest buffering the solution and, if so, what buffer would they use?

DR. F. HARTLEY replied that they had examined the adsorption of vitamin B_{12} on the filter. As the final solution had a concentration of the order of micrograms per ml. it was possible to pass a concentrated solution through the filter and to remove the adsorbed material by washing with a large amount of diluent. The changes in the *p*H values were not significant. They aimed to prepare a solution of *p*H between 4·5 and 6·5 which gave the maximum stability. All the experiments had been carried out at about 25°C. The solution of vitamin B_{12} was made isotonic by means of sodium chloride, and they had not found it necessary to use any special buffer. Difficulties had been reported in investigations on heating at 100°C. with a bactericide due to trace impurities in the phenolic bacteriostatics. As there was some decomposition caused by heat there was probably some decomposition at 100°C. and possibly even at 25°C. The experiments were being continued.

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART II.—THE BACTERICIDAL EFFECTS OF CERTAIN SUBSTANCES DURING THE SPRAY DRYING PROCESS

BY KENNETH BULLOCK AND E. A. RAWLINS

From the Department of Pharmacy, University of Manchester

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INTRODUCTION

IN previous communications from this Department[1,2] the production of spray-dried bacterial cultures has been described, and some of the effects of heat and of storage on these cultures have been examined. It has been shown that *Bacterium lactis aerogenes*, a non-sporing organism, suffers a high mortality on spray-drying, and that the surviving organisms rapidly lose their viability. On the other hand, the spores of *Bacillus subtilis* were not killed during the drying process and maintained their viability on storage. It was thought that the addition of a bactericide to the bacterial suspension before it was dried might result in the production of a sterile powder and, as the concentration of bactericide would rapidly be raised during the drying process, it appeared that sterility of the powder might be obtained by the use of very small initial concentrations of bactericide. Moreover, if a volatile bactericide were used, much or all of it might be volatilised during drying so that the resultant powders would contain traces at most. Such a process should be suitable for the production of injections and food products, and the process would be very useful for the sterilisation of thermolabile substances, as these have been shown to undergo no decomposition when spray-dried[3]. The experiments described in this paper show, however, that very few substances are capable of producing sterility when used for the above purpose, but the diversity of the results obtained prompts further inquiry. A critical examination has therefore been made of the factors involved when micro-organisms are spray-dried in the presence of potentially toxic substances and the significance of the observed results has been discussed.

EXPERIMENTAL TECHNIQUE

The Test Organism. When testing a process to ascertain if it can bring about sterility it is necessary to impose upon it the severest possible conditions. The experiments described in the present communication have therefore been performed using an organism known to exhibit a high resistance to disinfectant processes. For this purpose a suspension of spores of *B. subtilis* (Marburg) was prepared as previously described[2].

Preparation of Spray-dried Powders. The technique of preparing spray-dried powders containing micro-organisms has already been described[1,2]. In the earlier experiments presented in the present paper peptone-water was used as the substrate on which to dry the bacteria.

This was prepared as before[2] and inoculated with sufficient of the suspension of the test organism to give an expected count of 2×10^5 organisms per ml. The suspension was fed to the dryer and the resultant powder collected. It is known, however, that protein matter reduces the disinfectant action of some phenolic compounds[4] and of some other substances. Moreover, during the later stages of the drying process it might be expected that the organisms would become coated with a continuous film of the colloidal material which would reduce the effectiveness of gaseous bactericides. Some of the experiments were therefore repeated using lactose solution as the substrate in which to suspend the bacteria. The utility of lactose, however, is limited by the fact that at 130°C. it loses water of crystallisation and becomes hygroscopic, while at higher temperatures it melts. It is difficult, therefore, to prepare a spray-dried lactose powder which is free from stickiness and which can be sampled satisfactorily. In later experiments it was found that the spores could be dried using a solution of sodium acid phosphate as substrate, without suffering any significant mortality. The resultant powder was mobile and rather dense, making it easy to handle, the concentration of its solutions can be estimated chemically and it undergoes complete dehydration in the dryer. Several experiments were therefore carried out using sodium acid phosphate as substrate. Prior sterilisation of the various substrates was effected by autoclaving or, where this was inapplicable, by filtration. Non-volatile solid bactericides were added to the substrate before sterilising it, while volatile and liquid substances were added after sterilisation and immediately before drying, as also was the suspension of spores.

Reconstitution of the Bacterial Suspension and Estimation of Mortality During Drying

In order to estimate the mortality occurring during drying, portions of the spray-dried powder were dissolved in sterile water and viable counts were performed on the original and reconstituted suspensions. The relative strengths of the two suspensions were then compared by estimating their content of one non-volatile component. The following methods of estimation were used:—

(a) Peptone was estimated as before[2].

(b) In the case of lactose 0·001 per cent. of ferrous sulphate was incorporated in the original suspension and the iron was estimated colorimetrically by means of ammonium thioglycollate.

Suspensions containing sodium sulphate were similarly estimated.

(c) The B.P. assay processes were used for estimating sodium chloride, sodium phosphate, sodium acid phosphate, sodium nitrite, sodium carbonate, potassium chloride, and potassium chlorate, and the B.P.C. method for estimating calcium formate.

Potassium bromide was estimated by the method used for sodium chloride.

(d) In the case of the solution containing lactic acid and sodium acid

phosphate the latter was converted to the magnesium ammonium salt which was filtered off and ignited and the pyrophosphate weighed.

Method of Performing Viable Counts. Roll-tube counts were performed using graduated pipettes for mixing and measuring the bacterial suspensions. The technique employed was that previously described and tested[2]. In most cases viable counts were performed on 10^{-3} dilutions of the original and reconstituted suspensions, though in some of the earlier experiments lower dilutions were used. In all cases, however, the bactericides present were diluted sufficiently to ensure that they would have no inhibitory effect on the growth of the organisms in the culture medium.

The Spray-Drying of Organisms with Bactericides

Terminology. While the presence of viable organisms in a liquid or powder cannot be disproved without examining the whole of the material, the evidence provided by viable counts performed on small samples is sufficient to establish that the degree of contamination is low enough to be regarded as insignificant. The term "sterility" is, therefore, used in this paper to indicate that no viable organisms have been demonstrated by means of roll-tube counts.

The term "mortality" is used to represent the fall in viable count during spray-drying. It is estimated as a percentage of the number of organisms present in the original suspension.

The "inlet temperature" is the temperature of the air entering the main chamber of the drier.

The "substrate" is the substance, or mixture of substances, which, occurring in high concentration in the original suspension fed to the drier, forms the main bulk of the spray-dried powder.

The Effect of Drying on Salt Substrates. In our earlier experiments[2] the organisms were dried suspended in plain peptone water containing no added sodium chloride, as it was thought that this might itself exert a lethal effect on the organisms in the later stages of the drying process, since the concentration of the salt would then be high. An examination of the effect of drying on a substrate of sodium chloride alone was therefore made. Using a 5 per cent. solution of sodium chloride as substrate and inlet temperatures of 190°C. a mortality of about 50 per cent. was obtained. A 2 per cent. solution gave a slightly lower mortality. The 5 per cent. solution was also dried using a lower inlet temperature in order to increase, if possible, the period of contact of the spores with the concentrated solution of the salt: this resulted in a slightly higher mortality of 60·5 per cent. Raising the inlet temperature to 301°C. resulted in a 99·1 per cent. mortality. Since it is known[5] that salts vary in their bactericidal effect, both anions and cations showing specific activity, further dryings were carried out using a number of different salts as substrates. The results of these experiments are given in Table I.

Three different halides were tested, sodium chloride, potassium

chloride and potassium bromide, and there appears to be no significant difference between the mortalities they produced. Sodium sulphate produced a much lower mortality than the halides, possibly owing to the

TABLE I

Substrate	Inlet temperature	Mortality per cent.
Sodium chloride, 2 per cent	198°C.	41·5
" " 5 "	191°C.	54·8
" " " "	190°C.	45·2
" " " "	85°C.	60·5
" " " "	301°C.	99·1
Potassium chloride, 2·56 per cent.	195°C.	53·3
Potassium bromide, 4·0 per cent.	185°C.	48·5
Sodium sulphate, 4·80 per cent.	191°C.	20·5
Sodium dihydrogen phosphate, 5·34 per cent.	188°C.	3·8
Disodium hydrogen " 12·2 per cent.	185°C.	6·6
Sodium carbonate, 2·42 per cent.	200°C.	68·4
Sodium nitrite, 2·36 per cent.	187°C.	98·3
Potassium chlorate, 4·19 per cent.	200°C.	77·8
Boric acid, 3·00 per cent.	200°C.	60·2
Calcium formate, 4·45 per cent.	200°C.	73·6
Sodium chloride, 5 per cent., and polyoxyethylene stearate, 0·1 per cent.	202°C.	16·3
Sodium dihydrogen phosphate, 2 per cent., and sodium chloride, 2 per cent.	180°C.	27·4
Sodium thiosulphate, 5 per cent.	192°C.	38·1

formation of the anhydrous salt, which has a limited solubility at temperatures above 33°C. Oxidising and reducing agents are known to be toxic to bacteria, and potassium chlorate, sodium nitrite and calcium formate all produced fairly high mortalities. Boric acid and sodium carbonate produced mortalities of the same order, while sodium thiosulphate proved to be less toxic. It is interesting to note that when the spores were dried with sodium chloride as substrate in the presence of a polyoxyethylene stearate the spores were protected against the lethal action of the halide. This may be because the polyoxyethylene stearate is a waxy solid and on drying it forms a protective coating over the organisms. The most interesting result, however, is that of drying the spores on a phosphate substrate. Both sodium acid phosphate and sodium phosphate, alone among the salts tested, produced no mortality. Further work is being undertaken to elucidate the significance of this.

Phenolic Compounds. The mortalities produced by spray-drying the spores with various phenolic substances are recorded in Table II. In the work previously published, mortalities have been demonstrated varying between −15 per cent. and +12 per cent. when spray-drying *B. subtilis* spores in peptone water *in the absence of antiseptics. The number* of observations was too small to provide an accurate index of variation, but it would appear unwise to consider any mortality of less than 15 per cent. to be significant. It is evident, therefore, that both phenol and chlorocresol are ineffective in the presence of peptone and also when dried with lactose.

Resorcinol *gives the* highest *mortality* exhibited by the pure phenols, a finding in line with that of Twort and Baker[6], who found resorcinol to be the most effective phenol of those they tested for activity in bac-

tericidal mists. Phenylmercuric nitrate 0·002 per cent. shows about the same mortality as resorcinol 0·5 per cent.

TABLE II

Substrate	Bactericide	Inlet temperature	Mortality per cent.
Peptone, 4 per cent.	Phenol, 1 per cent.	165°C.	0·0
Peptone, 4 ,, ...	Phenol, 0·5 per cent.	178°C.	11·1
Peptone, 4 ,, ...	Phenol, 0·5 per cent.	178°C.	12·5
Peptone, 4 ,, ...	Chlorocresol, 0·2 per cent. ...	178°C.	6·9
Peptone, 4 ,, ...	Phenylmercuric nitrate, 0·002 per cent.	175°C.	32·0
Peptone, 4 ,, ...	Resorcinol, 0·5 per cent. ...	199°C.	29·6
Lactose, 4 ,, ...	Phenol, 0·5 per cent.	81°C.	16·2
Lactose, 4 ,, ...	Chlorocresol, 0·2 per cent. ...	148°C.	2·7
Lactose, 4 ,, ...	Phenylmercuric nitrate, 0·002 per cent.	155°C.	37·0
Sodium chloride, 2 per cent. ...	Phenol, 0·5 per cent.	200°C.	61·6
Sodium dihydrogen phosphate, 5 per cent.	Phenol, 0·5 ,, ...	185°C.	67·2

Surface-Active Substances. Surface-active agents may be classified as anionic, cationic, or non-ionic compounds. Studies of the bactericidal activity of the anionic and cationic compounds have been made by several workers[7,8,9], and some of the cationic compounds have been found very effective against Gram-positive organisms. Dunn[10], however, showed that their activity was greatly reduced by serum, and Baker, Harrison and Miller[7] found it to be influenced by *p*H. In Table III the results of spray-drying *B. subtilis* spores in the presence of surface active agents of each type are presented.

TABLE III

Substrate	Surface-active agent	Inlet temperature	Mortality per cent.
Peptone, 4 per cent.	Sodium lauryl sulphate, 0·5 per cent.	185°C.	4·6
Peptone, 4 ,, ...	CTAB.* 0·1 per cent.	191°C.	27·9
Lactose, 4 ,, ...	,, ,,	149°C.	32·1
Sodium dihydrogen phosphate, 5 per cent.	,, ,,	195°C.	87·9
Disodium hydrogen phosphate, 5 per cent.	,, ,,	194°C.	88·6
Lactose, 4 per cent.	Stergene, 0·5 per cent. ...	145°C.	6·6

* Cetyltrimethylammonium bromide.

Phenols Combined with Surface-Active Agents. Apart from their own germicidal activity, surface-active agents may increase the bactericidal effect of other substances by acting as solubilising or emulsifying agents and increasing the rate of penetration. Gershenfeld *et al.*[8] tested a number of detergents in conjunction with phenolic, mercurial and halogenated bactericides, and found that the effect of these was not increased. Valko and Dubois[9] showed that the bactericidal effect of cationic detergents could be nullified by anionic detergents, and Harper[11] has suggested that in order to obtain the maximum bactericidal effect an anionic detergent must always be used with an anionic bactericide and a cationic detergent with a cationic bactericide. Table IV shows

the results obtained on spray-drying *B. subtilis* spores in the presence of phenolic disinfectants together with surface-active agents.

TABLE IV

Substrate	Surface-active agent	Bactericide	Inlet temperature	Mortality per cent.
Peptone, 4 per cent.	Sodium lauryl sulphate, 0·5 per cent. ...	Phenol, 0·5 per cent.	185°C.	26·1
,, ,,	,, ,,	Chlorocresol, 0·2 per cent....	180°C.	17·9
,, ,,	,, ,,	Phenylmercuric nitrate, 0·002 per cent. ...	180°C.	53·8
,, ,,	CTAB, 0·01 per cent.	Chlorocresol, 0·2 per cent....	188°C.	0·0
,, ,,	,, ,,	Phenol, 0·5 per cent.	191°C.	57·8
Sodium chloride, 2 per cent.... ...	Polyoxythylene stearate, 0·1 per cent.	,, ,, ...	200°C.	0·0

Glycols and Hydroxyacids. The use of glycols for the promotion of aerial disinfection has now become an established practice, chiefly owing to the work of Robertson and his associates[12,13]. Puck[14] showed that the activity of these compounds was due to condensation of vapour molecules on the bacteria-carrying particles, thus demonstrating the necessity for the complete vaporisation of glycols. These workers also showed that the maximum activity of the glycols was exerted in atmospheres the relative humidity of which lay between 45 and 70 per cent. De Ome[15] showed that triethylene glycol was 100 times more effective than propylene glycol against *S. pullorum*. Lidwell, Lovelock and Raymond[16] found lactic acid vapour to be an efficient aerial germicide, but with this also the bactericidal activity decreased rapidly in atmospheres having relative humidities below 50 per cent. Table V shows the results obtained when *B. subtilis* spores were spray-dried

TABLE V

Substrate	Bactericide	Inlet temperature	Mortality per cent.
Peptone, 4 per cent. ... ... Sodium dihydrogen phosphate, 5 per cent.	Ethylene glycol, 0·5 per cent.	185°C.	13·5
,, ,, ...	Ethylene glycol, 1·0 per cent.	185°C.	29·0
,, ,, ...	Propylene glycol, 1·0 per cent.	200°C.	66·5
,, ,, ...	Triethylene glycol, 1·0 per cent.	192°C.	53·8
,, ,, ...	Polyethylene glycol, 1·0 per cent.	200°C.	39·2
,, ,, ...	Lactic acid, B.P., 1·0 per cent.	194°C.	88·7

in the presence of four members of the glycol series separately and also in the presence of lactic acid. The first drying was carried out using peptone as substrate, but thereafter it was found that sodium acid phosphate had no lethal effect on the spores and this was used for the remainder of the dryings in order to eliminate the protective action of the peptone colloids.

Lactic acid killed a high proportion of the spores and was superior in this respect to any of the glycols, but the maximum bactericidal activity of the latter was exerted by propylene glycol: triethylene glycol was less active, while the polyethylene glycol had less effect than

triethylene glycol. Ethylene glycol was the least active of the series and showed a negligible activity in the presence of peptone.

Gaseous Antiseptics. Douglas, Hill and Smith[17] showed that sodium hypochlorite solution when sprayed into the air could, even in very high dilution, rapidly kill *Bact. coli,* and these findings were later confirmed by Masterman[18], who concluded that the activity was due to liberation of gaseous hypochlorous acid. Formaldehyde, too, has long been used for the disinfection of unoccupied rooms, and Salle and Korzenovsky[19] showed that it could be used for the sterilisation of materials contaminated with bacteria. These workers also found that ethylene oxide, which has been employed for the fumigation of clothes and seeds, shows low bactericidal activity. Table VI records the mortalities produced by varying concentrations of these substances when solutions of them were spray-dried with *B.* subtilis spores.

TABLE VI

Substrate	Bactericide	Inlet temperature	Mortality per cent.
Peptone, 4 per cent.	Chloroform, 0·5 per cent. ...	168°C.	16·4
,, ,, ...	Milton, 1·0 per cent.	190°C.	6·4
Sodium dihydrogen phosphate, 5 per cent.	,, 0·1 ,, ...	198°C.	100·0
Sodium dihydrogen phosphate, 5 per cent.	,, 0·01 ,, ...	200°C.	20·8
Disodium hydrogen phosphate, 5 per cent.	,, 0·1 ,, ...	196°C.	47·9
Peptone, 4 per cent.	Formalin, B.P., 2·5 per cent....	175°C.	100·0
,, ,, ...	Formalin, B.P., 2·0 per cent....	195°C.	100·0
,, ,, ...	Formalin, B.P., 1·0 per cent....	195°C.	100·0
,, ,, ...	Formalin, B.P., 0·75 per cent....	185°C.	97·0
,, ,, ...	Formalin, B.P., 0·5 per cent....	190°C.	74·3
,, ,, ...	Formalin, B.P., 0·25 per cent....	193°C.	48·5
,, ,, ...	Formalin, B.P., 0·05 per cent....	185°C.	3·0
,, ,, ...	Formalin, B.P., 0·005 per cent.	193°C.	0·0
Lactose, 4 per cent.	Formalin, B.P., 0·5 per cent....	159°C.	100·0
,, ,, ...	Formalin, B.P., 0·05 per cent....	155°C.	100·0
,, ,, ...	Formalin, B.P., 0·005 per cent.	155°C.	67·1
Sodium dihydrogen phosphate, 5 per cent.	Ethylene chlorhydrin, 0·5 per cent.	185°C.	14·4
,, ,, ...	Ethylene oxide, 1·0 per cent....	190°C.	58·8
Disodium hydrogen phosphate, 5 per cent.	,, ,, ...	190°C.	41·8

The sodium hypochlorite solution employed (Milton) contained 1 per cent. of sodium hypochlorite: a 0·1 per cent. dilution of this solution produced complete sterility when sodium acid phosphate was used as substrate, while with sodium phosphate it produced a mortality of 47·9 per cent. In the presence of peptone the hypochlorite was completely inactivated, even when using a 1 per cent. dilution. Formaldehyde was used in the form of Liquor Formaldehydi B.P. Using lactose as substrate a 0·05 per cent. dilution of Liquor Formaldehydi was sufficient to produce sterility, and even a 0·005 per cent. dilution produced a mortality of 67 per cent. Formaldehyde showed the greatest bactericidal activity of any of the substances tested. As might be expected, it was inactivated to a considerable extent by peptone, but in the presence of this a 1 per cent. dilution still produced a 100 per cent. mortality. Ethylene oxide killed 58·8 per cent. of the spores when they were dried on sodium acid phosphate. Since ethylene oxide is more rapidly hydrolysed to ethylene

glycol in acid solution, the experiment was repeated using disodium phosphate as substrate. The mortality produced was, however, slightly lower than that obtained with the acid phosphate. Ethylene chlorhydrin was also employed as it was thought that it might hydrolyse during the drying, producing ethylene glycol and hydrochloric acid gas, but the mortality produced was actually lower than that given by ethylene glycol alone. Chloroform is often used as a preservative, but according to Bunyea[20] its vapour has little germicidal action: it was found to be ineffective when solutions containing it were spray-dried.

Although sodium hypochlorite and formaldehyde produced sterile powders, the utility of these is governed to some extent by the concentration of bactericide that they contain. Estimates of this concentration were therefore made. The available chlorine in the powder prepared from a 5 per cent. solution of sodium acid phosphate containing 1 per cent. of Milton was estimated by dissolving a weighed quantity of the powder in water, adding acetic acid and potassium iodide and comparing the colour with that of standard dilutions of Milton treated similarly, using a photoelectric absorptiometer for the estimation. No detectable amount of chlorine was found in the powder. Since the hypochlorite might be expected to be less stable in acid than in alkaline solution the experiment was repeated using disodium phosphate as the substrate, but again no detectable amount of chlorine was found. The formaldehyde in the powder was estimated colorimetrically by means of chromotropic acid and, using an initial concentration of 0·05 per cent. of formalin, the amount found in the powder was less than 0·01 per cent.

Discussion

In considering the utilisation of spray-drying as a sterilisation process due regard must be paid to the mechanisms involved in both the drying and the disinfection processes It appears probable that the solutions, on leaving the jet of the dryer, are evaporated to dryness very rapidly, in the space of a few seconds[1]. If, therefore, it be assumed that non-volatile substances can exert a bactericidal action only in solution, it is clear that these must have a very rapid action if they are to be used as sterilising agents in the spray-drier. On the other hand, during this period of drying, the solution containing the bactericide is subjected to an increasing degree of concentration which will increase its lethal activity and at the same time bring the bactericide into close contact with the suspended organisms. This enhanced activity, however, will largely be governed by the solubility and concentration exponent of the disinfectant, both of which vary with temperature. The lethal action of substances having a large value for the concentration exponent (' n '), will increase rapidly with increase in concentration, but a limit is set to the latter by the solubility of the substance at the temperature reached in the experiments. For most bactericidal substances the solubility increases with rise in temperature. The value of ' n,' on the other hand, may increase or decrease according to the substance used. Thus the values of

'n' for phenol have been shown by Tilley[21] to decrease with increasing temperature, while those for resorcinol increase. The solubility of resorcinol is also much greater than that of phenol and one might expect, therefore, that resorcinol would prove more effective than phenol in the spray-drier. The results given in Table II show that this was the case. The effect of chlorocresol was negligible and phenylmercuric nitrate, although a more efficient bactericide at lower concentrations than phenol, proved of little value; probably because the former compounds are not very soluble in water. While, however, the relative efficiencies of the phenols were in accordance with expectation, the mortalities produced were all low, except in the experiments where phenol was used with a salt substrate. Using phenol with sodium chloride, a mortality of 61·6 per cent. was obtained, which is approximately equal to the sum of the mortalities produced by these substances separately. A mortality of the same order, was, however, produced using phenol with sodium acid phosphate, a salt which itself has no lethal action. The enhancement of the activity of the phenol might, in the latter case, be due to the acidity of the substrate but the effect of the salts may also be to lower the solubility of the phenol in the aqueous vehicle and to alter the partition of the phenol between that and the lipoid constituents of the cell-wall.

Neither the anionic nor the non-ionic surface-active agents exhibited any significant lethal activity during spray-drying and the cationic compound, cetyltrimethylammonium bromide, produced only a low mortality when dried on peptone or lactose. As cationic surface-active agents are more active in alkaline medium than in acid medium[7] attempts were made to assess this effect by spray-drying the spores with cetyltrimethylammonium bromide using sodium acid phosphate and disodium phosphate as substrates. In each case, however, the activity of the quaternary compound was greatly increased, mortalities of the order of 88 per cent. being obtained, and no significant difference can be attributed to the effect of *p*H. The apparent increase in activity in the phosphate solutions may be due to "salting out" of the detergent or to the protective action of peptone and lactose. It does not appear from the results given in Table IV that the surface-active agents have markedly increased the activity of the phenols, though some increases are observable. Cetyltrimethylammonium bromide gave a diffusible precipitate with chlorocresol and peptone, which may account for the inactivation of the chlorocresol. Polyoxyethylene stearate, on the other hand, gave no precipitate with phenol but nullified the lethal action not only of this but also of the sodium chloride.

Besides the influence of concentration of bactericide on disinfection during spray-drying, the substrate on which the organisms are dried may also affect the process. The rapid evaporation of the solvent concentrates not only the molecules of the bactericide around the organisms but also many more molecules of the substrate. Substrates which do not react chemically with the bactericide at low concentrations may more readily do so when the concentration is increased. Substrates such as

colloids, which show surface activity, may adsorb the bactericide if the substrate is thrown out of solution before completion of drying, and the effective number of molecules of bactericide which can be adsorbed by the bacteria themselves may be too low to have any significant lethal effect. It would appear, therefore, that the possibility of obtaining a sterile powder by spray-drying in the presence of a non-volatile bactericide is not great if colloidal matter is also present, though it is difficult to assess the importance of this effect from the experimental evidence because all the substrates used may have had some influence on the course of the disinfection.

