REVIEW ARTICLE

A NEW CONCEPT OF THE PHYSIOLOGICAL ROLE OF VITAMIN B_{12}

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THE isolation of vitamin B_{12} from liver or fermentation broth sources has always involved one important and apparently inevitable step, namely, the separation of the vitamin from associated protein-like material. The nature of the binding is unknown. That it involves more than physical admixture has been evident since the early attempts to crystallize the vitamin were continually thwarted by the presence of protein-like impurity. Thus Lester Smith and Cuthbertson in 1948[1] had difficulty in freeing the vitamin from bound material in purified concentrates from liver. These workers identified some of the amino acids in the complexes of vitamins B_{12} and B_{12b} but, after the crystallization of the pure vitamin, they do not appear to have pursued the nature of the natural binding in liver extracts any further.

The effectiveness of crude liver preparations when given by mouth in the treatment of pernicious (Addisonian) anaemia has long been known. Recently Robinson and others[2] have shown most of the vitamin B_{12} in crude liver extracts to be bound; the material is not bound to protein since the bound vitamin can be dialysed through Cellophane. The possibility of a complex formation with a peptide of low molecular weight was not excluded. In contrast to crude liver preparations the crystalline vitamin is ineffective, even in large doses, in the oral therapy of pernicious anaemia. After its isolation it seemed for a time that the vitamin and the growth factor present in animal proteins, and necessary for supplementing the vegetable protein diets of animals, might prove to be identical. Later it became evident that this was not so.

These and other considerations led the authors to believe that for both animal growth and pernicious anaemia a vitamin B_{12}—peptide complex was the active substance since it was more readily absorbed and more nutritionally acceptable than the uncombined vitamin. Such a complex could be either ingested, or synthesized in the blood stream by the combination of the injected vitamin and available low molecular weight protein or peptide at blood pH, and also might not require a high degree of specificity in the nature of the bound material.

The generally accepted theory of action of vitamin B_{12} in oral therapy is that of Castle who postulated that the vitamin must be bound to some protein-like material which is derived from the alimentary tract and which facilitates absorption of the vitamin. Ternberg and Eakin[3] were the first to demonstrate that the vitamin combined with a principle in normal human gastric juice to form a non-dialysable complex. The vitamin in

this combination was not available to micro-organisms that need it as a growth factor which implies that the vitamin was binding preferentially to high molecular weight material. Glass, however, has recognised that the transport of a large protein molecule across the gut wall necessitates introducing some additional factor ("acceptor factor"). If Ternberg and Eakin's suggestion, that the vitamin B_{12}-binding principle in gastric juice is identical with intrinsic factor and is non-dialysable, were so, then low molecular weight proteins or peptides would be entirely inadequate to effect absorption of the vitamin. But the inconsistent results which have been obtained with "intrinsic factors" have challenged the validity of this suggestion. It also does not appear to agree with the accepted principles of the digestion process, which is normally concerned with the provision of relatively small molecules for absorption. Thus molecular size might be an important criterion for absorption of the vitamin in a utilizable form.

Species specificity has been observed[4,5], for instance rat gastric juice has intrinsic factor activity (i.e., results in the absorption of oral cyano-cobalamin) for the rat only. But, gastric juice is such a complex mixture that molecular size could still be a more important consideration than species specificity. If this should be so then vitamin B_{12}-peptide complexes released from mould or bacterial cells might be as active clinically as those released from liver cells. Thus, instead of isolating the pure vitamin from liver or fermentation liquors, and combining it with animal intrinsic factors, it should be possible to isolate the vitamin in a natural, combined state with improved absorption characteristics and therefore more effective orally than the pure vitamin.

During the past few years this possibility has been investigated in these laboratories. Encouraging clinical results have confirmed the oral effectiveness of vitamin B_{12}-peptide complexes prepared in the laboratory from fermentation sources[117]. The present article reviews the theoretical aspects on which the oral therapy of pernicious anaemia has been based. It endeavours to show the inconsistencies and contradictions inherent in the currently accepted approach to this problem. The new approach, which already has appreciable clinical support, is here correlated with the wider aspects of the role of vitamin B_{12} in animal nutrition.

Aetiology of "Addisonian" Pernicious Anaemia

Since the early nineteenth century it has been believed that pernicious anaemia is associated with imperfect assimilation of nourishment. Fenwick in 1877 and 1880 demonstrated that the digestive power was absent from an extract of the gastric mucosa of a patient who had died from pernicious anaemia. That achlorhydria accompanied pernicious anaemia was established by Cahn and von Mehring in 1886[6] and Faber and Bloch in 1900[7]. Lazarus in 1898 considered that the megaloblastic state of the bone marrow might be due to an arrest of maturation. These early observations were overlooked until 1926 when Minot and Murphy[8] demonstrated that liver administered orally could effect remission in pernicious anaemia. On the basis of a series of well known studies

(for a review see Castle[9]), Castle in 1929 put forward his classical hypothesis which linked the loss of the haemopoietically active principle in liver with the underlying gastric defect. Essentially it was established that normal human gastric juice contained a factor, the "intrinsic factor", which when given orally together with an "extrinsic factor", found in meat, was able to cause haematologic remission in pernicious anaemia. Castle and Minot in 1936[10] assumed that the gastric (intrinsic) factor reacted with the food (extrinsic) factor to produce the haematologically active principle, which was stored in the liver, kidney and other tissues. Since the discovery of vitamin B_{12} by Folkers and colleagues and by Lester Smith in 1948 and the demonstration that the crystalline vitamin caused remission in pernicious anaemia, *either* when injected parenterally or when given orally together with gastric juice, it has been thought that the role of intrinsic factor was to promote the intestinal absorption of the vitamin in a specific manner[11]. That is to say, vitamin B_{12} is considered to be the extrinsic factor. But according to Glass and others[12], the intestinal absorption of the vitamin—even in the presence of normal amounts of intrinsic factor—may be surprisingly small. Thus an increase in the amount of ingested vitamin from 0·5 to 50 μg. results only in an increase in hepatic accumulation from 0·45 to 1·5 μg. These authors, therefore postulated an intramural "intestinal acceptor of the vitamin" and the failure of increasing doses to be absorbed could be explained by the saturation of this acceptor. The limited capacity for absorption of the vitamin even in the presence of intrinsic factor, may explain some of the difficulties in treating sufferers from pernicious anaemia by the oral route.

According to present concepts (see e.g., Nutrition Reviews, 1955) "the absorption of vitamin B_{12} by the human intestine is a complicated problem involving the presence of a facilitating substance (intrinsic factor) and possibly an intestinal acceptor substance". Thus, if vitamin B_{12} is the extrinsic factor, then two additional factors must be involved in its transfer across the intestinal mucosa of a normal individual.

Before passing on to the consideration of the role of intrinsic factor in pernicious anaemia, some clinical results which have been obtained with oral crystalline vitamin B_{12} alone will first be considered.

ORAL ABSORPTION OF VITAMIN B_{12} WITHOUT A SOURCE OF "INTRINSIC FACTOR"

When crystalline vitamin B_{12} became available it was tested orally and found to be relatively ineffective. However, when given with normal gastric juice, it proved to be nearly as effective as by injection[11]. This led to the idea that extrinsic factor and the vitamin are the same.

Ungley[13] has summarized the results of several workers. He concluded that daily oral doses of 5 or 10 μg. of the crystalline vitamin (21 cases) either had no detectable effect, or more often, gave a trivial response similar to that produced by a single injection of 1 or 2 μg. With daily doses of from 25 to 450 μg. (27 cases) results were extremely variable. With few exceptions absorption appears to have been less than one per

cent. Large doses of 3,000 μg. were effective even without a source of intrinsic factor according to Ungley[14] and in 21 patients this dose was equivalent to an injection of 40 μg.

Reisner and colleagues[15] found that it was possible to maintain 43 persons with pernicious anaemia for periods of 12 to 28 months on doses of 1,000 μg. at weekly or two-weekly intervals. Chalmers[16], having previously shown that large doses of the crystalline vitamin were effective orally[17], studied a further 14 patients with pernicious anaemia in relapse. With daily oral doses of 50 to 200 μg. of the vitamin first thing each morning when fasting, results were good in studies of up to 2 years and most patients obtained complete remission with a return of serum vitamin levels to within normal range (200–1,000 μμg./ml.). Estren and Wasserman[18] claimed maximal responses in three of nine patients, sub-maximal in five and one failure at a dosage of "5–16·8 μg. of vitamin B_{12} . . . one to three times daily". The degree of response in the sub-maximal cases is difficult to assess as details of progress and duration of treatment are shown in two cases only.

Glass, Goldbloom and Boyd[19], using the method of hepatic uptake of radioactive vitamin B_{12} found that people of over 60 years with gastric hypo- or anacidity had a significantly lower intestinal absorption of the vitamin than those of the same age with normal or hyper-acidic gastric secretion. Glass, Pack, Mersheimer, Kusnick and Laughton[20] had likewise previously shown that patients with total gastrectomy, who frequently develop pernicious anaemia, observed no hepatic uptake of the radioactive crystalline vitamin after oral administration. The defect could be corrected by adding a source of "intrinsic factor". This reduced absorption of the crystalline vitamin is in agreement with the incidence of incipient megaloblastic erythropoiesis found in such elderly achlorhydric patients despite the absence of manifest symptoms of pernicious anaemia[21].

The oral absorption of the crystalline vitamin is appreciably greater in the normal subject than in the patient with pernicious anaemia. Using the technique of Welch and Nichol[22], which consists in giving orally a dose of 0·5 μg. of radioactive vitamin B_{12} to a fasting subject and then measuring the radioactivity of all the stools passed during the next 5–6 days, Callender Turnbull and Wakisaka[23] found that in ten subjects with normal haematological findings a mean of 31 per cent of the radioactivity was present in the faeces. In 13 patients with pernicious anaemia a mean of 89 per cent was recovered. In general it can be stated that, while patients showing a poor absorption by this diagnostic test are not necessarily suffering from pernicious anaemia, those with the disease normally show a low absorption of the crystalline vitamin.

Intrinsic Factor

Castle's hypothesis led inevitably to an enormous amount of work in the search for the intrinsic factor. Since the discovery of crystalline vitamin B_{12} in 1948, Castle has modified his original theory in so far as he has equated the vitamin with the extrinsic (or food) factor. Extensive reviews

on the subject of intrinsic factor have been made by Ungley[13] and more recently by Gräsbeck[24] and the following summary serves to indicate the present confused state of knowledge.

In the pig, intrinsic factor is not only present in the gastric juice[25], but also in the pure duodenal juice[26]. According to Ungley, preparations of every portion of the gastric intestinal tract of the pig (except the fundus (corpus) of the stomach) are haemopoietically effective when administered with vitamin B_{12}. Experimental work has been further complicated by the presence of both intrinsic and extrinsic factors[27,28] in the stomach and intestine of the normal animal. Even normal human gastric juice apparently contains the vitamin[29].

In man, the intrinsic factor is found in gastric juice but not in saliva and probably not in pure duodenal juice[30]. Likewise Schilling and others[31] have demonstrated, that the stomach is the only site in man for the production of intrinsic factor. Moreover, in contrast with the pig, it is the fundus (or corpus) and the cardiac end which are active, not the pyloric region[32–33]. Thus pernicious anaemia is found together with characteristic atrophy of the fundus region[34–36]. Histological evidence shows that the atrophic changes in the stomach persist during remission.

Properties of Intrinsic Factor

The properties of "intrinsic factor" from gastric juice have never been clearly defined and its behaviour has varied according to the stage of fractionation and the composition of the medium[13]. Ungley has pointed out that it is even more difficult to gauge the properties of intrinsic factor derived from preparations of stomach and intestine where, besides being associated with numerous impurities, it may be bound to the vitamin. He has concluded that "intrinsic factor" is unstable and that attempts at concentration usually involve considerable loss of activity.

Thus Seitz filtration, at acid pH, of normal gastric juice apparently led to considerable loss of activity in one instance[37] but Seitz filtered, neutral gastric juice was found active for potentiating the megaloblast-ripening effect of the vitamin in marrow culture[38]. The haemopoietic activity of an acid (pH 4·6) extract of stomach was destroyed by heating at 60 to 70° for 30 minutes[39]. Intrinsic factor in pyloric mucosa extract and in gastric juice was not destroyed by alkalinity at pH 9·8 for 30 minutes at room temperature.

Although intrinsic factor in gastric juice is said to be inactivated by 70 per cent ethanol[40], Wilkinson and Klein[41] used 92 per cent ethanol to precipitate a haematopoietically active material from stomach press juice. Prusoff and others[42] found that most of the "intrinsic factor" activity of desiccated stomach was in the fraction precipitated by 35 to 55 per cent saturated ammonium sulphate.

It is generally agreed that intrinsic factor will not pass through a semipermeable membrane such as Cellophane[43]. Helmer and Fouts[44] recommended ultrafiltration of gastric juice as a preliminary to concentration. Bethell and colleagues[28] used dialysis to remove the vitamin from

extracts of duodenal mucosa. Goldhamer and Kyer[45] also found that intrinsic factor was not dialysable.

It was found that the glandular mucoprotein fraction of gastric juice potentiated to some degree the effect of orally administered vitamin B_{12}[46] and the mucoprotein nature of intrinsic factor has since become widely accepted.

Hall, Morgan and Campbell[47] demonstrated that the intrinsic factor of gastric juice is effective even when given 2 hours after the vitamin. Even before the vitamin was discovered, Castle and Ham[27] found that when beef was given as long as 6 hours before gastric juice there was still a haemopoietic response. The daily oral administration of buffered mixtures of gastric juice and beef at pH 1·8 or 2·5 did not lead to the increased blood production observed when such mixtures were given at pH 5 or 7[48,49]. At the time this was thought to mean that the high acidity was preventing an essential combination between "intrinsic" and "extrinsic" factors which could take place only *in vivo* and which presumably occurred in the upper part of the small intestine. The finding of Callender and Lajtha[38] that the vitamin is inactive in bone marrow culture unless potentiated by gastric juice suggests that "the vitamin B_{12}—intrinsic factor complex may be the active form of the vitamin"[13].

Vitamin B_{12}—Binding Principle

Ternberg and Eakin[3] were the first to demonstrate that normal human gastric juice as well as hog stomach preparations contained a principle "apoerythein" which combined stoichiometrically with crystalline vitamin B_{12} to form a complex which was nondialysable and non-dissociable on dialysis. The vitamin in this complex was not available to micro-organisms (*E. coli*, *L. leichmannii* and *L. lactis* Dorner) that need it as a growth factor, a property which can be used for the assay of the binding principle. Heated gastric juice lost its capacity to bind the vitamin and heat released the latter from the complex. Eakin (cited by Hall[50]) found the concentration of apoerythein to be low in the gastric juice of pernicious anaemia patients and this led to the suggestion that apoerythein was identical with Castle's intrinsic factor or a component of it.

Several methods for assaying the binding capacity of the vitamin were subsequently developed—all based upon one of the following principles outlined by Ungley[14].

(i) Only the unbound vitamin can be utilised by micro-organisms. (Microbial Growth Inhibition technique). (ii) Only the free vitamin is dialysable. (iii) Only the free vitamin can be adsorbed on to certain micro-organisms (Microbial Adsorption Inhibition). (iv) Free and bound vitamin behave differently on electrophoresis. Most, if not all, of the work on the preparation of intrinsic factors, has been based on these principles. Much depends, however, upon what is meant by the arbitrary terms "free" and "bound" and these terms are discussed later.

Latner and others[51–53] claimed the isolation in a chemically pure form of the "intrinsic factor" which from ultracentrifugal studies had a molecular weight of about 15,000.

Glass[54], however, has disputed this, and has criticized the method (faecal excretion of radioactive vitamin) used for the measurement of intrinsic factor activity. More recently, Thompson and Latner[55] have claimed a preparation that was able to produce a haematopoietic response when given in a 4 mg. dose together with 5 μg. of the vitamin daily for ten days.

Hog bile is rich in a heat-stable vitamin binder[56]. Human duodenal juice has been reported to bind the vitamin[57], but to have little or no intrinsic factor activity[30]. The latter authors demonstrated that saliva did not possess intrinsic factor activity, yet Beerstecher and Altgelt[58] showed that it contained apoerythein. It is by no means certain, however, that apoerythein is identical with intrinsic factor. Doubt has been cast on the validity of deductions based on activity being associated with vitamin B_{12}-binding power, because the vitamin binds itself to many substances without "intrinsic factor" activity. This applies to egg-white lysozyme, sow's milk protein[59] and even to some fractions from pig's duodenum[50]. Wijmenga's[60] purified cobalamin protein is another example.

Purified Intrinsic Factor Preparations and their Activity

Prusoff, Welch, Heinle and Meacham[61] further purified an intrinsic factor preparation, "Ventriculin", from hog stomach employing saline extraction and ammonium sulphate fractionation. The fractions were tested both clinically and for binding capacity by the dialysis and microbial growth inhibition methods. No correlation was found of the ability to bind the vitamin and the clinical "intrinsic factor"; the fractions with the lowest vitamin-binding power had the greatest clinical effect! Again, Everse, Lens and Wijmenga[62] report that the clinical "intrinsic factor" effect of fractions of human gastric juice and of hog stomach does not correlate with the vitamin B_{12}-binding power measured by the microbial growth inhibition technique. Further, though most patients with pernicious anaemia lack vitamin B_{12}-binding capacity in their gastric juice, occasional patients have been found to possess this activity[63–64]. However, presumably the lack of "intrinsic factor" in gastric juice, need not be absolute[65]. Again, Virtanen and Tanksanen[66] isolated a substance from calf stomach which had no vitamin B_{12}-binding power but still evoked some haematologic response.

Wijmenga, Thompson, Stern and O'Connell[67] added the crystalline vitamin to a clinically active hog stomach preparation and isolated a pure conjugated cyanocobalamin protein, the protein part of which they considered to be identical with the vitamin-binding factor in hog gastric mucosa (and probably in human gastric juice also). Electrophoresis, alcohol fractionation and ammonium sulphate fractionation gave two red coloured products with molecular weights of 128,000 and 100,000 respectively. Later[60], these authors purified the cobalamin protein further and claimed that the vitamin content was as high as 18·5 μg./mg. Assuming that one molecule of vitamin is bound per molecule of protein the molecular weight is 70,000. The purified complex, however, failed

to show any clinical intrinsic factor activity after daily oral dose administration of amounts corresponding to 5 μg. of bound vitamin.

In the Lederle Laboratories, Williams, Ellenbogen and Esposito[68] prepared a complex which was haematopoietically active in a daily dose of 1–2 mg. The complex was prepared by means of alcohol and salt solution precipitations and by enzymic digestion. An end-product was obtained by dialysis and ultra-filtration which was known as the ultra filtration residue (U.F.R.) This consisted of two components, a low molecular weight component (about 5,000) comprising 70 per cent of the material and a heterogeneous high molecular weight component (of 100,000 and 500,000) comprising the remaining protein. Incomplete clinical trials showed that the lower M.W. component had the more intrinsic factor activity. The vitamin B_{12}-binding capacity of the U.F.R. fraction was 220 μg./g. which would mean that, if all binding capactiy were to be ascribed to the lower M.W. component, 1,000 molecules would be necessary for the binding of one vitamin molecule. From this and other considerations, the authors concluded that vitamin-binding is not a property of "intrinsic factor". The components of U.F.R. were mucoproteins or mucopolypeptides.

In contrast with these experiments which indicate that "intrinsic factor" activity is a phenomenon different from vitamin B_{12}-binding capacity, Hoff-Jorgensen and Landboe-Christensen[69] and Noer[70] compared the anatomic localization in the hog stomach of the vitamin-binding factor (determined by the microbial adsorption technique) and intrinsic factor activity and found the localization to be the same. The microbial adsorption inhibition technique is based on a concept of Burkholder[71] who showed that concentrates of hog stomach were able to prevent bacterial uptake of the vitamin by binding it. He found that vitamin-binding paralleled the clinical "intrinsic factor" activity of his preparations and suggested that "intrinsic factor" acted by inhibiting the uptake of the vitamin by the intestinal flora. The strain of *E. coli* which the Danish team used was one which had been isolated by Hoff-Jorgensen, Skouby and Gad Andresen[72] from the faeces of a patient with pernicious anaemia. Their later studies showed that the vitamin-binding capacity in the human stomach is strongest in the fundus[73] which is the principal site of "intrinsic factor" activity in man according to Fox and Castle[32].

