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The rheology of some oil-in-water emulsions stabilized by condensed complex films

F. A. J. TALMAN, P. J. DAVIES AND E. M. ROWAN

The rheology of emulsions containing oleyl, lauryl or cetostearyl alcohol as the oil-soluble component has been examined. As the concentration of fatty alcohol was increased, three main types of behaviour were found. These could not be explained by the particle size distribution, phase weight ratio or internal phase viscosity. It is suggested that migration of oil-soluble component and subsequent formation of viscous gels in the aqueous phase accounted for the observed behaviour. The effects of temperature, type of oil phase and chemical constitution of water-soluble components, have also been examined.

IN 1940, Schulman & Cockbain showed that oil-in-water emulsions employing a combination of oil- and water-soluble surface-active materials as emulgent, are stabilized by an electrically charged, condensed, intermolecular complex of both components at the interface.

Calculations based on the observed globule size distributions showed that the concentrations of both film-forming components used to produce a series of emulsions containing cetostearyl alcohol were always considerably in excess of those required to form a close-packed monolayer. Since the consistency of these emulsions ranged from thin fluids to semi-solid creams it seemed doubtful whether the interfacial film viscosity played as dominant a role in determining the viscosity of emulsions as was proposed by Schulman & Cockbain (1940). Similar doubts have been expressed by Shotton (1966).

Many factors are known to influence the rheological properties of emulsions; these have been reviewed by Sherman (1964). In particular the control of consistency, by varying the concentration of cetostearyl alcohol in an emulsion, is common pharmaceutical practice. Axon (1956) has shown that increasing concentrations of cetyl alcohol produced increases in both the yield value and plastic viscosity of some emulsions.

The work reported here is part of an investigation of the mechanisms by which cetostearyl and similar alcohols control emulsion rheology, and also the effect of internal phase viscosity, solubility of the fatty alcohol in the disperse phase, chemical constitution of water-soluble component, and other variables.

Experimental

Materials. Polyoxyethylene sorbitan monolaurate (Sorbester Q12) and mono-oleate (Sorbester Q17) (from Howards Ltd., Ilford, Essex), isopropyl myristate, oleyl and lauryl alcohols were commercial grades and were used without further purification. Potassium laurate was prepared by neutralization of the acid. All other materials conformed to the requirements of the B.P. or B.P.C.

Solubility determinations. These were made by the cooling curve method, the results being given in Table 1.

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TABLE 1. SOLUBILITY OF CETOSTEARYL ALCOHOL (% W/W) IN VARIOUS OILS

Oils	Temperature, ° C			
	20	25	30	35
Liquid paraffin	0·75	2·0	4·5	14·5
Light liquid paraffin	1·0	2·5	6·25	18·25
Arachis oil	4·0	5·75	8·75	14·0
Castor oil	5·5	9·5	14·0	21·75
Isopropyl myristate	9·0	14·0	23·5	38·5

N.B. The solubility of lauryl and oleyl alcohols is in excess of 30% w/w at 20°. These materials are miscible in all proportions with the oils above 25°.

Preparation of emulsions. Variations in preparation, storage and testing techniques have been shown to affect the properties of emulsions (Cockton & Wynn, 1952; Heinrich & Clements, 1960; Boylan, DeKay & Banker, 1962). A carefully standardized technique has been used to minimize these effects. Except where indicated, the following general formula has been used: liquid paraffin, 50·0 g; oil-soluble component, 0·1 to 10·0 g; water-soluble component, 0·5 g; distilled water to 100·0 g.

Solutions of oil- and water-soluble components, dissolved in the appropriate phases, were mixed at 60°, made up to weight, and homogenized by an Ormerod plate-valve homogenizer, model URF/1 (Ormerod Engineers Ltd., Rochdale, Lancs.). Unless otherwise noted in the text, samples were stored for 24 hr before rheological examination at 25 $\pm$0·1° in the water bath used to regulate the viscometer plate temperature.

Globule size analysis. A Timbrell Double-Image Micrometer, model A.10 (Fleming Instruments Ltd., Stevenage, Herts.), was used (Barnett & Timbrell, 1962). The emulsions were diluted with 50% v/v aqueous glycerin to give 50 to 100 globules per field, a sample was mounted in a Thoma Haemocytometer Cell, and at least 1000 globules were sized.

A number of globule size distribution functions for emulsions have been proposed (Jellinek, 1950; Schwarz & Bezemer, 1956; Rajagopal, 1959a). Our data showed sufficient conformity to a log-normal distribution for valid comparison of samples to be made without the correction for truncation errors used by Rajagopal (1959b).

Sampling and rheological examination. Flow curves were drawn by a Houston X-Y recorder, model EHR 93–4 (Advance Electronics Ltd., Hainault, Ilford, Essex) coupled, with appropriate gain and impedance matching, to a Ferranti-Shirley cone and plate viscometer (Ferranti Ltd., Moston, Manchester). The latter was fitted with a 3·5 cm radius cone, angle 20·5 mins, and with a 1200 g cm torque spring. The plate temperature was never more than 0·15° below the sample storage temperature, and the effects of draughts were minimized by screening the measuring unit. The cone and plate were equilibrated to the same temperature before use. Silicone fluids, standardized by the method of British Standard 188: 1957, were employed to check the manufacturer's calibration data.

Some change of rheological properties due to sample disturbance is inevitable during transference from the storage container to the viscometer

plate and during subsequent raising of this plate to the operational position. In an attempt to minimize this effect all samples were drawn into a dip tube and carefully ejected onto the plate. Separate samples were used for each examination.

A sweep-time of 600 sec and a maximum speed of 100 rev/min were used to examine all samples. Under these conditions, where time-dependent flow characteristics were exhibited, an equilibrium value of apparent viscosity was attained at the completion of the up-curve. This value of apparent viscosity (η_{100}) at 100 rev/min (1800 sec^{-1}) has been recorded as one parameter which, irrespective of rheological behaviour, was descriptive of all emulsions.

At low shear rates the up-curves for a number of samples have shown either a spur as described by Levy (1962) or an abrupt change of slope. The shear stress at these points has been reported as the static yield value (SYV). The variation in type of time-dependent behaviour exhibited by our preparations (Fig. 1), often within a closely related series, has precluded the inclusion of values for dynamic yield value and hysteresis loop area.

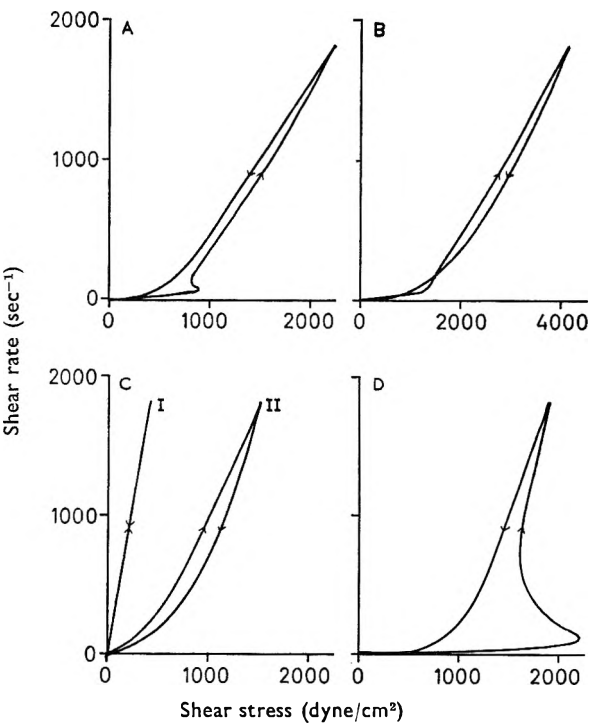

FIG. 1. Rheograms for products of stated composition. A. Cetostearyl alcohol (2·5% w/w)/cetomacrogol 1000 (0·5% w/w). B. Cetostearyl alcohol (4·0% w/w)/sodium lauryl sulphate (0·5% w/w). C. (I) Oleyl alcohol (7·5% w/w)/cetomacrogol 1000 (0·5% w/w), (II) lauryl alcohol (6·0% w/w)/potassium laurate (0·5% w/w). D. Gel containing cetostearyl alcohol (10·0% w/w)/sodium lauryl sulphate (0·5% w/w).

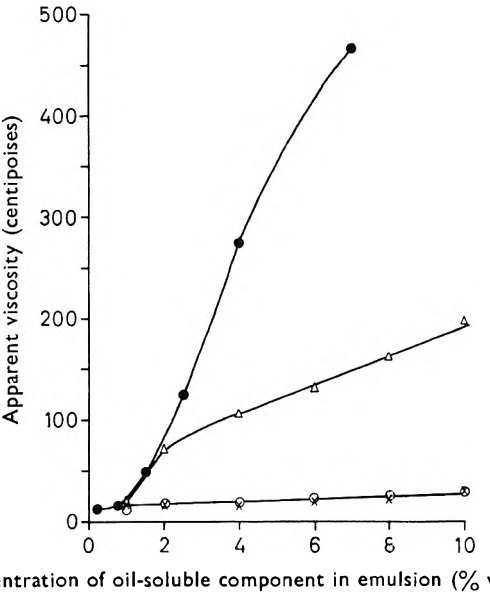

FIG. 2. Effect of oil-soluble component concentration on apparent viscosity in emulsions containing 0·5% w/w water-soluble component. ●, Cetostearyl alcohol/cetomacrogol 1000. △, Lauryl alcohol/sodium lauryl sulphate. ×, Lauryl alcohol/cetomacrogol 1000. ○, Oleyl alcohol/cetomacrogol 1000.

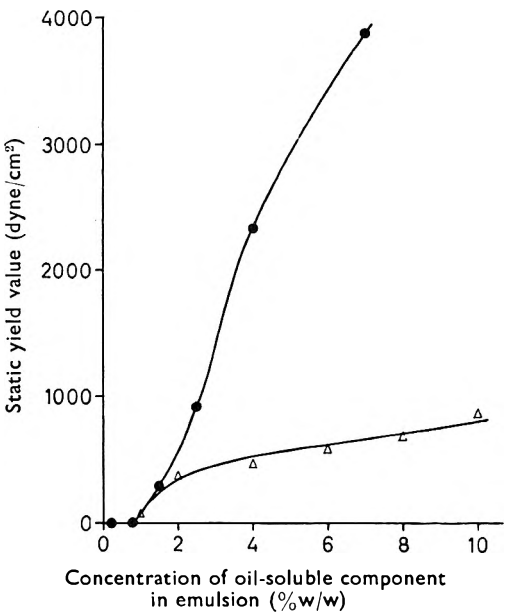

FIG. 3. Effect of oil-soluble component concentration on static yield value in emulsions containing 0·5% w/w water-soluble component. ●, Cetostearyl alcohol/cetomacrogol 1000. △, Lauryl alcohol/sodium lauryl sulphate.

Results and discussion

Figs 2 and 3 show the three main types of behaviour which were observed when oleyl, cetostearyl or lauryl alcohols were used as the oil-soluble component of emulsions.

All emulsions prepared with oleyl alcohol were fluids of low apparent viscosity (Table 2). Increasing the concentration of this material pro-

TABLE 2. APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF EMULSIONS CONTAINING VARYING CONCENTRATIONS OF OLEYL, LAURYL OR CETOSTEARYL ALCOHOL WITH 0·5% W/W WATER-SOLUBLE COMPONENT

Oil-soluble component (% w/w)	Water-soluble component									
	Cetomacrogol 1000		Sorbester Q12		Cetrimide		Sodium lauryl sulphate		Potassium laurate	
	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
Oleyl alcohol										
1·0	11	0	10	0	13	0	15	0	14	0
2·0	16	0	11	0	11	0	11	0	17	0
4·0	18	0	13	0	13	0	15	0	16	0
6·0	21	0	15	0	14	0	19	0	21	0
8·0	25	0	18	0	20	0	23	0	22	0
10·0	27	0	22	0	21	0	30	0	27	0
Lauryl alcohol										
1·0	15	0	15	0	39	151	19	63	13	0
2·0	15	0	15	0	64	352	69	364	17	0
4·0	16	0	19	0	58	477	105	452	25	0
6·0	18	0	20	0	74	503	130	565	85	*
8·0	21	0	23	0	79	691	161	678	165	*
10·0	28	0	25	0	94	754	198	854	210	*
Cetostearyl alcohol										
0·25	12	0	12	0	11	0	14	0	10	0
0·75	15	0	13	0	15	0	18	0	11	0
1·5	51	276	29	126	46	251	53	251	28	151
2·5	126	905	76	1131	130	1005	103	477	42	754
4·0	275	2337	176	1407	269	2412	230	1344	218	2638
7·0	468	3895	336	2638	638	6659	679	6533	†	†

* Pseudoplastic.
† Values too high for measurement with the large cone.

duced only a slight rise in apparent viscosity, which could be attributed to the increase in total disperse phase. Measurable static yield values were not obtained. The water-soluble component used had little effect on the properties of the emulsions.

Low concentrations of cetostearyl alcohol produced fluid emulsions similar to those obtained by the use of oleyl alcohol. As the content of cetostearyl alcohol was raised, a concentration was reached at which the apparent viscosity/concentration curve diverged markedly from that given by emulsions containing oleyl alcohol. Above this divergence concentration static yield values were observed. For emulsions prepared with the water-soluble components given in Table 2, the divergence concentration was identical with the saturation concentration, this latter being the concentration of cetostearyl alcohol in the whole emulsion which was just sufficient to cause saturation of the disperse phase. Slightly higher apparent viscosities and static yield values were found in the case of emulsions containing ionic water-soluble components. At temperatures of 28°, 30° and 35°, or when oils other than liquid paraffin or light

TABLE 3. EFFECT OF TEMPERATURE ON APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF EMULSIONS CONTAINING 50% W/W LIQUID PARAFFIN, 0·5% W/W CETOMACROGOL 1000 AND VARIOUS CONCENTRATIONS OF CETOSTEARYL ALCOHOL

Cetostearyl alcohol (% w/w)	Temperature, ° C									
	20		25		28		30		35	
	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
0·25	10	0	10	0	8	0	10	0	13	0
0·50	10	0	11	0	10	0	—	—	11	0
0·75	23	0	17	0	8	0	17	0	12	0
1·0	27	38	19	0	13	0	15	0	11	0
1·25	57	138	33	63	21	38	20	0	—	—
1·5	103	377	61	364	48	176	33	75	—	—
1·75	—	—	74	389	—	—	44	188	—	—
2·0	128	665	87	489	65	402	56	339	23	88
2·5	—	—	130	804	103	628	82	477	—	—
3·0	216	1206	189	1181	—	—	131	867	92	528
4·0	272	1470	243	1432	245	1583	216	1281	143	979
5·0	364	2600	359	2714	347	2671	328	2525	242	1809
5·5	388	2864	374	2814	—	—	349	2814	—	—
6·0	—	—	377	2864	380	2965	—	—	309	2512
7·0	—	—	—	—	—	—	—	—	423	3568
7·5	—	—	565	4208	—	—	478	3769	—	—

TABLE 4. APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF EMULSIONS CONTAINING CETOSTEARYL ALCOHOL AND 0·5% W/W CETOMACROGOL 1000 WITH DIFFERENT OIL PHASES

Liquid paraffin	Cetostearyl alcohol % w/w	0·25	0·5	0·75	1·0	1·25	1·5	1·75	2·0	3·5	—
	η_{100}	14	18	24	29	58	78	105	125	290	—
	SYV	0	0	0	75	276	440	565	704	2060	—
Light liquid paraffin	Cetostearyl alcohol % w/w	0·25	0·5	0·75	1·0	1·25	1·5	1·75	2·0	3·5	—
	η_{100}	14	15	18	21	37	56	71	105	261	—
	SYV	0	0	0	0	88	264	339	628	1885	—
Isopropyl myristate	Cetostearyl alcohol % w/w	4·75	6·0	6·5	6·75	7·0	7·25	7·5	7·75	8·0	9·75
	η_{100}	31	60	76	114	131	155	183	214	237	395
	SYV	0	276	415	653	817	1005	1281	1533	1771	3317
Arachis oil	Cetostearyl alcohol % w/w	0·25	1·75	2·0	2·75	3·0	3·25	3·75	5·0	7·5	10·0
	η_{100}	20	24	28	60	94	94	143	268	464	591
	SYV	0	0	75	377	533	533	1030	2588	4259	6433
Castor oil (1) (constant oil concentration)	Cetostearyl alcohol % w/w	2·0	4·0	4·25	4·5	4·75	5·0	5·25	5·5	7·5	10·0
	η_{100}	19	85	89	107	129	139	151	166	239	416
	SYV	0	276	377	565	766	879	980	1156	2299	3593
Castor oil (2) (constant total disperse phase concentration)	Castor oil % w/w	48·0	47·0	46·0	45·25	44·75	44·0	43·0	—	—	—
	Cetostearyl alcohol % w/w	2·0	3·0	4·0	4·75	5·25	6·0	7·0	—	—	—
	η_{100}	14	27	57	102	133	198	275	—	—	—
	SYV	0	0	138	616	854	1206	1709	—	—	—

liquid paraffin were used, the divergence concentration was less than the appropriate saturation concentration (Tables 3 and 4). As cetostearyl alcohol was found to be more soluble in the disperse phase under these conditions, the phase weight ratio increased correspondingly for a series of emulsions which included samples at and above the saturation concentration. The difference between the divergence and saturation concentrations was reduced, but not eliminated, with castor oil emulsions of constant phase weight ratio.

At 25°, lauryl alcohol, like oleyl, was miscible in all proportions with liquid paraffin. The rheological behaviour of emulsions containing lauryl alcohol was dependent on the type of water-soluble component. Fluid products of low apparent viscosity were obtained with non-ionic components, but ionic materials produced more viscous emulsions, some of which exhibited static yield values (Table 2). Their apparent viscosities and static yield values were intermediate between those of comparable products based on cetostearyl or oleyl alcohol.

Only minor differences in globule size and size distribution were found for a representative selection of emulsions (Table 5). These differences

TABLE 5. EXAMPLES OF GLOBULE SIZE DISTRIBUTION DATA FOR EMULSIONS CONTAINING 50% W/W LIQUID PARAFFIN, 0·5% W/W WATER-SOLUBLE COMPONENT AND THE STATED CONCENTRATION OF OIL-SOLUBLE COMPONENT

Water-soluble component	Oil-soluble component	Oil-soluble component (% w/w)	Geometric mean diameter (μ)	Standard deviation of mean ln. diameter
Cetomacrogol 1000	cetostearyl alcohol	0·25	1·93	0·477
Cetomacrogol 1000	cetostearyl alcohol	1·5	1·80	0·396
Cetomacrogol 1000	cetostearyl alcohol	4·0	1·75	0·387
Sorbester Q12 ..	oleyl alcohol	2·0	2·15	0·502
Sorbester Q12 ..	oleyl alcohol	6·0	2·08	0·493
Sorbester Q12 ..	oleyl alcohol	10·0	2·25	0·525
Sodium lauryl sulphate ..	oleyl alcohol	2·0	1·88	0·502
Potassium laurate ..	oleyl alcohol	2·0	1·80	0·544
Sorbester Q12 ..	lauryl alcohol	2·0	1·80	0·477
Cetrimide	cetostearyl alcohol	4·0	1·53	0·454

could not account for the observed pattern of results, although they may affect the absolute values of apparent viscosity and static yield value of individual preparations. The qualitatively similar behaviour of emulsions with constant oil and constant total disperse phase concentration led to the conclusion that phase weight ratio effects were not responsible for the rheological behaviour of our emulsions.

A comparison of the data for preparations based on a disperse phase of isopropyl myristate or castor oil (viscosities of 3 and 600 centipoises respectively) showed that internal phase viscosity was not a major factor determining the flow properties of our emulsions. Similar results for concentrated emulsions have been reported by Toms (1941) and by Shotton & White (1960). Nawab & Mason (1958) suggested that emulsion globules may behave like rigid spheres when stabilized by an interfacial film. Under these conditions the flow properties of emulsions would not be much influenced by internal phase viscosity. For this reason we rejected the possibility that increased viscosity of oil globules,

due to precipitation of excess cetostearyl alcohol, was responsible for the pronounced change in rheological properties above the divergence concentration. We have also failed to observe the lattice arrangement of particles characteristic of such systems during microscopical examination of our emulsions.

Time-dependent rheological properties are known to be associated with structure formation in the dispersing medium (Woodman & Marsden, 1966); such properties were a feature of a number of our emulsions. This, coupled with the apparent absence of cetostearyl alcohol from the internal phase, prompted us to investigate the possible modification of external phase viscosity. Many authors (Matalon, Salton & Cohen, 1951; Epstein, Wilson & others, 1954; Becher & Del Vecchio, 1964) have reported complex formation between fatty alcohols and surface-active agents in aqueous solution. We found viscous gels were formed by the interaction of cetostearyl alcohol, and in some instances lauryl alcohol, with solutions of the water-soluble components used in these studies. Oleyl alcohol did not form gels under these conditions. The viscosity of the aerosol emulsions examined by Sanders (1966) may be attributed to the use of preformed viscous gels of this type. The rheograms of the gels we have investigated exhibited hysteresis loops; their apparent viscosities and static yield values are given in Table 6. Generally, the flow data for the gels reflected the behaviour of corresponding emulsions.

TABLE 6. APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF GELS CONTAINING 10% W/W CETOSTEARYL OR LAURYL ALCOHOLS AND 0·5% W/W WATER-SOLUBLE COMPONENT

Water-soluble component	Cetostearyl alcohol		Lauryl alcohol	
	η_{100}	SYV	η_{100}	SYV
Cetomacrogol 1000	50	1319	20	370
Sorbester Q12	34	942	17	339
Cetrimide	118	1922	162	2763
Sodium lauryl sulphate	107	1972	58	704
Potassium laurate	129	2525	187	3995

The formation of gels would necessitate the migration of fatty alcohol from the oil to the aqueous phase. Migration of sorbitan monolaurate between emulsion phases has been reported by Sherman (1963). During homogenization the intense agitation and enormous extension of the interface provide the most favourable conditions for transfer of oil-soluble component, although this could continue during storage. Apparently, the process was virtually completed within 24 hr, as prolonged storage caused only insignificant changes in the flow properties of our emulsions.

Since lauryl alcohol was completely miscible with liquid paraffin at 25°, the equilibrium concentration of the alcohol in both phases was probably governed by those factors affecting a typical partitioning process. A more complicated transfer mechanism may be involved where emulsions contained cetostearyl alcohol in excess of its solubility in the disperse

phase. The close similarity of the divergence and saturation concentrations for some emulsions suggests that equilibrium was attained when such excess cetostearyl alcohol had been transferred to the aqueous phase. In those cases where the divergence concentration was less than the saturation concentration, it would appear that migration of cetostearyl alcohol continued below the saturation concentration, perhaps by a partitioning process similar to that proposed for lauryl alcohol.

