REVIEW ARTICLE

THE HORMONES OF THE ANTERIOR LOBE OF THE PITUITARY GLAND

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Modern advances in our knowledge of the hormones of the anterior pituitary have been stimulated by the development of techniques for hypophysectomy by various people using various animals. In 1927, P. E. Smith[1] announced that rats could be conveniently hypophysectomised, and that this operation caused inhibition of growth and atrophy of the thyroid, adrenal cortex and gonads. All these effects could be reversed by the implantation of rat pituitaries. Much recent work has been based on this.

Great progress has been made in the fractionation of anterior pituitary extracts. Preparations of follicle stimulating hormone, luteinising hormone, prolactin, thyrotrophin, corticotrophin, and growth hormone have been almost completely freed of other forms of activity. It seems possible that all the effects of extracts of the anterior lobe may be due to these six substances, acting either independently of one another or together. Other hormones have been postulated with ketogenic, parathyrotropic, glycotropic, diabetogenic, pancreatrophic, and other effects, but it seems likely that the effects attributed to these hormones were really due to one or more of the above six substances.

The separation of the hormones has been achieved by fractional precipitation with organic solvents and inorganic salts, and by adjustment of the *p*H. A detailed account of this work has been given by Li and Evans[2]. Some of the main data are summarised in Table I. An extract prepared with 66 per cent. acid acetone contains prolactin and adrenocorticotrophic hormone (ACTH) and little of the other hormones. Prolactin is almost insoluble in salt-free water. Gonadotrophins from different species

TABLE I

Hormone	Follicle stimulating Hormone		Luteinising Hormone		Prolactin	Thyrotrophin	Adreno-cortico-trophic Hormone	Growth
Animal	Sheep	Pig	Sheep	Pig	Sheep Ox	Ox	Sheep Pig	Ox
Molecular weight ...	70,000	—	40,000	100,000	26,500	10,000	20,000	44,250
Isoelectric *p*H	4·5	4·8	4·6	7·45	5·73	—	4·65	6·85
Per cent. saturation with $(NH_4)_2SO_4$ for precipitation	55-70	50-90	37-40	33-90	0	30-50	20	20-50
Solubility in 66 per cent. acid acetone	0	0	0	0	+	0	+	0
Sugar in molecule ...	+	+	+	+	0	+	0	0

appear to be different substances. In extracts of the pig's pituitary they can be separated by adjustment of the pH and in extracts of the sheep's pituitary they can be separated by precipitation with ammonium sulphate. Prolactin and adrenocorticotrophic hormone have seemed to have similar properties when obtained from different species, but the meaning of this fact is in doubt owing to recent evidence that the latter can be obtained in the form of quite small molecules.

Gonadotrophins

There are several different gonadotrophic substances, all of them probably glycoproteins, except luteotrophin which has been found to be identical with prolactin. It is important that these substances should not be confused with one another. Follicle stimulating hormone (FSH) usually denotes a substance obtained from the pituitary gland and should not be applied to the substance found in pregnant mare serum, although this also stimulates follicles. The two substances have quite different effects on male rats.

In the following list, follicle stimulating hormone, luteinising hormone (LH) and luteotrophin come from the pituitary, while human chorionic gonadotrophin (CG) and equine gonadotrophin (PMS) come from the placenta, and one or other of the last two is the main active constituent of most commercial gonadotrophins. The account of follicle stimulating hormone and luteinising hormone is based largely on that of Greep, van Dyke and Chow[3].

1. *The follicle stimulating hormone from the anterior pituitary (FSH, Thylakentrin).* In the female this stimulates the growth of follicles and increases the weight of the ovaries. If pure, it does not cause luteinisation directly. In intact animals luteinisation may occur eventually owing to the liberation of luteinising hormone from the animal's own pituitary. It used to be believed that follicle stimulating hormone caused the release of œstrogens from the ovaries, but there is evidence that pure preparations do not do this in hypophysectomised rats and that a small amount of luteinising hormone must be present for this effect to occur; pure follicle stimulating hormone thus has no effect on the uterus. In the male, it causes development of the seminiferous tubules, but not the release of androgens. The gonadotrophins in the urine in female castrates, or at the menopause or in male urine, are probably mainly follicle stimulating hormone mixed with a little luteinising hormone. There is no standard preparation. Highly purified preparations have been made from pig's pituitary by van Dyke and his colleagues[3] and from sheep's pituitary by Li, Evans and Simpson[4] in California.

2. *The luteinising hormone, or interstitial cell stimulating hormone of the anterior pituitary (LH, ICSH, Metakentrin).* This stimulates the interstitial cells in the gonads of either sex of rat. In the male, this causes the release of androgens with secondary effects upon other organs such as the prostate and seminal vesicles. In the female, the effect depends very much on the presence of follicles in the ovary. In young hypophysectomised female rats there are no follicles and, though the effect on the interstitial cells can be detected histologically, there is little or no

increase in the weight of the ovary and no release of œstrogens. If such rats are first treated with follicle stimulating hormone to form follicles, the injection of luteinising hormone causes ovulation, luteinisation, and the release of œstrogens. This is sometimes referred to as a synergistic effect. Follicle stimulating hormone and luteinising hormone both may increase the weight of the ovaries, but a proper combination of them both has most effect. Under the action of the œstrogens released there is a striking increase in the weight of the uterus. There is no standard preparation of luteinising hormone. The hormone has been isolated as a single homogeneous protein from pigs' pituitaries by Chow *et al.*[7], and from sheep's pituitaries by Li, Simpson and Evans[6]. These two proteins were quite different from one another (*cf.* Table I).

3. *Luteotrophin (Prolactin).* Corpora lutea formed under the action of the two preceding hormones do not secrete progesterone until stimulated to do so by a third substance present in extracts of the anterior pituitary. This substance has been called luteotrophin, but it seems to be identical with prolactin, which is discussed in a separate section[7,8].

4. *Human chorionic gonadotrophin (CG).* This is formed in the placenta and is present in blood, urine and placental extracts during pregnancy, or in other conditions when chorionic tissue is present, as it is in chorionepitheliomata and hydatidiform moles. Its actions on rats are similar to those of luteinising hormone. It is not the same substance as luteinising hormone as obtained from the pituitary of sheep or pigs, but might possibly be identical with human luteinising hormone which has not been much studied. In monkeys (rhesus) its action on the ovary is depressant, and in man it has little stimulant action unless it is combined with follicle stimulating hormone or equine gonadotrophin (PMS)[9]. Such a combination is sold as "synapoidin" and has been shown to produce corpora lutea and hæmorrhagic follicles in women, but human chorionic gonadotrophin has no clear use in therapeutics yet. The international standard preparation contains 10 units per mg. A preparation containing 8,500 IU per mg. was made by Katzman *et al*[10]. Claesson *et al*[11] claim to have isolated the pure hormone.

5. *Equine gonadotrophin (PMS).* This is formed in the equine placenta and obtained from pregnant mare serum. It differs from all the other gonadotrophins in the fact that it is not excreted in the urine. Its main effect on the ovary is like that of follicle stimulating hormone (stimulation of follicles), but large doses cause luteinisation even in hypophysectomised rats. Its main effect on the testes is more like those of luteinising hormone and human chorionic gonadotrophin (stimulation of the interstitial cells with the liberation of androgens). The international standard contains 4 units per mg. A preparation containing 12,000 to 13,500 I.U. per mg. has been made[12].

Assays[13]. Estimates of gonadotrophins in blood, urine and tissue extracts are sometimes required in physiological and clinical research. Estimates of human chorionic gonadotrophin and equine gonadotrophin are also of commercial importance. These last two hormones are sometimes present during pregnancy in such overwhelming amounts that

blood or urine can be used directly without extraction, but more often it is necessary to make an extract in order to reduce the bulk and remove toxic substances and other hormones which would interfere with the test. The methods used include precipitation with alcohol or acetone, adsorption on various reagents, salting out with sulphates, and precipitation with tannic acid[14]. One simple method which has been found satisfactory for human chorionic gonadotrophin and for the gonadotrophins in urine after the menopause, involves adsorption on kaolin as recommended by Scott[15] followed by precipitation with acetone[16,17]. The adsorption leaves most of the salts behind and the acetone removes œstrogens. A litre of urine can thus be concentrated to about 400 mg. of solid material containing all the gonadotrophin, and quantities corresponding to 200 ml. of urine or more injected into each animal.

Standard preparations are available for equine gonadotrophin and human chorionic gonadotrophin, but not for follicle stimulating hormone and luteinising hormone. It is therefore difficult to design really satisfactory assays of these, and most workers use animal units. It might perhaps be justifiable to use the standards for equine gonadotrophin and human chorionic gonadotrophin for the assay of follicle stimulating hormone and luteinising hormone respectively, using female rats. This would not necessarily give consistent results, since the active principles in the standard and unknown preparations would be different, but it might easily be more reliable than the use of animal units, which are notoriously variable.

The results of experiments on normal animals are liable to be complicated by the release of gonadotrophins from the animal's own pituitary. Results with hypophysectomised animals are easier to interpret, but even then the assay of mixtures of gonadotrophins is difficult because they often increase and may decrease one another's actions.

Assay of follicle stimulating hormone and luteinising hormone. According to Greep *et al.*[3] the ventral lobe of the prostate of young hypophysectomised male rats provides a test for luteinising hormone which is unaffected by the presence of follicle stimulating hormone in the solutions used. The hormone is injected for 4 days and the glands are then weighed. The specificity of this test appears to be unique. No other case is known in which the weight of an organ is increased by one of these hormones and not by the other. Greep *et al.*[3] use similar rats for the assay of follicle stimulating hormone, but assess the results by weighing the testes. This test is not quite specific, since luteinising hormone also increases the weight of the testes. Female rats give comparable results. Follicle stimulating hormone alone has a large effect on the weight of the ovaries and no effect on the weight of the uterus. Luteinising hormone has no effect on the weight of either ovary or uterus unless the animal has first been exposed to follicle stimulating hormone, but if follicles are first formed by the action of follicle stimulating hormone the effect of luteinising hormone is best shown by its enormous effect on the weight of the uterus.

The most popular way of estimating the gonadotrophins present in

non-pregnant human urine is by their effect on the weight of the uterus of a normal young mouse, a unit being defined as that quantity necessary to produce a given effect. The great advantage of this method is that it is very sensitive and gives positive results with most urines. Both follicle stimulating hormone and luteinising hormone may be present and the result depends on the proportion of one to the other, so that the result of the assay has no precise meaning; it may, nevertheless, lead to interesting conclusions! By estimating gonadotrophins in the urine it is possible to distinguish primary gonadal failure from secondary gonadal failure due to failure of the pituitary[18]. In Turner's syndrome there are large amounts of gonadotrophin in the urine; the pituitary is striving to stimulate unresponsive ovaries. In Simmond's disease small quantities are excreted because the pituitary itself is failing. Menopause urine may contain 200 to 400 mouse units measured in this way and no appreciable amount of œstrogen. Such an effect can be observed after the injection of untreated urine, but generally it is necessary to concentrate the urine and remove œstrogens. Concentrated extracts of menopause urine have effects on the weight of both the uterus and ovaries of young rats or mice and by weighing both organs it is possible to detect qualitative differences in urine from different sources[19]. Fevold[20] uses the weight of the ovaries to estimate follicle stimulating hormone and that of the seminal vesicles for luteinising hormone. It has already been mentioned that Greep *et al.*[3] used the weight of the testes for the former and the weight of the prostate for the latter. These workers agree in using the primary effect on the gonads to estimate follicle stimulating hormone, and the secondary effect on the accessory genital organs to estimate luteinising hormone, but the interpretation of the results is complicated if both hormones are present. This is well illustrated by the fact that menopause urine has a large effect on the weight of the uterus. It is agreed that the main hormone present is follicle stimulating hormone and that the effect on the uterus is mainly due to luteinising hormone. If the extracts of urine were free of luteinising hormone and injected into hypophysectomised rats they would presumably have no action on the uterus at all, but this experiment has not been done, and it is possible that human follicle stimulating hormone differs from the substances isolated from animals and that this conclusion is false. The position may be clarified when methods of separating human pituitary gonadotrophins are worked out. The methods used to separate sheep's gonadotrophins are different from those used to separate pig's gonadotrophins and neither method is necessarily applicable to human gonadotrophins.

Assay of human chorionic gonadotrophin. The effect of this hormone on the ovaries of mice forms the basis of the Aschheim-Zondek test for pregnancy. Tests have also been based on observations of ovarian weight, ovarian hyperæmia, ovulation in rabbits or in the clawed toad *Xenopus*[13,17]. The release of sperm by male toads is also used as an index[21,22]. In these tests the result depends on the primary effect of the hormone on the gonads and is little, if at all, affected by the presence of œstrogens in the injected fluids. When observations are made on the

uterine weight or on vaginal smears the result depends on the release of œstrogens from the animal's own ovary and is useless unless œstrogens have been completely excluded by suitable methods of extraction. The weight of the prostate of young rats provides a convenient and accurate method which is not affected by œstrogens and does not involve extraction[17]. Using the prostatic weight method a study of the chorionic gonadotrophin levels in blood and urine has been made in normal pregnancy, diabetic pregnancy and cases of pre-eclamptic toxæmia. The curve for the excretion in normal pregnancy has two distinct phases. In the first trimester very high figures, e.g., 20,000 to 40,000 I.U. per 24 hours are obtained, and there is great variation between individual patients. Toward the end of the first trimester the excretion falls and in the second and third trimesters the excretion remains in the range 4,000 to 11,000 I.U. per 24 hours ($P=0{\cdot}99$). The mean value for this period of pregnancy was found to be 7,400 I.U. per 24 hours and the variation between individual patients was much less marked than in the first trimester[17].

In the second and third trimesters of pregnancy an excretion consistently above 11,000 I.U. per 24 hours must be regarded as pathological. This was found in a proportion of pregnant diabetics[23,24]. In cases of pre-eclamptic toxæmia, urinary and serum levels in mild and moderate cases were within normal limits but in severe and fulminating cases serum and urinary levels were often abnormally high. Cases of essential hypertension in pregnancy showed no abnormality in the levels either in blood or urine. Throughout these studies the concentration in the serum was shown to be quantitatively similar to the concentration in the urine. The mean renal clearance in normal pregnancy was found to be 0·95 ml./min. $\pm{\cdot}04$ (standard error of the mean). Severe cases of pre-eclampsia and pregnant diabetic women had a significantly lower renal clearance than was found in normal women[19].

The administration of stilbœstrol to normal and diabetic women depresses the urinary excretion of chorionic gonadotrophin[24]. This depression, however, is evanescent and with continued therapy an "escape" phenomenon occurs, the excretion returning approximately to its original level. A similar effect with stilbœstrol has been noted on the serum concentration of chorionic gonadotrophin.

Assay of equine gonadotrophin. The most popular method of assaying this hormone is by its effect upon ovarian weight. Other methods depend upon vaginal smears, corpus luteum formation, the weight of the uterus or seminal vesicles, and ovulation[25,26].

PROLACTIN

It was first shown by Stricker and Grueter[27] that the anterior pituitary had some influence upon the mammary gland. These workers injected anterior lobe extracts into pseudopregnant rabbits, either normal or oophorectomised, on the 10th day of their pseudopregnancy and observed enlargement and development of the mammae followed by a profuse secretion of milk. They were unable to produce lactation in immature rabbits by the administration of pituitary extracts, and concluded that

morphological changes must occur in the gland before lactation will ensue. Since this pioneer work these observations have been extended to numerous other species[28,29], and it has also been shown that hypophysectomy performed during pregnancy will prevent lactation[30]. It has now been definitely confirmed that the anterior pituitary elaborates a substance known as prolactin, which is the essential factor in the initiation of lactation and is probably also important in its maintenance[31]. It exercises a direct effect on the mammary tissue as its actions are still produced in hypophysectomised, oophorectomised or adrenalectomised animals. Other names for prolactin are galactin or mammotrophin.

Actions of Prolactin. Normal lactation is affected by the pituitary, gonads, adrenal cortex and thyroid. The first step is the development of the mammary tissue during pregnancy, under the action of gradually increasing quantities of œstrogens and progesterone coming at first from the ovaries and later from the placenta. Although there is some species variation, it is now generally agreed that œstrogen acts mainly on the duct system and progesterone mainly on the lobulo-alveolar system. Œstrogens and progesterone together produce complete differentiation of the mammary tissue, but the actual flow of milk in the post-partum period is due to the liberation of prolactin from the anterior lobe of the pituitary. Prolactin itself cannot induce milk secretion unless the breast has been previously primed by œstrogens and progesterone. Nelson[32] believes that the suppression of lactation during pregnancy results from the high œstrogen titre present in body fluids, and stresses the inhibitory action of œstrogens on prolactin secretion. Meites and Turner[33], however, have recently questioned this view and suggest that œstrogens may actually stimulate prolactin secretion, and that during pregnancy progesterone inhibits this stimulant action of œstrogens.

Prolactin stimulates the crop glands of pigeons and doves, and this action is employed as a means of assaying the hormone[31]. In these birds rapid proliferation of the epithelial lining of the crop glands occurs; the epithelium becomes heaped up and shows numerous mitotic figures. In addition, prolactin increases the production of " crop milk," which is a caseous fluid consisting mainly of desquamated epithelial cells.

Prolactin undoubtedly has a stimulant action on mammary secretion, but there has been some controversy as to whether it causes actual growth of mammary tissue. Corner[28] produced both mammary growth and lactation in the rat by the administration of pituitary extract rich in prolactin. Using the delicate technique of intraduct injection Lyons[34] obtained evidence of hyperplastic changes in alveoli of oophorectomised virgin rabbits pretreated with œstrogen and progesterone. He concludes that in this species prolactin can actually cause tissue growth.

Prolactin has been used by Folley and Young[35] to increase the milk yields of lactating cows. These investigators believe that adrenocorticotrophic hormone helps to maintain milk production in combination with prolactin. Prolactin also stimulates maternal instincts in rats[31] and broodiness in hens. In pigeons it causes growth of the body as a whole, the most prominent effect being splanchnomegaly.

Evidence has recently accumulated that the gonadotrophic hormone, luteotrophin, is identical with prolactin. Luteotrophin stimulates and maintains the activity of corpora lutea and causes the secretion of progesterone. It inhibits the effect of œstrogens in producing cornification of the vagina in hypophysectomised rats and causes mucification of the vaginal mucosa instead. This has been made the basis of a method of assay[7]. Similar effects are produced by prolactin, and Cutuly[36,37] has shown that prolactin will maintain pregnancy in pregnant rats hypophysectomised on the 9th day of gestation. This effect is presumably due to its action on corpora lutea.

Assay. The most popular method of assay of lactogenic preparations depends on the increase in combined *weight of the crop glands* in pigeons[31]. Numerous investigators have called attention to the necessity for rigid standardisation of technique when this procedure is employed. The following factors have been found to influence the response—season of the year, body weight of the birds, strain and race of pigeon, environmental temperature, route of injection and volume of solution injected. In addition, all assays should be expressed in terms of the international standard of prolactin. Variants of this test are the *minimum stimulation method*[38] in which the crop gland is dissected out and merely examined against the light for a positive reaction, and the sensitive *local intradermal* or *micro method* introduced by Lyons and Page[39] in which the solutions under test are injected immediately over the crop gland. Prolactin may also be assayed by the induction of lactation in oophorectomised guinea-pigs[40] or rabbits, but these methods are less quantitative, more variable and less reliable than those employing pigeons.

This hormone may be assayed by the mucification reaction of the vaginal mucosa[7] or by the production of traumatic placentomata in the uteri of rats[8]. Prolactin is the only pituitary hormone for which there is an international standard preparation. By definition 1 unit = 0·1 mg. of this preparation.

Chemistry. Purified preparations of prolactin were first obtained by Lyons[41] and the hormone was isolated in pure form by Li *et al.*[42] Electrophoretic and solubility studies have shown that prolactin behaved like a pure protein. The molecular weight is estimated as 26,500 and the *iso*-electric *p*H is 5·5. The activity is 30 international units per mg.

