REVIEW ARTICLE

TISSUE MAST CELLS AND TISSUE AMINES

By G. B. West, B.Pharm., D.Sc., Ph.D., F.P.S.

Reader in Pharmacology, School of Pharmacy, University of London, Brunswick Square, W.C.1

Historical Introduction

The first description of tissue mast cells was made in 1877 during an investigation of the staining of fresh tissues by the new aniline dyes then being produced by the German chemical industry. Ehrlich[1] observed that some cells in the connective tissue of animals contained granules which changed the colour of toluidine blue and certain other dyes as staining proceeded. The numbers of such cells were greater when chronic inflammation or conditions characterised by increased local nutrition were present. For this reason, Ehrlich considered these cells to be overnourished connective tissue elements and called them "Mast cells" (Mastzellen = well-fed cells). In choosing the name of mast cell, he was also influenced by his finding of a high mast cell content in connective tissue subjected to lymph stasis; as we now know, in the selective lymphatic blockage of elephantiasis, the hypertrophied connective tissue is packed with mast cells and a similar histological picture, though on a smaller scale, is seen in young well-vascularised keloids.

Two years after his first discovery, Ehrlich[2] found similar cells in the blood, but whereas the blood mast cells, or basophils, take origin in the bone marrow and with the other leucocytes enter the peripheral blood, the more common tissue mast cells are born, live and die in the connective tissue. The similarity in the two kinds of cell lies in their content of water-soluble cytoplasmic granules which have a strong affinity for basic dyes, several of which change colour as staining occurs (metachromasia).

Besides discovering and naming the mast cells, Ehrlich also described their morphology, staining properties and distribution, but he then left it to others to elucidate the chemical nature of the granules. But, for the next 60 years, research on this subject remained almost entirely histological and then in 1937 Swedish workers solved one of the riddles of the metachromatic granules of the mast cell. Jorpes and his colleagues in Stockholm had for long been working on the powerful anticoagulant material first isolated from dog liver and hence called heparin. On finding that heparin stained metachromatically with toluidine blue, these workers[3] searched the tissues for metachromatism as a possible clue to the site of formation of heparin. And so it was that Jorpes was able to show that there is a good correlation of the mast cell count of a particular tissue and the amount of heparin that can be extracted from it. At that time mast cells were thought to be perivascular in location so as to produce heparin which pours into the blood stream. But a later study of the movement of the perivascular mast cells revealed that they migrate from the blood

vessels and hence they probably produce a secretion for both blood and tissues. Their function may be less concerned with blood clotting than with the maintenance and repair of the connective tissue.

The simultaneous release of both heparin and histamine was found by Rocha e Silva[4] to occur from the liver of the dog after anaphylactic shock, and it was suggested that perhaps both substances originated from the same cell. Chemical histamine liberators, some of which are fluorescent, were first tried to test this hypothesis. After lethal doses of such substances into rats, the fluorescent material was found to be sharply localised to the mast cells which thereafter broke up and released histamine[5]. Analysis of the tissues then indicated a good correlation of the mast cell content of a particular tissue and the amount of histamine that can be extracted from it. This is particularly evident when pathological tissues rich in mast cells are examined. For example, when the mast cell content of the skin is raised by painting it repeatedly with a carcinogenic hydrocarbon, the histamine content of the skin increases proportionately.

It seems, however, that the production of heparin and histamine are but part of the function of the mast cell, since mast cells are plentiful in lower organisms which lack a blood-vascular system. Even in higher animals, the mast cells appear to be associated with the tissues rather than with the blood vessels. There are other functions which are probably fundamental between mast cells and the connective tissue, and more work along these lines is clearly indicated. Asboe-Hansen[6] has recently described mast cells as unicellular endocrine glands which react as part of the mesenchymal system in time of stress. Such a definition places in a nutshell our present-day views on the importance of these mast cells to the organism in general.

Morphology of Tissue Mast Cells

Mast cells vary greatly in size and shape, depending in part upon their age and in part upon the structure and water content of the surrounding tissue. Usually, they measure from 8 to 20 μ in length and appear to be so distended with granules that the nucleus is obscured (as in the rat); in other species, like the hamster, the nucleus is usually visible, being round and about 5 μ in diameter. The cells may be spherical, spindle-shaped, or even stellate in appearance. In certain species, the smaller spindle-shaped cells predominate along the blood vessels while the larger, and probably older, spherical cells are found in extravascular areas. Stellate mast cells are found in the dermis of human skin. Considerable mingling of the several kinds may, however, occur in any one location. Each species has its own particular pattern of distribution for which, as yet, there is no adequate explanation.

The origin of mast cells has yet to be firmly established. They are recognised late in embryonic life but their origin in the embryo is uncertain. For example, tissue mast cells can be identified in the tip of the tail of new-born rats at a time when these cells are virtually absent from the skin (a tissue in which they abound in the adult). Once formed, they rapidly increase in number, size and granulation, and in the adult are

commonly found in the loose reticular adventitia of small blood vessels and in similar tissues which underlie epithelial, serous and synovial membranes. They are especially numerous in loose connective tissue undergoing fibrillogenesis and are without doubt mesenchymal cells. In the adult they may originate from fibroblasts. Dynamic changes occur in adult connective tissue following injury, and fibroplasia is succeeded by the appearance of mast cells. As recognisable collagen begins to take its shape, mast cells appear in the reactive zone. Should fibroplasia be protracted, as in chronic inflammation, the mast cell population further increases. In conditions of chronic lymphatic obstruction in which tissue spaces remain loaded with protein-rich oedema fluid, both mast cell hyperplasia and connective tissue hyperplasia become extreme. The endpoint of this sequence is reached with the formation of a vascular scar tissue in which neither fibroblasts nor mast cells are now present.

Mitotic figures have been observed by Hunt and Hunt in adult mast cells in rat mesentery after small intraperitoneal doses of histamine liberators. These authors also showed that large doses of the liberators disrupted the mast cells in the mesentery but produced mitotic figures in those of the subcutaneous connective tissue. After disintegration, they appeared to be replaced as a result of heteroplastic differentiation of mesenchymal cells or lymphocytes. It would be important to establish beyond question whether differentiated mast cells normally undergo mitosis[8].

It is possible that the granules in the cytoplasm of tissue mast cells are giant mitochondria. This suggestion is in fact supported by their physical behaviour and histochemical properties[9–11]. Since the granules are extremely soluble in water in some species, like the rabbit and in fish, many workers have failed to appreciate the high content of mast cells in certain tissues when watery fixatives are used. The first prerequisite for the histological demonstration of mast cells is therefore adequate fixation. The second determining factor is a suitable basic dye which changes colour as staining proceeds (metachromasia). Even well-fixed mast cell granules usually fail to stain with a commonly used histological technique—the haematoxylin-eosin method.

Recent advances in the complex cytochemistry of mast cells indicate that they contain, in addition to the amines discussed later, albumen, glycoprotein, polysaccharides, and phospholipids. Mast cells may be the site of active metabolic processes since enzymes such as acid and alkaline phosphatases, lipase and cytochrome oxidase can be detected in homogenates of cell concentrates. Heparin, usually localised in the granules, is a polymer consisting of disaccharide units each containing glycuronic acid and an amino sugar, glucosamine. It resembles another naturally occurring polymer of great physiological interest, namely hyaluronic acid. Whereas the amino sugar in hyaluronic acid is acetylated, that in heparin is sulphated, and it is the sulphate moiety which confers on the heparin molecule its anticoagulant property. The more sulphate groups which are incorporated in the molecule, the greater is the anticoagulant activity and also the intensity of metachromasia. Natural

heparin is probably a mixture of the di- and tri-sulphuric acid esters of the polymer. Heparin monosulphate has been found in mast cells and is probably the agent responsible for the positive test given by the cells after treatment with periodic acid and Schiff's reagent. It may be that the mast cells secrete a heparin-like substance into the tissues where it is desulphated and then acetylated into hyaluronic acid. In this way, it is possible that the mast cells produce the precursors of the mucinous interfibrillary cement.

Distribution of Tissue Mast Cells

The distribution of tissue mast cells in normal and pathological tissues of various species has been described in detail by Michels[12] and more recently by Asboe-Hansen[6] and Arvy[13]. In general, mast cells are present in large numbers in the subcutaneous connective tissue, lung pleura, mesentery, scrotum, uterus and thymus of mammals. They occur predominantly in the loose tissue around small blood vessels of warm-blooded vertebrates but are virtually absent from the central nervous system, though many are present in the sheaths of peripheral nerves. Parenchymatous organs are poor in mast cells but many can be found in the connective tissue of their capsules. Noteworthy exceptions to this are the widespread distribution of tissue mast cells throughout the parenchyma of the liver of the dog, and in the lung of the cat and ox. The rabbit is exceptional among laboratory animals in having few tissue mast cells but many blood mast cells.

The skin and ears of most mammals are rich in tissue mast cells, but whereas most of the large densely-staining cells are located in the outermost layers of the skin of many mammals, the reverse is true for the rat. In this latter species, the innermost layers of the skin contain the numerous large cells.

In such low forms of life as the sponges, starfishes, sea-urchins and molluscs, there are cells in the mesenchyme with basophilic metachromatic granules in their cytoplasm—by definition, mast cells. At the level of the crustaceans, mast cells can readily be demonstrated around the arterioles of crayfish. In the salmon, they are present in the gut-wall but not in the blood. In contrast, mast cells are common in the tissues and in the blood of reptiles and amphibia.

The mast cell content of the skin of the mouse can be increased by painting-on a carcinogenic hydrocarbon. Administration of oestrogens likewise may increase the mast cell population of tissue areas such as the fringe of the uterus in the female or the scrotum in the male. Such procedures also allow a study of the components of tissue mast cells. The common mast cell lesion in man is the skin disease of childhood, urticaria pigmentosa, in which focal collections of mast cells are found in the dermis, and may also occur in the liver and spleen[14]. Occasionally urticaria pigmentosa presents as a solitary tumour-like nodule, very rarely as a generalised mastocytoma. Such tumours are relatively common in dog where lymph nodes in the skin are usually involved. In chronic myeloid

leukaemia, blood mast cells are greatly increased in number but tissue mast cells are unaltered.

Pharmacology of Tissue Mast Cells

Heparin

Swedish workers have long been interested in anticoagulants and credit goes to Jorpes and his colleagues for having traced the source of tissue heparin. For example, Holmgren and Wilander[15] analysed ox liver, an organ rich in both mast cells and sulphuric acid esters, and found a strong anticoagulant substance which gave a pronounced metachromatic staining reaction. Much less anticoagulant activity was found in sheep liver which is sparsely supplied with mast cells and contains only traces of sulphuric acid esters. The anticoagulant activity of ox lung was likewise greater than that of pig lung (see Table I). Since heparin appeared to be the only naturally occurring mucopolysaccharide with strong anticoagulant action, Jorpes determined the activity of such tissues in terms of a standard heparin prepared from dog liver.

TABLE I

HEPARIN CONTENT (I.U./G.) OF TISSUES OF DIFFERENT SPECIES COMPARED WITH RELATIVE MAST-CELL CONTENT AND HISTAMINE CONTENT (μG./G.)

Tissue	Species	Heparin*	Mast cells	Histamine
Liver	Sheep	10	+	11
	Ox	32	++	25
Lung	Pig	21	++	28
	Ox	34	+++	40

* Based on results of Holmgren and Wilander. [15]

The metachromatic reaction, once said to be specific for sulphated mucopolysaccharides, may occur with a number of acid polyelectrolytes which do not contain sulphate groups. Cartilage stains metachromatically but it has no anticoagulant activity and no mast cells. The theory that mast cells produce heparin[16] is based on the metachromasia of the granules, the fact that heparin may be extracted from the tissues rich in mast cells, and a proportionality between the mast cell count and heparin content of a tissue (Table II). Recently, mast cell granules have been isolated from mouse connective tissue and shown to possess powerful anticoagulant properties[17].

TABLE II

HEPARIN CONTENT (I.U./G.) OF TISSUES COMPARED WITH RELATIVE MAST-CELL CONTENT AND HISTAMINE CONTENT (μG./G.)

Tissue	Heparin	Mast cells	Histamine
Rat liver	0	0	1
Pig aorta	0	0	1
Ox-liver parenchyma	4	+	5
Ox aorta	4	+	10
Rat subcutaneous tissue	6	++	16
Ox inferior vena cava	11	++	20
Ox-liver capsule	54	+++	40

Heparin is released into the bloodstream of the dog in peptone shock, in anaphylactic shock, and in the shock which follows the injection of chemical histamine-liberators. The blood becomes incoagulable and crystalline heparin has been recovered from the blood. Lymph in the thoracic duct also contains much heparin derived from the liver mast cells which disrupt under such treatment. In other species, like the rabbit and the guinea pig, little or no anticoagulant activity can be demonstrated in the blood when similar shock is produced[18], and the same is true for the rat which possesses an enormous population of perivascular mast cells in its connective tissues. The reason for these species differences is not exactly known. In the rat, the released heparin may not be allowed to leave the tissue, since Riley, Shepherd, West and Stroud[19] were able to show that complete disruption of tissue mast cells (and loss of histamine) produces a loss of only 50 per cent of the heparin in the tissue. By histological methods these authors were able to show that some of the released metachromatic material had been disposed of locally by macrophages and fibroblasts[20], whilst some had adhered to adjacent connective tissue fibrils and cells. The results suggest that the function of heparin may be concerned rather with events in the tissues than with the coagulability of the circulating blood.

A significant function of heparin may be chylomicron dissolution. For example, when heparin is injected into dogs, the passage of neutral fat through the capillary walls is facilitated. Submucous and subserous layers of the digestive tract of mammals are generally well supplied with mast cells and these may be predominantly involved in fat metabolism and in the depositing of fat in the gut vessels. Constantinides[21] attempted to relate the species difference in mast cell numbers and susceptibility to experimental atherosclerosis. The rabbit with few tissue mast cells is particularly susceptible to atherosclerosis brought about by cholesterol feeding, while the rat with numerous mast cells is refractory.

Histamine

In their classical paper on chemical histamine liberators, MacIntosh and Paton[22] showed that histamine can be released from tissues by many basic substances such as diamines, diamidines, or diguanidines. Since all these substances bear at least some structural resemblance to histamine itself, the authors suggested that histamine is displaced by these substances from its normal location in the tissues. It is now known that alkali or even tap water are also good histamine releasers, and it is probable that histamine is held, preformed in a loose complex in the granules of mast cells, and it is necessary to disturb only one component of the complex to permit the histamine to escape.

Histamine most probably is derived from the amino acid histidine by simple decarboxylation. A study of the distribution of histidine decarboxylase shows that the enzyme may be concentrated in the tissue mast cells. Radioactive histidine, for example, is decarboxylated to form radioactive histamine, and this is concentrated in the regions of the mast cells. On the other hand, radioactive histamine is not taken up by mast cells[23].

Histamine is generally recognised as a strong stimulant of the parietal cells of the gastric mucosa and of the smooth muscle in the gut wall, yet in certain species it is difficult to identify more than a few mast cells in this region. Histamine is also a strong vasodilator substance capable of increasing the permeability of capillary walls and of causing stasis in these vessels, and mast cells reside in these locations.

Rocha e Silva[4] was one of the first to show that a simultaneous release of heparin and histamine occurs from the liver of the dog after anaphylactic shock, and it thus seemed logical that both substances may originate from the same cell. To test this possibility, certain chemical substances capable of releasing histamine were first studied. When a rat was killed quickly by an intravenous dose of a fluorescent liberator, the fluorescence was at first sharply localised to the mast cells, especially those within the loose

TABLE III

THE EFFECT OF HISTAMINE LIBERATORS ON THE HISTAMINE CONTENT (μG./G.) AND RELATIVE MAST-CELL CONTENT OF SOME RAT TISSUES

Tissue	Control		Stilbamidine		Tubocurarine	
	Histamine	Mast cells	Histamine	Mast cells	Histamine	Mast cells
Omentum	20	++	10	0 to +	2	0
Subcutaneous tissues	19	++	10	0 to+	3	0
Mesentery	8	+	6	0 to +	4	+

TABLE IV

COMPARISON OF THE MAST-CELL COUNT (PER H.P. FIELD) AND HISTAMINE CONTENT (μG./G.) IN THE SKIN OF VARIOUS SPECIES

	Guinea pig	Rabbit	Man	Dog	Cat	Rat	Mouse	Hamster
Mast cells	3	2	7	40	26	30	40	50
Histamine	2	2	7	8	14	22	38	54

TABLE V

COMPARISON OF THE RELATIVE MAST-CELL CONTENT AND HISTAMINE CONTENT (μG./G.) OF SOME TISSUES OF RAT AND HAMSTER

Tissue	Rat		Hamster	
	Mast cells	Histamine	Mast cells	Histamine
Ears	+++	58	++++	112
Bristle region	+++	62	++++	94
Abdominal skin	++	35	+++	54
Cheek pouch or area	++	43	++	24
Feet skin	++	40	+	12
Stomach	+	30	+	19
Spleen	0	2	+	13

tissues of the peritoneum[24]. Thereafter the mast cells broke up and the histamine content of the tissue fell[5]. Other histamine liberators such as tubocurarine produced greater disruption of mast cells, and a greater release of histamine (Table III). Subsequent work showed that, as with heparin, there is a good correlation of the histamine content of a particular tissue and the number of mast cells which it contains. For example, the

abdominal skin of the hamster contains much histamine and many mast cells whereas that of the guinea pig or rabbit is deficient in both (Table IV). Even within one particular species there is an association between the numbers of mast cells of various areas of skin and the corresponding histamine content (Table V). As mast cells form and increase in number so the histamine content of that tissue rises (Table VI). Foetal tissues, for example, contain few mast cells and little histamine whereas similar tissues in the adult contain many cells and much histamine. Graham and her colleagues[25] obtained similar results with a chemical, instead of a biological, method of assay for histamine.

TABLE VI

HISTAMINE CONTENT (μG./G.) AND RELATIVE MAST-CELL CONTENT OF SOME TISSUES OF YOUNG AND ADULT ANIMALS

Tissue	Species	Young		Adult	
		Histamine	Mast cells	Histamine	Mast cells
Skin	Cat	2	0	20	++
Lung	Cow	7	+	25	++
Lung	Man	6	+	25	++
Liver	Cow	2	0	25	++

TABLE VII

HISTAMINE CONTENT (μG./G.) OF SEVERAL MAST-CELL TUMOURS IN DOGS COMPARED WITH THEIR HEPARIN CONTENT (I.U./G.)

Dog	Age (year)	Sex	Site	Histamine (control 9)	Heparin (control 11)
Terrier	12	M	Flank	179	211
Boxer	5	M	Flank	295	110
Labrador	12	M	Thigh	550	174
Labrador	10	F	Leg	700	130
Terrier	10	M	Flank	765	61

When pathological tissues rich in mast cells were examined, they were found to be rich in heparin as well as in histamine[26]. In Table VII are recorded some values for dog mast-cell tumours which generally are localised to skin sites. These were obtained at post-mortem examination, usually within a few minutes of the death of the animal. On occasions, the primary tumour has been removed and the animal allowed to recover. But within two months, it has recurred at another site and then the animal has been killed. Such examples provide additional data on the relationship between histamine, heparin and mast cells. It will be seen from Table VIII that there is a wider variation in the heparin values than in the histamine values, and tumours containing weakly-staining mast cells usually contain less material with anticoagulant properties than those with mast cells which stain intensely with toluidine blue. If metastases are found in other areas, these too may be filled with mast cells. Four such instances are shown in Table IX. Lymph nodes are usually involved but not the kidneys. In such instances, one can predict from the histamine values alone where metastases have occurred, and these can be confirmed later by histological examination.

In man, the lesions of urticaria pigmentosa have been shown to contain much more histamine and heparin than the adjacent skin. Light stroking of one of these lesions rapidly produces a reactive weal limited to the lesion itself, most probably as a result of the released histamine. Mast cells are known to be increased in such examples of urticaria pigmentosa.

TABLE VIII

HISTAMINE CONTENT (μG./G.) OF MAST-CELL TUMOURS IN DOGS COMPARED WITH THE HEPARIN CONTENT (I.U./G.)

Recurrences occurred about 2 months after each primary tumour was removed

Dog	Primary		Recurrence	
	Histamine	Heparin	Histamine	Heparin
Terrier	500	147	790	54
Spaniel	520	110	650	357
Scottie	661	21	277	21
Terrier	1290	434	629	590

TABLE IX

HISTAMINE CONTENT (μG./G.) OF MAST-CELL TUMOURS AND OTHER TISSUES FROM VARIOUS DOGS COMPARED WITH THEIR HEPARIN CONTENT (I.U./G.)

Tissue	Control		Spaniel		Terrier		Scottie		Mastiff	
	Histamine	Heparin	Histamine	Heparin	Histamine	Heparin	Histamine	Heparin	Histamine	Heparin
Skin tumour	—	—	650	357	629	590	277	21	219	368
Lymph node	4	9	600	293	600	290	139	24	234	78
Spleen	5	7	70	80	194	312	6	5	5	8
Liver	25	20	60	50	40	62	22	21	24	34
Lung	25	13	30	29	140	40	24	19	30	10
Normal skin	9	11	10	8	12	11	8	8	44	3
Kidney	1	6	1	5	1	4	1	9	1	4

Present evidence suggests that histamine is located in the mast cell granule. West[27] assayed mouse mast cell granules, obtained by Köksal's method, and found more histamine in the granules than in any other fraction. Similar results have been obtained for other species. From the results of their experiments using skin preparations, Riley and West[28] are convinced that the bulk of the extractable histamine is located in the tissue mast cells. Simple dissection of the components of skin and ears of several species show a similar regional distribution for mast cells and heparin on the one hand, and histamine on the other.