In 1954 Gad Andresen reported the isolation, from a commercial intrinsic factor preparation (Bendogen R), of the vitamin B_{12}-binding factor in a pure form and believed the cobalamin protein to be identical with that isolated by Wijmenga and his associates. In contrast to the latter substance, however, the complex caused complete haematological remission when given in a daily dose of 1·2 mg. corresponding to 10 μg. of bound vitamin.

Absorption studies by Baker and Mollin[74], using radioactive vitamin B_{12}, indicated that the reaction between it and intrinsic factor is a stoichiometric one, which would argue in favour of the identity of "intrinsic factor" and the binding principle, since Ternberg and Eakin[3] found the binding by "apoerythein" to be more or less stoichiometric. However,

earlier studies[75,76] than those of Baker and Mollin had indicated that the reaction between "intrinsic factor" and the vitamin is not stoichiometric. Raine[77] found that the pure intrinsic factor of Latner and co-workers bound vitamin B_{12} in relation to the vitamin concentration and not stoichiometrically. Thus the question of whether "intrinsic factor" and the vitamin B_{12}-binding principle are identical is not yet settled.

Commercial "Intrinsic Factor" Preparations

In clinically healthy individuals, intrinsic factor preparations were extremely variable in their effects[78]. Nevertheless, satisfactory treatment of pernicious anaemia by administration of crude preparations of hog's stomach has been achieved for years. Thus, Wilkinson[79] reported that of 441 patients treated for six or more years with desiccated hog's stomach, only fifteen relapsed through neglect of treatment and they responded again promptly when treatment was given. Although the heterologous nature of "intrinsic factor" preparations has often been blamed as a possible cause of their failure—and the latest results by Schwartz, Lous and Meulengracht[80] of Copenhagen would seem to support such a view—this can hardly be a serious factor. The belief has been expressed (*Lancet annotation*, 1957, **1**, 775) that the cause of the failure of commercial preparations might lie in their method of preparation as well as in any possible resistance or inhibition arising because of the heterologous source of "intrinsic factor". This editorial view point is in line with the belief expressed later in this article that the majority of even the successful preparations which are being made at present are probably effective more by fortuitous contamination with low molecular weight material of an active nature than by their own essential nature. Hence if the mode of preparation is at fault, a new approach to the problem would seem to be necessary. However, before discussing this there are other relevant topics to be considered; one of these concerns the arbitrary terms "bound" and "free" vitamin B_{12}.

"BOUND" AND "FREE" VITAMIN B_{12}

Discussions of the oral absorption of vitamin B_{12} assume that the "free" vitamin in animal foods is available for combination with an intrinsic factor to form the anti-pernicious anaemia principle. That is to say, it is considered that the "free" vitamin in such foods is identical with Castle's "extrinsic factor". It is doubtful, however, if much vitamin B_{12} is "free" at all, judging by the extraction procedures which are necessary to release it from tissues and foods for microbiological assay. Even in blood serum it mostly occurs "bound" but it can be converted to the "free" form by heating at 100° in acid medium for 15 minutes. It cannot be emphasized too strongly, that these terms are quite arbitrary. "Free" vitamin B_{12} is by no means synonymous with pure uncombined cyanocobalamin; the latter does not seem to occur naturally. The term "free" is generally taken to mean assayable by micro-organisms and, presumably, this means that some, undefined, form of the vitamin is capable of diffusing through the cell walls of the assay organism. However, even when attached to

quite large molecules, the vitamin may still be assayed microbiologically with certain organisms and indeed the assay value given is specific for the vitamin part only. Even in mixtures such as blood sera and liver extracts the values for "free" vitamin B_{12} apparently correspond to the amount of pure uncombined vitamins present, when assayed against organisms like *Euglena gracilis* z strain[81] or *Ochromonas*. Other organisms such as *E. coli* give higher values[82] and this is taken to indicate a greater specificity for vitamin B_{12} by *Euglena* and *Ochromonas*. Certainly the vitamin content of these complex forms is more accurately determined by the latter organisms, but the higher values obtained with *E. coli* may be due to an additional stimulus to growth by the peptide portion of the so-called "free" vitamin.

Normal Absorption of Vitamin B_{12}

Since the vitamin in foods is normally bound to protein material which is unavailable to micro-organisms and requires a pre-digestion, it may be considered that in the animal also the vitamin or "extrinsic factor" is likewise unavailable without prior digestion. On Castle's theory (that the crystalline vitamin is the "extrinsic factor") this digestion would involve splitting the pure cyanocobalamin from the protein complex in order that it could then combine with intrinsic factor in the gastric juice. It would seem equally feasible, however, that partial digestion such as occurs in the release of "free" vitamin from "bound" vitamin in sera and foodstuffs could take place. Even if some pure cyanocobalamin were released to combine with intrinsic factor this would not exclude the likelihood of the larger amount of "free" vitamin B_{12} (i.e., vitamin bound to low M.W. peptide material of a diffusible nature) coming from the food and entering the portal blood stream in that form. Thus neither "extrinsic factor" nor "intrinsic factor" would have a separate existence but would both be combined as a vitamin B_{12} peptide complex. This would argue against a specific "intrinsic factor" and, if such a hypothesis were true, then non-specific peptide complexes might be active orally in persons with pernicious anaemia.

Extraction of "Free" Vitamin B_{12} from Fermented Broths and Foods

In the normal extraction from fermented broths, an acid treatment at low pH values (e.g., pH 2·0 for 1 hour) or a heat treatment as used by Smith and Ball[83] is necessary to release all the vitamin from the cells in a "free" assayable state. When the vitamin is "free", i.e., diffusible, it will assay with some organisms as does pure cyanocobalamin. The vitamin must be combined in some way with protein-like materials within the cell since it does not diffuse into the medium to any appreciable extent during fermentation.

It is interesting to speculate on how this "freeing" of the "bound" vitamin occurs since it may have an analogy in human (or animal) nutrition. It could be that the vitamin B_{12}-protein complex of high molecular weight and undetectable on assay, is hydrolysed by the acid at some intermediate peptide bond(s) or is broken down from a polymer to a monomer, the vitamin remaining attached to its neighbouring peptide

portion. This type of extraction/hydrolysis might occur in the normal stomach which, of course, contains acid. The food is usually less acid than pH 2 but there would be a longer time of digestion and an augmenting effect of the enzymes of proteolysis. It is of interest to note that although achlorhydric patients do not necessarily become anaemic in the "pernicious" sense, according to Schilling and others[31] they absorb less of the vitamin than normal subjects.

The failure of pernicious anaemia patients to make use of vitamin B_{12} produced in the large intestine (colon) from which absorption of (diffusible) materials is known to occur normally, is probably due also to the vitamin being retained within the micro-organism which will normally be excreted in the faeces long before autolysis, thus preventing the patient from utilizing the vitamin.

The Role of Vitamin B_{12} in Nutrition

Animal Nutrition

Attempts in America to eliminate scarce and expensive animal proteins from the diets of farm animals led to the appearance of symptoms of nutritional deficiency. The growth rates of chicks and pigs were adversely affected and hens produced eggs of low hatching potentiality. Raising the level of certain sources of vegetable protein, such as that from soya bean, worsened the position. The deficiency signs could be prevented or cured by the addition of fish-meal or cow manure[84,85]. It was later shown that the factors in cow manure concentrate and fish solubles had very similar properties[86]. In 1946 Cary and others[87] demonstrated that liver extracts which also contained a rat growth factor were effective in pernicious anaemia. About the same time Zucker and Zucker[88,89] discovered a rat growth factor "zoopherin" in animal proteins. Thus it appeared likely that the factors were identical. That synthesis by micro-organisms seemed to be involved was suggested by the activity of cow manure and this was further supported by the observation that a factor was produced by the fermentation of food materials in chick faeces during warm weather, since chicks could manage without animal protein in their food at that time. Stokstad and others[90] confirmed the identity of the animal protein and anti-pernicious anaemia factors, when they showed that an organism isolated from hen faeces could synthesize a factor effective in promoting chick growth and in treating pernicious anaemia in man.

When crystalline vitamin B_{12} became available it was claimed that given orally or by injection, it had the same effect for chicks as the animal-protein factor[91,92]. Subsequent work, showed that the crystalline vitamin together with vegetable protein cannot completely replace animal protein from natural sources. Thus, Stokstad, Jukes, Pierce, Page and Franklin[93] found that a concentrate derived from fermentation liquors of *Streptomyces aureofaciens* gave a greater growth response than crystalline vitamin B_{12}. They concluded that chicks require an unidentified factor in addition to the vitamin. Ershoff[94] fed immature rats massive doses of thyroid together with a purified ration containing casein as the dietary protein. A marked retardation occurred both in the body and gonadal

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weight which was completely counteracted by administration of liver residues. Crystalline vitamin B_{12} was without effect.

Heuser and Norris showed in 1944 that growth was retarded in chicks not receiving animal protein foods. After the discovery of vitamin B_{12} it was shown that soybean cereal rations are deficient in the vitamin and the increased growth effect of animal proteins was thought to be due to the vitamin. But the work of Heuser and Norris[95] showed that this could not be so since, even with the addition of more vitamin to vegetable protein feeds than was added to animal protein feeds, there was a greater growth increase with the animal protein feeds. There was also a greater growth with vegetable foods and antibiotics, possibly due to the latter stimulating the intestinal flora to synthesize animal protein factor or to the presence of A.P.F. in the antibiotic concentrate. Also Briggs and Beeson[96] showed that a combination of vitamin B_{12} and streptomycin potentiated growth. This same augmenting effect of antibiotics in the presence of the vitamin has been confirmed with both penicillin and aureomycin[97].

In rats, Sherman, Schilt and Shaefer[98] showed that 3 per cent fish solubles gave double the increase in growth when compared with that produced by optimum levels of the vitamin. This magnitude of response must result from unidentified factors in fish solubles. It is interesting to note that antibiotics were not sources of unidentified factors.

Moulds and bacteria have been grown on solid or semi-solid vegetable media to produce animal protein factor to replace fish meal, fish solubles and liver extract[99,100]. These organisms produced vitamin B_{12} and other factors. It is well-known that antibiotics alter the intestinal flora and that such an alteration in animals might account for the production and utilization of additional growth factors. It is conceivable that some of these additional growth factors might be more readily absorbable forms of vitamin B_{12}, i.e., forms in which the material bound to the vitamin is of low molecular weight (peptide). That the form of the vitamin is important is shown also by the work of Coates and others[101] who found that the vitamin in sow's milk is only poorly utilized by the chick but is readily available to the piglet.

Human Nutrition

After the therapeutic value of the vitamin had been established its ability to improve partially the biological value of vegetable proteins was demonstrated in various animal species. But there has, however, been a delay in obtaining proof of its "animal protein factor" activity in human nutrition. A distinct growth promoting effect has been claimed when given as a dietary supplement to children in growth failure[102] which has not been confirmed[103,104]. Nevertheless, human dietary deficiency of the vitamin, that is a clinical condition apparently not due to an absence of "intrinsic factor", has recently been described among persons in Britain living entirely on vegetable foods[105,106].

A megaloblastic anaemia in which nutrition plays at least some part is reported by Foy and others in East Africa. In their latest paper they

show that those who respond to treatment either with penicillin or with relatively small oral doses (80 μg.) of vitamin, have low serum levels (20–100 $\mu\mu$g./ml.) of the vitamin. The diet of these people is poor in animal protein and rich in carbohydrates and gastric function is usually unimpaired. There would seem to be two possible explanations of this phenomenon. Firstly it may be that penicillin encourages the growth, in the intestine, of organisms which produce vitamin B_{12} in sufficient quantities to overcome the nutritional deficiency. Alternatively this diet may encourage the growth of organisms that compete for the available vitamin, and these organisms are presumably sensitive to penicillin.

In food, vitamin B_{12} is usually associated with animal protein; it has been detected in only one or two vegetable foods, such as groundnuts and, if any purely dietary deficiency ever occurs it is usually among vegetarians. Wokes, Badenoch and Sinclair[105–109] have investigated vegans who eat no animal protein at all not even dairy products; and groundnuts are often absent from their diets. Even so some of these people seem to escape deficiency symptoms for as long as five years on the diet. The commonest and earliest symptoms are in the mouth, a sore tongue being often prevalent. In a group of 150 vegans, 27 per cent had oral symptoms and paraesthesiae developed in 20 per cent. Amenorrhoea and other menstrual disturbances were common and appeared early in women aged between fifteen and forty-five years. Serum B_{12} levels were also low 45–193 $\mu\mu$g./ml. compared with the value (200–320 $\mu\mu$g./ml.) obtained for the controls. Haemoglobin levels were normal but red cell counts in some were a little low. Wokes and others point out that vegetables contain comparatively large amounts of folic acid; and since folic acid is known to precipitate the onset of neurological symptoms in classical pernicious anaemia, it is conceivable that in the diet it may have a similar effect on vegans who have become deficient in vitamin B_{12}.

Wokes[108] reported further data on children and stated that vitamin B_{12} requirements become more critical after early weaning, when dietary supplementation with vitamin B_{12} may be more effective. Wokes considers that possibly the reason why so many people in the Middle and Far East living on diets low in protein do not develop symptoms of vitamin B_{12} deficiency is due to their widespread use of fermented and germinated food. In vitamin B_{12}-deficiency in vegans there can be no shortage of "intrinsic factor" because the symptoms are relived by the administration of animal protein such as milk, which is a good source of the vitamin.

Further evidence that normal digestion is the only factor missing in pernicious anaemia comes from the work of Bonsdorff and his colleagues on fish tape-worm anaemia. This worm resides in the lower part of the intestine and has been shown[110] to be a rich source of cyanocobalamin. The dried worm alone is effective in curing the megaloblastic tape-worm anaemia and together with normal gastric juice it is effective in Addisonian pernicious anaemia. These experiments show that in the normal human intestine the vitamin must be in a readily assimilable form in order to diffuse readily through the body of the tape-worm.

In commenting upon both animal protein factor in animals and upon vitamin B_{12} deficiency in humans, it only remains to be pointed out that if a diffusible peptide complex of the vitamin were the truly requisite factor in animal nutrition, many if not all of the discrepancies could be more easily explained. Certainly the production by micro-organisms, either in the animal directly or via animal protein food, of bound forms of the vitamin which in the normal animal could be broken down to a more assimilable form, though still as a vitamin complex, is a feasible explanation both of the effectiveness of antibiotics and for the inadequate effect of the crystalline vitamin.

The New Approach

The theories of absorption of vitamin B_{12} have been critically examined. A new theory[117] is now put forward which is based on the assumption that a vitamin B_{12}-peptide(s) complex, and not the crystalline vitamin, is the active anti-pernicious anaemia principle. There is evidence for this in the poor absorption of pure uncombined cyanocobalamin given by mouth to pernicious anaemia patients, in the non-maturation of megaloblasts *in vitro* by the crystalline vitamin and in the effectiveness of oral liver therapy without added intrinsic factor. Murphy[111] has cautioned that "further critical study is necessary before vitamin B_{12} is accepted as a complete substitute for either whole liver or liver extracts" and it is significant that more vitamin is excreted when injected as cyanocobalamin than when injected as a purified liver extract (cobalamin-peptide).

In most foodstuffs, the vitamin occurs in a form which is non-assimilable unless it is broken down by proteolysis. Indeed the analysis of the "free" vitamin in foodstuffs involves appreciable preliminary hydrolytic procedures. In the normal human subject a preliminary digestion of protein occurs in the stomach at acid pH. This could result in the release of the pure vitamin from its protein combination, when it could recombine with protein degradation products of low molecular weight in the intestine. Alternatively, and the more likely, the vitamin may remain attached to protein while the latter is degraded to a point at which it could be either directly absorbed or further broken down in the intestine and then finally absorbed into the portal system, through the cellular wall of the intestine.

On such a theory it would be reasonable to expect the activity of combined forms of the vitamin given by mouth where the peptide portion is not homologous. Such results have indeed been obtained with both crude[112,113] and with purified[114] heterologous intrinsic factor preparations. The numerous failures might well be attributable to binding with protein of incorrect molecular size.

It is hard to conceive that, if for oral absorption a high degree of specificity is required in the protein part of the active complex, this specificity is likely to be achieved when cyanocobalamin is injected into the blood where at the pH of blood ready combination is possible with a wide variety of related materials of differing molecular weight. Specificity for particular amino acids is not unreasonable, however.

That vitamin B_{12} alone is insufficient to mature megaloblasts *in vitro*, whilst with normal gastric juice it does, indicates that some form of complex is essential. The true nature is unknown but, it would seem likely that the complex must diffuse as such into the portal blood stream through the gut wall and thence travel to the liver. From the liver it is likely that it is released again into the blood stream by the enzyme, erythropoietin[115], and that it then diffuses from the blood into the cells of the bone marrow to take part in haematopoiesis.

Some of the inconsistencies in intrinsic factor preparations may be partly explained by the variations in molecular size of the bound protein components in such mixtures. Thus vitamin B_{12}, if bound to high molecular weight material, even though the latter be comparatively pure, would not be expected to show oral activity in the patient with pernicious anaemia, e.g., the sow's milk protein complex of Gregory and Holdsworth[59] or the purified cobalamin protein (hog) of Wijmenga, Thompson, Stern and O'Connell[67]. These preparations had molecular weights of 55,000 and 70,000 respectively. Positive results obtained by using intrinsic factor preparations might be attributable to low molecular weight impurities, peptides and even amino acids being often extremely difficult to remove from some proteins according to Saidel[116].

Earlier work has led to attempts to prepare vitamin B_{12} complexes which have, as their protein component, a moiety of high molecular weight with non-diffusible properties. Good evidence for the new approach would be obtained if it could be shown that a non-specific bound form of the vitamin say one derived from a mould or bacterial source, were effective in promoting oral absorption of the vitamin (as the complex) in pernicious anaemia patients, particularly in maintaining them on small doses. Evidence that the vitamin is absorbed in a form similar to that in which it is ingested would be obtained if the vitamin B_{12}-peptide were to mature megaloblasts *in vitro*, a property which the crystalline vitamin does not possess.

If such a mould or bacterial B_{12}-peptide were effective as the anti-pernicious anaemia principle, it might seem reasonable to expect that it would be equally effective as the animal protein factor. For as the crystalline vitamin is not very effective *per se* in the oral therapy of pernicious anaemia, it is likewise not completely effective in replacing the animal protein factor in animal nutrition. However, it is well established that crude bacterial fermentations of the vitamin have animal protein factor activity and it would thus seem reasonable that a complex derived from these sources might be fully effective nutritionally for animals.

The above theory with special reference to pernicious anaemia, has already been tested experimentally and the results to date[117] fully support the novel and unorthodox approach to this problem.

A brief outline is given below of the isolation and properties of a vitamin B_{12}-peptide complex which possesses none of the properties which previously have been claimed to be necessary for absorption of the vitamin by the human intestinal tract.

PROPERTIES OF THE COMPLEX

The active vitamin B_{12}-peptide complex (H.P.P./1) was derived initially from fermentation of a Streptomyces mutant under standard conditions. The cobalamin-peptide was released from the protein complexes within the cells and concentrated and purified by a series of steps involving ion exchange chromatography, counter-current solvent extraction, activated-carbon treatment and ammonium-sulphate precipitation. A red precipitate was finally obtained which possessed the following properties:

The vitamin B_{12} portion of the complex was estimated spectrophotometrically at 550 mμ, at which wavelength the interference of the peptide portion is very small. The ratio of peptide to vitamin B_{12} on a weight basis was 6·8 : 1 and, assuming a 1 : 1 molecular ratio of vitamin to peptide, the molecular weight of the complex would be about 10,000.

The true figure was probably less than this in view of the fact that the complex was dialysable and ultrafiltrable through Cellophane and collodion membranes. The complex did not appear to dialyse, however, against ammonium sulphate solution under conditions which enabled the uncombined vitamin to do so.