Clinical Applications. Prolactin is at present of more value in veterinary than in human medicine. Most attempts to demonstrate it in the urine of lactating women have met with little success but Lyons and Page[39] using acetone precipitation to extract the urine claimed to have demonstrated prolactin in 8 cases using their intradermal method of assay. Using a similar assay method, Meites and Turner[43] extracted post-partum urine by alcohol precipitation and by dialysis followed by evaporation. Prolactin was demonstrated in all the cases studied. It was concluded that a relationship probably exists between the quantity of lactogenic hormone in the urine and the level of milk secretion. In the therapeutic field the evidence is conflicting. Winson[44] and Kenney and King[45] obtained encouraging results when prolactin was administered to puer-

peral cases. In the majority of women a significant increase in the milk yield was obtained. Stewart and Pratt[46], however, observed no effect on milk secretion even when large doses of prolactin were administered to lactating women.

Thyrotrophin

In 1888, Rogowitsch[47] described enlargement of the pituitary after thyroidectomy. This was the first hint of a relation between the two glands. In 1914, Adler[48] found that the destruction of the hypophysis delayed the development of tadpoles and at about the same time Gudernatsch[49] found that the administration of thyroid accelerated their development. Allen[50] showed that the thyroids of the hypophysectomised tadpoles showed signs of inactivity and the Smiths[51] showed that these thyroids could be reactivated by the administration of anterior pituitary. This evidence of a stimulant action of the pituitary on the thyroid was soon extended to other species.

The first solutions of thyrotrophin were made by Loeb and Bassett[52]. The hormone can be extracted with water from fresh gland or acetone-dried gland and purified by fractionation with acetone or salts, or by adsorption. It is not precipitated by 8 per cent. trichloracetic acid and may have quite a low proportion of protein-like material. Since preparations have been made with very little effect in tests for any of the other five hormones discussed here, there is no doubt that thyrotrophin can be separated from these other substances. The preparation made by White and Ciereszko[53] was a white powder easily soluble in water, and homogeneous in the Tiselius apparatus and the ultracentrifuge with an apparent molecular weight of about 10,000. 1 μg. of this powder produced a histological response on the thyroid of a 3-day-old chick when given once per day for 5 days.

Under the action of thyrotrophin the thyroid discharges its colloid so that the iodine content of the gland falls, but the cells increase in height and multiply so that the weight of the gland increases. There is a great increase in mitotic figures[54] and in the oxygen consumption of the tissue[55,56]. This active tissue has great avidity for circulating iodine, as can be shown by the fact that, if radioactive iodine is injected, it accumulates in the thyroid which has been stimulated by thyrotrophin even more rapidly than in the normal thyroid. The hormone increases the turnover of iodine in the gland, which makes thyroxine more rapidly and liberates it into the circulation. This causes various secondary effects such as a rise in the metabolic rate and heart rate, increased sensitivity to oxygen, creatinuria and increased calcium excretion. When continued injections of thyrotrophin are given they lose their effect owing to the development of antihormones[57,58,59]. Thyrotrophin also causes exophthalmos, especially in guinea-pigs. This effect is not produced by thyroxine and cannot in any case be secondary to an action on the thyroid, since it occurs in animals which have been deprived of this gland[60,61].

The rate of liberation of thyrotrophin from the pituitary depends on the concentration of thyroxine in the blood. If this is reduced by drugs, such as thiouracil, which inhibit the formation of thyroxine, the rate of

liberation of thyrotrophin increases and the thyroid increases in size. This effect can be prevented by the injection of thyroxine, which inhibits the release of thyrotrophin[62].

Assay. Methods of assay of thyrotrophin are numerous and four main experimental animals have been used. These are the chick, the guinea-pig, the rat and the tadpole.

The thyroid of the 1-day-old chick is one of the most sensitive methods of assay available. Smelser[63] states that it is 10 times more sensitive than the thyroid of the guinea-pig. The end-point may depend on the increase in weight of the gland[64] or on the histological changes[65]. Rawson *et al.*[66] calculate the mean acinar cell heights expressing the results by a process analogous to the Price-Jones curve for red blood cells. Dorfman[67] uses the iodine content of the chick thyroid. This has the advantage of technical simplicity if radio-active iodine is employed.

The guinea-pig thyroid has also been used frequently for the assay of thyrotrophin. Loeb[68] based his observations on the " mitotic index," i.e., the increase in mitosis produced by the administration of thyrotrophin. Most other workers have used either histological signs of stimulation[69,70] or the resulting increase in weight[71,64]. Recently, De Robertis and Del Conte[72] have introduced a new and extremely sensitive method depending on the determination of the number of colloid droplets in the cells of the guinea-pig thyroid, the gland being prepared by a freez-drying procedure. This method is claimed to be several hundred times more sensitive than any other. De Robertis[73] has applied this method to the assay of thyrotrophic hormone in human blood.

Evans[74] employs histological changes in the thyroid of hypophysectomised female rats as an indication of the degree of thyroid stimulation. A similar method was used by Hertz and Oastler[75] in a study of thyrotrophin in clinical states. The original method of Collip and Anderson[59] depending on the increase in metabolic rate produced by thyrotrophic extracts in hypophysectomised rats is not now widely used. Dvoskin[76] found that the administration of extracts containing thyrotrophic activity caused the formation of intracellular colloid droplets in the thyroid epithelium of hypophysectomised rats but unfortunately, the specificity of this test was doubtful.

D'Angelo, Gordan and Charipper[77] have used the starved tadpole for the assay of thyrotrophin. The administration of graded doses of thyrotrophic extract induced metamorphosis characterised by progressive loss of body weight, increase in hind limb length and activation of the thyroid gland.

Clinical. Injections of thyrotrophin produce a condition which resembles Graves' disease not only in the secondary effects due to thyroxine, but also in the appearance of the thyroid gland itself, and perhaps also in the exophthalmos[61]. In Graves' disease the gland also shows increased avidity for iodine and this fact has been made the basis of various tests of thyroid function.

Several attempts have been made to demonstrate increased amounts of thyrotrophin in the blood or urine of cases of Graves' disease, but these attempts have generally been unsuccessful[65,75]. De Robertis[73], however,

used the very sensitive method of assay depending on the presence of colloid droplets in the cells of the guinea-pig thyroid and found evidence of thyrotrophin in the blood of some cases of Graves' disease and some cases of myxœdema. He interprets the results by assuming that in some cases the disease is primarily in the pituitary and in others primarily in the thyroid. These interesting results require further confirmation.

ADRENOCORTICOTROPHIC HORMONE

The hormone in pituitary extracts which stimulates the adrenal cortex is called adrenocorticotrophic hormone (ACTH) or corticotrophin. Proteins with high activity of this kind were prepared in 1943 by salt fractionation of sheep pituitary[78] and by *iso*-electric precipitation from hog pituitary[79]. Another method combining both techniques gave a higher yield[80]. These protein preparations had a molecular weight of about 20,000 and were similar to one another, but more active preparations with lower molecular weights have been made[81,82,83] and it seems probable that the protein acts as a carrier for an active molecule which is thought to be a polypeptide, containing 7 aminoacids or less. The commercial preparations available contain protein and are generally contaminated with posterior lobe hormones. Doses are commonly given in terms of mg. of Armour's standard preparation.

Actions. It acts primarily on the adrenal cortex causing a loss of ascorbic acid and cholesterol, an increase of the weight of the gland and histological changes, mainly in the zona fasciculata. The zona glomerulosa is said to be affected in the rat by changes in mineral metabolism but not by this hormone[84,85].

It also causes the release of steroids from the adrenals, and various compounds can be detected either chemically or biologically in the urine. These substances may be divided into three classes according to their effects.

(1) Substances such as desoxycorticosterone which have no oxygen in position 11 cause the retention of sodium and chlorine with initial loss of potassium and have little or no other known effects. Death following adrenalectomy is mainly due to loss of sodium, and it is therefore clear that this action is essential to life. The typical changes in the urinary excretion of minerals have been produced by preparations containing this hormone, and this fact suggests that it releases these substances of this type, but there is no direct evidence of this; the interpretation of the results is complicated by the presence of small amounts of posterior lobe hormones in the preparations used.

(2) Substances with oxygen in position 11 (" 11-oxysteroids ") have much less effect on mineral metabolism, but some of them have other effects. The presence of a hydroxyl group in position 17 seems to increase these effects, which are particularly marked in cortisone (Kendall's compound E). They cause a loss of protein and an increase of the carbohydrates in the body. There is a negative nitrogen balance and an increased loss of uric acid in the urine. The excretion of creatinine is not

changed and the effect can conveniently be followed by estimating the ratio of uric acid to creatinine in the urine. Protein is lost from lymphatic tissue which decreases in weight. Lymphocytes and eosinophils disappear from the circulating blood and are presumably destroyed; on the other hand, the neutrophil leucocytes increase in the blood[86]. The blood sugar rises, sugar tolerance falls and glycosuria may occur. These effects resemble diabetes, and these hormones are sometimes said to cause an insulin-resistant diabetes. On the other hand, they increase the storage of glycogen, and in this they are opposed to pancreatic diabetes. These effects are also produced by adrenocorticotrophic hormone which, therefore, releases steroids of this type. According to Conn *et al*[87,88], the amount of sugar in the urine after adrenocorticotrophic hormone may be so large that it is necessary to assume that the utilisation of sugar in the body is decreased; the amount of extra sugar that could be formed from protein would not be enough to account for the results. These same workers found that adrenocorticotrophic hormone caused a fall in the blood glutathione, and that the injection of glutathione diminished the glycosuria[89]. There is, at present, no evidence that the doses of adrenocorticotrophic hormone used clinically can cause permanent diabetes.

(3) Various androgens are found in cortical extracts. Some of the symptoms of Cushing's disease have been attributed to the release of these androgens, but there is no direct evidence that this occurs. Adrenocorticotrophic hormone has been found to cause a rise[91], and cortisone a fall[95,98], in urinary 17-ketosteroids, which probably consist largely of androgens. These facts support the theory that adrenocorticotrophic hormone causes the release of a 17-ketosteroid from the adrenals, or of some other substance (not cortisone) which is converted into a 17-ketosteroid in the body.

Assay. Most methods depend on observations of the rats' adrenals after hypophysectomy. This operation causes lipoids to disappear from the cortex. One test depends on the repair of this change. Another test depends on the maintenance of the weight of the rats' adrenals after hypophysectomy[2]. The most popular method depends on the estimation of ascorbic acid in the adrenals[90]. This test can estimate about 0·5 μg. of the standard preparation and detect adrenocorticotrophic hormone in the blood following an injection[91] or during Addison's disease[92].

Release. There is evidence that adrenaline stimulates the adrenal cortex[93] probably by causing the release of adrenocorticotrophic hormone from the pituitary. It is also released in various conditions of stress such as hæmorrhage, scalding, exposure to cold, etc.[94].

Clinical use. Various workers have described the actions on man[91,95,96,97]. A wide variety of diseases are being treated experimentally with it, but it is still too soon to assess its range of usefulness. The discovery of the dramatic effect of cortisone in producing complete, though temporary, relief of the symptoms of rheumatoid arthritis led to the trial of adrenocorticotrophic hormone in this condition[98], and there is no doubt that it is very effective when injected in a dose of 10 mg. equivalents of

standard hormone every 6 hours[97]. The effect is a dramatic one. Pain and stiffness disappear from the joints in a few hours, appetite improves and weight increases; there is definite evidence of healing in the synovial membranes, and a rapid fall in the sedimentation rate and a rise in the concentration of hæmoglobin in the blood. The daily dosage is less than that of cortisone, and there is some evidence that the factor of safety is larger. Similar results have been obtained in rheumatic fever[99]. Adrenocorticotrophic hormone is also used to test the functional state of the adrenal cortex. It causes various measurable effects due to the release of corticoids; if these effects do not occur the cortex must be defective and a diagnosis of Addison's disease may be made. In Thorn's test[96] a count of the eosinophils is made by a special technique. If this does not fall the cortex is abnormal.

Growth Hormone

From a study of growth abnormalities in man it has been known for many years that the anterior lobe of the pituitary exerts a profound influence on skeletal growth. In 1885, Pierre Marie[100] described the clinical condition of acromegaly. This disease is believed to result from the excessive elaboration of growth hormone during adult life and occurs usually in association with an eosinophil adenoma of the anterior lobe. A similar pituitary lesion occurring in pre-adult life before ossification is complete produces gigantism. Conversely, pituitary failure during childhood or adolescence results in various forms of dwarfism. In 1910, Crowe, Cushing and Homans[101] hypophysectomised dogs and found that the growth of these animals was retarded. In 1921 Evans and Long[102] produced experimental gigantism in rats after prolonged treatment with extracts of ox pituitary. Putnam, Benedict and Teel[103] observed gigantism, acromegaly and splanchomegaly in bulldogs after the administration of pituitary extracts and Smith[104] showed that hypophysectomised rats ceased to grow and that a resumption of growth could be obtained by giving anterior lobe extracts.

Action. The action of this hormone in skeletal growth is independent of other endrocrine glands in that it causes growth in the absence of the adrenals, thyroid or gonads[105,106]. It does not depend on the improvement of appetite[107]. It increases the weight of most organs, including the liver, kidneys and ovaries, but it has a particularly large effect in increasing the weight of the thymus[108,109]. These effects depend upon an increase in the amount of protein and water in the organs; the amount of fat is generally diminished. The formation of new protein thus seems to play a fundamental part in the response. As might have been expected from these facts, there is a decrease in the non-protein nitrogen in the blood and in the excretion of urea. Nitrogen is retained in the body and used for the synthesis of protein[108,110,111].

Growth hormone also increases the weight of the bones. This effect is accompanied by an increase in the concentration of inorganic phosphate in the plasma of men, dogs or rats[112]. There is a rise in alkaline phosphatase content of the bones[110] and of the plasma[113], but no change was

detected in the D-amino acid oxidase in liver or kidney, or in the succinic acid dehydrogenase in muscle[114]. These effects are best seen in young animals, but older animals can also respond, as is shown when acromegaly develops after growth has ceased. Becks *et al.*[115] found that the administration of purified growth hormone to hypophysectomised rats reawakened osteogenic and chondrogenic processes in the epiphyseal cartilage of the tibia even after a post-operative interval of a year or more. The changes produced were similar to those in the young normal growing rat.

Evidence is accumulating that the diabetogenic hormone of the anterior pituitary does not exist as a separate entity and that this effect is probably mediated by the growth hormone and the adrenocorticotrophic hormone[116]. Pure growth hormone, prepared by the method of Li *et al.*[117], has been found to be actively diabetogenic in adult intact cats. Young[109,118] believes that a close relation exists between the growth stimulating and diabetogenic actions of anterior lobe extracts. Prolonged administration of actively diabetogenic extracts to puppies initially causes acceleration of growth uncomplicated by diabetes mellitus. If, however, the treatment is continued for several months the animals cease to grow and a diabetic condition supervenes. Milman and Russell[119] administered pure growth hormone to fasting partially depancreatised or alloxan diabetic rats and observed pronounced hyperglycæmia. This effect was not evident in normal rats, which do not develop diabetes so easily as cats or dogs.

Chemistry. Li *et al.*[117] isolated the growth hormone in pure form from alkaline extracts of ox anterior pituitary. The hormone was found to be a protein of molecular weight 44,250. It was relatively insoluble in water. The *iso*electric *p*H was 6·85 and the hormone behaved as a homogeneous substance as shown by the results of experiments involving electrophoresis, diffusion and solubility. The hormonal activity was destroyed by treatment with pepsin and trypsin. Amino and tyrosine groups appeared necessary for the action of the growth hormone. The method used by Wilhelmi *et al.*[120], involved the use of alcohol precipitation at low temperatures, the method being similar to that used by Cohn in fractionating plasma proteins. The chief advantages of this technique are its relative ease and the very high yields of the hormone obtained. It was found, however, that the crystals so prepared exhibited two components on electrophoretic examination and therefore the preparation is less pure than that of Li *et al.*[117].

Assay. Three main methods of assay are available for the growth hormone[2].

1. *Body growth of normal rats.* Six-month-old female rats which have almost stopped growth are used. These animals can be made to grow by the administration of growth hormone. The chief disadvantage of this test is its relative insensitivity, and a long period of injections (up to 20 days) is required[121]. It seems however to be an accurate method[122].

2. *Body growth of hypophysectomised rats.* This test is more sensitive than the preceding. Young female rats 28 to 30 days at operation are used and injections are commenced 10 to 14 days after hypophysectomy.

Injections are continued for 10 days. When the weight gain is plotted against the logarithm of the dose a straight line is obtained.[123].

3. *Tibia of hypophysectomised rats.* In 1943, Evans, Simpson, Marx and Kilbrick[124] described a new method of assay of growth hormone depending on the increase in width of the proximal epiphyseal cartilage of the tibia in hypophysectomised rats. This test is claimed to be 3 times as sensitive as that depending on the body growth in hypophysectomised animals. In addition, injections are given for only 3 days. The technique is similar to that employed in the " line test " for vitamin D and if the width of the uncalcified cartilage is plotted against the logarithm of the dose a straight line is obtained. Kinsell *et al.*[125] detected growth hormone in acromegalic blood by this method.

Summary

Six active substances are found in extracts of the anterior pituitary. All of them have been isolated as pure, or nearly pure, proteins. Much progress has been made recently in the development of sensitive methods of assay, and if all these methods are as good as they claim to be, it is now possible to estimate all these hormones in the blood or urine of patients when their concentrations are abnormally high. On the other hand, there are no standard preparations of these substances. Since the gonadotrophic proteins isolated from sheep are not the same as the gonadotrophic proteins isolated from pigs, there should perhaps be a set of standards for each species, but there is no evidence for this at present.

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RESEARCH PAPERS

THE COMBINED EFFECT OF PENICILLIN AND OF SULPHONAMIDES IN INFECTIONS WITH GRAM-NEGATIVE ORGANISMS

PARTS I AND II.

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Received December 10, 1949

PART I

INTRODUCTORY

THE synergism between penicillin and various sulphonamide compounds against Gram-positive organisms *in vitro* and *in vivo* was demonstrated by several workers[1-9]. Regarding the Gram-negative bacilli, very little was done to show how they are affected by the combined action of penicillin and the sulphonamides. T'ung[10] demonstrated a synergistic action between penicillin and sulphathiazole against *Brucella melitensis in vitro;* Bigger[11] reported the same phenomenon against *Salmonella typhi in vivo* and Kolmer showed a similar effect against *Salm. typhi* infection in mice.

The object of this work was to demonstrate on some common pathogenic Gram-negative bacilli, the effect of penicillin when used in combination with different sulphonamides, compared with the effect of either alone. The organisms tested were *Bacterium coli, Salm. typhi, Salm. enteritidis, Shigella flexneri* and *Proteus vulgaris.*

MATERIAL AND METHOD

A. In vitro *experiments.* The sulphonamide compounds used were sulphathiazole, sulphadiazine, sulphamerazine, sulphamezathine and sulphapyrazine. A 1/1000 solution of each was made in a meat extract nutrient broth* which was used as culture medium. The sodium salt of crystalline penicillin was used, it was dissolved in sterile saline solution and diluted to the required concentration at the time of the experiment. The test inoculum was added to the culture medium in a volume of 0·1 ml. after suitable dilution of a 24-hours culture. The susceptibility of the particular strain tested to penicillin and to the different sulphonamides was first estimated. To determine the effect of the two chemotherapeutic substances together, a series of test tubes with the culture medium were prepared, and an inhibitory concentration of penicillin was made up in 9 ml. of culture medium in the first tube. 4·5 ml. from this tube was then transferred to the second tube in the series which contained the same volume of plain broth, thus getting half the penicillin concentration of the first tube. 4·5 ml. from the second tube was transferred to the third and the process was repeated to give serial dilutions of penicillin until such a concentration was reached as was known to be

* As supplied by Burroughs Wellcome and Co.

non-inhibitory to the inoculum used. One tube in each series was left without penicillin for control. A second series of tubes were prepared in which the sulphonamide compound was added in a concentration which did not inhibit the growth of the organism completely. Serial dilutions of penicillin were then made up in the same way as in the first series. The inoculum was then added to each tube in 0·1 ml. of broth. The tubes were incubated at 37°C. for 24 hours. The results were then recorded by comparing the turbidity of the culture medium in each tube caused by the growth of the organism with that of the control tube. For convenience, numbers were given to indicate different grades of turbidity, thus indicating the degree of growth; 4 to indicate full growth i.e., as in the control tube, 0 to indicate a clear medium i.e., no growth, and 1, 2 and 3 to indicate increasing grades of turbidity corresponding to increasing degrees of growth.