Although many physico-chemical factors undoubtedly influence the migration process, the extent to which this occurs, and the "intrinsic" strength of the gel so formed, appear to be major factors determining the rheological properties of emulsions stabilized by a condensed complex interfacial film.

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Influence of dextrose on the viability of *Bacillus subtilis*

G. RICHARDSON

Dextrose causes an increase in viable count of *Bacillus subtilis* and the effect is greater for spores than vegetative cells. The proportion of spores in which germination is initiated is not influenced by dextrose. During germination the ability of dextrose to affect the viable count is completely lost but re-appears as the germinated spore changes into a dividing vegetative cell. When spores are incubated in broth before plating in nutrient agar there is an increase in viable count followed by a fall before the increase in count due to division is detected. When plated in dextrose agar this initial rise is not seen and there is a fall in count before onset of division. The magnitude of the increase in count caused by dextrose is dependent on the method of plating. For maximum recovery of viable organisms the combination of roll-rube method with dextrose agar is to be preferred to surface plating with or without dextrose in the counting medium.

IT has been shown previously (Richardson, 1965) that in the presence of 0·5% dextrose in the counting medium there is an increase in viable count of up to 75% for suspensions of *Bacillus subtilis* spores. The point in growth at which the presence of dextrose enables a cell or spore, otherwise non-viable, to produce a colony has been sought. Initially attempts were made to discover whether there was any difference between the magnitude of increase in count for spore, and that for vegetative cell suspensions.

Experimental

In addition to methods and materials previously described (Richardson, 1965), the following techniques were used.

Vegetative cell suspensions. These were prepared from an overnight broth culture of *B. subtilis* N.C.T.C. 8236 by the method of Adams (1966). Microscopical examination showed the suspension to be spore-free and to consist almost entirely of single cells. The suspension was used on the day of preparation. The diluent for counting vegetative cell suspensions was nutrient broth.

Roll-tube counts. These were determined using Astell roll-tubes. Tubes were incubated in an inverted position, with bungs removed to permit free diffusion of air into the tubes.

Surface viable counts. These were determined by spreading 0·5 ml inocula on the surfaces of well-dried plates, which were then incubated after a further short drying period (Roberts, 1961).

The complete counting experiment, involving the plating of 10 samples each of spores and 10 samples of vegetative cells, in roll-tubes and on plates with and without dextrose, was repeated on two further occasions.

Dextrose agar. Viable counts were compared in nutrient agar and in nutrient agar containing 0·5% dextrose. This was added to the medium before autoclaving. No change in dextrose concentration could be detected after autoclaving using Sumner's dinitrosalicylic acid reagent (Hawk, Oser & Summerson, 1952).

Dextrose effect. The observed effect of dextrose is termed the

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"dextrose effect" and is calculated from the viable count in presence of dextrose divided by the viable count in the absence of dextrose.

Change in dextrose effect during germination. Spores were incubated at 37° in nutrient broth and in 5 mM alanine solution. Samples were plated in roll-tubes without further dilution over a period of 5 hr to determine the change in dextrose effect during this time.

Influence of dextrose when present during incubation before plating. A comparison was made between the viable counts of spores incubated in nutrient broth and in nutrient broth containing dextrose, sampling at intervals for a period of 2 hr and plating in roll-tubes in dextrose-free medium. In this series of experiments dilution in broth at 37° was made before plating to inactivate the dextrose carried over to the plating medium (actual final concentration of 5–0·5 ppm was shown to have no effect on count). Samples were also plated in dextrose agar.

Nephelometry. The rates of germination and the subsequent growth pattern were followed by making nephelometric measurements on suspensions in nutrient broth (Oxoid CM1) or alanine solution (5 mM), with and without dextrose and held in a shaking water bath at 37°. Measurements were made in $\frac{3}{4}$ in tubes in an EEL nephelometer standardized to read 100 against an appropriate Perspex standard. In practice this corresponded to a spore count of about 5×10^6/ml and a check was made to establish that the meter reading varied linearly with concentration over the whole range. Eight replicate samples were incubated simultaneously and the mean values of the eight determinations were used in presenting the data graphically.

Results and discussion

It was found that $\frac{1}{4}$-strength Ringer was not satisfactory as a diluent in the vegetative cell counts; these did not show a Poisson distribution and were considered unreliable. Even with broth as a diluent, counts showed a high degree of variation and this may account for the variation in level of significance observed within the replicates from vegetative cell counts (Tables 1 and 2).

TABLE 1. THE EFFECT OF DEXTROSE IN THE COUNTING MEDIUM ON THE VIABLE COUNT OF *B. subtilis*

Method	Inoculum	Mean of ten counts in:		Significance of difference n = 10 + 10; 18 d.f.		Mean dextrose effect (count in presence of dextrose / count in absence of dextrose)
		Nutrient agar	Dextrose agar	*t*	P	
Roll-tubes	Spores	76 88 63	140 155 105	15·4 16·8 6·9	<0·001 <0·001 <0·001	1·75
	Vegetative cells	119 133 61	176 152 83	12·8 2·0 4·6	<0·001 0·05–0·1 <0·001	1·33
Surface plates	Spores	74 88 76	109 110 88	7·1 4·6 2·6	<0·001 <0·001 0·02–0·05	1·30
	Vegetative cells	163 127 85	153 118 77	1·2 1·5 2·1	0·2–0·3 0·1–0·2 0·05–0·1	0·92

TABLE 2. THE RELATIONSHIP BETWEEN ROLL-TUBE AND SURFACE VIABLE COUNTS OF *B. subtilis*

Medium	Inoculum	Mean of ten counts from: Roll-tubes	Mean of ten counts from: Surface plates	Significance of difference n = 10 + 10; 18 d.f. t	Significance of difference n = 10 + 10; 18 d.f. P	Mean plating effect (count in roll-tubes / count on surface plates)
Dextrose agar	Spores	140	109	5·8	<0·001	1·29
		155	110	8·9	<0·001	
		105	88	2·4	0·02–0·05	
	Vegetative cells	176	153	2·4	0·02–0·05	1·17
		152	118	4·8	<0·001	
		83	77	1·5	0·01–0·2	
Nutrient agar	Spores	76	74	0·6	0·5–0·6	0·95
		88	88	0·0	>0·9	
		63	76	2·8	0·01–0·02	
	Vegetative cells	119	163	5·2	<0·001	0·83
		133	127	0·7	0·4–0·5	
		61	85	5·2	<0·001	

EFFECT OF DEXTROSE

Magnitude. From the figures in Table 1 it is seen that when counting was by the roll-tube method, the effect of dextrose in the counting medium was greater for spores than for vegetative cells (an average increase of 75% as against 33%). With the surface viable count there was a smaller increase for spores on the dextrose medium (30%); for vegetative cells a slight decrease in count was noted. It seems possible that the lower counts obtained on dextrose plates may be due to over-crowding of colonies. Colonies on dextrose agar appeared more quickly and were much larger than colonies on nutrient agar.

Influence of dextrose on the change in opacity during growth. When the influence of dextrose on growth was observed nephelometrically the results were inconclusive. The change in opacity which occurs during initiation of spore germination was affected very little by the presence of dextrose. Minimum opacity occurred perhaps slightly sooner in the

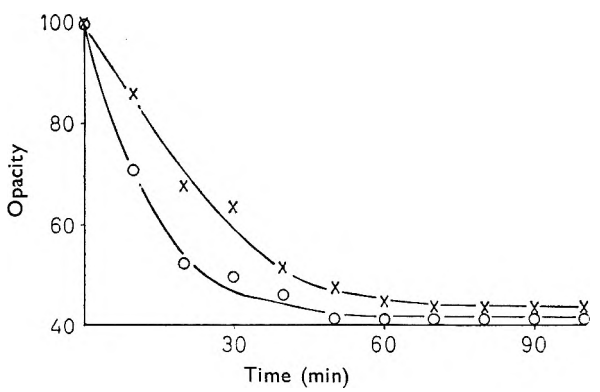

FIG. 1. The change in opacity of *B. subtilis* spore suspensions on incubation in alanine solution. ×, No dextrose; ○, dextrose 0·5%.

presence of dextrose but the % reduction in opacity was similar in each instance. This pattern occurred whether germination was induced by alanine solution (Fig. 1) or by nutrient broth (Fig. 2). The only marked

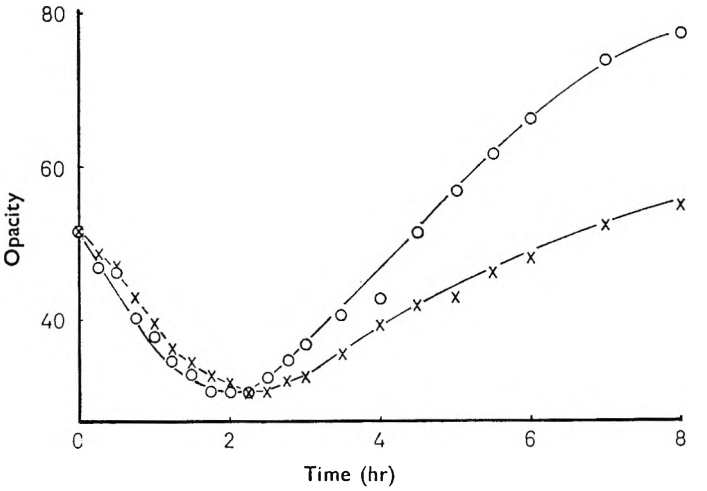

FIG. 2. The change in opacity of *B. subtilis* spore suspensions on incubation in nutrient broth. ×, No dextrose; ○, dextrose 0·5%.

difference was seen after completion of germination when there was a greater increase in opacity in the presence of dextrose. This difference was also seen with a vegetative cell inoculum (Fig. 3).

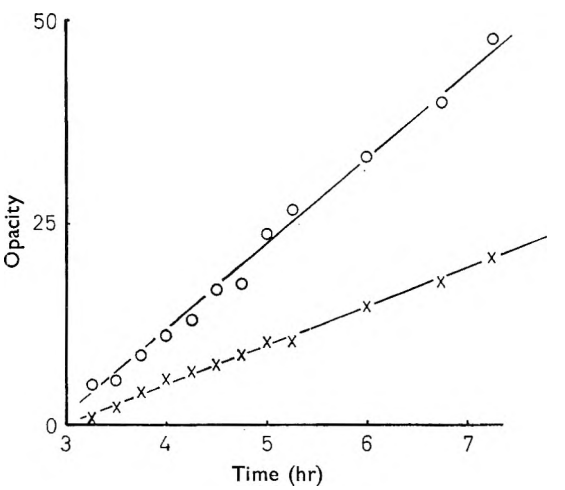

FIG. 3. The change in opacity of *B. subtilis* vegetative cell suspensions on incubation in nutrient broth. ×, No dextrose; ○, dextrose 0·5%.

From these results it is not possible to distinguish between an increase in rate of growth due to a quicker metabolic rate (including reduced

generation time) or an increase in the initial number of cells able to reproduce. The latter alternative would be shown in Fig. 2 by parallelism between the two lines but only if all potentially viable cells simultaneously became susceptible to the presence of dextrose (e.g. at a particular point in growth of a synchronous culture) and also if dextrose did not also cause an increase in rate of growth. Thus the observed effect of dextrose can only be interpreted in general terms as an acceleration in rate of increase in cell mass.

The difference in the ability of dextrose to affect the viability of spores and vegetative cells might be attributed to a simple quantitative difference in metabolism or to a more fundamental difference between the utilization of dextrose by spores and vegetative cells. To investigate this point further, the change in magnitude of dextrose effect during the processes of germination and growth was followed more closely. The clear pattern emerged (Fig. 4) that as germination proceeded the ability of dextrose to

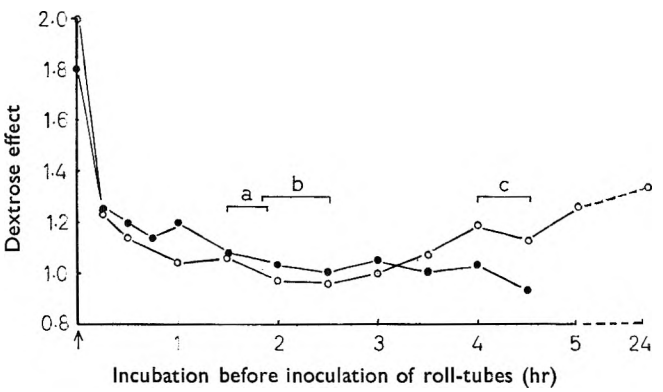

FIG. 4. The change in dextrose effect during growth of *B. subtilis* spores. ○, Incubation in broth; ●, incubation in alanine solution. (↑) Spore inoculum. (a) germination complete, (b) appearance of vegetative cells, (c) count rises due to division. Dextrose effect = count in presence of dextrose/count in absence of dextrose.

affect the count was completely lost, the value for the dextrose effect becoming 1·0. This loss occurred within 1–2 hr by which time germination was complete, although virtually no division had occurred. Subsequently, as the germinated cells began to divide, the influence of dextrose could again be detected, the dextrose effect reaching a maximum value during the first few divisions.

The same pattern was observed when incubation was in broth or dextrose broth, but, with incubation in alanine solution after the initial reduction in dextrose effect, the cells did not proceed to division and there was no subsequent gain in effect.

It seems likely therefore that two separate types of dextrose activity are involved, each associated with a different phase of growth and characterized by different magnitudes of the dextrose effect. The results as plotted in Fig. 4 show an interesting resemblance to the results of Blumenthal

(1965), who showed changes in the path of dextrose metabolism in *B. cereus* during the change from spore to vegetative cell.

Point of action of dextrose. The previous results show that dextrose must be present at or during initiation of germination in order to have maximum effect. An attempt was therefore made to discover whether there was a critical point during the germination process up to which dextrose, if present, could have some effect on the subsequent viability of the cell. To this end, spores were incubated with and without dextrose and then plated in dextrose-free medium. Mean results from five replicate experiments are shown in Fig. 5. Exposure to dextrose during initial

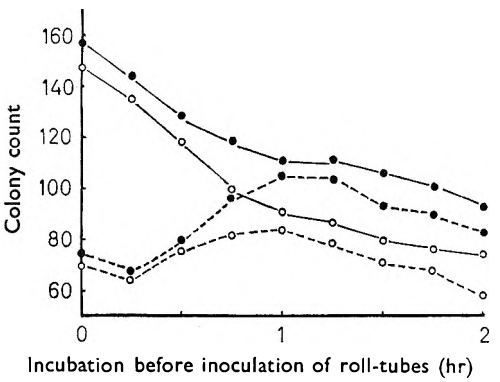

FIG. 5. The effect of dextrose during growth on the viable count of *B. subtilis* when present in the incubation medium before counting and when added to the counting medium. ●, Dextrose in incubation medium; ○, no dextrose in incubation medium; —— dextrose in counting medium; – – – no dextrose in counting medium.

incubation resulted in an increase in the subsequent viable count, becoming significant after about 30 min. The increase reached a maximum as germination was completed in about 90 min and then remained constant. However, the variation between replicates became excessive towards the end of the incubation period so that too great a reliance could not be placed on the later portions of the curves. It is felt that this excessive variation is an indication of the extent to which newly emergent cells are susceptible to environmental influences. Thus when dilution intervenes between incubation and plating there may be a removal of extracellular enzymes secreted during germination, the loss of which would adversely affect the further growth of the cell. This could also account for the fall in viable count during the early stages of germination mentioned below.

Interpretation of the change in the magnitude of the dextrose effect during germination and growth must take into account the associated changes in viable count (Fig. 5). Rather surprisingly it was found consistently during the early stages of incubation and before division could be detected microscopically, that the count showed a rise followed by a fall until the onset of division resulted in a second rise. This pattern was observed only when the cells were plated in dextrose-free medium.

When plated in dextrose agar a continuous fall occurred without the initial rise, until the fall was reversed by division. Thus the reduction in dextrose effect during the change from spore to vegetative cell is partly accounted for by an increase in count which occurs on incubation in the absence of dextrose and is not due solely to the loss of some dextrose-sensitive system during germination.

The ability of dextrose to affect viability disappears rapidly during germination. Thus it is necessary for dextrose to be present before or during initiation to achieve a maximum effect. However, dextrose does not cause activation of germination. Microscopical examination of spores germinating in alanine or broth showed that virtually all spores initiated the germination process (change in phase-contrast appearance) whether dextrose was present or not. In this respect the effect of dextrose is analogous to heat-activation in that, if the stimulus is applied before germination, the effect is observed not immediately but in the later stages of development. If the stimulus is applied later the effect is reduced (dextrose) or reversed (heat). The similarities of activation by heat and by reducing agents have previously been commented on by Keynan, Evenchik & others (1964).

If dextrose is present in the medium at the time of germination (spore inoculum) its effect is greater than if added after germination (vegetative cell inoculum). From the results it may be inferred that the presence of dextrose during initiation enables an additional number of spores to proceed through the stage of outgrowth or early division to form a viable clone. It is probable that two distinct mechanisms are involved, one taking effect during germination and the other during cell division.

EFFECT OF METHOD OF PLATING

When dextrose agar was used as the counting medium for both spores and vegetative cells the roll-tube method gave higher counts than the surface viable count. When nutrient agar was used roll-tube counts were equal to or lower than surface counts (Table 2). Thus dextrose encourages the development of submerged colonies or inhibits the development of surface colonies. The significance of this observation is not apparent but it is obvious that for highest counts of *B. subtilis* the combination of the roll-tube method and dextrose agar is required.

Acknowledgement. The technical assistance of Miss P. Collins is gratefully acknowledged.

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Replenishment by 5-hydroxytryptophan of the amine stores in the central 5-hydroxytryptamine neurons after depletion induced by reserpine or by an inhibitor of monoamine synthesis

H. CORRODI, K. FUXE AND T. HÖKFELT

A combined biochemical and histochemical analysis of central 5-hydroxytryptamine distribution has produced further evidence to support the theory that 5-hydroxytryptophan is specifically taken up and decarboxylated in 5-hydroxytryptamine neurons of rat brain. After treatment with an inhibitor of monoamine synthesis, α-propyldopacetamide, but not after treatment with reserpine, the intraneuronal 5-hydroxytryptamine stores could be replenished by injected 5-hydroxytryptophan. This appears to indicate that the uptake-storage mechanism of amines in the 5-hydroxytryptamine neurons is intact after α-propyldopacetamide. Pretreatment with a monoamine oxidase inhibitor, nialamide, allowed repletion of the intraneuronal 5-hydroxytryptamine stores by 5-hydroxytryptophan even after treatment with reserpine. This further stresses the importance of monoamine oxidase in regulating the amine levels of the 5-hydroxytryptamine neurons.

IN previous experiments (Corrodi, Fuxe & Hökfelt, 1966; Corrodi & Fuxe, 1967) it was shown that L-3,4-dihydroxyphenylalanine (dopa) can replenish the intraneuronal catecholamine stores in the brain after depletion by the methyl ester of α-methyltyrosine (H 44/68), which is a tyrosine hydroxylase inhibitor (see Andén, Corrodi & others, 1966; Corrodi & Hanson, 1966), but not after depletion by reserpine, which blocks the uptake-storage mechanism of the amine granules (Dahlström, Fuxe & Hillarp, 1965; Carlsson, 1966). When the monoamine oxidase inhibitor nialamide was injected before dopa, a replenishment of the intraneuronal catecholamine stores occurred even after depletion by reserpine. In the dopamine neurons, levels above normal were obtained, whereas the amine stores in the noradrenaline neurons were only partly replenished. The exceptionally high amounts of dopamine formed after nialamide-dopa treatment in the brains of rats pretreated with H 44/68 or reserpine, were found to be localized partly in the cells around the capillary walls (pericytes and endothelial cells).

The present study was made to establish how the 5-hydroxytryptamine (5-HT) neurons, depleted either by reserpine or an inhibitor of monoamine synthesis, react to 5-hydroxytryptophan (5-HTP) with or without nialamide pretreatment. The catecholamine and 5-HT biosynthesis was inhibited by α-propyldopacetamide (H22/54) (Carlsson, Corrodi & Waldeck, 1963).

Experimental

MATERIAL AND METHODS

About 100 male Sprague–Dawley rats (150–250 g) were treated either with reserpine (5 mg/kg, i.p.) or α-propyldopacetamide (500 mg/kg, i.p.) to lower the 5-HT stores in the brain. The reserpinized rats were injected 3 hr later with 5-HTP (50 mg/kg, i.p.), half these rats being treated also

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with nialamide (100 mg/kg, i.p.) ½ hr before the 5-HTP injection. The group of rats receiving α-propyldopacetamide were treated with 5-HTP (20 or 100 mg/kg, i.p.), half of the rats treated with the lower dose of 5-HTP (20 mg/kg) being pretreated with nialamide (100 mg/kg, i.p.) ½ hr before the 5-HTP injection. Rats were killed ½, 1 and 2 hr after the 5-HTP injection by decapitation under light chloroform anaesthesia. The brains and spinal cords of some of the animals were taken for histochemical analysis of dopamine, noradrenaline and 5-HT according to the procedure previously described (Dahlström & Fuxe, 1964; Hamberger, Malmfors & Sachs, 1965) but as modified by Fuxe & Jonsson (1967). The brains and spinal cords of others were taken for biochemical analysis of 5-HT (Bertler, 1961). Control rats received saline instead of 5-HTP. Some rats pretreated with reserpine or reserpine-nialamide as described above were also injected with doses of 20 or 100 mg/kg of 5-HTP (i.p.) 1 or 2 hr before killing. Some of the rats treated with α-propyldopacetamide-nialamide as described above received the higher dose of 5-HTP (100 mg/kg). These animals, however, were used only for histochemical analysis.

Results

REPLETION AFTER RESERPINE

Histochemistry. In the rats treated with reserpine alone, practically no yellow fluorescent terminals were observed in the spinal cord and in various parts of the brain (e.g. in the medulla oblongata and the hypothalamus). The 5-HT cell bodies also did not show any definite yellow fluorescence. Furthermore, there were marked reductions in the number and fluorescence intensity of the noradrenaline and dopamine nerve terminals of the brain, and the catecholamine cell bodies became almost completely non-fluorescent. After reserpine-nialamide treatment, the fluorescence microscopical picture did not change except after 2 hr, when a weak yellow fluorescence began to appear in the 5-HT cell bodies of the lower brain stem and a very weak to weak yellow fluorescence of the 5-HT non-terminal axons and terminals became visible.

After administration of 5-HTP (20–100 mg/kg) to reserpinized rats, no restoration of fluorescence was observed in the 5-HT neurons, either in the terminals or in the cell bodies. The only change observed was that with the highest doses of 5-HTP (50–100 mg/kg) the endothelial sheath and the pericytes around the capillary walls showed a weak yellow fluorescence. The yellow fluorescence in the pericytes was most pronounced 30 min after injection. The small amounts of 5-HT found biochemically in the reserpine-5-HTP treated rats (see Fig. 1B) are probably localized in these cells.