The ultra-violet and visible absorption spectrum of H.P.P./1 possessed absorption maxima at 277 mμ, 361 mμ, and 550 mμ—i.e., in the same positions as those of vitamin B_{12}. The maximum at about 277 mμ was appreciably greater than the corresponding peak value for the pure vitamin.

Both paper chromatography and paper electrophoresis showed the presence of only one vitamin B_{12}-peptide. Acid hydrolysis of the peptide component by 6N HCl at 100° for 24 hours yielded a mixture of amino acids, of which the following were identified by paper chromatographic separation and appropriate staining: glutamic acid, aspartic acid, glycine, alanine, valine, proline, serine, threonine, arginine, cystine (or cysteine), leucine, *iso*leucine, phenylalanine, lysine, and histidine. Methionine and hydroxyproline were not detected.

Alkaline hydrolysis, followed by paper chromatography, established, with specific reagents—e.g., Ehrlich's reagent—the presence of the essential amino acid tryptophane.

For accuracy in administration of the doses of active complex required, the vitamin content was estimated both spectrophotometrically and by direct microbiological assay using *Ochromonas malhamensis* as the test organism. That the vitamin in H.P.P./1 was directly assayable by organisms that require the vitamin as a growth factor shows that, according to generally accepted terminology, the vitamin was "free" and not "bound". The vitamin in H.P.P./1 also matured megaloblasts *in vitro*, a property not shared by the crystalline vitamin.

PRELIMINARY CLINICAL REPORTS

Four males and two females, aged 42–72, with newly diagnosed pernicious anaemia were treated exclusively with H.P.P./1. A seventh case, which had begun to develop signs of subacute combined degeneration of the cord on treatment with another oral preparation containing intrinsic factor, is described separately.

Pernicious anaemia was diagnosed by an examination of peripheral blood, a histamine-alcohol test-meal, and biopsy of the sternal marrow (haemoglobin 100 per cent = 14·8 g./100 ml.).

Reticulocyte-counts were made for at least three days before treatment to exclude the possibility of a coincident spontaneous remission.

The treatment was carried out in two consecutive uninterrupted stages: (1) correction of the deficiency of vitamin B_{12}, and (2) maintenance of the patient on a lower dosage. Ultimately the success or failure of any preparation is judged on the second stage, for a good initial response to oral therapy is no guarantee of successful maintenance over long periods.

TABLE I

Case No.*	Duration of treatment (days)	Vitamin B_{12} (as peptide complex) (μg.)		Most recent count	
		Total dose	Average daily dose	Haemoglobin	Haematocrit
1	516	6,440	12·5	103	45
2	536	7,270	13·4	97	41
3	521	5,420	10·4	109	48
4	445	5,610	12·6	98	43
5	430	4,930	11·5	107	52

* The numbers here refer to those same cases reported in ref. 117.

Initial dose. As it is generally agreed that the liver contains 1–2 mg. of vitamin B_{12} in health, it was decided to give the first case 780 μg. of H.P.P./1 (vitamin B_{12} 100 μg.) daily for eight days, and then half that amount for fourteen more days (total dose of vitamin B_{12} 1,500 μg.). Dosage was varied slightly in subsequent cases but never exceeded this amount in the first three weeks' treatment.

Maintenance dose. As already mentioned Ungley[13] considered that a daily oral dose of 5–10 μg. of crystalline vitamin B_{12} had no detectable effect on the blood-picture. Thus, if a patient could be maintained in health within this range of dosage, it would be reasonable to presume that the preparation was effective. Accordingly 78 μg. of H.P.P./1 (vitamin B_{12} 10 μg.) was generally given as a daily maintenance dose.

Results

There was a good haematological and clinical response, which has been maintained in all six cases. A reticulocyte crisis with maxima of 11–17 per cent developed at about the end of the first week in every case, and the average daily increases in red cells and haemoglobin in the first 28 days' treatment were 47,000 per c.mm. and 1·07 per cent respectively. No case up to now has developed signs either of cord deterioration or of iron deficiency on treatment.

A preliminary report on six cases has already appeared[117] and the most recent results on these patients are summarized in Table I. Case No. 6, after 50 days treatment with the oral preparation, elected at her own request to have parenteral treatment "like her sister", who also suffered from pernicious anaemia and had been receiving injections for years. Up to the time of transference, her maintenance on oral therapy had been completely satisfactory.

It will be seen that 5 cases have now been maintained exclusively on H.P.P./1 for periods ranging from 430 to 536 days and that the average daily maintenance dose has never exceeded 13·4 μg. of vitamin B_{12}.

A further case, a woman, aged 52, with early subacute combined degeneration of the cord was treated with the oral peptide complex H.P.P./1.

For the first three weeks she received 2,340 μg. of H.P.P./1 (vitamin B_{12} 300 μg.) daily and thereafter 1,560 μg. of H.P.P./1 (vitamin B_{12} 200 μg.) daily for eight more weeks. Oral treatment then had to be abandoned owing to shortage of experimental material, and the patient was given 1,000 μg. of parenteral crystalline vitamin B_{12} weekly.

During the 11 weeks' oral therapy, however, an unequivocal subjective improvement took place both subjectively and objectively. During both oral and parenteral treatment, her blood-count remained essentially unaltered.

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RESEARCH PAPERS

THE ANTI-INFLAMMATORY ACTIVITY OF GLYCYRRHETINIC ACID AND DERIVATIVES

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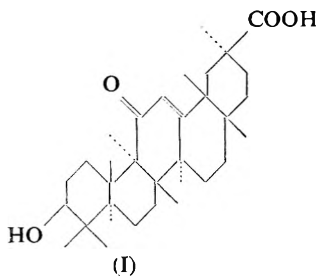
The anti-inflammatory activities of different fractions of glycyrrhetinic acid or glycyrrhetic acid and some of its derivatives have been assessed in laboratory animals. Some, but not all, preparations have been found to be active using four established methods for testing anti-inflammatory drugs. The findings provide a scientific basis for the clinical use of these compounds in inflammatory diseases, and may explain the discrepancies in the early clinical trials with this drug.

LIQUORICE has been used medicinally for generations, mainly as a demulcent and sweetening agent. It has only recently been discovered that some of its derivatives have an anti-inflammatory action. Revers[1] found that large doses of liquorice extract were effective in the treatment of stomach ulcers, and this was confirmed by Molhuysen and others[2]. The main water soluble constituent of liquorice is "glycyrrhizin", from which the aglycone "glycyrrhetinic acid" may be obtained by hydrolysis. The main component of crude glycyrrhetinic acid is the 18 β form, having the structure and configuration (I).



(I)

Glycyrrhetic Acid
Glycyrrhetinic Acid

The structure shows a resemblance to that of hydrocortisone, but the acid has no glucocorticoid action[3].

A number of isomers have been described[4]. We have found that not all fractions of glycyrrhetinic acid have an anti-inflammatory activity. This fact might account for the conflicting results of the early clinical trials with this compound in skin diseases. Adamson and Tillman[5] reported on the use of glycyrrhetinic acid in skin diseases. A number of small-scale trials followed with adverse results[6–8]. These may have been due to the

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material used, for the method of extraction is important. Evans[9], Annan[10], Chakravorti[11], Sommerville[12] and Colin-Jones and others[13,14], using materials which we had found to be biologically active, proved that they had valuable anti-inflammatory properties in a variety of dermatoses. Encouraging results have also been obtained in veterinary practice[15–18].

Similar conclusions have been made by Benigni and Franco[19] who found that the 18 β and so-called 18 γ isomers of glycyrrhetinic acid were active bacteriostatic agents while the 18 α form was inactive.

Little is known about the mode of action of glycyrrhetinic acid. But, Atherden[20] has shown that it inhibits the metabolism of progesterone and 11-deoxycorticosterone by rat-liver homogenates.

There have been few publications on the anti-inflammatory activity of glycyrrhetinic acid in induced inflammatory conditions using laboratory methods. Cornforth and Long[21] showed that certain fractions of liquorice suppressed, like hydrocortisone, the tuberculin reaction in B.C.G. sensitised guinea pigs. Somers[22] found glycyrrhetinic acid to be active in the cotton pellet test of Meier and others[23] and this was confirmed by D'Arcy and Kellett[24] and by Logemann, Lauria and Tosolini[25]. This paper describes the anti-inflammatory activity of glycyrrhetinic acid by four established methods for assessing anti-inflammatory drugs using laboratory animals. They prove that some, but not all, fractions of glycyrrhetinic acid are active anti-inflammatory agents, thus providing a scientific basis for the use of the active fractions in inflammatory conditions.

Materials

The samples of glycyrrhetinic acid and derivatives were those which had been prepared by Professor E. E. Turner, F.R.S., from material supplied through the courtesy of Dr. S. Gottfried of Biorex Laboratories, Ltd. Cortisone and hydrocortisone were used as saline suspensions in the form of the commercial preparations of the acetates Cortelan and Cortef.

METHODS

The anti-inflammatory activity of glycyrrhetinic acid has been determined experimentally in animals by the following methods.

The Cotton Pellet Method of Meier, Schuler and Desaulles[23]

Small cotton wool pellets, when implanted under the skin of the rat become infiltrated with granulation tissue. An anti-inflammatory drug reduces the deposition of this granulation tissue so that the increase in weight of the pellet is reduced. Four cotton wool dental pellets weighing about 5 mg. are weighed and implanted under the skin of the anaesthetised rat, one in each groin and axilla. These pellets are left *in situ* for 6 days, the rats being injected daily subcutaneously or intramuscularly with the preparation under test. The rats are then killed, the pellets removed, extraneous tissue trimmed off and the pellets dried overnight in an oven at 60°. The pellets are again weighed and the amount of granulation tissue is taken as the difference between the initial and final weights. Groups of male wistar rats weighing about 180 g. are used for each

preparation and compared with uninjected controls. The mean gain in weight for the pellets in each group is calculated together with its standard error. A statistical assessment of the significance is made by the Students "t" test.

Inhibition of the Tuberculin Reaction in B.C.G. Sensitised Guinea Pigs

The method was first described by Long and Miles[26], and Cornforth and Long[21], who found that certain fractions of liquorice extract were as active as cortisone. We have modified the technique slightly to allow for variations in the response in different areas of the skin. White male guinea pigs are sensitised to tuberculin by injecting, intradermally, 3 weeks previously, 0·2 ml. of a 1 in 20 dilution of B.C.G. vaccine. For the test they are randomly distributed into groups of four, and injected subcutaneously, three times a day, for 5 days with the preparation under test. On the sixth day 0·2 ml. of a dilution of Tuberculin B.P. in saline is injected intradermally, into the previously depilated skin on the back at three dose levels, (*a*) 100 units/ml., (*b*) 400 units/ml. and (*c*) 1600 units/ml. The doses are injected into the different areas of the back in a randomised order according to a 3×3 Latin Square design[27]; each guinea pig giving three responses at each of the three dose levels. On the following day the diameters of the wheals are measured in the groups and the means plotted against the logarithm of the doses.

The Rat Foot Test

The method was described by Selye[28]. For the test, groups of rats are injected subcutaneously daily for 6 days with the test drug, and on the last day 0·2 ml. of a 3 per cent solution of formaldehyde is injected into the plantar aponeurosis of the right rear foot. The degree of swelling is determined on the following day by comparing the volume of the injected foot with the volume of the uninjected foot, using the microburette described by Buttle and others[27]. The response is the difference between the volume of the injected and the uninjected foot and when expressed as a percentage of the uninjected foot may be compared with the percentage increase of the control.

The Granuloma Pouch Method

This method was also described by Selye[30]. For the test, 25 ml. of air is slowly injected through a fine hypodermic needle under the skin of the back of the rat. Into the air sac which is formed, there is injected 0·5 ml. of a 1 per cent solution of croton oil in arachis oil. A suspension of the drug being tested is injected subcutaneously daily for 5 days, in another site. The rat is killed on the sixth day and the pouch dissected. An anti-inflammatory drug reduces the thickening of the wall of the pouch and the exudation of fluid into the sac.

RESULTS

Cotton Pellet Method

Glycyrrhetinic acid depressed the formation of granulation tissue induced by subcutaneously implanted cotton pellets in rats. The response

is related to the dose[31] (Table I). Providing the dose was sufficient, glycyrrhetinic acid was as active as hydrocortisone. Figure 1 shows the results of a typical comparison of glycyrrhetinic acid and hydrocortisone in this test. Weight for weight glycyrrhetinic acid is between $\frac{1}{5}$th and $\frac{1}{10}$th

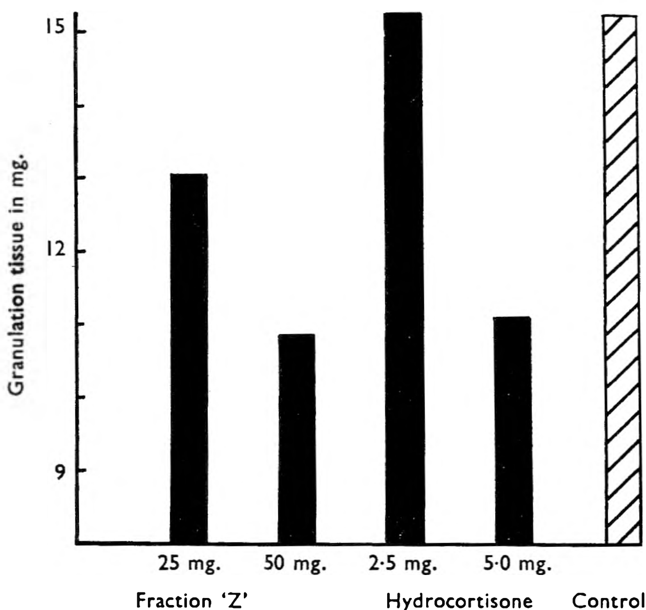

FIG. 1. The effect of glycyrrhetinic acid Fraction 'Z' and hydrocortisone on the formation of granulation tissue in cotton wool pellets.

the activity of hydrocortisone. However, the low solubility of glycyrrhetinic acid is a limiting factor, much of the injected material remaining at the site of the injection. It is for this reason that no direct quantitative comparison with hydrocortisone can be validly made. A number of derivatives of glycyrrhetinic acid were tested and the glycyrrhetinic acid

TABLE I

DEPRESSION OF FORMATION OF GRANULATION TISSUE BY GLYCYRRHETINIC ACID (FRACTION D) IN THE COTTON PELLET TEST IN THE RAT

Treatment	No. of rats	Granulation tissue mg.	Standard error mg.	Per cent of controls
Controls	10	14·1	±0·471	—
Glycyrrhetinic acid Fraction "D" 6·25 mg. ..	10	12·4	±0·636	88
Glycyrrhetinic acid Fraction "D" 12·5 mg. ..	10	11·3	±0·393	80
Glycyrrhetinic acid Fraction "D" 25 mg.	10	10·1	±0·608	72
Glycyrrhetinic acid Fraction "D" 50 mg.	10	9·4	±0·649	67

hydrogen succinate, the glycyrrhetinic acid propionate, the piperazine salt of glycyrrhetinic acid and the acyl derivative were found to be active (Tables II and III). The soluble sodium salt of the glycyrrhetinic acid hydrogen succinate was particularly active as seen in Table IV.

Inhibition of the Tuberculin Reaction in B.C.G. Sensitised Guinea Pigs

The results of a typical experiment are shown in Table V. They show that doses of 5 mg./kg. of glycyrrhetinic acid injected subcutaneously suppressed the tuberculin reaction in the guinea pig in the same way as 4 mg./kg. of cortisone when compared with untreated controls. Similar results were obtained with certain fractions of liquorice by Cornforth and

TABLE II

DEPRESSION OF THE FORMATION OF GRANULATION TISSUE BY A NUMBER OF DERIVATIVES OF GLYCYRRHETINIC ACID IN THE COTTON PELLET TEST IN THE RAT

Treatment	No. of pellets	Granulation tissue mg.	Standard error mg.	Per cent of controls	Significance to controls
Controls—saline	48	9·5	±0·65	—	—
Hydrocortisone 2·5 mg.	24	5·6	±0·27	59	P <0·001
Glycyrrhetinic acid hydrogen succinate 12·5 mg.	24	6·0	±0·028	63	P < 0·001
Glycyrrhetinic acid propionate 12·5 mg. ..	24	6·7	±0·38	70	P = 0·001
Glycyrrhetinic acid Fraction "Z" 12·5 mg. ..	24	6·8	±0·4	72	P = 0·001
Piperazine salt of glycyrrhetinic acid 6·75 mg.	24	6·8	±0·39	72	P = 0·0015

TABLE III

DEPRESSION OF THE FORMATION OF GRANULATION TISSUE BY A NUMBER OF PREPARATIONS AND DERIVATIVES OF GLYCYRRHETINIC ACID IN THE COTTON PELLET TEST IN THE RAT

Treatment	No. of pellets	Granulation tissue mg.	Standard error mg.	Per cent of controls	Significance to controls
Controls—saline	16	11·0	±0·85	—	—
Glycyrrhetinic acid methyl ester 12·5 mg. ..	16	9·3	±0·83	85	P > 0·05
Glycyrrhetinic acid Fraction "D" 12·5 mg. ..	16	8·3	±0·54	75	P = 0·02
Glycyrrhetinic acid Fraction "S" 12·5 mg. ..	16	8·6	±0·62	78	P = 0·05
Acyl derivative of glycyrrhetinic acid 12·5 mg.	16	7·6	±0·45	69	P = 0·005

TABLE IV

DEPRESSION OF THE FORMATION OF GRANULATION TISSUE BY THE SODIUM SALT OF GLYCYRRHETINIC ACID HYDROGEN SUCCINATE IN THE COTTON PELLET TEST IN THE RAT

Treatment	No. of pellets	Granulation tissue mg.	Standard error mg.	Per cent of controls	Significance to controls
Controls	16	13·3	±0·34	—	—
Sodium salt of glycyrrhetinic acid hydrogen succinate 12·5 mg.	16	7·8	±0·19	58·6	P < 0·001

Long[21]. They have stated, however, that this activity was not due to "glycyrrhetinic acid" which they found to be inactive in the test[32]. This discrepancy could be due to the material they used, for not all samples of so-called "glycyrrhetinic acid" are active in the test. We have ourselves tested a sample of "commercially pure" glycyrrhetinic acid and found it to be inactive in the test (Table VI).

Rat Foot Test

Glycyrrhetinic acid was active in this test when given daily for 6 days before the injection of formaldehyde into the foot. Table VII shows the

results obtained in comparison with hydrocortisone. A greater depression of the swelling was obtained with increasing doses of glycyrrhetinic acid, but this was not so with hydrocortisone where increasing the dose decreased the protection. Similar findings were made with hydrocortisone

TABLE V

INHIBITION OF TUBERCULIN REACTION IN B.C.G. INJECTED GUINEA PIGS

Treatment	No. of wheals per dose	Mean wheal diameter in mm. Units of tuberculin 20	80	320	Significance to controls Units of Tuberculin 20	80	320
Controls	12	11	16	18·5	—	—	—
Cortisone 4 mg./kg. ..	12	11	13	15	The inflammatory response was too small to demonstrate the anti-inflammatory activity of the drug	P = 0·02	P = 0·01
Glycyrrhetinic acid Fraction "D" 5 mg./kg. ..	12	10·5	12·5	15		P = 0·01	P = 0·01

TABLE VI

INHIBITION OF TUBERCULIN REACTION IN B.C.G. INJECTED GUINEA PIGS

Treatment	No. of wheals per dose	Mean wheal diameter in mm. Units of Tuberculin 20	80	320
Controls	12	7·0	9·3	12·2
Cortisone 5 mg./kg.	12	6·0	6·6	9·0
Glycyrrhetinic acid Fraction "Y" 25 mg./kg.	12	8·3	9·8	14·1

TABLE VII

REDUCTION IN SWELLING OF RAT FOOT INJECTED WITH FORMALDEHYDE BY GLYCYRRHETINIC ACID FRACTION "Z" AND HYDROCORTISONE

Treatment and dose per 200 g. rat	No. of rats	Mean volume of foot ml. Left	Right	Mean difference ml. and standard error	Mean increase as per cent of controls
Control	4	1·50	1·97	0·47 ± 0·07	—
Glycyrrhetinic acid Fraction "Z" 25 mg.	4	1·52	1·90	0·38 ± 0·11	81
Glycyrrhetinic acid Fraction "Z" 50 mg.	4	1·37	1·67	0·30 ± 0·07	64
Glycyrrhetinic acid Fraction "Z" 100 mg.	4	1·45	1·70	0·25 ± 0·08	53
Hydrocortisone 2·5 mg.	4	1·45	1·60	0·15 ± 0·03	32
Hydrocortisone 5·0 mg.	4	1·52	1·80	0·28 ± 0·09	59
Hydrocortisone 10·0 mg.	4	1·55	1·90	0·35 ± 0·06	75

by Cooper and others[33], who reported that after repeated doses of hydrocortisone the oedema produced by silver nitrate injected into the paw of the mouse was increased.