Since these two substances carry opposite charges, it might be assumed that they co-exist in the granules in the form of a salt—the heparinate of histamine. But this simple explanation is not entirely satisfactory. For example, Sanyal and West[29] obtained a complex formation between heparin and histamine in which the proportions of each substance (about 20 times as much heparin as histamine) were similar to those found in extracts of tissues rich in mast cells. The staining properties and chromatographic behaviour of the complex resembled those of the naturally-occurring mast cell granules, but the histamine was not releasable by the powerful chemical histamine liberators. Further work to elucidate the linkages involved is thus necessary.

The release of histamine can occur with little or no visible alteration to the mast cells. For example, West[30] has reported up to a 50 per cent release in the rat after injections of weak histamine liberators with only traces of degranulation of the cells. This reversible change most probably portrays the physiological release of histamine. Higher concentrations of powerful histamine liberators which include water[31] cause irreversible damage to the mast cells, followed in the recovery phase, by the appearance of new cells from the connective tissue precursors in the adventitia of small blood vessels, the milk spots of the omentum, and the septa of fat cells[32]. Such histamine liberators release large amounts of histamine from most tissues, the chief exception being the gut[33].

This is the story in the rat but in other species the release of much histamine may be achieved only with difficulty. Maximally tolerated doses of most histamine liberators release only about 50 per cent of the tissue histamine in the hamster and mouse and correspondingly tissue mast cells degranulate but do not disrupt[34,35]. Such a result is illustrated in Figure 1 where the effect of histamine liberators on tissue mast cells in the hamster and rat are compared; it will be seen that the mast cells in the hamster are resistant to the action of the liberators whilst those in the rat completely disrupt and disappear. In a similar way, most of the liberators do not affect tissue mast cells in the guinea pig, though anaphylactic shock in this species is usually accompanied by mast cell disruption.

The histamine-releasing power of the antibiotic, polymyxin B, has been described by Bushby and Green[36]. Although it releases maximal amounts of histamine from many tissues of the rat, accompanied by complete disruption of the mast cells, it fails to release much 5-hydroxytryptamine from similar areas[37]. Reserpine treatment on the other hand releases 5-HT but has little effect on the tissue histamine or mast cells. Bhattacharya and Lewis[38] were the first workers to show that the histamine liberator, compound 48/80, also releases 5-HT from perfused tissues of the rat and subsequent work proved that this release can occur in the whole animal. There seems clear evidence, however, that the release of one amine is not dependent upon the release of the other and most probably the major amount of each amine does not originate from the same mast cell.

Hyaluronic Acid

Hyaluronic acid is a high polymer composed of equimolecular quantities of acetyl glucosamine and glycuronic acid residues joined by beta-glycosidic links. As stated earlier, its chemical structure differs from that of heparin in being sulphate-free, in acetylation of the amino sugar, and in the type of glycosidic linkage. It is one of the best characterised of the extracellular substances of the connective tissue, being a component of the ground substance which partly supports capillary walls and acts as a barrier to the free passage of materials to and from the capillaries, tissue cells and environment.

In 1954, Asboe-Hansen[6] reported that a parallelism exists between the mast cell content and the hyaluronic acid content of many normal and

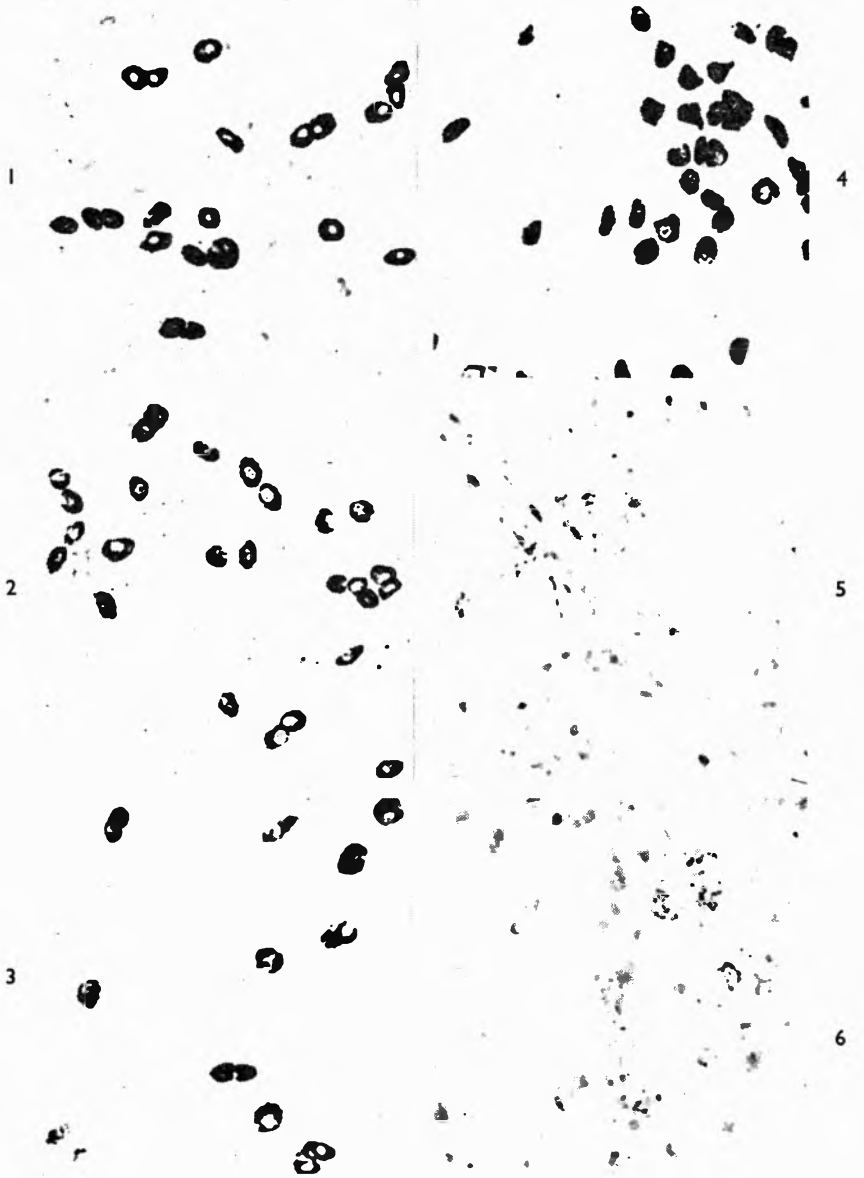

FIG. 1. Mast cells in the subcutaneous connective tissue of the hamster (1 – 3) and of the rat (4 – 6): toluidine blue, × 205; (1) and (4), normal animals: (2) and (5), after treatment with doses of compound 48/80: (3) and (6) after treatment with polymyxin B. Note that the tissue mast cells in the hamster are resistant to the action of the histamine liberators, whilst those in the rat are not.

pathological tissues. He has in fact proposed that mast cells under varying hormonal influences secrete hyaluronic acid, perhaps by way of a heparin precursor. Hyaluronidase is said to be the specific enzyme for the depolymerisation and hydrolysis of hyaluronic acid. When hyaluronidase is injected subcutaneously into the feet of rats, a localised oedema develops in an hour. But if the tissue mast cells are first disrupted by treatment with either compound 48/80 or polymyxin B, then the action of hyaluronidase may be considerably reduced. It is possible, therefore, that hyaluronidase is a histamine liberator or that some histamine liberators also release hyaluronic acid from the tissues. Hyaluronidase does not destroy the metachromasia exhibited by the granules of mast cells, so a simple explanation of these facts is not as yet possible.

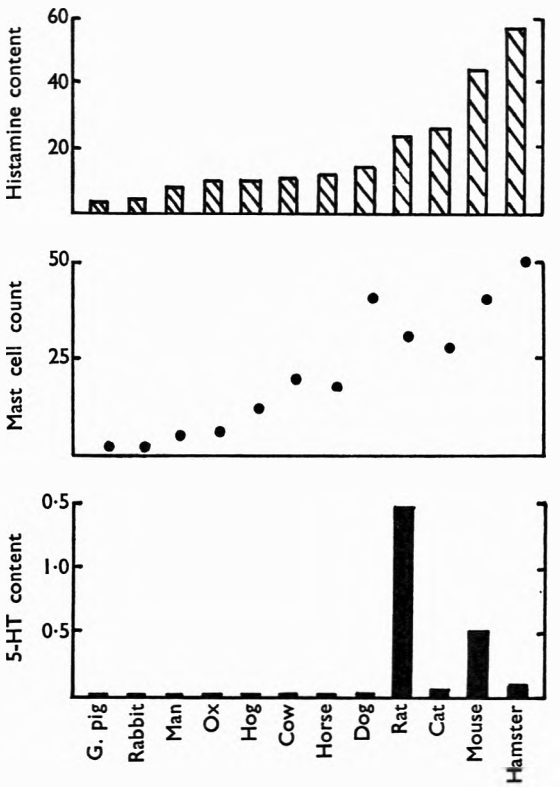

FIG. 2. Comparison of the histamine content (μg./g.) of the abdominal skin of different species with the mast cell count (cells per H.P. field) and 5-HT content (μg./g.)

5-Hydroxytryptamine (5-HT)

This amine is formed from the amino acid, tryptophan, which after hydroxylation is decarboxylated. The distribution of 5-hydroxytryptophan decarboxylase has been well studied and results suggest that the enzyme is not concentrated in mast cell-rich tissues. However, Benditt

and others[39] have reported the isolation of 5-HT from peritoneal washings in the rat; such washings are rich in mast cells and histamine. Extensive studies by Parratt and West[40] indicated that 5-HT is not concentrated in tissue mast cells of the guinea pig, rabbit, man, ox, hog, cow, horse, dog cat or hamster, though it may be associated with mast cells in the rat or mouse (Fig. 2). It is only in the rat and mouse that much 5-HT is contained in the skin. Later work[41] showed that, in 10 out of 17 specified regions of rat skin, there was a relation between the 5-HT and histamine contents and the mast cell population, but in the other 7 regions there was relatively much less 5-HT (Table X). In fact, it is easy to release 5-HT from rat tissues leaving mast cells and tissue histamine little affected, and conversely easy to release histamine from rat tissues and disrupt mast cells leaving tissue 5-HT almost unchanged[37]. The effect on the tissue amines is illustrated in Figure 3. Thus it seems most unlikely that tissue mast cells even in the rat contain any considerable quantity of 5-HT.

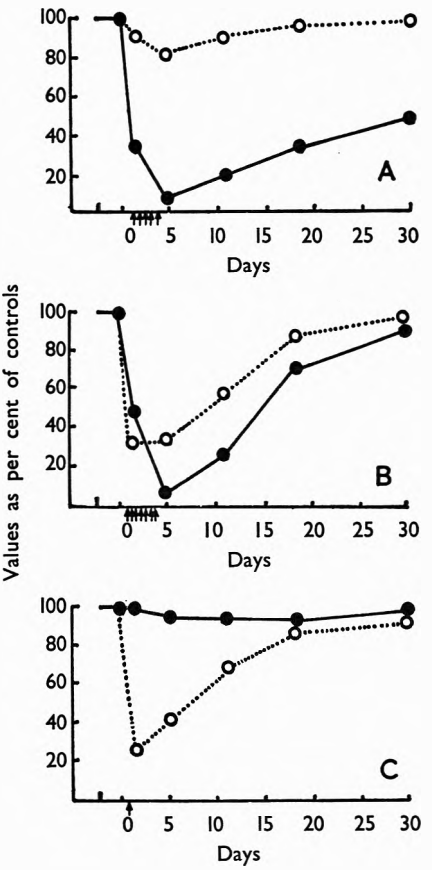

FIG. 3. Changes in the 5-HT (○---○) and histamine (●—●) contents of the abdominal skin of the rat after injections of (A) 5 doses of polymyxin B, (B) 7 doses of compound 48/80, (C) a single dose of reserpine (at the arrows). All values are expressed as percentages of the control levels before injection. Note the effects of polymyxin are almost the reverse of that of reserpine.

In other species, it is also possible to obtain results opposing the view that 5-HT is associated with mast cells and histamine. For example, pathological specimens of mast cell lesions of the dog, cat, cow and man have failed to give a 5-HT reaction when the tissue histamine content has been exceptionally high[42]. In Table XI, the results of assays on two dog mast-cell tumours clearly indicate this lack of association between mast cells and 5-HT, and the same is true for the cat (Table XII). It is of particular interest that the histamine content of the spleen of the cat exceeded 1 mg./g. of fresh tissue and the mast cell count was correspondingly high. In this species, it is possible that the primary tumour originates in the spleen, and not in the skin as in the dog. In guinea pigs, tissue mast cells are partly disrupted during the anaphylactic reaction and histamine is found in the

TABLE X

HISTAMINE CONTENT (μG./G.) OF VARIOUS REGIONS OF SKIN OF THE RAT COMPARED WITH THE RELATIVE MAST-CELL CONTENT AND THE 5-HT CONTENT (μG./G.)

Region	Mast cells	Histamine	5-HT	Ratio Histamine/5-HT
Lips and nose	+ + + +	72	4·36	17
Submental	+ + +	54	2·60	21
Throat	+ +	48	4·20	11
Eyelids	+ + +	48	2·20	22
Cheek	+ +	43	2·20	19
Ear base	+ +	41	3·20	13
Groin	+ +	38	1·80	21
Nipples	+ +	33	2·47	13
Abdomen	+ +	30	2·60	12
Anus	+ +	24	2·10	11
Scrotum	+ + +	63	1·40	45
Bristle area	+ + +	62	2·00	31
Hindlegs, dorsal skin	+ + +	52	1·31	40
Forelegs, dorsal skin	+ + +	48	1·32	36
Ear margin	+ + +	48	1·40	34
Tail margin	+	19	0·43	44
Tail base	+	19	0·36	53

TABLE XI

MAST-CELL COUNTS (PER H.P. FIELD) OF SOME TISSUES OF DOGS WITH MAST-CELL TUMOURS COMPARED WITH THE HISTAMINE AND 5-HT CONTENTS (μG./G.)

Tissue	11 year Spaniel ♀			6 year Spaniel ♂			Control dog		
	Mast cells	Histamine	5-HT	Mast cells	Histamine	5-HT	Mast cells	Histamine	5-HT
Inguinal skin tumour	201	678	0·03	112	800	0·03	5	10	0·03
Lymph node	223	788	0·03	58	200	0·03	2	4	0·03
Spleen	141	495	1·00	27	150	1·16	2	7	2·30
Liver	38	245	0·08	14	100	0·22	10	24	0·27

TABLE XII

MAST-CELL COUNTS (PER H.P. FIELD) OF TISSUES OF A CAT WITH A MAST-CELL TUMOUR COMPARED WITH THE HISTAMINE AND 5-HT CONTENTS (μG./G.)

Tissue	3 year castrated ♂			Control cat		
	Mast cells	Histamine	5-HT	Mast cells	Histamine	5-HT
Inguinal skin tumour	42	80	0·03	4	24	0·08
Lymph node	33	285	0·03	2	11	0·06
Spleen	81	1230	1·20	1	2	4·25
Liver	56	14	0·08	8	1	0·28

TABLE XIII

COMPARISON OF THE EFFECTS OF ANAPHYLAXIS AND HISTAMINE LIBERATORS IN VARIOUS SPECIES. ACTIONS RECORDED ARE THE DISRUPTION OF TISSUE MAST-CELLS, AND THE RELEASE OF HISTAMINE OR OF 5-HT FROM THE TARGET ORGANS

Species	Anaphylaxis			Histamine liberators		
	Mast cells	Histamine	5-HT	Mast cells	Histamine	5-HT
Dog	Disruption	Release	0	Disruption	Release	Slight
Guinea pig	Disruption	Release	Slight	Slight	Slight	0
Rat	Slight	Slight	Slight	Disruption	Release	Release
Mouse	Slight	Slight	Slight	Slight	Slight	Slight
Hamster	0	0	0	Slight	Slight	Slight
Rabbit	Slight	Release	Release	Slight	Release	Release

blood, but only traces of 5-HT are released[43]. In dogs, tissue mast cells are partly disrupted during anaphylaxis and histamine is released (with heparin) from the liver into the thoracic duct and hepatic vein, but 5-HT release is not seen[44] despite the fact that a relatively high 5-HT amount is known to be present in the liver, the target organ in that species. Histamine liberators likewise can release histamine from dog liver and disrupt mast cells without affecting the tissue 5-HT levels. Some of these results are tabulated in Table XIII and illustrate how mast cells in different species react in different ways to different agents or procedures.

5-HT is known to be present in the blood platelets, the spleen, and cells of the enterochromaffin system (particularly in the gut). It is thought to be released (along with histamine) from the platelets during injury. It generally exerts a vasoconstrictor action on arterioles and also an effect on capillary permeability. Two of the consistent symptoms of human argentaffinoma (tumours of the enterochromaffin tissue) are diarrhoea and oedema with flushing attacks, and it is now certain that 5-HT stimulates intestinal movement in most species and may be a local hormone within the gut wall. 5-HT is a potent stimulator of peristalsis in guinea pigs, if placed inside the lumen of the gut. It does not appear to be associated in this region with tissue mast cells but with argentaffin cells, and when argentaffin cells are scarce, as in the rat, there are generally argyrophil cells, which stain with silver salts but not with chromate.

When dextran or eggwhite is injected into rats, oedema of the extremities develops on the first injection. This anaphylactoid reaction is the result of a release of 5-HT (with some histamine) from such areas of skin. The reaction is prevented by depletion of skin 5-HT by previous treatment with reserpine, but it is not affected by depletion of only histamine[45]. The skin of other species does not contain 5-HT and injections of dextran or eggwhite do not produce local oedema on the first injection[46].

Difficulties Concerning the Relation of Tissue Amines to Tissue Mast Cells

Evidence is now so convincing that heparin and the bulk of the histamine in a tissue are normally contained in the mast cells that interest turns to possible exceptions to the rule. One such unusual location for histamine is in the mucosa of the pyloric portion of hog stomach. Here some cell other than the mast cell must be binding the histamine since mast cells are scarce in the inner layers of the mucosa where the histamine content is high. An obvious choice for the binding component is the mucin of the mucous cells, lining the lower two-thirds of the pyloric glands[47]. Indeed, gastric mucin can be shown *in vitro* to bind histamine. Like the granular material of the mast cells, this mucin also stains metachromatically with toluidine blue. Metachromasia with toluidine blue, however, is not in itself an indication of the ability of the tissue to bind histamine, since cartilage, for example, binds very little histamine. There is little evidence, as yet, to suggest a functional relation between mast cells and the gastric mucin.

In the rat intestine, a similar difficulty arises. Even those mast cells which normally reside in this region are more resistant to the action of the now traditional histamine liberators than are, say, mast cells in the skin. Tissue mast cells in other species may be likewise resistant to the damaging action of these liberators. It seems likely that an extensive investigation into the chemical structure and properties of the heparin of different laboratory species might provide some clue to this problem.

Eosinophils for long were thought to be the chief source of histamine in the body[48]. Recently the relationship of eosinophils to histamine has been found to be less close. Thus the highest amounts of histamine have been found in tissues when the predominant cell has been the mast cell, and there is proportionately less histamine when eosinophils appear in increasing numbers. This occasional association of eosinophils with mast cells may itself indicate the true function of the eosinophils in the histamine problem. In general, eosinophils are found in mast cell lesions when the mast cells are in the process of disruption. For example, eosinophils appear in lesions of urticaria pigmentosa in man when these have been subjected to mechanical irritation and they can be found in mast cell tumours when dogs have been treated with histamine liberators. Further, there is apparently developed in the lungs of anaphylactically shocked guinea pigs some factor that draws eosinophils to the site[49]. This factor is certainly not 5-HT since guinea pig lung contains only traces of this amine, but it might be histamine. It is therefore of particular interest that the isolated granules of eosinophils have been shown to possess antihistamine properties[50]. Perhaps the eosinophil is concerned more with detoxification and disposal of histamine than with its elaboration.

Objections to the theory that mast cells secrete hyaluronic acid are based on the facts that hyaluronidase does not destroy the metachromasia of mast cell granules and that there is a poor correlation of the mast cell counts and the hyaluronic acid content of certain tissues. Thus mast cell tumours and organ capsules exceptionally rich in mast cells contain little ground substance and conversely embryonic tissue rich in ground substance has few mast cells.

Mention has been made already of the fact that no parallelism exists in many species between the mast cell count and 5-HT content of the skin and other tissues. Even within one species such as the rat, 5-HT may be concentrated in areas where mast cells are few in number. Treatment with drugs (like polymyxin B and reserpine) likewise illustrates how disruption of mast cells and 5-HT release may be independent of each other. Feldberg and Smith[51] have already shown that large doses of 5-HT can release histamine from rat tissues and damage mast cells.

In a study of the physiological phenomena influencing the mast cell population in rats, Parratt and West[40] found that the histamine content of the skin is considerably raised at birth yet there is no corresponding increase in mast cell numbers. At weaning time, both the histamine and 5-HT concentrations in the skin are doubled (Fig. 4) but again the mast cell population is unaltered. Similarly during lactation, the histamine and 5-HT contents of certain tissues are raised with no increase in mast cells.

When thyroxine is administered daily to rats for 14 days or more, the skin 5-HT level is raised but this increase is associated with only a slight increase in the histamine content and mast cell population. Thyroxine-treated animals are very sensitive to histamine liberators (for example, dextran), but even the lethal action of these liberators is associated only with slight swelling and degranulation of the mast cells. No adequate explanation of these results is at once apparent.