B. In vivo *experiments.* White mice with an average weight of 20 g. were used. The drugs were given each in one dose at the time of infection; penicillin was given subcutaneously in 0·1 ml. of saline solution and the sulphonamides were given by mouth in 1 ml. of mucilage of tragacanth. The animals were infected by the intraperitoneal injection of a multiple of the average lethal dose of the organisms suspended in 0·5 ml. of mucin. One batch of animals was always left without treatment for control, other batches received penicillin alone, the sulphonamides alone, and the last received both chemotherapeutic substances together. The doses of penicillin and of the sulphonamides used were those shown to be only partially protective in the preliminary experiments.

Recording of the results. The number of mice surviving in each group was recorded daily up to 7 days, when the experiment was ended. The relative value of the different lines of treatment was estimated by comparing the average survival period per mouse in each group, obtained by adding together the number of days survived by each mouse and dividing the sum by the number of mice in the group.

THE COMBINED EFFECT ON *BACT. COLI.*

A. In vitro *experiments.* The result of the combined effect of various concentrations of penicillin and of 0·1 mg./ml. of each of the sulphonamide compounds is shown in Table I.

From Table I it is seen that these sulphonamides produce different effects. Sulphadiazine, sulphapyrazine and sulphathiazole were equally effective and were all more potent than sulphamezathine, while sulphamerazine was the least effective of all the drugs used. When they were used in combination with penicillin, a much more pronounced antibacterial action resulted, more with sulphadiazine and with sulphapyrazine than with the others. Thus 50 units of penicillin/ml. were required for complete inhibition of this inoculum of *Bact. coli,* while only 3·125 units/ml., i.e., 1/16th of the original concentration, were required to inhibit the growth of the same inoculum when sulphadiazine or sulphapyrazine was present in the medium as well.

TABLE I

Sulphonamide 0·1 mg./ml.	Penicillin concentration in units/ml.							
	100	50	25	12·5	6·25	3·125	1·56	0
———	0	0	3	4	4	4	4	4
Sulphadiazine	0	0	0	0	0	0	1	2
Sulphapyrazine	0	0	0	0	0	0	1	2
Sulphathiazole	0	0	0	0	0	1	2	2
Sulphamezathine	0	0	0	0	0	2	3	3
Sulphamerazine	0	0	0	0	0	3	4	4

Sulphathiazole used in the same concentration with penicillin showed the same phenomenon, although to a less marked degree; the inhibitory penicillin concentration being reduced from 50 units to 6·25 units/ml. In the 6th tube of this series, although growth was not completely inhibited, it was definitely much less than in the corresponding tube which contained penicillin only (3·125 units/ml.).

It is more interesting to observe the effect of the combination of sulphamezathine or of sulphamerazine with penicillin. Sulphamezathine was only slightly effective by itself and sulphamerazine exerted no bacteriostatic action, but when these drugs were present with a non-inhibitory concentration of penicillin, growth was completely inhibited. This effect was observed in the tubes containing 25, 12·5 and 6·25 units of penicillin/ml.

The advantage of this combination was also observed when these sulphonamides were used in a smaller concentration, 0·05 mg. per ml., this latter concentration was still less effective by itself on the growth of the inoculum. Table II shows the result of an experiment similar to the previous one, where the sulphonamides were used in this lower concentration.

TABLE II

Sulphonamide 0·05 mg./ml.	Penicillin concentration in units/ml.							
	100	50	25	12·5	6·25	3·125	1·56	0
———	0	0	3	4	4	4	4	4
Sulphadiazine	0	0	0	0	0	2	2	3
Sulphapyrazine	0	0	0	0	0	2	3	3
Sulphathiazole	0	0	0	0	0	2	3	3
Sulphamezathine	0	0	0	3	4	4	4	4
Sulphamerazine	0	0	0	0	3	4	4	4

Sulphadiazine, sulphapyrazine and sulphathiazole were the best drugs for showing this " synergistic " effect with penicillin. In the case of the tubes containing penicillin alone in concentrations of 12·5 and 6·25

units/ml., maximum growth occurred in 24 hours as in the control tube. When sulphadiazine, sulphapyrazine or sulphathiazole was added to these tubes, growth was completely inhibited, although the sulphonamide concentrations alone was only slightly inhibitory. Sulphamerazine and sulphamezathine on the other hand, did not exert any bacteriostatic effect on this inoculum when used in a concentration of 0·05 mg./ml. Nevertheless, a combination of either with an equally ineffective concentration of penicillin resulted in complete inhibition of the growth. From these observations it could be concluded that penicillin and any of these sulphonamide compounds acting together, exerted a greater antibacterial effect than either alone. Moreover, concentrations of either substance which, when used alone, were non-inhibitory or only slightly inhibitory, resulted, when used together, in complete inhibition of the growth.

B. In vivo *experiments.* Penicillin in a comparatively big dose, 2,000 units, did not show an appreciable therapeutic value against *Bact. coli* infections in mice. The different sulphonamides, however, showed variable protective effects against this infection; sulphadiazine and sulphapyrazine being the best, then sulphathiazole, sulphamerazine and sulphamezathine. The value of the combined use of doses of penicillin and of the different sulphonamide compounds is demonstrated in Table III where the average survival periods of several groups of animals under different schemes of treatment are recorded.

TABLE III

Treatment		Number of Mice Used	Average Survival Period Days
Penicillin	Sulphonamide		
—— ——	Sulphadiazine 0·1 mg.	18	1·7
2000 units	Sulphadiazine 0·1 mg.	12	4·1
—— ——	Sulphapyrazine 0·1 mg.	12	1·7
2000 units	Sulphapyrazine 0·1 mg.	12	5·0
—— ——	Sulphathiazole 0·5 mg.	30	2·3
2000 units	Sulphathiazole 0·5 mg.	24	5·7
—— ——	Sulphamerazine 0·5 mg.	12	3·1
2000 units	Sulphamerazine 0·5 mg.	6	5·8
—— ——	Sulphamezathine 0·5 mg.	6	1·7
2000 units	Sulphamezathine 0·5 mg.	6	5·5
2000 units	—— —— ——	60	1·4
Untreated controls	... ...	60	0·4

From the results in Table III it is seen that the average survival period per mouse was 1·7 days in the groups treated with sulphadazine or with sulphapyrazine or with sulphamezathine; when each of these drugs was combined with penicillin, the survival times were increased by: 2·4, 3·0 and 3·2 times respectively. Similarly, the average survival time was increased by 2·5 times when sulphathiazole was used with penicillin than when the same dose of sulphathiazole was used alone.

It is important to emphasise here that the marked increase in the average survival period was not the only improvement observed in those

animals having the combined therapy, but the general condition of the mice as judged by their activity and food consumption showed a very noticeable difference; the mice which received combined therapy and survived the experiment were very active and appeared quite normal; on the other hand the survivors from the groups receiving single treatment were very ill and much less active. This difference does not appear from the Tables as all these latter were counted as survivors.

Part II.

The Combined Effect on *Salmonella Typhi.*

A. In vitro *experiments.* The combined effect of penicillin and of the different sulphonamides on *Salm. typhi* showed the same synergistic phenomenon as against *Bact. coli.* Table IV shows the result of the presence of the sulphonamides in a concentration of 0·2 mg./ml. in the culture medium together with various concentrations of penicillin, compared with the effect of either alone on the growth of the organisms.

TABLE IV

Sulphonamide 0·2 mg./ml.	Penicillin concentration in units/ml.							
	20	10	5	2·5	1·25	0·6	0·3	0
———	0	0	4	4	4	4	4	4
Sulphathiazole	0	0	0	0	0	1	1	1
Sulphapyrazine	0	0	0	0	1	1	1	1
Sulphadiazine	0	0	0	0	2	2	2	2
Sulphamezathine	0	0	0	0	0	2	2	2
Sulphamerazine	0	0	0	0	1	1	3	3

Inoculum 20,000 organisms approximately. Tubes were incubated for 24 hours at 37°C.

Sulphathiazole and sulphapyrazine in this concentration were more active than the other sulphonamide compounds, and their combined effect with penicillin resulted in a greater degree of inhibition than when sulphadiazine, sulphamezathine or sulphamerazine were combined with penicillin. Although 10 units of penicillin/ml. were required to prevent the growth of this inoculum, only 1·25 units/ml. were sufficient to prevent the growth when sulphathiazole was present in the culture medium as well. A similar result was obtained when the same concentration of sulphamezathine was present instead of sulphathiazole. When sulphadiazine or sulphamerazine was present in the same concentration with penicillin, the inhibitory concentration of the latter was reduced from 10 to 2·5 units/ml.

When the sulphonamides were used in one half the previous concentration, although their individual bacteriostatic effect was weaker, they showed nearly the same inhibitory action when combined with penicillin (Table V).

Again, the inhibitory penicillin concentration was reduced from 10 units to 1·25 units/ml. when 0·1 mg. of sulphathiazole/ml. was present

TABLE V

Sulphonamide 0·1 mg./ml.	Penicillin concentration in units/ml.							
	20	10	5	2·5	1·25	0·6	0·3	0
———	0	0	2	3	4	4	4	4
Sulphathiazole	0	0	0	0	0	2	2	2
Sulphapyrazine	0	0	0	0	2	2	2	2
Sulphadiazine	0	0	0	0	2	3	3	3
Sulphamezathine	0	0	0	0	1	2	3	3
Sulphamerazine	0	0	0	0	1	3	3	3

with penicillin. Sulphadiazine, sulphamerazine and sulphamezathine were less active than sulphathiazole, but they also showed a "synergistic" action with penicillin. Apart from the reduction in the inhibitory penicillin concentration, it is seen that in the tubes containing 1·25 units of penicillin/ml., although the growth was not completely inhibited, it was definitely less than in the corresponding tube containing penicillin or one of these sulphonamides alone.

Therefore, the presence of these sulphonamides in partially bacteriostatic concentrations, with penicillin in non-inhibitory concentrations, resulted in complete prevention of the growth of the organism. When comparatively small concentrations of penicillin are combined with the sulphonamides, growth may not be completely prevented, but it was less when both substances were present together than when either substance was present alone.

B. In vivo *experiments.* The mice were infected by an intraperitoneal injection of 100 times the minimal lethal dose of *S. typhi.* Different groups of animals were treated with penicillin, a sulphonamide compound, or a combination of both and the average survival period per mouse in these groups was compared. The results recorded in Table VI show clearly the advantage of the combined therapy.

TABLE VI

Treatment		Number of mice used	Average survival period days
Penicillin	Sulphonamide		
——— ———	Sulphadiazine 1 mg.	12	1·7
2000 units	Sulphadiazine 1 mg.	12	6·3
——— ———	Sulphapyrazine 2 mg.	12	1·2
2000 units	Sulphapyrazine 2 mg.	12	6·1
——— ———	Sulphamezathine 2 mg.	12	1·9
2000 units	Sulphamezathine 2 mg.	12	6·0
——— ———	Sulphathiazole 5 mg.	12	2·4
2000 units	Sulphathiazole 5 mg.	12	6·5
——— ———	Sulphamerazine 5 mg.	12	0·7
2000 units	Sulphamerazine 5 mg.	12	6·1
2000 units	... ...	12	1·4
Untreated controls	... ...	30	0·2

In all the cases, the average survival period resulting from the combined therapy was 6 days or more out of a maximum of 7 days, whereas in no case was it as much as 2·5 days when similar sulphonamide doses were used alone. The increase in the survival time as a result of the combined therapy was 3·7 times in the case of sulphadiazine, 3·1 times with sulphamezathine and 2·7 times with sulphathiazole. A dose of 2 mg. of sulphapyrazine or of 5 mg. of sulphamerazine exerted a small protective action, less than that of 2,000 units of penicillin, but still when penicillin was given together with either of these sulphonamide doses, the average survival time of the mice was 4·3 times as long as that resulting from penicillin alone.

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THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS. PART III. 5:6-DISUBSTITUTED BENZIMINAZOLES AS PRODUCTS OF ACID HYDROLYSIS OF VITAMIN B_{12}

By G. R. Beaven, E. R. Holiday, E. A. Johnson, B. E. Ellis, P. Mamalis, V. Petrow and B. Sturgeon.

This Journal, 1949, **1,** 957

Corrections

Page 960, figure 3. Replace 1:5:6-Trimethylbenziminazole by 5:6-Dimethylbenziminazole. Replace 5:6-Dimethylbenziminazole by 1:5:6-Trimethylbenziminazole.

THE PENETRATION OF PHENYLMERCURIC DINAPHTHYL-METHANE DISULPHONATE INTO SKIN AND MUSCLE TISSUE

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Received November 1, 1949

DURING recent years various crystalloid salts of phenylmercuric hydroxide, notably the nitrate and acetate, have attained wide clinical use for the treatment of infections of the skin [1,2,3,4,5,6]. Although these salts have an extraordinarily high antibacterial activity which is not significantly impaired by the presence of serum, pus and tissue debris [3,7] yet they possess little or no capacity for penetration of and substantive fixation to skin and muscle.

The present communication relates to a colloidal salt of phenylmercuric hydroxide which, by virtue of the surface active and hydrotropic properties of the colloidal anion, rapidly penetrates living skin and fixes itself substantively to the subdermal connective tissue. The advantages of penetrative and protein substantive properties in a bactericide for topical dermatological application are twofold. The reagent penetrates to the site of organisms which have invaded the skin and hair follicles in depth and builds up a high concentration in these regions ; the irreversibility of the adsorption process retards absorption of the organomercurial into the general circulation.

Phenylmercuric dinaphthylmethane disulphonate is a white amorphous salt containing 40·8 per cent. of organically bound mercury. Although almost insoluble in water it rapidly dissolves in aqueous solutions of alkali metal dinaphthylmethane disulphonates to yield stable colourless solutions which may be buffered to any desired *p*H value. In these solutions the solutes are highly ionised and behave as typical colloidal electrolytes. Colloidal electrolytes possess two properties by virtue of which they influence biological activity, viz., their strong tendency to adsorb at interfaces and their power to form charged hydrated colloidal aggregates (micelles). Micelles, owing to their structure, adsorb ions carrying a charge opposite to that upon the micelle and in addition are able, by the process of molecular adsorption, to solubilise many organic substances which are normally insoluble in water. Micellar solubilisation is known to facilitate transport of sparingly soluble substances at a rate which may be enormously greater than the normal process of solution and diffusion. Unless otherwise stated the solution used in the present work contained 0·1 per cent. of phenylmercuric dinaphthylmethane disulphonate, 1·9 per cent. of potassium dinaphthylmethane disulphonate and 1·0 per cent. of the potassium dihydrogen phosphate-disodium hydrogen phosphate buffer system in water at *p*H 7·0.

Two methods have been employed for the determination of the extent of penetration after application to skin and muscle. Sections were made in planes perpendicular to the plane of penetration and these sections developed histochemically in order to visualise the area containing

phenylmercuric ion ; sections were made in planes parallel to the plane of penetration and submitted to quantitative chemical analysis for mercury.

Penetration of Phenylmercuric Dinaphthylmethane Disulphonate into Dead Muscle Tissue

Longissimus dorsi muscles, from freshly killed rabbits, were cut into cubes having *ca.* 15 mm. edges and these were immersed in the 1 : 1000 solution at 20°C. for 24 or 48 hours. The treated cubes were washed in running water for 6 hours in order to remove excess of reagent and then immersed in isotonic formol-saline for 24 hours. After washing for 24 hours in running water the cubes were embedded in gelatin and 40μ sections cut on the carbon-dioxide freezing microtome. Sections were developed in order to visualise (a) phenylmercuric ion and (b) dinaphthylmethane disulphonate ion. (a) The sections were dried between filter papers and flooded for 5 minutes on slides with a freshly made 0·2 per cent. carbon tetrachloride solution of dithizone. They were then immersed for 3 minutes in 10N aqueous ammonia and finally left floating in 2N ammonia overnight. This procedure removes excess of dithizone, leaving the insoluble phenylmercuric complex of dithizone as a deep brown peripheral margin surrounding a white central area. (b) The freshly cut wet sections were flooded for 3 minutes with a 0·5 per cent. aqueous solution of crystal violet, washed for 15 minutes in running water, immersed in 1 per cent. w/v sulphuric acid for 15 minutes and then left floating in a large volume of water overnight to remove excess of the stain. The insoluble dinaphthylmethane disulphonic acid salt of the crystal violet base remains as a bright purple peripheral margin surrounding the colourless central area of the section.

The depth of penetration of each ion into the muscle was measured by taking 10 readings from each of 10 sections, the mean value being recorded. The effect of the cation of the solubilising salt upon the depth of penetration is shown in Table I : all the solutions contained 0·1 per cent. of phenylmercuric dinaphthylmethane disulphonate and 1·9 per cent. of the alkali metal dinaphthylmethane disulphonate (solubilising salt) and 1·0 per cent. of the phosphate buffer system at *p*H 7·0 (Fig. 1).

TABLE I

Solubilising Salt cation	Penetration of Phenyl-mercuric ion		Penetration of Dinaphthyl-methane disulphonate ion	
	24 hours	48 hours	24 hours	48 hours
Sodium	0·40 mm.	0·66 mm.	0·42 mm.	0·82 mm.
Ammonium	0·48 mm.	0·78 mm.	0·44 mm.	0·90 mm.
Lithium	0·51 mm.	0·95 mm.	0·58 mm.	1·18 mm.
Potassium	0·52 mm.	1·04 mm.	0·62 mm.	1·20 mm.

The depth of penetration in all cases was substantially the same with solutions at *p*H values of 6·0, 7·0 and 8·0 ; at *p*H 4·0, however, there was less penetration.

Other muscle cubes immersed in the same solution for 48 hours at 20°C. were washed by immersion for 6 hours in slowly stirred distilled water and then mounted in gelatin in a metal mould in order to preserve the original cubic shape. Layers 1 mm. thick were removed from 5 faces of the cube and the remainder of the cube sectioned in a plane parallel to the remaining 6th surface, commencing from this surface. Sections of 80μ thickness were cut, these burnt with sulphuric acid and hydrogen peroxide to destroy all organic matter and the resulting mercuric sulphate determined by chemical analysis. The weight of each section was computed from its physical dimensions and its density, the density of the muscle being determined by the method of flotation using normal cubes and aqueous solutions of sodium chloride of varying specific gravity.

Typical results from separate determinations with muscle taken from three different rabbits I, II and III are shown in Table II. In the case of muscle cubes from rabbit No. III, the cubes IIIa, after treatment, were given the standard wash of 6 hours while the cubes IIIb were given a prolonged wash in large volumes of repeatedly changed distilled water for 48 hours in order to determine the extent of fixation of the compound to the muscle tissues, i.e. the degree of irreversibility of the adsorption process.