The fluorescence microscopical picture was dramatically changed by pretreatment with nialamide before the 5-HTP injection. In the reserpine-nialamide treated rats, 5-HTP in doses of 50–100 mg/kg i.p. produced a moderate to bright yellow fluorescence of the 5-HT nerve terminals in many areas in the brain (e.g. in the medulla oblongata and pons) and in the spinal cord. The catecholamine nerve terminals, however, did not

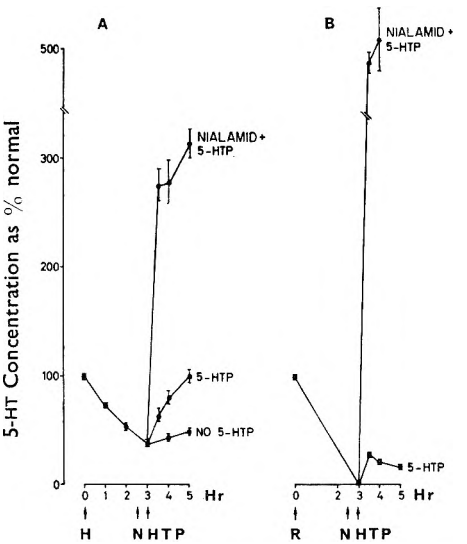

FIG. 1. Effect of 5-HTP with or without nialamide pretreatment on brain 5-HT concentrations in rats pretreated with α-propyldopacetamide (500 mg/kg, i.p.) in (A), or reserpine (5 mg/kg, i.p.) in (B). Each point is an average of 3–4 separate experiments and is expressed as a percentage of normal values ± s.e.m. Arrows indicate time after α-propylacetamide (H) or reserpine (R) when (A) nialamide (N) (100 mg/kg, i.p.) and 5-HTP (HTP) (20 mg/kg, i.p.), or (B) nialamide (100 mg/kg, i.p.) and 5-HTP (50 mg/kg, i.p.), were injected. The normal content of 5-HT was found to be 0·45 ± 0·009 μg/g wet weight (12 experiments).

show any signs of yellow fluorescence. Furthermore, the 5-HT cell bodies, but not the catecholamine cell bodies, showed a moderate to bright yellow fluorescence. Thus, the 5-HT neurons possessed a fluorescence intensity which was clearly above normal. The cells in the capillary walls showed a bright yellow fluorescence and this was observed in all parts of the brain. Yellowish background fluorescence was also noticed especially with the highest dose of 5-HTP (100 mg/kg). This fluorescence may be due to the diffuse presence of the amino-acid itself in the brain tissue. Similar results have been obtained previously in nialamide-5-HTP treated rats (Fuxe, 1965). After the dose of 20 mg/kg of 5-HTP, however, the main observation was an increase in the number and intensity of yellow fluorescence of the 5-HT nerve terminals and cell bodies. The cells in the capillary walls showed only a weak yellow fluorescence.

Biochemical findings. Biochemical results (Fig. 1B) also revealed dramatic changes after 5-HTP administration to reserpine-nialamide pretreated rats when compared with reserpine pretreatment alone. There was an increase in the 5-HT content of the brain from practically zero to a level five-fold the normal value. From the histochemical findings it is probable (see discussion) that these high amounts of 5-HT are present partly intraneuronally in the 5-HT neurons and partly in the pericytes and the endothelial cells along the capillary vessels.

REPLETION AFTER α-PROPYLDOPACETAMIDE

Histochemical findings. After treatment with α-propyldopacetamide alone, there was a large decrease in the number and fluorescence intensity of the 5-HT nerve terminals. The 5-HT cell bodies, which normally emit only a weak yellow fluorescence (Dahlström & Fuxe, 1964), showed a weaker yellow fluorescence or no specific fluorescence at all. In addition the catecholamine cell bodies and terminals showed markedly decreased fluorescence intensity. The brains from the rats treated with α-propyldopacetamide-nialamide showed a similar fluorescence microscopical picture as those from animals receiving α-propyldopacetamide alone.

When 5-HTP (20 mg/kg) was given to rats pretreated with α-propyldopacetamide, the intensity of the yellow fluorescence in the 5-HT nerve terminals and cell bodies was restored to normal levels after 1 hr. The cells in the capillary walls remained practically non-fluorescent. A dose of 100 mg/kg of 5-HTP caused a noticeable restoration of fluorescence in the 5-HT nerve terminals and cell bodies after only 30 min. The cells around the capillary walls now also showed a distinct yellow fluorescence and a yellowish fluorescence of low intensity was seen diffusely in the brain tissues. This fluorescence microscopical picture did not change to any marked degree during the following 1½ hr. The catecholamine neurons were not affected by the 5-hydroxytryptophan injection.

Biochemical findings. Treatment with α-propyldopacetamide produced a decrease in 5-HT levels to about 40% of their normal values (see Fig. 1A and 2). A progressive restoration of the 5-HT contents in brains of α-propyldopacetamide-treated rats occurred after a dose of 20 mg/kg of

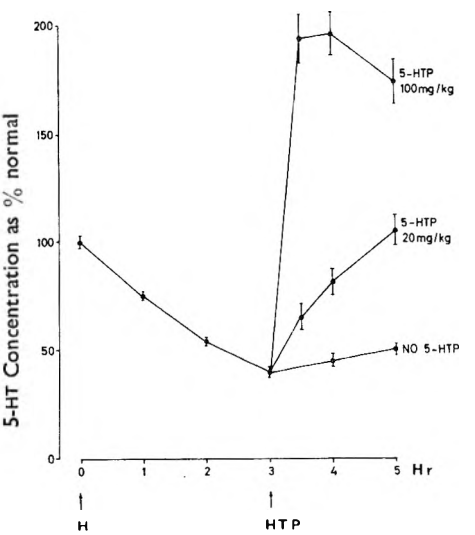

FIG. 2. Effects of two different doses of 5-HTP on brain 5-HT concentrations in rats pretreated with α-propyldopacetamide (500 mg/kg, i.p.). Each point is an average of normal values ± s.e.m. Arrow indicates time after α-propyldopacetamide(H) when 5-HTP was injected.

5-HTP. Normal levels were reached after 2 hr. If a high dose of 5-HTP (100 mg/kg) was injected, the 5-HT content of the brain increased to twice the normal value within 30 min. In view of the histochemical findings, these amounts of 5-HT were probably present in the pericytes and the endothelial cells of the capillary walls.

When the low dose (20 mg/kg) of 5-HTP was given to α-propyldopacetamide-pretreated rats in which monoamine oxidase had been inhibited with nialamide there was a marked restoration of the amine levels in the 5-HT nerve terminals and cell bodies after only 30 min. In fact, 5-HT terminals were even observed in large numbers in the reticular formation, the tectum and some of the cortical areas, where normally they are difficult to detect. The catecholamine neurons were not affected. A distinct yellow fluorescence also appeared in the pericytes and in the endothelial cells, and there was a slight increase in yellow background fluorescence. If a high dose of 5-HTP (100 mg/kg) was used there was no further increase in the intensity of the yellow fluorescence present intraneuronally but the yellow fluorescence of the pericytes and endothelial cells became brighter and an increased background fluorescence was observed. Nialamide pretreatment produced a marked increase in the 5-HT levels of the brain when compared with α-propyldopacetamide-5-HTP treated animals. After the low dose of 5-HTP (20 mg/kg) levels three times the normal value of 5-HT were obtained within 30 min in nialamide-pretreated rats. In view of the histochemical findings this peak level of 5-HT probably lies both in the 5-HT nerve terminals and in the cells of the capillary walls.

Discussion

There is good evidence that most of the 5-HTP decarboxylase is present in the monoamine neurons and is highly active there (Andén, Magnusson & Rosengren, 1965; Heller, Seiden & others, 1965; Andén, Dahlström & others, 1966). Thus, the restoration of yellow fluorescence in the 5-HT neurons after 5-HTP is probably due to 5-HT, formed intraneuronally by decarboxylation (Fuxe, 1965). Furthermore, the specific yellow fluorescence observed in the pericytes and the endothelial cells is also probably due to 5-HT and not to 5-HTP, since dopa-5-HTP decarboxylase is present in these cells (Bertler, Falck & Rosengren, 1964). The present combined biochemical and histochemical study provides further evidence supporting the view that 5-HT is responsible for the yellow fluorescence observed in these structures. Thus, changes in the 5-HT levels determined biochemically were always parallelled by corresponding changes in the yellow fluorescence in 5-HT nerve terminals and cells of the capillary walls. The diffuse yellowish fluorescence observed after high doses of 5-HTP in the brain tissue, however, is probably due to the amino-acid itself.

The fact that after depletion of 5-HT stores by α-propyldopacetamide a low dose of 5-HTP (20 mg/kg) was able to refill the intraneuronal 5-HT stores, provides evidence that α-propyldopacetamide acts by blocking only the first rate-limiting step in monoamine biosynthesis (Carlsson & others, 1963) and does not block the uptake-storage mechanism of the

5-HT granules. After depletion induced by reserpine, not even a high dose of 5-HTP could effect any repletion of the amine in the 5-HT neurons. The 5-HT formed appeared to be present extraneuronally. It is evident, however, that after pretreatment with nialamide, an injection of 5-HTP causes a marked and rapid replenishment of the amine stores in the 5-HT neurons to levels above normal, irrespective of whether the initial depletion had been caused by α-propyldopacetamide or by reserpine. These results agree with previous findings that monoamine oxidase plays an important role in the regulation of intraneuronal 5-HT levels (Carlsson, Lindqvist & Magnusson, 1959; Dahlström & Fuxe, 1964). Furthermore, regardless of whether the uptake-storage mechanism of the 5-HT storage granules is blocked or not, large amounts of 5-HT can be formed and accumulated in the 5-HT neurons after 5-HTP, provided that the monoamine-oxidase has been inhibited. Thus the 5-HT neurons behave like the dopamine neurons, since in the latter pretreatment with nialamide leads to the formation and accumulation of large amounts of dopamine from dopa (Corrodi & Fuxe, 1967). In addition, a large proportion of the high level of 5-HT formed in brain after 5-HTP and nialamide pretreatment is present in extra-neuronal stores, situated mainly in the pericytes and endothelial cells. The present findings also further underline the view (Fuxe, 1965) that there is a fairly specific uptake into, and/or a specific decarboxylation of 5-HTP in the 5-HT neurons, since the catecholamine neurons were hardly affected by the 5-HTP treatment.

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The effects of posterior pituitary hormones on isometric tension and isotonic shortening of the pig myometrium

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Isotonic recording showed that uteri in the luteal stage of the oestrus cycle were more sensitive to oxytocin and vasopressin than those in the follicular stage. The uterine horn was more sensitive than the cervix. A comparison of isotonic and isometric recording revealed that the former exaggerated the response to oxytocin. Under isometric conditions there was no difference in sensitivity to oxytocin between progesterone-dominated and oestrogen-dominated uteri, but both were more sensitive than immature uteri. Tension developed in response to oxytocin was greatest in oestrogen-dominated, less in progesterone-dominated and least in immature uteri.

THE influence of the female sex hormones on the response of the uterus to the hormones of the posterior pituitary gland has been most clearly elucidated in the rabbit. In this species, oestrogens augment, and progesterone blocks the response of the myometrium to posterior pituitary hormones (Robson, 1933a, b; Csapo, 1955, 1956; Schofield, 1957). Complete "progesterone block" has not been demonstrated in any other species, where the uterus contracts in response to oxytocin at all stages of pregnancy; the evidence has been reviewed by Caldeyro-Barcia & Sereno (1961).

On the basis of *in vitro* experiments using isotonic levers, it has been shown that the uterus of the non-pregnant sow responds to oxytocin at all stages of the oestrus cycle and is apparently most sensitive during the luteal stage (Adams, 1940; Knifton, 1962). The present work was undertaken to study further this apparent anomaly, by measuring the responses of strips of sow and immature gilt uteri to synthetic preparations of the posterior pituitary hormones under isotonic and isometric conditions.

Experimental

METHODS

Uteri from freshly slaughtered non-pregnant pigs were collected from a nearby abbatoir and strips assembled for recording as previously described (Knifton, 1966). Krebs solution gassed with oxygen 95% and carbon dioxide 5% was used in all experiments.

Isotonic recording. Oxytocin (Syntocinon, Sandoz) was added to the 10 ml isolated tissue baths in increasing concentrations from 2×10^{-7} to 2×10^{-2} units, so that each uterus was tested with 6 concentrations of drug, each differing by the order of 10, and the sensitivity of each strip was assessed by the number of applications of drug which induced a change in the spontaneous motility (Knifton, 1962). After a resting period of 30 min, vasopressin (lysine-8-vasopressin, Sandoz) was added to the baths in concentrations increasing by the order of 10 from 2×10^{-6} to 2×10^{-1} units, in a similar sequence to that described for oxytocin. The load on the uteri was 2·0 g.

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Isometric recording. The response to varying the frequency of electrical stimulation (staircase effect, Knifton, 1966) was recorded and the strips rested for 1 hr, during which spontaneous contractions developed. (This effect was used in each experiment together with histological data to determine whether the uterus was oestrogen- or progesterone-dominated.) Oxytocin was then added in increasing concentrations from 0·02 to 100·0 mU, so that each uterus was tested with 5 different concentrations of drug. The sensitivity of each uterus to oxytocin was assessed in the manner described for isotonic recording. In addition, responses were recorded, for each dose of drug, as the maximum tension developed during the period of drug contact.

In some cases the responses were also recorded with an isotonic lever on the same tissue, to compare the isometric tension and isotonic shortening of the uterus as a function of the dose of oxytocin.

Hormone dominance. The respective influence of the female sex hormones on the myometrium was assessed in each instance on the basis of histological data (Corner, 1921; Burger, 1952) and the type of staircase recorded (Knifton, 1966). This evidence enabled the uteri to be subsequently classified into immature, oestrogen-dominated and progesterone-dominated groups.

Results

Isotonic recording. The mean percentage responses to oxytocin of sow uteri and immature gilt uteri are summarized in Fig. 1. The sow uterus

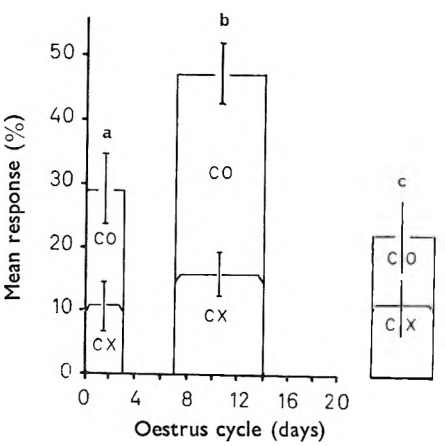

FIG. 1. The mean response (%) to six doses of oxytocin of the cervix (CX) and uterine horn (CO) of (a) 12 uteri from sows at oestrus stage, (b) 18 uteri from sows at luteal stage, (c) 9 uteri from immature gilts. The vertical bars indicate s.e. of the mean.

(horn and cervix) responds to oxytocin during both the follicular and luteal stages of the oestrus cycle. There is no difference in the sensitivity of cervical strips at different stages of the cycle, but the sensitivity of cornual strips during the luteal stage is greater ($P < 0{\cdot}01$) than during the follicular stage.

During both stages of the cycle the cornual strips are significantly more sensitive ($P < 0.01$) to oxytocin than the cervical strips. There is no difference in sensitivity between gilt cornual and cervical strips.

The mean percentage responses of the uteri to vasopressin are summarized in Fig. 2, and the differences in sensitivity between the groups are the same as described for oxytocin.

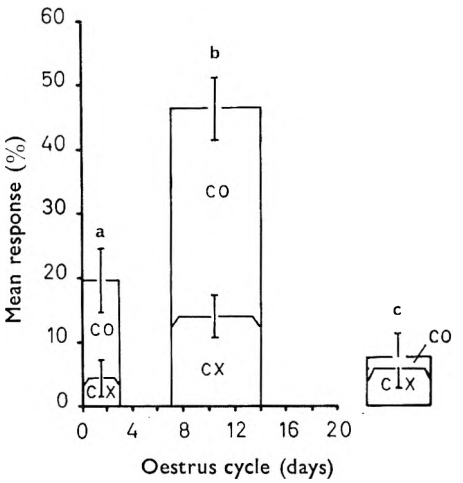

FIG. 2. The mean response (%) to six doses of vasopressin of the cervix (CX) and the uterine horn (CO) of (a) 11 uteri from sows at oestrus stage, (b) 18 uteri from sows at luteal stage, (c) 9 uteri from immature gilts. The vertical bars indicate s.e. of the mean.

Isometric recording. The results of these experiments in which the response to oxytocin was measured isometrically and then isotonically on the same strips revealed that a better dose-response effect was obtained with isometric levers. In some instances, the strips showed maximal isotonic shortening, but the same dose of oxytocin caused no detectable isometric response.

The results of recording isometrically the responses to oxytocin of uterine strips under the predominating influence of different female sex hormones are summarized in Fig. 3. The sow uteri developed significantly greater tension ($P < 0.01$) in response to the doses of oxytocin of 2·0 to 100·0 mU than the immature gilt uteri, and the responses of the oestrogen-dominated sow uteri were significantly greater ($P < 0.01$) than those of the progesterone-dominated group. These results correlate well with the results of measurements of tension developed during spontaneous contractions of the different groups of uteri. For each uterine strip, the greatest tension developed in spontaneous motility before the addition of oxytocin was measured, and the mean value for each group of uteri is shown in Table 1. The tension in the oestrogen-dominated group is significantly greater ($P < 0.01$) than the tension in the progesterone-dominated group and the difference in tension between the oestrogen-dominated sow uteri and the

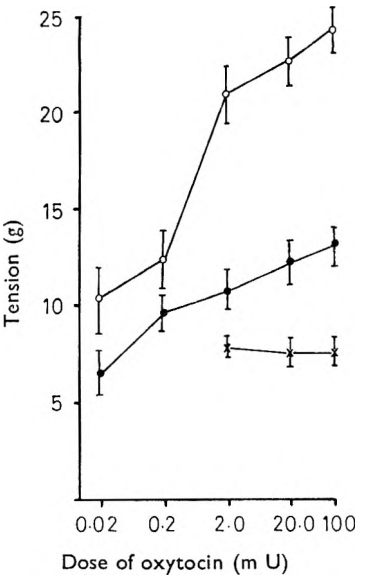

FIG. 3. The isometric response to oxytocin of cornual strips from immature gilts (×), progesterone-dominated (●) and oestrogen-dominated (○) sow uteri. Each point represents the mean response of 8 immature gilt uteri, 14 progesterone-dominated and 14 oestrogen-dominated sow uteri. The vertical bars indicate s.e. of the mean.

TABLE 1. MAXIMUM TENSION OF UTERINE STRIPS DURING SPONTANEOUS MOTILITY

	Oestrogen-dominated	Progesterone-dominated	Immature gilts
Mean tension (g)	19·1	6·4	7·4
s.e	1·7	0·6	0·5
No. of uteri	14	14	8

gilt uteri is also highly significant. There is no significant difference however between the maximum spontaneous tension of the progesterone-dominated sow uteri and the gilt uteri.

An analysis of the responses to oxytocin made by calculating the mean percentage response of each group of uteri to the series of doses of oxytocin gives a comparison of the sensitivity of the different groups to the hormone (Knifton, 1962). When the present results are analysed in this way (Table 2), both the oestrogen and progesterone-dominated groups of sow

TABLE 2. MEAN PERCENTAGE RESPONSE OF UTERINE STRIPS TO 5 DOSES OF OXYTOCIN
Responses recorded with isometric levers

	Oestrogen-dominated	Progesterone-dominated	Immature gilts
Mean response (%) ..	71·4	81·4	57·5
s.e.	12·1	10·4	17·5
No. of uteri	14	14	8

uteri are more sensitive to oxytocin than the immature gilt uteri ($P = 0.05$), but there is no difference in sensitivity between the two groups of sow uteri.

Discussion

In an earlier study of the response of the pig uterus to oxytocin (Knifton, 1962), Dale's solution was used in the tissue baths and the response to Pitocin (Parke-Davis) was measured isotonically. The results of the isotonic measurements in the present study do not differ from the earlier results despite the use of Krebs solution and synthetic oxytocin. The most significant feature of these results is that the uteri in the luteal stage of the oestrus cycle are more sensitive to oxytocin than those in the follicular stage; the predominating female sex hormones during these stages of the cycle are progesterone and oestrogen respectively (Knifton, 1966).

The isometric recordings produced a different result however, in that there was no difference in sensitivity to oxytocin between the oestrogen and progesterone-dominated sow uteri, but both were more sensitive than the immature uteri. This finding confirms that of other work, where it was shown that there was no difference in sensitivity to electrical stimulation of oestrogen and progesterone-dominated sow uteri (Knifton, 1966). The present study has also shown that the uterus can respond isotonically to doses of oxytocin which fail to elicit an isometric response.

It has been shown that in the rabbit, the membrane potential of the progesterone-dominated myometrium is greater than that of the oestrogen-dominated myometrium (Goto & Csapo, 1959; Kuriyama & Csapo, 1961; Marshall & Csapo, 1961). This is in accord with the evidence that in this species progesterone blocks the depolarizing, and hence contractile, effect of oxytocin (Schofield, 1963). In the rat, however, Jung (1964) found no difference in membrane potential between oestrogen and progesterone-dominated uteri. This accords with the evidence that progesterone does not appear to affect the sensitivity of the rat myometrium to posterior pituitary hormones (Reynolds, 1949). Oestrogens however do increase the sensitivity of the rat myometrium (Follett & Bentley, 1964). Similar findings with progesterone have been reported in the guinea-pig (Bell & Robson, 1937), cat (Robson & Schild, 1938) and woman (Moir, 1944; Bickers & Woods, 1949). The present results are thus in accord with those in several other species.

When the response of the uterus to oxytocin is assessed by the tension developed to different doses, however, the present study reveals marked differences between the immature, the oestrogen-dominated and the progesterone-dominated uterus. It has been shown that oestrogens regulate the concentration of contractile protein in the myometrium (Csapo, 1950; Needham & Cawkwell, 1957). Thus the more marked response of the mature uterus when compared with the immature uterus is presumably due to a greater concentration of actomyosin in the former. Csapo (cited by Reynolds, 1951) found no difference in the concentration of actomyosin in the sow myometrium at different stages of the oestrus cycle. In the present study however, the tension developed in response to oxytocin in the progesterone-dominated uteri was significantly less than the tension in the oestrogen-dominated uteri and it is concluded that this is due to progesterone "block", despite the fact that progesterone does

not appear to reduce the sensitivity (i.e. threshold dose) of the pig uterus to oxytocin. It has been shown, however, that progesterone affects the myometrium by various mechanisms (Daniel, 1964). The present studies with oxytocin also substantiate the conclusions drawn from experiments in which the tension developed in the pig uterus in response to electrical stimulation was measured (Knifton, 1966). It has also been shown that oestrogens produce a greater response to posterior pituitary hormones of the bovine uterus *in vivo* when compared with the response of the uterus in the luteal stage of the cycle (Fitzpatrick, 1960).