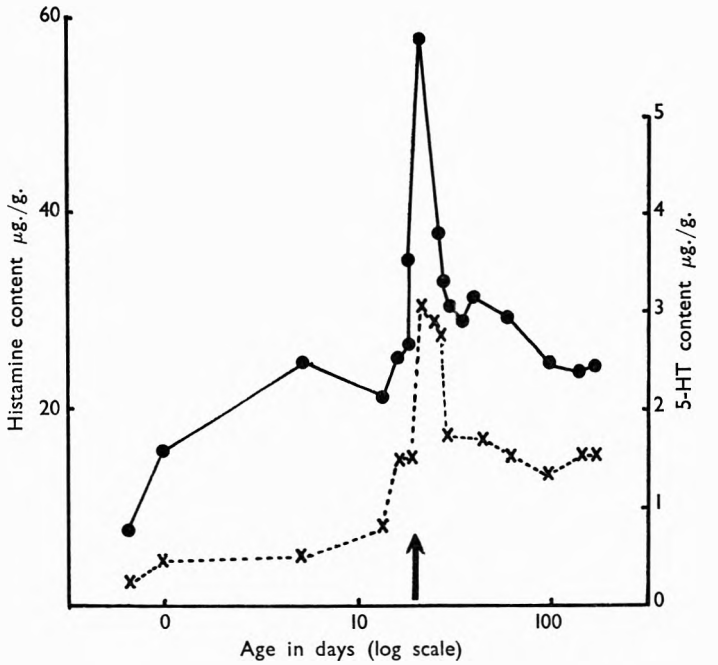

FIG. 4. Influence of age on the histamine content (●—●μg./g.) and 5-HT content (×---×μg./g.) of the abdominal skin of the rat. Note the abrupt increase in both amines at weaning time (indicated by the arrow), although the number of tissue mast cells is not increased at this time period.

BIOLOGICAL SIGNIFICANCE OF TISSUE MAST CELLS

The tremendous number of mast cells especially along blood vessels is sufficient cytological evidence to credit an important role to these cells. From Ehrlich's time onwards, histologists have suggested that the function of the mast cell is concerned in some way with connective tissue and particularly with the formation of its fibrils. Formation of the first mast cells in the embryo is preceded by a general metachromatism of the tissues, probably produced by the connective tissues themselves. Thereafter the tissue mast cells undergo cyclic changes, disappearing in areas of acute tissue injury and reappearing when connective tissue fibrils begin to be laid down and the ground substance shrinks. This behaviour on the part of the mast cell suggests that they alternately store and release substances, one

of which may be the mucopolysaccharide of the ground substance. Heparin, which may be the sulphated precursor of hyaluronic acid, has been used by Morrione[52] to precipitate fibrils from sollubilised collagen.

The activation of the reticulo-endothelial system, which follows the release of amines from mast cells and which is manifest by an increased phagocytic capacity of the endothelial cells, is only part of a more widespread mobilisation of the entire loose mesenchyme. This mobilisation begins around the small blood vessels and extends away from them into the tissues. The changes may thus be due to a flooding of the tissues with protein-rich oedema fluid, formed as a result of the increased vascular permeability[53]. The general appearance of these active cells closely resembles that seen in the inflammatory process.

Mast cell production appears to be under some hormonal control though the precise mechanism has yet to be elucidated. Pituitary thyrotropin is said to increase their numbers whereas ACTH and cortisone generally produce a decrease. Different dosages and routes of administration may account for the apparently contradictory effects obtained by certain workers. Oestrogens have been shown by Arvy[54] to increase the numbers of tissue mast cells particularly in the thymus, gut and subcutaneous connective tissue of mice. Although the number of mast cells increases in hypothyroidism and becomes normal in hypothyroid subjects treated with thyroxine, the true role of the thyroid gland in the control of mast cells and in histamine metabolism in particular is not clear. Early experiments showed that removal of the thyroid protected guinea pigs against the effects of anaphylactic shock whereas large doses of thyroxine had the opposite effect. Thyroid hormone, however, markedly influences the sensitivity of tissues of the rat both to extrinsic histamine and to released histamine whether liberated by primary release (compound 48/80, polymyxin B, eggwhite or dextran) or by anaphylaxis. Thyroid does not increase the amine release but appears to decrease the ability of the animal to destroy the released amine. Dysfunction of the thyroid gland may ameliorate certain allergic reactions in man and it would be interesting to know how far these changes are due to an increase in the sensitivity of the tissues.

Mast cells appear to be involved in physiological processes by the formation and release of certain agents. The presence of heparin in these cells is now well established but its release may not be their primary function. Mast cells also release histamine and possibly 5-HT—two substances which increase capillary permeability. The function of these two amines might therefore be inter-related. But when a large number of species are examined, there appears to be no relation, except in the rat and mouse, between the histamine level and mast cell content on the one hand and the 5-HT level on the other. The rat and the mouse have some 5-HT in areas rich in mast cells and it is possible therefore that in these two species 5-HT may act as a defence agent and perhaps take over part of the function of histamine. In periods of stress, for example at weaning, lactation, and in cold months, the levels of 5-HT are raised and so also are those of histamine. It is significant perhaps that similar changes in

the histamine level occur in other species when the skin is subjected to injury.

At least one action of 5-HT in the skin of the rat is the marked effect of increasing capillary permeability and in this species it is much more active than histamine[55]. Of the species studied so far by Sparrow and Wilhelm[56], only in the rat is 5-HT more active than histamine in increasing capillary permeability, and only in the rat (and to a minor extent, in the mouse) is 5-HT present in the skin in substantial amounts. It is unlikely that these two factors are coincidental. Neither can it be by chance that the only species developing the full anaphylactoid reaction is the one which contains much 5-HT in its skin. It may be that this ability of 5-HT to increase capillary permeability in minute doses is related to a defence function.

When histamine liberators are given to rats, oedema develops in the extremities and capillary changes occur leading to accumulation of the circulating colloidal dye. These changes show a characteristic distribution and occur in skin regions such as those of the feet and face. These regions are generally rich in histamine but there are at least three other areas which do not exhibit the full phenomena and yet have much histamine. These areas are the ear margin, the dorsal skin of the foot, and the scrotum. They contain relatively less 5-HT than do other areas with similar histamine concentrations and this fact alone may account for the lack of blueing of such regions, as manifested by oedema and exudation of circulating dye.

It was noted earlier that rat and mouse mast cells may contain a part of the tissue 5-HT. One of the reasons why the cells of these two species bind 5-HT as well as histamine is probably to be found in the type of heparin, and a biochemical study of this problem is urgently needed. Rat mast cells appear to contain slight 5-hydroxytryptophan decarboxylase activity as well as histidine decarboxylase activity so that both amines could be ready-made within the cell. This result explains why the tissue amines recover their normal levels only slowly after depletion by chemical liberators. If the intact mast cell is the only prerequisite for amine formation, then after a few weeks when the mast cell population is normal again the amines will be manufactured. Recovery of the levels of the tissue amines is dependent not only on the binding by heparin of exogenous amines but also on the presence of active decarboxylases.

Lastly, the question arises why human mast cells do not concentrate 5-HT. It may be too toxic for the sensory organs in the skin for it to remain there. It is known that the 5-HT concentration in wasp venom exceeds the pain-producing threshold concentration of 5-HT when applied to human skin as at the base of a blister, and a similar comment may be made about the stings of nettles[42].

The function of histamine and 5-HT in the tissues remains an enigma, and any attack on this problem is bound to be an indirect one. Already the new tools are available to enable each amine to be released separately. Many facts suggest that the function of histamine is concerned with the defence of the body, since its release is followed by a temporary mobilisation of the loose mesenchyme. It is remarkable that in every species

studied (except the rat) histamine is concentrated in the outer layers of the skin—cell layers which come into contact with the outside world. In the rat, 5-HT is concentrated in these outer layers of the skin and probably takes over part of the defence function of histamine in this species.

Conclusions

Although mast cells have been the subject of numerous publications since they were first clearly described by Ehrlich some 80 years ago, it is only recently that they have come to interest the pharmacologist. Modern work in Scandinavia showed one functional activity of mast cells in producing heparin, and this seemed to provide an obvious and teleologically satisfactory explanation of the perivascular location of these cells. Mast cells are perivascular in location because they produce heparin which they pour into the blood-stream. Yet it was a study of this very relation of mast cells to blood vessels which led to the discovery in this country of a completely different function of the cells, that of producing histamine. This was achieved using simple extraction and assay procedures, specific histamine antagonists to check the specificity of the responses, and various chemical substances which selectively disrupt mast cells and release histamine from tissues. These histamine liberators have not only paved the way for locating the site of storage of histamine but they have also enabled experiments to be carried out on animals whose tissues have been made deficient in histamine. In this way, a fresh attack has been made on the function of mast cells, so that they now appear to be less concerned with blood clotting (as was at first thought) than with the maintenance and repair of the connective tissues. Besides, in certain regions of the body mast cells may also contain 5-hydroxytryptamine, a substance which like histamine increases capillary permeability. Whatever other materials remain to be discovered in mast cells, it is certain that they are the site of active metabolic processes, since several enzymes can be detected in homogenates of cell concentrates.

Tissue mast cells seem to undergo cyclic changes, disappearing in areas of acute tissue injury and reappearing when connective tissue fibrils begin to be laid down. This behaviour suggests that mast cells alternately form, store and release certain substances. When such a release occurs, the tissues are flooded with a protein-rich oedema fluid, formed as a result of the increased vascular permeability by histamine and possibly by 5-HT also. This oedema fluid not only assists in the removal of foreign materials but also provides the tissues with some of the materials ready for the job of repair. Thus I believe that mast cells act as part of the defence mechanism of the body whereby they react quickly to any kind of cell injury. However, this cannot be the full story as animals whose mast cells have been disrupted by potent histamine liberators show few abnormal reactions when subjected to stress or injury. More work along this line is clearly indicated.

Finally, it seems that the tissue mast cells and the amines they produce or store are under hormonal influence. Over-activity of some of the

endocrine glands not only increases the mast cell population and the histamine and 5-HT contents of particular tissues but it also renders the animals hypersensitive to various agents, such as egg white, dextran, histamine liberators, adrenaline and particular antibiotics. The reaction in such hypersensitive animals provides the opportunity to evaluate experimentally the possible mechanism of particular allergies in man and further work in this direction is now taking place. The new association between histamine (and possibly 5-HT) and the heparin-containing mast cells of the tissues should become increasingly important in the future in assigning the various functional roles these substances play in a range of physiological and pathological reactions.

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References

1. Erhlich, *Arch. mikr. Anat.*, 1877, **13**, 263.
2. Erhlich, *Arch. anat. physiol., Lpz.*, 1879, **3**, 166.
3. Jorpes, Holmgren and Wilander, *Z. mikr.-anat. Forsch.*, 1937, **42**, 279.
4. Rocha e Silva, *Brit. med. J.*, 1952, **1**, 779.
5. Riley and West, *J. Physiol.*, 1953, **120**, 528.
6. Asboe-Hansen, *Intern. Rev. Cytol.*, 1954, **3**, 399.
7. Hunt and Hunt, *Proc. Soc. exp. Biol., N.Y.*, 1957, **94**, 166.
8. Padawer, *Trans. N.Y. Acad. Sci.*, 1957, **19**, 690.
9. Julén, Snellman and Sylvén, *Acta physiol. scand.*, 1950, **19**, 289.
10. Snellman, Sylvén and Julén, *Biochim. biophys. acta*, 1957, **7**, 98.
11. Bloom and Friberg, *Experientia*, 1953, **9**, 310.
12. Michels, *The Mast Cells*, in *Handbook of Haematology*, Hoeber, New York, 1938, **1**, p. 232.
13. Arvy, *Rev. Haematol.*, 1955, **10**, 55.
14. Gardner and Tice, *Pedriatrics*, 1958, **21**, 805.
15. Holmgren and Wilander, *Z. mikr.-anat. Forsch.*, 1937, **42**, 242.
16. Jorpes, *Heparin in the Treatment of Thrombosis*, 2nd ed., O.U.P., London, 1946.
17. Köksal, *Nature, Lond.*, 1953, **172**, 733.
18. Adams, *J. Pharm. Pharmacol.*, 1953, **9**, 580.
19. Riley, Shepherd, West and Stroud, *Nature, Lond.*, 1955, **176**, 1123.
20. Higginbotham, *Ann. N.Y. Acad. Sci.*, 1958, **73**, 186.
21. Constantinides, *Science*, 1953, **117**, 505.
22. MacIntosh and Paton, *J. Physiol.*, 1949, **109**, 190.
23. Schayer, *Amer. J. Physiol.*, 1956, **186**, 199.
24. Riley, *J. Path. Bact.*, 1953, **65**, 471.
25. Graham, Lowry, Wahl and Priebat, *J. exp. Med.*, 1955, **102**, 307.
26. Cass, Riley, West, Head and Stroud, *Nature, Lond.*, 1954, **174**, 318.
27. West, *J. Pharm. Pharmacol.*, 1955, **7**, 80.
28. Riley and West, *Amer. Arch. Derm.*, 1956, **74**, 471.
29. Sanyal and West, *Nature, Lond.*, 1956, **178**, 1293.
30. West, Abstr. XX int. physiol. Congr., Brussels, 1956, p. 964.
31. Fawcett, *J. exp. Med.*, 1954, **100**, 217.
32. Riley and West, *J. Path. Bact.*, 1955, **69**, 269.
33. Feldberg and Talesnik, *J. Physiol.*, 1953, **120**, 550.
34. Riley and West, *Arch. int. Pharmacodyn.*, 1955, **102**, 304.
35. Parratt and West, *ibid.*, 1957, **113**, 209.
36. Bushby and Green, *Brit. J. Pharmacol.*, 1955, **10**, 215.
37. Parratt and West, *J. Physiol.*, 1957, **137**, 179.
38. Bhattacharya and Lewis, *Brit. J. Pharmacol.*, 1956, **11**, 202.
39. Benditt, Wong, Arase and Roeper, *Proc. Soc. exp. Biol., N.Y.*, 1955, **90**, 303.

40. Parratt and West, *J. Physiol.*, 1957, **137**, 169.
41. Parratt and West, *ibid.*, 1957, **140**, 105.
42. West, *Int. Arch. Allergy*, 1957, **10**, 257.
43. Sanyal and West, *Nature, Lond.*, 1957, **180**, 1417.
44. Sanyal and West, *J. Physiol.*, 1958, **144**, 525.
45. Parratt and West, *ibid.*, 1957, **139**, 27.
46. Parratt and West, *Amer. Arch. Derm.*, 1957, **76**, 336.
47. Riley and West, *Experientia*, 1956, **12**, 153.
48. Code, *Physiol. Rev.*, 1952, **32**, 47.
49. Samter, Kofoed and Piefer, *Blood*, 1953, **8**, 1078.
50. Vercauteren, *Enzymologia*, 1953, **16**, 1.
51. Feldberg and Smith, *Brit. J. Pharmacol.*, 1953, **8**, 406.
52. Morrione, *J. exp. Med.*, 1952, **96**, 216.
53. Feldberg, *J. Pharm. Pharmacol.*, 1954, **6**, 281.
54. Arvy, *Nature, Lond.*, 1955, **175**, 506.
55. Rowley and Benditt, *J. exp. Med.*, 1956, **103**, 399.
56. Sparrow and Wilhelm, *J. Physiol.*, 1957, **137**, 51.

RESEARCH PAPERS

THE METABOLISM OF OUABAIN IN THE RAT

By Elizabeth Cox, G. Roxburgh and S. E. Wright

From the Department of Pharmacy and Pharmacology, University of Sydney, Sydney, Australia

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The urinary and biliary excretion of ouabain by rats has been estimated by paper chromatographic, colorimetric and biological methods of assay. After a dose of 1 μg./g., ouabain was excreted to the extent of approximately 90 per cent in bile and 4 per cent in urine. No metabolites of ouabain retaining the unsaturated lactone ring could be detected at this dose, but after extremely high doses traces of such metabolites were present. The influence of the polarity of the molecule on its excretion is discussed.

Ouabain (g-strophanthin) is one of the most rapidly eliminated cardiac glycosides, but little is known about the mechanism of its destruction or excretion. Hatcher and Eggleston[1] examined its fate in the rat, which is highly resistant to ouabain, and concluded that the liver played an important role in its elimination. Farah[2], also using rats, estimated by biological methods that 80 to 85 per cent of the glycoside was excreted in the bile 2–4 hours after administration. There is, however, no information available as to whether the glycoside is excreted unchanged or as a metabolite, and no quantitative estimation of the amount excreted in urine has been published.

In this study we have re-examined the biliary excretion of ouabain in rats, using paper chromatography to separate and identify the excretory products, and estimated the amounts excreted in bile by colorimetric methods. The urinary excretion of the glycoside has also been examined by paper chromatography and the embryonic chick heart assay method of Lehman and Paff[3].

Methods

Biliary Excretion

Male albino rats (300–450 g.) were anaesthetised with urethane and the bile duct cannulated with the shaft of a hypodermic needle attached to fine polythene tubing. Doses of 1 μg./g. were injected into the femoral vein and the bile collected for 5 hours (average yield = 3 ml.).

Urinary Excretion

Male albino rats (250–350 g.) were injected intraperitoneally with ouabain at doses of 1 or 2 μg./g. and the overnight sample of the urine of each pair of rats was precipitated with lead acetate[4] before extraction.

Extraction of Ouabain

Because of the high water solubility of ouabain it was necessary to saturate aqueous solutions with ammonium sulphate before continuous

extraction in a liquid-liquid extractor with 10 per cent methanol in chloroform for 6 hours. Using this method, recoveries from water were never less than 91 per cent.

From bile. The bile from one rat was diluted to about 15 ml. with water and extracted with chloroform in a liquid-liquid extractor for 1½ hours to remove chloroform-soluble bile constituents which interfered with subsequent paper chromatography. Trial experiments showed that no ouabain or recognisable metabolite was removed under these conditions. The diluted bile was then fully saturated with ammonium sulphate and re-extracted with 10 per cent methanol in chloroform for 6 hours. The chloroform-methanol extract was evaporated to a small volume for paper chromatography.

From urine. After treatment with lead acetate urine was saturated with ammonium sulphate and extracted for 6 hours with 10 per cent methanol in chloroform. The chloroform-methanol extract, 30 ml., was then shaken with 3 successive quantities of 10 ml. of water to remove the oubain and leave toxic substances in the chloroform. The aqueous layers were combined and evaporated at low temperature and the residue dissolved in a small volume of methanol for paper chromatography.

Paper Chromatography

The system of Schenker, Hunger and Reichstein[5], consisting of water saturated with butanol, was used for bile and urine extracts.

Bile extracts. The chloroform-methanol extracts containing the glycoside and much pigment were streaked quantitatively across strips of Whatman paper No. 3 (1¼ in. by 15 in.) and developed with the butanol-rich phase for 24 hours by the downward method. A longitudinal strip ¼ in. wide was cut from the chromatogram and used to locate the glycoside with alkaline *m*-dinitrobenzene[6]. The corresponding areas in the remainder of the strip were cut away, dried at 60° for 1 hour and eluted in a small soxhlet extractor with 30 ml. of methanol. The methanol was then removed, and the residue estimated colorimetrically.

Urine extracts. The extract of the urine of two rats was streaked on to strips of Whatman paper No. 4 (1¼ in. by 15 in.) previously saturated with the aqueous phase. The paper was developed with the butanol-rich phase in horizontal tanks at 20–24° for 12 hours. The glycoside bands were located as described for bile and the areas of the chromatograms containing active material were dried at 60° for 1 hour and eluted with methanol. The methanol was removed and the residue used for biological estimations.

Estimation

In bile extracts. Because of the comparatively large amounts of glycoside extracted in bile it was possible to use colorimetric methods of estimation. The Raymond reagent was used according to the method of Anderson and Chen[7], but modified as follows. The eluate from the

chromatogram was dissolved in 5 ml. of ethanol and a convenient aliquot, usually 3 ml., of this solution was pipetted into a colorimeter tube. Then 0·5 ml. of a 1 per cent ethanolic solution of *m*-dinitrobenzene was added the tube cooled in an ice bath and 0·5 ml. of 20 per cent aqueous sodium hydroxide added. The solution was thoroughly mixed and retained in the ice bath for 10 minutes by which time the blue colour reached a maximum and remained stable for 1–2 minutes. The optical density was measured using an E.E.L. colorimeter (red filter 607). Standards were prepared and read at the same time under the same conditions.

In urine extracts. The small amount of cardioactive material present in the extracts of the urine of each pair of rats could not be estimated colorimetrically. The urine extract was diluted with Ringer's solution to give an expected ouabain concentration of about 0·5–1 μg./ml. and estimated by the embryonic chick heart method of Lehman and Paff[3].

Recoveries

Although complete recoveries using known quantities of ouabain were not obtained, results (Table I) were sufficiently consistent to allow the mean recovery figures obtained for bile (83 per cent—limit of error 78–88 per cent, P = 0·95) and for urine (58 per cent—limit of error 53–63 per cent, P = 0·95) to be used as correction factors to obtain a close approximation of the ouabain content of the bile or urine obtained in extraction experiments.

TABLE I

RECOVERY OF OUABAIN FROM BLANK BILE AND URINE

Bile			Urine		
Ouabain added (μg.)	Ouabain recovered (μg.)	Recovery per cent	Ouabain added (μg.)	Ouabain recovered (μg.)	Recovery per cent P = 0·95
400	323	81	100	60	57–63
400	346	87	100	61	57–65
400	319	80	100	51	46–56
400	292	73	100	66	60–72
400	352	88	100	59	54–64
400	346	87	100	49	43–55
Mean recovery = 83 per cent (Limits of error 78–88, P = 0·95)			Mean recovery = 58 per cent (Limits of error 53–63, P = 0·95)		

RESULTS

The paper chromatograms prepared from bile and from urine extracts after doses of 1 μg. and 2 μg./g. showed the presence of one band only (see Fig. 1a). This was identified as ouabain by eluting from the paper and rechromatographing with the genuine glycoside using three different systems: butanol saturated with water[5]; butanol: toluene, 50: 50, saturated with water[5]; and tetrahydrofurane: chloroform, 50: 50, saturated with formamide[8]. The amounts of ouabain recovered from bile in the first 5 hours after injection, and from urine in the first 24 hours for both dose levels, are shown in Table II.