TABLE II

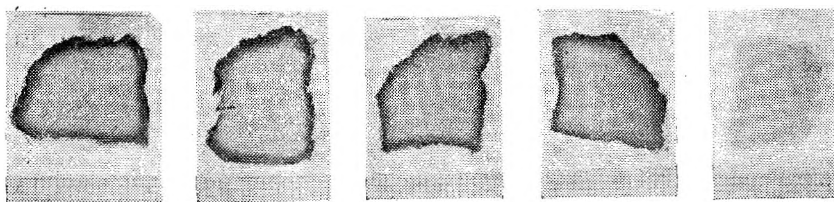
Section	Hg Found (Micrograms)				g. of compound per kg. of Tissue			
	I	II	IIIa	IIIb	I	II	IIIa	IIIb
1st (Top)	110	274	220	204	14·2	14·1	12·7	10·0
2nd	73	245	160	144	9·4	12·6	9·3	7·1
3rd	54	—	118	100	7·0	—	6·8	4·9
4th	43	—	110	98	5·6	—	6·3	4·8
5th	42	104	96	96	5·4	5·4	5·6	4·7
6th	35	—	88	90	4·5	—	5·2	4·4
7th	34	—	76	74	4·4	—	4·3	3·6
8th	30	81	66	72	3·9	4·2	4·2	3·5
9th	28	—	63	66	3·6	—	3·8	3·2
10th	24	78	52	60	3·1	4·0	3·5	2·9

Area of sections : I, 16 × 13 mm. ; II, 29 × 18 mm. ; IIIa, 31 × 15 mm. ; IIIb, 25 × 22 mm. Thickness of sections : 0·080 mm. Muscle density : 1·14 g./c.c.

Cubes of muscle tissue immersed for 48 hours in 1 : 1000 aqueous phenylmercuric acetate and then given standard 6 hours rinse prior to sectioning showed, by the histochemical method, a penetration in low concentration to a depth of 0·28 mm. When the intact muscle cubes were given a prolonged wash of 24 hours the sections, after developing with dithizone, appeared completely white with no peripheral margin showing complete absence of phenylmercuric ion. This was confirmed by the method of quantitative chemical analysis.

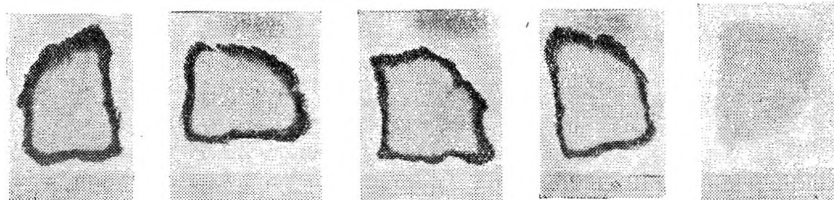
Two important properties of phenylmercuric dinaphthylmethane

disulphonate present themselves from Table II : (i) it is substantive to muscle tissue, i.e. the compound is adsorbed upon the tissue to build up a concentration in the latter greater than that in the bathing solution, (ii) the adsorbate is strongly resistant to removal by washing i.e. the adsorption process is not easily reversible. The low penetration of phenylmercuric acetate and its complete removal from the cubes by 24 hours washing presents evidence that the penetrating and substantive properties must be associated with the presence of the colloidal anion.



*p*H 4·5 *p*H 6·0 *p*H 7·0 *p*H 8·0 Control

Penetration of Phenylmercuric Ion.



*p*H 4·5 *p*H 6·0 *p*H 7·0 *p*H 8·0 Control

Penetration of Dinaphthylmethane Disulphonate Ion.

FIG. 1. Penetration of Phenylmercuric dinaphthylmethane disulphonate into rabbit muscle. Small squares are equivalent to 1 sq. mm.

PENETRATION OF PHENYLMERCURIC DINAPHTHYLMETHANE DISULPHONATE THROUGH LIVING SKIN INTO SUBCUTANEOUS CONNECTIVE TISSUE

The following is typical of experiments performed many times. An area of *ca.* 5 sq. cm. of a rabbit's back was shaved and the skin painted with the 1 : 1000 solution 3 times per day for 3 days. At the end of this time the skin was healthy, elastic to the touch and showed no sign of scaling or irritation. The animal was killed, the treated area dissected to a depth of *ca.* 1·5 cm. and this, after washing for 3 hours in water, cut into cubes in such a manner that one face of each cube consisted of the original skin. The cubes were embedded without distortion in a metal mould and frozen on the microtome stage with the skin uppermost and perfectly horizontal : sections (a) of 40μ thickness were made of the skin in a plane parallel to the surface of the latter. Other cubes were mounted on the microtome with the skin surface vertical and sections

(b) made in planes perpendicular to the surface of the skin. Sections (a) were stained and developed to show penetration and fixation of phenylmercury ion and also analysed for mercury content. Sections (b) after removing the thin strip of skin were stained and developed to show penetration of the reagent into, and fixation upon, the subcutaneous connective tissue. In all, 50 slides were examined in order to record depths of penetration.

The results are shown photographically in Fig. 2. The phenylmercury ion penetrates through the epidermis, the papillary and reticular layers of the corium and the subcutaneous adipose connective tissue—a total

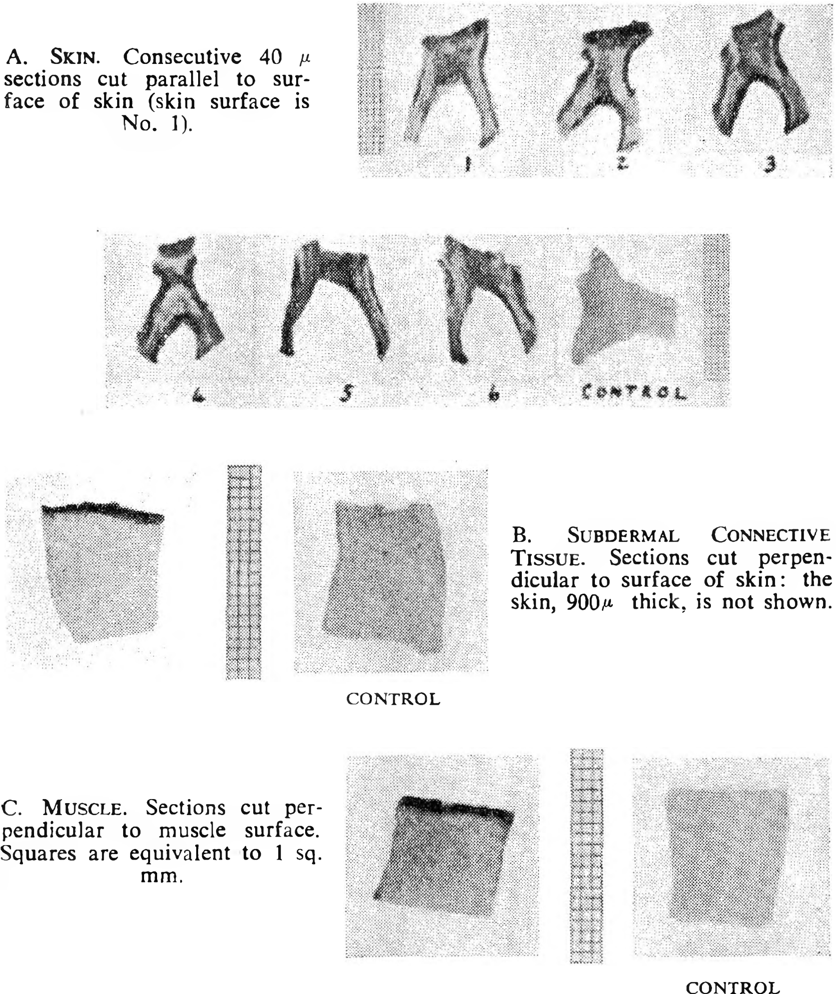


FIG. 2. Penetration of phenylmercuric dinaphthylmethane disulphonate through living skin into subdermal tissue and into exposed living muscle after treatment with a 1 in 1000 solution applied three times a day for three days. All the sections were developed with dithizone to show the fixation of the phenylmercuric ion.

in the rabbit of *ca.* 1500μ—and then for *ca.* 700μ into the subdermal muscular tissue, the total penetration being of the order of 2·2 mm. There is a heavy concentration of the reagent in and around the hair follicles.

In order to determine quantitatively the depth of penetration through living skin into subdermal tissue an area of *ca.* 10 cm.$\times$5 cm. of the lateral dorsal region of several rabbits was shaved. Lint dressings soaked in the 1 : 1000 solution were applied, these covered with oiled skin and cotton wool, and secured by bandaging ; a fresh soaked dressing was applied each morning for 3 consecutive days The animals were killed, the treated areas dissected to a depth of *ca.* 1·5 cm. and cubes of the muscles with the attached skin cut in the same manner as described above. The skin was stripped off the cubes, burnt with sulphuric acid and perhydrol, and the total mercury content assayed. The average thickness of the skin was computed from its area, weight and density, the density being determined by the method of flotation. The subdermal muscle was sectioned in planes parallel to the plane of the skin surface and the total mercury determined in each section. Table III records the results found in two typical experiments with two adult rabbits.

TABLE III

Tissue	Hg found (micrograms)		g. of compound per kg. of tissue	
	(a)	(b)	(a)	(b)
Skin	68	64	0·29	0·28
Subdermal Muscle				
1st Section	41	37	2·8	2·8
2nd ,, ...	38	36	2·6	2·7
3rd ,, ...	34	32	2·3	2·4
4th ,, ...	24	29	1·6	2·2
5th... ,, ...	18	20	1·2	1·5
6th... ,, ...	12	16	0·8	1·2
7th... ,, ...	8	10	0·5	0·7

Area of skin and Sections (a) 25$\times$16 mm. ; (b) 24$\times$15 mm. Thickness of skin : (a) 1·41 mm. ; (b) 1·50 mm. Density of skin : 1·03 g./c.c. Thickness of Muscle Sections : 0·080 mm. Density of Muscle : 1·14 g./c.c.

It is apparent that phenylmercuric dinaphthylmethane disulphonate passes through the living skin and enters deeply into the subdermal connective tissue and muscle ; at the 6th section of the latter, a depth of *ca.* 2·0 mm. below the surface of the skin, the concentration of the adsorbed substance is *ca.* 1 g./kg., i.e. the concentration of the compound in the solution that was applied. At the depth of the first section of the muscle, *ca.* 1·5 mm. below the skin surface, the concentration of the adsorbed substance is of the order of three times that existent in the applied solution.

Systemic Toxicity

Phenylmercuric dinaphthylmethane disulphonate solution was administered to 20 g. mice, in groups of 4 by the oral and the intraperitoneal route. Because of the well known delay in the appearance of toxic symptoms after the ingestion of heavy metal salts the animals were observed for 14 days before recording results.

Oral: LD100 80 mg./kg.; LD50 70 mg./kg.; LD0 50 mg./kg.

Intraperitoneal: LD100 50 mg./kg.; LD50 25 mg./kg.; LD0 10 mg./kg.

Guinea-pigs, in groups of 2, were given single oral doses of 5, 10, 20, 30, 40 and 50 mg./kg. of phenylmercuric dinaphthylmethane disulphonate and then kept on a diet of oats and fresh vegetables: the average initial weight of the animals was 300 g. After 6 weeks one animal which had received 50 mg./kg. was killed and the organs examined. The liver and kidneys were of normal size but contained numerous hæmorrhages and necrotic patches; sections of both organs after development with dithizone showed deposits of mercury. The spleen was of normal size; there were no histological changes but mercury deposits were present. At the end of 3 months the other 11 animals appeared normal and their average weight had increased to *ca.* 450 g. On autopsy no macroscopic or microscopic change was visible in the liver, kidneys or spleen. Sections of these organs developed with dithizone in all cases gave a negative test for mercury. (This reagent detects a concentration of mercuric, mercurous or phenylmercuric ion in section tissue of 1 mg./kg.)

Tissue Toxicity

Muscle cubes from freshly killed rabbits which had been immersed in solutions containing 0·1 per cent. of phenylmercuric dinaphthylmethane disulphonate plus 1·9 per cent. of potassium dinaphthylmethane disulphonate at *p*H values ranging between 6·0 and 8·0 for 48 hours at 20°C. maintained their original texture and retained the softness and elasticity of fresh normal muscle. Muscle thus treated is digested by pepsin and by trypsin almost to the same extent as untreated muscle. Complete gastrocnemius muscles of rabbits treated in this manner retained 90 per cent. of their normal elasticity as recorded on the kymograph.

Rabbit intestine which had been immersed in the 0·1 per cent. solution at *p*H 7·0 for 24 hours was found to be freely permeable to neutral red, glucose and fructose although rather less so than normal intestine.

Influence on the Opsono-Phagocytic Index.

Tubes containing 0·05 ml. of an emulsion of living *Streptococcus pyogenes*, 0·05 ml. of a suspension of washed rabbit leucocytes, 0·01 ml. of normal rabbit serum and varying amounts of solution were incubated for 15 minutes at 37°C. Slides were prepared and 50 polymorphonuclears examined for ingested bacteria.

TABLE IV

0·1 per cent. Solution	Number of Ingested Bacteria	Opsonic Index
ml.		
nil	200	4·0
0·01	185	3·7
0·025	174	3·5
0·05	160	3·2

Bactericidal and Antimycotic Activity of Phenylmercuric Dinaphthylmethane Disulphonate

The potentiating effect of the colloidal dinaphthylmethane disulphonic ion upon the bacteriostatic activity of the phenylmercuric ion in media containing up to 80 per cent. concentrations of serum has been reported in a previous communication[7]. The bactericidal activity recorded below is the time taken in minutes for the 1 : 1000 solution to effect sterilisation of a 24 hour subculture ; this time is given in parentheses after each organism. The organisms were grown in double strength broth for 24 hours and then an equal volume of 1 : 500 solution added and the solutions rapidly mixed. At intervals of 5 minutes a loopful was removed and inoculated into 50 ml. of fresh broth which was then incubated for 48 hours at 37°C.

Staphylococcus aureus (25), *Escherichia coli* (25), *Bacillus subtilis* (30), *Pseudomonas pyocyaneus* (20), *Bacillus proteus* (20), *Staphylococcus citreus* (25), *Staphylococcus albus* (25), *Streptococcus fæcalis* (20), *Pseudomonas fluorescens* (20), *Bacillus mesentericus* (20).

Czapek's medium reinforced with 0·1 per cent. of asparagine was used for the first 6 fungi in Table V, the serial dilutions being incubated at 22°C. for 7 days. Growth in the controls was slow for the first 3 days but then rapidly accelerated ; absence of turbidity was recorded as the maximum inhibitory dilution. In the case of *Monilia albicans* the medium used was Lab-Lemco made up in half strength beer-wort instead

TABLE V

Organism	Maximum Dilution in Multiples of 1000	
	Inhibitory	Sterilising
Rhizopus nigricans	10	—
Penicillium expansum	10	—
Penicillium citrinum	9	—
Penicillium notatum	16	—
Aspergillus fumigatus	7	—
Aspergillus niger	9	—
Monilia albicans	16	—
Microsporon aodouini	24	20
Microsporon lanosum (*felineum*) ...	24	20
Trichophyton mentagrophytes (*gypseum*)	24	20
Trichophyton purpureum	50	32
Epidermophyton inguinale (*floccosum*) ...	50	50

of water. The fungistatic and fungicidal activities against the last 5 dermatophytes were determined in 2 per cent. glucose broth containing 1 per cent. of peptone, the dilutions being incubated at 25°C. for 21 days.

Summary

1. The pharmacology of a bactericidal and mycoticidal colloidal electrolyte, phenylmercuric dinaphthylmethane disulphonate, is described.

2. The compound, applied topically in the form of its aqueous solution, penetrates the living epidermis, dermis and connective adipose tissue, entering into the subjacent muscle. The electrolyte is substantive to protein tissue, i.e., it is adsorbed upon living muscle to build up a higher concentration in the latter than that which exists in the bathing solution. The adsorption process is not easily reversible.

3. Phenylmercuric dinaphthylmethane disulphonate solutions do not cause any significant biochemical change in body tissue as shown by the macroscopic and microscopic appearance and the elasticity of tensor muscle, the permeability of intestine and the opsono-phagocytic index.

4. The toxicity of phenylmercuric dinaphthylmethane disulphonate is of the order of that of colloidal silver and less than that of silver nitrate.

The authors thank Dr. L. Syms for the antimycotic activities against the five dermatophytes in Table IV.

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THE DETERMINATION OF AMINO-COMPOUNDS OCCURRING AS IMPURITIES IN PHARMACEUTICAL CHEMICALS

PART III.

ARSANILIC ACID IN SODIUM *p*-GLYCOLLYL-AMINOPHENYLARSONATE AND IN CARBARSONE; SULPHATHIAZOLE IN SUCCINYLSULPHATHIAZOLE

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Received November 10, 1949

IN connection with the manufacture of sodium *p*-glycollyl-aminophenyl-arsonate (known by the trade name glycarsamide), carbarsone B.P., and succinylsulphathiazole. B.P., methods were required for the determination of the free amino-compounds. The British Pharmacopœia includes a limit test for sulphathiazole in succinylsulphathiazole which is based upon diazotisation and coupling with N-naphthyl-ethylene-diamine in acidified aqueous alcohol. Carbarsone and glycarsamide, unlike acetarsol and tryparsamide, are soluble in this solvent hence there was the possibility of applying this method to them also. Consideration was also given to the use of *p*-dimethylaminobenzaldehyde which has been used for the determination of various compounds containing primary aromatic amino-groups[1,2,3,4].

DIAZOTISATION AND COUPLING METHOD

It was found that optimum conditions are not used in the B.P. limit test on succinylsulphathiazole and hence these were determined in the usual way and included in particular the use of a higher acid concentration. A further modification was the substitution of sulphamic acid for urea for removing excess of nitrous acid. Identical results were obtained at 5°C. and at room temperature hence the latter was used for convenience. Under the conditions adopted no hydrolysis of succinylsulphathiazole or carbarsone was detectable; some degree of hydrolysis of glycarsamide was expected in view of that reported for acetarsol[5] and actually was equivalent to 0·02 per cent. of amino-compound under the test conditions. Although decomposition of carbarsone, which is a derivative of phenyl-urea, by nitrous acid might have been expected, none was found. Calibration curves (extinction × concentration) for both arsanilic acid and sulphathiazole were almost linear.

To a mixture of 25 ml. of alcohol (96 per cent.), 12·5 ml. of N hydrochloric acid and 7 ml. of water, cooled to room temperature, add 0·02 g. of finely powdered sample and shake until dissolved. Add 1 ml. of 0·25 per cent. sodium nitrite solution, mix and set aside for 3 minutes. Add 2·5 ml. of 4 per cent. sulphamic acid solution, mix, set aside for 4 minutes, add 1 ml. of 0·4 per cent. solution of N-naphthyl-ethylene-diamine hydrochloride, mix and dilute to 50 ml. Carry out a determination omitting the sample. Determine the extinction of each using

Ilford 605 filter, and read the amount of amino-compound from a calibration curve.

p-Dimethylaminobenzaldehyde Method

Methods for the determination of sulphonamides with *p*-dimethylaminobenzaldehyde have been described by Werner[2] and by Morris[3] and have the advantages of speed and simplicity. Optimum conditions were determined for the tests under consideration and, under these conditions, no hydrolysis occurred. Calibration curves again were almost linear.

Reagents. 1. p-*Dimethylaminobenzaldehyde Solution.* 2·5 per cent. solution of purified[6] *p*-dimethylaminobenzaldehyde in alcohol (95 per cent.).

2. *Citrate Buffer.* 0·75 M disodium hydrogen citrate solution. (39·4 g. of citric acid dissolved in 188 ml. of 2 N sodium hydroxide and the solution diluted with water to 250 ml.)

To a mixture of 9 ml. of N hydrochloric acid, 4 ml. of buffer, 17 ml. of water and 20 ml. of *p*-dimethylaminobenzaldehyde solution, cooled to room temperature, add 0·02 g. of finely powdered sample, shake until dissolved and, after 5 minutes, dilute to 50 ml. with water. Carry out a blank determination omitting the sample. Determine the extinction of each using Ilford 601 filter, and read the amount of amino-compound from a calibration curve.

Comparison of Methods

Table I shows substantial agreement between results obtained on all three substances by the two methods.