A further feature of the results is the relative insensitivity to posterior pituitary hormones of the cervix. This agrees with similar findings in the goat (Newton, 1934), rat, guinea-pig (Newton, 1937) and the rabbit (Bonnycastle & Ferguson, 1941).

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Acetylcholine in extracts and perfusates of urinary bladder

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Tissue extracts of the urinary bladder of the rat, cat and guinea-pig contain an acetylcholine-like substance which is considered to be stored predominantly in post-ganglionic nerve fibres. An acetylcholine-like substance was liberated into the fluid bathing the isolated bladder of the guinea-pig and its output was increased 200-fold after transmural stimulation. The acetylcholine-like substance, both from bladder extracts (rat, cat and guinea-pig) and perfusates (rat), was found at the same Rf value as acetylcholine chloride when separated by paper chromatography in three solvent systems. It is concluded that the substance was acetylcholine.

THE response of the urinary bladder to stimulation of its parasympathetic nerves is not blocked by antimuscarinic drugs such as atropine. This phenomenon has been reported in a number of species *in vivo* (Langley & Anderson, 1895; Henderson & Roepke, 1934; Edge, 1955; Vanov, 1965), as well as with *in vitro* preparations (Ursillo & Clark, 1956; Huković, Rand & Vanov, 1965; Chesher & Thorp, 1965). Several hypotheses to explain this anomaly have been proposed, amongst them the suggestion that the transmission process may include a non-cholinergic as well as a cholinergic component.

The evidence for the involvement of a cholinergic mechanism at the neuromuscular synapse of the bladder is convincing. Although the response to stimulation of the parasympathetic nerves to the bladder is resistant to blockade by high concentrations of antimuscarinic drugs, some initial reduction of the amplitude of contraction can usually be produced. A study by Ursillo (1961) of this atropine-sensitive portion of the response to nerve stimulation showed it to have the same degree of sensitivity to atropine as was found in tissues in which the antimuscarinic drugs produce a full blockade. The response of the bladder to stimulation of its parasympathetic nerves is potentiated by physostigmine, and the potentiated portion of the response can be abolished by atropine (Chesher & Thorp, 1965). The response of the bladder to parasympathetic nerve stimulation is abolished by hemicholinium (Huković & others, 1965) or botulinum toxin (Carpenter, 1963) if nerve stimulation is prolonged.

Acetylcholine-like activity has been reported to occur in tissue extracts of the bladder of the dog (Chang & Gaddum, 1933), and of the rat (Huković & others, 1965). These extracts were found to stimulate the frog rectus abdominis muscle preparation (Chang & Gaddum, 1933) and the guinea-pig ileum, and to depress the blood pressure of the pithed rat (Huković & others, 1965). The activity of the bladder extracts on the frog rectus abdominis muscle was potentiated by physostigmine, and on the guinea-pig ileum it resisted block by methysergide or diphenhydramine and was abolished by atropine or hyoscine. The activity of extracts was destroyed by boiling in alkali but resisted boiling in acid solution (Huković & others, 1965).

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These properties suggest that the active substance in the extracts is a choline ester, though they do not identify it as acetylcholine. Indeed, Chang & Gaddum (1933) pointed out that, when tested on the frog rectus abdominis muscle, extracts of dog bladder produced effects which differed sufficiently in their time relations from those of acetylcholine to suggest that they may really be due to some other choline ester.

Acetylcholine-like activity has also been demonstrated in perfusates of urinary bladder. Henderson & Roepke (1934) perfused the dog bladder *in situ* and reported the appearance of an acetylcholine-like substance in the perfusate after stimulation of the perivascular nerves. However, they did not exclude the possibility that this substance could have been liberated from autonomic ganglia. Carpenter & Rand (1965) reported acetylcholine-like activity in the fluid bathing the isolated rat bladder when the bladder was at rest, and a significantly higher activity after nerve stimulation by co-axial electrodes. In neither of these studies was the active substance in the perfusates identified as acetylcholine.

This paper describes experiments which confirm that extracts of bladder tissue from cat, guinea-pig and rat, and the perfusate of the rat and the guinea-pig bladder, contain a substance with the properties of a choline ester. When separated by paper chromatography, using three solvent systems, the acetylcholine-like activity of the extracts and the perfusates, and of acetylcholine chloride were found in the eluates having corresponding Rf values.

Experimental

Methylene blue staining. The bladders, rapidly excised after the animal had been stunned and bled, were gently stretched on glass cannulae with the serous surface outermost and stained by immersion for $\frac{1}{2}$ to 1 hr in a hypotonic methylene blue solution at 37° (Hillarp, 1946). After fixation in a solution of molybdate (8% w/v) the tissue was dehydrated, cleared and examined as a whole-mount under the light microscope. Better resolution, especially at higher magnifications, could be obtained by reducing the thickness of the whole-mount by removing the mucosa after dehydration.

Tissue extracts for acetylcholine estimation. Extracts were made by the methods of Beani & Bianchi (1963), using 0·01M citrate-phosphate buffer, pH 4, or with ice-cold 10% trichloroacetic acid, as described by Hebb, Krnjevic & Silver (1964). Tissue blanks for the trichloroacetic acid extracts were prepared as described by Hebb & others (1964), and those for the citrate-phosphate buffer extracts were prepared by boiling the tissue in 0·1N sodium hydroxide for 10 min. After cooling and neutralizing these blanks with 0·1N hydrochloric acid, an equal volume of 0·02M citrate-phosphate buffer, pH 4 was added. All tissue blanks were tested on the guinea-pig ileum to ensure the absence of acetylcholine-like activity.

The activity of the extracts was measured on the guinea-pig ileum preparation and check assays of some of the samples were made on the heart of the mollusc *Tapes watlingi*. The guinea-pig ileum was bathed in Tyrode solution (Feldberg, 1951) containing diphenhydramine 2×10^{-8} g/ml, maintained at 32° and aerated with oxygen, 95% and carbon dioxide, 5%. The *Tapes* heart was set up as described by Ladd & Thorburn (1955) and bathed in filtered sea water without aeration (Carroll & Chesher, 1965).

The acetylcholine equivalent was determined by matching the responses to the extract (diluted 1 : 5 or 1 : 10) with those produced by acetylcholine in tissue blank. In each instance, concentrations of acetylcholine were selected to produce responses both greater and smaller than those produced by the extract.

Extracts and tissue blanks were stored at −4° for up to 3 weeks before estimation of activity or application to chromatography paper was made. Five samples of acetylcholine added to tissue blank and stored under these conditions for 6 weeks were satisfactorily recovered when tested on the guinea-pig ileum preparation (% recovery = 96% ± 7·3).

Paper chromatography of tissue extracts. Ascending chromatograms on methanol washed and dried Whatman No. 3 mm paper were run in three solvent systems: (i) propanol–0·1N acetic acid (3 : 1); (ii) butanol–acetic acid–water (60 : 15 : 25); (iii) butanol–propanol–water (4 : 2 : 1). To each paper was applied 0·1 to 0·5 ml of citrate–phosphate buffer tissue extract from rat, cat or guinea-pig bladder. As a control, an equal volume of tissue blank and tissue blank containing acetylcholine (0·1 μg) were applied to the paper. The various extracts were developed in each of the three solvent systems. The samples were applied as a streak approximately 2 cm long, perpendicular to the direction of solvent flow, using the Agla micro-syringe. Chromatograms were run until the solvent front had moved 23 to 26 cm from the starting line. After drying, the papers were cut horizontally into sections, each encompassing 0·1 Rf unit, and the sections eluted in absolute ethanol for at least 30 min. The ethanol was then evaporated under reduced pressure at 55–60°, and the residue dissolved in 0·8 ml Tyrode solution. The eluates were tested on the guinea-pig ileum preparation using acetylcholine in Tyrode solution as control. For the testing of eluates, diphenhydramine was omitted from the Tyrode solution bathing the guinea-pig ileum.

The release of acetylcholine from the bladder (in vitro). The guinea-pig bladder was set up for transmural stimulation as described by Chesher & Thorp (1965), and incubated for 70 min at 37° in 25 ml of Tyrode solution containing mipafox, 10^{-5} g/ml. After washing by several changes of the bath fluid and adjusting the volume of Tyrode solution in the bath until it just covered the tissue, the bladder was stimulated by square wave pulses of 20 V, 2 msec duration, at a frequency of 20/sec. Stimulation was for 2 min followed by a 3 min rest period before another 2 min period of stimulation. After three or five such periods of stimulation (corresponding to 6 or 10 min total), the bath fluid was collected, its volume measured and its activity assayed on the guinea-pig ileum against a

standard solution of acetylcholine in Tyrode solution. For the determination of the resting output of acetylcholine, the bath fluid was collected for assay after the bladder had remained in it without stimulation for periods of 25 or 36 min.

Two perfusates, each of 8 ml vol, one from a bladder stimulated for 6 min and the other from a bladder allowed to rest for 36 min, were freeze-dried and the residues extracted with ethanol as described above.

Paper chromatography of bladder perfusates. The rat bladder was used for this study and was set up for transmural stimulation, incubated in mipafox and stimulated for periods of 2 min as described for the guinea-pig bladder. Extracts for chromatography were prepared by freeze-drying pooled samples of perfusate derived from a total of 100 min of stimulation of two or three bladders. Each of these pooled samples totalled approximately 80 ml of perfusate. To separate the acetylcholine-like substance from the inorganic salts in this volume of physiological solution, the freeze-dried residues were extracted with absolute ethanol. After centrifugation at approximately 4,000 rpm for 2 to 3 min to separate the ethanol-insoluble inorganic salts, the ethanolic solution was evaporated to small volume under reduced pressure at 55 to 60° and applied to chromatography paper. The same paper, method of application and solvent systems as described for the chromatography of tissue extracts were used. As controls, an equal volume of Tyrode solution, with or without added acetylcholine was freeze-dried, extracted with ethanol and applied to the paper. The developed chromatograms were eluted in the manner described for the tissue extracts, and the eluates were tested on the guinea-pig ileum preparation.

The responses of the guinea-pig ileum and the *Tapes* heart were recorded by means of a Thorp & Wilson (1965) isotonic transducer connected to a potentiometric pen writing recorder.

Results

As the aim of the present study was to identify the transmitter substance released by the post-ganglionic parasympathetic fibres to the smooth muscle of the bladder, a ganglion cell-free preparation was necessary in order to exclude the pre-ganglionic nerve endings as a possible source of acetylcholine-like activity. Whilst there is physiological and pharmacological evidence for the presence of intramural ganglion cells in the bladder of the guinea-pig (Chesher & Thorp, 1965; Chesher & James, 1966) and the cat (Gyermek, 1961) bladders, it seems that the rat bladder is devoid of ganglion cells (Huković & others, 1965). Histological confirmation of these observations was made in the present study.

Examination of whole-mounts of the bladder for ganglion cells. Ganglion cells were found in only two of ten rat bladders examined, and in two preparations only five to ten ganglion cells could be seen. This was in marked contrast to the bladder of the guinea-pig or cat. In ten cat and ten guinea-pig bladders abundant ganglion cells were seen. Although distributed in greatest number around the entrance of the ureters, ganglion

cells were found throughout the bladder musculature. From a rough count, using a low power magnification (× 200), of ganglion cells in two guinea-pig bladders, it was estimated that if the empty bladder were divided into two equal sections by a transverse cut, the urethral section would contain approximately 75% of the ganglion cell population.

Acetylcholine chloride equivalent of bladder extracts. The acetylcholine chloride equivalent of extracts of the bladder of rat, cat and guinea-pig, as determined on the guinea-pig ileum and the *Tapes* heart are shown in Tables 1–3. Some of the extracts were assayed on the two different preparations on different days, and were stored at −4° in the interim.

TABLE 1. ACETYLCHOLINE-EQUIVALENT DETERMINATIONS OF EXTRACTS OF URINARY BLADDER OF THE GUINEA-PIG

Tissue	Wet wt. (mg)	*Extraction method	Acetylcholine-equivalent estimates (μg/g) determined on (a) guinea-pig ileum	(b) *Tapes* heart	Mean
Whole bladder	520	TCA	—	1·9	1·9
	650	,,	—	1·2	1·2
	417	C-P	2·2; 1·8	2·5	2·2
	493	,,	1·4; 1·2	1·8	1·5
	388	,,	1·8; 1·9	—	1·9
	779	,,	2·3; 2·9	—	2·6
	292	,,	4·5	—	4·5
	240	,,	2·6	—	2·6
	387	,,	1·9	—	1·9
	297	,,	2·9	—	2·9
	263	,,	3·8	—	3·8
Urethral sections, bladders A & B	200	TCA	5·0	7·5; 3·8	5·4
Fundus sections, bladders A & B	250	,,	3·1	3·5; 5·3	4·0
Urethral sections, bladders C & D	350	,,	1·8	3·4; 3·3	2·8
Fundus sections, bladders C & D	420	,,	2·7	3·9; 3·9	3·5
Urethral sections, bladders E & F	513	C-P	1·3	—	1·3
Fundus sections, bladders E & F	668	,,	0·9	—	0·9
Urethral sections, bladders G & H	439	,,	3·1	—	3·1
Fundus sections, bladders G & H	395	,,	2·8	—	2·8

* TCA = Trichloroacetic acid. C-P = Citrate-phosphate buffer.

TABLE 2. ACETYLCHOLINE-EQUIVALENT DETERMINATIONS OF EXTRACTS OF URINARY BLADDER OF THE CAT

Tissue	Wet wt. (mg)	*Extraction method	Acetylcholine-equivalent estimates (μg/g) determined on (a) guinea-pig ileum	(b) *Tapes* heart	Mean
Whole bladder	4,130	TCA	—	1·5	1·5
	4,540	,,	—	1·4	1·4
	3,710	,,	2·6	3·0	2·8
	4,030	,,	3·7	2·5	3·1
	3,190	,,	3·2	2·7	3·0
	2,610	,,	5·5	5·4	5·5
	871	C-P	2·2; 2·5	3·1	2·6
	960	,,	2·1; 2·3	—	2·2
	885	,,	2·1; 2·2	—	2·2
	845	,,	2·0; 2·5	—	2·3
Urethral sections, bladders A & B	719	C-P	2·3	—	2·3
Fundus sections, bladders A & B	684	,,	2·6	—	2·6
Urethral sections, bladders C & D	705	,,	4·0	—	4·0
Fundus sections, bladders C & D	725	,,	3·9	—	3·9

TCA = Trichloroacetic acid. C-P = Citrate-phosphate buffer.

TABLE 3. ACETYLCHOLINE-EQUIVALENT DETERMINATIONS OF EXTRACTS OF URINARY BLADDER OF THE RAT

Tissue	Wet wt. (mg)	*Extraction method	Acetylcholine-equivalent estimates (μg/g) determined on (a) guinea-pig ileum	(b) *Tapes* heart	Mean
Whole bladder	100	TCA	—	0·6	0·6
	100	"	6·3	5·0	5·7
	85	C-P	2·1; 4·1	3·3	3·2
	128	"	1·8	—	1·8
	69	"	3·2; 5·7	5·3	4·7
	93	"	3·6	—	3·6
	97	"	4·1	—	4·1
	90	"	5·1; 5·1	—	5·1
	125	"	3·7; 3·7	—	3·7
	79	"	3·0; 6·0	—	4·5
	102	"	6·1	—	6·1
	100	"	3·9	—	3·9
	110	"	1·9	—	1·9
	106	"	2·8; 2·5	—	2·7
	110	"	1·5; 2·5	—	2·0
	93	"	2·8; 3·1	—	3·0
	182	"	1·6; 1·6	—	1·6
	143	"	3·8; 2·9	—	3·4
	86	"	4·8; 5·9	—	5·4
	187	"	2·8	—	2·8
	135	"	2·5	—	2·5

TCA = Trichloroacetic acid. C-P = Citrate-phosphate buffer.

The means of all determinations, both on the guinea-pig ileum and the *Tapes* heart were (± standard deviation): rat, 3·4 ± 1·5; cat, 2·8 ± 1·1; guinea-pig, 2·7 ± 1·2 μg/g.

It will be seen from Tables 1–3 that there was good agreement in the values obtained from the determinations on the guinea-pig ileum with those obtained on the *Tapes* heart. Furthermore, the activity of the extracts prepared by the two methods employed also showed good agreement.

Acetylcholine equivalent of "ganglion-rich" and "ganglion-poor" areas. The bladders of 8 guinea-pigs and 4 cats were grouped in pairs and each bladder divided transversely into fundic ("ganglion-poor") and urethral ("ganglion-rich") halves. The two urethral and the two fundic sections of each pair of bladders were combined and extracts made to compare their acetylcholine equivalents.

The results shown in Tables 1 and 2 indicate that there was no detectable difference in the acetylcholine equivalents of these two bladder sections. Within each pair of bladders there was good agreement in the estimates of each of the combined halves, and the mean value of all the urethral section extracts did not differ significantly from the mean value of all the fundic section extracts.

These results indicated that any acetylcholine-like substance contained within the pre-ganglionic nerve endings did not contribute significantly to the total acetylcholine measured.

Pharmacological properties of the tissue extracts. Suitable concentrations of the bladder extracts stimulated the guinea-pig ileum and inhibited the spontaneous beating of the *Tapes* heart. The contraction of the guinea-pig ileum was reduced or could be abolished completely by suitable concentrations of atropine. Equal responses to acetylcholine in

tissue blank and to the bladder extract were reduced to the same extent by atropine. The activity of the extracts was abolished when they were boiled at pH 9·5 to 10, or when incubated with guinea-pig plasma. However, activity was not reduced when the extracts were boiled at pH 4, or incubated in guinea-pig plasma containing physostigmine ($1·6 \times 10^{-5}$ g/ml).

All of these properties suggest that the active substance is a choline ester. However, there were some qualitative differences between the contractions produced by the tissue extracts and those induced by acetylcholine in the tissue blank. The contraction induced by the extract was often of slower onset and took longer to reach a maximum. These differences were more apparent when the responses had been reduced by atropine (Fig. 1). The substance responsible for this difference

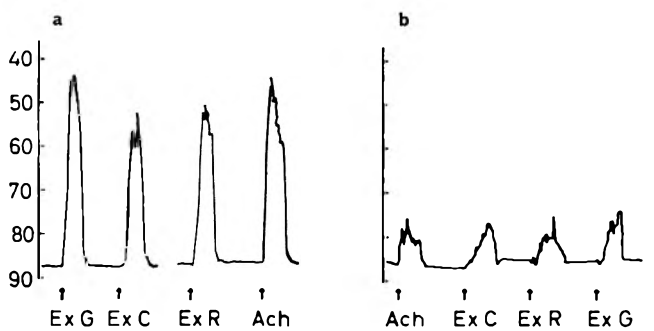

FIG. 1. Responses of the guinea-pig ileum, suspended in 2 ml Tyrode solution, to acetylcholine (Ach), $1·25 \times 10^{-9}$ g/ml, and to tissue extracts of the bladder of the rat (Ex R), the guinea-pig (Ex G) and the cat (Ex C). In (a) normal Tyrode solution; in (b) the Tyrode solution contained atropine, 5×10^{-9} g/ml. Note the slower response to the tissue extract when compared with the response to acetylcholine. Divisions on ordinate = 0·5 inch. Magnification × 30.

is presumably destroyed by the procedure used in the preparation of the tissue blanks, because acetylcholine in the tissue blank did not behave in this manner.

Paper chromatography of bladder extracts. Only citrate-phosphate buffer extracts were used for chromatography, and to control the possible influence on the Rf values of other substances in the extracts each paper was spotted with tissue blank containing acetylcholine chloride, and with inactive tissue blank. Fifteen chromatograms were developed, eluted and tested on the guinea-pig ileum. In butanol–propanol–water the approximate Rf values of the active eluates were 0·1–0·2 for acetylcholine and also for the extract and perfusate. In butanol–acetic acid–water the Rf values were 0·45–0·55 for these eluates and in propanol–acetic acid they were 0·4–0·5. The results show that the activity determined on the guinea-pig ileum is clearly confined to eluates derived from paper equivalent to 0·1 Rf unit and in each of the three solvent systems used the active eluates of both the extract and of acetylcholine corresponded to the same Rf values. Furthermore there was no species difference, the bladder extracts from rat, cat and guinea-pig all behaving similarly. No activity was found in control eluates.

The qualitative differences seen in the responses of the guinea-pig ileum to acetylcholine and to the tissue extracts were not seen with the active chromatographic eluates of the extracts. The responses to the active eluates of both acetylcholine and extract were reduced by atropine to the same extent (Fig. 2).

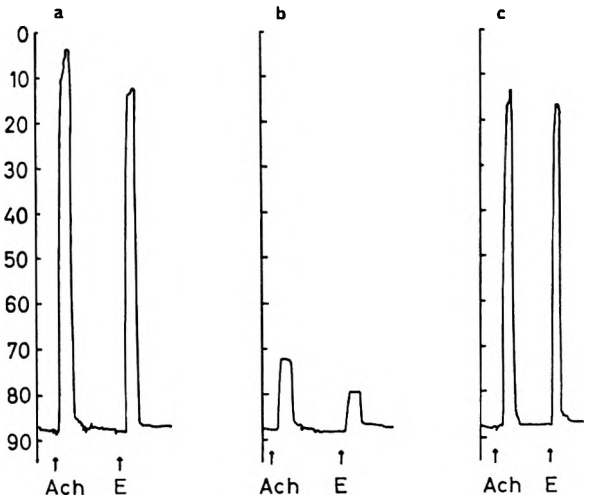

FIG. 2. Responses of the guinea-pig ileum to the active eluates from a chromatogram to acetylcholine (Ach) and a tissue extract of guinea-pig bladder (E). Eluates were obtained from paper strips of similar Rf value. In (a) normal Tyrode solution was used; in (b) and (c) the Tyrode solution contained atropine, $7{\cdot}5 \times 10^{-9}$ g/ml. In (c) the amounts of acetylcholine or tissue extract added to the guinea-pig ileum were increased tenfold. Divisions on ordinate = 0·5 inch. Magnification × 30.

The release of transmitter from the guinea-pig isolated bladder. Acetylcholine-like activity was detected in the perfusate of the resting guinea-pig bladder, and electrical stimulation of its intrinsic nerves increased the output of this substance approximately 200 times. The results of 16 determinations on six guinea-pig bladders are shown in Table 4.

TABLE 4. THE RELEASE OF ACETYLCHOLINE-LIKE SUBSTANCE INTO THE FLUID BATHING THE ISOLATED BLADDER OF THE GUINEA-PIG. DETERMINATIONS MADE ON THE GUINEA-PIG ILEUM

Resting output (ng/hr)	Stimulated output (ng/hr)
4*	400
5	600
8*	600
	1,300*
8*	2,000
8	2,400
10	2,500*
	3,000
10	3,000
Mean = $7{\cdot}6 \times 10^{-9}$ g/hr	Mean = $1{\cdot}8 \times 10^{-8}$ g/hr

* Freeze dried perfusates.