Apart from its effect on bactericides present the substrate may have a direct lethal action on the organisms as the concentration rises, and this may be observed in the results given in Table I of spray-drying the spores using various salts as substrates. Topley and Wilson[22] state that it is doubtful whether salts, except in high concentration, affect bacteria by virtue of their osmotic pressure, although Knaysi[23] demonstrated plasmolysis induced by a 25 per cent. sodium chloride. It does not appear, however, that the results obtained during spray-drying could be attributed to the effects of osmotic pressure alone, for although the initial solutions were approximately iso-osmotic the mortalities produced varied greatly and the phosphate showed no lethal effect at all A limiting factor would, of course, be the solubilities of the salts in water, but when these are compared with the observed mortalities no degree of correlation can be traced. It is not surprising to find this variation in the action of salts, for many workers[5] have noted it when working with solutions. The very great difference between the mortalities produced by the phosphates and the other salts is, however, somewhat unexpected. While a 0·85 per cent. solution of sodium chloride was shown by Flexner[24] to cause disintegration of the meningococcus, *B. subtilis* was shown by Fischer[25] to grow well in an infusion containing 9 per cent. of sodium chloride or 11 per cent. of potassium chloride, and it does not appear, therefore, that the halides are particularly lethal to this organism. A mixture of the phosphate was, in fact, found to have a greater inhibitory effect on the growth of *Bact. coli* than sodium chloride[26], yet when spray-dried, the halides killed half the spores of *B. subtilis* while the phosphates produced no apparent mortality. Whether this is associated with the special position which phosphates occupy in relation to bacterial metabolism is a matter which requires further investigation.

Volatile bactericides may act on organisms either in solution or in the gaseous state. Many of the substances which are actively bactericidal in mists or smokes probably act in solution and Puck[13] has shown that the very high efficiency of the glycols as aerial disinfectants is due to the mobility of the gaseous molecules which collide with the bacteria-carrying particles and dissolve in the condensed layer of water which those particles bear, quickly building up a lethal concentration of the glycol. The conditions necessary for this process cannot, however, be realised in the spray-drier, for, once dried, the bacteria-carrying particles are

carried forward in an atmosphere the relative humidity of which is too low to permit of condensation. It would appear, therefore, that the glycols exert their effect only during the period when the solution is being evaporated to dryness. This is confirmed by the comparatively low mortalities recorded in Table V and by the agreement of these results with the relative activities of the glycols in solution. Thus, although triethylene glycol has been shown to be a much more efficient aerial disinfectant than propylene glycol[14] it is less active in solution than the latter. Robertson[21] has ascribed the superiority of triethylene glycol to its lower aerial saturation value which causes it to condense more readily on to the bacteria-carrying particles but it is clear from the experimental results that no such effect of condensation could be demonstrated. Since, also, lactic acid shows a similar optimum humidity requirement to that of the glycols it seems probable that it acts in the same way and that its activity during spray-drying is confined to the actual period of drying. This is confirmed by a comparison of the phosphate contents of the original and reconstituted solutions, estimated gravimetrically, with the figures obtained by titrating them with alkali in the presence of excess sodium chloride. This shows that no appreciable amount of the lactic acid was vaporised and that therefore the high efficiency of the lactic acid must be due to the rapidity of its action.

The results obtained with sodium hypochlorite and formaldehyde, as shown in Table VI, are very different from the above for extremely low concentrations of these substances were sufficient to produce sterility. Masterman[16] has suggested that sodium hypochlorite acts as an aerial disinfectant owing to liberation of gaseous hypochlorous acid while Puck[13] maintains that the action of the latter is exerted in the same manner as that of the glycols. He contrasted the high efficiency of hypochlorous acid as an aerial germicide with that of chlorine which has little action and ascribed the difference to the much greater solubility of hypochlorous acid in water. Since both hypochlorous acid and formaldehyde in solution are much more lethal to bacteria than the glycols it is not unreasonable to suppose that the sterilisation produced by these substances was effected during the actual drying process but further work is proceeding to determine whether gases can exert a bactericidal action on dry bacteria, and if so to what extent.

It is evident, from the results given in Table VI, that both hypochlorite and formaldehyde could be used successfully for the production of sterile, spray-dried, powders. Certain factors however, militate against their use. They are both inactivated to some extent by protein matter: sodium hypochlorite is, in fact, completely inactivated even when a fairly heavy initial concentration is used. 0·4 per cent. of formaldehyde was sufficient to ensure sterility in the presence of peptone and only 0·02 per cent. was necessary when peptone was absent. The reactivity of these compounds and the ease with which they will combine with many substrates make them less useful than they might otherwise be. Where, however, such a consideration is inapplicable or unimportant the use of hypochlorite or

formaldehyde might find application, provided that the final concentration of these substances in the powder is low enough for the purpose required.

Summary

1. *B. subtilis* spores have been spray-dried, using various substrates with substances known to be bactericidal in solution. Only sodium hypochlorite and formaldehyde were found to be capable of producing sterility in the resultant powders. Phenols, synthetic detergents and glycols killed varying proportions of the spores, but none of compounds showed a high efficiency.

The reasons for this have been discussed.

2. Both sodium hypochlorite and formaldehyde were found to be largely inactivated by peptone, although in its presence formaldehyde produced sterile powders when used in an initial concentration of 0·4 per cent. In the absence of peptone an initial concentration of formaldehyde of 0·02 per cent. and of sodium hypochlorite of 0·001 per cent. was sufficient.

3. The final concentrations of sodium hypochlorite and formaldehyde in the spray-dried powders have been estimated. Using an initial concentration of 0·01 per cent. of sodium hypochlorite no detectable amount of available chlorine could be found in the powder, while an initial concentration of formaldehyde yielded a powder containing less than 0·01 per cent.

4. The effects of spray-drying the bacterial spores using various common salts as substrates have been examined. It was found that, whereas most salts exerted a lethal action on the spores, sodium phosphate and sodium acid phosphate produced no significant mortality. Further work is proceeding on the investigation of this phenomenon.

Our thanks are due to Mr. G. T. Bassil, M.Sc., for carrying out the estimation of formaldehyde in the powder.

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DISCUSSION

An abstract of the paper was read by Dr. Bullock.

MR. G. SYKES (Nottingham) asked for more details of the organism used by the authors. Some time ago they had had organisms of the *subtilis* group which were fairly highly resistant to moist heat. They could easily prepare a dry spore suspension which would stand up to dry heat at 145°C. for one hour, but not 150°C. However, this same organism in aqueous suspension died out fairly rapidly.

PROFESSOR H. BERRY (London) said that several years ago he had had a strain of *B. subtilis* which would stand up to $2\frac{1}{2}$ hours' boiling, but it had died out and he could not reproduce it. It had been grown on 1 in 10 nutrient agar and maintained over calcium chloride. Although he had kept the same strain he had not been able to reproduce the heat-resisting property. It would be interesting to hear the outcome of the authors' work on aerial bactericides. Moisture content was important as it was linked with the lethal action of the bactericides. There was a difficulty in testing when formaldehyde was used as it had to be inactivated. It acted in two stages, the first reversible and the second irreversible, and for this season it was difficult to prove that the formaldehyde had really killed the organisms.

MR. W. JONES (Manchester) asked whether the authors had considered the possibility of a recovery of viability on storing. They had found this to be so with *B. subtilis* spores; after 6 months' storage there was 50 to 80 per cent. recovery. Using a modified agar medium containing starch and phenol red, recovery was demonstrated after 48 hours.

DR. K. BULLOCK, in reply, said that the organism originally came from the National Collection of Type Cultures, Warburg strain. It was not killed by dry heat at 140°C. for 1 hour but was killed at 150°C. The powders were still being stored and it was hoped to give the results of storage experiments later on. As they were concerned with the dry state they had not done much work on wet heat. Formaldehyde and hypochlorite were not seriously put forward as methods of sterilisation—they were only last resorts to see whether any substance would effect sterilisation during spray-drying. He was sure that Mr. Rawlins had stored these powders for some time before reporting them to be sterile, and they had been tested at intervals. He was not sure, however, that Mr. Rawlins had used anything to inactivate the formaldehyde.

THE STABILITY OF INJECTION OF MORPHINE SULPHATE

By G. E. Foster, Miss J. Macdonald and T. D. Whittet

From the Wellcome Chemical Works, Dartford, and the Pharmaceutical Department, University College Hospital, London

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Opium preparations have been used medicinally for several hundred years but, until well into the nineteenth century, only crude preparations were available. Morphine, the most important opium alkaloid, was first isolated by Sertürner[1], a young German pharmacist, in 1805, but it was not until he published a further paper in 1817[2] that the importance of his work was appreciated.

The inclusion of a hypodermic injection of morphine acetate in the Additions, published in 1874, to the British Pharmacopœia 1865, constituted the first official recognition in this country of morphine for injection. The same salt was retained in the British Pharmacopœia 1885, but in the 1898 Pharmacopœia an injection of morphine tartrate was included and this appeared again, although its strength was reduced, in the 1914 Pharmacopœia. In all of these formulæ no directions were given for sterilisation of the injections, which were simply solutions of the appropriate salt in distilled water.

No formula for morphine injection appeared in the British Pharmacopœia 1932 but the British Pharmaceutical Codex 1934 included an injection containing 2·5 per cent. of morphine hydrochloride in water. This was sterilised by heating in an autoclave, by tyndallisation or by filtration. The B.P.C. 1934 formula was shown by Davis[3] to be unsatisfactory, particularly in allowing fungoid growth, and after testing several preservatives he suggested two formulæ. The first contained 2·5 per cent. of morphine hydrochloride with 0·05 per cent. of chlorocresol in distilled water, the injection to be prepared using aseptic precautions and sterilised by heating at 80°C. for one hour. The second contained 2·5 per cent. of morphine hydrochloride with 0·1 per cent. of chlorocresol and was sterilised by autoclaving. A modified formula based on this work was included in the second supplement, published in 1941, to the B.P.C. 1934.

Berry[4] reported that solutions of morphine salts may develop colour during storage or on heating. He concluded that although the hydrochloride, the sulphate and the tartrate of morphine were all used in medicine, it was the sulphate which gave the most stable solutions. He also found that the development of colour is dependent on the *p*H of the injection, the colour developing rapidly in alkaline solution and being retarded by acid. According to Berry a *p*H no higher than 3 is required if discoloration is to be prevented; he was of the opinion, however, that this *p*H was rather too low for an injection. The effect of *p*H on pain, at the site of injection, was investigated by Lupton[5] and was the subject of discussion at the 1945 meeting of The British Pharmaceutical Conference.

An alternative suggestion by Berry in the same paper was the inclusion of 0·05 per cent. of potassium or sodium metabisulphite which is very effective in preventing darkening in colour of the injection. In a footnote, however, he reported that later work had shown that certain qualities of rubber affect metabisulphite and reduce its protective action.

A solution of morphine sulphate, based upon Berry's formula, was introduced into Charing Cross Hospital in 1943 and found to be very satisfactory but, on storage for a few weeks, a brown colour was found to develop in solutions stored in clinbritic bottles. On examination of the solutions it was found that in many cases the bottoms of the rubber caps were bleached and it appeared that the caps were probably absorbing sulphur dioxide or sodium metabisulphite from the solutions. When the rubber caps were soaked in a 0·2 per cent. solution of sodium metabisulphite before use no further cases of darkening were noted. Similar results with injection of adrenaline have been reported by West and Whittet[6].

Morphine sulphate found a place in the Fourth Addendum, published in 1941, to the B.P. 1932 and an injection of morphine sulphate has been included in the B.P. 1948. It therefore seemed of interest to investigate the effect of sodium metabisulphite on the darkening of solutions of morphine sulphate and the effect of caps, treated and untreated with sodium metabisulphite, on such solutions when stored in vaccine bottles. There appears to be little data available on the correlation of colour change to loss of activity and it was thought desirable to ascertain whether such relationship does in fact exist.

Preparation of Morphine Injection. Samples of a solution of morphine sulphate 2·5 per cent. together with 0·2 per cent. of chlorocresol in water for injection were prepared, placed in clinbritic bottles, sealed and sterilised by heating at 98° to 100°C. for 30 minutes. These correspond to the B.P. 1948 injection of morphine and were used as controls. Even when freshly prepared they had a distinct brown colour.

Further samples were prepared exactly as above except that 0·1 per cent. of sodium metabisulphite was added. Half of these samples were stored in clinbritic vaccine bottles sealed with rubber caps which had been soaked for 48 hours in a 0·2 per cent. solution of sodium metabisulphite. The remainder were stored in bottles sealed with rubber caps which had not been so treated. All these solutions were water-white after sterilisation. These samples were stored under various conditions and were examined and tested from time to time.

Spectrographic Studies. Morphine, $C_{17}H_{19}O_3N$, readily undergoes oxidation and one of the main products is pseudomorphine, $C_{34}H_{36}O_6N_2$, isolated from opium by Pelletier[7]. This oxidation product is also known as oxydimorphine, dehydromorphine and oxymorphine and, as its formula indicates, it is formed by a reaction involving 2 molecules of morphine. The suggestion that some conversion of morphine into the comparatively inactive pseudomorphine may occur during sterilisation of solutions by heat[8] and the possibility of similar transformation occur-

ring in the body[9] has created a special interest in methods of detecting and estimating either alkaloid in the presence of the other. Dietzel and Huss[8] found that measurement of the ultra-violet absorption afforded a useful means of differentiating the two alkaloids and applied this technique to the study of solutions of morphine hydrochloride, under varying conditions of oxidation.

In preliminary experiments we were able to confirm the findings of Dietzel and Huss regarding the absorption spectra of the alkaloids. For this work we used a sample of morphine sulphate B.P. $(C_{17}H_{19}O_3N)_2,H_2SO_4,5H_2O$, and a sample of pseudomorphine prepared from morphine by the process of Dietzel and Huss[10] using potassium ferricyanide as oxidising agent. Figure I shows the ultra-violet absorption curves, measured with a Hilger medium quartz spectrograph, of the alkaloids using 0·1N sulphuric acid as solvent. It will be seen that morphine exhibits a well-defined curve with a maximum at $\lambda = 283$ mμ and a minimum at $\lambda = 262{\cdot}5$ mμ. In the case of pseudomorphine, however, only general absorption occurs and the minimum at $\lambda = 262{\cdot}5$ mμ has completely disappeared. Figure 2 shows the ultra-violet absorption curve of morphine sulphate injection, prepared as described above, containing chlorocresol and sodium metabisulphite. Although the chlorocresol and sodium metabisulphite cause some slight increase in intensity of absorption, amounting to about 10 per cent., the shape of the morphine curve is well maintained. It was therefore concluded that measurement of the absorption at $\lambda = 262{\cdot}5$ mμ and $\lambda = 283$ mμ would afford a satisfactory means of following the formation of pseudomorphine in our injections.

Samples of morphine injection, prepared and filled into clinbritic bottles as already described, were stored for 9 months after which their ultra-violet absorption spectra were measured. Examination of several samples immediately after preparation, when they were practically colourless with the exception of those containing no sodium metabisulphite, confirmed that their absorption spectra were substantially as recorded in Figures 1 and 2. After 9 months' storage at room temperature the samples containing sodium metabisulphite had developed a slight colour, there being very little difference between these with caps which had been treated with sodium metabisulphite and those with untreated caps. Samples stored in an incubator at 80°F. had darkened in colour to a greater degree. Under all storage conditions these samples containing no sodium metabisulphite exhibited the greatest degree of discoloration.

For the purpose of measuring their ultra-violet absorption all samples were diluted to contain 0·025 per cent. of morphine sulphate and determinations were made using 1 cm. cells. Table I summarises the results.

These spectrographic figures gave little, if any, indication of the formation of pseudomorphine in the injections although, under the experimental conditions, it was expected that the presence of 5 per cent. or more of pseudomorphine would have been detectable.

TABLE I

ULTRA-VIOLET ABSORPTION OF SAMPLES, AFTER 9 MONTHS' STORAGE, DILUTED TO CONTAIN THE EQUIVALENT OF 0·025 PER CENT. OF MORPHINE SULPHATE

Sample	Log $\frac{I_0}{I}$ for 1 cm. cell at $\lambda = 283$ mμ	Log $\frac{I_0}{I}$ for 1 cm. cell at $\lambda = 262{\cdot}5$ mμ
2·5 per cent. morphine sulphate (2.6.48)	0·95	0·25
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol (2.6.48)	1·00	0·30
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite (2.6.48)	1·00	0·35
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol steamed 30 minutes (9.7.48)	0·98	0·32
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite ; cap soaked ; steamed 30 minutes (9.7.48)	0·98	0·34
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite ; cap unsoaked ; steamed 30 minutes (9.7.48)	0·95	0·30
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol ; steamed 30 minutes (8.4.49)	1·00	0·40
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite ; cap unsoaked ; steamed 30 minutes (8.4.49)	1·00	0·35
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite ; steamed 30 minutes ; cap soaked ; stored in inverted position (8.4.49)	0·95	0·40
2·5 per cent. morphine sulphate + 0·2 chlorocresol, steamed 30 minutes inverted and incubated at 80°F. (8.4.49)	1·00	0·30

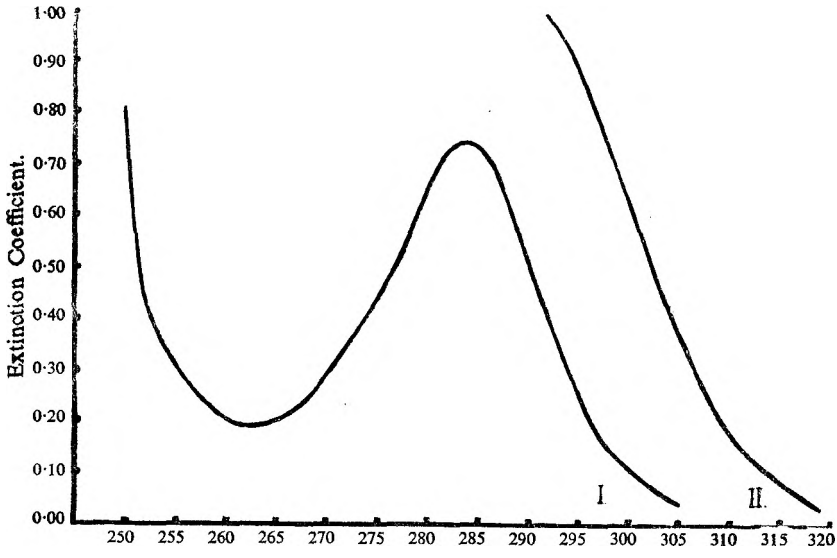

FIG. 1. Ultra-violet absorption curves of morphine and pseudomorphine: I, morphine sulphate 2·289 mg./10 ml. of 0·1N sulphuric acid, II, pseudomorphine 2·372 mg./10 ml. of 0·1N sulphuric acid.

Colorimetric Estimation of Pseudomorphine. Thörn and Ågren[11] recently reported that both morphine and pseudomorphine give colours with aromatic aldehydes in the presence of sulphuric acid. Using a 1 per

cent. solution of vanillin in sulphuric acid (95 per cent.), they found that solutions of pseudomorphine gave a characteristic green colour having maximum absorption at $\lambda = 600$ mμ while morphine afforded a reaction mixture exhibiting little absorption in this region. The reaction was claimed to be suitable for the estimation of pseudomorphine in

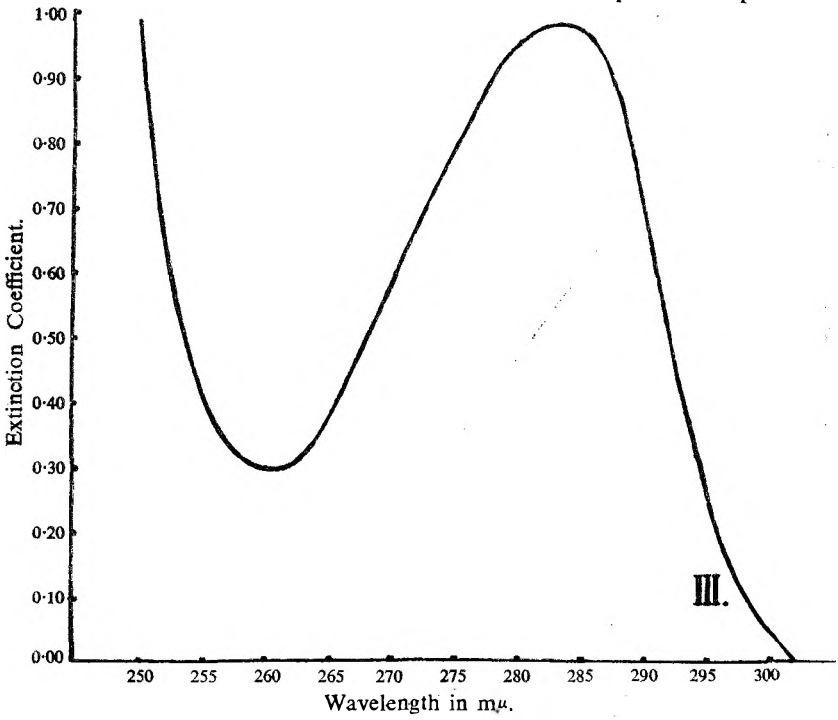

FIG. 2. Ultra-violet absorption curve of 2·5 per cent. morphine sulphate solution containing 0·1 per cent. of sodium metabisulphite and 0·2 per cent. chlorocresol; steamed for 30 minutes (diluted 1 in 100).

morphine injections. For this purpose 0·5 ml. of injection to be tested is placed in a dry test tube and treated with 10 ml. of vanillin reagent, which is slowly added from a pipette with continuous shaking and cooling. The mixture is heated in a boiling water-bath for 20 minutes, cooled in running water and the absorption at $\lambda = 600$ mμ measured. Thörn and Ågren[11] have published absorption curves for the reaction mixtures obtained both with morphine and pseudomorphine.

On trying the vanillin reagent we at once confirmed its sensitivity for the detection of pseudomorphine. A series of standard solutions was prepared by adding known amounts of pseudomorphine to samples of our morphine injection, containing 2·5 per cent. of morphine sulphate, 0·2 per cent. of chlorocresol and 0·1 per cent. of sodium metabisulphite. The vanillin reaction was then carried out on each sample. The technique which we adopted was very similar to that of Thörn and Ågren but we cooled the test tube in ice during the addition of the reagent and the subsequent mixing operation. Readings on each reaction mixture

were then taken with a Spekker absorptiometer using No. 607 Ilford filters and employing a water cell as the blank. Figure 3 shows the calibration curve constructed from the figures obtained. Different batches of vanillin reagent often gave different absorptiometer readings and we found it desirable to use freshly prepared reagent and to construct a calibration curve for each batch.

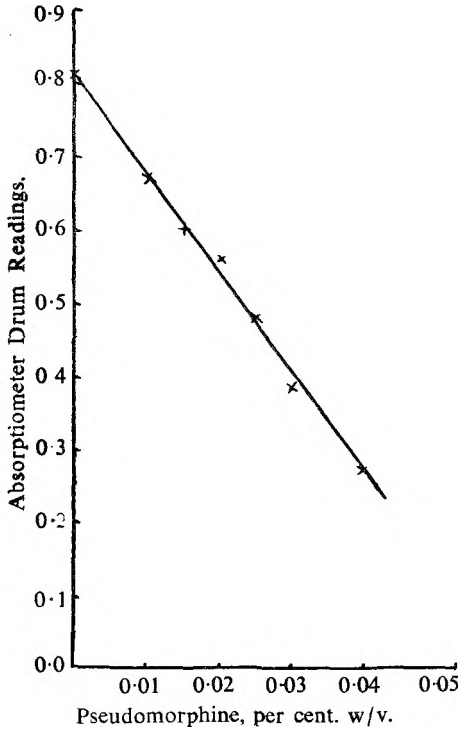

FIG. 3. Calibration curve for pseudomorphine in morphine injection; with Ilford 607 and heat resisting H503 filters.

Samples of morphine injection, prepared as for the spectrographic investigation, were divided into two groups which were stored at room temperature and in an incubator at 55°C. respectively. When freshly prepared the maximum amount of pseudomorphine estimated by the vanillin reagent in any sample tested did not exceed 0·002 per cent. At the end of three months samples when tested gave the results summarised in Table II.