TABLE I
Comparison of methods

Compound	Sample	Amino-compound per cent.	
		Diazotisation and Coupling	*p*-Dimethylamino-benzaldehyde
Succinylsulphathiazole	1	0·23	0·21
	2	0·48	0·47
	3	0·36	0·36
	4	0·30	0·29
Carbarsone...	1	0·48	0·50
	2	0·77	0·81
	3	0·62	0·64
	4	0·74	0·74
Glycarsamide	1	0·16	0·14
	2	0·22	0·21
	3	0·29	0·31
	4	0·27	0·29

The *p*-dimethylaminobenzaldehyde method is simpler and more rapid, but photo-electric matching is necessary because the colour is yellow and the reagent blank relatively large. In the diazotisation and coupling method the blank is almost negligible and the purple colour is suitable for visual comparison. Neither method was found suitable without modification for the determination of sulphathiazole in phthalylsulphathiazole

owing to the exceedingly low solubility of the latter and a rather high rate of hydrolysis.

Proposed Limit Tests

Limit tests for free amino-compound in carbarsone and glycarsamide with limits of 1 per cent. and 0·5 per cent. respectively may be based on the diazotisation and coupling procedure. The same test, which differs from the official test in incorporating optimum conditions is applicable also to succinylsulphathiazole. Dissolve 0·02 g. of substance in a mixture of 25 ml. of alcohol (96 per cent.), 12·5 ml. of N hydrochloric acid and 7 ml. of water, previously cooled to room temperature. Add 1 ml. of 0·25 per cent. sodium nitrite solution, mix, and set aside for 3 minutes. Add 2·5 ml. of 4 per cent. sulphamic acid solution, mix, set aside for 4 minutes, add 1 ml. of 0·4 per cent. solution of N-naphthylethylenediamine hydrochloride, mix and dilute to 50 ml. The colour produced is not greater than that produced when the appropriate amount of arsanilic acid or sulphathiazole is treated similarly.

Summary

1. Two methods are described for the determination of free amino-compounds in carbarsone, glycarsamide and succinylsulphathiazole.

2. Limit tests, with a uniform method, are proposed.

Thanks are due to Dr. E. F. Hersant for helpful comments and to the Directors of May and Baker, Ltd., for permission to publish this paper.

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A NOTE ON BACTERICIDES IN SOLUTIONS FOR INJECTION

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Received December 3, 1949

One of the methods described in the British Pharmacopœia for sterilising aqueous solutions is "heating with a bactericide." The bactericides recommended are 0·2 per cent. solution of chlorocresol (*p*-chloro-*m*-cresol) or 0·002 per cent. solution of phenylmercuric nitrate. Davis and Davison[1] have shown by means of the filtration sterility test that relatively low concentrations of bacterial spores may survive after heating with 0·2 per cent. solution of chlorocresol. In the discussion on this paper, Hartley[2] pointed out that chlorocresol in concentrations of 0·2 per cent. or less was inadequate for sterilisation and asked the authors to suggest a more suitable concentration.

Wien[3] determined the chronic toxicity of 0·25 per cent. solution of chlorocresol and of 0·002 per cent. solution of phenylmercuric nitrate by experiments on rabbits. The purpose of the experiments described in this paper was to determine the chronic toxicity of higher concentrations so that if the present concentrations are found inadequate, appropriate concentrations may be recommended.

Chlorocresol

Chronic Toxicity as determined by experiments on rabbits. In the following experiments 0·4 per cent solution in distilled water, prepared by dissolving in hot water and cooling to room temperature, was used. The experiment was made on two rabbits. Each received daily 5 ml. of the 0·4 per cent solution subcutaneously. A third rabbit used as control received daily 5 ml. of distilled water subcutaneously. The urine was examined every other day by qualitative tests for albumen and blood. Once a week the urine was examined for deposit of epithelial cells or casts. All the results were negative. The blood was examined once a week and no pathological abnormalities were found in the red or white cell counts. The variations in the white cell count were the same as in the control animal. Values are given for each week in Table I. The appearance of the rabbits remained normal in every way. There was no diarrhœa except in the case of the control rabbit which recovered in a day with careful feeding.

Histological examination of tissues. The liver and kidneys were examined. Sections were stained with eosin. Microscopic examination showed that all the specimens were normal. The skin at the site of injection showed no degenerative changes.

Effect of intracisternal injection on the pressure and cell content of the cerebrospinal fluid. The general experimental procedure was similar to that described in the literature (Bedford[3,4]). 1 ml. of 4 per cent. solution was introduced into the cisterna magna of three dogs. The animals, after recovery from the anæsthetic, were allowed to survive for 6 hours. The results are shown in Table III.

TABLE I

CHLOROCRESOL SUBCUTANEOUSLY IN RABBITS

RABBIT I (CONTROL)

	1 week	2 weeks	3 weeks	4 weeks
Weight (kg.)	1·75	1·65	1·65	1·75
Hæmoglobin, per cent.	92	90	86	82
Red cells /cu.mm	6,000,000	6,000,000	6,350,000	6,300,000
Colour index	0·8	0·75	0·68	0·65
White cells cu./mm	9,000	7,200	15,000	9,400
Lymphocytes, per cent.	68	70	67	70
Granulocytes, per cent.	32	30	33	30

RABBIT II

	1 week	2 weeks	3 weeks	4 weeks
Weight (kg.)	1·8	1·75	1·8	1·7
Hæmoglobin, per cent.	95	88	90	81
Red cells, cu./mm	6,430,000	6,350,000	6,750,000	6,550,000
Colour index	0·74	0·59	0·67	0·6
White cells /cu.mm	11,000	14,000	11,600	8,800
Lymphocytes, per cent.	66	52	55	66
Granulocytes, per cent.	34	48	45	34

RABBIT III

	1 week	2 weeks	3 weeks	4 weeks
Weight (kg.)	2·0	2·1	2·1	2·15
Hæmoglobin, per cent.	85	75	73	73
Red cells /cu.mm	6,650,000	5,900,000	6,500,000	5,650,000
Colour index	0·67	0·64	0·57	0·65
White cells /cu.mm	14,000	13,800	8,600	9,600
Lymphocytes, per cent.	68	67	67	70
Granulocytes, per cent.	32	33	33	30

PHENYLMERCURIC NITRATE

The basic salt $C_6H_5HgNo_3C_6H_5Hg(OH)$ was used. A 0·004 per cent. solution was obtained by dissolving in hot distilled water and cooling to room temperature.

Chronic toxicity as determined by experiments on rabbits. Two rabbits were used. Each received daily for 4 weeks 5 ml. of the solution subcutaneously. A third rabbit was used as control and received daily 5 ml. of distilled water subcutaneously. The urine was examined every day for albumen and blood and once a week for deposits or casts. All the results were negative. Blood examinations are shown in Table II.

Pathological examination of the tissues. The kidney and liver, and the skin at the site of the injections were examined. There were no degenerative changes.

Effect of intracisternal injection on the pressure and cell content of the cerebrospinal fluid. Two dogs were used for this experiment. Results are comparable with those with chlorocresol and are shown in Table III.

TABLE II

PHENYLMERCURIC NITRATE SUBCUTANEOUSLY IN RABBITS

RABBIT IV (CONTROL)

	1 week	2 weeks	3 weeks	4 weeks
Weight (kg.)	1·5	1·7	1·7	1·75
Hæmoglobin, per cent.	67	80	70	70
Red cells /cu.mm	5,800,000	6,000,000	5,400,000	5,400,000
Colour index	0·6	0·67	0·65	0·65
White cells /cu.mm	6,800	5,400	9,400	9,400
Lymphocytes, per cent.	70	55	70	68
Granulocytes, per cent.	30	45	30	32

RABBIT V

	1 week	2 weeks	3 weeks	4 weeks
Weight (kg.)	1·5	1·65	1·65	1·65
Hæmoglobin, per cent.	80	75	80	74
Red cells /cu.mm	6,100,000	5,900,000	5,900,000	5,600,000
Colour index	0·65	0·63	0·68	0·66
White cells /cu.mm	7,000	8,400	7,000	7,000
Lymphocytes, per cent.	55	65	70	65
Granulocytes, per cent.	45	35	30	35

RABBIT VI

	1 week	2 weeks	3 weeks	4 weeks
Weight (kg.)	1·45	1·6	1·6	1·65
Hæmoglobin, per cent.	71	73	75	75
Red cells /cu.mm	5,550,000	5,200,000	6,200,000	5,750,000
Colour index	0·64	0·7	0·6	0·65
White cells /cu.mm	9,800	5,800	6,200	4,400
Lymphocytes, per cent.	70	70	66	68
Granulocytes, per cent.	30	30	34	32

TABLE III

THE EFFECT OF INTRACISTERNAL INJECTIONS ON THE PRESSURE AND CELL CONTENT OF THE CEREBROSPINAL FLUID

Drug	Concentration per cent.	Weight of dog (kg.)	Pressure of cerebrospinal fluid		White cells per c. mm. after 6 hours
			Initial	After 6 hours	
Chlorocresol ...	0·2	7·85	90	130	4,000*
	0·4	6·0	75	135	4,800*
	0·4	11·0	100	260	10,500*
Phenylmercuric nitrate	0·002	4·3	70	130	3,600*
	0·004	7·7	80	220	2,000*

* All polymorphonuclear.

DISCUSSION

The chronic toxicity of concentrations higher than those recommended in the British Pharmacopœia has been studied in rabbits. There were no abnormalities in the urine, body weight, red and white cell counts or general health of the animals. There was no sign of necrosis at the site of injection.

Therefore, if the present concentrations are not adequate as claimed by Davis and Davison[1], it may be possible to recommend more appropriate concentrations.

Although, according to the British Pharmacopœia instructions, the method of sterilisation by heating with a bactericide is not to be used for the sterilisation of intrathecal and intracisternal injections, this recommendation is not based on any well-known experimental data.

The few experiments done on dogs demonstrate that the introduction into the subarachnoid space of chlorocresol or phenylmercuric nitrate excites powerful cell reactions and causes a rise in the pressure of cerebrospinal fluid, thus justifying on the grounds of actual experiments on animals, the Pharmacopœial instructions prohibiting the use of the method of sterilisation by heating with a bactericide for the sterilisation of intrathecal and intracisternal injections.

Summary

1. Daily injections for 4 weeks of 5 ml. of 0·4 per cent. solution of *p*-chloro-*m*-cresol were given to two rabbits. There were no abnormalities in the urine, body weight, red and white cell contents or general health of the animals.

2. Rabbits receiving daily injections (5 ml.) of 0·004 per cent. solution of phenylmercuric nitrate showed no abnormalities.

3. Pathological examination of kidney, liver and skin at the site of injection showed no degenerative changes or other abnormalities with either bactericide.

4. Intracisternal injections of solutions of *p*-chloro-*m*-cresol and phenylmercuric nitrate produce striking cell reactions in the cerebrospinal fluid and raise its pressure.

Assistance from Professor Macdonald and Dr. Bedford in regard to the necessary injections is gratefully acknowledged.

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THE EVALUATION OF THE BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL AND SOME OF ITS MONOALKYL ETHERS AGAINST *BACTERIUM COLI*

PART IX

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Received October 17, 1947

THE technique described in Part I[1] of this series of papers has been used to determine the course of disinfection of *Bact. coli* against ethylene glycol monohexyl ether[2]; this showed itself to be an asymmetrical sigmoid curve. In order to apply the methods of statistical analysis to the experimental data, it was first of all necessary to transform the results to a relationship which could be treated as though it were linear over the whole or an adequate section of its course. Success was achieved by converting the percentage survivors to probits and plotting them against log survivor time[3,4]. It was further shown that regressions from replicate experiments, when regarded as linear, could be taken as parallel, thereby enabling a mean regression to be calculated[5]. From the results of experiments conducted at different concentrations at 20°C.[6] and at 30°C.[7], it was possible to assign characteristic regression coefficients to every disinfectant substance used; it was shown that the values varied with the temperature. However, such a regression gives no indication of the bactericidal efficiency of the disinfectant, but it does, nevertheless, afford a means of assessing it.

The analogy of ED50 in estimating the activity of substances used pharmacologically, has been employed, and LT50's (the times to kill 50 per cent. of the initial inocula) have been calculated from the regression equation[8]. Determination of the time taken to reach a certain mortality level, or the comparison of concentrations of disinfectants sterilising in the same time (as in the Rideal-Walker and the Food and Drug Administration methods), gives no indication of the effect of dilution or of temperature on the disinfectant. The dilution factor is considered in the present Part and the effect of temperature in Part X.

THE CONCENTRATION EXPONENT AND ITS METHOD OF DETERMINATION

The significance of the concentration exponent n. The loss of efficiency of a disinfectant on dilution is proportional to some power of the concentration depending on the nature of the disinfectant. Watson[9] recalled that chemical reaction rates were often proportional to an exponent of the concentration and he assumed that a disinfection reaction behaved similarly. Different classes of disinfectants have different concentration exponents and hence their relative disinfection rates vary on dilution; disinfectants behave similarly on dilution only when their exponents are the same. A numerical example will make this clear. The concentration exponent, n, for mercuric chloride is generally taken as 1·0 (Gregerson[10]). This means that when any concentration of the disinfectant is doubled, the lethal time (a measure of its activity) for a standard inoculum will be

decreased by 2^n times, i.e., 2^1 (where $n=1$), or in other words, by half. Again, n for phenol is approximately 6 (Jordan and Jacobs[11]); a twofold increase in concentration results in 2^6, i.e., 64 times decrease in the disinfection time. A threefold increase in concentration would result in a 3^6, i.e., 729 times decrease in disinfection time, and so on. The germicidal ability of aqueous dilutions of substances like phenol, having high concentration exponents will increase at a rapid rate with increasing concentration, and decrease equally rapidly with increasing dilution. Substances possessing low exponents, like mercuric chloride, will decrease in activity more slowly with increasing dilution. From the list prepared by Chick[12], supplemented by Rahn[13], and from the observations of Tilley[14], Withell[15], Brownlee and Tonkin[16] and Hoffmann and Rahn[17], it is evident that a wide range of concentration exponents exists amongst disinfectant substances.

The effect of concentration of the bactericide on disinfection rate. Chick[18] put forward the following empirical relationship connecting concentration and the time for disinfection: $\frac{1}{c_o - c_n} \log \frac{c_n \, t_n}{c_o \, t_o} = a$ constant, where t_o and t_n were the times for disinfection corresponding to concentration c_o and c_n. Watson[9] modified Chick's formula and showed that the relation of the death time t to the concentration c, was more suitably expressed by the equation $c^n t = constant$; because of the logarithmic or exponential nature of this relationship, n is referred to as the "concentration exponent." It is also known as the "coefficient of dilution" of the disinfectant.

It must be realised that Watson's equation is arbitrary and cannot be absolutely accurate over all ranges of concentrations of the germicide. Every disinfectant possesses a threshold value below which no effect can be detected under the experimental conditions; hence c can have a finite value whilst t can be infinite. Jordan and Jacobs[19,20] from experiments on the disinfection action of phenol on *Bact. coli,* were able to secure evidence that n increased as c approached the threshold value. These same workers[21] constructed graphs of the virtual sterilisation times (*v.s.t.*) against phenol and showed that with the more concentrated solutions of disinfectant the curves became asymptotic to the abscissa at the higher temperatures used.

Watson's equation can also be put into the form $n \log c + \log t =$ constant, and then n can be calculated from the disinfection times t_1 and t_2 at two different concentrations c_1 and c_2, thus:

$$n = \frac{\log t_2 - \log t_1}{\log c_1 - \log c_2}$$

When log t is plotted against log c a straight line should result if the relationship be true within the range of concentrations tested; the magnitude of the slope of the regression gives the value of the concentration exponent n.

The use of death rates for the determination of n. The original relationship as postulated by Chick[18] was based on the times for complete disinfection as determined by end-point methods; the calculations

of Reichel[22,23], Gegenbauer and Reichel[24], Gregerson[10], Gegenbauer[25] and Tilley[26] were based on this technique. As experimental evidence accumulated it was believed that the velocity of the disinfection process was constant throughout its course, and hence the death rate k (as determined by counting methods) could be substituted for the death time; Watson's equation then became $\frac{c^n}{k}$ = constant, from which

$$n = \frac{\log k_1 - \log k_2}{\log c_1 - \log c_2}$$

Death rates were used by Ikeda[28], Watson[9], Paul, Birstein and Reuss[29,30], Hobbs and Wilson[31] and Withell[32] for determining n. This method is satisfactory so long as the death rate of the process is constant throughout its course. Counting methods, although somewhat more laborious than end-point methods, have the advantage that they yield several points on the death curve, thereby enabling the death time to be estimated more accurately; a death time can then be assigned to any desired level of mortality. When the death rate does not vary the velocity constant is indirectly proportional to the death time, and under certain circumstances, for example when disinfection is rapid, constant values of k are often obtained during intermediate intervals of time. However, when the process is retarded (for instance, by using less concentrated solutions) the death rate at intermediate stages in the process may be shown to vary. Such variations can only be detected by counting methods; end-point methods give only the overall reaction velocity. When the death rate varies it is difficult to decide which value to use in the equation for the determination of n and hence its employment does not give conclusive estimations.

The use of intermediate mortality levels for the determination of n. Counting methods enable the times for any level of mortality to be determined. Mention has been made in Part VIII[8] of this series of communications, of the times for different mortality levels used by research workers for the comparison of bactericidal activity. It has been argued (Withell[15]) that the value of t in Watson's equation need not necessarily be the extinction time, but that the times for other mortality levels more suitable for comparison purposes or more accurately determinable, might be substituted. However, Jordon and Jacobs[19] emphasised that the use of times for selected mortality levels must first be tried in Watson's formula to ascertain whether the equation is obeyed or not. A criterion is the relationship between the logarithms of the times for the fixed decrease in mortality (50 per cent., 99 per cent., etc.) and the logarithms of the concentrations of the disinfectant. If this is linear over the complete range of concentrations then the equation is obeyed. Nevertheless, even if Watson's equation is obeyed, the values of n may still differ when different mortality levels are used for the substitution in the equation.

Relationship between the log decrease in mortality time-log concentration regression, and the probit-log time regression. A rectilinear relationship between the logarithm of the decrease in mortality

time and the logarithm of the concentration of disinfectant is coupled with parallel probit-log time regressions; when this holds true, then any level of mortality may be chosen for the determination of n (over the range of parallelism established) because $log\ t_2 - log\ t_1 = constant$ (where t_2 and t_1 are the times for any mortality level in the range). Jordan and Jacobs[19] criticised Withell's[15] substitution of LT50 for t in Watson's equation, in that he did not prove parallelism of the probit-log time regressions over the range of concentrations of disinfectant considered. Jordan and Jacobs[19] also showed that the relationship of log LT50 to the logarithm of the concentration in some of Withell's experiments was curvilinear and could not therefore be used to give accurate and reliable values of n; a similar effect was observed when the log LT50's from their own results were plotted against the appropriate log concentrations. A rectilinear relationship, however, did exist over a smaller range embracing the lower concentrations of disinfectant, and here the probit-log time regressions were roughly parallel. Later results by these workers[20] have shown that this regression could be regarded no longer as bilinear, but that of a very asymmetrical sigmoid curve. The concentration exponent calculated from this portion of the curve did not differ significantly from that calculated when the *v.s.t.'s* were used. They further demonstrated that when the times for 99 per cent., 99·9 per cent. and 99·999999 per cent. mortality levels were used, the relationship became rectlinear over the whole range of concentrations of disinfectant used. The substitution of any mortality time down to 99 per cent. mortality for the extinction time in Watson's equation was therefore equally justified. These workers[21] also demonstrated that within the ranges of concentrations chosen, linear relationships existed between log *v.s.t.'s* and log phenol concentrations for experiments conducted at several temperatures.

Jordon and Jacobs[19] found that the value of n varied with the time for the degree of mortality (99 per cent., 99·9 per cent. or 99·999999 per cent.) chosen for the calculations. They preferred to use the value of the time from the highest mortality level because it was nearest to that which could be obtained from a technique based on extinction time—the complete sterility demanded in practice. Owing to the inaccuracies of end-point methods, n's calculated from extinction times themselves, cannot be considered reliable.