The response of the guinea-pig ileum to the perfusate, unlike that to tissue extract, was indistinguishable from the response to acetylcholine. Responses to acetylcholine and to the perfusate were reduced to the same extent by atropine (Fig. 3).

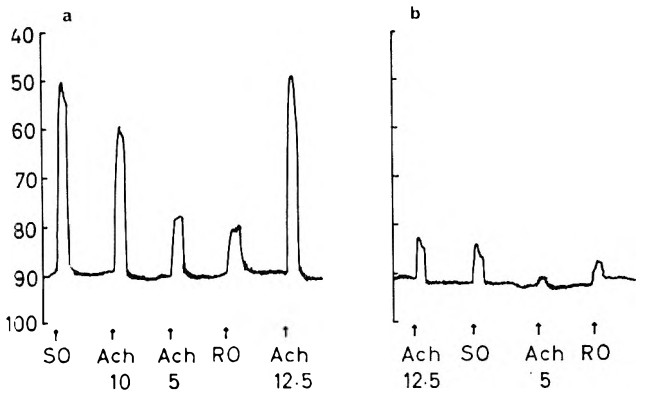

FIG. 3. (a) The responses of the guinea-pig ileum to perfusates from the guinea-pig bladder. Extracts of the freeze-dried residue of perfusates containing the transmitter output from 6 min transmural stimulation or of 36 min resting release were each dissolved in 1 ml of Tyrode solution. SO = 0·2 ml of a 1:10 dilution of the stimulated output extract. RO = 0·2 ml of the resting output extract. Ach = acetylcholine chloride, g/ml $\times 10^{-10}$. In (b) the Tyrode solution contained atropine, 5×10^{-10} g/ml. Divisions on ordinate = 0·5 inch. Magnification × 30.

On standing for 2–3 hr at room temperature all the perfusates lost most of their activity.

Paper chromatography of bladder perfusates. The rat bladder was used. The virtual absence of ganglion cells in this preparation excludes the pre-ganglionic nerve endings as a possible source of the acetylcholine-like activity.

As the preparation of the perfusates for chromatography involved freeze-drying, the effect of this treatment on the activity recovered was tested. The perfusates from two guinea-pig bladders were freeze-dried and the residues, extracted as described previously, were tested on the guinea-pig ileum. The results (Table 4, see *) were in good agreement with those obtained by the direct assay of perfusates. Furthermore, these experiments showed that the active substance, like acetylcholine, is soluble in absolute ethanol.

Three chromatograms were developed and the approximate Rf values of the acetylcholine-like substance in the perfusate were found to be the same as for acetylcholine chloride in each of the three solvent systems used. Control extracts of Tyrode solution were inactive. The response of the guinea-pig ileum to the active eluate of both the perfusate and the acetylcholine was reduced by atropine to the same extent. An increase in the concentration of both agonists overcame the blockade by atropine (Fig. 4).

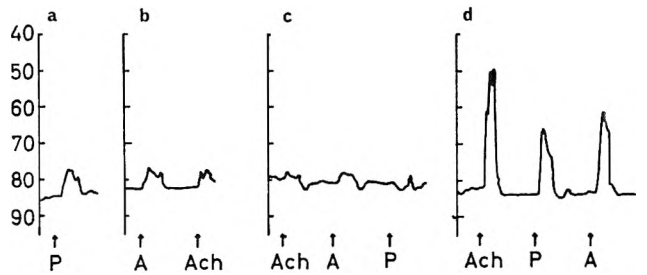

FIG. 4. After chromatography of a sample of perfusate from the rat bladder following transmural stimulation, and of acetylcholine added to perfusion fluid, eluates were made from strips of paper corresponding to the Rf value of acetylcholine. P = 0·1 ml of the eluate from the chromatogram of rat bladder perfusate. A = 0·1 ml of 1 : 10 dilution of the eluate from the chromatogram of acetylcholine in perfusion solution. Ach = acetylcholine, 5×10^{-10} g/ml. In (a) and (b) ileum was bathed in normal Tyrode solution; in (c) and (d) in the presence of atropine (5×10^{-10} g/ml). In (d) the amounts of A or Ach added to the ileum were increased tenfold, and of P were increased threefold. Divisions on ordinate = 0·5 inch. Magnification × 30.

Discussion

The results presented here provide evidence for the presence of acetylcholine-like activity in tissue extracts of the urinary bladder of the guinea-pig, the cat and the rat. The extracts, prepared by two procedures, had properties similar to those previously described by Chang & Gaddum (1933) for the dog bladder and by Huković & others (1965) for the rat bladder.

The differences in the time relations of the response of guinea-pig ileum to tissue extracts or to acetylcholine in the tissue blank appear to be similar to those reported by Chang & Gaddum (1933), who tested extracts of the dog bladder on the frog rectus abdominis muscle. This difference may be due to an interfering substance in the tissue extracts which reduced the rate of contraction of the guinea-pig ileum to acetylcholine. This effect was more apparent when the response of the guinea-pig ileum to acetylcholine had been reduced by atropine. This interfering substance was destroyed by the treatment used to prepare the tissue blanks, and was separated from the acetylcholine-like substance by paper chromatography. However, as it could not be detected in the perfusates of bladder after transmural stimulation, and its effect on the ileum was one of inhibition, this substance does not appear to be associated with the transmission of excitation at the neuromuscular synapse of the bladder.

The acetylcholine-like activity was found in approximately equivalent concentrations in the "ganglion rich" (urethral) and the "ganglion poor" (fundic) segments of the cat and guinea-pig bladder, and in the ganglion cell-free rat bladder. Therefore, assuming that the ester is found predominantly in nervous tissue, the concentrations reported represent ester stored in the post-ganglionic fibres.

The estimate of the acetylcholine-equivalent of the active substance found in the perfusate from the guinea-pig bladder, both at rest and after nervous stimulation, were in agreement with that reported for the rat bladder by Carpenter & Rand (1965). The participation of this substance in the neuromuscular transmission of the bladder is suggested by the 200-fold increase in output following nervous stimulation. Furthermore, Carpenter & Rand (1965) have shown that the transmural stimulation of the rat bladder does not release the acetylcholine-like substance if the post-ganglionic nerve fibres have degenerated.

After separation by paper chromatography, the acetylcholine-like substance of the rat, cat and guinea-pig bladder extracts and of the perfusates collected after transmural stimulation of the rat bladder was found in the eluates corresponding to the same Rf value as those containing acetylcholine chloride. The solvent systems chosen have been shown to provide a satisfactory separation of the choline esters (Whittaker, 1963; Cobbin, Leeder & Pollard, 1965).

It is concluded that the chromatographic and pharmacological properties of the acetylcholine-like substance in extracts and perfusates of the bladder are compatible with it being acetylcholine.

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A modification of a method for the evaluation of topical anaesthesia in the earthworm

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The method of Block, Potts & Finney (1964) for the evaluation of local anaesthetic activity using the response of the earthworm tail to hydrochloric acid has been found to give false negative results with control solutions. This was because the acid progressively damaged and thus desensitized the tail. This has been overcome by arranging the test so that no tail is immersed in the acid more than three times during an assay.

THE method of evaluating local anaesthetic activity using the response to acid of the tail of the earthworm, as described by Block, Potts & Finney (1964), although originally designed for qualitative screening purposes, was also recommended for quantitative comparison of the potencies of local anaesthetic agents.

Adhering *strictly* to this method, we evaluated several local anaesthetic agents and found that positive anaesthetic responses were yielded by control worms immersed in Ringer solution alone (Tables 1 and 2). Although sensation was not abolished during the initial three exposures to the acid, over 30% of the acid dippings produced no detectable reactions. In view of this a step-by-step review of the procedure was made.

Experimental

The conditions were re-examined and were found to conform to those of Block & others (1964) in genus and size of worm,* preparation and pH of the Ringer solution, normality of the hydrochloric acid solution and storage of the worms.

During all determinations, the initial responses were pronounced and unmistakable, but after a few dippings into the acid this sharpness diminished and was accompanied by an apparent change in the gross aspects of the skin of the tails which appeared dry and hard. The tails of worms were therefore examined microscopically after exposure to 0·0125 N hydrochloric acid. One group was dipped in the acid at 1 min intervals for 12 min; a second group was dipped twice at 1 and 3 min, while a third group was untreated and served as a control.

Photomicrographs showed that after two dippings in the acid the ectoderm had, to a large extent, been removed. That remaining was ragged and the cell morphology was disturbed. Some of the bundles in the circular muscle layer were swollen but the longitudinal layer had not been affected. After exposure of the tail to the acid at 1 min intervals for 12 min, the cuticle and ectoderm had been removed and the muscle bundles within the circular layer were swollen and oedematous. There was some degree of oedema in the longitudinal layer also.

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* They were identified specifically as *Lumbricus terrestris*.

MODIFIED PROCEDURE

As a result of these findings, we modified the original procedure.

Eathworms of the same genus and size were stored in a minimum of 250 ml of Ringer solution which was aerated while it contained the worms —a period of not less than 2 nor more than 3 hr (for periods in excess of this the condition of the animals progressively deteriorates).

Immediately upon removal from the Ringer solution, at least 18 worms were dipped in the acid solution to determine the acceptability of the responses. After testing, they were immersed in the experimental anaesthetic solution for 1 min and then divided into three equal groups. The tails of the first group were dipped into the acid solution at 1 and 3 min, after removal from the anaesthetic solution. Those of the second group were similarly dipped at 5 and 8 min, and those of the third group at 12 and 16 min. Thus, no tail was dipped into the acid on more than three occasions.

The data were then treated in a manner which differed from that recommended by Block & others (1964), purely on personal preference. The total number of "no responses", elicited by exposure to a given concentration of an anaesthetic solution, was divided by the total number of applied stimuli and the resultant product was multiplied by 1,000 to yield a value we called the Anaesthetic Index (see Table 3). These indices were plotted against concentration, %, and the ED50 for each anaesthetic agent was determined by the method of Litchfield & Wilcoxon (1949).

Results

The results of testing some anaesthetic agents using the modified method, have been summarized in Table 3. The differences between these results and those in Table 1 are obvious. Even though benzocaine still exhibited the greatest, and lignocaine the least potency, the ranges of gradient activity varied widely.

TABLE 1. RESULTS OF DETERMINING LOCAL ANAESTHETIC EFFICACY BY THE METHOD OF BLOCK & OTHERS (1964)

Drug	Conc. %	No. of worms	Total no. of stimuli	No. of "no responses"
Lignocaine	0·25	10	120	73
	0·50	10	120	103
	1·00	10	120	119
Diperodon	0·25	10	120	110
	0·50	10	120	111
	1·00	10	120	120
Benzocaine	0·0001	8	96	74
	0·001	10	120	109
	0·01	10	120	114
	0·10	10	120	120
	0·25	10	120	119
	0·50	10	120	118
	1·00	10	120	118
Ringer solution*		10	120	28

* NaCl, 3·67; Dextose, 1·33; $NaHCO_3$, 0·20; NaH_2PO_4, 0·67; 15% KCl, 0·62 ml; 16% $CaCl_2$, 0·20 ml; distilled water to 1 litre. Final pH, 6·6.

TABLE 2. DISTRIBUTION OF NEGATIVE REACTIONS OBSERVED WHILE ASSESSING PCTENCY BY THE METHOD OF BLOCK & OTHERS (1964)

Drug	Conc. (%)	Number of "no responses" at (min)											
		1	2	3	4	5	6	7	8	9	10	11	12
Lignocaine	0·25	0	0	1	4	4	8	9	8	10	9	10	10
	0·50	6	8	7	7	9	8	9	9	10	10	10	10
	1·00	10	10	10	10	10	10	10	9	10	10	10	10
Diperodon	0·25	6	7	9	9	10	10	10	10	10	10	10	10
	0·50	9	8	7	9	9	10	9	10	10	10	10	10
	1·00	10	10	10	10	10	10	10	10	10	10	10	10
Benzocaine	0·0001	1	3	3	6	6	7	7	7	7	7	7	7
	0·001	5	5	8	10	10	10	10	10	10	10	10	10
	0·01	5	9	10	10	10	10	10	10	10	10	10	10
	0·10	10	10	10	10	10	10	10	10	10	10	10	10
	0·25	10	10	10	9	10	10	10	10	10	10	10	10
	0·50	10	9	9	10	10	10	10	10	10	10	10	10
	1·00	9	9	10	10	10	10	10	10	10	10	10	10
Ringer solution		0	0	0	1	2	3	3	3	3	5	4	4

TABLE 3. RESULTS OF DETERMINING LOCAL ANAESTHETIC EFFICACY AS DETERMINED BY THE MODIFIED PROCEDURE

Drug	Conc. (%)	No. of worms	Total no. of stimuli	No. of "no responses"	Anaesthetic* Index
Lignocaine	1·0	18	36	7	190
	2·0	18	36	19	520
	3·0	18	36	23	630
	4·0	36	72	49	680
	6·0	36	72	60	830
Diperodon	0·01	36	72	3	40
	0·05	36	72	13	180
	0·10	36	72	20	270
	0·50	36	72	44	610
	1·00	36	72	47	650
Benzocaine	0·01	36	72	6	80
	0·05	36	72	20	270
	0·10	36	72	58	800
Ringer solution ..		36	72	2	28

* See page 457.

It was determined that the ED50 of lignocaine equalled 2·30 (1·81 to 2·92)%; that of diperodon equalled 0·14 (0·11 to 0·18)%; and that of benzocaine equalled 0·07 (0·06 to 0·08)%. These were calculated with a 95% limit of probability.

Discussion

Our observations would appear to explain the progressive lack of response noted in worms which had received repeated exposure to acid, since this degree of tissue destruction would also involve receptor sites. According to Block & others (1964), concentrations of acid higher than 0·0125N caused damage but worms of the genus *Lumbricus* tolerated this degree of acidity. It would appear that this statement should be qualified and that some limitation should be placed on time and frequency of acid exposure.

TABLE 4. DISTRIBUTION OF NEGATIVE REACTIONS OBSERVED IN ASSESSING POTENCY BY THE MODIFIED PROCEDURE

Drug	Conc. (%)	Number of "no responses" at (min) 1	3	5	8	12	16
Lignocaine	1·0	1	1	1	2	1	1
	2·0	3	3	5	4	2	2
	3·0	3	3	4	5	3	5
	4·0	4	5	8	8	12	12
	6·0	6	6	12	12	12	12
Diperodon	0·01	1	2	0	0	0	1
	0·05	1	1	1	3	2	5
	0·10	1	5	2	4	1	7
	0·50	6	8	4	7	2	7
	1·00	5	7	6	12	8	8
Benzocaine	0·01	0	0	0	1	2	3
	0·05	0	4	2	3	7	4
	0·10	7	9	9	11	11	11
Ringer solution ..		0	1	0	1	0	0

The fact that the modification had yielded different results, however, does not of itself support a conclusion that these were more valid than those obtained by the original method.

Attention was first directed to the responses yielded by the control animals which had been exposed to the Ringer solution alone. Using the modified method, a 2·8% incidence of negative responses was elicited by this group (Table 3); compared with 23% after the original method (Table 1). Data illustrating the distribution of "no responses" in these instances have been presented in Tables 2 and 4.

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Degraded and undegraded carrageenans and antipeptic activity

W. ANDERSON AND J. E. HARTHILL

The antipeptic activities of a series of undegraded κ- and λ-carrageenans and degraded carrageenans have been examined by two methods. The activities differ quantitatively. Compared with the κ-carrageenans, the λ-carrageenans, as a group, have greater activity and this is associated with low 3,6-anhydro-D-galactose and high ester sulphate content. Within the series of undegraded carrageenans, both κ and λ, differences in antipeptic activity are significantly and positively related to differences in sulphate content. In addition to the effect of sulphate, 3,6-anhydrogalactose content appears to be inversely related to activity but in the λ- series only; differences in viscosity are without effect. Initial degradation without loss of sulphate resulted in loss of activity but further degradation, also without loss of sulphate did not reduce activity further. Antipeptic activity appears to depend on a combination of structural features of which the differences between κ-λ carrageenans, molecular size and polyanionic properties are aspects.

THE addition of sulphated polysaccharides to gastric juice or to solutions of pepsin results in a decrease in demonstrable proteolytic activity. This has been stated to be due to complex formation between the sulphated polysaccharide and the enzyme in the instance of chondroitin sulphate (Levey & Sheinfeld, 1954). For degraded carrageenan (Anderson, 1961), protection of the substrate rather than direct anti-enzyme action was shown to be principally responsible for the decrease in peptic activity.

Because of the interaction between sulphated polysaccharides and protein (substrate or enzyme, or both) at appropriate pH, which depends on the polyionic nature of the substances concerned, it has been inferred that ester sulphate is the part of the carrageenan molecule responsible for the magnitude of antipeptic activity. Indeed, it has been shown that for certain sulphated polysaccharides, sulphate content can be related to activity (Hawkins & Leonard, 1962; Ravin, Baldinus & Mazur, 1962). However, comparison of published work suggests that sulphate content is not the only part of the molecule concerned in antipeptic activity. The relationships are even less clear where different methods of measuring peptic activity have been used.

In the present work, the relationship between antipeptic activity and sulphate content, and other aspects of carrageenan structure, is examined using two methods of measuring antipeptic activity.

Experimental

MATERIALS AND METHODS

Carrageenans. Undegraded and degraded carrageenans were used, and were obtained from the principal seaweed sources, *Chondrus*, *Gigartina* and *Eucheuma* species. Of the undegraded carrageenans, unfractionated samples and samples fractionated into κ- and λ-components were used. With the exception of a degraded carrageenan from *Eucheuma spinosum*—C16—(Anderson, 1961), and Rees' carrageenan (Rees, 1963) kindly

From the Department of Pharmacy, University of Strathclyde, Glasgow.

supplied by Dr. D. A. Rees, Chemistry Department, University of Edinburgh), all other carageenans (Black, Blakemore & others 1965) and the data in Table 1 were provided by the Arthur D. Little Research Institute, through the courtesy of Dr. E. T. Dewar.

Gastric juice. Gastric juice was obtained from duodenal ulcer patients undergoing the augmented histamine test and was used fresh. (It was provided through the courtesy of Dr. A. I. M. Glen, Western Infirmary, Glasgow.) Insoluble material was removed by centrifugation. Occasional juices devoid of acidity or discoloured were not used.

PEPTIC ACTIVITY

Method A was a modification of the method of Anson (1938). Two concentrations of carrageenan were used: 20 or 80 mg carrageenan was dissolved in 2 ml hydrochloric acid solution (pH 1·6) and this was added to 2 ml pepsin solution (20 mg/100 ml; pH 1·6; Armour crystalline porcine pepsin) giving concentrations of 0·5 and 2% of carrageenan respectively in the 4 ml of acid-pepsin at pH 1·6. This solution was incubated at 37° for 10 min and 2 ml haemoglobin solution (1·2%; pH 1·6; Armour bovine haemoglobin for proteolytic enzyme assay) was added, followed by incubation at 37° for 30 min. After incubation, 20 ml of 10% trichloroacetic acid was added, the flasks allowed to stand in the water bath for 1 hr and the contents filtered (Whatman No. 3). 5 ml filtrate was added to 10 ml N sodium hydroxide followed by 3 ml of a 1 : 2 aqueous dilution of Folin-Ciocalteu reagent. The extinction developing in 10 min was read at 660 mμ. Appropriate blanks and controls were included.

Method B was a modification of the method of Hunt (1948). Three concentrations of carrageenan were used: 8, 12 or 20 mg carrageenan was dissolved in 2 ml hydrochloric acid solution (pH 2·1) and 2 ml of centrifuged supernatant of the gastric juice added. (8 mg carrageenan in this 4 ml volume gave 0·2%.) 1 ml of this solution was used in the test for peptic activity. Appropriate controls and blanks were included. The remainder of the method was as described by Hunt. Extinction values were converted to Hunt units.

Antipeptic activity. This term was calculated from $(1 - I/S) \times 100$ where I and S represent peptic activity with and without inhibitor respectively, corrected for blanks as necessary. Antipeptic activity is therefore the amount of inhibition, %.

Results and discussion

A representative group of carrageenans from species of the red seaweed families *Chondrus*, *Gigartina*, *Eucheuma* and *Polyides* has been examined for antipeptic activity by two methods which differ both in enzyme and substrate. Method A uses a purified enzyme, whilst method B uses fresh human gastric juice from duodenal ulcer patients. Two methods were used to increase the likelihood of finding a pattern of antipeptic activity amongst the carrageenans and, hence, not only a basis for a standard method of assessing their antipeptic activity, but also information

about their mode of action. These two methods have been used in, or have formed the basis of, most reports on antipeptic activity.

The carrageenans were selected from a number of such substances which became available during an extensive survey (Black & others, 1965) of carrageenan as a possible therapeutic agent for the treatment of peptic ulcer. κ- and λ-Components were studied because they differ in structure and properties (Rees, 1966). Undegraded and degraded carrageenans were examined because, although degradation usually reduces antipeptic activity, it is possible in therapy and in pharmacological investigation to use greater amounts of the less viscous, more rapidly soluble degraded product, thus obtaining greater activity and more information than would otherwise be possible with the highly viscous, slowly soluble, native

TABLE 1. SOURCES AND PROPERTIES OF THE CARRAGEENANS USED

	Code	SO_3Na (%)		η_{inh} (dl/g)		3,6-anhydro-D-galactose $C_6H_8O_4$ (%)	
		κ	λ	κ	λ	κ	λ
Undegraded							
Chondrus crispus	CMI[1]	27·6	32·2	8·6	9·4	24·8	9·8
" "	CSE[2]	29·8	32·3	14·3	13·8	25·2	9·1
" "	CBC[3]	29·6	34·6	11·6	14·4	22·9	4·7
" "	CNS[4]	28·4	34·9	20·8	21·7	25·3	4·1
" "	CY[5]	28·2	37·3	13·7	16·2	22·2	3·6
" "	CRF[6]	—	38·8	—	15·1	—	4·2
" "	CCB[7]	—	47·6	—	13·1	—	4·5
	CNB[8]	43·6 (unfractionated)				18·8	
Rees' carrageenan	R-λ[9]	—	41·1	—	23·2	—	1·5
Gigartina stallata	GS[10]	30·1	28·3	15·0	13·6	22·6	15·9
G. radula	GR[11]	31·0	35·6	15·7	15·7	23·2	9·6
G. pistillata	GP[12]	35·0	43·6	10·5	20·6	12·8	4·2
Eucheuma spinosum	ES[13]	37·7		7·2		19·0	
Polyides rotundus	PR[14]	36·3		5·1		2·3	
Degraded							
(*E. spinosum*)	C16[15]	36·1		0·3		21·0	
C. crispus	CY-λ-D1[16]	30·5		6·4			
" "	"-D2	29·7		3·0			
" "	"-D3	33·5		1·2			
" "	"-D4	38·2		3·8			
" "	"-D5	39·2		2·3			
" "	"-D6	37·3		1·35			
" "	"-D7	38·2		1·04			
" "	"-D8	37·2		0·66			
" "	"-D9	39·2		0·45			
" "	"-D10	39·2		2·80			
" "	"-D11	39·4		1·72			
G. pistillata	GP-λ-D2	37·7		2·6			
" "	"-D3	42·5		2·23			
" "	"-D4	40·6		1·58			
" "	"-D5	40·9		1·04			
" "	"-D6	40·9		0·72			
" "	"-D7	40·1		0·65			
E. spinosum	ED3	37·2		1·37			
" "	" 4	35·8		0·94			
" "	" 5	37·5		0·68			
" "	" 6	37·7		0·54			

Inherent viscosity (logarithmic viscosity number), $\eta_{inh} = c^{-1}\ln(\eta_{soln}/\eta_{solv})$dl/g where c = g solute in 100 ml solution, was measured at 25° in an Ostwald viscometer (M2 BSU/M) using 0·1M sodium chloride as solvent; for undegraded carrageenans, c = 0·02; for degraded carrageenans, c = 0·2.