Some rats were given intravenous doses of 2 μg./g. but the chromatograms of the urinary extracts of these animals did not show significant differences from those obtained after intraperitoneal doses.

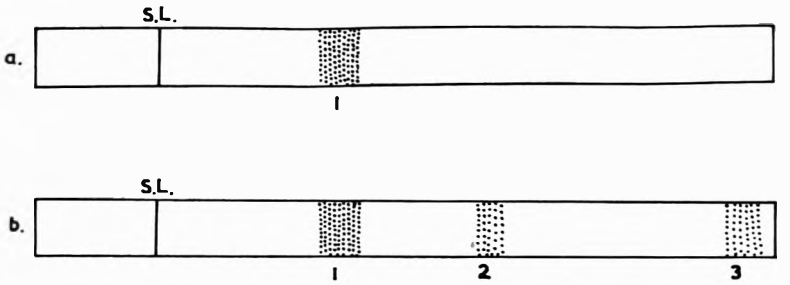

FIG. 1. (a) Chromatograms of bile extracts after doses of 1 μg./g., and of urine extracts after doses of 1 μg. and 2 μg. g. (b) Chromatograms of urine extracts after dose of 10 μg./g.

Band 1 = Ouabain.
Band 2 and 3 = Metabolites of ouabain.

When doses of 10 μg./g. were given intraperitoneally, three bands were detected with *m*-dinitrobenzene on the chromatograms of the chloroform extracts of urine (Fig. 1b). The ouabain band was very intense, but the second and third bands which appear to be metabolites of ouabain of a less polar nature were very faint. No quantitative estimations were attempted at this level, and no information was obtained about the possible nature of the metabolites.

TABLE II

EXCRETION OF OUABAIN IN BILE AND URINE

	Dose level	No experiments	Mean ouabain recovered per cent	Mean ouabain recovered (corrected) per cent	Limit of error P = 0·95
Bile	1 μg./g.	8	73	88	83–93
Urine	1 μg./g.	6	2·4	4·6	3·6–4·4
	2 μg./g.	3	2·8	4·8	4·4–5·2

DISCUSSION

These results confirm the work of Farah[2] in demonstrating that ouabain is excreted mainly in the bile and that the liver is therefore the chief organ of excretion. Furthermore, we have shown that ouabain is excreted almost entirely unchanged, as approximately 93 per cent of the dose can be accounted for in rat bile and urine as original glycoside. It is only after exceptionally high doses that metabolites of ouabain appear in urine.

The high biliary excretion of ouabain, a very polar water-soluble glycoside, is in accord with the results obtained by Cox and Wright[9] for the biliary excretion of the polar digitalis glycosides lanatoside A and lanatoside C. These glycosides have partition coefficients in favour of water and approximately 70–75 per cent of the dose is excreted in bile

without chemical modification. Ouabain, as indicated by its high water solubility and behaviour on paper chromatograms, is still more polar than the lanatosides and is excreted, also unchanged, to the extent of about 90 per cent in bile. Evidently, the polarity of the molecule is a major factor in determining the rate and extent of excretion of cardiac glycosides by the liver.

The biliary excretion of ouabain and the lanatosides (which are all non-cumulative glycosides in man) contrasts with that of the cumulative glycoside digitoxin. This glycoside is relatively non-polar and only 10 per cent of the dose can be recovered in rat bile in the 5 hours after injection[9]. Furthermore, the biliary excretory products of digitoxin in the rat consist of both unchanged glycoside and its metabolite, 12-β-hydroxy digitoxin (digoxin)[9]. There is evidence[10] that the digitoxin excreted in human bile is reabsorbed from the intestine and recirculated in the body. Some of the digoxin produced by metabolism of digitoxin may also be reabsorbed, but in the rat at least some is excreted in the faeces[11]. Ouabain, because of its highly polar nature, is not absorbed to any appreciable extent from the intestine[1] and hence would not recirculate after being excreted in bile.

Acknowledgements. This work is supported by a grant from the National Health and Medical Research Council of Australia. The authors wish to thank Mr. David Oldfield for assistance with biological assays.

References

1. Hatcher and Eggleston, *J. Pharmacol.*, 1919, **12**, 405.
2. Farah, *ibid.*, 1946, **86**, 248.
3. Lehman and Paff, *ibid.*, 1942, **75**, 207.
4. Shepheard, Thorp and Wright, *ibid.*, 1954, **112**, 133.
5. Schenker, Hunger and Reichstein, *Helv. Chim. Acta*, 1954, **37**, 680.
6. Raymond, *Analyst*, 1938, **63**, 478.
7. Anderson and Chen, *J. Amer. pharm. Ass., Sci. Ed.*, 1946, **35**, 353.
8. Kaiser, *Chem. Ber.*, 1955, **88**, 556.
9. Cox and Wright, *J. Pharmacol.*, 1959, **126**, 117.
10. Okita, Talso, Curry, Smith and Geiling, *ibid.*, 1955, **115**, 371.
11. Repke, *Naturwiss*, 1958, **45**, 94.

THE SYNTHESIS OF SOME POTENTIAL ANTIMETABOLITES OF PHENYLALANINE

PART I. THE SYNTHESIS OF SOME $\gamma\gamma$-DIALKYL-α-AMINOBUTYRIC ACIDS

BY B. J. MEAKIN, F. R. MUMFORD AND E. R. WARD

From the School of Chemistry, The Leicester College of Technology and Commerce

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Seven new dialkylaminobutyric acids have been synthesised as potential antiviral and antibacterial agents. None showed any significant activity against the Newcastle disease virus *in vitro*.

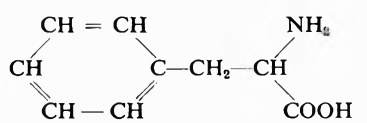
It is now a well established fact, that the natural amino acids are essential metabolites for many micro-organisms, but until recently, little work had been done with viruses. Many structural analogues of the natural amino acids have been synthesised, and a number of compounds with antibacterial activity discovered, but there is little systematic work of this nature with viruses.

The work of Dickinson and Thompson[1] indicated that phenylalanine was probably an essential metabolite for the Influenza A virus and further work by Dickinson (personal communication) suggests that this is also true for the Newcastle disease virus. This provides a possible point of attack in the search for compounds chemotherapeutically active against the smaller or so-called, true viruses. This suggestion is further supported by the evidence of antiviral acitivity in (*a*) the *ortho*, *meta* and *para* fluorophenylalanines[2], (*b*) the 2-substituted-3,4-dihydroxyphenylalanines[3], (*c*) α-amino-β-phenyl-ethane-sulphonic acid[4] and (*d*) β-(1-naphthyl) alanine[5].

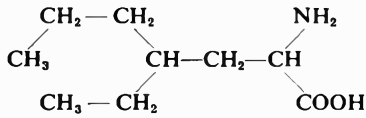
Dickinson[1] found the activity of β-phenylserine against the Influenza A virus to be reduced by modification of the amino, hydroxyl, or carboxyl groups, except in the methyl and ethyl esters, thus indicating that gross alterations of the polar parts of the molecule reduce activity. The increase in activity of the esters may be caused by an increase in the penetration of the compound into the cells owing to a decrease in the polarity of the molecule.

With these facts in mind, it seemed that the most reasonable point at which to modify the phenylalanine molecule, was the aromatic ring.

A variety of substitutions in the aromatic ring have been carried out by many workers in the past, but those compounds corresponding to an opened phenyl ring do not appear to have been reported in the literature and such compounds might fulfil the requirements of an antiviral antagonist of phenylalanine.



β-Phenylalanine



γ-Ethyl-γ-propyl-α-aminobutyr c acid

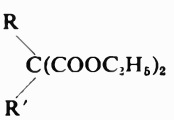
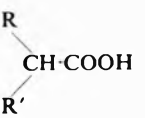
Compounds equivalent to opened 5, 6, 7, 8 and 9 membered rings have been synthesised and tested for activity against the Newcastle disease virus.

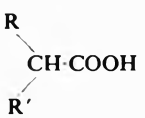
Preparation

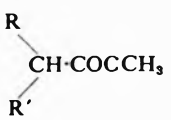
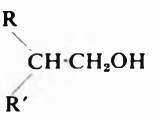
Acetamidomalonic ester and related acylamino esters have found extensive use in the synthesis of the natural[6–13] and also the synthetic amino acids containing an aromatic or heterocyclic nucleus[14–17] whereas new aliphatic amino acids have received little attention. The parent of the series, the γγ-dimethyl compound is the naturally occurring amino acid leucine and this has been prepared by the condensation of β-methyl allyl chloride with acetamidomalonic ester in 50 per cent yield[12], and its acetyl derivative from *iso*butyl bromide, the latter in 30 per cent yield[13]. The method seemed applicable to the synthesis of this series of compounds, which has now been accomplished by the condensation of the appropriate alkyl bromides with acetamidomalonic ester involving the following reaction sequence.

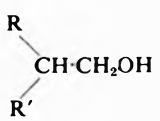
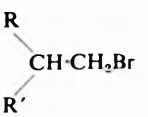
1. $RBr + CH_2(COOC_2H_5)_2 \xrightarrow{NaOC_2H_5} R{\cdot}CH(COOC_2H_5)_2$

2. $R'Br + R{\cdot}CH(COOC_2H_5)_2 \xrightarrow{NaOC_2H_5}$ 

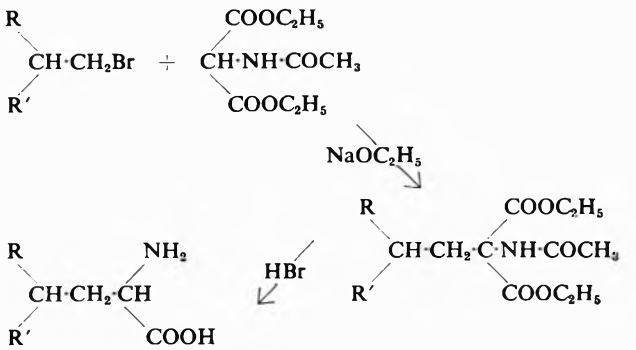
3.  $\xrightarrow[\text{Heat}]{\text{Hydrolyse}}$ 

4.  $\xrightarrow{CH_2N_2}$ 

5.  $\xrightarrow{LiAlH_4}$ 

6.  $\xrightarrow{PBr_3}$ 

7.

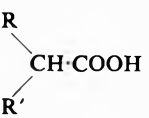


The synthesis of the αα-dialkyl acetic acids was by the standard procedure, preliminary work indicating, that there was no detriment to the yield, if the dialkyl malonic ester was not purified before hydrolysis and decarboxylation.

The methyl esters of the αα-dialkyl acetic acids were conveniently prepared by treating the acid the an excess of freshly prepared ethereal diazomethane[18].

TABLE I

αα-DIALKYL ACETIC ACIDS



R	R′	B.p. found	B.p. reported	n_D^{25}	Yield per cent	M.W. found	M.W. required	Reference
CH_3	C_3H_7	192–3°	192–3°	1·4110	66	117·2	116	24
CH_3	C_4H_9	209–10°	209°	1·4180	64	126·5	130	25
C_2H_5	C_3H_7	208–10°	210°	1·4158	68	129·0	130	26
C_3H_7	C_3H_7	221–2°	219–22°	1·4205	50	143·9	144	27
C_3H_7	C_4H_9	238–40°	238–40°	1·4210	66	158·8	158	28
C_4H_9	C_4H_9	252–55°	255°	1·4213	70	173·5	172	29, 30

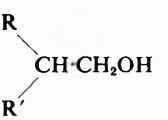
The primary alcohols were obtained by the reduction of the appropriately substituted acetic acid, or its methyl ester with lithium aluminium hydride. Brown[19], in his review of the applications of lithium aluminium hydride, states that the reduction of the carboxylic acid generally give a much lower yield of alcohol, than the reduction of the ester, but Sarel and Newman[20] have shown that with some acids, the reverse is true. Our work with αα-dipropyl acetic acid and α-ethyl-α-propyl acetic acid has shown that, in these instances, the yields by the two routes are comparable, being 82 and 84 per cent from the ester and 94 and 81 per cent from the acid respectively. Consequently, the overall yield from the acid is much greater if the ester stage is omitted, the yields from αα-dipropyl acetic acid via the methyl ester being 66 per cent and similarly from α-ethyl-α-propyl acetic acid, 59 per cent. Therefore, in the synthesis of the other alcohols, the substituted acetic acid methyl ester was not prepared.

Another synthesis of these $\beta\beta$-dialkyl ethanols according to Gilmore and Catlin[21], involving the action of gaseous formaldehyde on the Grignard reagent formed from the appropriate secondary bromide was attempted. It was found that the Grignard compound formed only with great difficulty, and the yield of alcohol obtained was very low.

The action of phosphorous tribromide on the appropriate alcohols under anhydrous conditions as described by Noller and Dinsmore[22] to form the required $\beta\beta$-dialkyl-α-bromo-ethanes was found to be the most convenient for their synthesis. The modification of Tseng, Hsu and Hu[23] whereby the reaction mixture is heated on a steam bath for one to two hours after the completion of addition produced a slight increase in the yield of bromide.

TABLE II

$\beta\beta$-DIALKYL ETHANOLS



R	R′	Compd. reduced by $LiAlH_4$	Yield per cent	B.p. found	B.p. reported	n_D^{25}	Reference
CH_3	C_2H_7	Acid	82	148°	148°	1·4153	31
CH_3	C_4H_9	Acid	90	163–4°	162–4°	1·4205	32
C_2H_5	C_3H_7	Acid	81	165–7°	164–6°	1·4222	33
		Methyl ester	84				
C_3H_7	C_3H_7	Acid	94	92° at 23 mm	179°	1·4272	34, 35
		Methyl ester	81	177–8°			
*C_3H_7	C_4H_9	Acid	92	103–4° at 22 mm.	—	1·4295	—
C_4H_9	C_4H_9	Acid	98	140° at 54 mm. 219–220°	218–19°	1·4285	36

* β-Propylhexanol has not been reported in the literature to date. Analysis gives C, 74·3 per cent and H, 13·8 per cent, $C_9H_{20}O$ requires C, 74·9 per cent and H, 13·9 per cent.

Five of the seven bromides do not appear to have been reported in the literature (see Table III). The substituted acetamidomalonic esters were prepared in the manner described by Snyder and others[7,10,13], refluxing being continued until the reaction mixture was neutral to moist litmus. Often only a small amount of sodium bromide was precipitated from the reaction mixture, and the compounds themselves could not be obtained crystalline. In each instance, a viscid brown oil was obtained which did not solidify, even on prolonged vacuum desiccation over phosphorus pentoxide, nor could it be recrystallised from a variety of solvents.

The crude α-alkyl-α-acetamidodiethyl malonates were hydrolysed by refluxing with 48 per cent hydrobromic acid for 24 hours and the resulting amino acids obtained by precipitation at their isoelectric points (pH 5–6) with ammonia.

Biological Results

The seven amino acids were tested against the Newcastle disease virus in tissue culture, using monolayers of chick embryo. The results showed

that none of the compounds possessed any significant activity at concentrations of 0·3 and 1·4 m.g. per ml.

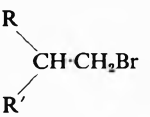
No severely toxic effects were exhibited towards the host cells at the higher concentration.

Experimental

The details of individual compounds are given in Tables I, II, III and IV. Typical syntheses are described.

TABLE III

ββ-Dialkyl-α-bromo-ethanes



R	R′	Yield per cent	B.p. found	B.p. reported	n_D^{25}	Analysis found C	Analysis found H	Analysis required C	Analysis required H	Reference
CH_3	C_3H_7	63	141–2°	142°	1·4475	—	—	—	—	37, 38, 39
CH_3	C_4H_9	50	75–5° at 8 mm.	—	1·4461	46·7	8·5	46·9	8·4	—
C_2H_5	C_3H_7	57	63–4° at 25 mm.	—	1·4421	46·6	8·1	46·9	8·4	—
C_2H_5	C_4H_9	73	75–7° at 16 mm.	80° at 18 mm.	1·4465	—	—	—	—	40, 41
C_3H_7	C_3H_7	65	80–2° at 18 mm.	—	1·4461	50·0	9·1	49·8	8·8	—
C_3H_7	C_4H_9	45	83° at 19 mm.	—	1·4515	52·8	9·3	52·2	9·2	—
C_4H_9	C_4H_9	51	130–2° at 60 mm.	—	1·4490	53·9	9·3	54·3	9·5	—

n-*Propylmalonic acid diethyl ester.* Sodium (46 g., 2·0 mol.) in dry ethanol (1·5 l.) was treated with malonic ester (320 g., 2·0 mol.) the mixture heated to reflux and *n*-propyl bromide (246 g., 2·0 mol.) added slowly over one hour. Refluxing was continued for six hours, until the reaction mixture was neutral to moist litmus, and the majority of the solvent then removed by distillation. The residue was cooled, diluted with water (200 ml.) the upper layer separated, and the aqueous layer extracted with ether (50 ml.). The organic liquors were dried over anhydrous sodium sulphate, the ether removed and the residue distilled to yield *n*-propyl malonic acid diethyl ester (370 g. 91 per cent) b.p. 117–118° at 22 mm.

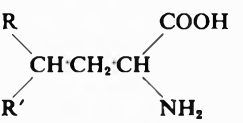
α-*Ethyl*-α-n-*propylacetic acid.* Sodium (23 g., 1·0 mol.) in dry ethanol (1·0 l.) was treated as above with *n*-propylmalonic acid, diethyl ester (202 g., 1·0 mol.) followed by ethyl bromide (109 g., 1·0 mol.), and the mixture refluxed for 19 hours until neutral. The reaction mixture was worked up to yield the crude ester, which was hydrolysed by dropping slowly into a stirred, refluxing solution of potassium hydroxide (140 g., 2·5 mol.) in ethanol (1·0 l.). After refluxing overnight, the solvent was removed over a water bath, the soapy residue dissolved in the minimum

quantity of water, cooled in ice and acidified with concentrated hydrochloric acid. The upper layer was separated and the aqueous residue extracted successively with ether (50 ml. portions) until no residue remained on evaporating a portion on a steam bath. The combined organic liquors were evaporated on a steam bath and the sticky brown residue was heated on a metal bath at 180° for 6 hours. The organic layer was separated from the small amount of water which had formed, dried over anhydrous sodium sulphate and fractionated to yield α-ethyl-α-*n*-propylacetic acid (94·5 g., 70 per cent) b.p. 208–210°.

α-*Ethyl*-α-n-*propylacetic acid methyl ester.* α-Ethyl-α-*n*-propylacetic acid (23·6 g.) was treated with an excess of an ethereal solution of diazomethane, from *p*-tolylsulphonyl-methyl-nitrosoamide, and the mixture stood overnight. The ether was removed and the residue shaken with 10 per cent aqueous sodium hydroxide (25 ml.) and twice with water (25 ml.). After drying over anhydrous sodium sulphate the residue was distilled to yield α-ethyl-α-*n*-propyl-acetic acid methyl ester (18·3 g., 71 per cent) b.p. 157°.

TABLE IV

γγ-DIALKYL-α-AMINOBUTYRIC ACIDS



R	R′	Reflux* time hours	Yield† per cent	M.p.‡	Analyses found C	found H	found N	required C	required H	required N
CH_3	C_3H_7	48	40	211–2°	60·2	10·7	8·6	60·3	10·7	8·8
CH_3	C_4H_9	48	50	226°	62·6	11·1	8·4	62·4	10·9	8·1
C_2H_5	C_3H_7	48	49	220–3°	61·5	10·9	8·1	62·4	10·9	8·1
C_2H_5	C_4H_9	60	48	227–8°	64·2	11·4	7·7	64·2	11·3	7·5
C_3H_7	C_3H_7	72	33	221–2°	64·5	11·1	7·3	64·2	11·3	7·5
C_3H_7	C_4H_9	72	46	229–31°	65·6	11·4	7·0	65·7	11·5	7·0
C_4H_9	C_4H_9	72	40	229–30°	66·7	11·6	6·4	66·7	11·6	6·5

* The reflux time is for the condensation between acetamidodiethyl malonate and the appropriate alkyl bromide.
† The yield is calculated from the appropriate alkyl bromide
‡ Melting points all occurred with decomposition.

β-*Ethylpentanol. Method A.* Lithium aluminium hydride (4·0 g., 0·1 mol.) was stirred into a slurry with ether (200 ml.), previously dried over lithium aluminium hydride, and α-ethyl-α-*n*-propyl-acetic acid methyl ester (18·3 g., 0·125 mol.), in an equal volume of dry ether, was added at a rate which just maintained reflux. After the completion of addition, the mixture was refluxed for a further 30 minutes, and the complex decomposed by the addition of wet ether, and then iced dilute hydrochloric acid. The ethereal layer was separated, washed with 10 per cent aqueous sodium bicarbonate (20 ml.), water (20 ml.) and dried over anhydrous sodium sulphate. After removal of the ether, the residue was distilled to yield β-ethylpentanol (12·5 g., 83 per cent), b.p. 165–167°.

β-Ethylpentanol. Method B. Lithium aluminium hydride (20 g., 0·5 mol.) in dry ether (500 ml.) was treated as above with α-ethyl-α-*n*-propylacetic acid (65 g., 0·5 mol.). Isolation of the product yielded β-ethylpentanol (47·1 g., 81 per cent), b.p. 165–167°.