It was thought of interest to carry out colorimetric determinations on some of the samples which had been

TABLE II

PSEUDOMORPHINE CONTENT, ESTIMATED BY VANILLIN REAGENT, IN MORPHINE INJECTION AFTER 3 MONTHS STORAGE

Sample	Pseudomorphine content, calculated from Absorptiometer readings	
	Stored at room *temperature*	Stored at 55°C.
	per cent.	per cent.
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol steamed for 30 minutes (12.12.49)	0·0029	0·0083
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite, steamed 30 minutes caps unsoaked (12.12.49)	0·0016	0·0030
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite, steamed 30 minutes caps soaked (12.12.49)	0·0015	0·0022
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite, steamed 30 minutes caps unsoaked, inverted (12.12.49)	0·0014	—

opened after 9 months and had been subsequently stored at room temperature for a further 3 months. The results are shown in Table III.

TABLE III

PSEUDOMORPHINE CONTENT, ESTIMATED BY VANILLIN REAGENT, OF MORPHINE INJECTIONS, OPENED AFTER 9 MONTHS, RESEALED AND STORED FOR A FURTHER 3 MONTHS AT ROOM TEMPERATURE

Bottle	Contents	Pseudomorphine content, calculated from absorptiometer readings
		per cent.
H	M.S.C. Caps soaked	0·012
E	M.S.C. Caps soaked	0·013
B	M.S.C. Inverted caps soaked	0·018
C	M.S.C. Caps unsoaked	0·021
U	T.M.S.C. Inverted caps unsoaked	0·024
R	M.S.C. Inverted caps unsoaked	0·027
A	M.S.C. Inverted caps unsoaked	0·030
Y	M.S.C. Inverted caps unsoaked	0·033
V	M.S.C. Caps unsoaked	0·033
Q	T.M.S.C. Inverted caps soaked	0·035
K	T.M.S.C. Caps soaked	0·035
W	T.M.S.C. Caps unsoaked	0·037
X	M.C.	0·035
T	M.C.	0·036
D	M.C. Inverted	0·043
S	T.M.C.	0·055
Z	T.M.C. Inverted	0·056

M = 2·5 per cent. morphine sulphate solution. S = 0·1 per cent. sodium metabisulphite solution
C = 0·2 per cent. chlorocresol solution. T – Stored at 80°F. for first 9 months.

Pharmacological Tests. In view of the small amount of pseudomorphine found in the injection, even when exhibiting discoloration, it was felt desirable to test two of the most discoloured samples, which had been stored at 80°F. for 9 months, for analgesic activity. Mr. A. F. Green, of the Wellcome Research Laboratories, Beckenham, carried out these experiments and we are indebted to him for the results which are summarised in Table IV.

TABLE IV

ANALGESIC TESTS ON MORPHINE INJECTIONS

The morphine was injected subcutaneously in rats using a quantal heat response

Solution	Dose of morphine sulphate mg/K.		
	2·0	*1·5*	*1·125*
Standard 2·5 per cent. morphine sulphate	28/30	21/30	17/30
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol steamed 30 minutes. Incubated at 80°F.	26/30	20/30	13/30
2·5 per cent. morphine sulphate + 0·2 per cent. chlorocresol + 0·1 per cent. sodium metabisulphite, steamed 30 minutes caps soaked. Incubated at 80°F.	27/30	19/30	17/30

There did not appear to be any significant difference between the activities of the injections and of the laboratory standard morphine sulphate

solution. Any observed differences were obviously within the experimental error of the tests.

Effect of Rubber Caps on Metabisulphite Solutions

Since the results of storage tests in this instance were indecisive with respect to absorption of metabisulphite the following tests were performed:—One litre of 0·1 per cent. sodium metabisulphite solution was prepared and divided into two parts. One half was placed in a corked flask and one dozen unused clinbritic rubber caps was added. The other half was placed in a similar flask and kept as a control. Another batch of caps was placed in distilled water as a control on the appearance of the caps. The flasks were stored at room temperature.

5 ml. quantities of the solution from the caps and the control solution were titrated regularly against 0·01N iodine solution. The results are shown in the Table V and are plotted in the graph (Fig. 4). They show

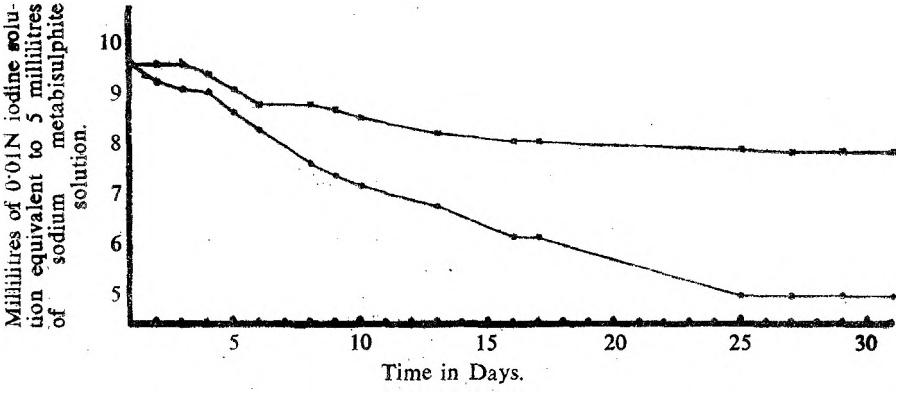

Fig. 4. The effect of storing clinbritic rubber caps in sodium metabisulphite solution (0·1 per cent.). Upper graph, solution only; lower graph, solution containing caps.

that there is considerable absorption of metabisulphite for between 18 to 26 days after which the loss in strength of the solution containing the caps was practically the same as that of the control (unfortunately it was not possible to take daily readings between the 18th and 26th days) probably indicating that the caps had become saturated with metabisulphite solution.

The caps stored in sodium metabisulphite were bleached almost white and were very much lighter than the control caps stored in water, although these were lighter than unused caps. The solution containing the caps had a slight odour of rubber with practically none of sulphur dioxide, whereas the control sodium metabisulphite solution had a very marked odour of sulphur dioxide.

From the readings obtained with the control solution it appears that

there is a slight deterioration of sodium metabisulphite in simple solution.

The above results suggest that it might be advisable either to increase the strength of sodium metabisulphite used for the preliminary soaking of the caps or to increase the duration of the soaking.

TABLE V

TITRATION OF SODIUM METABISULPHITE SOLUTIONS (0·1 PER CENT.) AGAINST 0·01 N IODINE. 5 ML. OF SODIUM METABISULPHITE SOLUTION USED.

Day	Test solution	Control solution
1	10·10	10·10
2	9·75	10·10
3	9·60	10·10
4	9·55	9·90
5	9·15	9·60
6	8·55	9·30
8	8·15	9·30
9	7·90	9·20
10	7·70	9·05
13	7·30	8·75
16	6·70	8·60
17	6·70	8·60
25	5·55	8·45
27	5·55	8·40
29	5·55	8·40
31	5·55	8·40

DISCUSSION

The work described in the present communication has confirmed that the B.P. injection of morphine sulphate progressively darkens in colour on storage. This colour change is accelerated at tropical temperatures. Addition of 0·1 per cent. of sodium metabisulphite retards, but does not entirely prevent, the development of colour.

As a result of our spectrographic and colorimetric studies it appears that the development of colour in the injection is not due to the formation of pseudomorphine, which occurs as colourless crystals yielding colourless solutions in dilute mineral acids. Further evidence in support of this conclusion emerged when an old sample of morphine hydrochloride injection, adjusted to *p*H 3·2 in order to retard colour formation, was examined. This injection contained 1·6 per cent. of morphine hydrochloride and 0·1 per cent. of chlorocresol and had been sterilised by heating in an autoclave. A colorimetric assay indicated that it contained 0·1 per cent. of pseudomorphine although it had become only slightly discoloured during 7 years' storage at room temperature.

The small amount, not exceeding 0·05 per cent., of pseudomorphine which we found in our injections is in harmony with the results obtained by Dietzel and Huss[8] with morphine hydrochloride solutions. These workers, using a spectrographic technique, failed to detect pseudomorphine in injections, adjusted to *p*H 3·24, after 120 minutes' heating in a boiling water-bath. Under the same conditions very little change occurred when nitrogen was passed through the solution. When nitrogen was replaced by oxygen, pseudomorphine was progressively formed. A similar change was brought about by heating in an autoclave at 150°C.

The vanillin colour reaction, introduced by Thörn and Ågren[11], has been found much more sensitive for the detection of pseudomorphine than the spectrographic method. The latter, however, affords a useful indication of the morphine content of an injection by virtue of the prominent absorption band with maximum at $\lambda = 283$ mμ. With injections which have become discoloured the vanillin reagent affords brownish green colours instead of the pure green yielded by pseudomorphine. For this reason our results have been recorded as "pseudomorphine content, calculated from absorptiometer readings," as it is appreciated that some absorption in the region of $\lambda = 600$ mμ might be due to products other than pseudomorphine. Nevertheless, for purposes of following the changes in morphine injection on storage the solution of vanillin in sulphuric acid (95 per cent.) is a valuable reagent.

One of the chief purposes of this investigation was to examine the effect on the stability of morphine injection containing sodium metabisulphite, of treating the rubber caps of the containers with sodium metabisulphite. In our experiments using clinbritic vaccine bottles very little, if any, difference between treated and untreated caps was observed. This was in marked contrast to the experience at Charing Cross Hospital during the war, and we have concluded that the composition of the rubber has much to do with the behaviour of the caps.

It appears that with the rubber caps now supplied with clinbritic bottles there is little danger of the protective action of sodium metabisulphite being seriously reduced but, in view of the earlier experience of Berry[4] and of West and Whittet[6], the preliminary soaking in sodium metabisulphite solution as directed by the Pharmacopœia would seem a wise precaution, unless the caps are known to be satisfactory.

We are well aware that the quality of commercial morphine salts varies, and even if two batches of salt are obtained from the same manufacturer they may yield solutions which discolour at different rates under identical conditions. It is against this background that our results must be assessed but we are of the opinion that the addition of sodium metabisulphite to morphine injection provides the best means so far available of preventing discoloration. This discoloration appears pharmacologically to be of little importance but pharmaceutically its prevention is most desirable.

Summary

1. Samples of morphine injection, containing 2·5 per cent. of morphine sulphate and 0·2 per cent. of chlorocresol have been prepared, filled into clinbritic vaccine bottles and sterilised by steaming for 30 minutes. Some samples contained, in addition, 0·1 per cent. of sodium metabisulphite.

2. The clinbritic bottles were divided into two groups, closed by rubber caps which had and had not been soaked in 0·2 per cent. sodium metabisulphite solution respectively.

3. Samples of injection were stored at room temperature at 80°F. and at 55°C. and examined at intervals.

4. Spectrographic measurements and colorimetric estimations, using

the vanillin reagent of Thörn and Ågren, have been made in order to follow the changes in morphine injection on storage. The colorimetric method has proved useful for the detection of psuedomorphine.

5. The presence of sodium metabisulphite retards but does not entirely prevent the discoloration of morphine injection. This discoloration does not appear to be due to the formation of pseudomorphine.

6. A pharmacological test revealed no decrease in the analgesic activity of discoloured samples of morphine injection.

7. In the experiments recorded little, if any, difference in behaviour was observed between caps treated and untreated with sodium metabisulphite.

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DISCUSSION

An abstract of the paper was read by Mr. Whittet.

THE CHAIRMAN said that he had found discoloration in sealed glass ampoules, particularly with morphine sulphate, which was accompanied by a fall in *p*H, presumably due to the decomposition of the base and the setting free of a certain amount of sulphuric acid. Had the authors come across that particular type of discoloration?

DR. G. E. FOSTER (Dartford) pointed out that in the paper the vanillin colour reaction with pseudomorphine had an absorption curve with a maximum of 600 mμ. This statement might be misleading, as it depended on what was used as the blank, whether one obtained the maximum at the right wavelength or not. It was possible, if one used the vanillin reagent, but not if water was used as the blank. Recently, he had measured the absorption spectrum of a discoloured sample of morphine injection without sodium metabisulphite in the visible region of the spectrum, using water as the blank. When the result was compared with that obtained with a similar injection containing sodium metabisulphite, there was a difference in the two curves which actually corresponded to a maximum at about 375 to 400 mμ. By setting the spectrophotometer at that wavelength and using it as a colorimeter he had compared the actual discoloration in samples of injection of morphine under various conditions. The density readings were as follows:—without metabisulphite, 1·2; with metabisulphite, 0·36. Soaking the caps previously in metabisulphite solution and storing the bottles in an inverted position had little effect

on the result. Possibly, further examination of the absorption at 375 mμ would give more information about the discoloration.

PROFESSOR H. BERRY (London) said that the problem also involved the quality of rubber. Attempts had been made to achieve a standard mix. However, even if the mix was standardised it was still possible to obtain different results with the rubbers made from it. The lubricant used in moulding the rubber was a source of trouble. For instance, if a mixture of sodium laurylsulphate and lauryl alcohol was used it was very difficult to remove it from the rubber. Other sources of trouble were the antioxidants and accelerators used in rubber manufacture. The age of the sample of morphine salt was a further factor; an old sample would discolour much more readily than a fresh one.

DR. F. HARTLEY (London) said that he understood that the discoloration was held to be pharmacologically unimportant by the authors. He did not think that the authors' tests were adequate and there was the question of whether the discoloration had any influence on the toxicity of the morphine sulphate or whether it increased the obnoxious side effects shown by morphine. He doubted whether the discoloration was only of pharmaceutical importance.

MR. T. D. WHITTET referred to the Chairman's remarks about the effect of *p*H on the stability of morphine salts. Previous reports had demonstrated that morphine salts were more stable in acid solutions. The advantage of metabisulphite was that it exerted a protective action without producing such a low *p*H. The subject of rubber was certainly important. The quality had recently been improved and the rubber caps were now made from pale crepe rubber instead of a mixture of half pale crepe and half smoked sheet. This might account for the difference between the earlier results and those obtained recently. Another point which might account for the different results was that in the work on adrenaline with Dr. West, they had used a stronger solution of sodium metabisulphite and had soaked the caps for a longer time. Mr. Coulthard had mentioned that he had not been able to confirm that rubber caps did in fact remove metabisulphite from adrenaline solutions, but, on the other hand, Mr. Soulsby had encountered examples of discoloration in the injection when stored in rubber-capped bottles. He agreed that further pharmacological and toxicity tests on the morphine solutions would be advisable.

ANTAGONISM BETWEEN NON-IONIC DETERGENTS AND ANTISEPTICS

By Mlle. A. Bolle and A. Mirimanoff

From the Laboratory of Galenical Pharmacy the University of Geneva

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The question of detergents has been the subject of numerous papers in scientific literature, and their main properties have recently been summarised for the pharmacist by N. J. Harper[1]. From the point of view of their germicidal properties, cationic detergents are often powerful antiseptics; whereas non-ionic detergents have no such activity owing to the fact, noted by Glassman[2], that they do not denature proteins. As to the anionic derivatives, they have in an acid medium a certain bactericidal action against Gram-positive micro-organisms. We have shown in an earlier paper[3] that the toxicity of these substances did not depend on their surface-active or wetting properties, and that this toxicity varies considerably with the type of plant cell to which it is applied; certain fungi, for example, being more sensitive to the action of anionic wetting agents than the higher forms of plant cells. Finally, when an anionic wetting agent of the type of sodium laurylsulphate is added to an antiseptic—the composition of the latter can vary considerably—a synergistic action is frequently obtained, since the wetting agent increases the fungistatic action of the antiseptic. This does not occur with the higher plant cells and rarely with bacteria. Engler[4] observed that non-ionic detergents not only possess no proper fungistatic action in themselves, but they stimulate the growth of several kinds of moulds. Moreover, in the presence of antiseptics, the fungistatic action is clearly inhibited.

These incomplete observations have led us to take up this latter problem on a more general basis, in relation to the following two points: (a) non-ionic detergents are increasingly used in pharmacy as creams or ointments, (b) if added to an antiseptic preparation, what will be the real value of the latter?

Experimental

In such investigations there are three main variables: (a) the micro-organism; (b) the detergent; (c) the antiseptic.

To limit the number of experiments we restricted the first variable to 2 strains of fungi, the second to 7 detergents selected from amongst those most commonly employed, and the third to 3 antiseptics of very different chemical composition. In certain instances the tests were carried out for definite control purposes or to check either a working hypothesis or an interpretation of results. For most of our tests we worked with a liquid medium, using the Jaag medium, which is favourable to the development of mycelium and has the following composition:—sodium nitrate 3 g., potassium dihydrogen phosphate 1 g.,

potassium chloride 0·5 g., magnesium sulphate 0·5 g., ferrous sulphate 0·01 g., sucrose 50 g., water to 1,000 g.

A suspension of spores (*Penicillium* spp. *Aspergillus niger*) of constant age (10 days) and constant average density is added to 50 ml. flasks containing 10 ml. of Jaag medium. This is the control series. Into similar flasks either the selected antiseptic in progressive dilution or the wetting agent is poured, making a separate series for each. A last series of flasks contains the spores, the antiseptic and the wetting agent all together. The flasks are put in an incubator at 28°C. and observations made at the end of 10 days. It is evident that if the addition of a wetting agent or an antiseptic or a mixture of the two can change the Jaag medium, by diluting it, the control series must undergo a similar modification. Each series of tests is carried out with at least 2 flasks for each variable, and, in the series requiring the weighing of mycelium, 3 flasks are used.

Results

Our first experiments having shown qualitatively that the presence of certain non-ionic detergents favoured the development of mycelium, we have attempted to bring out this point by tests of a quantitative nature. For this purpose we compared the weights of the clumps of mycelium obtained from the control series with those of mycelium from the media with added detergent, at a 2 per cent. concentration. Without going into the details of these tests, of which only the comparative results are of any interest, we shall simply indicate the weight of the mycelium cultivated in the presence of a detergent as compared with that of the control mycelium, the weight of the latter being arbitrarily represented as unity. In classifying, in the order of their growing action, the detergents which favour the development of mycelium we obtain the following series, in which the figures represent the average of 3 weighings.

	Weights
Control	1
+ Carbowax 1500	1·5
+ Tween 80	1·5
+ Span 20	2
+ Crillex 6	2
+ Crillex 11	2
+ Crillex 16	2
+ Oleic acid	2
+ Tween 40	3

Interpretation of results is not quite so simple as might at first appear. Molho and Lacroix[5], in a paper which we received during our experiments, rightly remark that the methods for testing fungistatics are particularly delicate when they deal with the estimation of the weight of the mycelial growths.

These authors have, therefore, made use of a non-ionic wetting agent,

Tween 60, which is a sorbitol monostearate condensed with a long chain of ethylene polyoxide, to render homogeneous the suspension of spores in a liquid medium. The addition of 0·02 per cent. of this product gives good results but upsets the metabolism of the fungus. Observations must therefore be limited to a very definite period of time as this concentration of Tween retards the fungus up to the sixth day. After this period an acceleration of development is to be observed, which depends both on the form of the containers and on the aeration surface.

In our tests we used non-ionic detergents of 100 times higher concentration (2 per cent.) as our object was not so much to obtain a better homogeneity of suspension as to work with quantities equivalent to those used in pharmaceutical practice. The remarks of the French scientists nevertheless remain true, and it is important to work with well-defined conditions and within convenient time limits (10 days in our experience). Further, although our quantitative results very clearly showed the stimulating role of different non-ionic wetting substances on the growth of the *Aspergillus niger* organism (we also made qualitative tests on *Penicillium* spp. *Botrytis cinerea, Achorion quinckeanum*) the main purpose of the present work was to observe the behaviour of some of these detergents in the presence of antiseptics.

The results thus appear clearly and in a perfectly reproducible manner, for if the detergent inhibits the antiseptic at a given dilution of the latter the development of mycelium will take place and a greater quantity of antiseptic is required to obtain the necessary fungistatic effect. Tables I and II summarise the results of a great number of tests.

The following conclusions may be drawn:—

(1) All non-ionic detergents added by themselves to a culture medium have a stimulating action on the development of mycelium.

(2) The antiseptics used are partly inhibited by the detergents added to the culture medium. It should be noted that if inhibition is relatively slight for nipagin, with the detergents used, it is more pronounced for oxyquinoline sulphate and extremely high for G.4 (dioxydichlorodiphenylmethane). At a concentration of 1 in 10,000 this last product is fungistatic for *Aspergillus niger :* it is not so at 1 in 1,000 in the presence of Crillex, Spans and Tweens.

(3) Carbowax 1500 has no appreciable inhibitory action on the antiseptic. It is a polyethylene glycol polymer.

(4) Mannite, tested because of its chemical relationship with certain non-ionic detergents, produces no effect.

Cystein and gelatin have a strong inhibitory action on oxyquinoline sulphate (no doubt because of their SH function), but do not interfere with G.4.

(5) Oleic acid behaves in the same way as the detergents used. Since these frequently contain a certain quantity of free oleic acid, it would appear that this acid can be considered as the essential cause of this

TABLE I—*Aspergillus niger*. THE BEHAVIOUR OF THREE ANTISEPTICS IN THE PRESENCE OF DIFFERENT DETERGENTS AND OF SUBSTANCES BELIEVED INHIBITORY TO ANTISEPTICS

Added Antiseptic		Absence of surface-active agent	Carbowax 1500 2 per cent.	Crillex 6 2 per cent.	Crillex 11 2 per cent.	Crillex 16 2 per cent.	Span 20 2 per cent.	Tween 80 2 per cent.	Tween 60 2 per cent.	Oleic Ac. 2 per cent.	Sodium Oleate 2 per cent.	Mannite 2 per cent.	Cysteine 2 per cent.	Gelatine 2 per cent.
None		+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	+ +
Nipagin	0·2 per cent.	−			−	+			−					
L.A. "	0·1 per cent.	−			+ +	+			+ +					
"	0·05 per cent.	+			+ +	+ +			+ +					
"	0·1 per cent.	+ +			+ +	+ +			+ +					
Oxyquinoline sulphate	0·1 per cent.	−	−	−	−	−	−	−	−	−	−	−	+ +	−
"	0·05 per cent.	−	−	−	−	−	−	−	−	−	−	−	+ +	−
L.A. "	0·02 per cent.	−	−	+ +	+ +	+ +	−	+ +	+	+ +	−	−	+ +	+ +
"	0·01 per cent.	+	−	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	−	+ +	+ +
"	0·005 per cent.	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Dioxydichlorodiphenylmethane	0·1 per cent.	−	−		+ +	−	+ +	+ +	+ +	+ +	−	−	−	−
"	0·05 per cent.	−	−		+ +	+ +	+ +	+ +	+ +	+ +	+	−	−	−
"	0·02 per cent.	−	−		+ +	+ +	+ +	+ +	+ +	+ +	+	−	−	−
L.A. "	0·01 per cent.	−	+		+ +	+ +	+ +	+ +	+ +	+ +	+	−	−	−
"	0·005 per cent.	+	+		+ +	+ +	+ +	+ +	+ +	+ +	+	+	+	+

Dotted line shows zone of action of the antagonist. Broken line (L.A.) shows limit of action of the antiseptic alone.

+ + normal development, similar to that of the control.
+ development below that of the control.
+ + + development above that of the control.
− no development (fungistatic action).

TABLE II

COMPARISON BETWEEN *Aspergillus niger* AND *Penicillium* SPP.

Antiseptic Added	*Aspergillus niger*						*Penicillium* spp.					
	Absence of surface active agent	Crillex 16 2 per cent	Span 20 2 per cent.	Tween 80 2 per cent.	Sodium Oleate 2 per cent.	Cystein 2 per cent.	Absence of surface active agent	Crillex 16 2 per cent.	Span 20 2 per cent.	Tween 80 2 per cent.	Sodium Oleate 2 per cent.	Cystein 2 per cent.
None	+ +	+ + +	+ + + +	+ + +	+ +	+	+ +	+ +	+ +	+ +	+ + +	+
Oxyquinoline sulphate :—												
0·1 per cent.	−	−	−	−	−	+ +	−	−	−	−	−	+ +
0·05 per cent.	−	−	−	−	−	+ +	−	−	−	−	−	+ +
0·02 per cent.	−	+ +	−	+ +	−	+ +	−	−	−	+ +	−	+ +
L.A.												
0·01 per cent.	+	+ +	+ +	+ +	+	+ +	−	+	+	+ +	+	+ +
0·005 per cent.	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Dioxydichlorodiphenylmethane (G.4) :—												
0·1 per cent.	−	−	+ +	+ +	+ +	−	−	+ +	+ +	+ +	+ +	−
0·05 per cent.	−	+ +	+ +	+ +	+ +	−	−	+ +	+ +	+ +	+ +	−
0·02 per cent.	−	+ +	+ +	+ +	+ +	−	−	+ +	+ +	+ +	+ +	−
0·01 per cent.	−	+ +	+ +	+ +	+ +	−	−	+ +	+ +	+ +	+ +	−
L. A.												
0·005 per cent.	+	+ +	+ +	+ +	+ +	−	−	+ +	+ +	+ +	+ +	−

Dotted line indicates the zone of action of the antagonist and the broken line (L.A.) shows the limit of activity of the antiseptic alone.

phenomenon. Complementary tests, not shown in the Table, with a concentration of 2 in 10,000, have confirmed these results.