Granuloma Pouch Method

The effects here were dramatic, but difficult to assess quantitatively. In the control rats the croton oil caused gross inflammation in the sac. The wall was thickened and the blood vessels engorged. The sac contained a light brown fluid often blood stained and the underlying tissues showed

necrotic changes with pus formation. With hydrocortisone and glycyrrhetinic acid these changes were prevented, the wall of the sac remained thin and only slightly inflamed, there was no fluid in the sac and no necrosis of the underlying tissue (Table VIII).

TABLE VIII

ANTI-INFLAMMATORY ACTION OF HYDROCORTISONE AND GLYCYRRHETINIC ACID IN THE GRANULOMA POUCH TEST

Treatment	No. of rats	Mean volume fluid ml.	Result
Controls	8	1·3	Sac contained inflammatory exudate. Wall of sac thickened and grossly inflamed
Glycyrrhetinic acid Fraction "Z" 12·5 mg.	4	2·0	do.
Glycyrrhetinic acid Fraction "Δ" 12·5 mg.	4	0	Sac thin and only very slightly inflamed. No fluid present
Glycyrrhetinic acid Fraction "Δ" 5·0 mg.	4	0	
Glycyrrhetinic acid Fraction "Δ" 1·25 mg.	4	0	
Hydrocortisone 5 mg.	4	0	do.
Hydrocortisone 1·35 mg.	4	0	
Hydrocortisone 0·35 mg.	4	0	

CONCLUSIONS

These results, obtained in four different kinds of tests, show that glycyrrhetinic acid is an active anti-inflammatory agent. They have been obtained in carefully controlled experiments with laboratory animals where psychological factors can presumably be ruled out. In three of these tests the responses have been assessed by actual measurement and not by subjective comparisons which may be influenced by the observer. The statistical validity of the findings has been proved and they provide a scientific foundation for the use of the biologically active fractions of glycyrrhetinic acid in inflammatory conditions and explain the clinical effectiveness of the drug.

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DETERMINATION OF NARCOTINE AND PAPAVERINE BY INFRA-RED SPECTROPHOTOMETRY AND NON-AQUEOUS TITRATION

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An infra-red spectrophotometric method for the determination of narcotine in the presence of papaverine in carbon tetrachloride or chloroform solution is described. The carbonyl group of the lactone ring present in narcotine displays strong absorption at 1760 cm.$^{-1}$ and the intensity of the band observed at this wavelength provides an index of the concentration of the narcotic. By conventional non-aqueous titration the amount of both alkaloids may be estimated quantitatively and the papaverine content of a mixture obtained readily by difference.

THE determination of narcotine and papaverine has been the subject of several publications. Most of the methods reported use Anneler's procedure[1] for the preliminary separation of the two alkaloids before their determination by either gravimetric[2] or spectrophotometric analysis[3]. The process described by Anneler involves heating the substrate with both strong acid and alkali. This treatment was found to destroy some of the opium alkaloids and only low recoveries of narcotine were obtained[4].

Lee and Farmilo recently reported a method for the isolation of narcotine and papaverine from raw opium[5], the *iso*quinoline alkaloid being removed as the reineckate from a two-phase water-chloroform system. During this study it was observed that the infra-red spectrum of narcotine reineckate exhibits strong absorption at 1760 cm.$^{-1}$ (C = O stretching vibrations of the lactone carbonyl group of the molecule), whereas papaverine reineckate shows no band in this region.

A further study of the infra-red spectra of the alkaloidal bases in both chloroform and carbon tetrachloride solutions revealed the same phenomenon—see Figure 1. This observation suggested a simple method of determining narcotine by an infra-red technique without prior separation of the alkaloid from papaverine. Since, furthermore, the total amount of these alkaloids in a binary mixture can be determined by conventional non-aqueous titration procedures the weight of papaverine present may readily be obtained by difference.

Apparatus, Reagents and Solutions

Perkin-Elmer Model 21 Recording infra-red spectrophotometer; sodium chloride cells, 1 mm. light path; narcotine, T. and H. Smith, Ltd., M.P. 174°; papaverine, T. and H. Smith, Ltd., M.P. 146°; carbon tetrachloride, Fisher Spectroanalyzed Grade, chloroform, Fisher Spectroanalyzed Grade, glacial acetic acid, Fisher Reagent Grade, acetous perchloric acid, 0·05N.

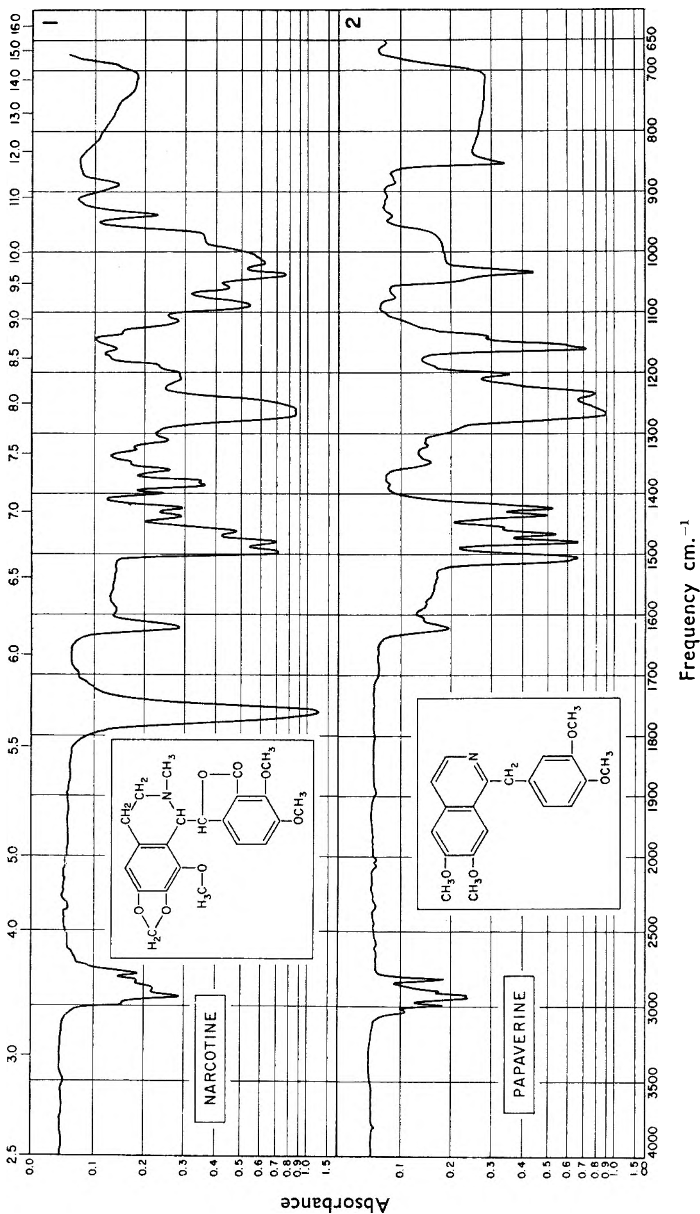

FIG. 1. Infra-red absorption spectra of narcotine and papaverine in carbon tetrachloride.

EXPERIMENTAL PROCEDURES AND DISCUSSION OF RESULTS

Preparation of Calibration Curve for Narcotine

Quantities of about 5, 10, 15, 20 and 25 mg. of narcotine were weighed accurately and dissolved in carbon tetrachloride or chloroform in 10-ml. volumetric flasks and the solutions made up to volume. The infra-red spectra of these preparations were measured from 1900 to 1650 cm.$^{-1}$ using slit schedule 927 and the instrument zeroed at 0·05 on the absorbance scale. The absorbance of each solution ($A_{corr.}$) was then computed by means of the following equation:

$$A_{corr.} = A_{1760\ cm.^{-1}} - \frac{A_{1900\ cm.^{-1}} + A_{1650\ cm.^{-1}}}{2} \quad .. \quad (1)$$

where A denotes the absorbance measured at the given wavelength.

The calibration curve thus obtained reveals that the absorbance of narcotine solutions at 1760 cm.$^{-1}$ is linear for concentrations up to approximately 2·5 mg./ml. when using cells of 1 mm. light path.

It was observed that on standing, solutions of narcotine in carbon tetrachloride gradually turned yellowish. Their absorbances at 1760 cm.$^{-1}$ remained, however, unchanged and since, furthermore, this solvent was found to serve advantageously for the extraction of narcotine and papaverine in a relatively pure state from crude opium[5] it was used throughout these experiments. Chloroform proved equally satisfactory for the infra-red measurements and may therefore also be employed as a solvent.

TABLE I

ANALYSIS OF SYNTHETIC MIXTURES OF NARCOTINE AND PAPAVERINE

Composition		Narcotine found (mg.)	Total volume of titrant used (ml.) (X)	0·05N Perchloric acid equivalent		Papaverine found (mg.)
Narcotine (mg.)	Papaverine (mg.)			Narcotine (ml.)* (Y)	Papaverine (ml.) (X–Y)	
23·9	8·0	23·0	1·60	1·11	0·49	8·3
30·4	8·0	29·7	1·94	1·44	0·50	8·5
47·0	20·0	48·0	3·43	2·32	1·11	18·8
105·0	40·0	101·0	7·37	4·89	2·42	41·0
138·0	40·0	140·0	9·10	6·77	2·33	39·5

* Calculated from infra-red data.

Determination of Narcotine and Papaverine in Binary Mixtures

Solutions containing both narcotine (0·4–3·0 mg./ml.) and papaverine (0·2–0·8 mg./ml.) were prepared by dissolving the alkaloids in carbon tetrachloride. Their infra-red spectra were recorded as described and the absorbances, measured at 1760 cm.$^{-1}$ in accordance with equation 1, expressed as narcotine with reference to a calibration graph. It was found that absorbances of the mixtures at the critical wavelength of 1760 cm.$^{-1}$ were independent of the amount of papaverine present and therefore provided an accurate index of the narcotine content.

For the determination of papaverine, solution aliquots were diluted with 20 ml. of glacial acetic acid and titrated with 0·05N acetous perchloric acid

in accordance with the procedure described by Levi and others[6]. The experimental data were expressed in terms of papaverine using the following equation:

$$W = (X - Y) \times N \times M$$

where W = mg. of papaverine present, X = ml. of titrant used, Y = ml. of titrant equivalent to weight of narcotine as derived from infra-red measurements, N = normality of titrant and M = molecular weight of papaverine = 339·4.

Results obtained by this method on mixtures of pure narcotine and papaverine are recorded in Table I. The compositions were chosen so as to cover fully the proportions in which the two alkaloids occur in raw opium and the experimental data suggest that the method described should find practical application in the field of drug and opium analysis.

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CHRONIC TOXICITY STUDIES ON FOOD COLOURS

Part IV. Observations on the Toxicity of Tartrazine, Amaranth and Sunset Yellow in Rats

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Tartrazine, amaranth and sunset yellow fed at concentrations of 0·03, 0·3, and 1·5 per cent of the diet for 64 weeks, did not increase mortality in male or female rats. Amaranth, at a level of 1·5 per cent, caused a significant decrease in growth rate in female, but not male, rats. This was attributed to an effect on food utilisation rather than on food consumption. Female rats fed amaranth at 0·3 and 1·5 per cent concentrations showed an increase in the weight of the liver. At the higher concentration there was also an increase in kidney weight. Histopathological studies were made on the lung, heart, liver, spleen, thyroid, pancreas, stomach, small intestine, kidney, urinary bladder, adrenal, testis, prostate, coagulating gland, ovary, uterus, and thymus. None of the changes observed were considered to be due to the ingestion of the food colours. There was no significant difference in tumour incidence between the control animals and the rats receiving the colours.

Provision is made in the Regulations under the Food and Drugs Act in Canada for the use of 15 coal tar colours in foods. Of the 15, amaranth, tartrazine and sunset yellow account for some 75 per cent of the total amount used. Because of this relatively high level of use and the renewed interest in their toxicity, it was decided to include them in our studies even though considerable toxicological data have been presented previously, especially for amaranth and tartrazine. Summaries of these data have been compiled[1].

As described for other food colours in earlier papers in this series[2–4], the effects of oral administration of amaranth, tartrazine, and sunset yellow on mortality, growth, food consumption, food efficiency, and organ weights were investigated. Histopathological, haematological and other studies were also done.

Methods

The colours were added to the normal laboratory diet to give concentrations of 0·03, 0·3, and 1·5 per cent for each dye. In the lower concentrations, alphacel (a non-nutritive cellulose) was added so that the level of dye plus alphacel was 1·5 per cent. The control diet contained 1·5 per cent alphacel. The rats were between 6 and 7 weeks of age at the start of the experiment and were distributed to give similar initial mean body weights among groups. All animals were kept in individual cages and were given free access to food and water. Groups of 15 males and 15 females were assigned to each level of each food colour, and 15 rats of each sex were given the control diet. Body weight, food consumption and food

efficiency data were recorded weekly. Necropsies were performed on the rats that died during the test. After 26 weeks on test, five rats of each group receiving 1·5 per cent colour were killed for histological examination. Haemoglobin estimations were done using a slight modification of the pyridine-haemochromogen method of Rimington[5].

At the end of the experiment, electrocardiograms and electroencephalograms were recorded from six rats of each sex on the control diet and three

TABLE I

CUMULATIVE NUMBER OF DEATHS OF RATS FED TARTRAZINE, SUNSET YELLOW AND AMARANTH

Treatment	Conc. of colour (per cent in diet)	No. rats on test	Time in weeks on test												
			16	20	24	28	32	36	40	44	48	52	56	60	64
Males															
Control ..		15	0	0	0	0	0	1	1	1	1	1	2	3	4
Tartrazine ..	0·03	15	0	0	0	0	0	0	0	0	1	1	1	1	1
	0·3	15	0	0	0	1	1	3	3	3	3	3	3	3	3
	1·5	15	0	0	0	*5	5	5	5	5	5	5	5	5	6
Sunset yellow	0·03	15	0	0	0	0	0	0	0	0	0	1	1	1	1
	0·3	15	0	0	0	0	0	0	0	0	1	2	2	3	3
	1·5	15	0	0	0	*5	5	5	5	5	5	5	5	5	6
Amaranth ..	0·03	15	0	0	0	0	1	1	2	2	3	3	4	4	5
	0·3	15	0	0	0	1	1	1	1	1	2	2	3	4	4
	1·5	15	0	0	0	*5	5	5	5	5	5	5	5	5	6
Females															
Control ..		15	0	0	1	1	1	1	2	2	3	3	3	3	5
Tartrazine ..	0·03	15	0	0	0	0	0	0	1	1	1	1	1	2	2
	0·3	15	0	0	0	0	0	0	0	0	0	0	0	0	1
	1·5	15	0	0	0	*5	5	5	5	5	5	5	5	5	5
Sunset yellow	0·03	15	0	0	0	0	0	0	0	0	0	0	0	0	0
	0·3	15	0	0	0	0	0	0	0	0	0	0	1	1	2
	1·5	15	0	0	0	*5	5	5	5	6	6	6	6	6	6
Amaranth ..	0·03	15	0	0	0	0	0	0	0	0	0	0	0	0	1
	0·3	15	0	0	0	0	0	0	1	1	1	2	2	2	2
	1·5	15	1	1	1	*5	5	5	5	5	5	5	6	6	6

* Five rats killed at 26 weeks.

rats of each sex on the 1·5 per cent level of each colour, lightly anaesthetised with pentobarbitone. Short hypodermic needles served as electrodes for both the ECG and the EEG. A Grass Electroencephalograph, Model III–D, was used to record the electric potentials.

After 64 weeks on test the surviving rats were anaesthetised with ether and a cursory gross examination was made of all organs and tissues. To facilitate fixation of tissues, the right auricle was then cut open and warm physiological saline was injected into the left ventricle; when the flow of saline from the right auricle was only slightly blood tinged a 5 per cent solution of formol-saline was injected into the left ventricle. A detailed gross examination of all tissues and organs was then made. The weights of certain organs were recorded. The tissues in which any gross pathological change was observed were studied histologically.

In addition, a detailed examination was made of haematoxylin-eosin stained paraffin sections of lung, heart, liver, spleen, thyroid, pancreas,

stomach, small intestine, kidney, urinary bladder, adrenal, testis, prostate, coagulating gland, ovary, uterus, and thymus.

Results and Discussion

Mortality

The mortality of control and test rats during the course of the experiment is shown in Table I. If the figures for males and females are combined, the mortality for the control rats at the end of the experiment was 30 per cent. For all rats on tartrazine and sunset yellow the mortality was 20 per cent, and for amaranth 27 per cent. These figures include

TABLE II

Mean body weight of rats fed tartrazine, sunset yellow and amaranth

Treatment	Dosage (per cent of diet)	Mean body weight (g. ± S.E.)					
		Initial	4 weeks	16 weeks	32 weeks	48 weeks	64 weeks
Males							
Control ..		129 ± 4	206 ± 4	289 ± 6	338 ± 8	364 ± 8	363 ± 17
Tartrazine ..	0·03	130 ± 3	205 ± 4	283 ± 5	330 ± 8	350 ± 10	351 ± 17
	0·3	123 ± 5	198 ± 5	283 ± 7	335 ± 6	352 ± 11	336 ± 13
	1·5	130 ± 5	202 ± 5	278 ± 7	322 ± 12	337 ± 18	336 ± 25
Sunset yellow	0·03	132 ± 4	200 ± 4	280 ± 6	320 ± 7	340 ± 13	349 ± 10
	0·3	131 ± 4	206 ± 4	293 ± 7	331 ± 11	351 ± 11	364 ± 10
	1·5	139 ± 5	208 ± 5	290 ± 6	344 ± 11	345 ± 14	362 ± 15
Amaranth ..	0·03	135 ± 3	203 ± 3	284 ± 4	344 ± 6	367 ± 11	389 ± 9
	0·3	135 ± 4	209 ± 5	293 ± 6	332 ± 9	347 ± 15	373 ± 13
	1·5	127 ± 3	198 ± 3	284 ± 5	336 ± 8	351 ± 8	359 ± 15
Females							
Control		105 ± 3	141 ± 3	185 ± 3	218 ± 4	230 ± 4	222 ± 9
Tartrazine ..	0·03	103 ± 3	139 ± 3	174 ± 3*	205 ± 5	223 ± 5	232 ± 7
	0·3	104 ± 3	140 ± 3	175 ± 4	211 ± 5	227 ± 5	224 ± 8
	1·5	106 ± 3	141 ± 3	177 ± 4	210 ± 7	209 ± 8*	209 ± 11
Sunset yellow	0·03	108 ± 2	143 ± 3	185 ± 4	217 ± 4	232 ± 4	238 ± 5
	0·3	107 ± 3	137 ± 2	180 ± 3	207 ± 3*	216 ± 2*	224 ± 7
	1·5	106 ± 3	141 ± 1	181 ± 3	206 ± 3*	230 ± 3	233 ± 6
Amaranth ..	0·03	103 ± 2	134 ± 2	175 ± 4	204 ± 3*	217 ± 3*	226 ± 8
	0·3	103 ± 3	135 ± 3	175 ± 4	207 ± 4	222 ± 4	224 ± 5
	1·5	103 ± 3	133 ± 2*	174 ± 3*	199 ± 3*	217 ± 3*	217 ± 3

* Significant at P = 0·05 or less.

those animals sacrificed at 26 weeks. If they are excluded the mortality rates are: tartrazine and sunset yellow 10 per cent, amaranth 18 per cent. Thus the dyes did not increase mortality when given at levels as high as 1·5 per cent of the diet.