It is possible that inaccurate observations, due to the great speed of the reaction caused by the concentrated solutions employed, might have been responsible for the departure from linearity of the log LT50-log concentration of disinfectant relationship in Withell's[15] results. If this were so then it would not be possible to determine n with any considerable accuracy from such concentrated solutions. With his highest concentrations of phenol, Withell[31,32,33] obtained LT50's of less than one minute in many of his calculations. The error attached to the estimation of these times must have been large and consequently the divergence from linearity in log LT50-log concentration of disinfectant relationships must not be taken as final on the basis of these observations.

When disinfection is rapid the log percentage survivor-time curve is likely to be rectilinear, suggesting a constant death rate of the disinfection process. The average of the intermediate death rates may then be taken as equal to the overall death rate, and in these circumstances the time for any mortality level may be used in the equation $c^n t$ = constant for calculating n. Jordan and Jacobs[19] plotted some of Withell's[33] results for the disinfection of *Bact. coli* against *para*chlor*meta*cresol, as log LT50 against log concentration of disinfectant and found that a rectilinear relationship existed, although many of the LT50's were less than 1 minute and in some instances less than 30 seconds. To what extent a linear relationship really holds can only be determined by experiment.

Calculation of the Concentration Exponents of Ethylene Glycol and its Monoalkyl Ethers at 20°C. and 30°C.

For all the compounds investigated, the mean log LT50's at each concentration (collected from Tables 2 and 4, Part VIII[8]) were plotted against log concentration. Every regression simulated rectilinearity and in some instances the fit was remarkably good. The magnitude of the slope of the regression gives the value for the concentration exponent n. A more accurate value of n is obtained by calculation of the regression coefficient; this method also affords a means of estimating the standard error of the slope and is therefore to be preferred. Table 1 sets out the relevant data for all the compounds. The slopes of the regressions have been calculated in the usual manner by the method of least squares. The error mean squares of these regressions have been computed and used to estimate the standard errors of each slope.

Discussion

Justification of the use of LT50 as the basis for the determination of n. Over the ranges of concentrations of disinfectants investigated, the standard errors of the log percentage concentration-log LT50 regressions are satisfactorily small, indicating that a rectilinear relationship may be assumed to exist. These results diverge to a certain extent from the relationship found by Jordan and Jacobs[19], who showed that taken over a wide range of concentrations of phenol, the regression of log percentage concentration-log LT50 was curvilinear. Perhaps if the present investigations had been conducted over a wider range of concentrations a similar result would have been observed. Nevertheless, the utilisation of LT50 in Watson's[9] formula, $c^n t$ =constant, is quite justified in the present series of experiments since the probit-log time regressions for the different concentrations of the same substance have previously been shown to be parallel (Parts VI[6] and VII[7]). Furthermore, concentrations which would give very small values of LT50 (and possibly subject to large experimental error), were avoided; from Table I it is seen that the values of LT50 rarely fell below 10 minutes, and the small standard errors indicate that these points have been satisfactorily estimated.

The magnitude of the concentration exponents. The values of n for ethylene glycol (15·8654 at 20°C. and 18·4582 at 30°C.) are extremely high; in fact they are the highest ever recorded for a disinfectant sub-

TABLE I

CALCULATION OF THE CONCENTRATION EXPONENTS OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS

(*a*) FOR EXPERIMENTS AT 20°C.

	Ethylene glycol		Monomethyl ether		Monoethyl ether		Monopropyl ether		Monobutyl ether		Monohexyl ether	
	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50
	1·860	2·237	1·628	2·734	1·398	2·493	0·892	2·101	0·544	2·123	$\bar{1}$·602	2·393
	1·875	2·108	1·653	2·357	1·439	2·025	0·954	1·738	0·574	1·847	$\bar{1}$·628	1·927
	1·889	1·811	1·677	2·008	1·477	1·739	1·000	1·432	0·602	1·484	$\bar{1}$·658	1·657
	1·903	1·546	1·699	1·786	1·512	1·218	1·079	0·884	0·628	1·384	$\bar{1}$·677	1·568
	1·917	0·982			1·544	1·977			0·653	0·989	$\bar{1}$·699	1·395
	1·929	1·374										
	1·954	0·793										
b ...	− 15·865418		− 13·187371		− 10·533373		− 6·449768		− 10·036182		− 9·817096	
s_b ...	± 1·256		± 1·882		± 0·863		± 0·680		± 1·158		± 1·293	

(*b*) FOR EXPERIMENTS AT 30°C.

	Ethylene glycol		Monomethyl ether		Monoethyl ether		Monopropyl ether		Monobutyl ether		Monohexyl ether	
	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50	log per cent. conc.	log LT50
	1·796	2·006	1·544	1·954	1·097	2·198	0·477	2·200	0·176	2·162	$\bar{1}$·512	1·991
	1·813	2·053	1·574	1·736	1·176	1·987	0·602	1·959	0·301	1·699	$\bar{1}$·544	1·803
	1·829	1·453	1·602	1·413	1·243	1·563	0·699	1·740	0·398	1·345	$\bar{1}$·574	1·616
	1·845	1·198	1·628	1·104	1·301	0·885	0·788	1·431	0·477	0·915	$\bar{1}$·602	1·248
											$\bar{1}$·627	0·951
b ...	− 18·458239		− 10·227064		− 6·289254		− 2·484941		− 4·061068		− 9·075544	
s_b ...	± 2·896		± 1·685		± 0·693		± 0·469		± 0·469		± 0·366	

(In this table the magnitude of the slope of the regression, b, equals the concentration exponent n.)

stance. The nearest approach to these figures are those for ethyl alcohol (11·3) and normal butyl alcohol (12·0) when *Bact. typhosum* was the test organism (Tilley[26]). The significance of high values of n is that substances possessing them lose their bactericidal efficiency very rapidly on dilution; this point has previously been discussed at the beginning of this paper. At both temperatures of the experiments the value of n for the other compounds decreased as the homologous series of the mono-alkyl ethers was ascended; the minimum value was reached in the monopropyl ether (in both sets of data) after which an increase was observed. These figures emphasise the necessity of a knowledge of the concentration exponent of a bactericide and the fallacy of assuming that compounds of similar chemical constitution have similar germicidal activities.

The effect of temperature on the concentration exponent. It is desirable to know whether the differences between the values of n at the two temperatures are significant or not. The calculations and results for this exercise are set out in Table II; the figures have been abstracted from the previous table. The method of the derivation of the terms is illustrated by reference to the ethylene glycol results. In this instance the 2 separate regressions account for 1·586034 and 0·452799 of the sum of squares of y_{20} and y_{30} leaving $(1{\cdot}779256 - 1{\cdot}586034) = 0{\cdot}193222$ and $(0{\cdot}529233 - 0{\cdot}452799) = 0{\cdot}076434$ for the 2 error items. Taking y_{20} and y_{30} together, this means that $1{\cdot}586034 + 0{\cdot}452799 = 2{\cdot}038833$ was taken out by the 2 regressions for 2 degrees of freedom in all, while there remained $0{\cdot}193222 + 0{\cdot}076434 = 0{\cdot}269656$ corresponding to the 7 error degrees of freedom.

In the analysis of variance the Common Regression is given by the *SS* pool. The Difference between regressions $= 2{\cdot}038833 - 2{\cdot}031481 = 0{\cdot}007352$ and the Error $= 0{\cdot}269656$. The mean squares have been calculated and a t test applied to test the significance of the difference between the 2 regressions. It is seen that the probability lies between 0·7 and 0·6, indicating that the difference is not significant. The other results were calculated in a similar manner.

For ethylene glycol, the monomethyl ether and the monohexyl ether, no significant difference between the values of n at the 2 temperatures could be detected. The values of n at 30°C. for the monoethyl, monopropyl and monobutyl ethers, however, were significantly lower than the corresponding values at 20°C. Tilley[26] secured similar observations for phenol and some of its derivatives, also for ethyl and normal butyl alcohols, against *Bact. typhosum* and *Staph. aureus* at several temperatures; in all these instances n decreased with rise in temperature. Jordan and Jacobs[19] showed that the relationship of log LT50 to log concentration of disinfectant in some of their own results as well as some of Withell's[15], was curvilinear and would therefore give different values of n over different sections of the regression. In a later communication they argued that n must increase as the concentration approached the threshold value and secured experimental evidence to show that threshold values did exist and that they varied with temperature. How n varies with the temperature cannot be forecast since n itself alters according to the

extent of the range of concentrations over which it is estimated, particularly so when this range borders on the threshold value.

A more satisfactory concentration exponent. Jordan and Jacobs[21] asserted that in the light of this evidence, Watson's[9] formula could not be

TABLE II

TEST OF SIGNIFICANCE OF THE DIFFERENCE BETWEEN n'S OF THE SAME COMPOUND AT 20°C. AND 30°C.

(a) SUMMARY OF TOTALS FROM CALCULATIONS OF LOG CONCENTRATION-LOG LT50 REGRESSIONS AT 20°C. AND 30°C. (ABSTRACTED FROM TABLE I.)

Item	Ethylene glycol			Monomethyl ether		
	20°C.	30°C.	Total	20°C.	30°C.	Total
$S[(x-\bar{x})(y-\bar{y})]$ ...	−0·099969	−0·024531	−0·124499	−0·037913	−0·040181	−0·078044
$S(x-x)^2$	0·006301	0·001329	0·007630	0·002811	0·003924	0·006735
$S(y-\bar{y})^2$	1·779256	0·529233	2·308489	0·515185	0·415485	0·930670
N	5	2	7	2	2	4
SS for individual regressions ...	1·586034	0·452799	2·038833	0·511347	0·410422	0·921769
b	−15·8654	−18·4583	−16·3170	−13·4874	−10·2271	−11·5877
SS pool	—	—	2·031481	—	—	0·904683

Item	Monoethyl ether			Monopropyl ether		
	20°C.	30°C.	Total	20°C.	30°C.	Total
$S[(x-\bar{x})(y-\bar{y})]$ ...	−0·140452	−0·145678	−0·286130	0·140483	−0·125574	−0·266057
$S(x-\bar{x})^2$	0·013334	0·023163	0·036497	0·021471	0·050534	0·072005
$S(y-y)^2$	1·490587	1·006395	2·496982	0·920581	0·320812	1·241393
N	3	2	5	3	2	5
SS for individual regressions ...	1·479433	0·916206	2·395639	0·919169	0·312044	1·231044
b	−10·5334	−6·2893	−7·7789	−6·4498	−2·4849	−3·7024
SS pool	—	—	2·237723	—	—	0·983447

Item	Monobutyl ether			Monohexyl ether		
	20°C.	30°C.	Total	20°C.	30°C.	Total
$S[(x-\bar{x})(y-y)]$ ...	−0·074338	−0·205222	−0·279560	−0·058343	−0·075445	−0·133785
$S(x-\bar{x})^2$	0·007409	0·050534	0·057943	0·005943	0·008313	0·014256
$\bar{S}(y-y)^2$	0·761985	0·840435	1·602420	0·605356	0·708874	1·314230
N	3	2	5	3	3	6
SS for individual regressions ...	0·745868	0·833420	1·579288	0·572759	0·684704	1·257463
b	−10·0362	−4·0611	−4·8247	−9·8171	−9·0755	−9·3847
SS pool	—	—	1·349189	—	—	1·255748

(b) ANALYSIS OF VARIANCE

Compound	Item	*SS*	*N*	Mean sq.	*t*	P
Ethylene glycol	Common regression	2·031481	1	2·031481	$t_{[7]} = \sqrt{\frac{\cdot 0007352}{0 \cdot 038522}}$	0·7—0·6
	Difference between regressions ...	0·007352	1	0·007352		
	Error	0·269656	7	0·038522	= 0·437	
Monomethyl ether	Common regression	0·904683	1	0·904683	$t_{[4]} = \sqrt{\frac{0 \cdot 017086}{0 \cdot 002225}}$	0·05
	Difference between regressions ...	0·017086	1	0·017066		
	Error	0·008901	4	0·002225	= 2·780	
Monoethyl ether	Common regression	0·237723	1	2·237723	$t_{[5]} = \sqrt{\frac{0 \cdot 157916}{0 \cdot 020268}}$	0·05—0·02
	Difference between regressions ...	0·157916	1	0·157916		
	Error	0·101343	5	0·020268	= 2·790	
Monopropyl ether	Common regression	0·983447	1	0·983447	$t_{[5]} = \sqrt{\frac{0 \cdot 247766}{0 \cdot 002036}}$	0·001
	Difference between regressions ...	0·247766	1	0·247766		
	Error	0·010150	5	0·002036	= 11·000	
Monobutyl ether	Common regression	1·349189	1	1·349189	$t_{[5]} = \sqrt{\frac{0 \cdot 230099}{0 \cdot 006029}}$	0·01—0·001
	Difference between regressions ...	0·230099	1	0·230099		
	Error	0·030147	5	0·006029	= 6·180	
Monohexyl ether	Common regression	1·255748	1	1·255748	$t_{[6]} = \sqrt{\frac{0 \cdot 001715}{0 \cdot 009461}}$	0·7—0·6
	Difference between regressions ...	0·001715	1	0·001715		
	Error	0·056767	6	0·009461	= 0·426	

considered entirely satisfactory and suggested a new method of treating disinfection data for the calculation of *n;* this method took into account the existence of the minimum effective concentration. Since the *v.s.t.*-concentration curves became asymptotic to the abscissa at the higher concentrations of disinfectants considered it was not possible to estimate with accuracy the concentration at which the *v.s.t.* was zero, i.e., the concentration at which the disinfection became almost instantaneous. A " maximum " effective concentration was arbitrarily chosen and assigned a *v.s.t.* of 10 minutes. Since the experimental technique made it difficult to determine *v.s.t.* values of less than 10 minutes, little accuracy would be forfeited by allotting this time to the maximum effective concentration; with improved technique the 10 minutes would be reduced. At each temperature there would be a different maximum effective concentration each having a *v.s.t.* of 10 minutes. At constant temperature (*v.s.t.*-10) plotted against the concentration should fall from infinity to zero as the concentration of disinfectant rose from the threshold value to the maximum effective concentration; sigmoid curves asymptotic to the ordinates at the minimum and maximum concentrations would therefore be obtained when log (*v.s.t.*-10) were plotted against concentration. A suitable equation was found to describe the curve; one of the constants of the formula partook of the nature of a concentration exponent which was constant over the full effective concentration range and for all temperatures.

SUMMARY

1. The conception of the concentration exponent has been expounded and the principles of the methods for its determination have been discussed.

2. The conditions for the legitimate substitution of an intermediate mortality level for the extinction time in Watson's[9] equation have been elucidated.

3. The concentration exponents of ethylene glycol and its monoalkyl ethers at 20°C. and 30°C. have been calculated from the log LT50-log concentration regressions. The standard errors of these regressions were all satisfactorily small.

4. The values of n for ethylene glycol (15·8654 at 20°C. and 18·4582 at 30°C.) are believed to be the highest ever recorded for a disinfectant substance.

5. At both temperatures of the experiments the value of n decreased as the homologous series of the monoalkyl ethers was ascended. The minimum value was reached in the monopropyl ether after which an increase was observed.

6. For ethylene glycol, the monomethyl ether and the monohexyl ether, no significant difference between the values of n at the 2 temperatures could be detected. The values of n at 30°C. for the monoethyl, monopropyl and monobutyl ethers were significantly lower than the corresponding values at 20°C.

7. Reference has been made to the proposal of Jordan and Jacobs[21] to establish a more satisfactory concentration exponent which was constant over a wide range of concentrations and for all temperatures.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Aneurine in Pharmaceutical Products, Determination of. R. Patrick and J. F. H. Wright. (*Analyst*, 1949, **74**, 303.) A modification of Holman's mercuric oxide oxidation of aneurine followed by the measurement of the fluorescence in aqueous acetone solution has been used for the determination of aneurine in aneurine tablets, vitamin "B group" tablets, injectable "B group" solutions, a multi-vitamin concentrate in aqueous alcohol and chocolate-malt based granules containing vitamins A, B_1, C and D and vanillin. The technique was simpler than the Jansen method and the precision and accuracy were higher; all reagents were stable for long periods, the purification of *iso*butyl alcohol was eliminated, and the acetone used was prepared by heating the commercial product with activated carbon under reflux and distilling. Alterations in the concentrations of potassium hydroxide and mercuric chloride were made to prevent the precipitation of mercuric oxychloride. An aqueous solution of 1-methyl-5-aminoacridine hydrochloride was preferred to quinine sulphate as standard, because of its greater stability. Fluorimeter readings were found to be directly proportional to aneurine concentrations over the range used, the results being based on measurements with a Klett instrument using a Corning 586 filter in the exciting beam and Corning 430 and 038 filters in front of the photo-cell. Reproducible results were obtained in the assay of tablets containing aneurine, the coefficient of variation being 1·6 per cent. With the chocolate-malt granules very poor results were obtained by simple acid extraction. Separation of the aneurine from interfering substances was obtained by adsorption on synthetic zeolite, previously activated by washing alternately with boiling 3 per cent. solution of acetic acid and with a 25 per cent. solution of potassium chloride in 0·1N hydrochloric acid until no precipitate appeared when the potassium chloride washings were made alkaline; the results obtained from nine samples of chocolate malt granules containing 0·1 mg. of aneurine per g. showed a coefficient of variation of 2·0 per cent. R. E. S.

Ephedrine, Colour Reaction of. H. Wachsmuth. (*J. Pharm. Belg.*, 1949, **4**, 186.) By warming a slightly alkaline solution of ephedrine with 1 drop of a 1 per cent. ninhydrin solution, a violet colour is produced. This may be extracted with amyl alcohol. The colour has a maximum absorption of 550 mμ. The reaction appears specific, since it is not given by some 40 alkaloids and bases which were tried. The sensitivity is about 1 in 5,000. G. M.

Picric Acid in Picrates, Colorimetric Determination of. R. Stöhr. (*Biochem. J.*, 1949, **44**, XXXV.) Dissolve quantities of picrates corresponding to 0·5 to 2·5 mg. of picric acid in 1 ml. of 20 per cent. anhydrous sodium carbonate solution or 1 per cent. sodium hydroxide solution in a 12·5 ml. marked test-tube, and add 5 ml. of 0·600 per cent. glucose solution (equal to 30 mg. of glucose) and water to a total volume of 11 ml. Heat in a boiling water-bath for 10 minutes, cool, and fill to the 12·5 ml. mark. Reading is made in the colorimeter against a standard solution prepared as follows: in a 12·5 ml. marked test-tube heat for 10 minutes in a boiling water-bath 5 ml. of 0·600 per cent. glucose solution, 5 ml. of water and 1 ml. of 20 per

cent. sodium carbonate, or 1 ml. of 1 per cent. sodium hydroxide. After cooling, add 1 ml. of 0·100 per cent. picramic acid (Egerer, *J. biol. Chem.*, 1918, **35**, 565) and fill with water to the 12·5 ml. mark. The author used a Klett colorimeter; height of the standard 20 mm. The method enables the determination of the molecular weight in organic basic substances in picrates of known constitution, whereas in picrates of unknown constitution the equivalent weight can be determined. S. L. W.