The results in Table II are of the same order, only the "growth" factor of the detergents by themselves being less marked. It is of interest to note that the inhibitory action of the antiseptic, weaker with oxyquinoline sulphate, is extremely energetic with G.4. This fact indicates that a detergent need not have stimulating properties to inhibit an antiseptic. It seems as if the detergent reacted chemically with the antiseptic to destroy or diminish the toxicity of the latter. One can correlate this fact to the "neutralisation" of oxyquinoline sulphate by cystein, a substance which is equally antagonistic to penicillin and to mercurial bactericides.

Discussion

Dubos and Davis[6], who seem to have been the first authors to use a non-ionic detergent (Tween 80) in a culture medium with a concentration of 0·1 per cent., had observed the favourable action of this product on the growth "in vitro" of a strain of tubercular bacilli. They attribute this diffuse growth in a culture medium to the surface-action properties of Tween 80 and observe that the non-surface-active esters of oleic acid are indifferent. Whitehill, Oleson and Subbarow[7] observe that free oleic acid can replace biotin as a growth-promoting factor for lactobacillus. Williams, Broquist and Snell[8] remark that oleic acid, although indispensable to the growth of certain micro-organisms, shows a definite toxicity according to the concentration employed and the *p*H to which it is used. On the other hand, the addition of Tween 40 eliminates the toxicity of oleic acid and gives rise to its growth-promoting action. These observations indicate that it is difficult to be precise about the mechanism of interference of these detergents, and that it varies, as one might expect, according to the nature of the metabolites and the culture medium.

Kodicek[9] has demonstrated that the more complete influence of fatty acids on the Gram-positive bacteria depends, amongst other things, on the presence of surface-active substances with which the acids can form complexes. These may be in competition with the substances of the cortex of the bacteria for inhibiting substances. To the best of our belief, facts of this nature concerning fungi have not been made known, and moreover one finds few observations on the subject of the interference produced by the wetting substance on an antiseptic. Mme. Chaix and L. Lacroix[10] have, however, pointed out that Tween 20 and 80 completely protect the cells of *Glaucoma pyriformis* (monocellular ciliated organism) against the action of gammexane. These authors attribute this phenomenon to physical protection.

In the case of our moulds we find it difficult to interpret the phenomena as the above-quoted bacteriologists have attempted to do, for it must not be forgotten that detergents are technical products whose chemical composition—and impurities—are not always precisely known. It seems,

as we have already pointed out, that the antagonistic action of detergents towards antiseptics is partly due to the presence of free, non-saturated, fatty acids side by side with an unesterfied fraction. This can be realised by comparing the action of pure oleic acid with that of sodium oleate. However, this does not explain the behaviour of Crillex 6, which is an ester of lauric acid (saturated), nor of Span 20 (sorbitol monolaurate). It would be useful to increase the number of tests by using chemically pure substances so as to give a more accurate interpretation of these phenomena. But this would be outside the scope of the present work which is devoted to pharmaceutical realities.

Summary

(1) The non-ionic detergents which have been examined in this paper favour the development of moulds in a liquid medium.

(2) With the exception of Carbowax 1500, these detergents decrease the fungistatic action of the antiseptic added to the culture medium. The degree of inhibition varies with the nature of the antiseptic.

(3) It seems that the fatty nature of these detergents is responsible for this phenomenon. Oleic acid by itself, even at a concentration of 2 per 1000, favours the development of mycelium and has an inhibitory effect on the antiseptic action. This may explain the inactivity of Carbowax 1500.

(4) These facts must be considered in the utilisation of non-ionic detergents in pharmacy and cosmetics. These preparations will tend to become mouldy rather quickly. In emulsions and antiseptic ointments with a non-ionic detergent base, control of fungistatic or bacteriostatic action seems indispensable.

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DISCUSSION

An abstract of the paper was read by Professor Mirimanoff.

DR. I. MICHAELS (London) asked whether the authors could give a summary of the technique of weighing the mycelium.

PROFESSOR H. BERRY (London) asked whether they had measured interfacial tension under the conditions of the experiments. This had been shown to be a very important factor by various workers.

DR. F. HARTLEY (London) commented on the use of " tweens " in microbiological assays, and asked whether the authors accepted the explanation that non-ionic detergents exerted not so much a direct stimulating action on the growth of the organism, but made the nutrient materials more readily available. The latter theory would link up with Professor Berry's point about the effect of interfacial tension between the different phases.

PROFESSOR A. MIRIMANOFF replied by first describing the technique of weighing the mycelium. The method was that described by the French authors Molho and Lacroix. With regard to the influence of the interfacial tension, a whole series of wetting agents were studied for their toxicity on micro-organisms and higher vegetative organisms, by plasmolysis and cytoplasmic movement. No relation had been found between interfacial tension and antiseptic action. For example, a non-ionic " tween " could have a much higher interfacial tension than an anionic agent and not possess toxicity, whereas the anionic agent could be toxic.

The stimulating action of the non-ionic detergents must primarily be a nutritive action, due to the presence of free oleic acid, which was difficult to prove in the commercial products. Substances such as carbowax, which do not contain this acid, do not possess this activity. There must be also a physicochemical action which was linked up with the experiments of Dubos in the study of tuberculosis. Kodicek had published some extremely interesting work on the subject, but its interpretation was very difficult.

THE TRYPTIC ACTIVITY OF PANCREATIN
A CRITICAL STUDY OF SOME ASSAY PROCESSES AND STANDARDS

By Kenneth Bullock and Jitendra Kumar Sen

From the Department of Pharmacy of the University of Manchester

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Introduction

The official process for the assay of trypsin in pancreatin suffers from certain serious defects. The chief defect, which in fact renders the results obtained by the present process almost valueless as a quantitative estimate of tryptic activity, is the presence of active enzyme in the blank. Experiments were therefore undertaken with a view to devising an amended process, really a rationalisation of the present process. These experiments indicated that the standard for tryptic activity required by the present British Pharmacopœia is several times higher than that required by the B.P. 1932. This led to the examination of a number of commercial samples of pancreatin with a view to deciding which standard is most suitable. The position was found to be complicated by the fact that manufacturers of weaker pancreatins are apparently in the habit of adding anything up to 80 per cent. of lactose before selling the material as pancreatin B.P.

The work described in the present paper may thus be classified under three headings.

1. A critical examination of the B.P. 1948 assay process with suggested amendments.

2. A comparison of the results obtained by assaying several samples of pancreatin for tryptic activity by the 1932 process, the 1948 process and the amended 1948 process.

3. An examination of the strength of commercial samples of pancreatin and an estimate of the strength which they would have possessed if they had not received an addition of lactose. The assay process for amylase is the same in the 1932 and 1948 Pharmacopœias. In a previous paper[1] it was shown that it is tryptic activity which limits the degree to which a sample of pancreatin may be diluted and still comply with the official standards for the three enzymes. This was so using the 1948 B.P. standard for lipase and the 1932 standard for trypsin. It is much more so if the 1948 or amended 1948 standard for trypsin is employed. For these reasons the following work is confined to the assay of trypsin and all methods, results, standards, etc., refer to that enzyme unless otherwise mentioned.

When trypsin is determined by methods involving formol titration three titration figures may be recorded. For purposes of clarity these three types of result will be given the following names in this paper.

I. The quantity of alkali required to adjust the mixture to *p*H 8·7

(*p*H 7·0, or pink to phenolphthalein as the case may be) after the digestion period and immediately before addition of the formaldehyde will be called the "neutralising titration." II. The quantity of alkali required to bring the mixture back to *p*H 8·7 (or pink to phenolphthalein) after addition of the formaldehyde will be called the "formol titration." III. The difference between the formol titration of the test and that of the blank will be called the "assay titration."

Experimental—Choice of Methods of Assay

Many methods have been used for the determination of tryptic activity. They may be classified on the basis of two considerations:—

1. The nature of the protein used as substrate. 2. The choice of the method for estimating the degree of digestion. The quantity of unchanged protein may be determined by precipitation and either matching of the resultant turbidity or filtering off and weighing, or filtering and estimating total nitrogen in the precipitate. Alternatively the -COOH groups or $-NH_2$ groups liberated during the digestion may be estimated chemically or the concentration of one particular amino acid liberated may be estimated, for example, colorimetrically.

The choice of casein as substrate in the official assay is a good one. It is easy to obtain and pancreatin is often used to "peptonise" milk.

The U.S.P. gives conditions so that at the end of digestion there should be no precipitation with acid; all the casein must have been changed. This is a vague end point. Turbidity methods also are relatively difficult and of low accuracy. Northrop[2] estimated either the weight or total nitrogen content of unchanged casein precipitated by acid. These methods are essential where the kinetics of the digestion are being studied but are lengthy tedious processes for simple assay work.

Of the colorimetric processes for determining a liberated amino acid that of Anson[3] has been widely used and highly praised. The difficulty is that the preparation of the substrate, hæmoglobin, is involved, and the product has to be carefully preserved so that the method is not very suitable for use as an official process.

Willstätter's[4] well-known method of determining -COOH groups liberated during digestion uses casein as substrate and necessitates the use of considerable quantities of alcohol. It does not seem to have any outstanding advantages over the formol titration method using casein as substrate and estimating the acidity released by blocking with formaldehyde the $-NH_2$ groups liberated by digestion. For these reasons only assay processes using this last method have been investigated in the work described in this paper.

Materials Used

Pancreatin. The various commercial samples of pancreatin examined were labelled as follows:—A1, A2, A3, from the same firm, Pancreatin; B. Pancreatin Triple B.P.; C. Pancreatinum B.P.; D. Pancreatinum B.P.; E. Triple Pancreatin B.P.; F. Pancreatin Triple U. S. P.

Casein. Of several samples of commercial casein examined none complied with the requirements of the 1948 Pharmacopœia. A sample of casein complying with the B.P. specification prepared by a slight modification of the method described in Biochemical Preparations[5] was very kindly supplied by Messrs. Benger's Food, Ltd., but it is at present not available on the market. Under these circumstances most of the work was carried out with B.D.H. "light white soluble casein." It is difficult to adjust the slightly turbid blank to *p*H 7·0 in the B.P. 1948 process but in the ammended process, using only phenolphthalein as indicator, B.D.H. casein gave perfectly satisfactory results. These results agreed exactly with those obtained by using the above specially prepared soluble casein over a wide range of pancreatin strengths. It seems a pity to specify a type of casein requiring special preparation when an available commercial brand is satisfactory.

A Critical Study of the 1948 B.P. Assay Process

1. *The Blank.* In the B.P. 1948, process there is no indication whatever that the blank should be titrated at once, on the contrary, by inference, it is kept at "laboratory temperature" for 20 minutes while the digest is being kept at 55°C. It soon became apparent that considerable digestion was occurring during the 20 minutes at laboratory temperature. To determine the extent of this digestion 5 experiments were carried out. One was at room temperature with previously boiled enzyme solution. In the other four, unboiled enzyme solution was used and the digests were kept for 20 minutes at 0°C., 17°C. (winter room temperature), 24°C. (summer room temperature) and 55°C. Table I shows the results obtained.

TABLE I

Digestion occurring in the test compared with that in the blank kept under varying conditions. Each digest and blank contained either 8 or 25 mg. of pancreatin A. All figures are ml. of 0·1N sodium hydroxide

	8 mg.				25 mg.		
Temperature °C.	Enzyme solution	Neutralising titration	Formol titration	Assay titration	Neutralising titration	Formol titration	Assay* titration
0	Unboiled	0·3	2·75	0·15	0·9	3·6	0·9
17	,,	1·0	3·1	0·5	2·2	5·1	2·4
24	,,	1·4	3·65	1·05	2·6	5·7	3·0
55	,,	3·5	7·4	4·8	4·6	9·3	6·6
20	Boiled	0·0	2·6	0·0	0·0	2·7	0·0

* *Calculated using the boiled enzyme experiment as blank.*

Unless the enzyme solution has previously been boiled, some digestion occurs during the 20 minute period even at 0°C. This degree of digestion increases with increasing temperature and is, of course, greater with

stronger pancreatins. The extent of digestion in the blank at room temperature may be more than one third that of the true assay titration and more than one half of the assay titration using as blank the mixture kept at 17°C. for 20 minutes. The difference in the blank at winter and at summer temperatures may be equivalent to 10 per cent. of the true assay titration or 16 per cent. of the result in the B.P. 1948 assay process. Since the formol titration of the casein solution alone, used in each digest, amounts to 2·2 or 2·3 ml. of 0·1N sodium hydroxide these difficulties can all be overcome by using previously boiled enzyme solution in the blank. If this is done no digestion occurs in the blank so that the conditions of storage are unimportant.

To stress the importance of this it may be mentioned that on one occasion 15 mg. of a pancreatin gave an assay titration of 5·0 while 30 mg. gave an assay titration of 5·2 ml. although the corresponding neutralising titrations were 8·5 and 12·0 and the formol titrations 13·5 and 17·2 respectively. This illustrates how very misleading results by the B.P. 1948 method can be.

2. *The use of neutral formaldehyde solution.* In the B.P. 1932 the solution of formaldehyde used was directed to be "previously neutralised to phenolphthalein." In the 1948 process this direction is omitted, presumably because the digest and blank are now adjusted before the formol titration to *p*H 7·0 and the official solution of formaldehyde is required to be "neutral or slightly acid to litmus." To bring 10 ml. of a commercial sample of Liquor Formaldehydi to *p*H 8·7 was found to require 0·8 ml. of 0·1N sodium hydroxide. Since this quantity is variable and increases the blank unnecessarily it was decided that in the modified process the requirement that the formaldehyde should be previously neutralised should be reintroduced.

3. *Variation in the degree of digestion with time and quantity of enzyme.* Schütz's[6] Law states that $x = k\sqrt{E}\,t$, where E is the quantity of enzyme present, at the time of digestion and x the resultant degree of digestion in that time. Schütz's Law was first shown to apply to tryptic digestion by Borissov[7]. Northrop[2] verified that the Schütz Law applies to most of the range of the digestion of casein by trypsin. Northrop[2] and Moelwyn Hughes[8] have discussed the derivation of Schütz's Law from the first order reaction equation provided that the following assumptions are made. 1. That the enzyme has considerable affinity for the products of digestion. 2. That the concentration of the products of digestion is large with respect to the quantity of enzyme. 3. That during the period under consideration the concentration of the substrate does not appreciably change. It is obvious that during the early part of the reaction condition 2 is not fulfilled while in the later stages of the reaction condition 3 is not fulfilled. Hence Schütz's constant k tends to fall off at the beginning and end of the reaction. It will be shown in the next section that a fall in *p*H accompanies digestion in the official process and this also accounts for some deviations from Schütz's Law.

The time course of the reaction is plotted in Figure 1. The fact that a plot of the assay titration against the square root of the time is almost a straight line shows how closely the modified form of Schütz's Law $x = k\sqrt{t}$ is followed.

The relationship between assay titration and quantity of enzyme in the digest for a 20-minute period of digestion is shown in Figure 2. Again Schütz's Law in the form $x = k\sqrt{E}$ is verified for all but the lower and higher assay figures since the plot of x against $\sqrt{E}$ is practically a straight line.

4. *The Effect of pH on Digestion by Trypsin.* The optimum *p*H for trypsin acting on casein has been shown by Northrop[9] to be in the neighbourhood of 8·6. The problem is complex for several reasons. Trypsin is probably not a single enzyme. Further the *p*H has at least two, in fact opposing, effects. As already mentioned Pace[10] has shown that the stability of trypsin decreases rapidly with increase of *p*H so that it becomes markedly unstable at *p*H values over 10. On the other hand, Northrop[11] has shown that trypsin reacts with negatively charged protein ions. If therefore the experiments are designed so that the alkali destruction of trypsin is not

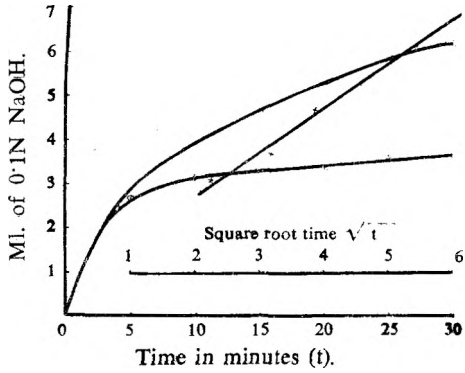

FIG. 1. Titration-time Curves of the Digestion of Casein by Trypsin. Top curve, assay titration against time; middle curve, assay titration against square root of time; lowest curve, neutralising titration against time.

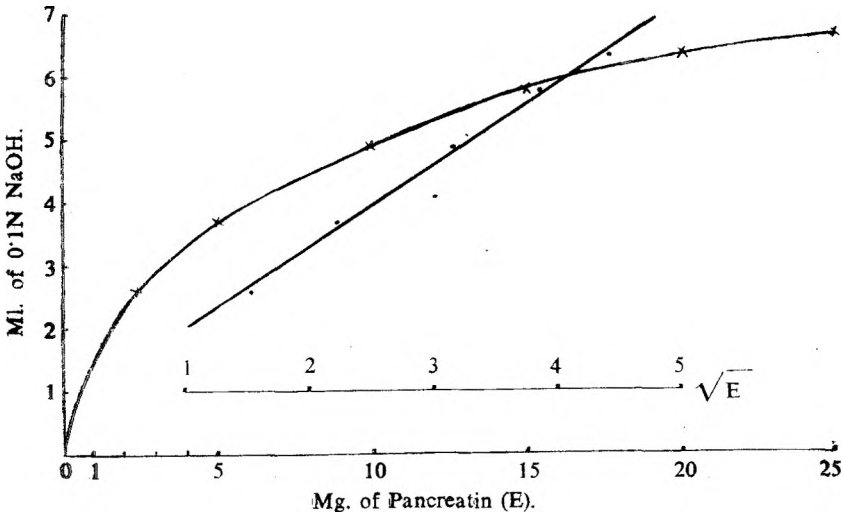

FIG. 2. Empirical Curve for Comparing Strength of Pancreatin.

a limiting factor the rate of digestion will increase with increasing *p*H and indeed will follow closely the titration curve of the casein. To find out the effects of these various factors on the process under consideration the *p*H of a number of solutions was determined with the glass electrode.

Most of the figures refer to experiments using the high strength pancreatin A. 5 ml. of a 0·16 per cent. solution, i.e., 8 mg. of pancreatin, was used per digest as this gave an assay titration of about 5 ml., approximately the upper limit to be recommended. From the results shown in Table II it is clear that in the digest there is a rapid fall from the initial *p*H of 8·7, which becomes more gradual as digestion proceeds, reaching *p*H 7·02 or lower with large degrees of digestion. According to graphs given by Northrop[9] a fall of *p*H from 8·6 to 7·0 corresponds to a loss of 25 per cent. of the activity of trypsin acting on casein. This increase in acidity is reflected in the increasing "neutralising titration" as digestion proceeds.

TABLE II

*p*H OF SOLUTIONS AND DIGESTS (GLASS ELECTRODE)

Solution	*p*H
Pancreatin solution (0·16 Pancreatin A)	6·77
,, ,, (0·5 per cent. Pancreatin D)	5·41
,, ,, (0·5 per cent. Pancreatin C)	6·03
4 per cent. Casein solution (adjusted to *p*H 8·7 using phenolphthalein)...	8·82
Digest (Pancreatin A) kept at room temperature for 0·0 minutes	8·72
,, ,, ,, ,, ,, 1 ,, ...	8·50
,, ,, ,, ,, ,, 10 ,, ...	7·87
,, ,, ,, ,, ,, 20 ,, ...	7·59
,, (Pancreatin D) ,, ,, ,, 20 ,, ...	8·25
,, (Pancreatin C) ,, ,, ,, 20 ,, ...	8·18
,, (Pancreatin A) kept at 55°C. for ... 10 ,, ...	7·12
,, ,, ,, ,, ... 20 ,, ...	7·02
,, (Pancreatin A) 0·5 per cent. kept at 55°C. for 20 ,, ...	6·02

That the increase in acidity is not directly proportional to the extent of digestion as measured by the assay titration is clearly shown in Figure 1. The change of *p*H during digestion results in the higher potency pancreatins giving lower assay titrations than would be the case if the digest could be maintained at constant *p*H. It also accounts for some of the deviations from Schütz's Law. It is clear that pancreatin can be compared in strength only by constructing an empirical curve as in Figure 2.

It was found that 100 ml. of 0·16 per cent. pancreatin A required 1·75 ml. of 0·1N sodium hydroxide to raise the *p*H from 6·77 to 8·7, but when duplicate assays were carried out with the two solutions no difference was observed in the results.

Some difficulty was experienced in adjusting the 4 per cent. B.D.H. soluble casein solution to *p*H 8·7 colorimetrically. The resultant solutions varied from *p*H 8·62 to 8·84 by the glass electrode, but again duplicate assays with the two substrate solutions resulted in identical results.

5. *Adjusting to pH 7·0 or pH 8·7 before the formol titration.* In the B.P. 1932 process the formol titration was carried out in the usual way the digest being neutralised to phenolphthalein, the neutral formaldehyde solution added and the digest again titrated to phenolphthalein, the second titration being the formol titration. In the B.P. 1948 process this procedure was varied, by Evers and Smith[12] presumably as a result of a paper by Northrop[13]. The digest and blank are brought to *p*H 7·0 and the formaldehyde solution added. The formol titration is the alkali then required to bring the *p*H to 8·7. This means that the buffer value between 7·0 and 8·7 is added to the formol titration. The buffer value of the test differs from that of the blank.

An examination of Northrop's[13] paper shows that the variation was introduced owing to the difficulty of determining the exact end-point in the case of small quantities of aminoacids. To find the effect of the variation in the assay process the following experiment was carried out. An assay was set up using a double quantity of test and blank. At the end of the digestion period each 100 ml. quantity was divided into 2 parts resulting in 2 exactly equal pairs of test and blank. The members of one pair were brought to *p*H 7·0 before addition of formaldehyde, while those of the second pair were adjusted to *p*H 8·7 before addition of the formaldehyde. All were finally titrated to *p*H 8·7. The results are shown in Table III.

TABLE III

DIFFERENCES IN ADJUSTING TO *p*H 7·0 OR 8·7 BEFORE ADDITION OF FORMALDEHYDE

		Ml. of 0·1N Sodium Hydroxide		
		Neutralising titration	Formol titration	Neutralising* formol
Adjusted to *p*H 7·0	Test	*−0·05	12·1	12·05
	Blank	−1·0	3·7	2·7
Adjusted to *p*H 8·7	Test	4·95	7·0	11·95
	Blank	0·05	2·6	2·65

* A minus sign denotes the addition of 0·1N hydrochloric acid.

It will be seen that the formol titration of the B.P. 1948 is equal to the true formol titration plus the neutralising titration, i.e., it includes the acidity developed during digestion with two absolutely arbitrary alterations, (1) if the blank or digest has a *p*H above 7·0 alkali equivalent to the acid necessary to bring it down to 7·0 is added, (2) if the digest should be below *p*H 7·0 then the alkali required to bring it up to *p*H 7·0 is deducted. Either the neutralising titration should be excluded or it should be added, on the grounds that the acidity is in fact produced by trypsin, without alteration.

It may be mentioned that the quantity of alkali, 3·5 ml., required to change the digest from *p*H 7·0 to 8·7 was exactly the same in the presence

and in the absence of the formaldehyde, indicating that the formol titration at *p*H 7·0 does not include any free-NH_2 groups missed by a formol titration starting at 8·7.

The neutralising titration may, of course, include alkali required to neutralise acid produced by the action of lipase upon fat if the casein and pancreatin are not absolutely fat free. Some commercial caseins do contain up to 1 per cent. of fat.

There are also theoretical considerations opposed to the inclusion of the neutralising titration in the formol titrations. The formol titration was especially designed to measure acidity liberated by neutralising the basicity of the free amino groups by addition of formaldehyde and to exclude acidity or alkalinity of the digest which might result from other causes.

Further, an examination of Figure I shows that the neutralising titration falls off with the degree of digestion at an even greater rate than does the assay titration. To include it in the assay titration would therefore emphasise the deviations from Schütz's Law and render the assay titration still less proportional to the quantity of trypsin present.

TABLE IV

Process	Ml. of 0·1N Sodium Hydroxide		
	Neutralising titration	Formol titration	Assay titration
B.P. 1948 process. 15 ml. of casein solution 30 ml. of water 5 ml. of enzyme solution. Heated rapidly to 55°C. (1 minute taken) 20 minutes at 55°C. ...	3·2	7·2	4·6
15 ml. of casein solution 30 ml. of water 5 ml. of enzyme solution. Placed in bath at 55°C. 20 minutes (5 minutes required to attain 55°C.)	3·4	7·2	4·6
15 ml. of casein solution 30 ml. of water heated to 55°C. Added 5 ml. of enzyme solution and keep in bath at 55°C. 20 minutes. (Temp. fell to 52°C. on addition of enzyme solution, regained 55°C. in 3 minutes) ...	3·5	7·8	5·2

6. *Adjusting the Digest to 55°C.* The B.P. 1948 process directs that the digest shall be rapidly heated to 55°C. Rapid heating may be variously interpreted. If the process is relatively slow then a variable amount of digestion occurs during the heating, if it is really rapid, for example, by heating over a gauze directly heated by a bunsen, it appears likely, as suggested by the results in Table IV that some enzyme is destroyed in the thin layer of liquid in contact with the hot glass.