Source: [1]Mud Island, Nova Scotia; [2]Sebasco Estates, Maine, U.S.A.; [3]Co. Claire, Eire; [4]Northumberland Str., Nova Scotia; [5]Yarmouth, Nova Scotia; [6]Roscoff, Finistere, France; [7]Casco Bay, Maine, U.S.A.; [8]North Brittany, France; [9]Dr. D. A. Rees, University of Edinburgh (Rees, 1963); [10]Millport, Scotland; [11]South Africa; [12]Povoa de Varzim, Portugal; [13]S. E. Asia; [14]Moosehead, Nova Scotia; [15]Anderson (1961); [16]A. D. Little Research Institute (all this series + GP and ED series).

TABLE 2. ANTIPEPTIC ACTIVITY OF UNDERGRADED AND DEGRADED CARRAGEENANS

Carageenan	Method A				Method B			
	carrageenan concentration (%)				carrageenan concentration (%)			
	0·5		2		0·2		0·3	
	κ	λ	κ	λ	κ	λ	κ	λ
Undegraded								
CMI	10	33	40	62	10	23	17	29
CSE	26	37	32	57	28	12	27	38
CBC	26	67	69	80	13	52	40	100
CNS	16	62	76	79	20	81	100	100
CY	23	74	36	79	27	57	14	100
CRF	—	—	—			36	—	100
CCB	—	53	—	72	—	17	—	84
CNB	56		81		12		94	
R-λ	—	67	—	70	—	100	—	100
GS	20	29	69	61	7	8	100	100
GR	17	25	68	69	6	24	34	94
GP	39	78	28	86	18	100	57	100
ES	35		71		21		90	
PR	60		62		23		37	
Degraded								
C16	41				11[1]*			
CY-λ-D1					27			
»-D2					25	[2]		
»-D3					55			
»-D4					53			
»-D5	39				7			
»-D6	28				4			
»-D7	46				9			
»-D8	39				7	[3]		
»-D9	41				8			
»-D10	37				14			
»-D11	43				8			
GP-λ-D2	51				12[2]			
»-D3	43				15			
»-D4	45				14			
»-D5	46				11	[3]		
»-D6	49				13			
»-D7	45				8			
ED3	34				21			
» 4	32				21	[4]		
» 5	40				15			
» 6	36				13			

All values are the mean of 2 results. Most undegraded carrageenans gave 100% inhibition at 0·5% in method B.
Concentrations are explained in the text.
* Method of degradation.
[1] Dilute HCl; [2] periodate; [3] hypochlorite; [4] H^+ ion-exchange).

material. Degradation was carried out (Black & others, 1965) with the retention of sulphate as a principal objective, because it is generally believed that the basis of the activity of these substances lies principally in the sulphate content. Also, a degraded κ-carrageenan from *E. spinosum* which combines low viscosity and high sulphate content has been shown to have promising therapeutic effect (Bonfils & Lambling, 1960; Demole, 1963; Evans, Nowell & Thomas, 1965). Degradation with sulphate retention also permits the effect of variation of molecular weight with constant polyanionic properties to be observed. The results are in Tables 1 and 2.

UNDEGRADED CARRAGEENANS

The λ-carrageenans (with the exception of λ-carrageenan from *Gigartina stellata*, GS-λ) have higher sulphate and lower 3,6-anhydrogalactose

content than the corresponding κ-carrageenans, whilst the logarithmic viscosity number, η_{inh}, is, with the exception of carrageenan from *G. pistillata*, broadly similar for corresponding κ-and λ-components (Table 1). *Eucheuma*, a naturally occurring κ-like carrageenan, and *Polyides* carrageenan, which differs in structural details from the κ- and λ-carrageenans from other species of seaweed (Black & others, 1965) and therefore appears unique, have relatively low viscosities.

Analysis of activities. The antipeptic activities of the pairs of κ- and λ-carrageenans from eight samples of seaweed (5 C. *crispus*, 3 *Gigartina* species) were analysed separately. The 64 pairs of results were considered as a doubly replicated $2^3 \times 8$ factorial experiment, yielding Table 3.

TABLE 3. ANALYSIS OF VARIANCE FOR THE ANTIPEPTIC ACTIVITY OF THE κ- AND λ-CARRAGEENANS FROM FIVE CHONDRUS AND THREE GIGARTINA SEAWEEDS

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F-ratio
M	42·8	1	42·8	
C	29,585·3	1	29,585·3	
F	24,255·0	1	24,255·0	302·7
S	21,539·0	7	3,077·0	
MC	780·1	1	780·1	
MF	420·51	1	420·51	5·25
MS	4,106·7	7	586·7	
CF	21·13	1	21·13	0·26
CS	12,294·2	7	1,756·3	
FS	11,083·0	7	1,583·3	
MCF	442·5	1	442·5	
MCS	3,095·9	7	442·3	
MFS	2,183·0	7	311·9	
CFS	4,128·4	7	589·8	
MCFS	2,906·5	7	415·2	
Total = treatments	(116,884)	(63)		
Error	5,128	64	80·125	
Total	122,012	127		

M corresponds to the method factor (2 levels); C to the concentration factor (2 levels); F to the fraction factor (2 levels); S to the seaweed factor (8 levels).
0·1% point for F (1,64) $\doteqdot$ 11·94 and for F (7,64) $\doteqdot$ 7·07; 1% point for F (1,64) $\doteqdot$ 7·07 and for F (7,64) $\doteqdot$ 2·94; 5% point for F (1,64) $\doteqdot$ 4·00 and for F (7,64) $\doteqdot$ 2·17.

Study of this Table and the percentage points of the F-distribution with the appropriate degrees of freedom shows that of the 15 treatment combinations, all but two, namely the effect of the method used and the interaction of concentration with fraction (i.e. κ or λ), will have significantly large F-ratio values. This may be attributed to the relatively small error sum of squares obtained by measuring the variation of the results within each of the 64 cells. Of these 64 cells, 18 contained an equal pair of results and this would reduce the error sum of squares considerably. Although there is no difference between the results of the two methods, it has to be remembered that different concentrations of carrageenan have been used in the two methods. The conclusions drawn are: that despite the size of the error sum of squares there is a highly significant difference in the effects of the two fractions, κ and λ; that there is no interaction between the concentration and the fraction used, i.e. the effect of the different fractions is the same regardless of concentration; and finally, that the interaction of method used and fraction studied is slightly significant. Since the antipeptic activity of undegraded carrageenan is probably

mediated by substrate occlusion (as for degraded carrageenan), this could indicate differences in structure of the complexes formed between the κ- and λ-carrageenans and the proteins used in the methods.

Again, considering only the eight pairs of undegraded carrageenans, a multiple regression analysis was performed by computer, treating 3,6-anhydrogalactose (*g*), sulphate (*s*) and viscosity (*v*) as independent variables, and the logarithm of antipeptic activity (*y*) as dependent variable (both methods, 3 concentrations) for both κ- and λ-carrageenans. The antipeptic activities of the higher concentration in method B were omitted from the calculation because the proportionately large number of duplicate activities of 100 would tend to blunt the perception of the analysis. The regressions of *y* on *s*, *y* on *s* and *v*, *y* on *s* and *g* and *y* on *g*, s and v were studied separately for both κ- and λ-carrageenans.

Sulphate content. The regressions of antipeptic activity on sulphate were very highly significant (level of significance lower than 0·1 of 1%): differences in sulphate content determined differences in antipeptic activity amongst both κ- and λ-carrageenans in both methods at all concentrations studied, high sulphate content being associated with high activity. Differences in viscosity made no significant contribution to this conclusion.

3,6-*Anhydrogalactose content.* Higher 3,6-anhydrogalactose content, on the other hand, had significant association with lower effect (after due allowance for the effect of sulphate) only in the λ-carrageenans in method B and at the higher concentration in method A. With κ-carrageenans, where 3,6-anhydrogalactose content is higher than in the more active λ-carrageenan (*t* test showed significance at 0·1 of 1%), differences in 3,6-anhydrogalactose content did not contribute to differences in activity independently of sulphate.

Further interpretations of differences in activity require more knowledge of carrageenan structure and the nature of the interaction with substrate protein on which inhibition depends.

Molecular size. An apparent absence of effect of molecular size (as indicated by viscosity) is almost certainly due to the similarity which exists in this series of undegraded carrageenans.

Unfractionated extract. The effect of the κ-component in an unfractionated extract cannot yet be discerned, but the antipeptic activity of CNB carrageenan (Table 1), for example, does not match the high sulphate content. That this might be due solely to the presence of κ- carrageenan in the unfractionated extract tends to be discounted by the antipeptic activity of CRF-λ and CCB-λ where high sulphate content, especially in CCB-λ, is not associated with the highest antipeptic activity. There would therefore be unexplained exceptions to a "sulphate rule." It is not likely that incomplete fractionation of the κ- and λ-components could account for the exceptions which probably have their origins in other structural differences. κ- and λ-Carrageenans [and the "third component" (Rees, 1966)], are stages in a biosynthetic process and cannot, therefore, under conditions of growth and harvest, be expected to be at all times identical.

Antipeptic activity. The effect on antipeptic activity of the method used to determine it is seen in the individual cases of R-λ and GP-λ (Table 2). The high activity at lower concentration in method B for these carrageenans was not reproduced in method A, suggesting that these carrageenans may have some particular affinity for the enzyme or substrate in method B. With these carrageenans, and also with CY-λ and CNS-λ to some extent, it was observed that when they were added to gastric juice, insoluble globule-like structures were formed and it was usually when this occurred that extinction of peptic activity occurred. 100% antipeptic activity was not observed in the absence of this occurrence. Differences were also observed in the size of the floccules formed in the reaction between the carrageenans and the substrate, large floccules tending to produce greater inhibition than small ones. Attention has been drawn to a similar occurrence in the reaction between fibrinogen and carrageenans (Anderson & Duncan, 1965). Molecular size probably influences antipeptic activity by determining such differences in the nature of the reaction with substrate protein. R-λ and GP-λ, together with CNS-λ and CY-λ, have the highest viscosities of the series (Table 1). This suggests that there may be an optimum association of sulphate content and molecular properties for activity.

The antipeptic activity of *Polyides* carrageenan with its unique structure (Black & others, 1965) shows that activity is not monopolized by the κ-λ carrageenans. The activity of *Eucheuma* carrageenan, which occurs naturally without a λ-component, indicates that, with adequate sulphate the κ- configuration and a high 3,6-anhydrogalactose content do not preclude marked antipeptic activity.

DEGRADED CARRAGEENANS

Undegraded carrageenans generally dissolve slowly and to a limited extent in water to give solutions of very high viscosity. This raises practical problems in the study of the actions and uses of carrageenans. The anti-ulcer action of carrageenan follows inhibition of peptic activity (Anderson, 1961), mucosal coating (Anderson & Watt, 1959) and anti-secretory activity (Anderson, Marcus & Watt, 1962), and for these actions rapid dissolution after administration is desirable. Reduction of molecular weight with retention of activity would therefore be desirable to increase solubility. Of the λ-carrageenans, GP-λ and CY-λ were chosen, on the grounds of activity and availability, for degradative study in which sulphate content was left intact as far as possible. *Eucheuma* carrageenan was also studied because of its relatively high sulphate content and naturally low viscosity (Table 1), and also because of its susceptibility to degradation under mild acid conditions (British Patent, 840,623) explained by Black & others (1965) as being possible on account of the presence of the acid-labile 3,6-anhydrogalactose in the molecule.

Results for η_{inh} (Table 1) show that much degradation could be effected in the series without serious loss of sulphate. However, with the exception of CY-λ-D3 and CY-λ-D4 (and to some extent CY-λ-D1 and CY-λ-D2) antipeptic activity was reduced as a result of degradation,

especially when measured by method B; it was also reduced, but to a lesser extent, in method A. Whether the retention of activity by CY-λ-D3 and -D4 is due to periodate degradation as opposed to hypochlorite degradation used for the others, is not known, but the antipeptic activity of GP-λ-D2, also periodate degraded, was about half that of CY-λ -D3 or -D4. CY-λ-D3 and -D4 have retained more sulphate than CY-λ-D1 or -D2. However, if this were the only factor CY-λ-D5 to -D9 might have been expected to be more active.

Degraded carrageenans were also examined at the higher concentrations in both methods, but the activities were either similar to, or less than, those at the lower concentrations. It is suspected that, at the higher concentrations, the degraded carrageenans may be forming trichloroacetic acid-soluble complexes with the substrate protein, with consequent interference with the estimation of the split products of the proteolysis. These results have therefore been omitted.

Otherwise adequate sulphate content turns out to be insufficient for high activity when certain λ-carrageenans are degraded by hypochlorite, and differences shown by the two methods of assessing activity are rather marked, suggesting that structures other than the sulphate are concerned in interaction with the different proteins in the two methods.

The analysis of the antipeptic activities of the undegraded carrageenans showed that such differences as existed in viscosity did not contribute to activity differences. However the reduction in viscosity effected by degradation, in the presence of substantially unaltered sulphate, suggests that marked changes in molecular size do affect antipeptic activity. The evidence therefore suggests that antipeptic activity is a function of the whole molecule, sulphate content and molecular size being principal features.

In use it may well be advantageous to employ degraded carrageenan the lower viscosity of which enables greater dosage to be used and faster dissolution to be achieved with more rapid distribution over the mucosa

Acknowledgement. We thank Dr. A. J. Howie, Department of Mathematics, who advised on the statistical analysis of the results.

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Mechanism of the vasoconstrictor action of isoprenaline on an isolated artery preparation

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It was not possible to demonstrate a vasodilator action of isoprenaline using the isolated artery segment from the rabbit ear. However, in high doses isoprenaline produced vasoconstriction. This vasoconstrictor response was blocked by α-receptor antagonists but not by β-receptor antagonists, except in high, non-specific doses. The response was not reduced by pretreatment of the rabbits with reserpine or by cocaine. The vasoconstriction produced by isoprenaline in this preparation therefore appears to be due to a direct action on the α-adrenotropic receptors.

AN isolated perfused segment of the central artery from the rabbit ear has been shown to constrict when its sympathetic periarterial nerves are stimulated and when noradrenaline, histamine or 5-hydroxytryptamine (5-HT) is injected into the perfusion fluid (de la Lande & Rand, 1965). Furthermore, these amines enhance the responses to sympathetic nerve stimulation. It was also shown that acetylcholine produces vasodilatation in the preparation, but only when the tone is raised by continuous sympathetic stimulation. Starr & West (1966) showed that bradykinin was also vasodilator when the tone was raised.

The main effect of isoprenaline on blood vessels is dilatation, as was first shown by Konzett in 1940. More recently, Furchgott (1959) showed that isoprenaline may produce a contraction of arterial smooth muscle, although it is about 100 times less potent than noradrenaline. Eckstein & Hamilton (1959) showed that isoprenaline produces vasoconstriction in the venous system and the work of Kaiser, Ross & Braunwald (1964) suggests that this is due to the stimulation of β-adrenotropic receptors.

In the present study, the actions of isoprenaline were investigated on the isolated artery from the rabbit ear. In this preparation, isoprenaline produces vasoconstriction which, on analysis, appears to be due to a direct action on α-receptors.

Experimental

METHODS

A segment of the artery from the rabbit ear was set up as described by de la Lande & Rand (1965). The preparation was perfused with McEwen solution (1956), which was gassed with carbon dioxide 5% in oxygen. The temperature of the perfusion fluid was maintained at 37° and the preparation was perfused at a rate of 6 ml/min with a constant volume flow inducer. Changes in perfusion pressure, which arose from changes in the resistance to flow through the arterial segment, were recorded with a mercury manometer and kymograph or a Statham pressure transducer and an Offner recorder. Injections and infusions of drugs were given via a rubber connection close to the cannula. Drugs were freshly prepared in McEwen solution and the volume injected was 0·05 to 0·15 ml. Infusions were given by means of a Palmer slow injection apparatus at a rate

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of 0·05 to 0·2 ml/min through a polythene catheter inserted into the rubber connecting tube. The periarterial sympathetic nerves were stimulated by means of bipolar platinum ring electrodes.

DRUGS

Drugs used were: isoprenaline hydrochloride (racemic isoprenaline), (−)-isoprenaline bitartrate, (−)-noradrenaline bitartrate, (−)-adrenaline tartrate, histamine acid phosphate, phentolamine hydrochloride, phenoxybenzamine hydrochloride, tolazoline hydrochloride, pronethalol hydrochloride, propranolol hydrochloride and cocaine hydrochloride. All doses are expressed in terms of these salts.

Results

ACTIONS OF CATECHOLAMINES ON THE ARTERIAL SEGMENT

Very low concentrations of noradrenaline and adrenaline produced constriction of the artery, the threshold doses being 0·1 to 1 ng; moderate vasoconstriction was produced by 1 to 5 ng. Noradrenaline and adrenaline were found to be approximately equipotent, confirming the results of de la Lande & Harvey (1965). Isoprenaline was without action in the dose range in which noradrenaline was effective, but in doses of 1 to 10 μg it too produced constriction. The vasoconstrictor potency of racemic isoprenaline was approximately 5,000 times less than that of noradrenaline. The vasoconstrictor potency of (−)-isoprenaline was about twice that of racemic isoprenaline.

EFFECT OF ISOPRENALINE ON SYMPATHETICALLY INDUCED VASOCONSTRICTION

In an attempt to demonstrate a vasodilator action of isoprenaline in the artery, injections were given during an arterial spasm produced by continuous stimulation of the sympathetic nerves. Doses of isoprenaline ranging from 1 ng to 20 μg were given during the periods of sustained vasoconstriction. Low doses had no effect: in no case was there any hint of vasodilatation. Higher doses produced only a further increase in vasoconstriction. The same findings were obtained when infusions of

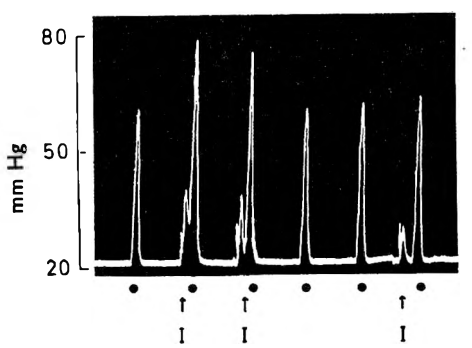

FIG. 1. Isolated artery segment. Periarterial stimulation (●) for 10 sec every 2 min using 1 msec pulses at 10/sec and supramaximal voltage. At I, isoprenaline (20 μg) was injected.

noradrenaline (2 to 20 ng/ml) or ergotamine (2 to 20 ng/ml) were given to produce a prolonged spasm.

Injections of isoprenaline before a short burst of sympathetic stimulation resulted in an increase in the size of the vasoconstrictor response. Fig. 1 shows that the increase in size depended on the interval between the isoprenaline injection and nerve stimulation, and did not persist beyond the duration of the vasoconstrictor effect of the isoprenaline. The increase is therefore merely due to summation of the two vasoconstrictor stimuli and thereby differs from the facilitation of sympathetic vasoconstriction produced by 5-HT as reported by de la Lande & Rand (1965).

Continuous infusions of isoprenaline were ineffective in diminishing vasoconstrictor responses to short bursts of nerve stimulation, and, if the concentration of isoprenaline was raised, the sympathetic vasoconstrictor responses were increased (Fig. 2).

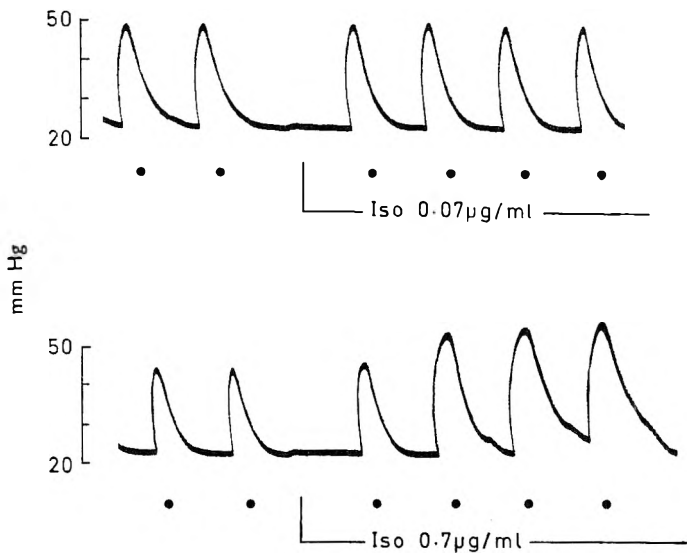

FIG. 2. Responses of the isolated artery segment to periarterial stimulation (●) for 10 sec every 2 min using 1 msec pulses at 10/sec and supramaximal voltage. Infusions of isoprenaline (Iso) are marked by the horizontal bars.

EFFECTS OF THE β-RECEPTOR ANTAGONIST PROPRANOLOL ON RESPONSES TO CATECHOLAMINES

Fig. 3 (upper panel) shows that propranolol (80 ng/ml) increased the vasoconstrictor responses to both isoprenaline and noradrenaline. When the concentration of propranolol was increased to 7 μg/ml, the response to isoprenaline was reduced, but there was a similar reduction in the response to noradrenaline (Fig. 3, lower panel).