α-Bromo-β-ethylpentane. β-Ethylpentanol (34·8 g., 0·3 mol.) was cooled to −10° and phosphorous tribromide (32·5 g., 0·13 mol.) was slowly added maintaining the temperature below 0°. After the completion of addition, the reaction mixture was stirred overnight and then heated on a steam bath for 2 hours. The mixture was poured into iced water (250 ml.), the lower layer separated and shaken with concentrated sulphuric acid (d. 1·84, 25 ml.). The organic layer was then washed successively with 25 ml. portions of water, aqueous 10 per cent sodium carbonate, and water, dried over anhydrous sodium sulphate, and distilled to yield *α-bromo-β-ethylpentane* (35 g., 65 per cent), b.p. 63–64° at 25 mm.

α-Acetamido-α-(β-ethylpentyl)malonic acid diethyl ester. Sodium (2·3 g., 0·1 mol.) in dry ethanol (250 ml.) was treated with α-acetamidomalonic acid diethyl ester (21·7 g., 0·1 mol.) the mixture heated to reflux, and α-bromo-β-ethylpentane (17·9 g., 0·1 mol.) was added over 30 minutes. The mixture was refluxed for 48 hours, until neutral to moist litmus, and the bulk of the ethanol removed over a steam bath. The residue was cooled, diluted with iced water (250 ml.) the upper layer separated and the aqueous residue extracted with ether (50 ml.). The combined organic liquors were dried over anhydrous sodium sulphate and the ether removed to yield a viscid brown oil (20·7 g.), which did not solidify on prolonged vacuum desiccation. All attempts to recrystallise the oil proved unsuccessful. The yield of oil was equivalent to 65 per cent of *α-acetamido-α-(β-ethylpentyl)malonic acid diethyl ester.*

γ-Ethyl-γ-propyl-α-aminobutyric acid. The whole of the crude α-acetamido-α-(β-ethylpentyl)malonic acid diethyl ester was refluxed with 48 per cent of hydrobromic acid (100 ml.) for 48 hours. After cooling, the resultant brown crystals were filtered off, and the residue evaporated under reduced pressure. Water was added and the mixture re-evaporated. This was repeated until the distillate was only faintly acidic.

The final concentrate and the crystals were dissolved in water (200 ml.) treated with decolourising charcoal, filtered and the volume reduced to half. The resulting colourless solution was adjusted to pH 5–6 with dilute ammonia, the bulky precipitate filtered off and recrystallised twice from water to yield *γ-ethyl-γ-propyl-α-aminobutyric acid* (8·5 g., 59 per cent) as colourless plates, m.p. 220–222° with decomposition.

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References

1. Dickinson and Thompson, *Brit. J. Pharmacol.*, 1957, **12**, 66.
2. Pearson, Lagerborg and Winzler, *Proc. Soc. exp. Biol. N.Y.*, 1952, **79**, 409.

3. Kaiser and Berger, *J. Amer. chem. Soc.*, 1957, **79**, 4365.
4. Ackermann, Kurtz, Kleinschmidt and Uhlendorf, *Proc. Soc. exp. Biol. N.Y.*, 1952, **80**, 362.
5. Rafelson, Pearson and Winzler, *Arch. Biochem.*, 1950, **29**, 69.
6. Redeman and Dunn, *J. biol. Chem.*, 1939, **130**, 341.
7. Snyder, Howe, Cannon and Nyman, *J. Amer. chem. Soc.*, 1943, **65**, 2211.
8. Albertson and Tuller, *ibid.*, 1945, **67**, 582.
9. Galat, *ibid.*, 1947, **69**, 965.
10. Snyder, Skelton and Lewis, *ibid.*, 1945, **67**, 310.
11. Atkinson and Scott, *J. chem. Soc.*, 1949, 1040.
12. Albertson and Archer, *J. Amer. chem. Soc.*, 1945, **67**, 308.
13. Snyder and Smith, *ibid.*, 1944, **66**, 354.
14. Elliot, Fuller and Harrington, *J. chem. Soc.*, 1948, 85.
15. Burchhalter and Stephens, *J. Amer. chem. Soc.*, 1951, **73**, 56.
16. Bixler and Nieman, *J. org. Chem.*, 1958, **23**, 575.
17. Mamalis, Petrow and Sturgeon, *J. chem., Soc.*, 1950, 1600.
18. de Boer and Backer, *Organic Syntheses*, Vol. 34, Wiley and Co. New York, 1954, p. 24.
19. Brown, *Organic Reactions*, Vol. VI, Wiley and Co., New York, 1951, p. 478.
20. Sarel and Newman, *J. Amer. chem. Soc.*, 1956, **78**, 5416.
21. Gilmore and Catlin, *Organic Syntheses*, Collective Vol. I, Wiley and Co., New York, 1941, p. 188.
22. Noller and Dinsmore, *ibid.*, Collective Vol. II, Wiley and Co., New York, 1943, p. 358.
23. Tseng, Hsu and Hu, *Science Quart. Nat. Univ. Peking*, 1935, **5**, 371.
24. Heilbron and Bunbury, *Dictionary of Organic Compounds*, Vol. II, Eyre and Spottiswood, London, 1934, p. 829.
25. *ibid.*, Vol. II, p. 642.
26. *ibid.*, Vol. II, p. 52.
27. *ibid.*, Vol. I, p. 672.
28. Sommaire, *Bull. Soc. Chim. France*, 1926, **33**, 190.
29. Levene and Cutcher, *J. biol. Chem.*, 1918, **33**, 509.
30. Tiffeneau, *Bull. Soc. Chim. France*, 1926, **33**, 188.
31. Heilbron and Bunbury, *Dictionary of Organic Compounds*, Vol. II, Eyre and Spottiswood, London, 1934, p. 732.
32. *ibid.*, Vol. II, p. 620.
33. Morgan, Hardy and Procter, *J. Soc. Chem. Ind.*, 1932, **51**, T1.
34. Koller and Kandler, *Monatsch.*, 1931, **58**, 213.
35. Bhathande, Phalniker and Bhide, *Chem. Abstr.*, 1941, **35**, 6930.
36. Franzen, *Chem. Ber.*, 1954, **87**, 1219.
37. Levene and Mikesa, *J. biol. Chem.*, 1929, **84**, 580.
38. Olivier, *Rec. Trav. Chim.*, 1936, **55**, 1027.
39. Rhehberg and Henze, *J. Amer. chem. Soc.*, 1941, **63**, 2785.
40. Weizmann, Bergmann and Haskelberg, *Chem. Ind.*, 1937, **56**, 587.
41. Rhinesmith, *J. Amer. chem. Soc.*, 1936, **58**, 596.

THE HISTAMINE-HEPARIN COMPLEX

By R. K. Sanyal and G. B. West

From the Departments of Pharmacology, Darbhanga Medical College, Bihar, India, and School of Pharmacy, University of London, Brunswick Square, London, W.C.1

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Some factors concerned in the formation *in vitro* of a complex between the base histamine and the acid heparin have been studied. More histamine is contained in the complex when precipitation is carried out in acid medium in the presence of adenosine triphosphate. Although the staining properties and morphological characters of the complex resemble those of natural mast cell granules, it has not been possible to release most of the histamine from the synthetic granules without dissolving them. 5-Hydroxytryptamine, noradrenaline and adrenaline failed to form complexes with heparin and did not alter the formation of the complex of histamine and heparin.

In recent years, tissue mast cells of mammals have been shown to contain both heparin and histamine[1,2]. Although there is a close relationship between the heparin content of a particular tissue and the amount of histamine that can be extracted from it, it is still uncertain how the histamine is held in these cells. Experiments have therefore been made to study the affinity of heparin for histamine under varying conditions. Since 5-hydroxytryptamine (5-HT) has been suggested as a constituent of mast cells in certain species[3], the affinity of heparin for 5-HT has also been studied. A preliminary note on this work has already been reported[4].

Methods

The following drugs were dissolved in normal saline. Ox heparin, 20 mg./ml., histamine base, histamine acid phosphate, histamine dihydrochloride, 5-HT creatinine sulphate, noradrenaline and adrenaline acid tartrates, and adenosine triphosphate (ATP), each 1 mg./ml. After mixing equal volumes of one of the amines and of heparin, the pH value of the mixture was determined and adjusted if necessary. Ethanol was then added to give a final concentration of 70 per cent (v/v). At other times, adenosine was included, and occasionally acetone was used for precipitation instead of the alcohol. The precipitate of heparin was separated by centrifugation, dissolved in normal saline and assayed for its amine content. The histamine content of both the complex and the supernatant was estimated on the isolated atropinised guinea pig ileum, checking the specificity of the response with mepyramine (10^{-7}). The 5-HT content was estimated on the isolated atropinised uterus or colon of a rat in oestrus, using 2-bromolysergic acid diethylamide to check specificity. The adrenaline content was measured on the blood pressure of an atropinised cat before and after dibenamine (10 mg./kg.).

In a few experiments, the precipitate was shaken with toluidine blue (0·1 per cent, w/v) in 70 per cent (v/v) ethanol, washed with 70 per cent

ethanol, and then spread on a microscope slide and covered with Xam neutral mountant.

For paper chromatographic studies, the chief solvent system used was 70 per cent (v/v) ethanol adjusted to pH 6·0. This pH value was chosen as more histamine was removed by the precipitate of heparin at acid pH values than at alkaline pH values. Other solvent systems used included *iso*propanol : water : acetic acid (53 : 46 : 1) and *n*-propanol : water (3 : 1). Spots each of 0·01 and 0·05 ml. of the mixture of amine and heparin were applied to Whatman No. 1 paper and ascending chromatograms run for 6 hours. The developing agents used were Pauly's reagent for histamine, Ehrlich's reagent for 5-HT, potassium iodate for noradrenaline and adrenaline, and toluidine blue for heparin. The intensities of the colours which developed were compared with those of known amounts of standard amine solutions. In some experiments, the active areas were eluted from the chromatograms with water and tested biologically.

Results

Heparin-Histamine Complex

Heparin formed complexes with all three histamine preparations. However, about twice as much histamine was removed from the solution when the acid phosphate or the dihydrochloride was used (67–70 per cent) than when the base was tested (36 per cent). Table I shows the mean

TABLE I

THE DISTRIBUTION PER CENT OF HISTAMINE IN THE SUPERNATANT AND IN THE PRECIPITATE OF HEPARIN WHEN ETHANOL IS ADDED TO THE MIXTURE OF HISTAMINE, 1 mg., AND HEPARIN, 20 mg., WITH AND WITHOUT ADENOSINE TRIPHOSPHATE, 1 mg.

Histamine preparation	With adenosine		Without adenosine	
	Supernatant	Precipitate	Supernatant	Precipitate
Base	70	30	64	36
Acid phosphate ..	10	90	33	67
Dihydrochloride ..	10	90	30	70

results of 18 experiments, and also records the results when adenosine triphosphate was included in the mixture. The presence of ATP allows more histamine to be removed from the mixture by the heparin when one of the histamine salts was used, but not with the base. In all experiments, the complex of heparin and histamine base was much finer than that of heparin and one of the histamine salts. Repeated washing of the complexes with ethanol or acetone failed to remove the histamine, but they were readily soluble in water, normal saline or weak acid, giving solutions with biological effects corresponding to those of the respective amine and free heparin.

The combination of heparin and histamine took place at all temperatures used (between 4 and 40°), the amount of histamine removed being fairly constant. But the pH value of the mixture before precipitation was significant, as more histamine was associated with the complex if the solution was acid. The optimal pH value was about 6·0 (Fig. 1). This

complex stained with toluidine blue and resembled in appearance and size natural mast cell granules isolated from mouse connective tissue[5]. In the solvent system of 70 per cent ethanol adjusted to pH 6·0, the mixture

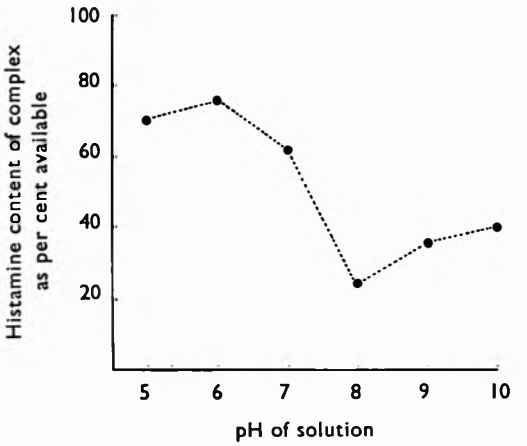

FIG. 1. The effect of pH on the histamine content of the heparin-histamine complex, formed by adding ethanol to a mixture of heparin, 20 mg., and histamine acid phosphate, 1 mg. Maximal removal of histamine from the solution occurs at pH 6·0.

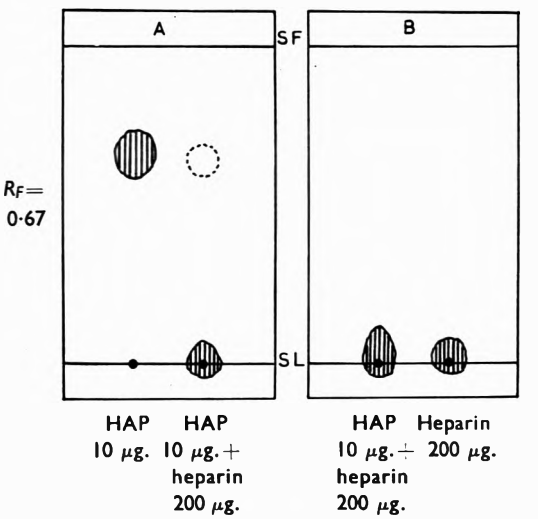

FIG. 2. Chromatographic separation of the heparin-histamine complex. Solvent is 70 per cent ethanol (pH 6·0). Ascending paper chromatograms 6 hour contract. Histamine (HAP) developed by Pauly reagent (A), heparin by aqueous toluidine blue (B). Note the two spots for histamine in the mixture of histamine and heparin, the stronger being at R_F value = 0. SL = starting line. SF = solvent front.

of heparin and histamine gave two spots for histamine, one at R_F value of 0·68 and one at 0. Histamine alone gave one spot at R_F 0·67, and heparin one at R_F 0. On elution, about 70 per cent of the total histamine

was found to be located with the heparin, the remainder running to the spot corresponding to free histamine (see Fig. 2).

Complexes of Heparin with Other Bases

When 5-HT, noradrenaline, or adrenaline was used instead of histamine, the heparin precipitate contained less than 1 per cent of the respective amine. The chromatographic behaviour of the mixtures in 70 per cent ethanol (pH 6·0) also resembled that of the respective constituents. When each of these three amines was added to the heparin and histamine mixture before precipitation with ethanol, there was no alteration in the composition of the complex with histamine.

Complexes of Histamine with Other Substances

When hog mucin was used in place of heparin, no histamine was attached to the precipitate of this mucopolysaccharide formed by the addition of ethanol. The histamine liberator, compound 48/80, failed to displace histamine from its complex with heparin, but when it was added to the mixture of heparin and histamine before the addition of ethanol the uptake of histamine was reduced from 72 per cent at pH 6·0 to 53 per cent. In a similar manner, the amino acid, lysine, slightly reduced the uptake of histamine by heparin.

Discussion

The results show firstly that histamine forms a complex *in vitro* with heparin, and secondly that the morphological characters and staining properties of the complex resemble those of naturally occurring granules. Its formation appears to be specific as noradrenaline, adrenaline and 5-HT all fail to combine with heparin under similar experimental conditions. Further, it can be shown that, when precipitated heparin is shaken with these amines, only histamine is taken out of solution. These results have been obtained with heparin of ox origin, and it is possible that different conclusions might be reached when heparin of a different source is used.

The preparations of heparin and histamine in the complex (namely, 20 parts to 1 part respectively) are similar to those found in extracts of tissues known to be rich in mast cells[6]. Yet it has not been possible so far to release most of the histamine from the synthetic complex except by dissolving it. Certain basic amino acids like lysine displace histamine from its attachment *in vivo* to the acidic residue of heparin by some kind of cation exchange[7], yet they fail to do so from the synthetic complex *in vitro*. Likewise, compound 48/80 is not effective in this respect. Both lysine and compound 48/80 slightly reduce the uptake of histamine by heparin when present in the mixture before precipitation[8], but further work is needed to determine the optimal conditions for this action.

In the presence of adenosine triphosphate, more histamine is removed from the mixture by heparin especially when the pH of the solution is around 6·0. In the tissues, biologically active amines like noradrenaline, adrenaline, 5-HT and histamine are associated with adenosine triphosphate which assists in their binding by providing energy bonds. It is

possible that natural mast cell granules use adenosine triphosphate in this manner and this would explain why its presence causes more histamine to be present in the synthetic complex. Adsorption of the histamine to the surface of the precipitated heparin is unlikely since a good adsorbent like kaolin removed not only histamine, but also noradrenaline, adrenaline and 5-HT, from solutions of these amines.

References

1. Riley and West, *J. Physiol.*, 1953, **120**, 528.
2. Graham, Lowry, Wahl and Priebat, *J. exp. Med.*, 1955, **102**, 307.
3. Benditt, Wong, Arase and Roeper, *Proc. Soc. exp. Biol. Med., N.Y.*, 1955, **90**, 303.
4. Sanyal and West, *Nature, Lond.*, 1956, **178**, 1293.
5. West, *J. Pharm. Pharmacol.*, 1955, **7**, 80.
6. Cass, Riley, West, Head and Strand, *Nature, Lond.*, 1954, **174**, 318.
7. Eldridge and Paton, *J. Physiol.*, 1954, **124**, 27*P*.
8. Werle and Amman, *Klin. Wschr.*, 1956, **34**, 624.

INFLUENCE OF COPPER AND EDTA ON THE ALKALINE OXIDATION OF ADRENALINE

By J. G. Lennart Harthon

From the Analytical Department, Research Laboratories, AB Bofors, Nobelkrut, Bofors, Sweden

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Adrenaline can be determined quantitatively in the presence of sulphite by measuring the maximum fluorescence obtained by oxidation in alkaline solution. The presence of copper in the solution quenches the fluorescence according to Stern-Volmer's law. Ethylenediaminetetra-acetic acid present in copper-free solutions has no influence on the fluorescence intensity curve. When copper is present as EDTA-complex the oxidation is strongly catalysed and the fluorescence maximum appears earlier the higher the concentration of complex. The reactions do not seem to be photochemically influenced.

Adrenaline in low concentrations can be quantitatively determined by measurement of the transient fluorescence from an unstable intermediate product, *N*-methyltrihydroxyindole, formed during the alkaline oxidation of adrenaline[1,2].

Copper ions are known to catalyse the oxidation of adrenaline[3,4] and so it is necessary to keep the concentration of copper as low as possible in pharmaceutical preparations containing adrenaline. Green, Mazur and Shorr[5] have studied the effect of iron on the oxidation of adrenaline at pH 7·4 and showed that this catalysed oxidation is accelerated tenfold in the presence of the chelating agent ethylenediaminetetra-acetic acid (EDTA).

The purpose of this preliminary investigation was to study how the presence of copper influenced the fluorescence obtained in the determination of adrenaline based on its oxidation in strongly alkaline solution and to what extent the simultaneous presence of EDTA changed the fluorescence-time relation. As the primary interest in this reaction was its use in the quantitative fluorimetric determination of adrenaline in anaesthetic solutions, all the adrenaline solutions also contained 0·05 per cent sodium metabisulphite. This means that the copper might be present in the monovalent state, either as a complex with sulphite $(CuSO_3)^-$ or hydroxide as experiments indicated that there was still sulphite present, even after the adrenaline was oxidised.

Experimental

All chemicals were selected to contain a low content of heavy metals. The water used was repeatedly distilled in all-glass apparatus and its copper content was less than 2 μg./l. Stock adrenaline solutions were prepared by dissolving adrenaline base (UCLAF, Paris) and sodium metabisulphite in diluted hydrochloric acid. The final working solutions were prepared so that they were 0·01 N in hydrochloric acid and contained 0·05 per cent sodium metabisulphite.

The alkaline solution for the oxidation consisted of 1·1 N sodium hydroxide in 50 per cent (v/v) ethanol with the content of oxygen which was obtained when the solution was equilibrated in air.

The mixture on which the fluorescence was measured was made from 20 ml. of oxidation solution to which was first added 3·00 ml. of water or the same amount of a solution containing the appropriate amounts of copper sulphate and EDTA, and then, rapidly, 2 ml. of adrenaline solution. Time was measured with a stop watch when the adrenaline solution had been added. The first reading was made 15 seconds later and then readings were taken every 10 seconds for about 6 minutes. The blank value of the solution was taken from the same solution about an hour later when fluorescence had ceased.

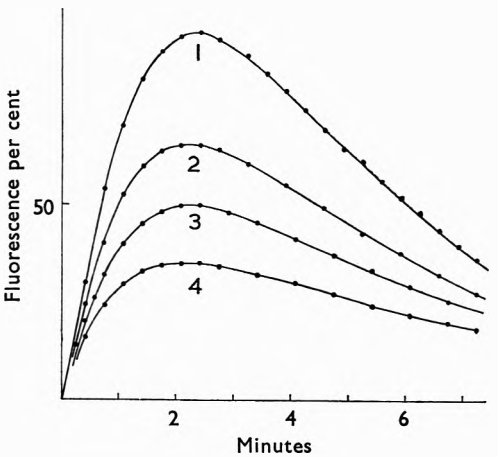

FIG. 1. Fluorescence-time relation for the alkaline oxidation of 0·8 μg./ml. of adrenaline (1). The same reaction for solutions containing in addition 0·08 μg./ml. of Cu (2); 0·2 μg./ml. of Cu (3); and 0·4 μg./ml. of Cu (4).

The fluorescence was measured in a Photovolt filter-fluorimeter Model 540 with photomultiplier attachment and a Hanovia S4 mercury vapour lamp as a source for the exciting radiation. The primary filter had its transmission maximum at 365 mμ and the secondary filter at 505 mμ. The fluorescence standard consisted of a solution of 2-ethoxy-6,9-diamino-acridinelactate (Rivanol).