Theophylline, Mercurimetric Detection and Determination of. J. Bosly. (*J. Pharm. Belg.*, 1949, **4**, 66.) The composition of the precipitate obtained by the addition of mercuric acetate to a solution of theophylline indicates that it is a mercury salt of theophylline, B_2Hg2H_2O. The reaction may be used qualitatively as a test for theophylline; 1 ml. of a 0·1 per cent. solution of theophylline gives with one drop of a 5 per cent. solution of mercuric acetate a crystalline precipitate, soluble in mineral acids or in a large excess of reagent. Using the microscope it is possible to detect 1 to 2 µg. of theophylline. For the detection of theophylline in theobromine, 0·6 g. of the latter is shaken for 15 minutes with 1 ml. of water, and the mixture is then filtered on a small glass filter. The solution is treated with one drop of mercuric acetate solution (0·5 per cent.) and after several hours is examined for the characteristic crystals. It is possible to detect 0·1 per cent. of theophylline in theobromine. For quantitative work, an excess of a freshly prepared and filtered solution of mercuric acetate is added to the solution under examination, and, after filtering, the excess of mercury in the filtrate is determined by titration with 0·1N thiocyanate. For the assay of theophylline ethylenediamine and theophylline monoethanolamine it is necessary first to neutralise the solution. When determining theophylline in theobromine or caffeine, it is essential that the volume of mercuric acetate added should not be more than 50 per cent. more than that required to precipitate the theophylline. G. M.

Thiomersalate, Polarographic Determination of. J. E. Page and J. G. Waller. (*Analyst*, 1949, **74**, 292.) A polarographic method has been developed for the measurement of small amounts of thiomersalate (sodium ethyl mercurithiosalicylate) in pharmaceutical preparations. The substance gave, in acid or neutral solution, a characteristic polarogram with two well-defined steps. Concentrated hydrochloric acid (1·0 ml.) and 1·0 ml. of 0·1 per cent. gelatin solution were added to a volume of solution containing between 0·1 and 1·0 mg. of thiomersalate and diluted to 10 ml. A 3 ml. portion of the diluted solution was transferred to the polarograph cell, nitrogen was bubbled through it for 10 minutes to remove oxygen and it was examined over the potential range 0 to $-0·8$ v.; the height of the step appearing at about $-0·5$ v. was then measured. For solutions containing suspended matter (e.g., alum-precipitated vaccines), either the precipitate was filtered off before addition of the acid or the final solution containing suspended matter was examined in a polarograph cell connected through an agar bridge to a saturated calomel electrode, the latter being used as anode. Determinations by the two procedures on vaccines containing 0·01 per cent. of thiomersalate agreed to within 10 per cent. The recoveries of added thiomersalate from vaccine preparations were very good but those from a crude prolactin preparation and from liver extract preparations were less satisfactory; for vaccines containing about 0·01 per cent. of thiomersalate the recovery was 90 per cent. or more. Antiseptics of the phenol type, such as *p*-chloro-*m*-cresol, most organic substances and metallic ions (except those of antimony, arsenic, bismuth, cadmium, tin, titanium and vanadium) did not interfere.

Thiomersalate could be determined in vaccine preparations, but not in whole blood or in ter- and quinquevalent antimony preparations, such as stibophen and sodium antimonyl gluconate. Determinations on liver extracts containing about 0·01 per cent. of thiomersalate were usually about 20 per cent. low, but as this loss was consistent, a calibration curve could be used. Phenylmercuric nitrate and acetate, mercurochrome, mercuric nitrate and mercuric chloride were examined in acid solution at negative potentials; only the phenylmercuric salts formed similar steps. In an ammoniacal cobalt buffer solution thiomersalate gave catalytic steps of similar shape to, but of a much lower height than, those given by cysteine. R. E. S.

d-Tubocurarine Chloride, Determination of. D. Klein and S. M. Jordan. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38,** 438.) A multiple chemical assay procedure has been developed for *d*-tubocurarine chloride in dilute solution based on three different properties of the substance. Measurement of optical rotation gives a roughly quantitative value, while direct determination of the extinction at 280·5 mμ and photometric determination of the pink Reineckate in alcoholic solution at 525 mμ yield values which agree with each other and with the known concentrations. The combination of all three procedures may be used to identify and determine *d*-tubocurarine chloride in solution.

S. L. W.

FIXED OILS, FATS AND WAXES

Ground-Nut Oil. K. Ramamurti and B. N. Banerjee. (*Indian J. med. Res.*, 1948, **36,** 371.) A study has been made of the quality and stability of ground-nut oils commercially available in India. There was no direct relationship between the colour and acidity of the oil although, broadly, they were related. As regards the extraction and refining processes it was found that the incorporation of broken, mouldy, and shrivelled grains raised the acidity from 3 to 4 times, while subjecting the seeds to heat-treatment before extraction of the oil nearly doubled the acidity. The process of refining an oil of high free fatty acid content did not increase its stability. The rate of hydrolysis of ground-nut oils using pancreatic lipase was studied in comparison with fresh cow ghee as a standard; fresh ground-nut oil showed a satisfactory hydrolysis rate and samples possessing up to 2 per cent. acidity gave a slight fall in the rate of hydrolysis, although with oils of higher acidity the fall was rapid and a highly rancid sample showed only slight hydrolysis. There was a gradual decrease in the rate of hydrolysis of the oil, with increasing acidity. The fall in the rate of hydrolysis of the oil after cooking was more marked if the acidity was higher than 2 per cent. of free fatty acids. The hydrolytic curve with pancreatic lipase showed that an acidity of less than 1 per cent. is desirable; such an oil can be prepared if damaged nuts are removed before crushing. The presence of a high free fatty acid content caused the inactivation of carotene and vitamin A. Vitamin A was more easily inactivated with fried oil than with raw oil; refining and hydrogenation of a high acidity ground-nut oil did not prevent the inactivation to any considerable extent. The addition of an anti-oxidant such as ethyl gallate was effective in prolonging the period of inactivation of vitamin A only so long as the free fatty acid content of the oil was below a certain limit; as the free fatty acid content of the oil increased the protection afforded by the anti-oxidant decreased. An oil containing less than 1 per cent. of free fatty acids caused little inactivation of carotene or vitamin A. The removal of the free acidity from a high-acidity oil did not improve the storage property and the addition of an anti-oxidant then failed to increase its

storage life. It was considered that ground-nut oils should only be used for edible purposes if containing less than 1 per cent. of free fatty acids.

R. E. S.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Cardio-active Toad Poisons. K. Meyer. (*Pharm. Acta Helvet.*, 1949, **24**, 222.) Toad venom has been used in Chinese medicine for centuries under the name of Ch'an Su. The author examined a purified extract from this material, using chromatography on alumina, and succeeded in isolating 7 compounds, as follows :

	Substance	Yield	M. Pt. °C.	$[\alpha]_D$ in Chloroform	Probable Formula
1	α-Sitosterin	1·5 g.	150–152	−39·9°	$C_{29}H_{50}O$
2	Cinobufagin	14·0 g.	216–217	− 3·6°	$C_{26}H_{34}O_6$
3	Bufalin	3·0 g.	244–248	− 8·7°	$C_{24}H_{34}O_4$
4	Bufotalin (impure)	1·0 g.	168–180	+ 1·7°	$C_{26}H_{36}O_6$
5	Cinobufotalin	1·1 g.	259–262	+10·7°	$C_{26}H_{34}O_7$
6	Gamabufotalin (as acetate) ...	0·9 g.	265–266	−10·4°	$C_{26}H_{36}O_7$
7	Telocinobufagin	0·15 g.	160–175 210–211	+ 4·4°	$C_{24}H_{34}O_5$

The four free bufogenins (Nos. 2, 3, 5 and 7) were tested biologically on cats, and gave values for the mean lethal dose ranging from 0.1 to 0.2 mg./kg. The identity of fraction 6 could not be confirmed with certainty, as sufficient data for comparison are not available. Compound 7, which appears to be different from all previously reported bufogenins, shows a double melting-point (from acetone). Cinobufotalin, which has also been reported in material from this source, was not obtained.

G. M.

ORGANIC CHEMISTRY

Phenolic Compounds, Adsorption of, on Aluminium Oxide. C. O. Björling. (*Farm. Revy*, 1949, **42**, 588.) The adsorption on aluminium oxide of three series of compounds, di-, mono-, and non-phenolic has been studied. The first series examined consisted of adrenaline, oxedrine and ephedrine; the second consisted of alkaloids (apomorphine, morphine, codeine, and dionine); while the third consisted of simple benzene derivatives (catechol, resorcinol, hydroquinone; guaiacol, phenol; veratrole, anisole). In all the series especially in the first and third, the (ortho) diphenolic compounds were adsorbed more strongly than the mono-phenolic substances, and these, in turn, more strongly than the non-phenolic ones. Adrenaline, apomorphine, and catechol were retained by the adsorbent, both in alcoholic and aqueous solution, and were desorbed only by acid. Oxedrine, morphine, and phenol and guaiacol were desorbed slowly and incompletely by alcohol, fully by water, and in the series stated were intermediate in behaviour between the di-phenols and the non-phenolic compounds which were easily removed from the column. The adsorption of the substances investigated seemed to be independent of their basic properties. With reference to acidic properties, the more acidic apomorphine was adsorbed more strongly than morphine and both much more than the non-acidic codeine and dionine:

in series 3 no clear connection could be traced apart from the fact that the acidic phenols were adsorbed more strongly than the non-acidic compounds. Experimental details of the technique are given for both macro- and micro-scale operations, the macro scale employing about 0·5 millimol. of the substance to be tested and 10 g. of the oxide in a tube, 1 cm. wide, while the micro scale used about 0·005 millimol, of the substance and 0·4 g. of the oxide in a column, 3 mm. wide. R. E. S.

TOXICOLOGY

Arsenic Contents of Human Organs after Fatal Arsenic Poisoning. F. Hansen and K. O. Møller. (*Acta Pharmacol. Toxicol.*, 1949, **5**, 135.) An account of an investigation into the relative arsenic contents, based on quantitative determinations, of isolated organs and blood from normal human subjects, from individuals treated with ordinary therapeutic doses of arsenic preparations and from individuals dying of acute arsenic poisoning. The material in the last-mentioned group consisted of organs obtained from 20 individuals who had swallowed arsenic trioxide, usually in the form of a cattle wash, either accidentally or intentionally, together with the results of analyses of a further 20 cases appearing in the literature. The figures for arsenic content in the blood of patients treated with inorganic arsenic preparations were based on those observed in 57 patients treated with neoarsphenamine, and figures were also obtained for arsenic content of blood and organs of two patients who died after treatment with organic arsenic preparations. The data obtained are set out in the following table:—

	Arsenic concentration in mg./100 g.		
	Liver	Kidneys	Blood
Normals	0·001—0·01	0·001—0·01	0—0·002
Treated with inorganic arsenic preparations ..			0·01—0·025
Treated with organic arsenic preparation ..	*ca.* 0·1(1)	*ca.* 0·1(1)	*ca.* 0·2(2)
Acute Poisoning[3] ..	1 to 50	0·5 to 15	0·1 to 1·5

(1) About 4 to 14 days after conclusion of treatment.
(2) About 8 days after conclusion of treatment.
(3) Death occurred ½ to 14 days after intake of poison. S. L. W.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

nor-Adrenaline from the Adrenal Gland, Isolation of. S. Bergstrom, U. S. von Euler and U. Hamberg. (*Acta. Chem. Scand.*, 1949 **3,** 305.) The isolation of *l*-nor-adrenaline from cattle adrenals is reported. It occurred together with *l*-adrenaline in the approximate proportions 1:4, the mixture of these bases being isolated from the crude protein-free extract by mean of ion-exchangers. The bases were then separated with counter-current distribution between 0·02N hydrochloric acid and phenol; after extraction of the phenol with ether, pure *l*-nor-adrenaline was isolated as the crystalline base by addition of ammonia. Ultimate analyses are given together with the ultra-violet absorption spectra and the X-ray powder diffraction patterns of the isolated product. These properties were identical with those found for a synthetic specimen. Colorimetric and biological tests also indicated that synthetic and natural samples were identical. R. E. S.

Gramicidin S, Tyrocidine, and Gramicidin, Diffusion Experiments on. K. O. Pedersen and R. L. M. Synge (*Acta chem. scand.*, 1949, **2,** 408). In an attempt to evaluate the molecular weight of these peptides, the diffusion constants were measured by means of the Lamm scale method (*Nova Acta Reg. Soc. Scient. Uppsal.*, 1937, **4,** (6) 10) the results being calculated according to the "area method" and according to the "moment method." The agreement between the results in acetic acid and in ethyl alcohol solutions suggested that the molecular states of the peptides in the different solvents were not greatly different. For gramicidin S, if a spherical unhydrated molecule is assumed having partial specific volume 0·81 the diffusion constants correspond to molecular weights in the range 1080 to 1880, suggesting that gramicidin S has the cyclodecapeptide structure. If the same degree of asymmetry/solvation was assumed for tyrocidine as for gramicidin S and the partial specific volume was taken as 1·75, the diffusion data indicated a molecular weight in the range 1900 to 5100. The diffusion of gramicidin was studied only in 70 per cent. (v/v) ethyl alcohol and, making the same assumption as with tyrocidine, partial specific volume 0·80, the molecular weight range indicated was 2800 to 5000. R. E. S.

Saponins, Rate of Hæmolysis by. A. Sols. (*Nature*, 1949, **164,** 111.) By increasing the density of a saponin solution, a suspension of erythrocytes can be superposed without mixing; on standing, sedimentation of the erythrocytes will occur. The rate of incorporation of the erythrocytes into the saponin solution is less than that of complete reaction between saponin and erythrocytes (cholesterol), hæmolysis continuing until the drug is exhausted. There will therefore be a linear relationship between the quantity of saponin and that of liberated hæmoglobin, which can be estimated by colour measurement after discarding the residual erythrocytes. A saponin solution of density *ca.* 1·020 was used and an equal volume of the suspension of erythrocytes was placed upon it. Practical details of the procedure are given. Digitonin liberated from 20 samples of oxalated human blood 90 (84 to 95) mg. of hæmoglobin per mg. of drug, from 3 samples of rat blood 88 to 92 mg., from 3 samples of guinea-pig blood 77 to 84 mg., and from 1 sample of rabbit blood 78 mg.; irregular results were obtained with some samples of defibrinated sheep blood. Sapindus saponin (Merck) liberated from human blood 200 mg. of hæmoglobin per mg. of drug. A slow reacting saponin (Kahlbaum) liberated 60 mg. of hæmoglobin per mg., the value at 37°C. being identical with that obtained at room temperature (17°C.). The hæmoglobin/saponin relation was found to be linear within wide limits. For small liberations of hæmoglobin a blank may be necessary although a hæmoglobin result of less than 15 mg. per cent. is of doubtful value. A slow reacting saponin in an extract of *Hepatica triloba* Chaix., gave the same linear relationship as the other saponins studied. R. E. S.

Vitamin A in Shark-Liver Oil, Protection by Anti-Oxidants. S. M. Bose and V. Subrahmanyan. (*Ind. J. med. Res.*, 1949, **37,** 11.) The influence of acidity and moisture and of diffused and direct sunlight on the protective action of a combination of *iso*butyl gallate and citric acid for shark liver oils has been studied. The oils were stored under varying conditions in sealed bottles at 40°C., and were examined at intervals, the normal analytical figures and vitamin A content being determined. For the study of the influence of diffused sunlight on the protection of vitamin A by anti-oxidants, samples of shark-liver oil (acid value 0·68), treated with and without anti-oxidants (0·02 per cent. of *iso*butyl gallate—0·01 per cent. of citric acid), were spread out into a number of closed Petri dishes. One batch of dishes containing both

control and treated oils was stored in a dark cupboard at room temperature (30° to 35°C.) while the other batch containing similar samples was stored in diffused light; dishes were removed at intervals for examination. Results indicated that a high free acidity adversely affected the keeping properties of the oils and lowered the efficiency of added anti-oxidants; the adverse affect of moisture was more marked with oils of high acidity than of low acidity. Considerable destruction of vitamin A in shark liver oils exposed to sunlight occurred and anti-oxidants were ineffective in preventing this; the protective power of anti-oxidants was reduced in the presence of diffused light although the difference between the rates of destruction of vitamin A in the control samples stored in diffused light and those stored in darkness, was small. The destruction of vitamin A in sunlight was considered to be due to increased temperature and to ultra-violet light. R. E. S.

BIOCHEMICAL ANALYSIS

Adrenaline: Separation from Mixtures. C. O. Björling. (*Farm. Revy.*, 1949, **43**, 601.) Adrenaline is preferentially adsorbed on aluminium oxide from aqueous solutions of *p*H greater than 6, and can be quantitatively eluted with hydrochloric acid. The efficiency of the method is such that the adrenaline can be estimated by the Folin-Ciocalteu reagent without risk of interference by other phenols which may be present. The sensitivity of this reagent permits the ready estimation of small amounts of adrenaline, so that only small volumes of sample are required. The method was applied to an aqueous solution of adrenaline hydrochloride (0·00385 per cent.) and procaine hydrochloride (2 per cent.) containing chlorbutol, sodium chloride and sodium metabisulphite. 0·1 g. of tri-sodium citrate was added to 10 ml. of sample to adjust the *p*H, and the solution drawn through a column of 1 g. of aluminium oxide in a tube of 5-mm. diameter. The column was carefully eluted with water to remove the procaine hydrochloride, which gives a colour with the reagent, and then with 0·1N hydrochloric acid to remove the adrenaline. Chlorbutol and sodium metabisulphite separately tested under similar conditions were found not to interfere. G. R. K.

nor-Adrenaline in Adrenaline, Determination of. M. E. Auerbach and E. Angell. (*Science*, 1949, **109**, 537.) A standard curve is first obtained as follows. To each of five 50 ml. glass-stoppered graduated cylinders add 1 ml. of standard adrenaline solution (prepared by dissolving 100 mg. of pure synthetic adrenaline in 2·5 ml. of borax solution (5 per cent.) and diluting to 100 ml. with water); to 4 of the cylinders add respectively 0·25, 0·50, 0·75 and 1·00 ml. of standard nor-adrenaline solution (prepared freshly by dissolving 40 mg. of pure *l*-nor-adrenaline in 5 ml. of borax solution (5 per cent.) and diluting to 200 ml. with water). To each cylinder add 1 ml. of buffer solution *p*H 9·6 (Clark and Lubs), swirl, add 0·5 ml. of solution of sodium β-naphthoquinone-4-sulphonate (0·5 per cent. and used within one hour of preparation), swirl and allow to stand at room temperature for 45 minutes. Add 0·15 ml. of aqueous solution (1 per cent.) of alkyl dimethyl-benzylammonium chloride (Roccal or Zephiran) followed by exactly 10 ml. of mixed solvent (toluene 85 parts, redistilled ethylene dichloride 15 parts mixed freshly daily, washed with borate buffer and filtered through dry paper). Shake thoroughly and set aside for 45 minutes with re-shaking at intervals. The solvent layer (purplish red in the presence of nor-adrenaline) should separate clear but may be clarified if necessary by centrifuging in stoppered tubes. The extracts are transferred to colorimeter tubes and the percentage

transmission determined in a suitable photo-electric colorimeter using a green glass filter (540 mμ), the instrument being set at 100 per cent. transmission for the extract from the cylinder containing only the adrenaline. On semi-logarithmic paper, a straight line should be obtained. For examination of samples of natural adrenaline, a weighed quantity is dissolved in solution of sodium borate (5 per cent.) and diluted with water such that the solution contains borax (approximately 1 per cent.) and adrenaline (approximately 2 mg./ml.) and the solution used in place of the mixture of adrenaline and nor-adrenaline solutions in the above procedure. Samples of U.S.P. Reference Standard epinephrine examined in this way contained respectively 16·3 and 17·5 per cent. of nor-adrenaline and 4 representative samples of epinephrine U.S.P. contained 10·5 to 18·5 per cent. of nor-adrenaline. The absorption spectrum of the coloured extract showed a very broad maximum at 530 to 560 mμ. The procedure used eliminates interference by dihydroxyphenylalanine and dihydroxyphenylethylamine and the borate buffer used forms a complex with the catechol group thus diminishing chromogenic side reactions. The application of the method to the assay of fresh adrenal glands is being examined. F. H.