From Figure 3 it can be calculated that a drop of temperature of the digest from 55°C. to 52°C. for 3 minutes would cause a drop in the assay titration of just less than 0·1 ml. On the other hand, as shown in Table V, a solution of trypsin maintained at 55°C. in the absence of substrate loses strength relatively rapidly, more than corresponds to 0·1 ml. in the assay titration in 3 minutes.

It is advisable, therefore, to preheat the casein solution, but not the enzyme solution, to 55°C. before mixing.

7. *Heat Inactivation of Trypsin in the Absence of Substrate.* It has been shown by Pace[9] that at 50°C. trypsin-kinase is heat-inactivated at a rate in fair agreement with the unimolecular reaction equation, and that the rate of inactivation varies, not only with temperature, but also with *p*H. The time of half-decomposition in minutes at 50°C. was found to be 277 at *p*H 6·0, 300 at *p*H 7·0 and 128 at *p*H 8·5.

A 0·16 per cent. solution of pancreatin A was kept at 55°C. and 5 ml. quantities assayed after 20 and 40 minutes. The results in Table V show a quite rapid rate of inactivation. Even in 3 minutes the destruction would correspond to a drop of approximately 0·15 ml. of 0·1N sodium hydroxide in the assay titration.

TABLE V

HEAT INACTIVATION OF TRYPSIN IN PANCREATIN SOLUTION. BLANK IN ALL CASES 2·6 ML. OF 0·1N SODIUM HYDROXIDE

	Neutralising titration	Formol titration	Assay titration
Time of storage of pancreatin solution at 55°C. prior to assay :—			
0 minutes	3·4	7·2	4·6
20 ,,	2·2	6·1	3·5
40 ,,	2·0	5·1	2·5
Time of storage of pancreatin solution at 20°C. prior to assay :—			
0 minutes	3·6	7·5	4·7
20 ,,	3·6	7·5	4·7
60 ,,	3·6	7·5	4·7
120 ,,	3·6	7·5	4·7
Overnight in refrigerator	3·6	7·5	4·7
3 days in refrigerator	3·7	7·2	4·6

The relative stability of the solution of pancreatin at 20°C. and *p*H 6·8 may be compared with the instability of pure solutions of trypsin at *p*H 7·8 and 25°C. reported by Schwert[14].

8. *The Upper Limit to the Assay Titration.* It is generally agreed that enzymes are most accurately assayed by determining only the initial rate of reaction. For this purpose not more than 25 per cent. or at most 50 per cent. of the substrate should be used up; or with a complex process such as the one under consideration not more than 25 per cent. or at most 50 per cent. of the possible reaction, measured in this case by the assay titration, should occur. To determine this upper limit digestions

TABLE VI

DIGESTION BROUGHT ABOUT BY 25 MG. OF PANCREATIN A PER DIGEST

Time of digestion	Neutralising titration	Formol titration	Assay titration
0 minutes	0·0	2·6	0·0
5·5 hrs. at 55°C. plus 16·5 hrs. at 37° C.	5·8	14·0	11·4
3·5 hrs. at 55°C. plus 16·5 hrs. at 37° C.	5·4	12·6	10·0
Room temperature (20°C.) 19 hours	5·2	12·4	9·8

using the strong pancreatin A were carried out for several hours at 55°C. and then overnight in the incubator at 37°C. The results are shown in Table VI.

It is clear that the practical upper limit to the assay titration is not much above 12 ml. of 0·1N sodium hydroxide. This means that in assay work the assay titration should not be much above 3 or 4 and certainly not above 6 ml. To assay a pancreatin giving an assay titration over 6 the assay should be repeated using a smaller amount of pancreatin. The extensive degree of digestion occurring during the assay was confirmed by precipitating unchanged casein at *p*H 4·8 and weighing. For assay titrations of 3·0 and 5·0 only 10·6 per cent. and 5·2 per cent. respectively of the total casein remained unchanged at the end of the assay.

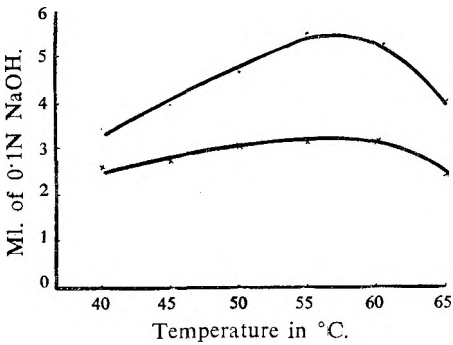

FIG. 3. Effect of temperature in the rate of heat inactivation of pancreatin. Upper curve, assay titration; lower curve, neutralising titration.

9. *Optimum temperature.* Owing to the complex nature of trypsin and the fact that a rise of temperature increases the rate of heat inactivation of the enzyme, the latter rate being also dependent on the *p*H, there is some confusion in the literature as to the optimum when using such crude preparations as pancreatin. The results shown in Figure 3 indicate that 55°C. is near the optimum for the modified official process with its 20 minutes digestion period.

MODIFIED ASSAY PROCESS FOR TRYPSIN IN PANCREATIN

Taking all the above findings into consideration the following modification of the B.P. 1948 process is recommended. Dissolve 4 g. accurately weighed, or purified casein in 90 ml. of water containing 1 ml. of 1·0N sodium hydroxide, adjust the *p*H of the solution to 8·7 and make up the volume to 100 ml. with water. In each of two flasks place 15 ml. of casein solution and 30 ml. of chloroform water, adjust the temperature to 55°C. and place in a water-bath at 55°C. To one flask add 5 ml. of a freshly prepared 0·15 per cent. solution of pancreatin unfiltered in chloroform water. To the other add 5 ml. of a portion of the same enzyme solution previously boiled and cooled. Maintain both flasks at 55°C. for 20 minutes. Cool rapidly to room temperature and to each flask add 0·75 ml. of solution of phenolphthalein 0·1 per cent. w/v. Bring both solutions to *p*H 8·7 by addition of 0·1N sodium hydroxide. Add 10 ml. of solution of formaldehyde previously neutralised to phenolphthalein to each flask and titrate both liquids with 0·1N sodium

hydroxide until the *p*H is again 8·7. The difference between the two titrations should not be less than 3·0 ml. and not more than 5·0 ml.

An Examination of Commercial Pancreatins by the B.P. 1932 Assay Process

In the first place a graph, Figure 4, was prepared relating quantity of enzyme with corresponding assay titration. This curve can now be used to compare the strengths of pancreatins with the B.P. limits or with each other.

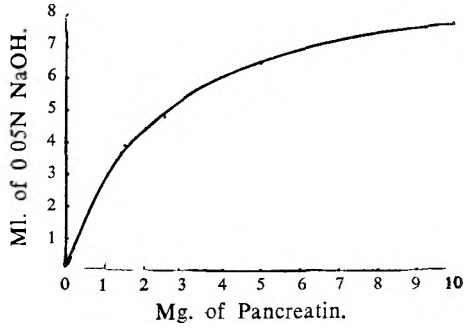

FIG. 4. Relation of the quantity of enzyme with the assay titration.

To understand how the figures in column 4 of Table VII were obtained, suppose that x mg. of a given pancreatin gave an assay titration of 5·2; from the graph of Figure 4, this corresponds to 2·88 mg. An assay titration of 4 corresponds to 1·68 mg. Therefore the quantity of the given pancreatin which should give a titration figure of 4 is $x \times 8/2{\cdot}88$; this is the figure in column 4. The figures in column 5 were obtained by dividing 50 by the figures in column 4.

It will be seen in Figure 4 that an assay titration of 8 is on the flat part of the curve. Approximately 8·8 times as strong a pancreatin is required to comply with the upper limit of the B.P. as compared with the lower limit. Only pancreatins A1 and A2 exceeded this upper limit although F is about equal to it.

These limits appear to be very wide.

TABLE VII

RESULTS OF ASSAYING 6 SAMPLES OF COMMERCIAL PANCREATIN. B.P. 1932 REQUIRES 50 MG. OF PANCREATIN TO GIVE AN ASSAY TITRATION BETWEEN 4·0 AND 8·0 ML. OF 0·05N ALKALI. ALL RESULTS AS ML. 0·05N SODIUM HYDROXIDE

Pancreatin	Mg. in digest	Assay titration	Mg. pancreatin equivalent 4·0 ml. assay titration	Strength in terms B.P. limit = 1·0
A 1	1·5	3·9	1·54	32·5
A 2	5	5·2	2·8	17·9
B	20	4·2	18·3	3·1
C	50	6·1	20·0	2·5
D	50	4·7	36·5	1·4
E	20	4·7	14·5	3·45
F	5	3·3	7·12	7·0
F	10	5·2	5·85	8·5

THE ASSAY OF COMMERCIAL SAMPLES OF PANCREATIN BY THE B.P. 1948 PROCESS

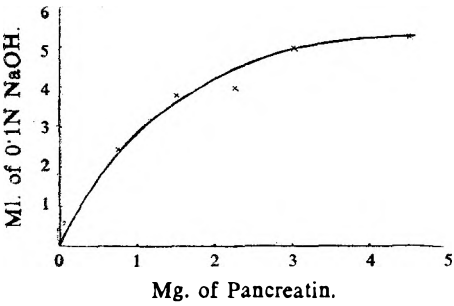

FIG. 5. Curve showing relation between the amount of pancreatin and the corresponding assay titration.

The curve relating mg. of pancreatin and the corresponding assay titration is shown in Figure 5. Using this graph as explained in the previous section the results of assaying 6 commercial samples of pancreatin are shown in Table VIII.

TABLE VIII

Pancreatin	Pancreatin in digest	Assay titration N/10 sodium hydroxide	Pancreatin equivalent assay titration 4·5 ml.	Strength in terms B.P. limit = 1·0
	mg.		mg.	
A 3	15	4·6	14·4	1·73
A 2	15	5·06	11·0	2·27
A 1	15	5·1	11·0	2·27
B	25	4·8	20·3	1·23
C	25	4·47	26·7	0·94
D	25	3·1	51·1	0·49

THE ASSAY OF COMMERCIAL SAMPLES OF PANCREATIN BY THE PROPOSED MODIFICATION OF THE B.P. 1948 PROCESS

The graph relating mg. of pancreatin per digest with the resulting assay titrations, using varying quantities of Pancreatin A only, is shown in Figure 6. A plot of the square roots of the quantities against the assay titrations is also shown and is approximately a straight line for assay titrations of 2·5 and over.

Lactose content and the true strength of commercial samples of Pancreatin. The Pharmacopœia at present allows the addition of lactose to commercial pancreatin in the preparation of pancreatin B.P. In discussing the quality of commercial pancreatins therefore two points are of interest, namely the strength of the pancreatins as manufactured and the strength of the pancreatins as sold. That the latter differ widely can clearly be seen from the first part of Table IX. There is, however, much less difference in the strengths of the pancreatins as manufactured. Table X shows the percentage of lactose in the various pancreatins

examined and also the results of assaying the samples so that the quantity of real pancreatin in each digest was 7·5 mg. (i.e.) an allowance has been made for the lactose present. From the second part of Table IX it will be seen that all except two of the pancreatins examined would have passed the proposed test provided that they had not received additions of up to 87·6 per cent. of lactose.

The Keeping Properties of Pancreatin. Three of the samples of pancreatin (A1, C and D) were retested after 12 months' storage in the manufacturer's container on a shelf in the laboratory. Three other samples (B, E and F) were retested after 6 months' similar storage. In all cases any deterioration was within the experimental limits

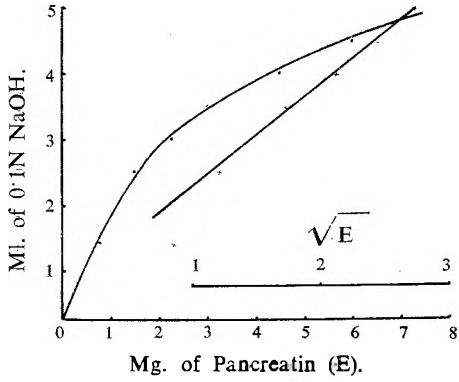

FIG. 6. Relation of the quantity and the square root of the quantity of pancreatin to the assay titration. Lower graph represents $\sqrt{E}$

TABLE IX

RESULTS OF ASSAYING COMMERCIAL SAMPLES OF PANCREATIN BY THE PROPOSED MODIFICATION OF THE B.P. 1948 ASSAY PROCESS

Sample No.	Pancreatin in digest	Assay titration	Pancreatin equivalent to assay titration of 3·0	Strength in terms of lower limit = 1·00
	mg.		mg.	
A1	7·5	5·0	2·19	3·42
B	7·5	1·5	24·2	0·31
C	7·5	0·8	56·2	0·13
D	7·5	0·6	1·30	0·06
E	7·5	1·8	16·1	0·47
F	7·5	2·7	9·95	0·75

Sample No.	True pancreatin per digest	Assay titration	Pancreatin equivalent to titration of 3·0	Strength in terms of lower limit (3·0) = 1·00
	mg.		mg.	
A1	7·5	5·0	2·19	3·42
B	7·5	3·5	5·1	1·47
C	7·5	2·5	10·05	0·71
D	7·5	3·1	7·25	1·03
E	7·5	3·4	5·63	1·33
F	7·5	2·7	9·95	0·75

of error of the method. It may be mentioned that the results recorded in Table IX were all obtained with the samples after storage.

TABLE X

LACTOSE CONTENT OF PANCREATINS AND TRUE STRENGTH OF COMMERCIAL PANCREATINS

Sample No.	Lactose	Pancreatin per digest	True pancreatin per digest	Assay titration
	per cent.	mg.	mg.	
A1	0·0	7·5	7·5	5·0
B	78·6	35·1	7·5	3·5
C	67·1	22·8	7·5	2·5
D	87·6	60·5	7·5	3·1
E	59·91	18·8	7·5	3·4
F	0·0	7·5	7·5	2·7

Lactose was estimated volumetrically using the method of Lane and Eynon in which methylene blue is used as indicator.

DISCUSSION

The B.P. 1932 assay process for trypsin in pancreatin had several faults. The digestion period of 1½ hours was unnecessarily long. More important, the use of fresh milk as a substrate was objectionable, not only because of the tedious nature of the preparative process but also on account of its notable variability in constitution. It might have been expected that variable results would be obtained from time to time and in different laboratories

The B.P. 1948 process amended as described in this paper is a definite improvement.

It has, however, been shown that during a typical digestion the *p*H of the digest falls from *p*H 8·7 to anything down to *p*H 6. This results in a progressive reduction in the activity of any given quantity of trypsin. At the optimum temperature of 55°C. there must be a steady even, if slight, destruction of the enzyme during the digestion period, again resulting in a progressive reduction in tryptic activity. Further, it has been shown that at the end of a typical digestion considerably more than 50 per cent. of the substrate has been digested. Indeed, for a satisfactory formol titration to be obtained there must be, of necessity, an accumulation of end products of digestion. It is not therefore surprising to find that, as shown in Figures 1, 2, 5 and 6, neither the graph of the time course of the digestion nor the graph of the relationship between quantity of enzyme and resultant assay titration is a straight line. Instead Schütz's Law is approximately, but only approximately, followed. Thus there is no simple mathematical relationship between the assay titration and the strength of a pancreatin. From Figure 6 it can be seen that while 2·25 mg. of pancreatin A1 give an assay titration of 3·0 ml., 7·5 mg., 3·3 times as much, is required to give a result of 5·0 ml.

The first part of Table IX shows that there is an undue variation in the strength of commercial pancreatins, one sample is approximately 50 times as active as another. This difference is shown by the second part of the table to be due mainly to the practice of adding lactose before sale. It appears to be the manufacturers of the weakest pancreatins who add the largest quantities of lactose. Indeed, if dilution with lactose had not been practised the strongest pancreatin would have been not quite 5 times as strong as the weakest.

There would appear to be two valid reasons for permitting the addition of lactose to pancreatin. In the first place the diluted preparation might be more suitable on account of its texture or other properties or because the larger quantities per dose were easier to handle. In that case it would be advisable to fix a not very high upper limit to prevent the dispensing of the presumably too strong material. Secondly, it might be necessary to permit the addition of lactose if variations in the strength of pancreatin as manufactured were large and unavoidable. In this case all batches could be diluted to a uniform standard within narrow limits. There might be something to be said for this but, in practice, as shown in the second part of Table IX there is not a very great difference in the strength of pancreatins as manufacturerd by the different firms but instead of being reduced, the difference is very greatly emphasised by the addition of lactose to the weaker materials.

If no lactose were added the lower limit of 3·0 suggested in the amended B.P. 1948 test would exclude only the two worst pancreatins.

From a comparison of the last columns in Tables VII, VIII and IX several conclusions can be drawn. The standard of the B.P. 1932 was so low that all the samples of commercial pancreatin complied with it while the best sample was 32 times stronger than the minimum and 3 or 4 times as strong as the maximum.

The products of 2 makers out of 4 complied with the requirement of the B.P. 1948, the best sample being just over twice as strong as required.

With regard to the amended test, which is really a rationalisation of the B.P. 1948 process, only one commercial pancreatin complied, being about 3·5 times as active as the minimum and equal to the maximum. If, however, the other samples had not been mixed with lactose only the two worst would have failed to comply and even these would have possessed over 70 per cent. of the required activity.

The minimum standard of the amended process appears to be about 10 times as high as the minimum of the B.P. 1932 when pancreatins of reasonable strength (A1 and F) are used.

If we assume that the purchasers of pancreatin B.P. should receive mainly pancreatin and not mainly lactose, and that the standard fixed should be one with which manufacturers could readily comply, and that it is desirable that the product should not vary from sample to sample to an undue degree, then the standards of the amended process are better than those of either the B.P. 1932 or B.P. 1948.

SUMMARY

1. Certain faults in the B.P. 1948 assay process for trypsin are pointed out. The chief is the presence of active enzyme in the blank.

2. An amended process, actually a rationalisation of the present process, is suggested.

3. A comparison of the standards of the B.P. 1932, the B.P. 1948 and the amended process is made.

4. It is found that commercial samples of pancreatin would be mainly of high quality if the weaker samples had not been further reduced in strength by addition of up to 80 per cent. of lactose.

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This work has been done at the request of the British Pharmacopœia Commission.

A NOTE ON THE B.P.1948 ASSAY PROCESS FOR TRYPSIN IN PANCREATIN

By G. E. FOSTER and W. SMITH

From the Control Laboratories, The Wellcome Chemical Works, Dartford, and the Analytical Laboratories, Allen and Hanburys Ltd., Ware

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THE British Pharmacopœia 1948 monograph on pancreatin includes an assay process for proteolytic activity which is different from the process used in the 1932 pharmacopœia. The latter depended upon the digestion of skimmed milk by pancreatin under standard conditions and a subsequent formol titration of the amino-acids formed, while the former employs casein as the substrate. The official process is based upon the work of N. Evers and W. Smith[1], who modified a process described by A. R. Smith[2] before this Conference in 1912. Since the publication of the B.P.1948 some disquieting results have been obtained which suggest that the standard of proteolytic activity, required by the new assay, is higher than that demanded by the B.P. 1932. It was the purpose of the present work to investigate this alleged difference.

For our purpose it was decided to assay a series of pancreatin samples (I) by the B.P.1932 process and (II) by the B.P.1948 process and make a critical comparison of the results. Work was carried out in our two independent laboratories, using as far as practicable the same materials. While the B.P.1932 process requires skimmed milk, easily prepared in the laboratory, it was found that casein of the quality required for the B.P.1948 process was unobtainable in this country. Evers and Smith used Hammarsten's casein, obtainable in Germany before the war, but only a small pre-war stock was available for the present work. Accordingly, it was decided to carry out experiments using two makes of purified light casein, representing the best quality of casein on the market. Control experiments were performed using Hammarsten's casein.

EXPERIMENTS USING THE B.P.1948 PROCESS

Assays were carried out strictly in accordance with the directions of the B.P.1948, to which reference should be made for experimental details. Preliminary work revealed that with some samples of pancreatin it is necessary to grind the preparation in a mortar under the chloroform water when preparing standard solutions. If this is not done low figures for the proteolytic activity result. Table I summarises the results obtained and for purposes of comparison these have been stated as (I) difference in 0·1N sodium hydroxide titration, as required by the B.P. and (II) difference in N sodium hydroxide titration calculated for 1 g. as suggested by Evers and Smith. The figures stated are in most cases the average of several determinations.

It will be seen that the results are influenced by the quality of casein

used and that there is some variation in the results obtained by independent workers. However, it is obvious that, of the samples examined, only "X" could be regarded as satisfying the B.P.1948 requirements.

TABLE I

B.P.1948 METHOD

Sample of Pancreatin	Casein used for assay	Difference as ml. of 0·1N sodium hydroxide		Difference for 1 g. as ml. of N sodium hydroxide	
		Lab. 1.	Lab. 2.	Lab. 1.	Lab. 2
X.	Light purified casein(1)	4·3	4·4	17·2	17·5
Y.	,,	1·6	1·7	6·4	6·8
Z.	,,	2·9	2·8	11·6	11·3
X.	Hammarsten's casein	4·8	5·2	19·2	20·9
Y.	,,	2·2	1·8	8·8	7·2
Z.	,,	3·4	2·8	13·8	11·3
X.	Light purified casein(2)	3·8	—	15·2	—
Z.	,,	2·0	—	8·0	—

The B.P. standard requires "difference as ml. of 0·1N sodium hydroxide" not less than 4·5, equivalent to 18ml. for 1 g.

With samples of pancreatin of low proteolytic activity the official process gives very small titration figures for the amino-acids formed by the digestion of the casein. Experiments were therefore carried out in an attempt to amend the process with a view to increasing the titration and thereby the accuracy of the assay when poor quality pancreatin was under examination. It was thought that this result might be achieved by either doubling the amount of pancreatin or by halving the strength of standard sodium hydroxide solution used for titration.

The results obtained with samples Y and Z and using these modifications of the official process are shown in Table II.

TABLE II

B.P.1948 METHOD BUT USING TWICE THE AMOUNT OF PANCREATIN SPECIFIED (i.e., 20 ML. OF SOLUTION INSTEAD OF 10 ML.). PURIFIED LIGHT CASEIN(1) USED

Sample of pancreatin	Difference as ml. of 0·1N sodium hydroxide	
	Lab. 1.	Lab. 2.
Y.	3·5	3·9
Z.	4·2	4·1

B.P.1948 METHOD BUT TITRATING WITH 0·05N SODIUM HYDROXIDE INSTEAD OF 0·1N. PURIFIED LIGHT CASEIN(1) USED

Samples of pancreatin	Difference as ml. of 0·05N sodium hydroxide	
	Lab. 1.	Lab. 2.
Y.	4·2	3·8
Z.	5·7	4·3

It was concluded that use of 0·05N sodium hydroxide increased the error of the determinations owing to difficulty in judging the end-point and, for this reason, doubling the amount of pancreatin used is a more satisfactory modification for the assay of poor samples.

In the case of a sample of high proteolytic activity the amount of pancreatin taken in the assay should be correspondingly decreased in order to give a titration figure for the amino-acids of 4·0 to 4·5 ml. of 0·1N sodium hydroxide. When treated in this way a sample labelled U.S.P. Pancreatin (triple strength) gave the following results—*Lab.* 1. 1 g. equivalent to 35·2 ml. N sodium hydroxide. *Lab.* 2. 1 g. equivalent to 29·2 ml. N sodium hydroxide.

Owing to the difficulty of seeing end-points in assays carried out with the purified light casein, it was thought of interest to explore the possibility of using electrometric methods. For this purpose assays were carried out by the B.P.1948 method except that no indicators were used; *p*H adjustments were made and end-points determined with a direct reading *p*H meter using a glass electrode. The results obtained on samples X, Y and Z were substantially the same as those recorded using indicators, as directed by the B.P. The electrometric method may be of value when caseins, affording opalescent solutions in sodium hydroxide, are used, but it has no advantage over the use of indicators when casein, of the quality of Hammarsten's, is available.

Experiments Using the B.P.1932 Process

Assays were carried out in accordance with the directions of the B.P.1932. The results are summarised in Table III.