EFFECTS OF α-RECEPTOR ANTAGONISTS ON RESPONSES TO CATECHOLAMINES

Phentolamine (16 ng/ml) blocked the vasoconstrictor responses to noradrenaline and isoprenaline. The recovery of the responses to the

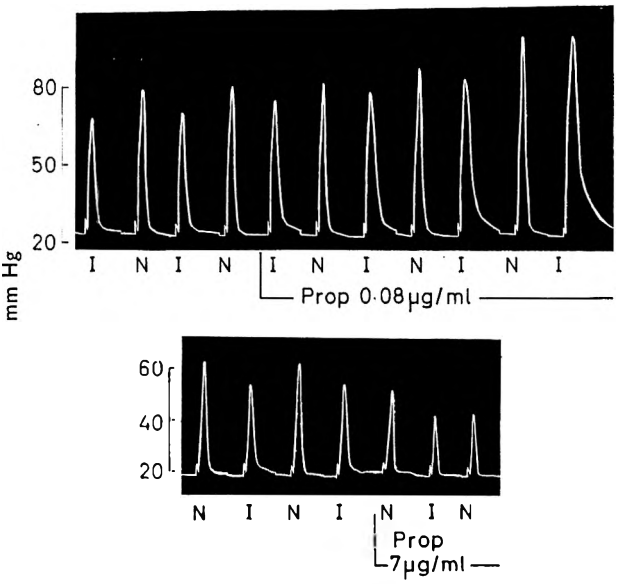

FIG. 3. Responses of isolated artery segments to injections of isoprenaline, 5 μg (I) and noradrenaline, 0·5 ng (N). Infusions of propranolol (Prop), 0·08 μg/ml and 7 μg/ml, are marked by the horizontal bars.

two drugs after blockade by phentolamine followed a similar time-course (Fig. 4). Similar results were obtained with phenoxybenzamine. In a concentration of 25 ng/ml, phenoxybenzamine blocked the responses to noradrenaline and isoprenaline without affecting the vasoconstrictor response to histamine (1 μg).

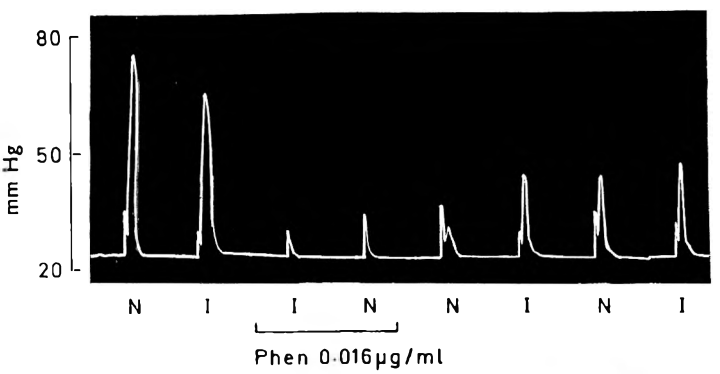

FIG. 4. Responses of the isolated artery segment to noradrenaline 0·002 μg (N) and isoprenaline 5 μg (I). An infusion of phentolamine (Phen), 0·016 μg/ml, is marked by the horizontal bar.

EFFECTS OF COCAINE AND RESERPINE

Observations were made with these drugs to determine whether isoprenaline may have been acting by releasing noradrenaline from stores within the adrenergic axons innervating the vascular smooth muscle.

Cocaine (0·2 to 0·5 μg/ml) enhanced the vascoconstrictor responses to nerve stimulation and potentiated the vasoconstrictor activity of both noradrenaline and isoprenaline as shown in Fig. 5. The vasoconstrictor action of histamine (1 μg) was also potentiated by cocaine.

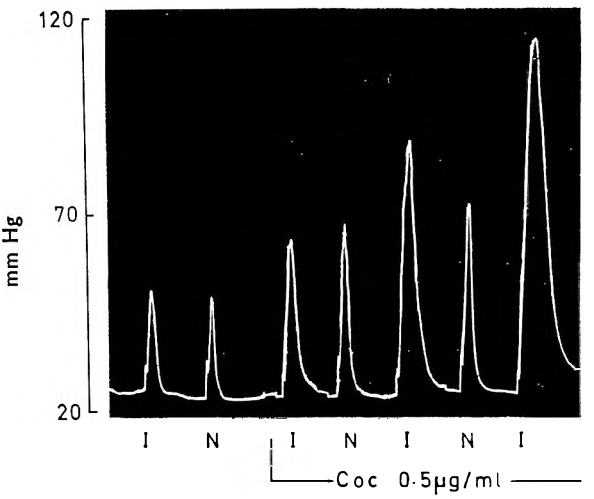

FIG. 5. Responses of the isolated artery segment to isoprenaline 7 μg (I), and to noradrenaline 0·002 μg (N). An infusion of cocaine (Coc), 0·5 μg/ml is marked by the horizontal bar.

Artery segments taken from rabbits treated with reserpine (2 mg/kg) 20 hr before setting up the isolated preparations showed slightly reduced vasoconstrictor responses to isoprenaline and noradrenaline. However, the relative potency of the two amines was unaffected. These observations indicate that isoprenaline acts directly on the receptors, since otherwise it would have been expected that cocaine or reserpine pre-treatment would decrease its vasoconstrictor activity.

Discussion

Vasodilator responses to acetylcholine and bradykinin have been demonstrated in the isolated artery preparation in which tone was maintained by vasoconstrictor stimulation (de la Lande & Rand, 1965; Starr & West, 1966). Thus the presence of drug receptors which mediate vasodilatation when combined with the appropriate agonist may be deduced. However, no vasodilator action of isoprenaline could be observed, which suggests either that β-receptors are absent in the preparation or that their stimulation does not lead to vasodilatation.

The only effect of isoprenaline on the arterial segment was to cause vasoconstriction, and this was additive with the vasoconstriction produced by nerve stimulation. Constriction of veins produced by isoprenaline

has been attributed by Kaiser & others (1964) to an action on β-receptors, but it seems unlikely that the vasoconstrictor action of isoprenaline observed in the arterial segment in our experiments is due to β-receptor stimulation since the action was not specifically depressed by propranolol. In fact, propranolol caused an equal degree of antagonism of isoprenaline and noradrenaline, as did phentolamine and phenoxybenzamine. These findings suggest that the vasoconstrictor action of isoprenaline is due to its combination with α-receptors. In accord with this is the finding that isoprenaline is several thousand-fold weaker than noradrenaline. The effects of propranolol on the actions of the two agonists indicate that it may combine with α-receptors as an antagonist, but it is weak in this respect.

The potentiation of the vasoconstrictor action of isoprenaline by low concentrations of propranolol might suggest at first sight that a vasodilator component of the response was being masked by blockade of β-receptors. However, there was an equal degree of potentiation of noradrenaline, and it is most unlikely that it too could be acting on β-receptors to produce a masked vasodilator component of response equivalent to that of isoprenaline.

The potentiation of the action of noradrenaline by propranolol and by cocaine may be explained by the blocking of its uptake into the adrenergic neurons in the tissue, since both drugs interfere with noradrenaline uptake mechanisms (Euler, 1967). However, this explanation cannot apply to isoprenaline because it is a poor substrate for catecholamine uptake mechanisms (Hertting, 1964).

From experiments with cocaine and reserpine it appears that the action of isoprenaline is directly on the receptors. Therefore blockade of reuptake of released noradrenaline cannot explain the potentiation of isoprenaline. The vasoconstrictor action of histamine was also potentiated by cocaine. The only explanation for the findings that we are able to offer is that cocaine sensitizes the arterial smooth muscle to vasoconstrictor drugs. Nevertheless, it should be pointed out that de la Lande (submitted for publication) has good evidence that cocaine does in fact potentiate the action of noradrenaline on the isolated artery segment by blocking its uptake.

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The effect of differing reserpine pretreatments on the cardiovascular response to tyramine

SIR,—Zaimis (1965, 1966) has shown that chronic treatment with small daily doses of reserpine in rats failed to cause a significant reduction in the cardiovascular response to tyramine although peripheral catecholamine levels were severely depleted. Since tyramine is thought to require endogenous catecholamines to exert at least some of its pharmacological effects (Burn & Rand, 1958; Muscholl, 1966) it was decided to investigate the responses to tyramine after chronic reserpine treatment. Four treatments were adopted: (A) Control animals received daily doses of vitamin C 20% w/v, 1 ml/kg intraperitoneally. (B) Reserpine 100 μg/kg daily in 20% w/v vitamin C intraperitoneally for three to four weeks. (C) Chronic treatment as in (B) followed by a single 5 mg/kg dose of reserpine intraperitoneally given 18 hr before the rats were used. (D) A single dose of 5 mg/kg reserpine intraperitoneally given 18 hr before the rats were used without prior chronic treatment.

All experiments were made using rats anaesthetized with pentobarbitone (60 mg/kg i.p.) and in which the blood pressure and heart rate were recorded. The results are shown in Table 1 and Fig. 1.

The cardiovascular responses to tyramine and bretylium were partly modified only by treatments (B) and (C) compared with control responses. The influence of these two treatments on the interaction of tyramine with the drugs listed in Table 1 did not indicate any qualitative differences in the nature of the response from that seen in control rats.

The effect of mecamylamine given after treatments (B) and (C) may indicate the presence of some residual sympathetic tone although it appears to be less than that obtained after treatment (A). It is possible therefore that after small daily doses of reserpine, sympathetic transmission, although impaired, still

TABLE 1. THE EFFECT OF DIFFERING RESERPINE PRETREATMENTS ON THE CARDIOVASCULAR RESPONSE TO TYRAMINE AND SOME OTHER DRUG-INDUCED RESPONSES IN ANAESTHETIZED RATS. Reserpine treatments: (A) 20% w/v vitamin C, 1 ml/kg daily (reserpine solvent); (B) reserpine, 100 μg/kg daily; (C) reserpine, 100 μg/kg daily + reserpine 5 mg/kg overnight; (D) reserpine, 5 mg/kg overnight.

Effect examined	Treatment			
	A	B	C	D
Mean initial resting blood pressure (mm Hg)	126·5 (140–110)	92·5 (110–70)	87 (110–82)	58 (75–45)
Mean initial heart rate (beats/min)	436 (468–396)	293 (336–276)	256 (276–228)	192 (220–156)
Pressor response to tyramine 25 μg i.v. (mm Hg)	29 (33–20)	19 (21–16)	14 (16–12)	0
Pressor response to tyramine after phentolamine 1 mg/kg + propranolol 2 mg/kg	Tyramine blocked			
Pressor response to tyramine 25 μg after desmethylimipramine 30 μg i.v.	Tyramine blocked			
Bretylium 6 mg/kg i.v.	Pressor + positive chronotropic effect			Pressor effect blocked + negative chronotropic effect
Pressor response to tyramine 25 μg after bretylium 5 mg/kg	Potentiated			"Restored"
Mecamylamine 1 mg/kg i.v.	Hypotensive + negative chronotropic effect			No effect

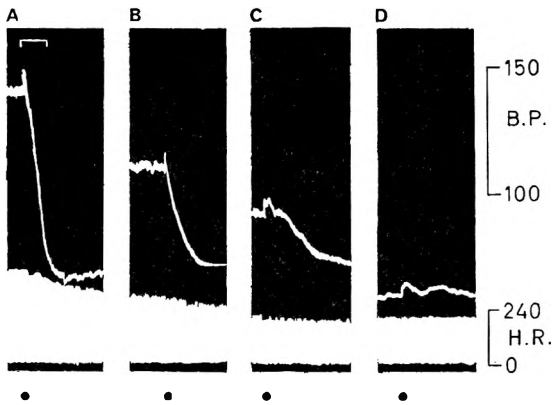

FIG. 1. The effect of mecamylamine 1 mg/kg i.v. (●) on the blood pressure (B.P.) (mm Hg) and heart rate (H.R.) (beats/min) of anaesthetized rats, after various reserpine pretreatments. Reserpine treatments: (A) 20% w/v vitamin C 1 ml/kg daily (reserpine solvent), (B) reserpine 100 μg/kg daily, (C) reserpine 100 μg/kg daily + reserpine 5 mg/kg overnight, (D) reserpine 5 mg/kg overnight. Time scale: 5 min.

functions to some extent despite the severe depletion of peripheral noradrenaline. This suggests the presence of small amounts of residual transmitter which could be sufficient to account for the pharmacological effect seen with tyramine. This is supported by the fact that increased sensitivity to noradrenaline exists in chronically treated rats (Zaimis, 1966) and that the action of the released noradrenaline is known to be enhanced by the tyramine itself (Muscholl, 1966). The single large dose of reserpine (treatment D) abolished the hypotensive effect of mecamylamine and the responses to tyramine.

The negative chronotropic effect of bretylium observed after treatment (D) is in accord with the results of Gaffney (1961) on the isolated reserpinized dog heart-lung preparation.

Irrespective of the form of treatment employed, bretylium potentiated tyramine; this effect is most probably due to the monoamine oxidase inhibitory property of this drug (Furchgott & Sanchez-Garcia, 1966; Clarke & Leach, unpublished).

Chronic treatment terminated with a large single dose of reserpine fails to abolish the effects of tyramine, bretylium or mecamylamine. These animals also appeared more active and showed less ptosis and diarrhoea than animals under treatment (D). It is possible that the persistence of low concentrations of reserpine in rat tissues during chronic treatments leads to the development of some form of "resistance" to this drug. Cass & Callingham (1964) noted an "escape" from the depleting action of reserpine after small daily doses and there is evidence (Carlsson, 1966) of a small labile pool of catecholamines which are more resistant to chronic reserpine treatment than the bulk of endogenous amines.

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The influence of blood pressure on the responses of the nictitating membrane of the cat to sympathetic stimulation

SIR,—We have observed in cats anaesthetized with chloralose that the responses of the nictitating membrane were reduced when the blood pressure was reduced to 45 mm Hg.

Cats were anaesthetized with chloralose, 7·5 ml/kg of a 1% w/v solution in 0·9% w/v saline, administered intravenously after induction with ether. Carotid arterial blood pressure was recorded by means of a mercury manometer and contractions of the nictitating membrane by a frontal writing lever (15 times magnification, 8 g tension). The responses of the membrane to intravenously administered noradrenaline (50 μg) and adrenaline (40 μg) and to post-ganglionic nerve stimulation (10 V; 1 msec duration; 1, 5, 10 and 20 pulses/sec for 20 sec)

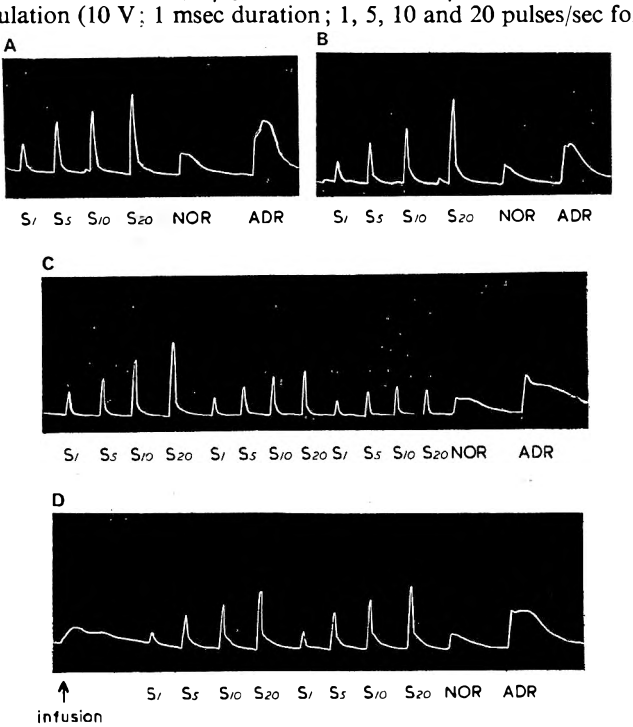

FIG. 1. The influence of blood pressure on the responses of the cat nictitating membrane to postganglionic stimulation of the ascending cervical sympathetic nerve (S) and to intravenous injections of 50 μg noradrenaline (NOR) and 40 μg adrenaline (ADR). Stimulus parameters 10 V, 1 msec duration at 1, 5, 10 and 20 pulses/sec for 20 sec. Between A and B the blood pressure was reduced by haemorrhage from 110 mm to 60 mm Hg. Between B and C the blood pressure was further reduced to 45 mm Hg. D shows the effect of restoring the blood pressure to 110 mm Hg by infusing the blood collected during haemorrhage.

were periodically recorded throughout the experiments, in which blood pressure was altered by changing the blood volume. Hypotension was produced by slow haemorrhage from a femoral arterial catheter and the blood pressure was restored by an intravenous infusion of heparinized cat blood. Typical results are shown in Fig. 1.

The mean resting arterial blood pressure of all cats used during these experiments was 100–120 mm Hg. In each case there was no significant change in any of the responses of the nictitating membrane until the blood pressure had been reduced to 45–60 mm Hg. A further reduction in pressure to 30–45 mm Hg resulted in a marked reduction in the responses of the membrane to nerve stimulation and only a slight reduction of the responses to injected catecholamines. When the cats were maintained in this state of hypotension the responses to injected adrenaline and noradrenaline remained unaltered whereas the responses to nerve stimulation became progressively weaker. Following re-establishment of the resting blood pressure there was an almost immediate return of all responses towards the control pre-haemorrhage levels.

We conclude that the reduction of blood pressure to 45–60 mm Hg or below, especially if prolonged, will reduce the responses of the cat nictitating membrane to nerve stimulation. Since the effect of blood pressure on the responses to intravenously administered catecholamines was minimal, interference with sympathetic nerve function is implicated.

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Potentiation of noradrenaline isomers by cocaine and desipramine in the isolated vas deferens of the rat

SIR,—A correlation of block of noradrenaline uptake and supersensitivity to this amine at the receptors of the effector organs, has received support from many authors (Hertting, Axelrod & Whitby, 1961; Muscholl, 1961; Thoenen, Huerlimann & Haefely, 1964).

The uptake of noradrenaline at nerve terminals is supposed to be stereospecific in favour of the D-(−)-isomer. Maickel, Beaven & Brodie (1963), Iversen (1963) and Euler & Lishajko (1964) have shown that D-(−)-noradrenaline is much more readily taken up into noradrenaline stores than is its L-(+)-isomer.

Block of uptake should result in a greater sensitization for those amines which are taken up readily than those which are scarcely taken up. Trendelenburg (1965) and Tye, Patil & LaPidus (1967) showed that cocaine sensitizes the nictitating membrane and the vascular system of the cat more to the D-(−)- than to the L-(+)-form.

In the investigations on which the present report is based, the phenomenon was examined *in vitro* on the isolated vas deferens of the rat, which is one of the most suitable preparations to assess noradrenaline supersensitivity (Ursillo & Jacobson, 1965; Cuenca & Valdecasas, 1965). In this preparation the D-(−)-form of noradrenaline is more active than the L-(+)-isomer (Patil, LaPidus & Tye, 1967). We found both cocaine and desipramine sensitized the preparation to the D-(−)-form whereas the L-(+)-form was unaffected by desipramine and slightly but not significantly potentiated by cocaine. This is shown in Fig. 1 where dose-response curves before and after cocaine or desipramine, obtained in four different preparations, are shown.

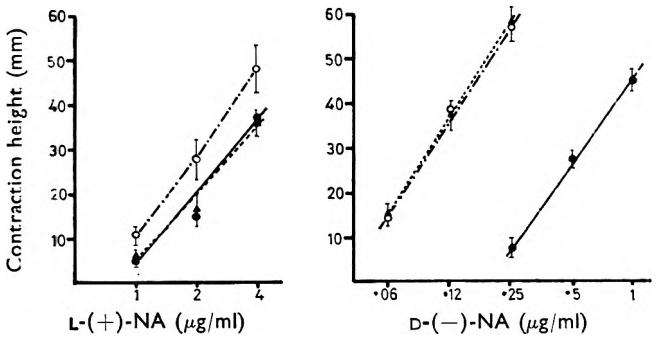

FIG. 1. Effect of cocaine and desipramine on isolated vas deferens of the rat to the contractions induced by L-(+) and D-(−)-noradrenaline (NA) before (●——●) and 15 min after addition to the bath of desipramine 10^{-7} g/litre (▲ - - - - ▲) or cocaine 10^{-6} g/litre (○ — - - — ○).

Desipramine and cocaine reduce the effect of indirectly-acting sympathomimetic amines. However, the ineffectiveness of desipramine and cocaine in potentiating the L-(+)-isomer cannot be attributed to a specific indirect action of this amine since Patil, LaPidus, Campbell & Tye (1967) showed that the L-(+)-isomer of noradrenaline did not lose its effectiveness in the vas deferens of rats pretreated with reserpine. Iversen (1965) advanced the hypothesis that there are two uptake mechanisms for concentrating noradrenaline at the sympathetic nerve terminals. $Uptake_1$ which operates at low doses of the amine, and $Uptake_2$ which operates at high doses. Desipramine and cocaine are good inhibitors of $Uptake_1$, whereas the second process is scarcely inhibited by high doses of desipramine or cocaine. It might be that the high doses of the L-(+)-isomer require the second process to effect their uptake. If this is so, no potentiation after exposure to cocaine and desipramine would be expected.

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The subcellular binding of propranolol in rat heart

SIR,—Propranolol is known to block β-receptor actions upon the heart (Black, Duncan & Shanks, 1965; Nakano & Kusakari, 1965). We now report the binding of [^{3}H]propranolol to various particles in the heart of the rat.

^{3}H-Labelled propranolol (250 and 500 μc; specific activity 0·94 mc/mg) was injected into the tail vein of male Sprague-Dawley rats. After 30 min, each rat was decapitated and the heart quickly removed, rinsed and chilled, and homogenized in 5 volumes of ice-cold isotonic sucrose solution (0·25 M). Subcellular fractionation in a continuous sucrose gradient and subsequent determination of radioactivity was made (Potter & Axelrod, 1963a,b). To prepare the gradient, 4·5 ml of 0·25 M sucrose was placed in the upper tube of an exponential gradient maker and 4·5 ml of 2·2 M sucrose into the lower flask. The tube containing the gradient was chilled, 0·5 ml of the heart homogenate was layered over the gradient and this was then centrifuged in a Spinco model L preparative ultracentrifuge at an average force of 125,000 *g* for 30 min. All homogenization and fractionation procedures were at 0°– 4°. Although the fractions were not verified by electron microscopy, the layers in the gradient tube after centrifugation corresponded visually to that described and illustrated by Potter & Axelrod (1963a).

Fig. 1A illustrates the distribution of tritium label in the density gradient tube after centrifugation of a homogenate of a heart removed from a rat 30 min after intravenous injection of [^{3}H]propranolol. Radioactivity was found to be predominantly associated with the "microsomal" fraction and to a lesser extent with the mitochondrial and muscle debris layers. However, when heart tissue from a non-injected rat was homogenized in 0·25 M sucrose with [^{3}H]-propranolol (0·5 μc), a distribution pattern similar to that illustrated in Fig. 1A resulted.