Growth, Food Consumption and Food Efficiency

For results discussed in this section, significant differences between mean values were determined by Student's "*t*" test. Mean body weights at selected intervals are given in Table II. A consistent lag in growth occurred for female rats in the group fed 1·5 per cent amaranth. At the 0·03 per cent level, the amaranth-fed females showed a significantly lower body weight at 32 and 48 weeks. There were three instances where female

rats on sunset yellow had a lower mean weight than did the control group and two isolated significant decreases for the females on tartrazine.

For the male rats there were no significant differences between any of the test groups and the control group as shown in Table II. A similar sex difference was noted in an earlier study[4]. This suggests that female rats may be more sensitive to some coal-tar dyes than males where an effect on growth is concerned.

Most of the groups of females showed significant decreases in food consumption at one time or another during the experiment, as seen in Table III. There was a continuous depression in food consumption for

TABLE III

MEAN FOOD CONSUMPTION OF RATS FED TARTRAZINE, SUNSET YELLOW AND AMARANTH

Treatment	Dosage (per cent of diet)	Mean food consumption (g./rat/day ± S.E.)					
		4 weeks	8 weeks	16 weeks	32 weeks	48 weeks	64 weeks
Males							
Control ..		16·1 ± 0·28	16·5 ± 0·19	16·9 ± 0·20	17·4 ± 0·25	18·4 ± 0·25	18·6 ± 0·26
Tartrazine	0·03	16·0 ± 0·25	16·4 ± 0·28	17·2 ± 0·30	17·8 ± 0·32	18·5 ± 0·36	18·7 ± 0·38
	0·3	15·3 ± 0·29	16·1 ± 0·28	16·8 ± 0·33	17·5 ± 0·22	18·1 ± 0·30	18·3 ± 0·30
	1·5	15·3 ± 0·24*	15·9 ± 0·22*	16·5 ± 0·23	17·4 ± 0·41	18·2 ± 0·43	19·0 ± 0·39
Sunset yellow	0·03	15·7 ± 0·30	16·4 ± 0·33	17·0 ± 0·38	17·3 ± 0·41	17·9 ± 0·42	18·5 ± 0·50
	0·3	16·0 ± 0·14	16·6 ± 0·19	17·3 ± 0·31	17·8 ± 0·33	18·3 ± 0·37	18·8 ± 0·38
	1·5	15·9 ± 0·32	16·6 ± 0·32	17·2 ± 0·36	18·2 ± 0·51	18·8 ± 0·46	19·2 ± 0·54
Amaranth	0·03	16·1 ± 0·29	16·6 ± 0·35	17·1 ± 0·45	18·0 ± 0·44	18·3 ± 0·42	19·1 ± 0·44
	0·3	16·3 ± 0·32	16·5 ± 0·30	16·6 ± 0·27	17·5 ± 0·26	18·1 ± 0·27	18·8 ± 0·33
	1·5	15·6 ± 0·17	16·1 ± 0·21	16·2 ± 0·21*	17·3 ± 0·26	18·1 ± 0·33	18·8 ± 0·41
Females							
Control ..		13·4 ± 0·22	13·5 ± 0·18	13·7 ± 0·19	13·9 ± 0·20	14·6 ± 0·14	14·9 ± 0·17
Tartrazine	0·03	12·9 ± 0·31	13·1 ± 0·22	13·5 ± 0·24	13·9 ± 0·38	14·6 ± 0·29	14·9 ± 0·27
	0·3	12·7 ± 0·21*	13·1 ± 0·19	13·2 ± 0·17	13·4 ± 0·17	14·4 ± 0·14	14·7 ± 0·21
	1·5	12·6 ± 0·21*	12·8 ± 0·17*	12·6 ± 0·10*	13·0 ± 0·11*	13·9 ± 0·12*	14·2 ± 0·16*
Sunset yellow	0·03	12·9 ± 0·23	13·0 ± 0·21	13·0 ± 0·20*	13·3 ± 0·18*	14·0 ± 0·15*	14·4 ± 0·19
	0·3	12·9 ± 0·13	13·1 ± 0·17	13·1 ± 0·22*	13·1 ± 0·22*	13·6 ± 0·20*	14·1 ± 0·14*
	1·5	12·9 ± 0·26	13·3 ± 0·21	13·1 ± 0·14*	13·5 ± 0·24	14·3 ± 0·31	14·6 ± 0·34
Amaranth	0·03	12·6 ± 0·21*	12·9 ± 0·23*	13·0 ± 0·31	13·6 ± 0·42	14·5 ± 0·45	14·9 ± 0·47
	0·3	12·9 ± 0·18	13·2 ± 0·20	12·9 ± 0·16*	13·3 ± 0·21*	14·3 ± 0·22	14·8 ± 0·30
	1·5	12·7 ± 0·22	12·9 ± 0·24	12·9 ± 0·26*	13·5 ± 0·38	14·4 ± 0·34	14·7 ± 0·40

* Significant at P = 0·05 or less.

the group fed 1·5 per cent tartrazine from the fourth week to the end of the test. However, this did not produce any significant decrease in body weight except at the 48 week interval. For sunset yellow and amaranth the differences appeared more randomly distributed. In general the groups on sunset yellow showed reduced food consumption from the sixteenth week onward while most of the differences in the rats receiving amaranth occurred within the first 16 weeks. With the exception of tartrazine these decreased food consumption values showed no correlation with the concentration of colour given in the diet. For the male rats there were only three instances where a significant drop in food consumption occurred. These were for the group fed 1·5 per cent tartrazine at 4 and 8 weeks, and for the high level amaranth group at 16 weeks.

The mean cumulative food efficiency is shown in Table IV. It appears that amaranth at 1·5 per cent in the diet interfered with the utilisation of food for growth in female rats. Although the decrease in food efficiency was not significant at 4 weeks there was a significant decrease from the eighth to the thirty-second week. In the latter half of the experiment, when food efficiency is low for all groups, this effect was not apparent.

TABLE IV

MEAN CUMULATIVE FOOD EFFICIENCY OF RATS FED TARTRAZINE, SUNSET YELLOW AND AMARANTH

Treatment	Dosage (per cent of diet)	Mean food efficiency (g. gained/100 g. food consumed ± S.E.)				
		4 weeks	8 weeks	16 weeks	32 weeks	64 weeks
Males						
Control		16·7 ± 0·65	11·8 ± 0·42	8·4 ± 0·22	5·4 ± 0·21	2·8 ± 0·18
Tartrazine	0·03	16·3 ± 0·46	10·9 ± 0·35	7·9 ± 0·19	5·0 ± 0·18	2·6 ± 0·19
	0·3	17·0 ± 0·49	11·9 ± 0·34	8·4 ± 0·18	5·6 ± 0·19	2·6 ± 0·11
	1·5	16·3 ± 0·68	11·3 ± 0·44	8·0 ± 0·19	5·0 ± 0·30	2·5 ± 0·31
Sunset yellow ..	0·03	15·3 ± 0·46	10·9 ± 0·33	7·8 ± 0·29	4·9 ± 0·19	2·6 ± 0·10
	0·3	16·0 ± 0·82	11·6 ± 0·59	8·4 ± 0·32	5·0 ± 0·18	2·9 ± 0·10
	1·5	15·2 ± 0·48	10·8 ± 0·36	7·8 ± 0·25	5·0 ± 0·26	2·6 ± 0·13
Amaranth	0·03	14·7 ± 0·55*	10·6 ± 0·44	7·8 ± 0·26	5·2 ± 0·17	3·0 ± 0·08
	0·3	15·6 ± 0·48	11·3 ± 0·39	8·5 ± 0·25	5·0 ± 0·24	2·8 ± 0·14
	1·5	15·5 ± 0·44	11·4 ± 0·25	8·6 ± 0·25	5·4 ± 0·29	2·8 ± 0·17
Females						
Control		9·4 ± 0·34	7·3 ± 0·28	5·2 ± 0·14	3·6 ± 0·11	1·7 ± 0·12
Tartrazine	0·03	9·4 ± 0·44	6·7 ± 0·29	4·7 ± 0·12*	3·3 ± 0·12	1·9 ± 0·08
	0·3	9·6 ± 0·39	6·7 ± 0·22	4·8 ± 0·15	3·5 ± 0·11	1·8 ± 0·08
	1·5	9·4 ± 0·41	6·9 ± 0·22	4·9 ± 0·18	3·4 ± 0·17	1·6 ± 0·20
Sunset yellow ..	0·03	9·3 ± 0·19	7·4 ± 0·25	5·3 ± 0·19	3·7 ± 0·12	2·0 ± 0·07
	0·3	8·1 ± 0·46*	6·9 ± 0·23	4·9 ± 0·17	3·4 ± 0·12	1·9 ± 0·12
	1·5	8·5 ± 0·65	6·9 ± 0·31	5·0 ± 0·19	3·3 ± 0·13	1·9 ± 0·05
Amaranth	0·03	8·4 ± 0·44	6·8 ± 0·23	4·9 ± 0·20	3·3 ± 0·11	1·9 ± 0·13
	0·3	8·5 ± 0·33	6·5 ± 0·29	4·9 ± 0·19	3·5 ± 0·13	1·9 ± 0·10
	1·5	8·2 ± 0·50	6·5 ± 0·25*	4·8 ± 0·11*	3·0 ± 0·07*	1·7 ± 0·06

* Significant at P = 0·05 or less.

Two other differences in food efficiency for female rats were noted (see Table IV) but it is difficult to explain the significance of these results. In the food efficiency data for males there was only one result that showed a significant difference from the control levels. This was at 4 weeks for rats on 0·03 per cent amaranth.

Electrocardiograms and Electroencephalograms

The three standard leads of ECG were recorded. The tracings were essentially normal with the exception that extrasystoles were observed in two rats (one male rat on tartrazine and one female rat on sunset yellow, see Fig. 1 A). No significant deviation of the electrical axis was observed in any rat. It may be noted, however, that the auriculo-ventricular conduction was slower in all the rats, ranging from 55 to 60 msec., as compared to the reported figure of 24 to 55 msec.[6–10] This might be related to the age of the rats, since Hundley and others[7] reported that the P–R interval increased with age in their rats. The P, R and T waves had

voltages two to three times higher than the values reported in the references cited. This may be due to a more sensitive response obtained with the recording machine used. The tracing of an exemplary ECG is presented in Figure 1 B. The mean heart rates and their standard errors of the control rats were 373 $\pm$ 13 beats per minute for males and 383 $\pm$ 13 for females. The figures for the treated rats were similar to those of the controls.

A bipolar EEG (frontal-occipital) was recorded from these rats, with the left ear grounded. All the tracings appeared normal. A typical EEG is shown in Figure 1 C.

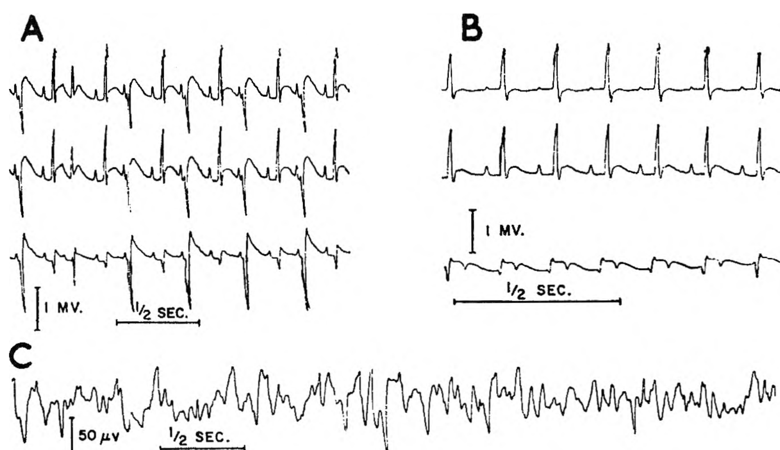

FIG. 1. A. The ECG of a female rat on 1·5 per cent sunset yellow, showing extrasystoles (septal).
B. The ECG of a control male rat. The heart rate was 375 beats per minute, the P-R interval, 55 msec., the QRS, 15 msec., the Q-Tc, 250 msec., the P_2, 0·23 mv., the R_2, 1·23 mv. and the T_2, 0·22 mv.
C. The EEG (frontal-occipital) of a control male rat.

Organ Weights

The mean weights (in mg./g. body weight) of some of the organs taken for histological examination are given in Table V. There were no significant weight changes in any of the organs of the male rats. There was a marked increase in liver weight for the female rats receiving 0·3 and 1·5 per cent amaranth. In the latter group this was accompanied by a significant increase in the weight of the kidneys. Such increases in organ weight may be the result of hyperactivity of the organs. There was a decrease in liver weight in two of the groups on sunset yellow and a decrease in the weight of the spleen for two groups on sunset yellow and one on amaranth. These changes were not correlated with the level of food colour in the diet and are difficult to interpret.

Pathology

A necropsy was performed on 39 rats that died during the test. Advanced autolysis precluded diagnosis in four cases. Respiratory tract

infections accounted for 28 deaths. Two animals died of starvation, one of meningitis, one of a ruptured right auricle and three as the result of neoplasms. The histopathological findings of the last three are included in Table VI.

Histopathology

Middle ear and respiratory infections have been found to be relatively numerous in older rats of the colony and in the groups under study these infections were observed in a large number of rats at autopsy (70 weeks of age). Only those changes which were considered excessive and capable of affecting the animal noticeably are listed in Table VI. There was no

TABLE V

ORGAN WEIGHTS OF RATS FED TARTRAZINE, SUNSET YELLOW AND AMARANTH

Treatment	Dosage (per cent of diet)	No. rats killed	Mean organ weight (mg./g. body weight ± S.E.)					
			Heart	Liver	Spleen	Kidneys	Gonads	Adrenals
Males								
Control ..		11	3·6 ± 0·25	27·4 ± 1·70	2·1 ± 0·08	6·9 ± 0·28	8·1 ± 0·27	0·10 ± 0·008
Tartrazine	0·03	14	3·6 ± 0·17	25·2 ± 0·56	2·1 ± 0·07	6·9 ± 0·17	8·1 ± 0·40	0·10 ± 0·007
	0·3	11	3·5 ± 0·15	24·6 ± 0·33	2·2 ± 0·06	7·0 ± 0·19	8·5 ± 0·31	0·10 ± 0·008
	1·5	8	3·6 ± 0·22	27·2 ± 1·72	2·1 ± 0·11	7·2 ± 0·27	8·5 ± 0·30	0·10 ± 0·019
Sunset yellow	0·03	14	3·4 ± 0·10	24·8 ± 0·23	2·1 ± 0·07	6·9 ± 0·16	8·3 ± 0·35	0·09 ± 0·005
	0·3	12	3·4 ± 0·10	25·3 ± 0·32	2·1 ± 0·04	7·2 ± 0·18	8·3 ± 0·28	0·09 ± 0·004
	1·5	9	3·5 ± 0·16	25·4 ± 0·81	2·2 ± 0·06	7·1 ± 0·14	8·8 ± 0·32	0·09 ± 0·005
Amaranth	0·03	10	3·2 ± 0·17	25·0 ± 0·71	2·0 ± 0·07	6·9 ± 0·25	8·0 ± 0·16	0·10 ± 0·01
	0·3	11	3·3 ± 0·11	28·5 ± 1·22	2·1 ± 0·07	6·7 ± 0·19	8·3 ± 0·35	0·08 ± 0·005
	1·5	9	3·4 ± 0·11	27·0 ± 1·31	2·1 ± 0·07	7·1 ± 0·23	8·4 ± 0·34	0·09 ± 0·005
Females								
Control ..		9	4·6 ± 0·25	30·0 ± 1·02	3·0 ± 0·12	8·4 ± 0·27	0·49 ± 0·05	0·23 ± 0·02
Tartrazine	0·03	13	4·5 ± 0·13	28·9 ± 0·68	2·8 ± 0·07	8·3 ± 0·23	0·42 ± 0·04	0·23 ± 0·01
	0·3	14	4·5 ± 0·12	28·3 ± 0·98	2·8 ± 0·08	8·3 ± 0·23	0·44 ± 0·02	0·25 ± 0·01
	1·5	10	5·0 ± 0·30	31·5 ± 2·44	2·8 ± 0·20	9·2 ± 0·52	0·50 ± 0·05	0·27 ± 0·02
Sunset yellow	0·03	15	4·3 ± 0·11	26·7 ± 0·38*	2·6 ± 0·05*	7·9 ± 0·28	0·40 ± 0·02	0·22 ± 0·01
	0·3	12	4·3 ± 0·18	25·8 ± 0·71*	2·7 ± 0·16	7·9 ± 0·20	0·43 ± 0·02	0·28 ± 0·03
	1·5	9	4·4 ± 0·14	27·5 ± 1·36	2·5 ± 0·08*	8·6 ± 0·31	0·42 ± 0·03	0·21 ± 0·01
Amaranth	0·03	13	4·1 ± 0·11	28·2 ± 1·63	2·6 ± 0·13*	8·0 ± 0·24	0·43 ± 0·03	0·24 ± 0·01
	0·3	13	4·6 ± 0·16	35·1 ± 0·79*	3·1 ± 0·08	8·8 ± 0·28	0·46 ± 0·04	0·23 ± 0·01
	1·5	9	4·9 ± 0·17	34·7 ± 0·84*	3·3 ± 0·24	9·8 ± 0·52*	0·50 ± 0·02	0·26 ± 0·02

* Significant at P = 0·05 or less.

difference in incidence and severity of respiratory lesions between the control and test groups. Chronic otitis media was observed in nearly 50 per cent of the animals. The disease was evenly distributed in the various groups.

Twenty-two animals exhibited pathological change in the adrenal cortex. This change was characterised by a small focal area of haemorrhage with rarefaction of the cytoplasm of parenchymal cells and some necrosis in and surrounding the area. This particular pathology was acute in nature and was not considered to be an effect of the food colours since it was observed in two of the control animals and since there was no correlation between the incidence and the concentration of colour fed.

Changes observed in the kidneys were difficult to assess as they occur normally in aged rats in this colony. In an attempt to permit an assessment of observed change and to correlate this with the possible effects of the food colours, an index of nephrosis and glomerulonephritis was established. Nephrosis was characterised by moderate flattening of proximal convoluted tubular epithelium, tubular dilatation and hyaline

TABLE VI

SUMMARY OF HISTOPATHOLOGICAL FINDINGS IN RATS FED FOOD COLOURS FOR 64 WEEKS

Treatment	Control		Sunset yellow						Tartrazine						Amaranth						
Dosage (per cent of diet) ..			0·03		0·3		1·5		0·03		0·3		1·5		0·03		0·3		1·5		
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	Totals
Number of rats on test ..	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	300
Number of survivors ..	11	10	14	15	12	13	9	9	14	13	12	14	9	10	10	14	11	13	9	9	231
Number examined	11	10	8	15	12	13	9	9	14	13	12	14	9	10	10	14	11	13	9	9	225
Glomerulonephritis.. ..	4	—	2	—	6	—	5	—	5	3	4	—	3	—	3	—	7	—	5	2	49
Nephrosis	11	5	3	—	9	1	7	1	8	3	10	3	5	1	8	1	10	2	8	2	98
Hydronephrosis	—	2	—	2	—	—	—	3	—	3	—	4	—	4	—	3	—	3	—	4	28
Cystic kidney	—	—	—	—	1	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	3
Hydroureter	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	1
Hydrouterus..	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	1	—	—	5
Pyometritis	—	—	—	—	—	3	—	—	—	—	—	1	—	—	—	—	—	—	—	—	4
Ovarian cyst..	—	1	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	2	5
Testicular atrophy	1	—	—	—	1	—	—	—	1	—	2	—	—	—	—	—	—	—	—	—	5
Myocarditis	—	—	—	—	—	—	—	1	1	—	1	—	—	—	—	—	—	—	1	—	4
Periaortitis	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	2	—	1	—	4
Pneumonic abscess	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
Pneumonia advanced ..	1	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	2
Tracheobronchitis	—	—	—	—	—	—	—	—	—	1	—	—	1	1	—	—	—	1	—	—	4
Cystic pancreatitis	—	—	—	—	—	—	—	—	—	—	1	—	—	1	—	—	1	1	—	—	4
Adrenal hemorrhage ..	—	2	—	1	1	4	1	—	—	3	2	4	—	—	—	2	—	—	—	2	22
Liver abscess	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	3
Tumours:																					
Uterus leiomyoma	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	1	—	—	2
Uterus polyp	—	2	—	—	—	2	—	2	—	—	—	4	—	2	—	—	—	3	—	2	17
Uterus leiomyosarcoma ..	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Rectum neurofibroma ..	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	1
Testis leydig tumour ..	—	—	1	—	—	—	1	—	—	—	1	—	—	—	—	—	—	—	—	—	3
Thyroid adenoma	—	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
Kidney tubular adenoma ..	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Skin papilloma	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Mammary fibroadenoma ..	—	—	—	—	—	—	—	—	—	3	—	—	—	—	—	1	—	—	—	—	4
Urinary bladder papilloma	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	1
Pancreas neurogenic sarcoma	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	1
Stomach adenocarcinoma ..	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	1
Liver hamartoma .. .. (congenital anomaly)	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1

cast collection. Glomerulonephritis was characterised by interstitial nephritis with collections of lymphocytes and monocytes and by glomerular change starting with a slight thickening of capsular epithelium. Advanced changes were typical of chronic glomerulonephritis.