RESULTS AND DISCUSSION

For adrenaline solutions containing up to 0·8 μg./ml. of adrenaline, the maximum fluorescence showed good proportionality against concentration. Increasing the metabisulphite content to 0·5 per cent had no influence on the results. The time from mixing the solutions until maximum fluorescence was reached was about 135 seconds and independent of the concentration of adrenaline.

Figure 1 shows the relation between fluorescence and time for 0·8 μg./ml. of adrenaline with an additional amount of copper increasing from 0·0 to 0·4 μg./ml. It is apparent that the addition of copper lowers the maximum fluorescence (I_0) for a given amount of adrenaline. A linear relation between fluorescence (I) and inhibitor concentration (Q) cannot be demonstrated but if I_0/I is plotted against Q (Fig. 2) a straight line is obtained for a copper concentration of up to 1·2 μg./ml. This indicates that Stern-Volmer's law $I/I_0 = 1/(1 + KQ)$ is obeyed and, calculated in molar quantities from the slope of the curve, $K = 2{\cdot}4 \times 10^5$

$1 \times$ mol.$^{-1}$ is obtained. The fulfilment of Stern-Volmer's law indicates that copper in this reaction acts as a fluorescence quencher.

The time after which fluorescence maximum is reached seems to be independent of the amount of copper added (Fig. 1). But the experimental arrangements did not give sufficient accuracy in the fluorescence-time relation to permit any conclusions about the catalytic activity of copper on the oxidation. This is to be the subject for further investigations.

The effect of an addition of EDTA in concentrations up to 8 mg./ml. was tested on copper-free adrenaline solutions. No influence was noted on either the maximum fluorescence or the progress of the fluorescence. The results are, however, different if copper is present too. Figure 3 shows the changes in the progress of fluorescence of an adrenaline solution containing copper at different concentrations of EDTA. The maximum fluorescence appears earlier and is increased in comparison with the same solution without EDTA. Additions of EDTA up to 8 mg./ml. were investigated on a solution containing 0·8 μg./ml. of adrenaline and 0·2 μg./ml. of Cu. The highest fluorescence was obtained at about 2 mg./ml. of EDTA and was about 85 per cent of the fluorescence for a copper-free solution. At higher concentrations of EDTA the maximum fluorescence was again slowly diminished.

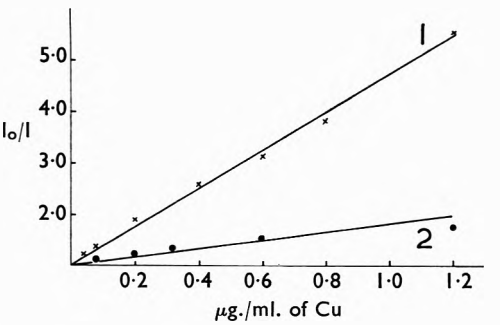

FIG. 2. Influence of increasing amounts of copper on the maximal fluorescence of adrenaline solutions (0·8 μg./ml.) containing (1) none or (2) 2 mg./ml. of EDTA.

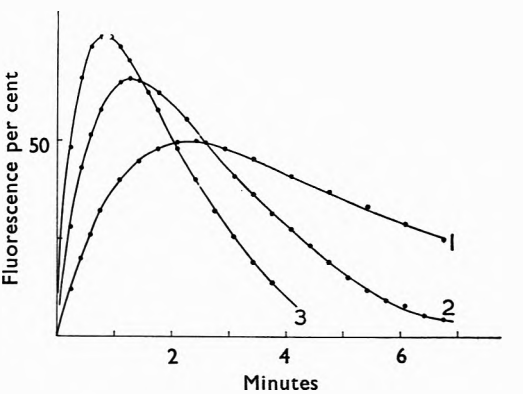

FIG. 3. Fluorescence-time relations for 0·8 μg./ml. of adrenaline and 0·2 μg./ml. of Cu containing (1) none; (2) 0·2; (3) 0·8 μg./ml. of EDTA.

The influence on the maximum fluorescence of different concentrations of copper at a constant content of EDTA (2 mg./ml.) is represented in Figure 2 and plotted in the same way as for EDTA-free solutions. It is evident that an increasing copper concentration decreases the fluorescence but not to the same extent as for EDTA-free solutions. The time for reaching fluorescence maximum is also shortened when copper and

EDTA are present together. In this experiment the following figures were obtained:

μg./ml. Cu	0·0	0·2	0·4	0·8	1·6
time for max in seconds ..	135	85	55	35	25

If it is supposed that the reaction products of these experiments are the same it is evident from Figure 3 that the simultaneous presence of copper and EDTA strongly catalyses the oxidation of adrenaline in alkaline solution. As the oxidation of adrenaline to adrenochrome is the rate determining reaction in the formation of the fluorescent substance[2], it is evident that this reaction is catalysed as the fluorescence maximum appears earlier. Figure 3, however, also indicates that the destruction of the fluorescent substance might be catalysed. The same phenomenon was observed by Ehrlén[6], when using this method for the determination of adrenaline in procaine solutions, and might have been due to some metal complex in his solutions. The reaction rates in these reactions will, however, be studied in more detail.

As copper can act as a photochemical catalyst, there was reason to see if the oxidation of adrenaline in these experiments was influenced in any way by the energy of the exciting radiation. This was performed in such a way that several fluorescence-time relations for solutions containing copper as well as copper-EDTA were recorded with different entrance slits on the fluorimeter. The slit was changed so that the fluorescence standard gave the same instrument deflection at a ten times lower sensitivity on the detector. In no case could any change in the course of the curves be noted. The oxidation of adrenaline under these conditions seems therefore not to be photochemically influenced.

References

1. Ehrlén, *Farm. Revy*, 1946, **45**, 753.
2. Heller, Setlow and Mylon, *Amer. J. Physiol.*, 1950, **161**, 268.
3. Chaix, Chauvet and Jezequel, *Biochem. Biophys. Acta*, 1950, **4**, 471.
4. Mørch, *Pharm. Weekbl.*, 1958, **93**, 141.
5. Green, Mazur and Shoor, *J. biol. Chem.*, 1956, **220**, 237.
6. Ehrlén, *Farm. Revy*, 1948, **47**, 242.

SOME PHYSICO-CHEMICAL STUDIES ON SALTS OF LONG CHAIN DICARBOXYLIC ACIDS

By P. H. Elworthy

From the Department of Physical Chemistry, School of Pharmacy, University of London, Brunswick Square, W.C.1.

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Conductimetric studies on the dipotassium salts of acids of the type $HOOC(CH_2)_nCOOH$ have been made on sebacic acid (K_2, $n = 8$), dodecanedicarboxylic acid (K_2, $n = 12$), and hexadecane dicarboxylic acid (K_2, $n = 16$). The equivalent conductance-concentration curve for K_2, $n = 16$ showed breaks at 11 and at about 33 mM $l.^{-1}$; diffusion studies indicated that the formation of small aggregates occurred at 10 mM $l.^{-1}$, the aggregates containing two molecules. The second concentration limit was believed to represent either a change in the electrical properties of the solute, or an increase in the amount of aggregate present. Certain tentative deductions about the length of the molecule in solution were made from the diffusion results.

Very few studies have been made on the salts of dicarboxylic acids of long chain length in solution. Danielsson[1] has studied conductivity, solubilisation, and pH of the dipotassium salts of sebacic, brassylic (K_2,$n = 11$), tetradecane dicarboxylic (K_2,$n = 14$,) and hexadecane dicarboxylic acids. Breaks in the conductivity-concentration curves were observed for the salts of the last two acids, and were believed to correspond to micelle formation. Solubilisation of decanol by K_2,$n = 14$ began at 0·35 M $l.^{-1}$ at 40°, and at 0·027 M $l.^{-1}$ for K_2,$n = 16$ at 60°. The final values assigned to the critical micelle concentrations (CMC) were: K_2,$n = 16$, — 0·04 M $l.^{-1}$ at 60°; K_2,$n = 14$, — 0·32 — 0·4 M $l.^{-1}$ at 40°, and 0·4 — 0·5 M $l.^{-1}$ at 60°.

No direct determination of micelle size was made; this is of interest in view of the introduction of a second polar group into the structure of normal soaps. In this paper conductance studies on solutions of the dipotassium salts of $n = 8$, $n = 12$, and $n = 16$ are reported, and also diffusion measurements on K_2,$n = 16$ to determine the micelle size. (For brevity all dipotassium salts are referred to as K_2,$n = \ldots.$)

Experimental

Materials. The methyl esters of dodecane and hexadecane dicarboxylic acids were converted to the potassium salts by refluxing for 4 hours with 20 per cent potassium hydroxide in 50 per cent aqueous ethanol. On cooling the potassium salts crystallised; the crystals were washed with ethanol, extracted continuously with ether for 48 hours to remove traces of the ester, recrystallised three times from ethanol containing the minimum amount of water, dried in a vacuum oven and stored under vacuum. K_2,$n = 8$ was prepared from recrystallised sebacic acid by boiling with a five times excess of 20 per cent potassium hydroxide in 50 per cent aqueous ethanol for 4 hours, allowing to crystallise, and

purifying in the same manner as the salts of the other acids. A portion of the free acid was prepared from each potassium salt.

Analysis figures were: $n = 8$, free acid molecular weight = 202·7 (202·2); $K_2,n = 8$, K = 28·04 per cent (28·09 per cent). $n = 12$, free acid molecular weight = 258·7 (259·3); $K_2,n = 12$, K = 23·35 per cent (23·38 per cent). $n = 16$, free acid molecular weight = 314·7 (314·5); $K_2,n = 16$, K = 20·03 per cent (20·02 per cent). Theoretical figures are given in brackets.

Density of K_2,n = 16 was determined using a displacement technique in dry benzene in a stoppered specific gravity bottle. Finely powdered solid was used, and was freed from included air by repeated evacuations under dry benzene. Repeat measurements gave 1·280 and 1·280 g.ml.$^{-1}$ at 25°.

Conductivities. These were determined with a Cambridge Instrument Co. conductivity bridge, the final reading being made by the method of Ward and Chitale[2], using an external resistance. All solutions were made in demineralised water to provide a small solvent correction, and to minimise any effect of carbon dioxide on the long chain salts. Both conductivity and diffusion experiments were performed at 25 ± 0·05°.

Diffusion Coefficients. These were measured on a Gouy diffusiometer of a type similar to that of Gosting[3] and Saunders[4]. All components were mounted on a vibration free 2 m. optical bench. The green line (5461 Å) was isolated by interference filters from a mercury vapour lamp and illuminated a slit of 12·5 μ width, which was mounted horizontally on the optical bench. The image of the slit was focused through the diffusion cell on to a photographic plate.

The diffusion cell[5], which is of a new type, was fitted with optically flat ($\lambda/2$) windows; it had the general form of a U-tube, the boundary being initially formed in a constricted part of one arm of the U-tube above the cell windows. After formation, the boundary was gently displaced downwards to the middle of the windows, where it was sharpened by flow of liquid through a 50 μ slit set in one wall of the cell. An experiment was started by stopping the outflow from the cell, and allowing diffusion to commence. The interference patterns consisted of a series of closely spaced fringes, which were photographed at timed intervals after the start of diffusion. After development, distances on the plates were measured with a Cambridge universal measuring machine to 0·0002 cm.

No correction had to be made for Δt in any experiment; there was a random variation of diffusion coefficient with time. (Δt is the time taken for an infinitely sharp boundary to reach the state of the existing boundary when flow from the cell is stopped.)

Results

The conductivity results are shown graphically in Figure 1 as a plot of the equivalent conductivity (Λ) against (normality)$^{\frac{1}{2}}$. The curves for $K_2,n = 8$ and $K_2,n = 12$ are almost parallel over the concentration range studied, while the $K_2,n = 16$ curve shows two anomalies. The

first, at A, lies between 11 and 12 mM $l.^{-1}$, and is a fairly sharp change of slope of the type usually associated with micelle formation. The second, in the region of B, 32–34 mM $l.^{-1}$, is a gradual change of slope, due either to a second CMC or to some change in the electrical properties of the solute.

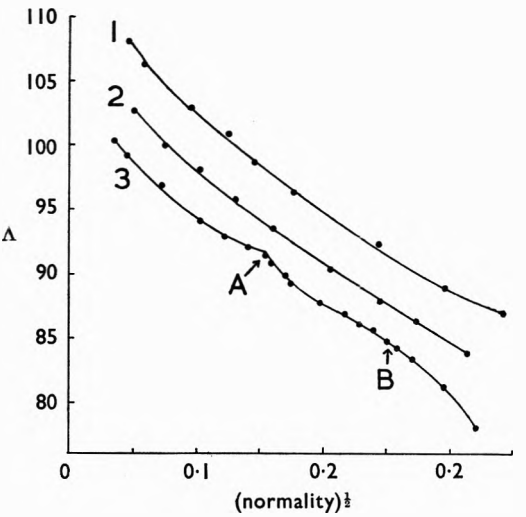

FIG. 1. Equivalent conductivity (Λ) — (normality)$^{\frac{1}{2}}$ curves for (1) K_2, $n = 8$; (2) K_2, $n = 12$; (3) K_2, $n = 16$.

The integral diffusion coefficients (diffusion from a solution into pure solvent, terminology of Stigter, Williams and Mysels[6]) are shown in Table I and Figure 2 (i).

TABLE I

INTEGRAL DIFFUSION COEFFICIENTS OF K_2, $n = 16$ IN WATER

Concn. mM $l.^{-1}$	4·01	5·11	5·75	7·73	10·00	12·00
jm	11·11	14·20	15·01	21·53	27·86	33·35
10^6D, $cm.^2sec.^{-1}$	8·62	8·60	8·62	8·63	8·58	8·42
Concn. mM $l.^{-1}$	15·00	19·70	28·08	37·86	44·00	
jm	43·04	52·46	78·21	103·5	123·5	
10^6D, $cm.^2sec.^{-1}$	8·35	8·14	8·03	8·00	8·05	

Figure 2 (i) shows that below a concentration of 10 mM $l.^{-1}$ there is only a slight variation of diffusion coefficient with concentration. This is the expected result for the diffusion of single molecules. Above 10 mM $l.^{-1}$ the slope of the curve changes rapidly, probably due to micelle formation. No patterns showing lines of anomalous intensity were observed above the CMC, and all patterns could be analysed for the diffusion of a single solute. This is probably the consequence of there being little refractive index difference per unit concentration between the solutions containing single molecules, and those containing micelles, as a plot of jm against concentration shows only a slight break at the CMC.

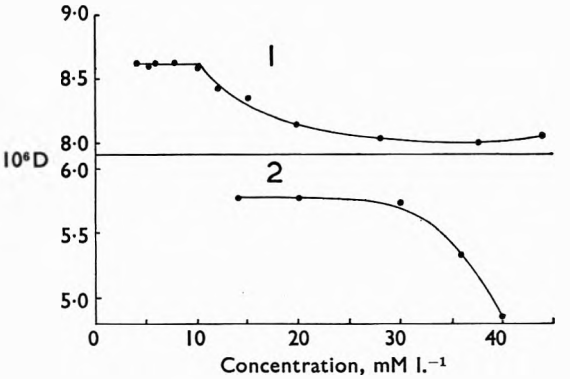

FIG. 2. (1) Plot of diffusion coefficient (D) against concentration for integral diffusions.
(2) Plot of diffusion coefficient (D) against mean concentration ($\bar{c}$) at $\Delta c = 6$ mM l.$^{-1}$ for differential diffusions.

Examples of the analysis of patterns from diffusion experiments at concentrations below and above the CMC are given in Tables II and III.

TABLE II

ANALYSIS OF A PATTERN AT CONCN. = 5·75 mML.$^{-1}$ (BELOW CMC)
$jm = 15{\cdot}01$ $t = 1202$ sec.

j	Y (cm.)	exp $(-z^2)$	C_t
1	0·2295	0·7202	0·319
2	0·2001	0·6254	0·320
3	0·1739	0·5457	0·319
4	0·1508	0·4740	0·318
5	0·1303	0·4085	0·319
6	0·1112	0·3485	0·319

Mean $C_t = 0{\cdot}319_0$

TABLE III

ANALYSIS OF PATTERN AT CONCN. = 19·70 mM L.$^{-1}$ (ABOVE CMC)
$jm = 52{\cdot}46$ $t = 1455$ sec.

j	Y (cm.)	exp $(-z^2)$	C_t
1	0·9123	0·8761	1·041
2	0·8686	0·8334	1·042
3	0·8286	0·7961	1·041
4	0·7942	0·7625	1·042
5	0·7634	0·7310	1·044
6	0·7316	0·7017	1·043
7	0·7027	0·6743	1·042
8	0·6752	0·6472	1·043
9	0·6477	0·6218	1·042
10	0·6224	0·5980	1·041

Mean $C_t = 1{\cdot}042_1$

In Tables II and III, j is the fringe number, the outermost fringe in a pattern being numbered zero. Y is the observed displacement of a fringe below the undeviated slit image, and $\exp(-z^2)$ is the theoretical displacement calculated from the theory of Kegeles and Gosting[7]. $C_t = Y/\exp(-z^2)$, and should be constant for single component diffusion.

Tables II and III show that the theory for single solute diffusion fits the patterns well for experiments both above and below the CMC.

Each value of the diffusion coefficient quoted is the mean of the results from at least four patterns. D is calculated from Longsworth's[8] equation:

$$D = \frac{jm.^2b.^2\lambda^2}{4\pi.C_t.^2t}$$

where *jm* is the difference in optical path length between the two liquids used in an experiment in wavelengths of light, *b* is the optical distance from the centre of the cell to the photographic plate in cm., and λ is the wavelength of light used.

The diffusion of colloidal electrolytes in water is complicated by electrical effects, as the ionic atmosphere around the larger anion is inclined to be in advance of the anion, causing an increase in the latter's diffusion rate. To obtain diffusion coefficients when electrical effects are decreased, differential diffusion experiments were performed by diffusion from a concentrated into a dilute solution. The results are given in Table IV.

TABLE IV

DIFFERENTIAL DIFFUSION COEFFICIENTS OF K_2,n = 16

$\bar{c}$	Δc	jm	10^6D, cm.^{2}sec.$^{-1}$
20	10	26·42	7·22
30	10	26·02	7·29
40	10	26·28	5·70
5	8	22·10	7·94
40	8	21·11	5·31
5	6	16·00	7·07
14	6	15·41	5·77
20	6	15·37	5·77
30	6	15·50	5·73
36	6	15·36	5·33
40	6	15·41	4·85
5	5	13·11	6·72
20	5	13·08	5·41
5	4	10·36	6·29
20	4	10·29	4·99
40	4	10·27	4·40

$\bar{c}$ = mean concentration of upper and lower solutions in mM l.$^{-1}$
Δc = concentration difference between solutions in mM l.$^{-1}$

Figure 3 shows the effect on the diffusion coefficient of decreasing the concentration difference (Δc) between the two solutions used in an experiment. In all cases the plots are linear. Results at $\bar{c}$ = 5 mM l.$^{-1}$ extrapolate to D = 4·67 $\times$ 10^{-6} cm.^{2}sec.$^{-1}$. Those at $\bar{c}$ = 14, 20, and 30 mM l.$^{-1}$ fall on the same line, giving D = 3·52 $\times$ 10^{-6} cm.^{2}sec.$^{-1}$. Although the results at c = 40 mM l.$^{-1}$ give a line with a smaller slope than the two preceding sets of results, the extrapolated value of D, 3·50 $\times$ 10^{-6}cm.^{2}sec.$^{-1}$, is almost identical with that from $\bar{c}$ = 14–30 mM l.$^{-1}$.

DISCUSSION

The first break in the equivalent conductance—(normality)$^{\frac{1}{2}}$ curve occurs between 11 and 12 mM l.$^{-1}$. It is suggested that these concentrations represent a concentration limit, where some discontinuity

occurs in the properties of the solution, e.g., micelle formation. Figure 2 (i) shows a sharp break in the D-concentration curve at 10 mM $l.^{-1}$, where the diffusion coefficient decreases sharply for a small increase in concentration. This effect is likely to be due to the formation of micelles, which would have a smaller diffusion coefficient than the single molecules which are present below the CMC. The two estimates of the CMC, 10 and 11–12 mM $l.^{-1}$, obtained from different methods, agree reasonably well.

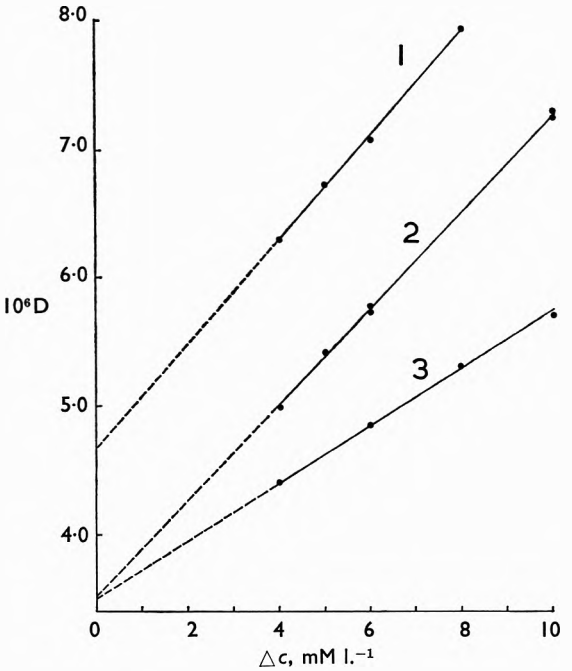

FIG. 3. Plots of differential diffusion coefficients (D) against $\Delta\bar{c}$ for various values of $\bar{c}$.
1, $\bar{c} = 5$; 2, $\bar{c} = 14 - 30$; 3, $\bar{c} = 40$.

