The Identification of a Lipid-Mobilizing Factor from Sheep Midbrain

By P. P. SHAH, ANNE R. TEE, P. D. ENGLISH,
B. T. REDMAN and J. BUNYAN

Beecham Research Laboratories, Nutritional Research Centre, Walton Oaks, Tadworth, Surrey, U.K.

(Received 1 June 1972)

The mass spectrum of a lipolytic substance from purified sheep midbrain extract indicated that it was isoprenaline, not *N-n*-propylnoradrenaline, noradrenaline or adrenaline. The identification of the lipid-mobilizing factor as isoprenaline was confirmed qualitatively by t.l.c. and g.l.c. and quantitatively by determination of its lipolytic activity and u.v. absorption and by fluorimetric determination of its catecholamine content.

The existence of a lipid-mobilizing substance in the anterior pituitary was first recognized by Best & Campbell (1936). Since then, several pituitary hormones have been shown to be lipolytic (Desbals *et al.*, 1970). Hollett (1968*a,b*, 1969) has partially purified a low-molecular-weight lipid-mobilizing substance from calf midbrain free from pituitary gland and infundibulum. Hollett (1969) claimed that the substance was indistinguishable from hypoxanthine by chromatographic, electrophoretic or spectrophotometric techniques. However, the factor could not have been hypoxanthine because the latter is not lipolytic. The present paper describes the isolation and characterization of a lipid-mobilizing factor from sheep midbrain (free from pituitary gland and infundibulum). This material was separated from hypoxanthine by the use of suitable chromatographic procedures.

Materials and Methods

Catecholamines and collagenase were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. *N-n*-Propylnoradrenaline was a gift from Boehringer, Ingelheim, Germany. Noradrenaline (I) was synthesized by the method described by Kawazu (1960). Sheep and calves were killed at a local slaughterhouse in batches of six to 50 and their heads were brought to these laboratories. The midbrains were removed within 2 h of death and placed immediately in acetone–0.01 M-HCl (4:1, v/v) at –5°C. Pig brains were removed whole at the slaughterhouse and stored there at 4°C for 24 h. The midbrains were removed immediately on arrival in the laboratory and treated as described for sheep and calf midbrain. The midbrain consisted of the superior and inferior corpora quadrigemina, the median geniculate body, the hypothalamus and the attachments to the cerebral peduncle and

the optic chiasma. The pituitary and its infundibulum and the pineal body were carefully excluded.

Preparation of the crude midbrain extract

The crude extract was prepared by the method of Hollett (1969), with an additional step to remove lipids.

Midbrains were homogenized with 9 vol. of acetone–0.1 M-HCl (4:1, v/v) at room temperature. The pH was adjusted to 4.0. The homogenate was stirred for 1 h at 0–4°C and left overnight at this temperature to precipitate protein and cell debris.

The residue was removed by centrifugation at approx. 1500*g* for 15 min at room temperature. The clear supernatant was concentrated under reduced pressure at 25°C to remove acetone and some of the water. The pH was adjusted to 4.0 and lipids were removed by extraction with chloroform. The lipid-free aqueous extract was concentrated under reduced pressure to 10–15 ml at 25°C, after adjustment of pH to 6.7.

Fractionation of the crude midbrain extract

Sephadex G-25 chromatography. The crude aqueous extract (10–15 ml, pH 6.7) was applied to a column (2.7 cm × 36 cm) of Sephadex G-25 (fine grade) that had been equilibrated with the elution medium, 6 mM-sodium phosphate buffer, pH 7.4, and eluted, at a flow rate of 60 ml/h.

Cellulose–water chromatography. Whatman CF 11 cellulose was washed five times with the suspending medium, the fines being decanted. Lipolytic material from the Sephadex G-25 column was concentrated to 10 ml, loaded on a cellulose column (2.4 cm × 65 cm) and eluted with deionized water, at a flow rate of 50 ml/h.

Cellulose–acetone–water chromatography. Lipolytic material from the previous cellulose column was

concentrated to 10ml, loaded on a cellulose column (2.4cm × 54cm) and eluted with acetone–water (4:1, v/v), at a flow rate of 50ml/h.