In establishing the index, the kidney changes were graded according to the degree or extent of nephrosis or glomerulonephritis. The data so obtained were examined by means of a rank sum test[11]. In none of the test groups was there any significant deviation from the controls.

The tumour incidence in the control group was 14 per cent and in the food colour groups it was 16 per cent. Amaranth had a tumour incidence of 11, tartrazine 19, and sunset yellow 18 per cent. Uterine polyps occur frequently in this colony of rats and accounted for approximately 50 per

cent of the neoplasms in this study. Excluding polyps, the tumour incidence was 5 per cent in 21 control animals and 9 per cent in the 204 test animals. For the individual colours the incidence was: sunset yellow 12, tartrazine 11, and amaranth 3 per cent. The differences in tumour incidence are not significant according to chi-square tests.

TABLE VII

HAEMATOLOGICAL FINDINGS IN RATS FED FOOD COLOURS FOR 64 WEEKS

Treatment	Dosage (per cent of diet)	Haemoglobin (g. per cent)	Red blood cells ($X 10^6$)	White blood cells
Males				
Control ..		18·0*	9·2**	12,490***
Tartrazine	0·03	17·7	9·8	11,770
	0·3	18·5	9·8	12,350
	1·5	18·0	9·4	12,100
Sunset yellow	0·03	18·0	9·3	13,600
	0·3	17·4	8·9	10,760
	1·5	17·6	9·0**	12,550***
Amaranth	0·03	18·3	9·7	12,900
	0·3	18·2	8·7	11,430
	1·5	18·1	9·9	12,540***
Females				
Control ..		17·7*	8·6**	11,920***
Tartrazine	0·03	17·6	8·5	11,440
	0·3	17·3	8·0	11,900
	1·5	18·0	9·0	10,950
Sunset yellow	0·03	17·7	9·3	11,120
	0·3	17·8*	8·9**	11,150
	1·5	17·5	8·8	†8870***
Amaranth	0·03	17·7	8·0	11,810
	0·3	17·5	8·5	11,990
	1·5	17·6	9·1	11,020***

* Readings on 9 rats. ** Readings on 8 rats. *** Two readings on each of 8 rats. † $P<0·01$.

Haematology

One week before termination of the experiment red and white blood cell counts and haemoglobin determinations were made on three animals in each group. In some groups in which the readings were suggestive of an altered blood picture extra readings were taken to ensure statistical comparison at a suitable level of confidence. The results are shown in Table VII. The only significant finding was a lowered white cell count for females on 1·5 per cent sunset yellow as compared to the controls.

TABLE VIII

DIFFERENTIAL CELL COUNTS IN THE BLOOD AND BONE MARROW OF SOME FEMALE RATS

(Each figure is the average reading for 8 rats)

	Blood per cent			Bone marrow per cent	
	Neutrophiles	Lymphocytes	Others	Myeloid	Erythroid
Control ..	33·1	64·8	2·1	68·0	32·0
Sunset yellow 1·5 per cent ..	29·5	68·0	2·5	70·0	30·0

Differential counts of the cells in the blood and bone marrow were made on female rats of the control and 1·5 per cent sunset yellow groups because of the lower white cell counts of the latter group. The results, as given in Table VIII, showed no significant difference between the two groups. The haematological findings suggest that these food colours did not adversely affect the blood cells of the animals.

Acknowledgements. Thanks are due to Miss Constance Cox for much help with statistical assessments and to Mrs. Elaine O'Grady and Miss C. Beliveau for technical assistance.

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THE DETECTION OF YELLOW PHOSPHORUS AND PHOSPHIDES IN BIOLOGICAL MATERIAL

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The micro test to detect poisoning by yellow phosphorus or zinc phosphide has been modified and improved so that increased specificity is obtained. This is achieved by paper electrophoresis. Chlorine is used in the place of solid calcium hypochlorite to oxidise silver phosphide to silver phosphate.

Yellow phosphorus is readily obtainable without restriction in the form of rat poison in Great Britain. The many cases of murder and suicide that have occurred even within the last five years in which death has resulted from the ingestion of this element show that it is still of great toxicological importance.

Death from phosphorus poisoning usually occurs in one of two ways—as a result of shock within 24–36 hours after ingestion, or as a result of liver failure several days later. In this latter type of case it is rare for a positive Mitscherlich phosphorescence to be obtained when the intestine contents are distilled and proof of the ingestion of yellow phosphorus was, until three years ago, rarely possible. In 1955, however, the introduction of a micro test[1] showed that in such cases yellow phosphorus could be detected in as little as 20 g. samples of liver.

The basis of the test is the micro distillation of the liver and passage of the resultant steam and phosphorus through a filter paper soaked in silver nitrate. Any silver phosphide shows itself as a black stain which can be oxidised to silver phosphate with bleaching powder and hence to a blue reduced phosphomolybdate.

There are three other compounds which could conceivably also react in this test. The first is phosphine which may be present either as a product of putrefaction or following ingestion of the rat poison zinc phosphide, zinc phosphide itself, and arsine. It is possible to differentiate phosphine and arsine from zinc phosphide in that the gases are liberated from neutral solution, even at room temperature. If zinc phosphide is present phosphine is liberated at room temperature only after acidification of the sample under test. Yellow phosphorus is unique in that it distils readily only after the sample has been heated.

It is the purpose of this paper to describe some modifications of the test which have resulted in an increased specificity and which enable a permanent record of the result of the test to be obtained.

There are four stages in the analysis which is now recommended. These are:—

(1) Passage of a stream of carbon dioxide through the liver sample and formation of insoluble silver phosphide in a Gutzeit type paper holder.

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Carbon dioxide is preferable theoretically to the stream of air recommended in the original paper because of the ready oxidation of phosphorus.

Carbon dioxide is, for the reasons described above, passed in three stages: (*a*) through the cold, neutral sample, (*b*) through the cold, acidified sample, (*c*) through the acidified sample heated to 100°.

(2) Oxidation of the silver phosphide to silver phosphate by the use of chlorine gas and removal of excess chlorine by aeration.

(3) Examination of the silver phosphate by electrophoresis: in this way phosphate is separated from any arsenate which might have interfered with the interpretation of the result and from the silver ion in the presence of which Hanes and Isherwood's spray for phosphates[2] cannot be used.

(4) The development, after electrophoresis of the paper strip with Hanes and Isherwood's reagent. This gives a record which can be photographed.

Experimental

The sample of liver, kidney or intestine contents suspended in water is put into a flask or tube fitted with the head illustrated in Figure 1. The space in the head is packed with glass wool impregnated with basic lead acetate. A strip of Whatman No. 1 filter paper (4 in. × 15 in.) is clamped between the flat faces of the perspex end. The small area of filter paper, approximately 0·4 cm. diameter, through which the distilling gases will pass is wetted by means of a capillary tube with a saturated solution of silver nitrate in methanol. The position of this area is chosen so that it lies between exactly similar spots prepared from phosphine and arsine controls, at right angles to the length of the paper, in a position suitable for subsequent examination in a paper electrophoresis apparatus. Carbon dioxide is passed into the apparatus at a slow rate (2–3 bubbles a second through a bubble counter) for 1½ hours. If no stain appears the sample is acidified and the passage of carbon dioxide continued. If still no stain appears the flask or tube is heated to 100° when any yellow phosphorus will distil. We have found it most convenient to use electric heating jackets fitting the tube or flask in use. Small tubes are used for 20 g. samples, flasks for larger quantities.

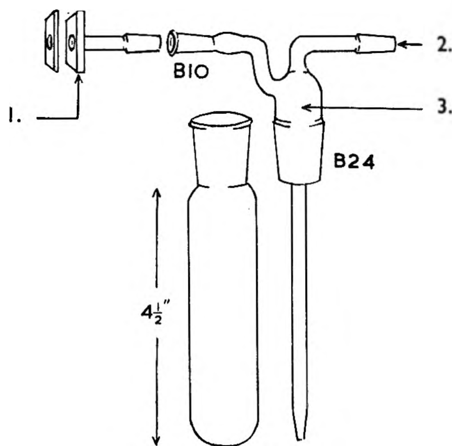

Fig. 1. Diagram of apparatus.

1. Perspex holder.
2. Carbon dioxide input.
3. Glass wool/basic lead acetate.

When distillation is complete the end holder is removed and placed on the B 10 socket fitted to a chlorine generator. The silver phosphide or

arsenide spots are rapidly decolourised and excess chlorine is removed by blowing the paper with a stream of cold air from an electric hair-drier for 10 minutes. The control spots are similarly treated. We have found this method of oxidation to be vastly superior to that described in the original method.

The strip of paper is then placed in a horizontal paper electrophoresis apparatus using 5 per cent aqueous acetic acid as the electrolyte. The paper is very carefully sprayed with the electrolyte, the area about the spots being sprayed last. The current (about 2 mA at 250 v.) is passed for approximately 3 hours giving a movement of 5½ in. for arsenate and 6½ in. for phosphate.

After completion the paper is dried at 100° and sprayed with the phosphate reagent. This is prepared by mixing 62·5 ml. of 4 per cent w/v ammonium molybdate with 25 ml. N hydrochloric acid, 12·5 ml. 60 per cent perchloric acid and then making up to 250 ml.

After heating at 85° for 7 minutes the paper is exposed to steam for a few seconds and immersed in a jar of hydrogen sulphide. The blue spots on a brown background appear immediately. We have found that the sensitivity of this reagent is approximately the same as that of molybdate-benzidene[3].

Comparison of the intensity of the silver phosphide or arsenide stains with the final blue colour is also possible.

We attempted in many experiments to separate phosphate and arsenate spots by paper chromatography but found that in the acid solvents which it was necessary to use because of the presence of the silver ion that a separation could not be achieved. These experiments, coupled with those using paper electrophoresis, showed that oxidation to phosphate and arsenate using chlorine was proceeding rapidly. Further experiments[4] showed that arc spectrographic analysis of the silver spots could also reveal the presence of arsenic provided more than one microgram of arsenic was present on the spot.

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TETRAHYDROAMINACRIN AS A DECURARISING AGENT

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Received June 30, 1958

Tetrahydroaminacrin has marked anticholinesterase activity. It is a mild antagonist of tubocurarine, and this may be shown on the rat phrenic nerve diaphragm preparation. This antagonism is more marked in rats and dogs. Other pharmacological properties are described. THA has been used successfully to decurarise patients who have received tubocurarine or gallamine. Usually the administration of atropine is not necessary and recurarisation does not occur.

Tetrahydroaminacrin (THA) was synthesised by Albert and others[1] and shown by Rubbo and others[2] to have no bacteriostatic properties. Later Shaw and Bentley[3] showed that the compound was a moderate antagonist of morphine but from experiments in the dog appeared to be too convulsant for use in man. These authors[4] also showed that smooth muscle was slightly contracted by THA and that it produced spontaneous contractions in the uterus of the guinea pig. On the intestinal musculature of rat or guinea pig, THA induced a 10 fold potentiation of the action of acetylcholine. Paradoxically, the drug, which itself has little action on the frog heart, abolishes the action of acetylcholine at that site. Thus, on one tissue, the intestine, it displays eserine-like activity and on another, the heart, it has atropine-like properties. Shaw and Bentley[4] also showed that THA was almost as powerful an anticholinesterase as eserine or neostigmine; it produced a 50 per cent inhibition of the hydrolysis of acetylcholine with a concentration of 10^{-7}M. This latter fact suggested that THA might act as a decurarising agent. With this purpose in mind a more complete investigation was undertaken.

Methods

Toxicity. The general effect and LD50 were observed on rats, mice, dogs and rabbits. The drugs were injected intramuscularly.

Circulatory system. The effect on blood pressure and heart rate was observed on cats anaesthetised with pentobarbitone.

Antihistaminic activity. This was observed on the guinea pig intestine, which was bathed in Tyrode's solution.

Anticurare action. (*a*) Rat phrenic nerve diaphragm preparation (Bulbring)[5], (*b*) intact dog and cat. For details see later.

Results

Toxicity

When THA is injected into rats and mice there is little effect other than some salivation until lethal doses are reached. At this stage the animal exhibits tremors, passes into clonic convulsions, which are not asphyxial and dies within a few minutes. The LD50 for mice is 33 mg./kg. with limits of 31 and 35 mg./kg.

Circulatory System

Doses up to 10 mg./kg. have little effect on the heart rate or blood pressure, a rise in pressure of up to 10 mm. Hg being recorded in some cases and a fall of similar magnitude in others. Robinson and McCaul (unpublished) found that in man the ECG was unaffected by THA, but in some cases it showed mild bradycardia presumably of vagal origin.

Antihistaminic Activity

THA has slight antihistaminic activity. A concentration of 10^{-3}M will prevent the action of histamine on the guinea pig ileum. The effect is, however, abolished in a few minutes when the drug is washed out.

Anticurare Action

(*a*) *Rat phrenic nerve-diaphragm preparation.* Figure 1 shows the moderate anticurare effect of THA at a dilution of 10^{-7}M. When the THA is added to the bath before the curare, the effects are variable. If the THA concentration is too high the preparation is damaged. On two occasions, however, the curare has had no effect in its presence at 10^{-7} and the contractions remained constant.

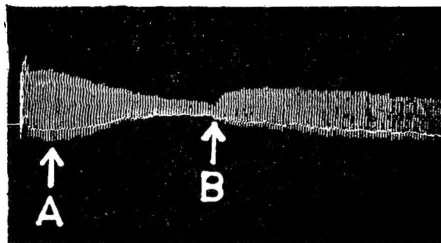

FIG. 1. The anticurare effect of THA. A. Tubocurarine 2×10^{-6}M. B. THA 10^{-7}M. Between A and B, 11 minutes. The preparation was stimulated once every ten seconds.

(*b*) *Intact animal.* (i) *Rat.* THA was given i.m. to 28 rats in doses from 5 to 40 mg./kg. Salivation occurred in 86 per cent of the animals. As the dose was increased the incidence of muscular tremors increased and a dose of 40 mg./kg. represented an LD100. Tubocurarine was given to 14 animals. It was found that 0·5 mg./kg. always produced death by respiratory paralysis.

Both drugs were given simultaneously to 17 animals to observe the antagonistic effect of THA against tubocurarine. The latter was given at a dose which always produced paralysis, viz., 0·5 mg./kg. It will be seen from Table I that about half the animals survived.

(ii) *Dog.* The dose of tubocurarine to produce muscular paralysis regularly was found to be 0·15 mg./kg. The LD100 was 0·25 mg./kg. THA when given alone to dogs in doses up to 5 mg./kg. i.m. produced no marked alterations in the animals behaviour. When given intravenously, however, doses of 2·0 mg./kg. cause respiratory stimulation together with marked panting but minimal or an absence of tremors or convulsions. This dose range also produced a bowel action in about half the animals to which it was given. Salivation did not occur in any of these animals when either compound was given alone.

THA and tubocurarine. Both compounds were given to 23 dogs of both sexes to observe the antagonising effect of THA in a dose range from 1·5 to 4·5 mg./kg. i.v. Hyoscine was given (0·5 mg./kg. i.m.) in some cases

to prevent salivation which was found to occur when THA was given after tubocurarine. Throughout these trials, when THA was given alone to the animal, no salivation resulted but when given to a curarised animal profuse salivation always occurred.

Tubocurarine followed by THA. Two dogs were given 0·15 mg./kg. tubocurarine i.v. This dose produced muscular paralysis. THA, 2·5 mg./kg., was then given intravenously when the signs of paralysis first became evident. This produced a dramatic return to normal level of muscle tone and restoration of respiration. A similar favourable result

TABLE I

SIMULTANEOUS ADMINISTRATION (I.M.) OF THA WITH 0·5 MG./KG. TUBOCURARINE

No. of rats	Dose THA mg./kg.	Results (No. died)
5	5	2
6	10*	3
1	20	1
2	25	1
1	35	—
2	20*	2

* Doses marked thus were administered against 1 mg./kg. dosages of tubocurarine.

was obtained in another dog which was given 0·2 mg./kg. of tubocurarine and 4·5 mg./kg. of THA. In this instance respiratory failure supervened before THA was given. Six animals were given 0·25 mg./kg. tubocurarine (lethal dose). When muscle hypotonia and failure of respiration was established 2·5 mg./kg. THA was given i.v. Respiration was immediately restored to normal. There was also a general improvement in muscle tone although the animals were still unco-ordinated in their movements for a few minutes.

Tubocurarine together with THA. Three dogs were given tubocurarine (0·15 mg./kg.) and THA (2·5 mg./kg.) mixed in the same syringe (i.v.). These animals showed neither loss of muscle tone nor depression of respiration. Three dogs were given a lethal dose of tubocurarine (0·25 mg./kg.) together with THA (2·5 mg./kg. i.v.). A slight degree of muscular paralysis was produced and the respirations were entirely diaphragmatic, but the animals did not die.

DISCUSSION

Tetrahydroaminacrin is a member of a series of compounds shown by Shaw and others[3] to reverse the narcotic activity of morphine, particularly in dogs. When further investigation of its pharmacodynamics was undertaken it was found to be a particularly effective anticholinesterase. It was now natural to investigate its anticurariform activity.

Its anticurare action on the rat phrenic nerve-diaphragm preparation was moderate. The action was variable, especially if the THA were added before the curare when it did prevent the action of curare on two occasions. However, it prevented curarisation in rats and dogs when the mixed drugs were administered simultaneously. Dogs with muscular and respiratory paralysis brought about by an injection of tubocurarine

were restored to almost normalcy by the intravenous administration of THA, provided respiratory paralysis had not proceeded too far.

In animals THA produces little salivation. Paradoxically salivation is sometimes seen in the curarised animal. This is not noted with therapeutic doses of THA and curare in man.

In animals death due to THA is accompanied by convulsions. Despite considerable use in clinical anaesthesia Robinson and McCaul (unpublished) have not noted convulsions in man.

Apart from its action on the central nervous system THA has little effect on other systems. The antihistaminic activity is weak. It has little effect on the heart or blood pressure.

Its decurarising action has been confirmed clinically by Robinson and McCaul (unpublished) who found that THA affected decurarisation of 200 patients who had received tubocurarine or gallamine. Unless large doses of THA were employed the patients were not atropinised. Recurarisation did not occur.

THA is also a mild non-specific respiratory stimulant. In many cases of respiratory failure occurring during operations on animals, breathing has been restarted by an intravenous injection of 5 to 10 mg./kg. This stimulant action has been confirmed clinically by Robinson and McCaul and by ourselves, the full details of which will be published later.