That this binding property of propranolol is not shared by (±)-[^{14}C]noradrenaline* (^{14}C-NA) and is not solely a result of our technique is demonstrated in Fig. 1B. It is seen that noradrenaline possesses markedly different binding

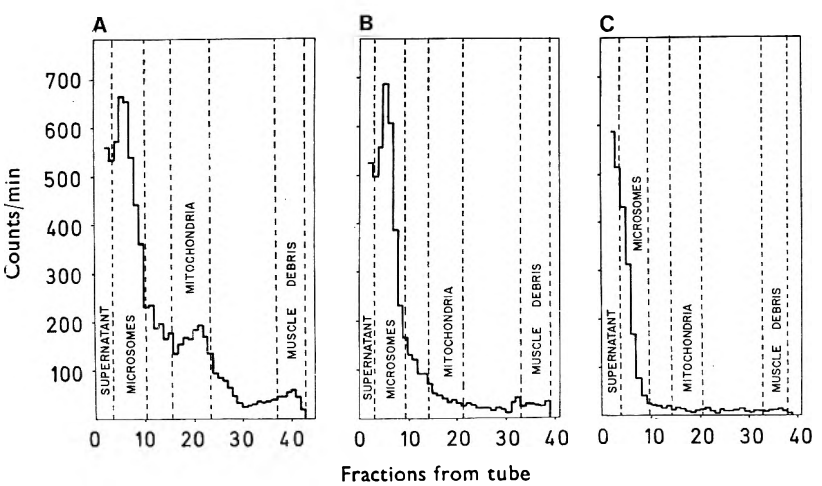

FIG. 1. Subcellular distribution of radioactivity in rat heart 30 min after intravenous injection of (A) [^{3}H]propranolol, (B) [^{14}C]noradrenaline, (C) density gradient fractionation of rat heart homogenized with [^{14}C]noradrenaline.

characteristics. Thirty min after intravenous injection, ^{14}C-NA (2 μc; specific activity 43 mc/mM) is localized almost exclusively in the "microsomal" fraction of rat heart, confirming the findings of Potter & Axelrod (1963a). Also, in contrast to propranolol, ^{14}C-NA (6×10^{-4} μc) shows no peak of radioactivity associated with any particulate fraction when mixed and homogenized with non-labelled heart tissue in sucrose (Fig. 1C).

To exclude the possibility that the larger quantity of propranolol used might have been responsible for general labelling of all particulate fractions, (±)-[^{3}H]noradrenaline* (specific activity 10·28 c/mM) was diluted with sufficient non-radioactive noradrenaline to have the same specific activity as that of [^{3}H]propranolol, and the same quantity of radioactivity was mixed and homogenized in 0·25 M sucrose with heart tissue from a non-injected rat. This distribution was likewise similar to Fig. 1C. Because of toxicity the *in vivo* experiment with a noradrenaline dose comparable to that of injected propranolol could not be made.

These experiments demonstrate the markedly different binding property of propranolol and noradrenaline. The distribution picture appears to indicate that noradrenaline is specifically and actively taken up by particles of microsomal size, which confirms other reports (Potter & Axelrod, 1963b; Sjöqvist, Titus & others, 1965; Taylor, Chidsey & others, 1966), whereas propranolol, which is lipid soluble, appears to bind indiscriminately to various membrane structures. In the *in vivo* experiment, the possibility cannot be excluded that the subcellular localization pattern of propranolol was actually different *in vivo* and that a subsequent redistribution might have occurred during homogenization.

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* 2-Amino-1-(3,4-dihydroxyphenyl)-[1-Label]ethanol.

Failure of dopamine to accumulate in central noradrenaline neurons after depletion with diethyldithiocarbamate

SIR,—Evidence has recently been obtained that treatment with the dopamine-β-oxidase inhibitor, disulfiram, results in an accumulation of dopamine in sympathetically innervated organs and that the dopamine accumulated in this way can be released on nerve stimulation (Thoenen, Haefely & others, 1966). Furthermore, in previous reports (see Carlsson, Fuxe & others, 1966) support has been obtained for the view that diethyldithiocarbamate inhibits dopamine-β-oxidase *in vivo*. Significant increases of dopamine, however, were observed only in the brain stem and in the adrenals. The low accumulation of dopamine in the central noradrenaline neurons may be due to breakdown by monoamine oxidase or to some other factor preventing the accumulation of dopamine in noradrenaline neurons. To further elucidate this problem a combined histochemical and biochemical study has been made to examine the effect of nialamide, a potent monoamine oxidase inhibitor, on central noradrenaline neurons depleted of this amine to a large extent, with the help of diethyldithiocarbamate.

Male, Sprague-Dawley rats (150–250 g) were treated three times with sodium diethyldithiocarbamate (500 mg/kg i.p. including water of crystallization) 10, 6 and 3 hr before death. Half the animals were also treated with nialamide (500 mg/kg, i.p.) $3\frac{1}{2}$ hr before death. Control rats received nialamide alone in the same way as described above. Some mice were also injected with diethyldithiocarbamate or diethyldithiocarbamate-nialamide as described, but only for behavioural studies. The animals were killed by decapitation under light chloroform anaesthesia. The brains of one and the same group were subjected to histochemical (see reviews by Hillarp, Fuxe & Dahlström, 1966; Corrodi & Jonsson, 1967) or biochemical analysis for dopamine, noradrenaline and 5-hydroxytryptamine (5-HT). Dopamine was measured by the method of Carlsson & Waldeck (1958) with the modification introduced by Carlsson & Lindqvist (1962) and noradrenaline by the method described by Bertler, Carlsson & Rosengren (1958). Usually dopamine and noradrenaline measurements were made on brain stem (di- and mesencephalon, pons and medulla oblongata) striatum and hemispheres.

After treatment with diethyldithiocarbamate alone there were marked to very marked decreases, mainly in intensity but also in number of noradrenaline nerve terminals in various parts of the brain. There always remained, however, weakly to strongly (hypothalamus) green-fluorescent noradrenaline nerve terminals in most of the areas examined. The fluorescent noradrenaline nerve cell bodies showed no certain decreases in intensity at the time-interval used. The dopamine nerve terminals and cell bodies of the brain were not affected by this treatment but remained strongly green-fluorescent. Biochemically, these effects were seen as marked decreases in the brain noradrenaline levels accompanied by an increase in the dopamine level of the brain stem (Table 1). The rats and mice were markedly sedated by this treatment. If nialamide was given $\frac{1}{2}$ hr before the last diethyldithiocarbamate injection there was only a small—if any—increase in the fluorescence intensity and number of the various noradrenaline nerve terminals of the brain, as compared to those of brains of rats treated with diethyldithiocarbamate alone. The dopamine neurons were hardly affected by the nialamide treatment. The 5-HT neurons showed distinct increases in fluorescence intensity and in number of fluorescent terminals. Biochemically, there was hardly any additional rise of dopamine in the brain stem by injection of nialamide to the diethyldithiocarbamate-treated mice.

TABLE 1. EFFECT OF DIETHYLDITHIOCARBAMATE ON CATECHOLAMINE LEVELS IN DIFFERENT AREAS OF THE BRAIN WITH OR WITHOUT MONOAMINE OXIDASE INHIBITION BEFORE THE LAST INJECTION OF THE DRUG.
Each experiment was performed on pooled tissue parts of 3 rats.

	Normal	Diethyldithiocarbamate 380 mg/kg × 3 i.p.		Diethyldithiocarbamate 380 mg/kg × 3 i.p. + nialamide 500 mg/kg	
		Ambient temperature			
	+22–23°	+22–23°	+29°	+22–23°	+29°
Brain area	Brain noradrenaline μg/g				
Stem	0·84 1·02	0·16	0·17	0·16	0·26
Hemispheres	0·36 0·38	0·03	0·03	0·03	0·05
Striatum	0·24 0·26	0·05	0·05	0·05	0·08
	Brain dopamine μg/g				
Stem	0·10 0·24	0·35	0·29	0·50	0·21
Hemispheres	0·08 0·18	0·11	0·04	0·14	0·11
Striatum	2·4 3·3	2·9	2·2	3·5	2·7

After dopamine-β-oxidase inhibition, only small amounts of dopamine were accumulated in the central noradrenaline neurons in spite of monoamine oxidase inhibition. This points to the importance of the β-hydroxyl group for a proper binding of the amine to the adenosine triphosphate-protein complex of the granules, as has been suggested previously (Musacchio, Kopin & Weise, 1965). Thus, since the dopamine formed probably cannot be sufficiently bound to the amine granules, it will lie in the axoplasm outside the granules. Since the uptake-concentration mechanism at the nerve cell membrane of the noradrenaline neurons seem to be even more efficient for dopamine than for noradrenaline (Burgen & Iversen, 1965), and since the monoamine oxidase is inhibited, there exist good possibilities for inhibition of the tyrosine-hydroxylase (Nagatsu, Levitt & Udenfriend, 1964), for example, which could cut off the synthesis and explain why dopamine is not accumulated in large amounts.

Treatment with diethyldithiocarbamate resulted in marked central nervous depression which could not be reversed by nialamide, but which was rather potentiated. The behavioural syndrome observed in mice after a large dose of nialamide was not complete in the diethyldithiocarbamate-treated mice. Thus, the general activation (e.g. continuous running) was not observed. The nialamide-syndrome is not prevented by a potent blocker of the first step in catecholamine biosynthesis whereas a blocker of both the tyrosine and the tryptophane hydroxylase causes a marked blockade of this syndrome (Corrodi, 1966). The present findings might suggest that the central noradrenaline neurons also are important for the nialamide syndrome, at least for its full development. However, the possibility must be considered that the central depressant action of diethyldithiocarbamate is largely due to some action other than inhibition of dopamine-β-oxidase.

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Brain dopamine and the amphetamine-reserpine interaction

SIR,—It seems that in rats the amphetamine excitatory response including stereotyped activity (continuous sniffing, licking and biting) is effected by some interaction or synergism of amphetamine with the brain catecholamines, dopamine and noradrenaline. It can be prevented by inhibition of the synthesis of dopa, the physiological precursor of these amines (Weissman, Koe & Tenen, 1966; Randrup & Munkvad, 1966a) and then restored by the injection of dopa (Randrup & Munkvad, 1966a; Hanson, 1966). In very large doses dopa alone can produce stereotyped activity (Randrup & Munkvad, 1966b; Ernst, 1965).

Further experiments showed that specific inhibition of the synthesis of noradrenaline did not affect the stereotyped activity induced by amphetamine or dopa. This activity, therefore, seems to depend exclusively on dopamine, while noradrenaline seems to be involved in other forms of activity such as locomotion and aggressive behaviour (Randrup & Scheel-Krüger, 1966; Scheel-Krüger & Randrup, 1967).

With this background it becomes necessary to explain why reserpine, which completely depletes the brain both of dopamine and noradrenaline, does not prevent the amphetamine excitatory response.

To investigate this problem we made some experiments on the influence of reserpine and amphetamine upon brain catecholamines. Male Wistar rats weighing 210 to 280 g were injected with various combinations of reserpine (7·5 mg/kg s.c. 20 to 20½ hr before death), the monoamine oxidase inhibitor, nialamide (100 to 500 mg/kg s.c. 2½ hr before death), and (+)-amphetamine sulphate (10 mg/kg s.c. 2 hr before death). The rats were killed by a blow on the back of the neck and the catecholamines together with their *O*-methylated metabolites were measured in brain (Häggendal, 1962, 1963; Scheel-Krüger & Randrup, 1967; Carlsson & Waldeck, 1964).

When reserpine was given alone, none of the four amines, noradrenaline, *O*-methylated noradrenaline, dopamine or *O*-methylated dopamine, could be detected in the rat brains (concentrations below 0·02, 0·01, 0·08 and 0·04 μg/g respectively), and even after the addition of nialamide in the highest dose (500 mg/kg, 5 experiments) they remained undetectable. When, however, amphetamine was added after reserpine and nialamide, *O*-methylated dopamine appeared in measurable amounts.

In five experiments, in which amphetamine was given after reserpine and the highest dose of nialamide, the amounts were 0·35, 0·24, 0·32, 0·50 and 0·07 μg/g tissue, respectively. In three experiments with lower doses of nialamide (100–200 mg/kg) the values were 0·10, 0·27 and 0·14 μg/g, respectively. The other three amines remained undetectable.

In all the experiments amphetamine produced the characteristic stereotyped behaviour. In the dose used (10 mg/kg) it also produces stereotypy when given after reserpine alone, nialamide alone or without pretreatment. In this laboratory 10 mg/kg s.c. is the standard dose used to produce amphetamine-stereotypy. The present experiments thus show that although this stereotypy-producing dose of amphetamine does not alter the level of dopamine in the brain of reserpinized rats, it does interfere with the turnover or metabolism of this amine.

Although this isolated effect of amphetamine upon *O*-methylated dopamine may seem surprising it is in agreement with recent findings about the influence of reserpine and amphetamine on the turnover and metabolism of catecholamines in brain (Andén, Roos & Werdinius, 1964; Glowinski, Axelrod & Iversen, 1966; Carlsson, Fuxe & others, 1966). Thus it has been found that the synthesis of dopamine is not affected by reserpine as evidenced by the undiminished level of the dopamine metabolites dihydroxyphenylacetic acid and homovanillic acid (Andén & others, 1964). Noradrenaline synthesis, however, seems to be inhibited (Stjärne, 1966; Scheel-Krüger & Randrup, 1967).

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Occurrence of a cardiac heteroside in *Bersama yangambiensis*

SIR,—The presence of a cardiotonic bufadienolide has been demonstrated in leaves of *Bersama abyssinica* (Lock, 1962). This note gives evidence for the occurrence of a similar compound in the bark of another plant of the Bersama group—*Bersama yangambiensis* (Toussaint, 1959).

Crude extracts were prepared from various parts of *Bersama yangambiensis* by a modification of the method of Euw & Reichstein (1950). Tested on guinea-pigs, bark extracts were found to be about three times more toxic than leaf extract. Purification was achieved by successive extraction with diethyl ether, dichlormethane and dichlormethane–ethanol (3:2; v/v) followed by separation into eight fractions on an alumina column by eluents of increasing polarity. The last fraction, which retained the sour taste of the dichlormethane extract, was eluted by 94% ethanol–water (6:4; v/v) and was further characterized. Its dry residue developed characteristic colours when treated with concentrated sulphuric acid and was demonstrated to retain the bulk of the cardiotonic activity.

This chromatographically pure extract, readily soluble in ethanol, was shown to contain the steroidal skeleton of cardiotonic heterosides by well-established reactions (Lieberman, Baljet) described by Paech & Tracey (1955).

The spectral analyses were sufficiently conclusive to ascribe the bufadienolide structure to the compound. The infrared spectrum in chloroformic solution shows absorption bands at 1640^{-1} and 1605 cm^{-1} (C=C) and 1720 cm^{-1} (C=O) characteristic of the group CH=CH−C=O of the six-membered lactone ring of bufadienolide (Jones & Herling, 1954). However, the ultraviolet spectrum gives an absorption band at 279 mμ but not the bufadienolide band at 300 mμ. This may be explained by the presence of a ketonic function in position 11 or 12 of the cyclopentanophenanthrene molecule (Hegedus, Tamm & Reichstein, 1955).

The extract also possesses the pharmacological properties of cardiotonic heteroside as defined by Chen (1963). This sour-tasting compound stops the frog heart preparation of Straub in systolic contraction (Fig. 1A) and the

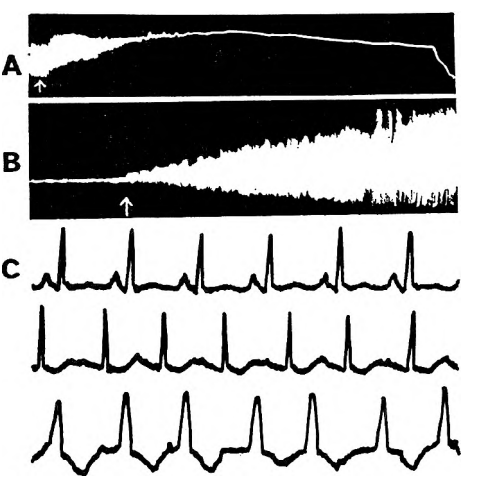

FIG. 1. A. Isolated frog heart perfused with a dilute solution of the Bersama extract. B. Rabbit auricle with addition of Bersama extract (about 200 μg is added to the 15 ml Locke solution in the organ bath). C. Electrocardiogram of a cat. Intravenous injection of 1·5 mg of a semi-purified extract at zero time (upper), 5 min after injection (middle) and 10 min after injection (lower record).

intravenous injection of the extract to the cat is followed 3–5 min later by auriculo-ventricular block, the degrees of the block increasing with time. Other rhythm disturbances including bigeminism were eventually observed (Fig. 1B). The action was over in 30 min but the maintenance of a myocardiac impregnation by the compound was evidenced by the faster and the more pronounced effect of a second injection.

Finally, the extract has a positive inotropic action on the isolated rabbit auricle preparation as described by Burn (1952) (Fig. 1C).

It is concluded that this evidence demonstrates the presence of a cardiotonic drug of bufadienolide type in *Bersama yangambiensis*.

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A simple method for measuring the amount of azovan blue exuded into the skin in response to an inflammatory stimulus

SIR,—The most characteristic sign of inflammation is the enhancement of vascular permeability. To indicate the increased permeability, the sulphonic acid dyes like azovan (Evans) blue or trypan blue, which become bound to serum proteins, are suitable. The dye-protein complex is exuded into the surrounding tissues during the enhancement of the permeability. In most reports the evaluation of the local staining effect was confined to a subjective visual estimation such as colour intensity or diameter of the coloured areas. Several methods exist for extracting the dye from minced tissues, but they are complicated, especially for routine examinations (Sachs & Lummis, 1955; Clausen & Lifson, 1956; Judah & Willoughby, 1962; Hladovec, Horáková & Mansfeld, 1961). Of these methods only the last two have been elaborated for the extraction of the dye from the skin.

We now describe a simple extraction method in which a methanolic solution of suramin (Bayer 205) is used to extract the azovan blue at room temperature from a piece of skin without maceration or mincing.

The strong linkage of suramin to protein has been known for a long time (Mayer, 1922). Jancsó (1955) showed that the suramin molecules competed with the dye molecules for the possession of the binding sites in the tissue and in this way high concentrations of suramin displaced the bound dye from the tissue structures. This competition arises because the two compounds are similar in chemical constitution, both being symmetrical naphthalene sulphonic acids.

The suramin solution slowly elutes the dye from the skin so that within 14 days all the dye passes into the clear alcoholic phase and can then be measured spectrophotometrically. An Optica-Milan spectrophotometer is suitable.

The spectrum peak of the tissue extract (620 mμ) corresponds to the spectrum peak of the pure azovan blue solution, whereas suramin alone shows no absorption over the wavelengths of the curve (475–675 mμ). Tissue extracts from rats to which dye had not been injected did not give extinction values. All the measurements were made at 620 mμ.

To establish the optimal concentration of suramin for the dye extraction, experiments were made with solutions of different suramin content. Rats were injected intravenously with 0·5 ml/100 g of 1% azovan blue solution, then the inflammatory reaction was induced by painting the skin of the hind paws with 4% mustard oil in liquid paraffin. The rats were killed by bleeding 20 min after the application of the inflammatory agent and the dorsal skin of the paws was removed, weighed and put into the suramin-methanol solution. For 100 mg of tissue 3 ml extracting solution was suitable. A solution containing 0·01% suramin did not extract any dye from the skin. The 0·1% solution extracted a large amount of dye within 2 weeks, but the skin was not completely decolourized. We then transferred the skin to 1·0% suramin solution in which it was completely decolourized in a few days and the solution turned pale blue. Taking the whole amount of azovan blue obtained by this double extraction procedure to represent 100% extraction it was established (4 experiments) that the 0·1% suramin solution extracted 88·4% of the dye contained in the skin and the 0·5 and 1% suramin solutions extracted 95·5 and 95·2%. Without changing the solution a higher extraction ratio could not be reached as tissue pieces floating in the fairly concentrated dye solution were stained by it. This was shown by the fact that this 4–5% of dye remaining could be eluted if the piece of skin was put into a few ml of pure methanol, instead of into a suramin solution.

As an example we examined the extent to which vascular permeability, i.e. the amount of exuded dye, was increased by mustard oil applications of increasing concentration. The dorsal skin of the hind paws of rats weighing 130–150 g was painted with 0·25, 1, 4 or 8% mustard oil solution after 50 mg/kg azovan blue dye had been injected intravenously. The animals were killed 20 min later and the dye was extracted from the skin of the paws with a 1% suramin solution (3 ml solution per 100 mg skin). After painting with 0·25% mustard oil a total amount of only 2 μg dye could be demonstrated, a quantity which agreed with the dye content obtained in the control paws not treated with the irritant. Hence, in such low concentrations mustard oil does not enhance the permeability, but with increasing concentrations of mustard oil there was increased recovery of azovan blue from the skin and there was a linear relation between concentration of irritant and vascular permeability as measured by the amount of azovan blue exuded. The figures were: 10 μg dye after 1% oil, 20 μg after 4% oil and 24 μg after 8% oil.

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Effect of anticoagulants on the capillary resistance of internal organs of rats

SIR,—It has been reported that 10 min after an intravenous injection of certain anticoagulants like sodium polyanethole sulphonate (Liquoid) or heparin, the capillary resistance of rat skin decreased (Gábor, Dux & Kiss, 1954; Gábor & Dux, 1954, Gábor, 1960) an effect confirmed for heparin by Kramár (1961).

A method has been developed recently by Dirner, Antal & Gábor (1966) for measuring the capillary resistance in internal organs and we have used it to measure the effect of anticoagulants.

The experiments were made on the ileum, colon and kidney of fasting rats, males or females, weighing 160–200 g, under urethane anaesthesia. Measurements were made before, and 10–20 min after the injection of the anticoagulants. Suction was applied for 30 sec by means of a plexiglass suctioning apparatus, 3 mm in diameter.

TABLE 1. EFFECT OF SODIUM POLYANETHOLE SULPHONATE (LIQUOID) AND OF HEPARIN (1 MG/KG I.P.) ON THE CAPILLARY RESISTANCE (EXPRESSED AS A MEAN FALL IN PRESSURE IN MM HG) OF INTERNAL ORGANS OF RATS

	Ileum[1]	Colon[2]	Kidney[3]
Sodium polyanethole sulphonate (15 animals)	200 (range 100–500) mm Hg	180 (range 100–300) mm Hg	180 (range 100–400) mm Hg
Heparin (15 animals)	150 (range 100–300) mm Hg	193 (range 100–400) mm Hg	140 (range 100–300) mm Hg

Initial internal pressure range:

[1] 200–700 mm Hg for the sodium polyanethole sulphonate- and 200–500 mm Hg for heparin-treated animals.

[2] 400–700 mm Hg for the sodium polyanethole sulphonate- and 600–700 mm Hg for heparin-treated animals.

[3] 200–700 mm Hg for the sodium polyanethole sulphonate- and 400–700 mm Hg for heparin-treated animals.

The results of the experiments are in Table 1. These results show that the capillary resistance decreased in all animals after the administration of sodium polyanethole sulphonate and heparin.

We then attempted to prevent the capillary resistance-lowering activity of heparin with specific antagonists like protamine sulphate or toluidine blue. In 5/5 animals the capillary resistance-lowering action of heparin did not occur after either protamine sulphate (30 mg/kg i.p.) or toluidine blue (10 mg/kg i.v.).

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