DEAE-Sephadex A-25 ion-exchange chromatography. The method of Caldwell (1969) was used to prepare a column (1.6cm × 45cm) of DEAE-Sephadex, but his gradient-elution technique was not employed. The buffer for suspension and elution was 0.01M-triethylammonium acetate, pH4.7. A solution of active material from the previous cellulose column (about 200mg of solid in 5ml) was loaded on the column and eluted, at a flow rate of 50ml/h.

Preparative t.l.c. The purified extract (see below) was fractionated on an air-dried layer of Whatman microcrystalline cellulose, 0.3mm thick, with butan-1-ol saturated with 1M-HCl as eluent.

Examination of the midbrain extracts

Determination of lipolytic activity. A suspension of isolated fat-cells was prepared by treating rat epididymal fat-pads with collagenase (Rodbell, 1964). Glucose was omitted from the medium. Lipolytic activity was measured by using a suspension of adipocytes, equivalent to 50mg of fresh tissue, in 1ml of sodium bicarbonate buffer (Cohen, 1957) containing 4% (w/v) bovine serum albumin. The suspension was incubated in air for 1h at 37°C in a shaking water bath. Stimulation of fatty acid release by the fraction under test was compared with that due to noradrenaline (1 μM, final concn.), which gave maximal stimulation. Free fatty acids were measured by the copper diphenylcarbazide method of Laurell & Tibbling (1967), as modified by Larsson (1967).

Thin-layer chromatography. A layer of 0.3mm of cellulose was used with water as solvent. Inosine (R_F 0.72) and hypoxanthine (R_F 0.55) were used as standards. Chromatograms were examined under a u.v. lamp. Amino acids were made visible by spraying the plates with 0.2% ninhydrin in ethanol and heating them at 110°C for 10min. Phenolic compounds were made visible as blue spots by spraying the plates with Folin–Ciocalteu reagent (Keith *et al.*, 1958).

Gas–liquid chromatography. To 1mg of catecholamine, base or hydrochloride, or an approximately equivalent amount of lipid-mobilizing factor, 3 drops of dimethylformamide were added, followed by 25 drops of hexamethyldisilazane. The mixture was heated at 80–90°C for 30min, evaporated to a small volume in a stream of O₂-free N₂ and then injected into a Pye 104 instrument with a dual flame-ionization detector. The glass column (2.7m × 0.4cm) was packed with 5% SE 30 on a support of Celite (85–100 mesh B.S.S.). Nitrogen was used as the carrier gas, at a flow rate of 50ml/min. The temperature was programmed to rise from 150°C to 250°C at 4°C/min.

Fluorimetric determination of catecholamines. The trihydroxyindole derivatives of the catecholamines and of lipid-mobilizing factor were made by the method of Crout (1961). The intensity of the fluorescence was measured on an Aminco Fluoro-microphotometer, with a primary filter transmitting light from 254nm to 420nm, and a secondary filter with a sharp cut-off, passing light above 485nm.

Mass spectrometry. An A.E.I. MS 902 instrument was used at an ionizing voltage of 70 eV. The temperature of the ion source was 220°C.

Ultraviolet and infrared spectra. A Perkin–Elmer model 137 u.v. spectrophotometer and a Perkin–Elmer model 257 grating i.r. spectrophotometer were used.

Cardiovascular experiments. Pithed adult male rats were maintained at 35°C and their parasympathetic nervous function was blocked by an intravenous injection of atropine sulphate (2mg/kg body wt.). Somatic function was blocked by an injection of tubocurarine chloride (3mg/kg body wt.). Test substances were injected intravenously, dissolved in 0.15M-NaCl with the aid of a trace of 1M-HCl. Blood pressure and heart rate were recorded.

Results

Activity of crude extracts from various sources

We examined calf brain for lipid-mobilizing factor by the method of Hollett (1969). However, it proved difficult to obtain a quantity sufficient for large-scale extraction. Pig brain was therefore tried because more abundant supplies were available, but, as shown in Table 1, it had lower activity than calf brain. Sheep brain was available in adequate quantity and it proved to have sufficient lipolytic activity for these studies. The lipolytic activities shown in Table 1 are only approximate, because a complete dose–response curve was not obtained for each preparation. In addition, hypoxanthine, which inhibits lipolysis, was probably also present in variable amounts in the crude extracts.