TABLE III
B.P.1932 METHOD

Sample of Pancreatin	ml. of 0·05N sodium hydroxide for A		ml. of 0·05N sodium hydroxide for B	
	Lab. 1	Lab. 2	Lab. 1	Lab. 2
X.	6·7	5·1	13·5	12·0
Y.	4·8	4·9	9·0	8·5
Z.	4·8	4·9	10·2	9·0

B.P. 1932 standard requires "A" not less than 4·9 and not more than 5·1 ml. 0·05N sodium hydroxide, "B" not less than 9 and not more than 13 ml. 0·05N sodium hydroxide.

According to these figures samples of pancreatin X and Z may be regarded as complying with the requirements of the B.P.1932 while Y barely conforms to the lower limit.

Preparation of Purified Casein (B.P. Appendix)

While this investigation was in progress, we also investigated the preparation of purified casein suitable for this assay. The original method of Hammarsten[3] was as follows:

Fat-free milk is diluted with 4 volumes of distilled water and sufficient

10 per cent. acetic acid added with constant stirring to make the excess acid about 1 per cent. over that required to coagulate the casein. After settling, the clear liquid is removed by siphoning and the curd thoroughly washed several times with cold water. The casein is dissolved in the minimum amount of dilute ammonia solution, the solution filtered and the casein reprecipitated with acetic acid. This process of dissolving and reprecipitating is repeated twice. After washing with water and alcohol the casein is extracted with ether in a Soxhlet apparatus and dried in a vacuum at 60° to 70°C.

It was found that a simpler procedure of precipitating casein gave a product which, though inferior to Hammarsten's casein, would satisfy the requirements of the B.P. Appendix I, p. 641. Skimmed milk is centrifuged to remove the extraneous matter and the milk heated to 37°C. The casein is precipitated by the cautious dropwise addition of dilute hydrochloric acid with constant stirring until the *p*H is 4·0. The curd is thoroughly washed by decantation first with hot water acidified with hydrochloric acid, then with several lots of hot water, followed by alcohol and finally with ether. The curd is dried by exposure on a stainless steel tray and finally sieved and powdered.

Samples of pancreatin X, Y and Z, were assayed using casein prepared as described above and the results were very similar to those obtained using Hammarsten's casein.

Discussion

The results obtained during this investigation leave no doubt that the B.P.1932 requires a much less stringent standard for the proteolytic activity of pancreatin than does the B.P.1948. Some estimate of the quantitative difference between the two standards may be arrived at by consideration of the data on sample Y. This specimen of pancreatin is one which only just complies with the requirements of the B.P.1932, which requires the "B" titration to be not less than 9 ml. of 0·05N sodium hydroxide. But according to the B.P.1948 method 1 g. of pancreatin (Y) is equivalent to 6·6 ml. (purified light casein(1)) or 8 ml. (Hammarsten's casein). As the B.P.1948 standard requires 1 g. of pancreatin to be equivalent to 18 ml. of N sodium hydroxide it would appear that the official standard is about $2\frac{1}{2}$ times as stringent as that of the B.P.1932.

Evers and Smith suggested that a reasonable limit for pancreatin would be that 1 g., when assayed by their method, should give a titration of not less than 15 ml. of N sodium hydroxide. This they stated was approximately equivalent to the B.P.1932 standard. The present work does not support the latter statement, though this appears to have been justified on the six samples tested at the time.

When Evers and Smith published their paper, there is no doubt that pancreatin complying with their suggested standard was readily available. At the present time much pancreatin is of lower activity. It therefore seems to us that if it is desirable that the official standard for proteolytic activity of pancreatin should be amended to bring it more in harmony

with the material at present available, this could be achieved by halving the required proteolytic activity and could best be brought about by doubling the amount of pancreatin employed in the assay. When proteolytic activities are to be compared the amount of samples taken should, of course, be adjusted to give titrations of 4·0 to 4·5 ml. of 0·1 N sodium hydroxide by the B.P.1948 method.

Our experience in this work has again emphasised the necessity to use casein, similar in quality to Hammarsten's, for assay purposes and it is hoped that the details, here published for preparing suitable material, will be of value to other workers in this field.

Summary

1. Samples of pancreatin have been assayed for proteolytic power using the B.P.1948 method and it has been found that the pancreatin, at present available, often fails to comply with the official standard.

2. Experiments using different samples of casein as substrate in the assay process established that the quality of the casein influences the results of the assays.

3. For satisfactory results to be obtained casein, similar in quality to Hammarsten's casein, is required. A process for the preparation of suitable casein is described.

4. Three samples of pancreatin have been assayed by the methods of the B.P.1948 and B.P.1932 respectively. It has been shown that the B.P.1948 standard for proteolytic activity is more stringent than that of the B.P.1932.

5. A suggestion is made for amending the official standard in order to bring it into harmony with the quality of the pancreatin at present available.

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DISCUSSION

The two papers dealing with pancreatin were discussed together, the first being presented by Dr. Bullock and the second by Dr. Foster.

THE CHAIRMAN said that it was felt that the B.P.1948 method for the assay of pancreatin was an improvement on the 1932 method, but he could confirm Dr. Foster's statement that manufacturers found it difficult to comply with the B.P. requirements. He agreed with Dr. Bullock's suggestion of reducing the amount of lactose but a certain proportion was desirable in any preparation of this kind.

DR. NORMAN EVERS (Ware) said that the most important observation in Dr. Bullock's paper concerned the digestion of the blank. It was to be

hoped that as a result of these two papers, a more satisfactory process would be obtained. The reason for adjusting the *p*H to 7·0 was that if one took a pure amino acid, such as aminoacetic acid, adjusted the *p*H to 8·7, and then added formaldehyde and titrated, one did not get 100 per cent. results. He did not think that Dr. Bullock and Mr. Sen had taken sufficient notice of the type of casein used, as Foster and Smith had shown that it was important. Certainly it led to greater accuracy to have a clear solution for the titration. From experience he could confirm that the quality of pancreatin was very much poorer than it had been before the war. He thought that the work showed the need for the B.P. to state limits of accuracy of analytical methods rather than the standard figure as at present.

DR. G. E. FOSTER, in reply, said that he was inclined to agree with Dr. Evers that this was an analytical process for which great accuracy could not be expected. It was not known whether pancreatin was one enzyme or many, and obviously any assay process must be empirical. What had to be decided was which method, in all circumstances, was the best. One of the merits of the paper by Bullock and Sen was the fact that the blank solution was boiled, and in this respect the B.P.1948 process should be amended. However, he was inclined to believe that the suggested difference in the titration technique was something which might, if adopted, lead in time to further trouble.

MR. J. K. SEN said that Dr. Foster did not seem able to find the actual defect in the B.P.1948 process, and his suggestion of doubling the amount of pancreatin to be taken to bring the weakest pancreatin to the B.P. standard, or halving the strength of the alkali to do so, seemed unscientific, to say the least. Six or seven different types of casein had been tried and it was found that "light white soluble casein" gave about the same results as the others. Several experiments had been carried out on adjusting the *p*H to 7·0 before titration, and it was found that adjustment to 8·7 after digestion did not give variable results, whilst the adjustment to *p*H 7 was difficult because it was done colorimetrically by matching against some standard buffer and was less accurate. Boiling the blank kept it constant, and made it easy to test different pancreatins and compare their strength. Experiments at different times had shown the accuracy to be ±1·0 per cent. which he thought was sufficient.

DR. K. BULLOCK said that if Dr. Foster would try out their process he would get repeatable results without difficulty. They agreed, of course, that the new B.P. standard was higher than previously, but it was impossible to say anything about it in one sense because the results would always depend on the temperature of the laboratory, and on a number of other factors. That undoubtedly explained some of Dr. Foster's difficulties in getting comparisons between the two laboratories. Boiling the blank was most important, and he could not conceive of an enzyme chemist who would use a blank with active enzyme in it which might amount to 30 per cent. or more of the total digestion in the actual test. The blank

must either be boiled or kept at a low temperature so that no digestion went on, and some did occur even at 0°C. On the question of the kind of casein, if one were going to adjust to *p*H 7, at the end before adding the formaldehyde one would require purified casein. If one were going to do the titration to phenolphthalein only, then, although it was of some advantage to have a clear solution, it was by no means a necessity, and it was possible to get repeatable and accurate results with ordinary light white casein. The suggestion of adjusting to *p*H 7 apparently originated in a paper by Northrop in which he estimated accurately certain quantities of free amino acids. The present B.P. process, however, was empirical and did not estimate accurately the amount of free amino acid. If the adjustment was made in a sample containing fat, then in the assay for trypsin one might well estimate some of the fatty acids which had been liberated. Secondly, one would also include acidity liberated by the digestion but not due to the removal of NH_2 groups; i.e., acidity which was not part of the formol titration at all. From Figure 1 of the paper, the lower curve, it would be seen that the acidity fell off much more rapidly with increasing quantities of pancreatin or with increasing time. It confused the issue, therefore, to include this acidity in the formol titration. Not only did the B.P. do that, but there was another factor which was introduced. If the blank was adjusted to *p*H 7 one would be starting the blank and the test differently, because during the digestion the *p*H fell so that the blank was not at *p*H 7 and had not the same buffer value as the test. By adjusting to *p*H 7, therefore, one was really adding some but not all of this acidity other than formol titration acidity. The amount of this added acidity was purely arbitrary.

He would be pleased if Dr. Foster would see whether the presence of lactose was not capable of explaining almost entirely the low quality of present day pancreatin. They themselves had found that when lactose-free, almost all the samples would comply with the standard which they suggested. It might be right to have some lactose in pancreatin, but the present situation was anomalous. There was a standard given by the B.P. and yet manufacturers were selling triple B.P. strength. The correct position would be for true pancreatin to be sold as such and then, if there was a demand for a lower strength, it could be sold as " half-strength " B.P., etc. The pure substance should be called pancreatin and the dilution should be called a dilution.

DR. G. E. FOSTER said that his paper was only intended to be in the nature of a note. They had been asked by one of the B.P. Committees to find out whether there was any substance in the complaints which had been received. There was a discrepancy between results obtained by the B.P.1932 process and those obtained by the 1948 process. The pharmacopœial assay process laid down a test and if one carried out the test exactly as directed and got the result given there, the substance passed the test and could be referred to as B.P. It did not specify the estimation of a particular substance.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Adrenaline, Determination of, in Mixtures. C. O. Björling and H. Hellberg. (*Farm. Revy.*, 1950, **49**, 69.) *Correction*: In the abstract on page 519 of this Journal the statement that " results are up to 50 per cent. too high " is incorrect, and is due to a misreading of the heading of the table in the original paper. The whole of the last sentence of this abstract should be deleted. C. H. H.

Alkaloids, Partition Chromatography of. R. Munier and M. Macheboeuf. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1144.) Partition chromatography of various alkaloids and alkaloidal bases has been studied: the technique is applicable if widely differing conditions of *p*H and development are used. In developing the spots obtained on filter-paper strip chromatograms, various reagents were tried. Ultra-violet light was useful and out of 44 alkaloids studied, cinchonine, quinidine, quinine, cinchonidine, narcotine, cotarnine, hydrastinine, boldine, berberine, corynantheine, chelidonine, papaverine, and piperine were detectable in amounts of less than 20μg.; iodine vapour could be used for mescaline, hordenine and ephedrine: iodine in potassium iodide solution gave a brown colour with many alkaloids. Dragendorff's reagent detected 0·2μg. of hyoscyamine and larger amounts of berberine, yohimbine, trigonelline, theobromine, theophylline, nicotinamide and betaine, although caffeine only showed in amounts of 50μg.; nicotine, sparteine, hydrastinine and cotarnine were red while the colour of the other alkaloids ranged from yellow to orange with this reagent. Potassium iodoplatinate showed up spots due to yohimbine, ephedrine, hydrastinine, trigonelline, hordenine, and nicotinamide in quantities from 10 to 20μg. Phosphomolybdic acid treatment followed by washing with water and reduction with stannous chloride solution was useful in detecting 20μg. of cicutine and mescaline although betaine, trigonelline, caffeine, theobromine, theophylline and nicotinamide gave negative results: phosphomolybdic acid solution alone showed up caffeine, theobromine, theophylline and trigonelline. A description of a chromatographic technique similar to that of Consden, Gordon and Martin is given; using this method boldine, sparteine, berberine, chelonidine, hydrastinine and cotarnine were run as bases dissolved in 20μg. amounts in chloroform; for the solvent phase, toluene, glycol, monochlorhydrin and ammonia were used. Caffeine, theobromine, theophylline, trigonelline and ephedrine could also be separated in an alkaline medium. Morphine, thebaine and codeine were run in acid medium as the free base while other examples are given of alkaloids run in acid medium as the salt. Detailed conditions of procedure are given together with diagrammatic representations of the developed chromatograms. R. E. S

Digitalis Glycosides ; Modified Bell and Krantz Method for the Assay of. E. E. Kennedy. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 25.) The following method is applicable to the assay of digitoxin, digoxin and lanatoside C. 10ml. of a methyl alcoholic solution of the glycoside containing about 0·4mg./ml. is mixed with 15ml. of picrate reagent and allowed to stand for exactly 30

minutes. The optical density of the mixture is read on a suitable spectrophotometer using 1-cm. cells at 495mμ and the amount of glycoside calculated from the standard graph. A blank consisting of a mixture of 10ml. of methyl alcohol and 15ml. of picrate reagent is used as a comparison solution. The picrate reagent is prepared by dissolving 1g. of trinitrophenol in 15ml. of methyl alcohol, adding 20ml. of a 10 per cent. solution of tetra-ethylammonium hydroxide and diluting to a 100ml. with water ; it should be freshly prepared. The standard graph is prepared by measuring the optical densities of solutions containing 0·2, 0·4, 0·6 and 0·8mg. of the appropriate reference standard in 10ml. of methyl alcohol treated in the same manner as the test solution. By reading the optical density at 495mμ instead of 525mμ the sensitivity of the method is increased two and a half times. The accuracy of the method is within 2 per cent. of the amount of glycoside present. G. R. K.

Fat Analysis; A Micro-method based on the Formation of Monolayer Films. K. K. Jones. (*Science*, 1950, **111**, 9.) Droplets of piston oil are placed on the cleaned surface of 0·3 per cent. sulphuric acid contained in a glass dish coated with hard paraffin, until the colour of the surface is green. The fat to be estimated is extracted from the original material and used as a solution in light petroleum. An aliquot portion of this solution is placed by means of a capillary pipette on the centre of the oil surface. The light petroleum evaporates, and the fat spreads against the piston oil until equilibrium is established. The area occupied by the fat is measured, and is a sensitive criterion of the amount of fat present; the conversion factor depends upon the constitution of the fat under examination.

G. R. K.

Imidazoline Derivatives, Identity Reaction of. H. Laubie. (*Bull. Trav. Soc. Pharm., Bordeaux*, 1950, **88**, 65.) To 1 ml. of a solution containing about 1 μg. of the compound, add 0.3 ml. of 5 per cent. solution of sodium nitroprusside and 0·5 ml. of N sodium hydroxide, then buffer the solution by the addition of 1 g. of sodium bicarbonate. After a few minutes the brownish-green colour changes to a violet. It has been shown that penicillin, at *p*H2, gives rise to a compound containing an imidazol nucleus; this compound however gives only a weak orange red colour after several hours. G. M.

Iodine and Bromine in Organic Compounds, Determination of. O. Michel and G. Deltour. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1125.) A technique is described which allows the determination of from 100 to 500 μg. of iodine, and from 50 to 500 μg. of bromine simultaneously in an organic compound, with an approximate error of ±4 per cent. To the organic substance, containing about 200 μg. of iodine, is added a small amount of sodium arsenite solution and the resulting mixture is heated to redness in a nickel crucible. On cooling the residue is dissolved quantitatively in 10 ml. amounts of warm distilled water, transferred to a suitable vessel and acidified with 5N sulphuric acid to congo red. 1 ml. of sodium nitrite solution (5 per cent.) is added and the resulting product is extracted with 5, 3 and 2ml. quantities of carbon disulphide. The carbon disulphide solution is separated, centrifuged to free from suspended material, and the depth of the violet colour is measured on a suitable spectrophotometer using as comparison solution a blank prepared in the same way as the test solution but without the iodine. In the case of compounds containing bromine and iodine the colour

due to bromine does not interfere with the above procedure. The estimation of bromine in compounds containing bromine and iodine follows the method of Leipert and Watzlawek (*Hoppe-Seyl. Z.*, 1934, **226,** 108) in which the substance is heated for 12 hours in a sealed tube at 100°C. with 5N sulphuric acid; the solution resulting from this method is divided into two parts. One part gives the total bromine following the method of Leipert and Watzlawek and includes some of the iodine; the other part is used for a determination of the iodine by the sodium arsenite method. The iodine result obtained in the second estimation is subtracted from the result (bromine plus iodine) obtained in the first estimation, to give the true bromine content. Recovery experiments on di-iodotyrosine, dibromotyrosine and known mixtures of these two compounds gave satisfactory results. R. E. S.

Phenolphthalein in Mineral Oil Emulsion, Determination of. A. T. Warner, J. E. Logun and R. L. Thatcher. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39,** 10.) About 10 ml. of the emulsion is shaken with two 30-ml. and four 20-ml. quantities of a 25 per cent. solution of sodium chloride containing 0·25 per cent. of hydrochloric acid. The aqueous layers are filtered, the filter paper washed and placed in a 500-ml. flask, to which is added the oily layer from the separating funnel, previously mixed with 30 ml. of sodium hydroxide solution. The funnel is washed with water and the washings are also added to the flask followed by 50 ml. of 0·1N iodine. The flask is stoppered and shaken until the mixture becomes white with a greenish-yellow tint, when 15ml. of hydrochloric acid is added and the excess of iodine titrated with 0·1N sodium thiosulphate ; each ml. of 0·1N sodium thiosulphate is equivalent to 0·003979g. of phenolphthalein. G. R. K.

Water in Glycols and Glycerol, Determination of. C. B. Jordan and V. O. Hatch. (*Anal. Chem.*, 1950, **22,** 177.) A method is described for determining the percentage of water in samples or solutions of glycols and glycerol, based on reflux distillation and salting out and using *n*-butyl alcohol as the refluxing medium, since it forms an azeotrope with water but not with glycol or glycerol and is capable of breaking any hydrates formed in aqueous glycol solutions. The sample under test is placed in a flask fitted with a side-inlet for a thermometer and a Dean and Stark (Barrett type) receiver trap with a water condenser having a calcium chloride tube inserted at the top; *n*-butyl alcohol (b.pt. 238° to 243°F.) is added and the flask is heated slowly until boiling is steady. For samples containing large percentages of water it is necessary to withdraw small portions of water from the trap at regular intervals; after 2·5 hours refluxing the volume of the lower layer in the trap is checked at 20 minute intervals and if there is no visible change in volume the water layer is withdrawn and the heating is increased up to a temperature 3°F. below the boiling point of the butyl alcohol. All the butyl alcohol and water now distilled from the flask is mixed in a measuring cylinder, the volume is recorded, anhydrous potassium carbonate is added and the volume of the top butyl alcohol layer is noted; further potassium carbonate is added until there is no increase in the volume of the alcoholic layer; this volume subtracted from the total distillate volume gives the amount of water present. For aqueous mixtures of glycols or polyhydric alcohols of unknown boiling-point the above procedure can be used with modification; after the 2·5-hour refluxing, or at the time when no increase in the water layer is noticed, the water layer is removed from the trap, the distillation is continued to 350°F., the whole of the liquid is withdrawn from the trap and salted out as

before. 40 samples of known composition, varying in water content from 1 to 95 per cent., were analysed with a maximum error of 0·5ml. on 100ml. quantities. The method was applicable to antifreeze solutions, mixtures of glycols and glycol-ethers and *n*-butyl alcohol and alcohols containing more than 4 carbon atoms. R. E. S.

Yohimbine, Quantitative Reaction for. L. S. Malowan. (*Analyst*, 1950, **75**, 338.) Dimethylaminobenzaldehyde gives coloured compounds with a series of indole derivatives in the presence of mineral acids which can be transformed to blue compounds in the presence of weak oxidants such as hydrogen peroxide or nitrous acid; yohimbine also gives this reaction. Dissolve a small amount of the alkaloid in about 1 ml. of concentrated hydrochloric acid. Add 4 drops of a 2 per cent. solution of *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid and warm. Add 2 drops of a 0·05 per cent. solution of sodium nitrite to the colourless test liquid. In the presence of yohimbine, a deep violet-blue ring appears after a short time and on shaking gently the whole solution becomes deep blue. 1 mg. of yohimbine gives a distinct reaction and tablets containing yohimbine can be treated in the same way, with the same result. R. E. S.

ORGANIC CHEMISTRY

Aldehydes, Direct Chlorination of. H. Guinot and G. Tabuteau. (*C.R. Acad. Sci., Paris*, 1950, **231**, 234.) Aliphatic aldehydes can be chlorinated, with a good yield of α-derivatives, by the direct action of chlorine in strongly hydrochloric acid solution. Under these conditions the oxidising action of the chlorine is greatly reduced, while substitution proceeds at a low temperature, thus avoiding condensation reactions. With acetaldehyde at a concentration of 4·5N, and 7N hydrochloric acid, formation of monochloracetaldehyde occurs at 18° to 20°C. In order to obtain the dichlorinated product a temperature of 35° to 40°C. is necessary, while, to obtain chloral, the temperature must be raised first to 80°C., the reaction being finished at 90°C. under reflux. The same method may also be applied to paraldehyde, yields of more than 90 per cent. of the various chloracetaldehydes being obtained. The reaction mixture may be separated either by fractionation at reduced pressure, or by extraction with ether. With *n.* propionaldehyde, a temperature of 15°C. gives the monochlorinated product; 30°C. gives α-dichloropropionic aldehyde. No further substitution is attained even at the boiling-point of the mixture. With *iso*propionic aldehyde, 20° to 25°C. gives the mono-derivatives, but no higher derivatives are obtained. G. M.

Curcumin and Curcuminoids. T. Pavolini, F. Gambarin and A. M. Grinzato. (*Ann. Chim. applic., Roma*, 1950, **40**, 280.) Curcumin, the yellow principle from turmeric, can be prepared by fusing acetylacetone with vanillic aldehyde and boric anhydride. The purified product melts at 178° to 180°C. A brown substance which the authors call curcumin brown is also obtained. Made in this way the product gives the characteristic red colour with boric acid and sodium hydroxide, whereas when the condensation is carried out with hydrochloric acid as in Heller's method α and β *iso*-curcumins are obtained which do not give colours with boric acid. Curcumin proper is the keto-enolic form while the *iso*-curcumins are the di-ketonic and di-enolic forms, there are also *cis* and *trans* forms known as rubro-curcumin and

roso-cyanin. By the same reaction the authors have prepared similar compounds from other aromatic aldehydes, which they call curcuminoids. From piperonylic aldehyde, dipiperonylacriloymethane (m.pt. 190° to 195°C.); from cinnamic aldehyde, dicinnamylacriloylmethane (m.pt. 189° to 190°C.), from benzoylacetone and vanillic aldehyde, benzoylfuroylmethane (m.pt. 152°C.); from cinnamoylacetone and vanillic aldehyde, cinnamoylferuloylmethane (m.pt. 137° to 140°C.); from acetylacetone and salicylic aldehyde, dicumaroylmethane in very small yield. H. D.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Adrenal Cortical Hormones, Synthetic Analogues of. G. P. Hager and R. M. Burgison. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 7.) 4 : 4′-bis-(Acetoxyacetyl)-$\alpha\alpha'$-diethylstilbene, $(CH_3.CO.O.CH_2.CO.C_6H_4.C(C_2H_5) :)_2$, was prepared from *p*-bromopropiophenone in the following way. *p*-Bromopropiophenone was converted to the hydrazone by heating with 85 per cent. hydrazine hydrate, with simultaneous removal of water, and the hydrazone oxidised with freshly precipitated mercuric oxide to *p*-bromophenyl ethyl diazomethane, which was converted to 4 : 4-dibromo-$\alpha\alpha'$-diethylstilbene by treatment with sulphur dioxide and heating the sulphone so formed. The *trans* and *cis* isomers of 4 : 4′-dibromo-$\alpha\alpha'$-diethylstilbene were obtained separately in yields of 52 and 16·8 per cent. respectively. The *trans* isomer was converted by way of the dicyanide, dicarboxylic acid, and di-acid chloride to the diazoacetyl compound, which was finally treated with glacial acetic acid and acetic anhydride to give the required product in 34 per cent. yield. G. R. K.

Chloromycetin, and Serine Derivatives, Analagous action of. C. Mentzer, P. Meunier, L. Molho-Lacroix and D. Billet. (*Bull. Soc. Chim. Biol.*, 1950, **32**, 55.) Chloromycetin may be considered as a derivative of *d*-serine, and it has already been shown that *d*-serine (but not *l*-serine) retards the growth of *E. coli*, while *l*-aspartic acid increases this inhibitory action and aminoacetic acid or dl-alanine annul it. The authors have now shown that similar effects are observed with chloromycetin as with *dl*-serine. *dl*-Phenylserine also has an inhibitory action on *E. coli*, but its behaviour in presence of the other amino acids indicates that its biological action is farther removed from that of chloromycetin than is that of *dl*-serine itself. G. M.