Acknowledgement. We would like to thank Monsanto (Aust.) Pty. Ltd. for their very generous supply of tetrahydroaminacrin.

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MICROCHEMICAL DIFFERENTIATION BETWEEN OPTICAL ISOMERS OF *N*-METHYLMORPHINAN ANALGESICS

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A simple method for differentiating between microgram quantities of optical isomers is described.

THE analgesic drug 3-hydroxy-*N*-methylmorphinan was brought into use in its racemic form in 1951 under the name of Dromoran. Subsequently, when it had been shown that the analgesic activity was associated with the *laevo* isomer only[1–3] manufacture of the racemic form was discontinued, and the *laevo* form was supplied under the same trade name. The three isomers were given the approved names of levorphanol, racemorphan, and dextrorphan, the three corresponding methyl ethers (3-methoxy-*N*-methylmorphinan) being designated levomethorphan, racemethorphan, and dextromethorphan. As these drugs were habit forming, they were placed under international control, and, in Great Britain, became subject to the provisions of the Dangerous Drugs Regulations. Both (+)-isomers were removed from control in 1954, when it was shown that they did not produce addiction. Since then, dextromethorphan has come into clinical use as an antitussive agent under the name of Romilar.

We thus have two drugs, of which the *laevo* and racemic forms are proscribed narcotic drugs, while the corresponding *dextro* isomers are free of control. As there are considerable penalties attached to the misuse of substances on the list of Dangerous Drugs, it is essential to have some method that will distinguish clearly between these isomers. The obvious method of measuring the optical rotations can of course be applied if sufficient material is available, but even modern high precision methods[4] cannot operate on quantities much below 1 mg.

Numerous methods have been described for the identification of these compounds, including X-ray diffraction patterns[5,6], ultra-violet spectra[7], infra-red spectra[8], paper chromatography[9,10], and crystal and colour tests[11–16]. Although several of these methods serve to distinguish the racemic compound from the optically active forms, none of them is capable of differentiating these (+) and (−) forms from one another. It is the purpose of this note to describe an extremely simple method for distinguishing between μg. quantities of these isomers.

EXPERIMENTAL PROCEDURE

The hanging microdrop technique developed by Clarke and Williams[17] is used. When a microdrop of a 5 per cent solution of sodium carbonate is added to a similar drop of a solution of 1 part of racemorphan in 100–500 parts of 1 per cent hydrochloric acid, crystals in the form of bunches of small plates appear, usually within half an hour. Under similar conditions, the (+) and (−) isomers give only amorphous precipitates, which do not crystallise even after standing for 48 hours.

To distinguish between the (+) and (−) forms the following procedure is adopted: place a microdrop of the test solution on a cover slip; add a microdrop of a 1 per cent solution of, say, the (−) isomer, made up from a known sample; then add a microdrop of a 5 per cent sodium carbonate solution. Seal, invert, and examine under the microscope in the usual way. If the test solution is the (−) isomer, the addition of further (−) isomer will not affect it, and the precipitate formed will be amorphous. If, however, it is the (+) isomer, the solution will now contain both the (+) and (−) forms, and crystals typical of the racemic compound will be formed.

The same method is used to distinguish between dextromethorphan and levomethorphan, except that in this case the reagent employed is a saturated solution of trinitrobenzoic acid. With this reagent racemethorphan forms rosettes of crystals, feathery or dense in appearance according to the concentration. These usually form within a quarter of an hour. Dextromethorphan and levomethorphan give oily amorphous precipitates which do not crystallise.

As the volume of a microdrop is 0·1 μl., the sensitivity of this test is about 0·2 μg.

Discussion

Although the racemic form of an alkaloid will often give crystals that are different in form from those of the optical isomers, a fact that has long been used to distinguish atropine from hyoscyamine, the alkaloidal reagents normally used yield identical crystals with both (+) and (−) isomers. An alkaloidal precipitating agent which was itself optically active might be expected to give rise to different forms of crystal with each isomer, but up to now no such reagent has been described. Substances such as tartaric acid which are used to resolve racemic bases cannot be made to give satisfactory crystals on the microscale.

The method described above is of general application, and can be used for differentiating between the optical isomers of any alkaloid, provided that there can be found a precipitating agent that will give crystals with the racemic form and not with the (+) and (−) isomers, and that a pure sample of one of these isomers is available for cross-testing. In the case of the N-methylmorphinan analgesics there is no difficulty on the latter score, as both levorphanol and dextromethorphan are obtainable commercially.

Acknowledgements. I wish to express my thanks to Dr. J. Marks, and to the officials of the Dangerous Drugs branch of the Home Office, for the help they have given to me. I acknowledge most gratefully gifts of drugs from Roche Products, Ltd. I am also much indebted to Mrs. A. Williams for technical assistance.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Adrenaline and Noradrenaline, Effect of Light on Fluorescence of Ethylenediamine Derivatives of. A. Goldfien and R. Karler. (*Science*, 1958, **127**, 1292.) After incubating a mixture of adrenaline and noradrenaline with ethylenediamine the fluorescence of the two compounds differs so that at 500 mμ the ratio of fluorescence of adrenaline to noradrenaline is 0·98 and at 550 mμ it is 4·4. This report shows that entirely different ratios can be obtained when the procedure is carried out in a room illuminated by light of different wavelengths. About 80 per cent of the fluorescence due to noradrenaline was lost if the procedure was carried out in daylight. Duplicate solutions of adrenaline and noradrenaline were prepared and samples were read at intervals of 5 mμ on the spectrophotofluorimeter, one procedure being carried out in a room illuminated by a 25-watt ruby lamp. Daylight, in addition to causing a loss of fluorescence of noradrenaline, also caused a small shift in the fluorescence maxima. Little effect was noted on the fluorescence of adrenaline. W. C. B.

Analgesics of the Morphine Group, Identification of. H. Baggesgaard Rasmussen, J. Berger, K. Folting and G. Espersen. (*Dansk Tidsskr. Farm.*, 1958, **32**, 81.) The picrates of morphine, codeine, ethylmorphine, nalorphine, dihydromorphinone, dihydrocodeinone and dihydrohydroxycodeinone were prepared, but they proved to be unsuitable for identification purposes as their melting ranges extended over an interval of 10–20°. A further difficulty was that some of the picrates contained water of crystallisation which could not be removed by drying over silica gel. Acetyl derivatives were found to be satisfactory for identification purposes. They were prepared by refluxing the morphine derivatives with acetic anhydride and sodium acetate, and isolated by pouring the reaction mixture into water and neutralising with sodium carbonate. The derivatives were purified by recrystallisation from ethanol, and gave reproducible melting points which are tabulated below. The bases may be recovered by precipitation with ammonium hydroxide solution, and, except in the case of morphine, the melting point provides an additional check on the identity of the compound.

Base	Melting point °C.	Melting point of acetyl derivative °C.
Morphine	—	173
Codeine	155–158·5	133·5
Ethylmorphine	87–90	130–131
Nalorphine	208–209	137·5–138·5
Dihydromorphinone	263–268	162·5–163
Dihydrocodeinone	197–200	154–155
Dihydrohydroxycodeinone	220–225	(1) 208 (2) 214

(1) = Diacetyl derivative. (2) = Monoacetyl derivative.

G. B.

Sulphonamides, Identification of, by Fluorescent Microscopy. I. Tschudi-Steiner. (*Pharm. Acta Helvet.*, 1958, **33**, 105.) This method depends upon the crystalline form and fluorescence when observed under a fluorescence microscope of Schiff's bases formed *in situ* on the microscope slide from sulphonamides with free primary aromatic amino groups and various aromatic aldehydes. The sulphonamides chosen were sulphadiazine, sulphamerazine, sulphadimidine and sulphasomidine and the aldehydes were *p*-dimethylaminobenzaldehyde, *p*-hydroxybenzaldehyde, salicylaldehyde, anisaldehyde, cinnamaldehyde and piperonal. These aldehydes gave fluorescent Schiff's bases with at least one of the four sulphonamides. The crystalline forms were also characteristic.
D. B. C.

Vitamin A, a New Spectrophotometric Method for the Assay of. I. M. Jakovljevic, (*Pharm. Weekbl.*, 1958, **93**, 585.) This depends upon the formation of a red colour with phosphotungstic acid in a chloroformic solution of the vitamin in the presence of a certain amount of acetic anhydride. 50 to 300 I.U. gives a suitable colour intensity. Cod-live oils and vitamin A concentrates are saponified with N alcoholic caustic potash solution, extracted with benzene, and an aliquot part of the dried benzene solution is evaporated in a stream of carbon dioxide below 40° and the residue dissolved in chloroform before the reagents are added. The colour formed is blue at first but changes to red and the mixture is allowed to remain in darkness for one hour for the colour to become stable, absorption maximum 538–540 mμ. The effect of varying the concentration of the reagents and the time and temperature of the reaction is examined.
D. B. C.

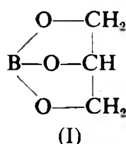
ESSENTIAL OILS

Mint Oils, Some New Constituents of. R. H. Reitsema. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 265.) Dihydrocarvone was shown to be present in some oils of the spearmint type. Its presence in oil from *Mentha niliaca* was demonstrated by reaction with 2:4-dinitrophenylhydrazine in ethanol, to form the dinitrophenylhydrazone. Similarly, dihydrocarvone was detected in oil from *M. crispa*, after removal of carvone. Jasmone was shown to be present in this oil after removal of carvone and alcohols. The oil from *M. aquatica* was found to contain about 40 per cent of menthofuran, determined by infra-red absorption at 733 cm.$^{-1}$, and pure menthofuran was isolated from the oil in a yield of 34 per cent. The oil of *M. sylvestris*, which had previously been reported to contain piperitone oxide, was found to contain the related compound diosphenol, a substance which is present in buchu leaves. By means of fractional distillation and chromatography it was shown that the oil also contains diosphenolene, piperitone, piperitenone, limonene and cineole. G. B.

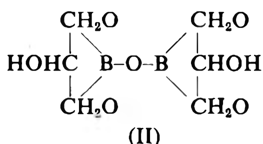
ORGANIC CHEMISTRY

Glyceryl Borate, Constitution of. W. Gerrard and E. F. Mooney. (*Chem. Ind.*, 1958, 227.) The authors have studied the formula for glyceryl borate put forward by Ahmad and Khundkar (I) and failed to prepare a structure corresponding to this formula, which shows a remarkable distortion of valency angles and which could not be constructed satisfactorily with Fischer-Herschelder models. It was considered that the difficulty would be overcome by the formation of a B–O–B structure. The product from boron acetate and glycerol was

a white amorphous powder which, contrary to Ahmad and Khundkar, could not be crystallised from acetone and was not soluble in ethyl acetate. The boron/glycerol ratio was closely 1 : 1 (found, B, 9·8 per cent) and the molecular weight determined ebullioscopically was 216. The infra-red spectra showed the presence of OH groups and of the B–O–B structure present in the tetra-acetyl diborate. With thionyl chloride, behaviour indicated the presence of a hydroxyl group, and the weight of water evolved after the formation of the polymer of high molecular weight corresponded to one hydroxyl group for each atom of boron. The structure II is suggested for glyceryl borate.



(I)



(II)

J. R. F.

Morphine, "Bound", Studies on the Structure of. J. M. Fujimoto and E. L. Way. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 273.) "Bound" morphine obtained from the urine of human addicts was shown, as follows, to be identical with a morphine conjugate from dog urine. After hydrolysis, a dinitrophenol derivative was prepared which gave an X-ray diffraction pattern identical with that of morphine dinitrophenyl ether. The identity of the glucuronic acid moiety was confirmed by examination of the infra-red spectrum and by the colour reaction with naphthoresorcinol and carbazole. Quantitative analysis indicated that the conjugates from human and dog urine were morphine glucuronates of identical composition. From an examination of the infra-red absorption spectrum and the pK values calculated from the two points of inflection of the titration curve it is postulated that at pH 5·8 the substance exists almost entirely in the form of a zwitterion. G. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Glycyrrhetinic Acid Inhibition of Metabolism of Steroids *In Vitro*. L. M. Atherden. (*Biochem. J.*, 1958, **69**, 75.) This paper may explain the effectiveness of glycyrrhetinic acid in dermatological conditions. It has been shown that glycyrrhetinic acid is *in vitro* a powerful inhibitor of the metabolism of both 11-deoxycorticosterone and progesterone by rat-liver homogenates, both in the absence and in the presence of added reduced diphosphopyridine nucleotide. With rat liver particle free supernatant the metabolism of progesterone was completely inhibited by 0·0001M glycyrrhetinic acid in the presence of triphosphopyridine nucleotide and in the absence of *iso*citrate. Evidence that the active moiety of glycyrrhetinic acid is the $\alpha\beta$-unsaturated ketone group was provided by the observation that metabolism of progesterone is unaffected by 0·005M 11-deoxyglycyrrhetinic acid, methyl vinyl ketone or *p*-benzoquinone. The 11-oxo group is required for the inhibition. This may explain why Groen and others found glycyrrhetinic acid to have a 11-deoxycorticosterone sparing effect in Addison's disease and that analogues of glycyrrhetinic acid without the 11-oxo group were ineffective. G. F. S.

Penicillin, Chloramphenicol and Tetracycline, Penetration of Human Red Cells by. K. C. Watson. (*J. Lab. clin. Med.*, 1958, **51**, 778.) Solutions of the antibiotics were added to packed human red cells and the mixtures were then incubated. After incubation the tubes were centrifuged and the supernatant was removed. The cells were then washed with sterile saline before being haemolised with distilled water and repeated freeze-thawing. Finally the disrupted cell suspensions were assayed for antibiotic content. Penicillin appeared to be able to penetrate the cell wall to approximately 10 per cent of the extracellular concentration. Similar results were obtained whether the penicillin solution was made in saline or in plasma showing that penetration is independent of any plasma factor. Tetracycline appeared to pass into the red cell to a greater extent than penicillin but chloramphenicol was found to be unable to pass the cell wall barrier in active form in the concentrations used. In a further series of experiments with penicillin the haemolised blood, obtained by the abovementioned procedure, was centrifuged and the supernatant haemoglobin solution removed. Assays carried out on both the haemoglobin solution and the deposited cell membranes showed that the antibiotic was contained only in the haemoglobin solution. W. C. B.

BIOCHEMICAL ANALYSIS

Antibiotics, A Modified Method for Evaluation of Clinical Usefulness of. I. Hoette and A. P. Struyk. (*J. Lab. clin. Med.*, 1958, **51**, 638.) A modified paper disc agar diffusion method is described for determining the sensitivity, to various antibiotics, of micro-organisms associated with infections. The method is designed so that the concentration of antibiotic at a zone diameter of 10 mm. is equivalent to that which can easily be obtained clinically as blood or urine level by the usual antibiotic therapy. Zone sizes greater than 10 mm. therefore indicate the suitability of the antibiotic for clinical treatment of the patient. The preparation of a special disc for differentiating between penicillinase and non-penicillinase staphylococci is described. Comparative experiments indicated that a good correlation exists between results obtained with the modified paper disc method and those obtained with the dilution method.

W. C. B.

Urinary Catechol Amines, Determination of. R. B. Johnson. (*J. Lab. clin. Med.*, 1958, **51**, 956.) A method is described for the estimation of urinary adrenaline and noradrenaline which incorporates a rapid chromatographic column isolation with a fluorimetric analysis, so giving an increased specificity. Collect 24-hour specimens of urine in bottles containing 10 ml. of 6N hydrochloric acid and 2 g. of oxalic acid crystals and store in a refrigerator. Measure the volume, adjust to 1500 ml. with distilled water, filter and collect 150 ml. of filtrate. Adjust to pH 1·5 with 6N hydrochloric acid and divide into three 50 ml. aliquots. One of these is used to determine recovery following the addition of 10 μg. of noradrenaline. Heat the aliquots in a boiling water bath for 15 minutes, cool and add 30 ml. of acetate buffer (pH 8·5) and adjust to pH 8·5 by slow addition of 25 per cent sodium carbonate solution. Pour immediately onto alumina columns (prepared by adding 5 ml. of 0·2M sodium acetate solution to 2·5 g. of alumina and washing into chromatographic columns 14 mm. by 30 cm.), and allow to pass rapidly. Wash the alumina with 10 ml. of acetate buffer and then with 50 ml. of distilled water. Add 0·25N sulphuric acid and allow the eluate to pass at a decreased speed. When acid to bromothymol blue collect 10 ml. and analyse this stable eluate for fluorescent substances as follows. Place 0·2 ml. (0·2 μg.) of working standards of adrenaline

and noradrenaline and 0·2 ml. of each eluate into a series of 15 ml. glass stoppered graduated tubes. Treat one tube of each as the blank adding 1 ml. of acetate buffer and 0·1 ml. of 0·25 per cent potassium ferricyanide. After exactly 2 minutes add 0·9 ml. of 20 per cent sodium hydroxide, mix and stand for 10 minutes. Do the same with the sample tubes but after the ferricyanide treatment add 1 ml. of a freshly prepared sodium ascorbate solution (9 parts of 5N sodium hydroxide and 1 part of 2 per cent ascorbic acid prepared 1 to 2 minutes before). 0·1 ml. of 2 per cent ascorbic acid is now added to the blanks and the timer set for 10 minutes. Add distilled water to 10 ml., mix and transfer aliquots to respective cuvettes. Compare the fluorescence in a photofluorimeter at the 10-minute period. Take three series of readings using primary filters at 365, 400 and 436 mμ and a secondary passing a narrow band at 510 mμ to measure emitted light. The adrenaline standard is set in all cases to read 50 and its blank zero. All other blanks and sample tubes are compared with this. For the calculation

$$\frac{\text{(Eluate sample minus eluate blank)}}{\text{(Nad. sample minus Nad. blank)}} \times 20 = \mu\text{g. per cent total catechol amines as noradrenaline.}$$

These values are determined for each wavelength. The method has a reproducibility of approximately $\pm$10 per cent. G. F. S.

PHARMACY

Gum Tragacanth Jellies, Conditions for the Preservation of. A. Taub, W. A. Meer and L. W. Clausen. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 235.) Experiments were carried out with jellies containing 2 per cent of tragacanth and 5 per cent of propylene glycol, which was used to wet the gum. Jellies ranging from pH 3 to pH 7 were prepared by the addition of McIlvaine's buffer solutions. Four test organisms were used to assess the suitability of added preservatives, the jellies being inoculated and samples removed at intervals for plating. To obtain consistent results it was necessary to sterilise the jellies before inoculating with the test organism, *Micrococcus pyogenes* var. *aureus, Bacillus subtilis, Escherichia coli* or *Candida albicans*. Benzoic acid (0·2 per cent) was ineffective as a preservative at pH 7, but satisfactory at pH 5 and below. Chlorbutol (0·5 per cent) was satisfactory at pH 5, but not sufficiently active to prevent the growth of *C. albicans* at pH 7, and the activity against this organism decreased on continued storage. Methyl hydroxybenzoate (0·2 per cent) with propyl hydroxybenzoate (0·05 per cent) was satisfactory at pH 7 and below. G. B.

PHARMACOLOGY AND THERAPEUTICS

Anti-inflammatory Activity of Compounds Obtained from Egg-yolk, Peanut Oil and Soybean Lecithin. O. H. Ganley, O. E. Graessle and H. J. Robinson. (*J. Lab. clin. Med.*, 1958, **51**, 709.) Various fractions of peanut oil, soybean lecithin and egg-yolk were tested for anti-inflammatory activity in three different anti-inflammatory assays. Crystalline fractions of all three substances in a dose of 3 μg./kg. i.p. were effective in inhibiting the swelling of joints produced in the guinea pig by a local joint anaphylaxis reaction. The active factor in all cases was identified as *N*(2-hydroxyethyl)-palmitamide and the synthetic compound was found to be as active as the natural crystalline

product. Ethanolamine, a degradation product of *N*(2-hydroxyethyl)-palmitamide was also found to be active while palmitic acid was inactive. By substituting various groupings of ethanolamine, pharmacological activity was shown to be associated with a high degree of chemical specificity. The fractions were inactive both in the pellet assay in which an inflammatory response is produced in rats by implanting cotton pellets impregnated with a non-virulent culture of *Micrococcus pyogenes* var. *aureus* and in the Evan's blue test in rats in which inflammation, produced by the subcutaneous implantation of cotton pellets, is reflected by an increased permeability of the capillaries to the dye. W. C. B.