The unaggregated solute shows a very large change of D with Δc (Figure 3, $c = 5$ mM $l.^{-1}$). This indicates that there are large electrical effects present during diffusion due to the influence of the smaller K^+ ions on the rate of diffusion of the long chain anion. By reducing the concentration difference between two solutions diffusing into each other, the electrical effects are decreased, and extrapolation to $\Delta c = 0$ should yield a diffusion coefficient with minimised ionic atmosphere effects.

For differential diffusions where both solutions used in an experiment have concentrations greater than the CMC, two species are expected to be present—single molecules and micelles. Generally, aggregation into micelles is governed by an equation of the mass action type; above the CMC the concentration of micelles increases more rapidly than that of single molecules. Two solutions both with concentrations greater than the CMC will contain roughly equal amounts of single molecules, and

will differ in their content of micelles. As the concentration difference between two such solutions is decreased, the diffusing species consists more and more of the micellar form. An extrapolation to $\Delta c = 0$ gives the diffusion coefficient of the micellar species; this diffusion coefficient is also obtained with minimised electrical effects.

A second concentration limit appears in the K_2,$n = 16$ curve of Figure 1 at 32–34 mM $l.^{-1}$. A plot of D against $\bar{c}$ at $\Delta c = 6$ mM $l.^{-1}$ also shows a break at about 33 mM $l.^{-1}$ (Fig. 2 (ii)), while no break is shown on the integral diffusion coefficient-concentration curve (Fig. 2 (i)). The size of the micelles above the second concentration limit is unchanged, as shown by the plot of D against Δc at $\bar{c} = 40$ mM $l.^{-1}$, which gives the same extrapolated diffusion coefficient within experimental error ($3{\cdot}50 \times 10^{-6}$cm.^{2}sec.$^{-1}$) as found for the 14–30 mM $l.^{-1}$ region ($3{\cdot}52 \times 10^{-6}$cm.^{2}sec.$^{-1}$). The second concentration limit is due either to an increase in the amount of solute aggregated, or to a change in the electrical properties of the micelle. It is impossible to decide between the two possibilities on the present evidence.

Size of Micelles. By comparison with diffusion coefficients of other soap micelles obtained by the same procedure, for example, potassium laurate[9], $D = 1{\cdot}43 \times 10^{-6}$cm.^{2}sec.$^{-1}$, containing 52 monomers; sodium dodecyl sulphate[10], $D = 0{\cdot}97 \times 10^{-6}$cm.^{2}sec.$^{-1}$ containing 157 monomers; the diffusion coefficients reported here for K_2,$n = 16$ indicate the micelles are small. The introduction of a second polar group, remote from the first, into the structure of normal soaps, appears to decrease the size of the micelles.

The Stokes-Einstein relationship can be used to calculate the diffusion coefficient of a sphere from its molecular weight:

$$D = \frac{RT}{6\eta\ \pi\ N(3M\bar{v}/4\pi N)^{\frac{1}{3}}}$$

where η is the viscosity of the solvent, M is the molecular weight, $\bar{v}$ is the partial specific volume of the solute, and the remaining symbols have their usual significance.

To interpret the observed diffusion coefficient in terms of particle size, two limiting cases are examined. Firstly, the particles are considered as spheres and their diffusion coefficients are calculated for aggregates containing one, two, and three monomers. Secondly, the particles are considered as ellipsoids. For particles which are larger than the solvent molecules, Perrin's[11] relationships may be applied to relate the ratio of the frictional coefficient of an ellipsoid to that of a sphere of the same molecular weight (f_e/f_o) to the ratio of the semi-axes of an ellipsoid. In this case the extended long chain anion approximates to a prolate ellipsoid, where a in the major semi-axis, and b is the minor semi-axis. The frictional ratio may be related to the ratio of the diffusion coefficient of a sphere, D_o, to that of an ellipsoid of the same molecular weight, D_e,

$$\frac{D_o}{D_e} = \frac{f_e}{f_o}$$

From measurements on molecular models of K_2,n = 16 values of a/b can be found, and the diffusion coefficients of the ellipsoids calculated. The results of the calculations are given in Table V.

TABLE V

CALCULATED VALUES OF THE DIFFUSION COEFFICIENT FOR PARTICLES OF DIFFERENT SHAPES

Number of monomers in particle	1	2	3
$10^6 D_o$ (sphere) cm.^{2}sec.$^{-1}$	4·91	3·90	3·41
$10^6 D_e$, fully extended, cm.^{2}sec.$^{-1}$ ..	3·59	3·12	—
10^6 D observed, cm.^{2}sec.$^{-1}$	4·67	3·51	—

D_o for three monomers is lower than the observed diffusion coefficient for the micelles, which falls between the limiting values for a micelle of two monomers. Due to the long hydrocarbon chain, the particles are unlikely to be spherical. The diffusion coefficient of the monomeric form also falls between the two limiting values.

Degree of extension of hydrocarbon chains. A very rough idea of the degree of extension of the hydrocarbon chain in the molecule in solution may be gained by using the observed diffusion coefficient to calculate the frictional ratio, and interpreting this in terms of a/b. For monomers a/b = 2·12, for double molecules a/b = 3·00. The volume of an ellipsoid will be given by

$$V = 4\pi ab^2/3$$

V can be calculated from the density of the solid, so both a and b can be found.

For monomers, a = 8·2 Å and b = 3·9 Å, while for double molecules, which are assumed to lie side by side, a = 13·0 Å and b = 4·3 Å. These results are in the correct order, as the hydrocarbon chain of the monomer would be expected to be contracted to the maximum extent, to minimise the area in contact with water; the double molecule has a smaller amount of hydrocarbon chain/molecule exposed to the water, if the two molecules lie side by side. It should therefore be able to lengthen more, as "bundling up" will cause a strain on the chain. These calculations are tentative, no account being taken of hydration.

Acknowledgements. I should like to thank Dr. L. Saunders for encouragement and useful discussions, and Dr. E. P. Taylor, of Allen and Hanbury's Ltd., Ware, for the gift of the methyl esters of n = 12 and n = 16.

REFERENCES

1. Danielsson, *Acta Acad. Aboensis Math. Phys.*, 1956, **20**, No. 15.
2. Ward and Chitale, *Proc. 2nd. Int. Conf. Surface Activity*, Butterworths, London, 1957, **1**, p. 352.
3. Gosting, Hanson, Kegeles, and Morris, *Rev, Sci. Instruments*, 1949, **20**, 209.
4. Saunders, *J. chem. Soc.*, 1953, 519.
5. Elworthy, *ibid.*, 1959, 1951.
6. Stigter, Williams and Mysels, *J. phys. Chem.*, 1955, **59**, 330.
7. Kegeles and Gosting, *J. Amer. chem. Soc.*, 1947, **69**, 2516.
8. Longsworth, *ibid.*, 1947, **69**, 2510.
9. Brudney and Saunders, *J. chem. Soc.*, 1955, 2916.
10. Brudney and Saunders, *J. Pharm. Pharmacol.*, 1955, **7**, 1012.
11. In Svedburg and Pederson, *The Ultracentrifuge*, Clarendon Press, Oxford, 1940, p. 41.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

***Aspidosperma olivaceum* M. Arg., Alkaloids of.** J. Schmutz and F. Hunziker. (*Pharm. Acta Helvet.*, 1958, **33**, 341.) The isolation of uleine, $C_{18}H_{22}N_2$, and of a newly discovered alkaloid olivacine, $C_{17}H_{14}N_2$ from the root- and stem-barks and leaves of *Aspidosperma olivaceum* M Arg. is described. Uleine had been previously shown by the authors to be a tetracyclic, oxygen-free, dihydrocarbazol alkaloid. The new alkaloid, olivacine, crystallizes from dilute methanol in yellow needles, m.p. 318°–324°, is optically inactive, has an intense blue fluorescence in very dilute alcoholic solution and shows an absence of *N*-methyl and *C*-alkyl. On chromic acid oxidation it yields a trace of acetic acid and another acid of unknown structure already obtained from the alkaloids or related species. The ultra-violet absorption spectrum indicates a polycyclic aromatic system. Addition of acid causes a bathochromic shift and a loss of fine structure, indicating an aromatically bound nitrogen atom. The alkaloid is a relatively strong base. The infra-red spectrum is very similar to that of u-alkaloid D, $C_{17}H_{16}N_2$ from *Aspidosperma ulei* Mgf., for which the authors assigned a carbazole structure. These two alkaloids also have a great many similar colour reactions which are given. D. B. C.

Holarrhidine, A New Alkaloid from *Holarrhena antidysenterica* Wall. L. Lábler and V. Černý. (*Coll. Czech. Chem. Comm.*, 1959, **24**, 370.) Cold extraction of the bark with ethanol-aqueous ammonia is reported to yield the total alkaloids in 1·39 per cent yield, which can be separated with light petroleum into insoluble (0·31) and soluble fractions (1·07 per cent). Holarrhimine (0·028 per cent) was isolated from the petrol-insoluble matter, which contains at least four alkaloids, by precipitation as the sparingly-soluble *p*-nitrobenzylidene derivative. Treatment of the mother liquors with cinnamic acid precipitated the sparingly-soluble holarrhidine cinnamate. Holarrhidine, m.p. 181 to 182°, $[\alpha]_D^{20}$–23° has the molecular formula, $C_{21}H_{36}ON_2$. Methoxyl and *N*-methyl groups are absent, and the molecule contains one ethylenic bond. The petrol-soluble fraction contains at least six alkaloids, including connessine, which on methylation with formic acid-formaldehyde yielded small amounts of *NNN'N'*-tetramethylholarrhimine. J. B. S.

***Lobelia cardinalis*, Chromatographic Investigation of the Basic Fraction from.** F. Kaczmarek and E. Steinegger. (*Pharm. Acta Helvet.*, 1958, **33**, 852.) Since it was thought that *L. cardinalis* contained only one alkaloid, lobinaline, some plants were grown from seed, harvested in bloom, and the crude alkaloidal content determined in the flowers, leaves and stalks. The average content was 0·46 per cent, and was largest in the flowers, smaller in the leaves and much smaller in the stems. Paper chromatography of the crude alkaloids revealed two spots, with R_F values respectively 0·5 and 0·86, which gave a positive reaction with Dragendorff's reagent. Both of these substances were isolated by fractional crystallization procedures, and the substance with $R_F = 0{\cdot}5$ was identical with lobinaline. The second substance of $R_F = 0{\cdot}86$ was a base and was designated Cardinalis-alkaloid 2. Its structure will be investigated. D. B. C.

ANALYTICAL

Barbituric Acids, Substituted, Non-aqueous Assay for. S. W. Goldstein and D. F. Dodgen. (*Drug Standards*, 1958, **26**, 113.) Samples of barbitone, amylobarbitone, aprobarbitone, methylphenobarbitone, phenobarbitone and vinbarbitone were assayed by non-aqueous titration against potassium hydroxide in anhydrous methanol, or lithium methoxide. Potassium hydroxide in methanol was readily prepared and relatively stable, but gave somewhat lower results than lithium methoxide. A method of titration with 0·1N lithium methoxide using dimethylformamide as solvent and thymol blue as indicator is recommended. When titrating with potassium hydroxide in methanol using thymol blue, a more definite end point was obtained in dimethylformamide than in chloroform-methanol. G. B.

Camphor, Gravimetric Estimation of, in Pharmaceutical Preparations. K. K. Kaistha. (*Drug Standards*, 1958, **26**, 83.) Liniment of camphor (1·5 g.), liniment of mustard (2 ml.) or acetic liniment of turpentine (2 ml. of oily distillate) was mixed with 2·5 ml. of aldehyde-free ethanol (80 per cent), 2 g. of semicarbazide hydrochloride and 1·5 g. of freshly fused potassium acetate and heated at 70° for 3 hours under reflux, shaking occasionally. Water was added, and after stirring and cooling, the precipitate was collected on a filter, washed with cold water and light petroleum, dried, and weighed, each g. of residue being equivalent to 0·727 g. of camphor. With suitable modifications the method was applied to the determination of camphor in liniments of aconite, belladonna and chloroform, concentrated camphor water, spirit of camphor, concentrated camphorated tincture of opium, and compound chloral pigment. Recoveries of camphor ranged from 96·9 to 102·75 per cent. G. B.

(±)-Canadine, Resolution of, by Paper Chromatography. O. F. Uffelie and M. M. Nijland. (*Pharm. Weekbl.*, 1958, **93**, 1045.) In this method ordinary Whatman No. 1 paper is used without special preparation, and the mobile phase is a 2 per cent aqueous solution of potassium dihydrogen phosphate in freshly boiled and cooled distilled water and other buffer solutions at pH 5. Quantities of 1 to 4 μg. of the racemic mixture can be separated. Canadine isolated from hydrastis gave one spot with the same R_F value as that of (−)-canadine. D. B. C.

Novobiocin, Carbamate-Ammonia Assay for. F. A. Bacher, G. V. Downing, Jr. and J. S. Wood, Jr. (*Analyt. Chem.*, 1958, **30**, 1993.) This method depends upon the release of ammonia from the carbamyl group in novobiocin by alkaline hydrolysis, and subsequent titration of the ammonia after its recovery in an ammonia distillation apparatus. The reaction is not quantitative on alkaline hydrolysis alone since a certain amount of carbamate ion may form which is stable in alkaline solution. To overcome this difficulty the solution is made acid with sulphuric acid to hydrolyse the carbamate ion to carbon dioxide and ammonia, and then alkaline again to recover the final traces of ammonia. About 50 mg. of novobiocin is required for each assay and the error is ± 1 per cent. A blank determination is performed and a correction procedure is described for ammonium ion and non-extractable amides and amines in the sample, but it was rarely found necessary to apply any correction. As little as 5 mg. of sample could be assayed, but only with considerable loss in precision. D. B. C.

Ointments, A Note on the Assay of, in Non-aqueous Solution. S. M. Wang, H. W. Starr and R. J. Hoffman. (*Drug Standards*, 1958, **26**, 116.) Ointments were treated with a solvent capable of dissolving the bases and providing solutions miscible with glacial acetic acid. The active ingredients were then determined by titration with perchloric-acetic acid, using methyl violet as indicator. Ointments prepared with paraffin, macrogol and hydrophilic ointment bases were successfully assayed using a mixture of five parts of chlorobenzene and one part of chloroform as solvent, and the colour of the bases did not affect the accuracy of the titration. Results are reported for ammoniated mercury, benzocaine and butamben picrate ointments. G. B.

Reserpine, A Rapid and Simple Colorimetric Determination of, in Pharmaceutical Preparations. A. W. M. Indemans, I. M. Jakovljevic, J. J. A. M. van der Langerijt. (*Pharm. Weekbl.*, 1959, **94**, 1.) This method is based on an orange colour reaction obtained by the extraction of reserpine with acetic acid. A reserpine solution in glacial acetic acid equal to 50 to 300 μg. is diluted to 6 ml. with glacial acetic acid, and 0·4 ml. of a 2 per cent solution of sodium nitrite is added. This is then heated on a boiling water bath for 5 minutes. After cooling the contents are transferred to a separating funnel, and 40 ml. of water is added. It is then shaken with 10 ml. of chloroform for 1 minute. The organic phase is separated and filtered, and absorption measured at 465 mμ. Readings are linear up to 300 μg. of reserpine per 10 ml. of chloroform. Reserpinic acid and methyl reserpinate also react to give a coloured compound but these are not removed from the aqueous phase by chloroform. B. R.

ORGANIC CHEMISTRY

1 : 6-Di-(2-bromoethylamino)-1 : 6-dideoxy-D-mannitol Dihydrobromide: a new Cytostatic Agent. L. Vargha and T. Horváth. (*Nature, Lond.*, 1959, **183**, 394.) The analogue of mannomustine (Degranol), 1 : 6-di-(2-bromoethylamino)-1 : 6-dideoxy-D-mannitol dihydrobromide has been synthesised from 1 : 6-diethylenimino-1 : 6-dideoxy-3 : 4-isopropylidene-D-mannitol and concentrated aqueous hydrobromic acid. It was obtained as a crystalline solid, m.p. 204–205°, easily soluble in water, and shows cytostatic activity in smaller doses than the chloric analogue, mannomustine. J. B. S.

BIOCHEMISTRY

BIOCHEMICAL ANALYSIS

Adrenaline and Noradrenaline in Urine, Differential Fluorimetric Estimation of. A. F. De Schaepdryver. (*Arch. int. Pharmacodyn.*, 1958, **115**, 233.) A modified method, based on the technique of von Euler and Floding, for differential fluorimetric estimation of adrenaline and noradrenaline in urine is described. W. C. B.

Adrenaline, Noradrenaline, and 5-Hydroxytryptamine Subjected to Various Simple Treatments, Relative Stability of. D. Joyce. (*Nature, Lond.*, 1958, **182**, 463.) Adrenaline and noradrenaline, which are often present in tissue extracts containing 5-hydroxytryptamine (5-HT) and interfere with the bioassay of the latter, may be destroyed without loss of 5-HT by the action of alkali. 5-HT was assayed upon the rat stomach preparation, and adrenaline and noradrenaline in

concentrations 50–200 times those which were found to abolish the response of the tissue to the stated amounts of 5-HT were inactivated at pH 11 after 30–60 minutes (room temperature). At 98°, 5 minutes at pH 7·8 was equally effective. Heating under more alkaline conditions leads to a loss of 5-HT activity. Similar results were obtained with human serum containing adrenaline, noradrenaline and 5-HT. J. B. S.

Calcium, Determination of, in Biological Material. A. A. Henly and R. A. Saunders. (*Analyst*, 1958, **83**, 584.) The calcium is precipitated at pH 5.0 (to avoid the co-precipitation of magnesium) by an ammonium oxalate-oxalic acid buffer, centrifuged and the calcium oxalate dissolved in 0·2N hydrochloric acid. A known amount of a standard EDTA-ethanolamine reagent and an indicator solution containing eriochrome black T are added, and the excess EDTA is back-titrated with a standard magnesium solution until the colour changes from blue to red. Faeces, tissues or foods are firstly ashed, and the ash dissolved in hydrochloric acid, and the pH adjusted to 5 before precipitation. The method is applicable directly to serum, urine or cerebrospinal fluid, using 1 ml. samples. The accuracy of the method was proved by adding known amounts of calcium to standardised body fluids and reassaying. The standard deviation of 50 analyses was $\pm$ 1 per cent of the mean value. D. B. C.

Catechol Amines, Urinary, A Rapid Quantitative Method for Chemical Estimation of, in the Diagnosis of Phaeochromocytoma. J. T. Wright. (*Lancet*, 1958, **2**, 1155.) The usual method for the extraction and preliminary purification of urinary catechols is to adsorb them on to alumina. The main disadvantages of this method is that the alumina is difficult to standardise and that the adjustments of pH throughout the procedure are critical. A new method is described where the catechol amines are extracted from solution as boric acid complexes by means of anion exchange resins. The method is simple, quick and economical to operate. Using four Dowex anion exchange columns, it is possible to screen four unknown urines for the presence of excess catechol amines or to obtain an accurate estimate of the catechol amine content of one urine in about 2 hours. Urine samples from 69 hypertensive patients have been examined by this method. A high urinary catechol amine content, indicating the presence of a phaeochromocytoma, was found in six cases, the diagnosis being confirmed at operation in each case. M. B.

Dopamine (3-Hydroxytyramine), Fluorimetric Method for the Determination of. A. Carlsson and B. Waldeck. (*Acta physiol. scand.*, 1958, **44**, 293.) Dopamine, a probable intermediate in the biosynthesis of adrenaline and noradrenaline, has been detected in many tissues and in urine. Only fluorimetric methods are sensitive enough for the estimation of this substance. However, in the ethylenediamine condensation method of Weil-Malherbe and Bone the fluorescent products of dopamine and adrenaline have almost the same characteristics. The method described in this paper is similar to the trihydroxyindole method for the estimation of adrenaline and noradrenaline. In this method the catechol amines are first oxidised to red indole derivatives, which are then rearranged in alkali to strongly fluorescent trihydroxyindoles. Utilising differences in the fluorescence characteristics at pH 5·3, micro quantities of dopamine can be detected and estimated in the presence of at least equal amounts of adrenaline or noradrenaline. M. B.

Glucose, Determination of, in Biological Fluids with Ethylenediaminetetra-acetic Acid. H. V. Street. (*Analyst*, 1958, **83**, 628.) This method depends upon the determination of the unreduced bivalent copper in the presence of cuprous oxide, after boiling the sample with excess alkaline cupric tartrate reagent, by titration with ethylenediaminetetra-acetic acid at pH 10. A freshly prepared saturated aqueous solution of murexide (ammonium purpurate) is used as indicator. This changes from yellow to reddish violet. With blood and cerebrospinal fluid a sodium tungstate reagent is used to precipitate protein and does not affect the titration. For blood, cerebrospinal fluid and urine the amount of sample required is 0·2 ml. Recovery experiments showed that the precision of the assay on blood was ± 5 per cent and on urine ± 2 per cent.

D. B. C.

PHARMACOLOGY AND THERAPEUTICS

Acetazolamide, Renal Colic and Anuria from. J. G. Yates-Bell. (*Brit. med. J.*, 1958, **2**, 1392). This is a report of a case of renal colic and anuria in a man of 54 who had been treated with acetazolamide (2 tablets daily for a week) for glaucoma. The diagnosis lay between bilateral non-opaque ureteric calculi and crystalluria. The obstruction to the ureteric catheter closely resembled the blockage experienced in sulphonamide crystalluria, but the diagnosis was not clinched until it was appreciated that acetazolamide was a sulphonamide derivative. The condition responded to the treatment for sulphonamide crystalluria. Previous reports of anuria and ureteric colic following acetazolamide administration are reported, though in no case have sulphonamide crystals been detected, nor has an expected phosphaturia been confirmed. S. L. W.