Dihydrostreptomycin Base, Crystalline. H. W. Rhodehamel Jr., S. L. McCormick and S. F. Kern. (*Science*, 1950, **111**, 233.) Dihydrostreptomycin base may be obtained by treatment of relatively pure dihydrostreptomycin sulphate with a strongly basic ion-exchange resin (for example, Amberlite IRA-400) or by titration with barium hydroxide solution to *p*H 12, and precipitation from the aqueous solution with acetone. The oily precipitate gradually crystallises in needles with some tendency to cluster in rosettes. Analysis for elements is in agreement with the formula $C_{21}H_{41}O_{12}N_7,H_2O$, and the product has the following characters:—chars at 240°C. and turns black without melting up to 300°C., *p*H of a 1 per cent. aqueous solution, 12 and biological potency (against *E. coli* by a turbidimetric test), 922 µg. per mg. G. B.

Sodium Azide Preservation of Blocking-type Rh Antiserum. M. Jayne, H. C. Batson and M. Brown. (*J. Lab. clin. Med.*, 1950, **35**, 988.) A study is reported of the preservative effects of sodium azide on unfortified and bovine albumin-fortified blocking-type D(Rh_0) anti-serum stored at refrigerator and room temperatures, and 37°C. over a period of 72 weeks. Sodium azide in a concentration of 0·1 per cent. was found to exert no deleterious effect on the blocking-type antibody and to be a satisfactory bacteriostatic agent in both unfortified and albumin-fortified serum specimens. The agglutinative activity of albumin-fortified serum specimens was much less affected by bacterial contaminants, temperature and time of storage than was that of unfortified samples. S. L. W.

BIOCHEMICAL ANALYSIS

Amino-Acids, Paper Chromatographic Identification of. E. M. Gal and D. M. Greenberg. (*Proc. Soc. exp. Biol. N.Y.*, 1949, **71**, 88.) The amino-acids examined were DL-valine, DL-leucine, and DL-phenylalanine and their *N*-ethyl, *N*-isopropyl, *N*-propyl and *N*-phenyl derivatives. They were separated in 25 μg. quantities with phenol-water and collidine-water solvents. After the solvent had reached a 20-cm. liquid front (within 20 hours), the paper strips were air-dried and examined by ultra-violet light, when the unsubstituted amino-acids showed as faint blue spots and the *N*-substituted derivatives as absorbing non-emitting spots. After treatment with a 0·15 per cent. ninhydrin solution in saturated butyl alcohol-water, all the acids responded although the spots given by the *N*-substituted acids were much less intense in colour than those given by the corresponding unsubstituted acids. Colour intensity and R_F values could be correlated with the rate of hydrolysis of the substituted and with the steric arrangement. During drying, the *N*-phenyl derivatives, which were very soluble in the solvents used, were lost from the papers. G. R. K.

Aureomycin in Blood and Urine, Estimation of. A. Saltzman. (*J. Lab. clin. Med.*, 1950, **35**, 123.) The antibiotic is separated from interfering substances by adsorption on a column of Decalso, which is washed with limited amounts of distilled water, ethyl alcohol and then air dried. Elution is performed with hot 5 per cent. sodium carbonate solution. In alkaline solutions, aureomycin fluoresces a bright blue under ultra-violet light. The fluorescence of the aureomycin in the eluate is directly measured in a fluorophotometer. The procedure requires only 1ml. of serum which need not be sterile. The sensitivity of the method (0·5 to 10 μg./ml.) is adequate for the usual range of serum values as found by microbiological methods. While the latter have better sensitivity, their accuracy, especially in the upper range, is somewhat less. Similar values are obtained by the application of both methods to blood and urine. The specificity of the procedure was tested with common drugs and with uræmic blood and no interference was found. S. L. W.

Chloromycetin in Serum or Plasma, Colorimetric Determination of. S. P. Bessman and S. Stevens. (*J. Lab. clin. Med.*, 1950, **35**, 129.) A microcolorimetric method is described for the determination of chloromycetin in 1 ml. samples of serum, based on the reduction of the aryl nitro group with stannous chloride, and subsequent diazotisation and coupling to produce a red complex. The method is about 4 times as sensitive as the method of Glazko and co-workers and has the advantages that the entire reaction can be carried

out in the colorimeter tube, no dilution is necessary, no filtration or centrifugation is required to remove the reducing agent, and adenine does not interfere with the determination. A comparison of the values for sera determination by this method and by microbiological methods indicates reasonable agreement, within the limits of error of the microbiological methods. S. L. W.

Heparin Assay, a new Technique for. D. C. McGoon. (*J. Lab. clin. Med.*, 1950, **35**, 111.) This method consists of testing short, heparin-induced prolongations of the clotting-time of recalcified citrated human blood with an accurate end-point which determines an incipient degree of clotting. This is made possible by employing a specially constructed hour-glass tube. These tubes are 17 cm. in length, with an inside diameter of 1 cm. At 7·5 cm. from each end an hour-glass constriction commences, narrowing at the centre to an inside diameter of 1 mm. In the centre of each half of the tube there is a small air-vent. These tubes are secured, the bottom openings corked, in a tilt rack which holds 10 tubes, air vents all facing in one direction. Tubes 1 and 10 are used as controls and 1 ml. of saline solution placed in each. Tubes 2 and 9 are used to determine clotting-time of blood containing a heparin solution of known concentration, say, 1 ml. of a normal saline dilution containing approximately 0·45 to 0·50 unit of heparin /ml. Tubes 3 to 8 are used to determine clotting-times of blood containing 6 different dilutions of the unknown heparin solution, and differing approximately 2 to 3 per cent. in concentration. 0·5 ml. of 1 per cent. solution of calcium chloride is added to each tube ; 1 ml. of blood is then added to each tube at nearly the same instant. The top openings of the tubes are then immediately stoppered and the tubes inverted 3 times, as rapidly as the blood solution will flow from one end to the other. The tubes are then inverted every 15 seconds. After from 3½ to 5 minutes the controls will suddenly stop flowing. If 15 seconds intervene between the two controls the longer is considered the control clotting-time ; if 30 seconds, their average. As the remaining tubes clot the time is recorded. From these data, the beginning time is subtracted from the time of clotting, and thus the clotting-time is calculated. The prolongation of clotting-time is then calculated by subtracting the control clotting-time from the actual clotting-time of the heparinised blood, and these values are recorded. The test should be repeated 3 times and the average values plotted on a graph. S. L. W.

Œstrogens Natural, Polarographic Estimation of. C. Heusghem. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1114.) A new method is given for the polarographic estimation of three natural œstrogens. In working out the details of the method a study has been made of the conditions necessary for the formation of the nitroso-derivatives of œstrone, œstradiol and œstriol and the solutions necessary for the reaction are given. For œstrone 50 to 250μg. in alcoholic solution is evaporated under reduced pressure, a specific reagent (acetic acid, 5; sulphuric acid, 5; nitric acid, 0·5) is added to produce the nitroso-derivative, the mixture shaken and placed in a thermostat at 35°C. for 30 minutes. The product is diluted with water and made alkaline by the addition of potassium hydroxide solution (20 per cent.), the solution is freed from oxygen by the passage of nitrogen and polarographed from a voltage of −0·3 to −1·05; polarographic curves are given for the various œstrogens. It was found experimentally that polarographic reduction was best carried out in alkaline solution and the strongly acid reaction solution was therefore made alkaline with a small amount of potassium hydroxide; using these conditions the solution obtained for polarographic estimation was stable for several hours. With the technique described, the

estimation is quantitative up to a maximum of 350μg. for œstrone and œstradiol and up to 500μg. for œstriol. Amounts of œstrogen as low as 5 to 20μg. can be determined. R. E. S.

Œstrone, Equilin and Equilenin in Mixture, Colorimetric Determination of. D. Banes. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 37.) Three separate colorimetric determinations are required. One portion of the mixture is treated with dibromoquinonechloroimide in slightly acid solution, when equilenin yields a stable red pigment extractable with chloroform, equilin slowly develops a similar product with approximately half the intensity, and œstrone gives practically no colour. A second portion is esterified with benzenesulphonyl chloride and the benzenesulphonates so obtained are treated with the same reagent, when equilin gives a stable violet pigment and equilenin and œstrone do not react. The third portion is heated with a reagent containing phenylsulphonic acid and ferric iron in sulphuric acid, when œstrone gives a deep orange-red colour with a green fluorescence, equilin a blue colour and equilenin an orange-pink colour. From optical density measurements of each determination and of suitable standard solutions of the pure ketosteroids, the composition of the original mixture can be calculated. The method was applied to solutions of the œstrogens in sesame oil, extracts from pregnant mares' urine and commercial preparations. Results showed good agreement with the infra-red spectro-photometric method. G. R. K.

Penicillin in Blood, Micromethod for Assay. G. Hildick-Smith and Mary Fell. (*J. Lab. clin. Med.*, 1949, **34**, 1687.) The method requires 0·2 ml. of blood, which can be conveniently collected from a single puncture wound of the finger using the collecting tube described. The serum is separated by centrifuging and added in diminishing amounts to a series of tubes containing a phenol red dextrose broth. The tubes are inoculated with a suitable culture of any penicillin-sensitive organism and incubated at 37°C. overnight. When the amount of penicillin in the blood is insufficient to inhibit the growth of the organism the dextrose broth becomes acid and changes colour. By running a series of control tubes using a penicillin solution of known strength, the concentration of penicillin in the serum can be readily calculated. Comparison with the results obtained by the Rammelkamp method shows the micromethod to be more accurate ; it has been used successfully for hundreds of routine assays of penicillin levels in body fluids. G. R. K.

Penicillin in Liquids ; the Agar-rope Technique for Estimating. H. W. Julius and W. J. Alsche. (*J. clin. Path.*, 1950, **3**, 51.) A technique is described for the rapid determination (after 2 to 4 hours) of penicillin or other antibiotics in liquids such as serum, body fluids or culture media. Accurately prepared agar discs, mechanically cut from an agar rope, containing heavy inocula of bacteria, are submerged in small quantities of graded dilutions of known, compared with unknown, penicillin-containing fluids. Staphylococci are the most suitable bacteria but the method can be adapted for the use of any other organism sensitive to penicillin. An indicator, such as phenol red, detects normal compared with suppressed bacterial activity. The sensitivity of the method is 0·04 unit/ml. (serum). The quantity of material wanted for the test is 2 ml. (serum of 0·4 unit/ml.) or considerably less. With streptococci the activity can be augmented to 0·01 unit/ml. S. L. W.

Progesterone, Paper Chromatographic Separation and Ultraviolet Analysis of Commercial Preparations of. A. L. Haskins, Jr., A. I. Sherman,

and W. M. Allen. (*J. biol. Chem.*, 1950, **182**, 429.) The preparations consisted of solutions of progesterone in sesame or cottonseed oil. Quantities of 0·005 to 0·1 ml. diluted 10 times with carbon tetrachloride were placed on paper strips and allowed to dry. The strips were developed for 16 to 18 hours with alcohol (80 per cent.) and again allowed to dry. The progesterone was found by the blue colour obtained by immersing one-third of the strip cut lengthwise in a saturated alcoholic solution of *m*-dinitrobenzene, heating until dry, immersing in 5N potassium hydroxide and again drying. Under controlled conditions the R_f was about 0·84. The progesterone was extracted from the remaining two-thirds of the strip by immersing the appropriate parts in 5 ml. of alcohol (95 per cent.), and the resulting solution was submitted to ultraviolet analysis. The amount of progesterone was calculated from the density at 240 mμ. Samples prepared with sesame oil yielded a strongly ultraviolet absorptive factor which interfered with the analysis of progesterone ; a correction factor is given. Deoxycortone acetate and testosterone propionate may be separated from sesame oil in the same manner. They also have a similar absorption band and give the same colour reaction with *m*-dinitrobenzene and potassium hydroxide. Physiological tests are necessary to identify progesterone completely. G. R. K.

Streptomycin, Turbidimetric Assay for, and its Critical Evaluation. E. J. Oswald and L. F. Knudsen. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 61.) A suitable dilution of the unknown preparation is heated in a water-bath at 37°C. for 4 hours with broth previously inoculated with an aqueous suspension of the test organism, *Klebsiella pneumoniæ*, 4 drops of solution of formaldehyde are added and the percentage light transmission read in a photoelectric colorimeter. The potency is then read from a graph prepared by plotting percentage transmission against concentration for a series of dilutions of a standard preparation. The aqueous suspension of the test organism was used as the source of inoculum in an attempt to minimise the day to day variation of the test ; it was found to maintain vitality for 2 weeks when stored in a refrigerator. The factors entering into the test are analysed statistically and a nomograph and formula for calculating the standard error are also given. Comparison of the method with the *B. subtilis* plate assay on about 1,000 samples showed that in general the turbidimetric method gave slightly lower but more precise results. G. R. K.

PHARMACY

NOTES AND FORMULÆ

Chloramphenicol (Chloromycetin). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950, **143**, 813.) Chloramphenicol is D-(−)-threo-1-(*p*-nitrophenyl-2-dichloroacetamido-1 : 3-propanediol ($C_{11}H_{12}Cl_2N_2O_5$). It occurs as a white to greyish-white or yellowish-white crystalline powder, or as needles or elongated plates, slightly soluble in water and freely soluble in alcohol; a saturated aqueous solution has *p*H 4·5 to 7·5. In ethyl acetate it is lævorotatory, but in alcohol dextrorotatory; a solution of 1·25 g. in 15 ml. of absolute alcohol has a specific rotation $[\alpha]_D^{25°C.}$ of + 18·5° ±1·5°. The extinction coefficient $E_{1\,cm.}^{1\ per\ cent.}$ at 2780 Å is 289 ±9. The identity tests comprise a test for absence of inorganic halides, a test for the presence of organic chlorine and a test for the presence of a nitrophenyl group, which consists of reduction with tin and hydrochloric acid, diazotisation and coupling with β-naphthol, when a brilliant orange precipitate is obtained. The amount of

heavy metals present is equivalent to not more than 25 p.p.m. of lead. Chloramphenicol is assayed by determining the optical density of a 0·002 per cent aqueous solution spectrophotometrically at 2780 Å, compared with water as a blank; it contains 97 to 103 per cent. of chloramphenicol. Large doses may produce nausea and vomiting, otherwise no toxic effects have been observed. The initial dose is 50 to 75 mg./kg. of body-weight by mouth, with subsequent doses of 0·25 to 0·5 g. 2 or 3 hourly. G. R. K.

Chloriodised Oil (Iodochlorol). (*New and Nonofficial Remedies: J. Amer. med. Ass.*, 1950, **142,** 990.) Chloriodised oil is an addition product of arachis oil and iodine monochloride. It is a pale yellow viscous oily liquid with a faint bland taste, almost insoluble in water, slightly soluble in alcohol and freely soluble in benzene, chloroform and ether; refractive index at 25°C, about 1·505, specific gravity, about 1·260, residue on ignition not more than 0·1 per cent. It is assayed by hydrolysing with methyl alcoholic potassium hydroxide, diluting with water, acidifying with hydrochloric acid, extracting with chloroform and titrating the aqueous layer with potassium iodate; chloriodised oil contains 26·5 to 28·5 per cent. of iodine. It is used as a radiopaque agent to assist visualisation of the bronchial tract, genito-urinary tract, soft tissue sinuses, fistulas, etc. G. R. K.

Dymenhydrinate (Dramamine). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950, **143,** 815.) Dimenhydrinate, 2-(benzohydryloxy)-*N*:*N*-dimethylethylamine 8-chlorotheophyllinate ($C_{17}H_{22}ON$, $C_7H_6O_2N_4Cl$) is a white, crystalline, odourless powder, m.pt. 102°C. to 107°C., soluble in alcohol, benzene and chloroform and sparingly soluble in ether and water; a saturated aqueous solution has *p*H 6·8 to 7·3. The base, obtained by treatment with ammonia and extraction with ether, responds to the identity tests for diphenhydramine hydrochloride (N.N.R.). The addition of dilute sulphuric acid to a solution of dimenhydrinate produces a white precipitate of 8-chlorotheophylline, which responds to the following tests: (a) the murexide test (U.S.P.), (b) when fused with sodium peroxide, a solution of the residue in water gives a white precipitate with dilute nitric acid and silver nitrate (distinction from theophylline), and (c) when dissolved in diluted ammonia, it gives a white precipitate of silver 8-chlorotheophylline with silver nitrate. Dimenhydrinate loses not more than 0·1 per cent. of its weight when dried *in vacuo* over phosphorus pentoxide at room temperature for 24 hours; sulphated ash, not more than 0·1 per cent. It contains 53·0 to 53·3 per cent. of diphenhydramine and 44·0 to 47·0 per cent. of 8-chlorotheophylline. It is assayed for diphenhydramine by liberating the base with ammonia, extracting with ether, treating with a known volume of hydrochloric acid and titrating the excess acid with sodium hydroxide. The content of 8-chlorotheophylline is determined by dissolving in water with the aid of ammonia and ammonium nitrate, precipitating the 8-chlorotheophylline as the silver salt by adding silver nitrate, filtering and titrating the excess silver nitrate with ammonium thiocyanate. Dimenhydrinate is a histamine antagonist. It exerts a temporary therapeutic and prophylactic action in motion sickness, particularly in sea-sickness and car-sickness. Dose: 50 to 100 mg. half-an-hour before departure, repeated before meals and at bedtime. The untoward effects are similar to those of diphenhydramine, drowsiness being frequently observed G. R. K.

Fluoro-Iodo X-Ray Contrast Media, Preparation of. S. G. Mittelstaedt and G. L. Jenkins. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39,** 4.) Search was made for an ideal X-Ray contrast medium which gave

clear concise shadows of the required parts, was concentrated and retained for sufficient time in structures such as the kidney or gall bladder and then excreted fairly rapidly without toxic effects. Ease of administration and rapid transport in the body were also required with stability under normal conditions and in the blood stream, and lastly, ease and low cost of production were desired. A number of derivatives were prepared of aromatic hydrocarbons with iodine ortho to fluorine. Those prepared were 3-iodo-4-fluorobenzoic acid and a number of salts of the acid. They all exhibited anæsthetic, hypnotic and analgesic effects. Most of them had low toxicity. Ethyl-3-iodofluorobenzoate and the sodium salt of the free acid were tested for use as contrast media. When used in concentrations necessary to produce a distinct shadow of the gastro-intestinal tract, they proved to be rather irritating. They appeared to be rapidly eliminated.

G. R. K.

Prophenpyridamine (Trimeton). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950, **142**, 817.) Prophenpyridamine is 1-phenyl-1-(2-pyridyl)-3-dimethylaminopropane, $CH(C_6H_5)(C_5H_4N).CH_2.CH_2.N(CH_3)_2$, and occurs as a slightly yellow, oily liquid with an amine-like odour, insoluble in water and soluble in alcohol, benzene, chloroform, ether and dilute acids; b.pt. 135°C. at 0·5 mm. and 181°C. at 13 mm.; refractive index, 1·5519 to 1·5521; specific gravity, 1·0081. It is identified by preparing the dipicrate, which melts at 198° to 204°C., and complies with a test for absence of primary amine. It contains 11·50 to 11·80 per cent. of nitrogen, determined by the Kjeldahl method, and 98·5 to 100·5 per cent. of prophenpyridamine, determined by potentiometric titration with 0·1 N hydrochloric acid. A 0·003 per cent. solution in alcohol exhibits an ultraviolet absorption maximum at 2630 Å ($E_1^{1\ \text{per cent.}}_{\text{cm.}} = 184 \pm 3$), a sharp inflection at 2690 Å and a minimum at 2380 Å. Prophenpyridamine is a histamine antagonist. The average adult dose is 25 to 50 mg.

G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Acetylsalicylic Acid, Effect of Buffering Agents on Absorption of. W. D. Paul, R. L. Dryer and J. I. Routh. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 21.) Two tablets each containing 5g. of acetylsalicylic acid and a buffering agent consisting of a mixture of magnesium carbonate and aluminium dihydroxyaminoacetate were given to each of 238 patients. Subsequently only one patient complained of gastric distress although 22 had experienced ill effects after taking unbuffered acetylsalicylic acid. The effect of buffering on the rate of absorption was investigated by determining the blood salicylate before and 10 and 20 minutes after administration of 10 grains of unbuffered and buffered acetylsalicylic acid to two series of 47 patients. The results indicated that the addition of the buffer doubled the blood salicylate. G. R. K.

Artane in the Treatment of Parkinsonism. B. K. Ellenbogen. (*Lancet*, 1950, **258**, 1034.) Initial results in a series of 12 patients with parkinsonism treated with artane over a period of 3 to 6 weeks suggest that it is a drug of low toxicity and high potency in overcoming mental hebetude, relaxing spasm, and reducing tremor. The initial dose of the drug was 2·5 mg., increased by this amount each day to a total of 12·5 mg. in 5 doses. Where this amount was exceeded no greater improvement was obtained and the patients developed side reactions, such as headache, giddiness, slight

cycloplegia, and dryness of the mouth. The drug does not cause convulsions even with massive doses. Where artane is effective it is so immediately and dramatically; the patient becomes cheerful, alert and more responsive. It was more successful in the post-encephalitic group, controlling oculogyric crises, than in the senile and idiopathic groups. S. L. W.

Emetine and Quaternary Emetine Derivatives, Chemical and Pharmacological Studies on. A. Lasslo and K. K. Kimura. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 43.) *N*-methylemetine-*N* : *N'*-dimethiodide, *N*-methylemetine-*N* : *N'*-diethoethylsulphate and *N*-benzoylemetine-*N'*-ethoethylsulphate were synthesised. The first of these was much less toxic than emetine hydrochloride when given to 28 albino rats ; twice daily intraperitoneal doses of 17·2 mg./kg. for 3 weeks produced no ill effects, and the substance appeared to be excreted unchanged. Lethal doses of the same substance killed by asphyxia in contrast to emetine which kills by direct action on the heart. All 3 quaternary derivatives exhibited curare-like action on the striated muscle of frogs, rabbits and mice. The bisquaternary derivative, *N*-methylemetine-*N* : *N'*-diethoethylsulphate was twice as potent in frogs and mice as *N*-benzoylemetine-*N'*-ethoethylsulphate, which has only one quaternary nitrogen atom, and has approximately one-third the activity of *d*-tubocurarine chloride in rabbits. G. R. K.

Ethylene Glycol Vapour, Chronic Intoxication by. F. M. Troisi. (*Brit. J. indust. Med.*, 1950, **7**, 65.) A description is given of the toxic manifestations caused by inhalation of ethylene glycol vapour in women workers engaged in a process involving the spreading of a mixture of ethylene glycol 40 per cent., boric acid 55 per cent., and ammonia 5 per cent. The mixture was maintained at 105°C. to facilitate application by brush, so that the women were exposed to continuous evolution of the vapour. In 9 out of 38 workers so engaged frequent attacks of sudden loss of consciousness lasting for 5 to 10 minutes occurred, and nystagmus was observed; 5 of these had an absolute lymphocytosis. Among the remaining workers a further 5 cases of nystagmus were discovered, but attacks of loss of consciousness did not occur. Of the 9 most sensitive women, 2 were transferred to another department and the attacks ceased. The remaining workers elected to remain at their occupation and after the installation of a recovery plant the attacks completely disappeared. It is recommended that systematic examination for nystagmus should be carried out among workers in contact with glycols or other solvents of the fatty or aromatic series. H. T. B.

Iron, Intravenously, in the Treatment of Anæmia. A. S. Ramsey. (*Brit. med. J.*, 1950, **1**, 1112.) Intravenous iron therapy is now a practical method of treating microcytic hypochromic anæmia, and is the method of choice for patients unable to tolerate iron orally or in cases of refractory iron-deficiency anæmia. These patients will respond dramatically to intravenous saccharated oxide of iron (reports of 11 cases treated with the proprietary ferrivenin are given). Toxic reactions are avoided when the dosage begins with 100 mg. daily for several days and is then gradually increased to 200 or 300 mg., a full course consisting of not more than 10 injections. Severe toxic reactions occur, however, when attempts are made to replace the requirements of iron by a single intravenous injection. The toxic effects closely resemble the symptoms of paroxysmal hæmoglobinuria caused by cold, and the author suggests as a cause the sudden increased activity of the cells of the reticulo-endothelial system. S. L. W.

2-Methylaminoheptane (Œnethyl), A Study of. F. E. Shaffer and P. K. Knoefel. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 12.) The action of 2-methylaminoheptane was compared with that of deoxyephedrine, by administration orally and parenterally to two normal men and intravenously to an unspecified number of dogs; it was concluded that as a vasopressor agent, 2-methylaminoheptane had one-fifth the activity of deoxyephedrine. Comparative acute lethal toxicity studies on mice, rats and dogs whose numbers are also unspecified revealed that 2-methylaminoheptane had a degree of toxicity similar to that of tuamine (2-aminoheptane) on subcutaneous and intravenous injection. Deoxyephedrine and 2-methylaminoheptane are equally toxic intravenously but the former is more toxic subcutaneously. Chronic toxicity studies indicated that the smallest dose at which a deleterious effect is noted is 23mg./kg. of body weight per animal per day for albino rats although it is possible that this is true only of female animals. G. R. K.