Dextromoramide (R875), Analgesic Activity of, in Student Volunteers. D. A. Cahal. (*Brit. J. Pharmacol.*, 1958, **13**, 30.) This compound, (+)-1-(3-methyl-4-morpholino-2:2-diphenylbutyryl)pyrrolidine, related to methadone, was found by Janssen to be a very potent analgesic in animal experiments. When injected into student volunteers it raised the threshold to ischaemic pain, the peak of analgesic activity being reached in two hours. Side effects were very marked even on the low dose (2·96 mg.), nausea and vomiting being very prevalent. Euphoria was absent, which is unusual in such a potent analgesic drug. There was evidence of an effect on voluntary muscle, many subjects experiencing muscular weakness, thirty minutes after the injection and hiccups and twitching two or three hours afterwards. Therapeutically the dose is limited by the severity of the side effects and should probably not exceed 6·0 mg. G. F. S.

Drugs, Absorption of, From The Rat Small Intestine. L. S. Schanker, D. J. Tocco, B. B. Brodie and C. A. M. Hogben. (*J. Pharmacol.*, 1958, **123**, 81.) The relative rates of absorption of a large number of drugs were measured by perfusing solutions of them through the entire length of the small intestine of the anaesthetised rat. The rate of absorption of those which were rapidly absorbed could be studied after a single perfusion of the small intestine. Others which were slowly absorbed were studied after continuous perfusion for 3 hours. No evidence for the existence of specific transport mechanisms was obtained indicating that absorption of drugs is a passive physical process. A relationship between the dissociation constant and the degree of absorption of compounds of widely different chemical structures was observed. Acidic drugs were rapidly absorbed if their pKa's were greater than 3. Basic drugs were rapidly absorbed if their pKa's were less that 8. The stronger acids and bases were relatively slowly absorbed and the absorption of very strong acids and bases was imperceptible. The authors conclude that absorption from the rat intestine can best be explained by assuming simple diffusion of unionised drug across a barrier which may be lipoid in nature. W. C. B.

Hypoglycin-A, an Hypoglycaemic Substance, Action of. P. C. Feng and S. J. Patrick. (*Brit. J. Pharmacol.*, 1958, **13**, 125.) The pharmacological and biochemical effects of hypoglycin-A were studied in animals. Hypoglycin-A is a substance isolated from the fruit of *Blighia sapida*, a tree growing commonly in the West Indies. Administration of lethal doses to kittens, guinea pigs and white rats initially caused drowsiness, lachrimation and secretion from the nose and mouth; vomiting was frequently observed in kittens. Autopsies performed immediately after death showed few gross pathological changes but suitable staining of histological sections showed a marked reduction in glycogen granules in the liver and a reduction of the granules of the alpha cells of the islets in the pancreas. Studies on the acute toxicity showed that previous fasting rendered rats much more susceptible to the toxic action of the compound. The most outstanding biochemical change produced by hypoglycin-A was a

pronounced hypoglaecemia which was preceded by exhaustion of liver glycogen. There were also smaller decreases in the glycogen stores of the heart, skeletal muscle and kidney without any increase in blood lactate or pyruvate. Hypoglycin-A lessened the effect of adrenaline on blood glucose and decreased both glucose tolerance and insulin sensitivity. It also decreased the oxygen consumption and carbon dioxide production of the intact rat. These effects are consistent with the hypothesis that the primary action of hypoglycin-A is the interference with glycogen production by the liver. Hypoglycaemia and low liver glycogen concentrations are found in patients suffering from "vomiting sickness". Evidence is therefore provided in support of the theory that the ingestion of the fruit of *Blighia sapida* is a cause of the disease in man.

W. C. B.

Noradrenaline, Relative Lack of Pharmacological Action of 3-Methoxy Analogue of. E. V. Evarts, L. Gillespie, T. C. Fleming and A. Sjoerdsma. (*Proc. Soc. exp. Biol., N.Y.*, 1958, **98**, 74.) The effects of 3-methoxynoradrenaline, an intermediate product in the metabolism of noradrenaline, were studied in the cat, the dog and in man. In cats under pentobarbitone anaesthesia, 3-methoxynoradrenaline in doses up to 2 mg. injected into the carotid artery was without effect on the transcallosal response. In unanaesthetised cats intravenous doses up to 10 mg./kg. were without effect on spontaneous cortical activity, recruiting responses, or the primary cortical responses to retinal photic stimulation or electrical stimulation of the lateral geniculate radiations. In a single study in the dog, 3-methoxynoradrenaline was shown to have only 1/500th or less of the pressor activity of noradrenaline. In two human subjects, the intravenous infusion of 3-methoxynoradrenaline in doses up to 5 mg. in 5 minutes did not produce any cardiovascular reactions nor were any psychological reactions observed. These findings suggest that *O*-methylation of noradrenaline *in vivo* results in its inactivation. W. C. B.

Nystatin Aerosol in Pulmonary Moniliasis. G. D. W. McKendrick and J. M. Medlock. (*Lancet*, 1958, **1**, 621.) A girl of 9 with fulminating influenzal pneumonia was given intramuscular penicillin and streptomycin, together with sulphadiazine by mouth. This prevented secondary staphylococcal infection. Bacteria, however, were superseded by *Candida albicans* and the extensive moniliasis which developed might well have proved fatal. As soon as *C. albicans* was seen in the sputum treatment with nystatin was started; 500,000 units was given 6-hourly by mouth for 8 days and 500,000 units 4-hourly by nebulizer into the oxygen tent for 4 days. The solution was made by mixing 500,000 units of Mycostatin with 15 ml. of distilled water to a fine suspension and atomising this at a flow of 6 litres a minute. The *C. albicans* quickly disappeared from the sputum and the patient's general condition began to improve from the first day of treatment and continued until she was eventually discharged 5 weeks after admission. It is thought that the inhalations were the decisive factor in recovery as nystatin is poorly absorbed from the intestinal tract.

S. L. W.

Pteroylglutamic Acid, Effect of, on the Serum Vitamin B_{12} Concentrations in Pernicious Anemia in Relapse. J. Bok, J. G. Faber, J. A. de Vries, W. F. Stenfert Kroese and H. O. Nieweg. (*J. Lab. clin. Med.*, 1958, **51**, 667.) After determining the vitamin B_{12} level in the serum of 13 patients with pernicious anaemia in relapse, pteroylglutamic acid (PGA) was administered orally in doses of 15 mg. per day for varying periods ranging from 4 to 8 days. The serum vitamin B_{12} level was again estimated on the last day of treatment. In 10

out of 11 cases which showed a haemopoietic response, a decrease of the serum vitamin B_{12} activity was demonstrated. In the remaining 2 cases, treatment with PGA did not increase blood formation and the serum vitamin B_{12} level showed no change. A correlation was established between the decrease in serum vitamin B_{12} level and the haemopoietic activity of PGA. W. C. B.

Pempidine (1:2:2:6:6-Pentamethylpiperidine). A New Hypotensive Drug. G. E. Lee, W. R. Wragg, S. J. Corne, N. D. Edge and H. W. Reading. (*Nature, Lond.*, 1958, **181**, 1717.) The preparation of 1:2:2:6:6-pentamethylpiperidine (pempidine) is described. This compound, which has been independently and contemporaneously developed and studied in another laboratory (see Spinks and Young, *Nature, Lond.*, 1958, **181**, 1397), was shown to possess about 1·4 times the activity and about eight times the duration of action of hexamethonium in causing relaxation of the preganglionically stimulated nictitating membrane of the anaesthetised cat. Further experiments, carried out on the anaesthetised cat and the isolated ileum of the guinea pig, localised the site of action of the drug at the autonomic ganglia. There was no evidence that large doses caused the release of histamine and neuromuscular block was produced only by very large doses. The *in vitro* anti-acetylcholinesterase action was shown to be negligible. Large doses of the compound were required to decrease the rate and amplitude of the contractions of the Langendorff rabbit heart. No adverse effects were observed on the blood picture of guinea pigs injected daily with the compound for 4 weeks and very large doses were required to cause a 50 per cent reduction in the growth rate of young rats. The compound was rapidly and well absorbed on oral administration. The pharmacological properties of pempidine appear to resemble those of the secondary amine, mecamylamine but it possesses potential advantages over mecamylamine in respect of tolerance, duration of action and excretion. W. C. B.

Pernicious Anaemia, Oral Treatment of. J. G. Heathcote and F. S. Mooney. (*Lancet*, 1958, **1**, 982.) The ineffectiveness of vitamin B_{12} by mouth in pernicious anaemia has been ascribed to the absence of an "intrinsic factor" in the gastric juice, necessary for its absorption. When given with normal gastric juice it is active in very small doses. The intrinsic factor has never been isolated and Heathcote and Mooney doubt its existence. They believe that the fundamental cause of pernicious anaemia is an inability to absorb vitamin B_{12}, due to the failure of simple proteolysis in the stomach through the absence of the secretion of gastric juice. For absorption the vitamin must be in the form of a simple peptide complex of low molecular weight, dialysable and assimilable by micro-organisms. Thus combined B_{12} in the food must first be digested, and pure crystalline B_{12} combined with a peptide before absorption in the intestine can occur. An active vitamin B_{12} peptide complex (H.P.P.) was prepared from a fermentation of Streptomyces, dialysable and ultra filtrable through Cellophane and collodion membranes. The preparation has been shown clinically to be the most effective oral preparation yet described. Six newly diagnosed cases of pernicious anaemia, treated exclusively with this oral preparation, showed a good haematological and clinical response which has been maintained from 140 to 290 days. A reticulocyte crisis consistently developed at the end of the first week and no case developed signs of cord deterioration. A seventh case showed early subacute combined degeneration of the cord, following treatment with another oral preparation. Oral treatment with HPP ameliorated the symptoms and an unequivocal subjective improvement took place. G. F. S.

Perphenazine in Post-operative Vomiting. C. F. Scurr and D. S. Robbie. (*Brit. med. J.*, 1958, **1**, 922.) The efficacy of perphenazine (1-(2-hydroxyethyl)-4-[3-(2-chloro-10-phenothiazinyl)-propyl]-piperazine in post-operative vomiting was investigated in 200 consecutive patients undergoing various operative procedures. Alternate patients received perphenazine 5 mg. by intramuscular injection in the right thigh at the end of operation, the remainder serving as controls. The vomiting rate was 21 per cent in the controls and 7 per cent in those receiving perphenazine. The mean recovery time was 17 minutes in the controls and 27 minutes in the perphenazine series. More females vomited than males, in the ratio of 2·5 : 1. There was no indication that any differences between the two groups regarding pre-medication, anaesthetic agent, or operation contributed significantly to the difference in vomiting rate. No side-effects of the drug were observed. The drug has some potentiating effect on barbiturates and other narcotics, but the effect is much less than with chlorpromazine. The main danger is that perhaps, like chlorpromazine, the drug can obscure the cause of vomiting due to organic disease. S. L. W.

Serotonin Antagonism, Comparative Study on, of Amide Derivatives of Lysergic Acid and of Ergot Alkaloids. A. Cerletti and W. Doepfner. (*J. Pharmacol.*, 1958, **122**, 124.) The antiserotonin activity of more than forty ergot derivatives and about thirty semi-synthetic derivatives of lysergic acid has been determined on the isolated rat uterus. The degree of specificity was assessed by determining simultaneously the acetylcholine antagonism of the different compounds. Doses of serotonin were given at 10 minute intervals and the antagonist applied once only during the interval. With the amide derivatives none reached the activity of (+)-lysergic acid diethylamide (LSD). The most active was the monoamylamide of lysergic acid. Three isomers of LSD and Lumi-LSD were practically ineffective. Dihydro-LSD maintained 50 per cent of the activity of the original product. With the natural and hydrogenated alkaloids of ergot, all the peptide alkaloids—ergotamine and ergotoxine derivatives—with the exception of dihydroergotamine, showed less than 10 per cent of the activity of LSD. The ergonovine group were quite active antagonists, the most active being the methyl derivative which had 61 per cent of the activity of LSD. Substances with antiserotonin activities higher than LSD were found within the group of LSD derivatives with different substitution on the ring structure of LSD. The most potent compound so far studied was 1-methyl-2-bromolysergic acid diethylamide. G. F. S.

Urocanoylcholine (Murexine), Pharmacological Properties of. M. J. Keyl and V. P. Whittaker. (*Brit. J. Pharmacol.*, 1958, **13**, 103.) Urocanoylcholine (2-β-imidazol-4(5)-ylacryloyloxyethyl) trimethylammonium bromide) is a naturally occurring choline ester first identified by Erspamer in three Mediterranean species of whelks. The compound has been found to have both ganglion stimulating and neuromuscular blocking actions. In the dog a small dose (50 μg./kg.) has a slight vasodepressor action and by increasing the dose to 500 μg./kg. this is followed by a vasopressor response antagonised by T.E.A. Neuromuscular blockade was due to depolarisation of the end plate region and as with decamethonium, cats were most sensitive and rats least sensitive. The action of urocanoylcholine was short-lasting in all species, but this was not due to hydrolysis by plasma esterases. G. F. S.

PHARMACOPOEIAS AND FORMULARIES

THE EXTRA PHARMACOPOEIA—MARTINDALE, VOL. I., 24th EDITION*

Reviewed by Justin L. Powers, Ph.D.,
Chairman of the Revision Committee of the U.S. National Formulary

If the galaxy of pharmaceutical information in this new edition of *The Extra Pharmacopoeia* is surpassed or even approached by any other book of comparable or larger size, this reviewer has never seen or heard of such a volume. Since 1883, when William Martindale produced the First Edition of *The Extra Pharmacopoeia* to provide an epitome of information on drugs used in Great Britain, it has kept pace throughout its 23 revisions with developments in the pharmaceutical field. Its scope has also consistently increased and for many years interest in it has extended quite beyond the United Kingdom and the Commonwealth nations. The twenty-fourth edition provides more information about foreign drugs, pharmacopoeias, and formularies than any of the earlier editions. This should add to its universal appeal which it is hoped may be matched by increased distribution in countries other than those for which it was originally designed.

The new edition represents a complete revision which is at once apparent from the added information on new drugs and the up-to-date documentation in the form of short abstracts of recent articles published in pharmaceutical and medical journals. The preface states that in order to accomplish such a thorough revision some deletions were necessary. It goes on to explain that the deletions are few since, once established, drugs take a long time to become obsolete. When one considers the amount of space given to botanical drugs, for example, which have not had official status in any modern pharmacopoeia for several decades, one may question whether the deletions are as extensive as they should have been. During recent years obsolescence of many drugs has begun earlier and proceeded more rapidly to complete oblivion than was true 30 or 40 years ago. The supplementary list of drugs, for example, contains information on many older items which seem to possess little, if any, reference value. In the few instances when information on obsolete drugs is needed, older editions of *The Extra Pharmacopoeia* could be used advantageously.

Despite the space allocated to material that may have little or no reference value, the presentation of information on new drugs is adequate and excellent. For example, new information on cortisone and corticotrophin has necessitated a fourfold increase in the number of pages of text since 1952. Other adrenocortical hormones which have become available since the publication of the last edition have been added. These include fludrocortisone, hydrocortisone, prednisolone, and prednisone. The example is typical of comparable expansion, including information on new drugs, found in all of the therapeutic groups in which significant developments have occurred during the past six years.

The basis of the arrangement of sections continues to be one of "planned inconsistency", which is partly alphabetical and partly pharmacological. The design of Volume I of the twenty-third edition, published in 1952, has not been followed throughout the new edition. Most of the deviations appear to be well

* Pages xxx–1695 (including index). The Pharmaceutical Press, London, 1958. 65s., postage 2s. (overseas 3s. 3d.).

conceived and to add to convenience by bringing together drugs in the same therapeutic categories and yet avoiding pharmacological groupings characteristic of textbooks. Examples of this type of arrangement in the present edition include the grouping of all antipyretics and analgesics under the main heading of "Acetylsalicylic Acid", placing information on sympathomimetics under "Adrenaline", following the information on amphetamine with that relating to other central stimulants and anorexic drugs, arranging the discussion of adrenocortical hormones following cortisone, and grouping the antihistaminics under promethazine hydrochloride.

The "planned inconsistency" design, however, causes the user of the book no great inconvenience if he once reconciles himself to the fact that a strictly alphabetical arrangement is not used. The general index, containing nearly 20,000 entries, is so well planned, with adequate cross indexing under every conceivable name, that one experiences no difficulty in locating quickly the information he seeks.

The arrangement of information on each principal drug begins with the main title, in English, followed by other names employed in foreign pharmacopoeias and elsewhere. The doses given come next and are usually those in the British Pharmacopoeia or the British Pharmaceutical Codex if the drug is described in either of these. Pharmaceutical information includes chemical and physical properties of pharmaceutical interest such as solubility data, melting points, and boiling points.

One of the most important features is the provision of concise descriptions of the principal toxic effects, antidotes, contra-indications, and uses of drugs under these respective headings. The pharmacological and therapeutic information is supplemented by abstracts of original papers or reviews. The information included in these abstracts is of especial value because the documentation enables one to refer to the original articles.

Antibiotics, immunological products, and radioactive isotopes have been placed in special sections, the latter of which is new and includes up-to-date information on the toxicity, hazards, precautions, and supply of isotopes employed in clinical and experimental medicine.

The format of the twenty-fourth edition of *The Extra Pharmacopoeia* is essentially the same as that of its recent predecessors. The use of slightly larger type for the abstracts and some other parts of the text is commendable and contributes to ease of reading. The wealth of authentic information presented so succinctly in compact form places the book in the unique position of having no counterpart as a usable and valuable pharmaceutical reference treatise. Those who contributed to the new edition are to be complimented upon a stupendous task completed in a most commendable manner.

BOOK REVIEW

KURZES LEHRBUCH DER PHARMAZEUTISCHEN CHEMIE, by K. Bodendorf. Pp. vii + 490 (including Index). Springer-Verlag, Berlin, 1958. DM. 34.50.

This, the fifth edition of Professor Dr. K. Bodendorf's short textbook of pharmaceutical chemistry, over-emphasises and indeed puts misplaced emphasis on the current importance of inorganic chemistry, at least to the British pharmacist, if not to his Continental counterpart. Thus no less than 190 pages of text out of a total of 471 are devoted to classical inorganic chemistry, introduced admittedly by a short chapter on the periodic classification, electron distribution in the elements, and valency. The idea of energy levels and electron shells is introduced, but one misses the discussion of the Bohr-Sommerfeld atom which is fundamental to any study of modern inorganic chemistry. Radioactivity, too, is dismissed in a few short pages, which are concerned mainly with natural radioactive series, and, whereas such elements as ^{14}C, ^{131}I and ^{32}P are mentioned, there is no serious discussion of their properties and uses. The remaining chapters devoted to organic chemistry follow the conventional pattern of any introductory course, dealing in turn with the main branches of aliphatic, carbocyclic (alicyclic and aromatic) and heterocyclic chemistry, in a manner such as to provide a useful course of general organic chemistry, well illustrated with pharmaceutical examples. Liberal use is made throughout of graphic formulae, which add considerably to the clarity of the text, but the choice of formulae which represent heterocyclic elements as *exocyclic* atoms is very much to be regretted in a students textbook. The general approach to stereochemistry, too, is weak for a textbook of this level, considering the importance of the subject to modern ideas of structure-action relationships. *cyclo*Hexane conformations and the stereochemistry of steroids are not discussed; graphic formula of such familiar substances as vitamins A and D are not represented as the all-*trans* structures which they are and which are essential to their activity. In other ways, however, the book gives a reasonably comprehensive cover of the simpler synthetic organic medicinals, and such natural products as vitamins, the simpler hormones and alkaloids, but the short section on antibiotics is disappointingly short, and could usefully have been extended. Despite the shortcomings enumerated, this volume will provide a useful introduction to pharmaceutical chemistry for those to whom the German text is no serious disadvantage.

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