Aureomycin, Chemical Assay **of.** J. Levine, E. A. Garlock, and H. Fischbach. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38,** 473.) This paper presents two physico-chemical methods of assay : fluorimetric, measuring the fluorescence developed by alkaline treatment, and colorimetric, measuring the intensity of the colour developed by acid treatment of aureomycin. The former works with a lower limit of 0·25 μg./ml. and the latter with a lower limit of 20 μg./ml. Time-rate studies are included for the separate steps in the procedures. The results obtained by the two methods are in very close agreement and are directly related to biological potency of the antibiotic as shown by a comparison with results obtained by the cup-plate bioassay.

S. L. W.

Peptide-Amino-Nitrogen, Determination by the Copper Method. H. K. Kerkkonen (*Acta chem. scand.*, 1948, **2,** 518.) The method of Pope and Stevens for the determination of amino-nitrogen based on the formation of soluble copper compounds between the amino-acid or digested protein and the excess of copper present in the form of copper phosphate, has been found to give correct figures with amino-acid mixtures and with completely hydrolysed proteins. With partially hydrolysed proteins, however, the values were too high, indicating that the soluble copper compounds of peptides formed were not similar to those of the amino-acids as suggested by Pope and Stevens. A number of determinations were carried out with synthetic peptides and with hydrolysates of zein and zein plastein. Solutions of glycyl-L-leucine, glycyl-glycine, L-leucyl-L-tyrosine and L-leucyl-glycyl-glycine were prepared containing about 10 to 20 mg. of total nitrogen per 25 ml., and on each of these solutions the amino-nitrogen was determined by the copper method and by Van Slyke's volumetric nitrous acid method (5 minutes shaking), the total nitrogen being estimated by the micro-Kjeldhal method. It was found that the peptides bind twice as much copper as the amino-acids, and, in calculating the results from the amino-nitrogen value obtained by the copper method, the factor 0.14 instead of 0.28 should therefore be used. R. E. S.

Sugar in Urine or Milk; Colorimetric Estimation of. Salah El-Dewi. (*Brit. med. J.*, 1949, **1,** 899.) Fine's method (*Brit. med. J.*, 1934, **2,** 167) is adapted to the colorimeter so as to give the amount of sugar in urine or milk within 2 g./l. (0·2 g. per cent.) of that determined by Folin's method.

For the determination of urine sugar mix 5 ml. of Benedict's qualitative reagent and 0·25 ml. of urine, heat in a boiling water-bath for 5 minutes, and centrifuge. If the supernatant fluid is not blue, repeat with 0·1 ml. of urine; if it is still not blue, dilute the urine 1 in 2 or more, and repeat. The procedures for the colorimetric reading of the blue colour of the supernatant solution, using either a Duboscq type of colorimeter or a photo-electric colorimeter (Lumetron) are given, and the amount of sugar (g./l.) can be directly read for the colorimetric reading of the standard from the Table or Figures set out in the paper. For the determination of milk sugar, the proteins of milk are precipitated by adding 1 ml. of sodium tungstate solution (10 per cent.), 1 ml. of 2/3 N sulphuric acid, and 2 ml. of water to 1 ml. of milk, thus diluting milk to 1 in 5. Filter through a filter paper; the filtrate should be clear. Add 0·5 ml. of the filtrate to 5 ml. of Benedict's qualitative reagent. The procedures for the colorimetric readings are given. S. L. W.

PHARMACOLOGY AND THERAPEUTICS

Acetaldehyde, Pharamacological Action of, on Human Organism. E. Asmussen, J. Hald and V. Larsen. (*Acta Pharmacol. Toxicol.*, 1948, **4**, 311.) The authors confirmed that the normal concentration of acetaldehyde in blood is from 0·020 to 0·040 mg. per cent. By intravenous infusion of a 5 per cent. solution of acetaldehyde into normal human subjects they were able to show that at concentrations of 0·2 to 0.7 mg. per cent. in the blood there was a marked increase in heart rate, ventilation and dead space, and a decrease in alveolar carbon dioxide. Qualitatively and quantitatively the symptoms were the same as those seen after alcohol intake in persons previously treated with antabuse, which results in a similar increase in the blood acetaldehyde. The experimental subjects who had previously experienced the antabuse and alcohol effect spontaneously reported that the effects were very similar to those experienced after the acetaldehyde infusions and in both instances the same characteristic "hang-over" feeling was noticed. Since the formation of acetaldehyde is limited by the combustion rate of alcohol in the organism, and as the rate of alcohol elimination is hardly altered after antabuse treatment, the authors consider it very probable that alcohol intake after antabuse could result in dangerous concentrations of acetaldehyde in the blood. S. L. W.

Adrenaline, Metabolism of. Z. M. Bacq. (*J. Pharmacol.*, 1949, **95**, 1.) A review of information recently published on the normal metabolism of adrenaline in the body (a bibliography containing 221 references is appended). From this review the author draws the following conclusions:—(1) that adrenaline may be excreted unchanged in small amounts by the kidneys and stored in the tissues and red blood cells; (2) that its deamination by amine-oxidase in the body is unlikely; (3) that an important fraction is sulpho-conjugated; (4) that another important fraction is simultaneously oxidised to indole substance; (5) that adrenochrome and its derivatives have important biochemical and physiological properties entirely different from those of adrenaline and deserving of further study. S. L. W.

***para*-Aminosalicylic Acid Therapy, Complications of.** M. Hemming and C. J. Stewart. (*Lancet*, 1949, **257**, 174.) The authors report a case of idiosyncrasy to *p*-aminosalicylic acid. Benadryl exerted a slight suppressive action on the condition and it is suggested that it was caused by a local liberation of histamine-like substances. After the administration of

6 g. of *p*-aminosalicylic acid twice daily for 24 days the patient became acutely ill and had an intense generalised skin irritation. Withdrawal of the *p*-aminosalicylic acid produced almost immediate relief of symptoms. The patient gave no history suggesting allergy. The drug was freshly prepared on alternate days as a solution in water flavoured with liquorice. In a second patient with amyloidosis, a course of 12 g. of *p*-aminosalicylic acid daily in 6 doses for 4 weeks caused an increase in the urinary protein excretion to 20 or 30 g. a day. After withdrawal of the drug, urinary protein excretion dropped to about 10 to 12 g. per day. There was no evidence that the diseased kidneys had been irreparably damaged by the *p*-aminosalicylic acid.

A. D. O.

Antabuse and Alcohol, Effect of, on Animals. V. Larsen. (*Acta Pharmacol. Toxicol.*, 1948, **4**, 321.) In experiments with rabbits most of the symptoms so characteristic in man after antabuse and alcohol are either lacking (peripheral vasomotor reactions and increase in heart rate) or very irregularly present (increase in ventilation). Only one symptom, the increase of acetaldehyde in blood is invariably present; this increase was about 1 to 2 mg. per cent., and was about 5 times as much as is seen after the same dose of alcohol given to untreated rabbits. S. L. W.

Arterenol (Nor-Adrenaline) and Epinephrine (Adrenaline), Acute Toxicity of Optical Isomers of. J. O. Hoppe, D. K. Seppelin and A. M. Lands. (*J. Pharmacol.*, 1949, **95**, 502.) The object of this investigation was to determine the acute intravenous toxicity in mice and rats of lævo, dextro and racemic arterenol in comparison with epinephrine. In rats, *l*-epinephrine was found to be 20 times and in mice 18·5 times as toxic as *d*-epinephrine, while it was 1·8 times in rats and 1·5 times in mice as toxic as the racemic mixture. The acute toxicity ratio between *l*- and *d*-arterenol is smaller than with epinephrine. *l*-Arterenol is 14 times as toxic in rats and 12 times as toxic in mice as the *d*-isomer. No significant differences were observed between the toxicities of *l*- and *dl*-arterenol in either rats or mice. There were striking species differences between the sensitivities to these amines as judged by lethal doses, the rats being approximately 50 times as sensitive to arterenol and 60 times as sensitive to epinephrine as mice. The three optical forms of epinephrine were approximately twice as toxic as the corresponding forms of arterenol. The dose-mortality curves were very steep in rats for arterenol and epinephrine and in mice for *l*- and *dl*-epinephrine, but they were flat in mice and for *d*-epinephrine and arterenol.

S. L. W.

Chloramphenicol in Experimental Cholera. R. L. Gould, A. S. Schlengman, E. B. Jackson. M. C. Manning, H. C. Batson and C. C. Campbell. (*J. Bact.*, 1949, **57**, 349.) Chloramphenicol (chloromycetin) was found to be an effective agent in the treatment of mice with experimental cholera provided it was given in adequate dosage within 2 hours of inoculation with *Vibrio comma*. Only two deaths occurred in 90 mice after 2·5 mg. of chloramphenicol intraperitoneally at times ranging from 1 hour before to 2 hours after inoculation, as compared with 50 deaths in 60 mice receiving no treatment. When the dose was increased to 0·5 or 2·0 mg. the number of deaths was 3 out of 178. The vomiting, diarrhoea and dehydration characteristic of cholera in man do not occur in mice infected with *V. comma* and might prevent absorption from doses given orally, so that parenteral injection would be necessary. It is suggested that the drug should be tried clinically, especially for prophylaxis, in any outbreak of human cholera. H. T. B.

Colchicine, Pilocarpine and Veratrine, Toxicities of. C. F. Poe and C. C. Johnson. (*Acta Pharmacol. Toxicol.*, 1949, **5**, 110.) The toxicities for white rats were determined by intraperitoneal injections of the alkaloids and the effects on bacteria were determined by observance of their action on the normal fermentative activities of *Escherichia* and *Aerobacter*. Colchicine was shown to be highly toxic for the white rat but only very slightly toxic for bacteria; pilocarpine showed only low toxicity for both bacteria and rat; and veratrine was fairly toxic for both. S. L. W.

Decamethonium Iodide, Comparison with *d*-Tubocurarine in Controlling Electrically Induced Convulsions. J A. Hobson and F. Prescott. (*Lancet*, 1949, **256,** 819.) The use of decamethonium iodide and thiopentone for modifying electrical convulsions is described. Decamethonium iodide has all the advantages of other curarising drugs. The traumatic complications of convulsion therapy are avoided; there is no serious rise in blood pressure; and it enables convulsion therapy to be given to certain patients in whom otherwise convulsions would be contraindicated. It is to be preferred to *d*-tubocurarine for this purpose as it has no tendency to produce histamine-like reactions and as the curarisation passes off more rapidly; several patients were able to sit up within 10 to 15 minutes after the convulsion modified with decamethonium iodide, whereas the time after giving *d*-tubocurarine is rarely less than half an hour. Further advantages are that it is miscible with thiopentone without precipitation, and, being a synthetic preparation, it should not need biological standardisation. Efficient facilities for controlled respiration should be at hand; the injection of stimulants or analeptics cannot replace the provision of a clear airway and rhythmic insufflation of oxygen. It is probable that the fatalities which have occurred in using curarising drugs to modify electrically induced convulsions have been due to the inefficient methods of artificial respiration used.

S. L. W.

Heparin, Biological Assay of. M. N. Lewis and F. De Maria. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38**, 441.) A method for the routine assay of the anticoagulant activity of heparin samples is described. Citrated beef plasma is recalcified with calcium chloride in the presence of increasing amounts of heparin and the percentage of the clot formation evaluated. As the heparin concentrations are selected so as to produce an inclusive curve, from 100 per cent. to 0 per cent. clotting, the most characteristic zone of clotting (50 per cent.) can be used in the computations. Two workers reading the same series will have practically identical curves. Experience has shown that the less calcium chloride and heparin used in the clotting system the greater the sensitivity of the method. S. L. W.

Methyl *n*-Propyl Ether. C. E. Sykes. (*Brit. med. J.*, 1949, **2**, 420.) Methyl *n*-propyl ether, metopryl, $C_3H_7.O.CH_3$, is a clear, colourless liquid with a characteristic but not unpleasant odour. It boils at 39°C., and has a specific gravity at 16°C. of 0·726 and an inflammability range similar to that of diethyl ether. Previous reports on its use as an inhalation anæsthetic have shown that, compared with diethyl ether, is causes less irritation of the respiratory tract and has a somewhat higher potency and wider margin of safety. In this report, its use on 20 patients undergoing a variety of operations confirmed its lack of irritation of the respiratory tract. G. R. K

Penicillin, Concentration in Cerebrospinal Fluid. W. P. Boger and W. W. Wilson. (*Amer. J. med. Sci.*, 1949, **217,** 593.) A study was made of the length of time necessary to demonstrated the presence of penicillin in

the cerebrospinal fluid after parenteral administration in large doses. 21 patients suffering from central nervous system syphilis were treated with a single intravenous dose of 500,000 units in aqueous solution. The penicillin diffused into the cerebrospinal fluid within 2 hours in 14 of 18 individuals, and in 3 hours in 15 out of 18. 3 patients failing to show the presence of the antibiotic in the cerebrospinal fluid after this dosage showed measurable quantities when caronamide was administered intravenously in conjunction with the penicillin. In the 18 patients the cerebrospinal concentrations of penicillin were doubled by the simultaneous administration of 3 g. of caronamide. Since significant levels of penicillin are attained in the cerebrospinal fluid of all the patients studied, in whom the barrier between the blood and cerebrospinal fluid was less permeable than is the case in patients suffering from meningitis, it is suggested that parenteral, and particularly intravenous, administration of penicillin has a place in the therapy of purulent meningitis, and that intrathecal injection is unnecessary in the majority of cases. H. T. B.

Posterior Pituitary Injection: Evaluation of Antidiuretic Activity. K. M. Lindquist and L. W. Rowe. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38**, 227.) The intravenous rabbit method has been found more accurate and rapid than the previously used rat and mouse methods. 3 mg./kg. of morphine sulphate is given subcutaneously and 1·5 g./kg. of urethane intraperitoneally. The jugular vein is then cannulated for infusion of warm saline solution and the bladder or urethra for collection of the urine. To start diuresis a single injection of 25 ml. of 20 per cent. glucose solution is given intravenously 30 minutes before the first saline infusion of 50 ml. Urine is then collected for 45 minutes and measured at 5-minute intervals, immediately after which the dose of posterior pituitary injection is given intravenously and the urine again collected for 45 minutes. This permits several injections of sample and standard for comparison in the course of an 8-hour day. The modification suggested by Fugo and Aragon (*Fed. Proc.*, 1947, **6**, 330) of giving water by stomach tube (5 per cent. of the rabbit's body weight) 3 hours before the start of the antidiuretic experiment does not seem to contribute to the accuracy or uniformity of the results and its omission is desirable. The present International Reference Standard, while equal to the U.S.P. Reference Standard in oxytocic and pressor activity, was found to be only about 75 per cent. as active by the rabbit antidiuretic method. Two and often 3 doses may be directly compared in the same animal for antidiuretic activity, but 4 doses will usually give an unreliable result. S. L. W.

Pyrogens, a Quantitative Assay Method for. W. C. Ott. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38**, 179.) A method for the quantitative determination of pyrogens in water and biological products, based on the relationship between the log dose and the average maximum temperature rise following intravenous injection into rabbits, is described. Evaluation is based on the standard curve having the equation "average maximum temperature rise in °C. $= 1{\cdot}00 + 0{\cdot}78$ (log. pyrogen units/kg.)," a "pyrogen unit" being the amount of pyrogen in 0·10 mg. of a standard preparation. Under the conditions of this test one pyrogen unit caused an average maximum rise of 1·0°C. in rectal temperature. The standard error of an assay value based on the average response of 4 rabbits was estimated at approximately $\pm$ 60 per cent. Tabular values are given for evaluation of assays conducted under conditions conforming to the statistics of the standard curve, and a

(*Continued on page* 127)

BOOK REVIEWS

"ANALAR" STANDARDS FOR LABORATORY CHEMICALS. Fourth Edition. Pp. 297 and Index. Issued jointly by The British Drug Houses, Ltd., and Hopkin and Williams, Ltd., London, 1949. 10s. 6d.

The name "Analar" is now so firmly established that the realisation that it is only of fifteen years standing comes as a surprise. The addition of 58 new items brings the number of monographs to nearly 300 and many of the older specifications have been critically revised. For example, it has been found that for some chemicals, improved analytical methods have shown that previous limits for impurities had been optimistically numerated; as far as possible, the quality of the reagents has been improved in order that the former figures might be retained and, where this could not be achieved, a higher but truer limit has now been given without, however, any actual deterioration in the quality of the chemical concerned. With an eye to the future, the authors have introduced the polarograph where this instrument is particularly suited to the determination of certain impurities. For the determination of water (or should we say "aquametry"?) in organic liquids other than acetone, the Karl Fischer procedure has been applied. Electrolytic deposition has been utilised for the cleaner separation of some metals prior to the determination of alkalis and alkaline earths and, in suitable cases, has been made to serve the purpose of assay at the same time. Refractive index and density are now recorded for a standard temperature of 20°C., save for the lower alcohols, where specific gravity at 15·5°C. has been retained. The book has grown from the slim volume we used to know into something approaching a textbook of practical analysis.

B. A. ELLIS.

ABSTRACTS (*Continued from page* 126)

procedure is illustrated for determination of sensitivity and slope of the dosage-response curve. Individual rabbits appear to differ widely and characteristically in their response to a standard pyrogen and animals are chosen to minimise the variability of response. S. L. W.

Thyroid; Effect on Toxicity of Arsenobenzenes. A. Dybing. (*Acta Pharmacol. Toxicol.*, 1948, **4**, 333.) In an endeavour to find new methods for the biological assay of thyroid preparations, various substances have been examined with a view to establishing a possible effect of thyroid administration on their toxicity. This paper deals with the effect on neoarsphenamine and oxophenarsine. Experiments on mice showed that administration of thyroid powder increases the toxicity of both these compounds. In animals receiving thyroid powder in doses of 10 mg. the toxicity of oxophenarsine was found to correspond to an LD50 of about 0·02 mg./g., while in untreated animals it was about 0·03 mg./g. Similar results were obtained in corresponding experiments with neoarsphenamine. The possibility of using this effect as a basis for biological assay of thyroid preparations was studied, but the uncertainty of the method seemed too great. S. L. W.

LETTERS TO THE EDITOR

Further Observations on the "Ninhydrin-reacting" Hydrolytic Fragment of Vitamin B_{12}.

SIR,—In the course of experiments on the nature of the "ninhydrin-reacting" hydrolytic fragment of vitamin B_{12}, Ellis, Petrow, and Snook[1] compared its behaviour with that of 2-aminopropanol on unidimensional paper chromatograms, and observed that the two substances had identical partition coefficients in four different solvent systems. They accordingly drew the conclusion that the two compounds may be identical, but pointed out that a final decision must rest on a rigid chemical comparison. Billman, *et al.*[2] have recently reported on the facile conversion of 2-aminoalcohols into the corresponding aminoacids by means of acid potassium permanganate. Accordingly we have examined the oxidation of microgram quantities of authentic 2-aminoalcohols with acid potassium permanganate, followed by chromatography of the oxidation products and development with the ninhydrin reagent. Very satisfactory results were obtained, the corresponding aminoacids being obtained and unambiguously identified in every instance, 2-aminopropanol, for example, giving alanine. Oxidation of the "ninhydrin-reacting" fragment of vitamin B_{12}, after its chromatographic separation from components α-, β- and γ and subsequent elution from the paper[3], was carried out employing the same technique, and the product examined by paper chromatography using *n*-butyl alcohol/acetic acid and *iso*butyric acid as the irrigation solvents. Treatment of each of the chromatograms with ninhydrin gave rise to the appearance of only one spot which did not correspond in position with that of alanine and was, moreover, bright yellow in colour, slowly changing to purple after *ca.* 3 hours at room temperature. It was not identical with either proline or hydroxyproline.

The "ninhydrin-reacting" fragment and 2-aminopropanol represent the only instance, to our knowledge, of different compounds which cannot be distinguished from each other on replicate chromatograms irrigated with as many as four different solvent systems. Such cases must indeed be rare but serve to emphasise that chromatographic studies, *per se*, may not be sufficient for identification unless supported by collateral evidence of a chemical character.

The authors thank the Directors of The British Drug Houses Ltd. for permission to publish these results.

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December 20, 1949.

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