Œstrogens, Further Observations on the Potency of. P. M. F. Bishop, N. A. Richards, D. J. N. Smith and W. L. M. Perry. (*Lancet*, 1950, **258**, 848.) The potencies of ethinylœstradiol and "equine conjugated œstrogen" were compared with the potency of stilbœstrol by giving the œstrogen daily by mouth in 14-day courses to amenorrhœic women and recording whether œstrogen-withdrawal bleeding took place. "Equine conjugated œstrogen" is the name used for an extract of œstrogenic substances from the urine of pregnant mares and consists of a mixture of conjugated œstrogens, including œstrone sulphate. The results of the investigation indicated that ethinylœstradiol was 20 to 25 times as potent as stilbœstrol and "equine conjugated œstrogen" half as potent. With the doses used to induce withdrawal bleeding there were few cases of nausea with ethinylœstradiol and only one with "equine conjugated œstrogen"; at higher dosage levels such as those necessary to produce hæmostasis in functional uterine bleeding, nausea usually developed with ethinylœstradiol and in 2 such patients "equine conjugated œstrogen" produced severe vomiting.

G. R. K.

Pethidine and Amidone (Methadone) Derivatives. P. O. Wolff. (*Bull. World Hlth. Org.*, 1949, **2**, 193.) The analgesic effects of modifications of the pethidine molecule are as follows. *Ester group:* Only the ethyl, *iso*-propyl, allyl and *n*-propyl esters are active. Corresponding amides are inactive, but ketones are more active than esters (for example, 1-methyl-4-*m*-hydroxyphenyl-4-propionylpiperidine, "keto-bemidone"). Acyl derivatives of 4-phenyl-4-hydroxy-1-methylpiperidine are active, especially the propionyl compound. Benzene ring substitution: 2′-methyl substitution increases potency and duration; 3′-acetoxypethidine is similar to pethidine and 3′-methoxypethidine is about half as active. This relationship is comparable with that of morphine, diamorphine and codeine. β-pethidines: *l*-*N*-nor-β-pethidine has similar antispasmodic properties to pethidine but is less sedative, and produces no noticeable euphoria. *N-group*: *N*-butyl and *N-iso*propyl derivatives are highly active. 3-alkyl substituted pethidines: The following are 4 to 8 times as potent as morphine: *dl*-, *d*- and *l*- 1:3-dimethyl-4-phenyl-4-propionoxypiperidine, and 1-methyl-3-ethyl-4-phenyl-4-propionoxypiperidine. Steric configuration affects the potency of certain derivatives, 3:3-diethyl-2:4-dioxopiperidine gives good or fair sedation, but is suspect of habit forming. The following amidone derivatives have been examined: *l*-amidone, which has about 50 times the analgesic effect of the *d*-compound, and is responsible for the addiction-producing effect of the

dl-substance; *dl*-methadol (6-dimethylamino-4:4-diphenyl-3-heptanol); *dl-iso*-methadone which is an addiction-producing drug; γ-dimethylamine-α:α-diphenylvaleric acid which has an atropine-like effect on the intestine of the anæsthetised dog; 1-dimethylamino-2-methyl-3:3-diphenyl-4-acetoxyheptane. 2-dimethylamino-4:4-diphenyl-5-hexylidene acetylimine, 2-dimethylamino-4:4-diphenyl-5-acetoxyheptane and 2-morpholino-4:4-diphenyl-5-acetoxyheptane which have a high therapeutic index; phenadoxone (6-morpholino-4:4-diphenyl-3-heptanone), a potent analgesic, the most important side effect of which is euphoria, and 6-dimethylamino-4:4-diphenyl-5-methyl-3-hexanone, which is half as active as amidone. It is suggested that all pethidine and amidone derivatives should be controlled legally as habit-forming drugs, restrictions on a particular compound being withdrawn when practice has shown that it does not produce addiction. G. B.

Priscol in Peripheral Vascular Disease. A. H. Douthwaite and T. R. L. Finnegan. (*Brit. med. J.*, 1950, **1**, 869.) The effects of priscol benzyl-imidazoline) were investigated in 10 normal subjects 4 with rheumatoid arthritis, 9 with Raynaud's disease, 24 with intermittent claudication of arteriosclerotic origin, 2 with claudication of thrombo-angiitis obliterans and 6 with arterial tension without claudication. It was found to have a more lasting vasodilator effect than other drugs commonly employed for this purpose, and to have an elective affinity for the smaller arteries and arterioles of the fingers, hands and toes. The best results are to be looked for where the element of spasm is maximal and organic blockage of the lumen is minimal. It is outstandingly successful in the treatment of Raynaud's disease, but the proportion of cases of arteriosclerotic intermittent claudication which benefit from the drug is surprisingly high. It would seem of little value in thrombo-angiitis obliterans or rheumatoid arthritis and should not be used in arterial hypertension. It is relatively non-toxic, but may give rise to pallor, sweating, weakness, palpitation and giddiness. This effect is known as " priscol shock," and it is advisable to test the patients' sensitivity by giving 25 mg. by mouth; if this has no unpleasant effect 50 mg. intravenously may be given. Of the 9 patients with Raynaud's disease 5 remained completely comfortable on a dose of 25 mg. four times a day and 4 by taking occasional doses. S. L. W.

Procaine Penicillin, Aqueous Suspension of. R. I. Cohen. (*Lancet*, 1950, **258**, 622.) In a trial of an aqueous suspension of penicillin G in 30 adult volunteers intramuscular injections caused no pain or discomfort, and there was no swelling or redness at the site of injection. An effective blood penicillin level of over 0·03 unit/ml. was maintained for 24 hours in 27 out of the 30 volunteers after a single intramuscular injection of 300,000 units. The aqueous suspension used contained the stable crystalline procaine salt of penicillin G with less than 1·5 per cent. of suspending agents; the suspended particles in the preparation were nearly all less than 40μ in diameter. Into a " single-dose " vial of aqueous suspension containing 330,000 units of penicillin 1·2 ml. of sterile water was injected and the vial shaken. As much as possible (about 1·5 ml.) was withdrawn into the syringe and the injection made into the triceps muscle at ordinary speed through a No. 14 needle. No difficulty was encountered in giving the injection, and the suspension was still easier to handle if 2 ml. instead of 1·2 ml. of diluent was added. S. L. W.

Sodium Phosphate in Lead Poisoning. C. D. Procter and H. S. Kahn. (*Amer. J. med. Sci.*, 1950, **219**, 316.) It has previously been pointed out that sodium phosphate appears theoretically to have the following

advantages in the treatment of chronic plumbism. It precipitates the lead in its most insoluble form, as $Pb_3(PO_4)_2$; the slight alkalosis may mobilise some of the lead in the bones; the excess of sodium ions disturbs the calcium balance so that calcium, and therefore lead, are drawn from the trabeculæ; mobilisation and detoxification are simultaneous; the buffer action of the phosphate decreases the amount of ionised lead. The authors therefore undertook an investigation of the therapeutic value of sodium phosphate in this condition. Clinical diagnosis was confirmed by determination of the urinary excretion of lead during a 24-hour period. The usual dosage was 30 to 60 gr. by mouth 3 times a day for about a week, although in some cases 5 to 10 gr. was given 3 times a day intravenously as a 5 per cent. solution. In all cases the urinary excretion of lead was markedly increased without producing any symptoms and the treatment reduced the 24-hour urinary lead level below about 150 μg./ml. which was regarded as the toxic limit. Sodium phosphate is considered to provide an effective and inexpensive treatment in chronic lead poisoning which is superior to any other form of therapy at present available. H. T. B.

Tubocurarine Iodide, Dimethyl Ether of, as a Curarising Agent in Anæsthesia. H. B. Wilson, H. E. Gordon and A. W. Raffan. (*Brit. med. J.*, 1950, **1**, 1296.) From the use of this curarising agent in 100 cases of major intrathoracic procedures the authors conclude that it is from 2 to 2½ times more potent than *d*-tubocurarine chloride, but that its duration of action on diaphragmatic activity is a little shorter and subsequent doses are required at intervals of 20 to 25 minutes. Clinically, there seems less release of histamine, as evidenced by maintenance of systolic and diastolic blood pressures and ease of inflation of collapsed lung tissues. In no case was there any cause for anxiety referable to the use of the curarising agent, neither was there any evidence of undue reactions to the drug or to its delayed detoxication. The dosage of the dimethyl tubocurarine employed was of the order of 1 mg./stone, with incremental doses of 3 mg. S. L. W.

Tubocurarine Iodide, Dimethyl Ether of, Pharmaco'ogy of. H. O. J. Collier. (*Brit. med. J.*, 1950, **1**, 1293.) Dimethyl tubocurarine is a more effective curarising agent than tubocurarine in the rat, guinea-pig, rabbit and cat, but is less active than tubocurarine in the mouse. In the mouse, rat and rabbit dimethyl tubocurarine is antagonised by neostigmine. Weight for weight it is no more active than tubocurarine in liberating histamine or in blocking autonomic ganglia in the cat. Its repeated administration to the rabbit or rat does not appear to give rise to increased sensitiveness or tolerance. Dimethyl tubocurarine has been successfully administered to the rat with ether and with thiopentone. A combination of dimethyl tubocurarine and ether has a greater effect than either separately. From the experimental evidence it seems likely that dimethyl tubocurarine can be used with success in man in association with an anæsthetic, though it would appear that in man its action is of slightly shorter duration than that of tubocurarine. It depresses the respiration relatively less than *d*-tubocurarine or decamethonium iodide. S. L. W.

BACTERIOLOGY AND CLINICAL TESTS

Antiobiotics, *in vitro* Sensitivity of *Bacillus proteus* and *Pseudomonas æruginosa* to. P. F. Frank, C. Wilcox and M. Finland. (*J. Lab. clin. Med.*, 1950, **35**, 205.) Experiments similar to those described for coli-

form organisms, were carried out. For *B. proteus*, chloramphenicol and penicillin were most effective, rather high concentrations of streptomycin or aureomycin were required for inhibition of growth and polymixin, aerosporin and bacitracin were ineffective. 28 per cent. of strains were resistant to penicillin, and 11 per cent. to streptomycin. For *Ps. æruginosa* the following is the order of decreasing activity :—aerosporin, polymixin, streptomycin, aureomycin and chloramphenicol. Bacitracin and penicillin were not effective. 14 per cent. of strains were resistant to streptomycin. G. B.

Antibiotics, *in vitro* Sensitivity of Coliform Bacilli to. P. F. Frank, C. Wilcox and M. Finland. (*J. Lab. clin. Med.*, 1950, **35**, 188.) A number of strains of *Escherichia coli*, *Aerobacter aerogenes* and *Klebsiella pneumoniæ* were cultured from patients with active infectious processes. Routine tests for sensitivity to the antibiotics were carried out by a tube dilution method. When several strains of the same organism were tested simultaneously against all the antibiotics, a plate dilution method was used. Polymixin and aerosporin were most active against all three species, aureomycin and chloramphenicol being somewhat less effective. Streptomycin was as effective as chloramphenicol against two-thirds of the strains, the remaining strains being less sensitive. A higher concentration of penicillin was required for inhibition, and bacitracin was practically ineffective. 16 per cent. of the strains of *E. coli*, 24 per cent. of the strains of *A. aerogenes* and 9 per cent. of those of *K. pneumoniæ* were resistant to streptomycin. *K. penumoniæ* was the most sensitive and *A. aerogenes* the most resistant to the antibiotics. G. B.

Ethylene Oxide for Sterilisation. A. T. Wilson, and P. Bruno. (*J. exp. Med.*, 1950. **91**, 449.) Ethylene oxide gas has been used widely in industry as a sterilising agent and the authors have investigated the use of liquid ethylene oxide as a sterilising agent for bacteriological broth, milk and serum in cases where heat or filtration cannot be used. The liquid vapourises at 10·7°C.; it is kept in a stoppered bottle in the cold and measured by a chilled pipette or syringe, the latter being preferable because the substance is toxic. It was found that ethylene oxide destroys a number of Gram-positive and Gram-negative organisms, aerobic and anaerobic spore-forming bacilli, fungi, and vaccinia virus, including organisms present in culture media contaminated with floor-sweepings, fæces, throat-washings and soil. No organisms were encountered which withstood the action of 1 volume per cent. of liquid ethylene oxide in the culture medium, and 0·5 per cent. is often adequate. The time required to effect sterilisation depended on the amount of ethylene oxide used. 0·5 per cent. sterilised milk contaminated with a Group A streptococcus in 4 hours, and broth inoculated with the same organism in 6 hours. 24 hours after culture media were subjected to treatment with the liquid, they again became fully capable of supporting bacterial growth. Sterilisation of media by ethylene oxide is accompanied by a rise in *p*H which may necessitate adjustment with sterile acid to recover optimal conditions for bacterial growth. A mixture of ethylene oxide and broth was toxic to mice for 6 hours, after which toxicity disappeared rapidly even on repeated intraperitoneal inoculation. The substance combines slowly with water to form ethylene glycol and with many acids. It also reacts with carboxyl, amino, sulphydryl and phenolic groups but no evidence of interference with any essential growth factors was obtained. H. T. B.

LETTERS TO THE EDITOR

Assay of Alkaloidal Salts

SIR,—Reimers, Gottlieb and Christensen[1] have described the assay of alkaloidal salts by adsorption of the anions on activated alumina from solution in aqueous alcohol of 90 per cent. v/v or less, followed by elution of the alkaloidal bases with the same solvent, and titration against decinormal acid. Van Os[2], in recommending the method, stipulates that the alumina should be free from alkali. With several alkaloids we have recently had occasion to assay, however, we have found that the alumina may advantageously be replaced by a mild alkali insoluble in alcohol (90 per cent.), such as anhydrous sodium carbonate, sodium bicarbonate, or fused and powdered borax. The manipulation is in other respects as described by Reimers and associates, though the reaction in our procedure is, of course, not one of adsorption, but simply a neutralization of the anion.

Probably all the assays which are practicable by the alumina method can be reproduced by this procedure. Sodium carbonate tends to give results up to 2 per cent. too high, probably because a small amount may be carried through by the alcohol. With borax, a somewhat slower reaction, up to 30 minutes for the passage of 5 ml. of solution and 30 ml. of washings, should be allowed for. The method gives good results in some cases where the alumina technique failed, e.g., apomorphine hydrochloride on a sodium bicarbonate column gave an assay of 100 per cent. The manipulation is much less laborious than the U.S.P. assay of apomorphine hydrochloride tablets, with which it is chemically comparable.

Brownlee[3] describes the assay of galenicals on a column of alumina, which brings about decolorisation in addition to the essential reaction. (It may be incidentally noted that Brownlee, using alcohol of less than 90 per cent. strength, found an appreciable amount of alkali extracted from the alumina.) In such cases, the use of an adsorbent in conjunction with the alkali will clearly be of advantage.

We are indebted to the Government Chemist for permission to communicate these results.

Government Laboratory, Clements Inn Passage, Strand, London, W.C.2.
September 5, 1950.

E. G. KELLETT.
D. L. T. CADMAN.

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Noradrenaline and the Adrenal Glands of the Domestic Fowl

SIR,—Investigations have been carried out in this laboratory to determine the nature of extracts of adrenal glands of various mammals and birds. Most of the mammalian extracts contain a mixture of adrenaline and noradrenaline, with the former amine predominating. One exception to this finding is the rabbit, where noradrenaline is detected very occasionally in minute amounts. We have now completed a series of experiments and find that in extracts from fowls the predominating amine is noradrenaline.

The fowls used in this work ranged from day-olds to birds of about five months, with body weights ranging from 110 g. up to over 2 kg. The

adrenal glands were removed as quickly as possible after death, weighed and ground up in a mortar with sand and acid. The mixture was filtered

TABLE I.

MEAN VALUES FOR NORADRENALINE AND ADRENALINE CONTENT OF SUPRARENAL GLANDS.

Animal	mg./g. fresh tissue		approximate percentage of adrenaline in the mixture
	adrenaline	noradrenaline	
Rabbit	0·485	traces	100
Rat ...	1·1	0·13	90
Cattle	2·0	0·6	77
Guinea-pig ...	0·26	0·08	77
Cat . ..,	0·8	0- 0·65	55- 100
Dog .	0·8	0-0'85	49 - 100
Fowl ...	2·01	8·09	20

and the filtrate tested for adrenaline and noradrenaline on the following pharmacological preparations; the blood pressure and nictitating membrane of the spinal cat[1], the isolated rectum of the chick[2], and the isolated ileum of the rabbit. The mean value of extracts from 18 fowls was 8·09 mg. of noradrenaline per g. of fresh tissue of 2·01 mg. of adrenaline per g. (see Table I).

The significance of this finding is not yet clear. It is not that noradrenaline is more active on the blood pressure of the fowl than is adrenaline, for comparisons show that the latter amine is about twice as potent as the former (just as it is in the rabbit where the glands contain only adrenaline). Perhaps it may be that the adrenal medulla is not strongly stimulated very frequently and that the small adrenaline content is sufficient for its immediate needs. Also the methylating process in the fowl may be a slow one. It is certainly of interest that intravenous doses of dibenamine do not reverse the vasoconstrictor action of adrenaline in the fowl[3]. A similar resistance to adrenergic blockage by ergot was noted many years ago. Another interesting point is that in man the adrenal glands contain about 75 per cent. of adrenaline and 25 per cent. of noradrenaline, and yet in medullary tumours (pheochromocytoma) noradrenaline predominates, with adrenaline only about 10 per cent. of the mixture.

Pharmacological Laboratory, G. B. WEST.
Department of Materia Medica,
Medical School, Dundee.

September 19, 1950.

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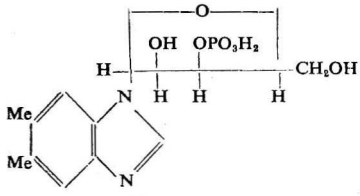
The Mode of Linkage of Component α in Vitamin B_{12}

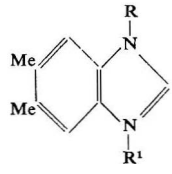
Sm,-A comparison of the absorption spectra of *component* α (1)1,2,3,4, and vitamin B_{12} (II) reveals an anomaly. Thus whereas (I) shows a well-resolved fine structure band or " notch" at λ=2850 Å in dilute acid and λ=2880 A

in dilute alkali, (II) shows only an inflection in this region which is, moreover, unchanged in position over the *pH* range 2-12. Some factor or factors are therefore operative in the" *component* α combination" ex:sting in (II) which affect the contribution made by the benziminazole chromophore of (I) to the spectrum of B_{12}. A consideration of the ways in which the chromophoric character of (I) can be affected sufficiently to abolish the resolution of this" notch" without interfering with the wavelength position of the main benziminazole band and its subsidiary features leads, by a process of elimination, to the conclusion that the nitrogen atom in position 3(N3) of (I) is involved in some form of combination within the B_{12} molecule. This combination, on theoretical grounds, would appear to be a linkage of covalent (III) or co-ordinate (IV) character.

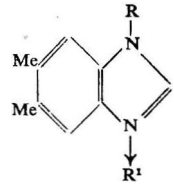
Spectroscopic measurements of quaternary derivatives of benziminazole eliminate (III) from further consideration. Platinic chloride complexes of I-substituted benziminazoles, however, which possess the structural feature (IV), show anomalou spectral characteristics in marked accord with tbose established for the benziminazole contribution in (II).



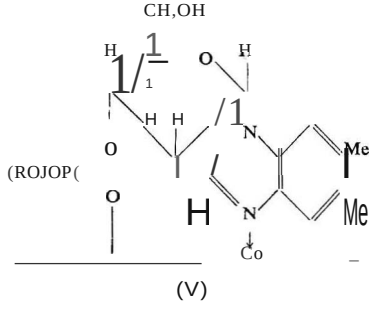
(I)



(III)



(IV)



(V)

Studies of the action of cyanide on the spectrum of B_{12} lead to the conclusion that tbe reversible formation of a purple" B_{12}-cyanide" complex is dependent upon co-ordination of cyanide ion with coblit. As this co-ordination leads to a partial abolition of the anomaly observed in the *component* α contribution to the B_{12} spectrum, i.e., to greater resolution of the inflection at $\lambda = 2885$ A, it is concluded that N3 in (1) is prob3.bly linked directly to cobalt by a co-ordinate link as shown in (V). The cobalt-containing complex represented graphically in (V) as a straight line is visualised as a planar structure spatially akin to the porphyrins, the benziminazole ring lying perpendicularly to this plane.

A full account of this work is being submitted for publication in your Journal.

It is a pleasure to acknowledge the encouragement of the Directors of The British Drug Houses, Ltd., in this work.

M.R.C. Spectrographic Unit,
The London Hospital, E.1
Research Department,
The British Drug Houses, Ltd.,
London, N.!.

G. H. BEAVEN,
E. R. HOLIDAY,
E. A. JOHNSON,

B. ELLIS,
V. PETROW.

September 22, 1950

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Conversion of Vitamin B'2b into Vitamin B_{12}

SIR.-The spectra',2 of vitamins B_{12} and B_{12} resemble each other in general character, but differ considerably in the position and magnitude of particular features. A structural similarity is thus evident between the two compounds[S] which is reflected in certain of their chemical reactions. On treatment with cyanide ion[4] in aqueous solution, both compounds form purple cyanide complexes having the *same* absorption spectrum, which is markedly different from those of the two vitamins. On removing cyanide from the complex, vitamin B_{12} reverts to its original state, as shown by its absorption spectrum, microbiological activity, chromatographic behaviour[5], and general crystallographic appearance. On removing the cyanide from the B_{12} -cyanide product, however, the spectrum does not revert to that of B'2b' but is strikingly similar to that of B_{12}. The regenerated material, moreover, shows further properties siilila to those of B'2' viz. microbiological activity against ***Lactobacillus*** *lactis* Dorner and behaviour on paper chromatography[5]. These observations lead to the conclusion that B_{12}'b may be converted, through intermediate formation of a cyanide complex, into a substance which, as far as can be ascertained at present, is identical with vitamin B_{12}. As B'2 appears to form the major constituent of the B'2 group of factors in materials derived from the *in vitro* fermentation of micro-organisms, the above conversion assumes preparative importance.

A full account of this work is being submitted for publication in your Journal.

It is a pleasure to acknowledge the encouragement of the Directors of The British Drug Houses, Ltd., in this work.

Research Department,
The British Drug Houses, Ltd., London, N.1
M.R.C. Spectrographic Unit,
London Hospital, E.!.
September 22, 1950.

B. ELLIS.
V. PETROW.
G. H. BEAVEN.
E. R. HOLIDAY.
E. A. JOHNSON.

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4 : 4'-Diamino-2 : 2'-dihydroxydiphenyl Sulphone

SIR,-The recent publication by Amstutz[1] of the synthesis of 4: 4'-diamino·2 : 2'-dihydroxydiphenyl sulphone as a potential antitubercular drug, although unaccompanied by any pharmacological data, prompts us to place on record the preparation of the identical compound in these laboratories.

2: 2'-Diethoxy-4: 4'-dinitrodiphneyl sulphide, obtained in 30 per cent. yield by the condensation of sodium 2-ethoxy-4-nitrothiophenoxide with 4-chloro-3-ethoxynitrobenzene in alcoholic solution, was oxidised with excess of potassium permanganate to yield the corresponding sulphone; combined reduction and de-ethylation of this suiphone with hydriodic acid gave, according to the conditions employed, either 4: 4'-diamino-2 : 2'-diethoxydiphenyl sulphone (I), m.pt *269°* C. (Found: C, 48'1; H, 4·2; N, 7'3; S, 8·3 per cent. C16HlSOsN2S requires C, 48'5; H, 4'0; N, 7'1; S, 8'1 per cent.) or 4:4'-diamino-2: 2'-diphydroxydiphenyl sulphone (II); m.pt *179°* to 181·C. (Amstutz, 180° to 184°C.).

Preliminary examinations of the sulphones 1 and II against *M.tuberculosis* (H37Rv) in Dubos medium was kindly conducted by Professor G. A. H. Bptt1e of this School and inhibitory dilutions of 1: 16,000 and I: 256,000 respectively were recorded. The oral toxicity of 4: 4'-diamino-2 : 2'-dihydroxy-diphenyl sulphone was of a low order and a limited investigation of its *in vivo* activity in mice infected with *M.murium (N.C.T.C.* 5676) has met with promising results.

Pharmaceutical Chemistry Research Laboratories,
School of Pharmacy, University of London.
17, Bloomsbury Square, W.c.!:
October t2, 1950.

W. H. LINNEIL.
J. B. STENLAKE.

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