Anileridine Hydrochloride as an Analgesic and Sedative. R. C. Therien, L. W. Lee, E. M. Malashock and N. B. Davis. (*J. Amer. med. Ass.*, 1958, **168**, 2098.) Anileridine hydrochloride is a substituted pethidine, chemically known as ethyl-1-(4-aminophenethyl)-4-phenyl*iso*nipecotate dihydrochloride. It is an analgesic agent with a potency approaching that of morphine and greater than that of pethidine. It has a prompt onset of action (15 to 30 minutes) and a long duration of analgesia (5 to 6 hours). The side-effects such as general depression, depression of respiration, and lowering of blood pressure, are considerably milder than those produced by morphine and somewhat milder than those of pethidine. The clinical effects of anileridine were studied in 2,500 administrations given to more than 600 patients. It was used both as premedication for general anaesthesia in surgery, in doses averaging 50 mg. orally or subcutaneously, and as a post-operative sedative and analgesic, in doses varying from 25 to 75 mg. Its effects resemble those of morphine and pethidine, but euphoria was uncommon and no evidence of addiction was seen, even in a patient who received 552 doses during the course of treatment of metastatic carcinoma of the colon. Nausea occurred in 8 patients and sickness in 6. Respiratory depression caused by excessive doses in a few patients was easily counteracted by levallorphan in doses of 1 mg. for every 25 mg. of anileridine hydrochloride. S. L. W.

Atropine Eye-drops, Toxic Psychosis Following. J. P. Baker and J. D. Farley. (*Brit. med. J.*, 1958, **2**, 1390.) This is a report of a case of acute confusional psychosis in a woman of 33 following routine daily instillations of 1 per cent atropine sulphate drops over a period of 3½ weeks in the treatment of retinal

detachment. The psychosis was accompanied by myocardial changes. Other peripheral signs of atropine intoxication were absent. A remission of the confusional state followed discontinuation of the eye-drops and a short course of electroplexy; paranoid trends and emotional lability persisted for a further few days. The psychotic symptoms and myocardial change were later reproduced in the same patient following a small test dose of atropine given by injection. The origin of states of minor emotional disturbance in patients undergoing eye surgery with prolonged atropinisation warrant closer investigation. E.C.G. changes appear to be useful in establishing a diagnosis of atropine sensitivity where other peripheral signs of atropine intoxication are lacking.

S. L. W.

Bephenium Hydroxynaphthoate in the Treatment of Hookworm. L. G. Goodwin, L. G. Jayewardene and O. D. Standen. (*Brit. med. J.*, 1958, **2**, 1572.) Bephenium (benzyldimethyl-2-phenoxyethylammonium) was compared with tetrachlorethylene in the treatment of 284 cases of hookworm infection in Ceylon. Of the three salts employed—bromide, embonate and hydroxynaphthoate—the hydroxynaphthoate was selected as the most suitable, administered as tablets or as a sweetened and flavoured suspension in water. The drug has a bitter taste but was accepted by both adults and children. A single dose of 2 or 3 g. of base (1·73 g. of hydroxynaphthoate equals 1 g. of bephenium base), or multiple doses given on successive days or three times on the same day, compared favourably in effectiveness with tetrachlorethylene. The bephenium was given early in the morning on an empty stomach and no food allowed for 2 hours; if given in three doses on one day, the second and third doses were spaced between meals. No purge was given after the dose. Bephenium hydroxynaphthoate was found particularly suitable for the treatment of patients with advanced anaemia, diarrhoea, and heavy hookworm infections because of its low toxicity, even with large doses, and because no purge is necessary. In view of its bitter flavour, however, it is desirable to withhold the treatment from patients with severe vomiting and dehydration until the fluid balance has been restored by intravenous infusion. Bephenium hydroxynaphthoate was also effective against roundworm, which was present as a concurrent infection in 85 of the patients treated with this drug; tetrachlorethylene was ineffective against roundworm in 16 patients treated. S. L. W.

Bephenium Salts; Excretion in Urine. E. W. Rogers (*Brit. med. J.*, 1958, **2**, 1576.) From a study conducted on 18 healthy volunteers in Britain, given doses of bephenium bromide, embonate and hydroxynaphthoate, it was shown that the urinary excretion of bephenium and its metabolites in man is low. The percentage of dose recovered in 24 hours following administration of hydroxynaphthoate, 2·5 g. of base daily for 3 or 4 successive days, varied between 0·05 and 0·27. Side-effects from the hydroxynaphthoate were slight; mild diarrhoea sometimes occurred and there were some complaints of borborygmi. Side-effects ceased completely 24 hours after the dose. A technique for the determination of bephenium in urine by means of a modification of the methyl orange dye-lake procedure is described. S. L. W.

Cyanocobalamin in Pernicious Anaemia: Intramuscular or Oral? E. H. Hemsted and J. Mills. (*Lancet*, 1958, **2**, 1302.) Over a period of 2 years a group of 71 patients suffering from pernicious anaemia, taking a daily dose of 100 μg. of cyanocobalamin by mouth, were maintained at least as well as a

group of 84 patients receiving a monthly intramuscular injection of 100 μg. The oral preparation was given as an elixir containing 50 μg. per teaspoonful, and each patient was instructed to take two teaspoonfuls each morning on awakening and to take no food or drink for one hour afterwards. It had previously been shown that during a period of 5 years no relapse occurred in any of 43 patients treated throughout with 100 μg. of cyanocobalamin by intramuscular injection once a month, and that no advantage was gained by increasing the dose beyond that amount. S. L. W.

1 : 6-Di-(2-bromoethylamino)-1 : 6-dideoxy-D-mannitol Dihydrobromide, Effect of on Tumours of Laboratory Animals. J. Baló, G. Kendrey, J. Juhász and I. Beszynyák. (*Nature, Lond.*, 1959, **183**, 395.) The effects of 1 : 6-di-(2-bromoethylamino)-1 : 6-dideoxy-D-mannitol dihydrobromide and mannomustine have been compared on different tumours in rat and mouse. The compounds were administered once daily intraperitoneally after the appearance of palpable inoculated tumours. The bromo compound inhibits the growth of both rat and mouse tumours to a greater extent than mannomustine. Effective dose levels are 3 to 5 mg./kg. in mice and 2 to 3 mg./kg. in rats for the bromo compound compared with 20 mg./kg. and 15 mg./kg. for the chloro compound (mannomustine) in mice and rats respectively. Changes in the bone marrow and the blood are described. J. B. S.

Hydrocortisone Hemisuccinate by Inhalation in Asthma. J. M. Smith. (*Lancet*, 1958, **2**, 1248.) A controlled trial of hydrocortisone hemisuccinate solution, 5 mg. daily by inhalation for a month, was conducted on 57 children with asthma. Twenty-nine of the children were given hydrocortisone and 28 an apparently identical placebo. It was considered that in order to be of real value the treatment must be shown to be beneficial to at least 50 per cent more children than might benefit from the inhalation of an inert solution. As it was estimated that 15 per cent would be likely to benefit from the placebo, the hydrocortisone had to benefit 65 per cent or more to satisfy the criteria. The treatment failed to benefit 50 per cent more children than did the placebo; over the whole period of the investigation the hydrocortisone benefited 21 per cent of 29 children, and the placebo 14 per cent of 28 children. S. L. W.

Noradrenaline Infusion, Fall of Blood Pressure after, and its Treatment by Pressor Agents. J. H. Burn and M. J. Rand. (*Brit. med. J.*, 1959, **1**, 394.) The experiments were carried out on spinal cats to which, on the two preceding days, resperpine (3 mg./kg.) had been administered intraperitoneally. After reserpine, the store of noradrenaline in the heart and blood vessels disappears and under these conditions an intravenous infusion of noradrenaline caused a rise in blood pressure which was not sustained but which gradually returned to the resting level in spite of the fact that the rate of infusion remained the same. After the infusion the rise in blood pressure normally produced by a rapid injection of noradrenaline did not occur but the response to ephedrine, which is abolished after treatment with reserpine, was restored by the infusion. The authors explain these results by suggesting that during an infusion, noradrenaline is taken up and held in a store from which amines like ephedrine can release it. Apparently because of the increase in the store of noradrenaline, the vessels become insensitive to further injections of this and other directly acting amines. Sympathomimetic amines may be divided into three classes: those which, like noradrenaline, work only by direct action, those which, like tyramine and

ephedrine, appear to act only by liberating noradrenaline from the store and which are, therefore, most active at the termination of a noradrenaline infusion which has been filling the store and finally, those which are intermediate having some direct action as well as some action in discharging the store. In experiments in which both blood pressure and spleen volume were recorded, the actions of six pressor amines were examined. Three of these—methedrine, mephine and vonedrine appeared to act only by liberating noradrenaline from the store, while the other three—aramine, vasoxine and propadrine, were shown to have both a direct action and an action through discharging the store. It is well known that on stopping a drip of noradrenaline, the patient's blood pressure often falls to a low level. Noradrenaline is known to depress ganglionic transmission and the absence of sympathetic tone after an infusion may in part be due to this action. The main conclusion arising from this investigation, however, is that when this occurs, the correct procedure is to inject one of those amines believed to act by discharging the noradrenaline store, rather than to resume the drip. W. C. B.

Polyoestradiol Phosphate, A Long Lasting Oestrogen. O. Fernö, H. Fex, B. Högberg, T. Linderot and S. Veige, (*Acta. chem. scand.*, 1959, **12**, 1675.) This is a high molecular weight polyester of oestradiol-17β and phosphoric acid. The chemical and biological properties are described. The molecular weight was 26,000, indicating a molecule of approximately 80 oestradiol moieties. Enzyme studies showed it to be a powerful inhibitor of acid phosphatase, alkaline phosphatase and hyalurodinase. Pharmacologically the acute intravenous LD50 in mice was 240 mg./kg. and the chronic LD50 by the subcutaneous route 700 mg./kg. The oestrogenic activity in spayed mice showed the log dose to be linearly related to the log duration of oestrogenic activity. Doses of 7μg. and 14 μg. gave a mean duration of 17 and 26·4 days respectively. The compound was inactive orally. G. F. S.

Prednisolone in the Treatment of Chronic Asthma. H. M. Brown. (*Lancet*, 1958, **2**, 1245.) Ninety patients with chronic asthma, in whom bronchodilator drugs had failed, were treated with prednisolone. Complete relief of bronchospasm was obtained in 57 cases, partial relief in 10, slight relief in 7, and no relief in 16. Except when bronchospasm was very severe the dose given was 5 mg. three times daily for a week, followed by reassessment and adjustment of dosage sufficiently to keep the patient free from wheezing. Experience showed that if there was no improvement in the course of two weeks on 15 or 20 mg. of prednisolone daily, in the absence of purulent sputum, no significant benefit would be derived from continuing treatment. The elderly bronchitic with severe bronchospasm rarely benefited. The success of the treatment is dependent on the presence of large numbers of eosinophils in the sputum. In the absence of an eosinophilic sputum a satisfactory response is unlikely and the use of prednisolone is contraindicated. A rapid method of determining eosinophils in the sputum is as follows. Place a small piece of sputum on a slide, spread out with forceps (*not* into a thin film), apply one drop of Leishman strain and one drop of distilled water, put a cover-slip on top and examine under a 2/3 in. objective in daylight in from 10 minutes to several hours. The treatment never does more than suppress the asthmatic state; it does not abolish eosinophils in the sputum though the number is much reduced. S. L. W.

Vitamin B_{12}, Site of Absorption of, in Man. C. C. Booth and D. L. Mollin. (*Lancet*, 1959, **1**, 18.) A study has been made of the distribution of radioactive vitamin B_{12} in the small intestine of patients undergoing abdominal operations and of the absorption of B_{12} after small intestinal operations. Three hours after an oral dose of 1 μg. of ^{56}Co-labelled vitamin B_{12} little or no radioactive material was found in the plasma. There was no radioactivity in the proximal small intestine, all the radioactivity being in the distal portion. Peak plasma activity occurred at 8 to 12 hours after the dose. In patients who had undergone resection or short circuiting of the small intestine, absorption was normal in one who had had 8 feet of jejunum resected while in nine patients whose ileum had been resected absorption of B_{12} was subnormal. Absorption was not improved by the administration of intrinsic factor derived from hog stomach. Examination of patients who had had resection of the ileum from 1 to 6 years previously showed evidence of megaloblastic anaemia which responded to injections of vitamin B_{12}. The results show that absorption of vitamin B_{12} occurs in the ileum, and this is supported by experiments previously reported in animals. It is suggested that patients whose ileum has been resected or short circuited should be kept under close haematological supervision or treated prophylactically with vitamin B_{12}. G. F. S.

APPLIED BACTERIOLOGY

Dihydrostreptomycin and Anaerobiosis—Indirect Evidence for Two Sites of Action of Dihydrostreptomycin. G. M. Williamson. (*J. gen. Microbiol*, 1958, **19**, 584.) The activity of streptomycin and dihydrostreptomycin is known to be decreased under anaerobic conditions. In an attempt to find some reason for the greater resistance to dihydrostreptomycin of a facultive anaerobe when growing anaerobically experiments were carried out under carefully controlled environmental conditions with organisms whose growth rate and general metabolic behaviour was known. The sensitivity to the antibiotic of four strains of *Escherichia coli* and of *Aerobacter aerogenes* when growing under aerobic and under anaerobic conditions in heavily buffered enriched medium was compared. Each comparison covered a series of pH values from 5·5 to 8·0. The results showed that the decreased activity of dihydrostreptomycin appeared to be related to the enzymic make up of the organism in respect to carbohydrate metabolism. It could not be due to a slower rate of growth of the organism under anaerobic conditions nor could it be accounted for by acidic end products of anaerobic growth. In a further series of experiments a comparison was made of the behaviour of *A. aerogenes* under identical conditions, but in a simple medium. The results obtained suggested that dihydrostreptomycin was more effective against synthesising than against energy producing mechanisms.

W. C. B.

Sorbic Acid and Other Preservatives, Fungistatic Effect of. C. Trolle-Lassen. (*Arch. Pharm. Chem.*, 1958, **65**, 679.) The fungistatic activity of six substances against strains of *Aspergillus*, *Mucor* and *Penicillium* spp. was determined at pH 3, 5, 7 and 9, using a serial dilution technique. Benzalkonium chloride and phenylmercuric acetate proved to be the most potent substances tested, while methyl parahydroxybenzoate had a lower activity but was less affected by changes in pH. Sorbic and benzoic acids showed good fungistatic activity at pH 3–5, but were relatively inactive in alkaline solution. Sodium propionate was the least effective substance examined. G. B.

***Staphylococcus aureus*, Dissemination from Woollen Blankets.** K. F. Anderson and R. A. W. Sheppard. (*Lancet*, 1959, **1**, 514.) A series of experiments were carried out to demonstrate the dissemination of *Staph. aureus* from an infected blanket. A new 100 per cent wool blanket, free from pathogenic staphylococci, was infected with a 6-hour broth culture of *Staph. aureus* phage-type 80/81, using a wooden swab-stick lightly drawn across the blanket. The experiments were carried out in an empty room, the air of which was shown to be free of coagulase-positive staphylococci. In Experiment 1 the infected blanket was lightly shaken at waist height over a corresponding marked area on the floor and 16 culture plates were placed at distances of from 1 to 4 feet round the outside of the marked area, the plates were then exposed and 15-minute precipitation samples collected; in Experiment 2, 4 new plates were placed in position at points 8 feet from the marked area, which was then swept with a new broom, precipitation samples being again collected over a 15-minute period; in Experiment 3, 4 plates were placed in position at bed-level at a distance of 4 feet from the marked area, the blanket being shaken as before and 15-minute samples collected. *Staph. aureus* was isolated from 4 plates exposed during Experiment 1, from 2 plates in Experiment 2, and from 3 plates in Experiment 3. Two weeks after the experiments colonies of the test staphylococcus could still be recovered from the infected blanket. While hospital blankets cannot be solely incriminated, these experiments show that they cannot be excluded from measures to reduce the number of pathogenic staphylococci in hospital wards.

S. L. W.

2:4:5-Trichlorophenyl Ester with an Antimycotic Action. L. Hepding, H. M. Henning and U. Jahn. (*Arzneimitt.-Forsch.*, 1958, **8**, 525.) The fungistatic action of carbamic acid esters of chlorinated phenols is described with special reference to the phenylcarbamic acid ester of 2:4:5-trichlorophenol which seemed to be most effective, combining marked antimycotic action with bacteriostatic properties. Although not quite as active as the parent phenol, the substance was better tolerated and less irritant to mucosa and skin. The fungistatic, fungicidal, bacteriostatic and bactericidal activities against several organisms were investigated, and also the virucidal activity against the Newcastle-Allantois virus which was appreciable. The inhibition of the oxygen-uptake of various bacteria and the effect on the growth of fibroblasts from embryonic chick hearts were investigated in order to ascertain the effect on living cells. The acute and chronic toxicities were investigated using mice and rats respectively. By the oral route, acute toxicity was too low to ascertain the LD50 dose under the conditions described, and thus the substance was far better tolerated than the free phenol. The local toxicity was tested on the ear-muscle and conjunctiva of rabbits and chronic irritability was tested on the skin of guinea pigs. The progress of healing of induced or artificial infection on rabbits' ears was followed and gave promising evidence of usefulness clinically. Lastly, the compounding of the substance into pharmaceutical preparations was investigated.

D. B. C.

BOOK REVIEWS

HETEROCYCLIC CHEMISTRY. By Adrien Albert. Pp. viii + 424 (including Index). Athlone Press, of the University of London, 1959. 45s.

Those who are familiar with the work of Professor Albert will not be disappointed with his original approach to heterocyclic chemistry. The subject matter is divided for convenience into hetero-paraffinic, hetero-ethylenic and hetero-aromatic chemistry, and covers both preparative methods and properties, but with the emphasis very heavily on the latter. The approach to hetero-aromatic chemistry, which occupies a considerable part of the book, is refreshing, and provides a classification, based on the distribution of π-electrons in the ring, which simplifies the study of hetero-aromatics. The main subdivision is into (*a*) π-deficient *N*-hetero-aromatics—compounds such as pyridine, having nitrogen as the sole hetero-element, and a deficit of π-electrons elsewhere, (*b*) π-excessive *N*-hetero-aromatics, (e.g. pyrrole), and (*c*) π-excessive *O*- and *S*-hetero-aromatics. The correlation of structure and properties is much assisted by liberal use of formulae showing the most probable electron distributions, a feature which helps to emphasise the generality of this method of approach. Special chapters on spectra, ionisation constants, oxidation-reduction potentials, dipole moments and on the interpretation of complex formulae in terms of physical and chemical properties add considerably to the value of the book, which also includes a large bibliography and many excellent suggestions for further reading. The text also contains appropriate references to important biological, medical and technical applications. It should provide a most useful source of reference for honours students in chemistry and pharmacy and research workers alike, at a very economic price.

J. B. STENLAKE.

APPLIED PHARMACOLOGY (*Clark*). Ninth Edition. By Andrew Wilson and H. O. Schild. Pp. xii + 750 (including Index and 165 illustrations). J. and A. Churchill, Ltd., London, 1959. 50s.

Clark's Applied Pharmacology needs no description and it is very welcome that Professor Wilson and Dr. Schild have again brought it up to date. The appearance and layout have been little changed, and the many inescapable new drugs have been deftly included with an increase of less than ten per cent in the number of pages. There have been many small alterations; particularly the chapter on general principles of drug action has been refreshed and is a joy to read. There is also a very useful new section, written by Dr. Hannah Steinberg, on methods of studying drugs which affect mental activity. In parts of the text there has been a slight and regrettable tendency to make it less easy to trace statements to their origin; for instance, dates no longer appear beside the names of some authors whose statements are quoted, and so it has become more difficult to find these references for further information. It is also a pity that references in the text to tables which appear up to 14 pages away no longer indicate where in fact these tables are. But these are most minor blemishes on an excellent production. Much of A. J. Clark's original text has been preserved, and it is a measure of its quality that so much can remain unaltered. The revisions and additions are better than in the eighth edition, and the present volume admirably fills a gap which was developing as its predecessor became out of date.

MILES WEATHERALL.

METHODEN DER ORGANISCHEN CHEMIE (*Houben-Weyl*). Fourth Edition. Edited by Eugen Müller. Volume XI, Part 2. Spezielle chemische Methoden. Stickstoffverbindung II (Unwandlung von Aminen) and Stickstoffverbindungen III. Pp. xlviii + 840 (including Index). Georg Thieme Verlag, Stuttgart, 1958. Moleskin, DM.155.00.

The extensive literature on nitrogenous derivatives has necessitated the division of Volume XI into two parts, issued as sub-volumes. Volume XI/1, which has already been reviewed, dealt solely with the preparation of amines, and such of their reactions as led to the formation of other amines. In the first part of Volume XI/2, the survey of amines is completed with a review of their actions which lead to their conversion to other derivatives, such as acid amides, amidines, nitramines, and amine oxides. Deamination with nitrous acid and its derivatives has been given special attention, and the chapter forms a useful survey of these reactions, which is not to be found elsewhere. The chapter on elimination of the amino group in the preparation of olefines and in substitution reactions also reflects current interests. The second part of Volume XI/2 summarises the literature on 1,2- and 1,3-alkylenimines, amino acids and their derivatives, lactams, quaternary ammonium compounds and certain nitrogen-sulphur compounds, including sulphamido acids, thioamido acids and their derivatives. The latter, and certain of the other contributions again are particularly valuable in providing surveys which are not readily available elsewhere. The volume as a whole is well up to the standard of the series, being liberally illustrated with formulae and adequately referenced, many of the references being of recent date.

J. B. STENLAKE.