Purification of the crude midbrain extract

The chromatographic procedures are set out in Scheme 1. Lipolytic activity was eluted from Sephadex G-25 in a fraction that also contained amino acids and some material absorbing at 250–255nm. This material was examined by t.l.c. and shown to contain hypoxanthine (identified by i.r. and u.v. spectra).

The cellulose–water system then removed about one-half of the hypoxanthine and most of the sodium phosphate buffer from the lipolytic fraction. Amino acids were still present. The cellulose–acetone–water system then removed more hypo-

Table 1. *Lipolytic activities of crude midbrain extracts after chromatography on Sephadex G-25*

For experimental details see the text.

Source of midbrains	Batch no.	Wt. of midbrain examined (g)	Lipolytic activity equiv. to noradrenaline ($\mu\text{g/g}$ of tissue)
Calves	1	18	0.28
	2	18	0.45
	3	88	0.03
Pigs	1	23	0.03
	2	8	0.11
	3	1100	0.10
	4	2130	0.02
	5	1660	0.02
	6	2047	0.03
Sheep	1	111	0.12
	2	125	0.25
	3	221	0.21
	4	223	0.16
	5	396	0.10
	6	195	0.06
	7	243	1.05
	8	2380	0.11

xanthine and ninhydrin-positive material from the lipolytic fraction. At this stage, the dry weight of the lipolytic material was about 200mg.

A portion of the active material was then put through DEAE-Sephadex, which removed all the material absorbing at 250–255nm. The purified lipolytic material weighed 96mg and contained amino acids and two substances that fluoresced under the u.v. lamp.

Passage through a second cellulose–acetone–water column removed most of the amino acids. The lipolytic fraction then weighed 2–3mg and was very faintly ninhydrin-positive. The fluorescent substances were still present. Lipolytic activity was equivalent to about 1 mg of noradrenaline. This fraction will be referred to as the purified extract.

Comparison of lipid-mobilizing factor and catecholamines

The relationship of the factor to the catecholamines was investigated because of the well-known presence of the lipolytic catecholamine, noradrenaline, in midbrain (Popov *et al.*, 1967). Further, adrenaline and higher homologues (Černhorský *et al.*, 1966) are also lipolytic.

The purified extract was unstable in dilute solution, its lipolytic activity surviving for only a few hours at room temperature or a few days at -20°C . It was also sensitive to light. In these properties it is similar to the catecholamines.

The purified extract was submitted to t.l.c. on cellulose with butan-1-ol saturated with 1M-HCl as solvent. Spraying with the Folin–Ciocalteu reagent revealed components at R_F values 0.32, 0.39 and 0.47. Only the component with R_F 0.47 was lipolytic. Catecholamines and related substances were investigated in the same system. Results are shown in Table 2. Noradrenaline and adrenaline had R_F values 0.23 and 0.30 respectively, and were further distinguished from the factor by their appearance under u.v. after spraying with Na_2CO_3 (Table 2). Roberts & Broadley (1967) found that one of the artifacts of chromatography of adrenaline was adnamine (II). In case noradrenaline could break down in a similar way, noradnamine (I) was synthesized and tested. However, although noradnamine (R_F 0.50) ran close to the factor, it behaved differently when sprayed with Na_2CO_3 and was not lipolytic even at $35\mu\text{M}$ (Table 2). Metadrenaline and normetadrenaline had R_F values 0.39 and 0.33 respectively and were not lipolytic. After the Na_2CO_3 spray, these spots appeared dark blue under u.v. At the concentrations we were able to use the non-lipolytic components of the purified extract at R_F values 0.39 and 0.32 did not produce colours with Na_2CO_3 .

A larger quantity of the factor was prepared free of the two non-lipolytic components by preparative t.l.c. in the butan-1-ol–HCl system described above. Pharmacological studies in the rat showed that the factor decreased blood pressure and increased heart

Chromatographic sequence	Elution patterns
Crude extract from 120 g of midbrain (15 sheep)	
↓	
Sephadex G-25 column	135–220 ml ninhydrin-positive material 180–295 ml lipolytic activity 205–295 ml hypoxanthine
Fraction, 180–295 ml (containing 53 mg of hypoxanthine)	
↓	
Cellulose column (water solvent)	100–200 ml ninhydrin-positive material 105–340 ml lipolytic activity 200–340 ml hypoxanthine (not fully eluted)
Fraction, 105–240 ml (containing 29 mg of hypoxanthine)	
↓	
Cellulose column (acetone–water solvent)	200–250 ml weakly ninhydrin-positive material 250–600 ml strongly ninhydrin-positive material 170–350 ml lipolytic activity 210–350 ml hypoxanthine
Fraction, 200–250 ml (containing 13 mg of hypoxanthine)	
↓	
DEAE-Sephadex A-25 column	45–54 ml ninhydrin-positive material 45–57 ml lipolytic activity 57–71 ml hypoxanthine
Fraction, 48–54 ml (containing no hypoxanthine)	
↓	
Cellulose column (acetone–water solvent)	53–68 ml weakly ninhydrin-positive material 68–140 ml strongly ninhydrin-positive material 47–62 ml lipolytic activity
Fraction, 53–59 ml (purified extract), examined further (see text)	

Scheme 1. Flow sheet for the purification of the crude extract from sheep brain (batch 7, see Table 1)

Each fraction to be chromatographed further was evaporated to a small volume before loading. For experimental details see the text.

rate. These effects were not altered by pretreatment with the histamine antagonist mepyramine (2-[(2-dimethylaminoethyl) (*p*-methoxybenzyl) amino]pyridine) or the 5-hydroxytryptamine antagonist methysergide (*N*-[1-(hydroxymethyl)propyl]-1-methyl α -(+)-lysergamide), thus ruling out the presence of

histamine and 5-hydroxytryptamine. Propranolol (a β -adrenoreceptor antagonist) blocked the fall in blood pressure and the increase in heart rate due to the factor. A larger dose of the factor induced a slight rise in blood pressure, but this effect was blocked by pretreatment with phenoxybenzamine (an

Table 2. *Properties of lipid-mobilizing factor and catecholamines*

Cellulose t.l.c. plates were developed with butan-1-ol saturated with 1 M-HCl and sprayed with aq. 20% (w/v) Na_2CO_3 . All spots showed blue with Folin-Ciocalteu reagent. Other experimental details are given in the text.

Compound	R_F	Appearance under u.v. after Na_2CO_3 spray	Lipolysis	
			Activity	Concn. tested (μM)
Purified extract*	0.32	None	Inactive	
	0.39	None	Inactive	
	0.47	White	Active	
Noradrenaline	0.23	Yellow	Active	1
Adrenaline	0.30	Yellow	Active	1
Normetadrenaline	0.33	Dark blue	Inactive	40
Metadrenaline	0.39	Dark blue	Inactive	40
Isoprenaline	0.47	White	Active	1
<i>N-n</i> -Propylnoradrenaline	0.47	Not tested	Active	1
Noradnamine	0.50	Bright pink	Inactive	35

* The crude midbrain extract after purification according to Scheme 1.

α -adrenoreceptor antagonist). These results indicated that the factor contained some sympathomimetic substance that behaved mainly like isoprenaline.

Isoprenaline had the same R_F value as the lipolytic component of the factor (Table 2). The two substances also ran as one spot when co-chromatographed.

Physical measurements

Lipid-mobilizing factor, freed from non-lipolytic components, showed u.v. absorption at 215–220 nm and 283 nm, similar to isoprenaline.

The mass spectrum of the factor showed ions at m/e 72, 123, 124, 176, 178, 191, 193 and 211. The m/e ion 211 seemed to be the molecular ion. Mass measurement of the ion m/e 193 gave the formula $C_{11}H_{15}NO_2$. The mass spectrum of isoprenaline (III) was very similar. Therefore the m/e 193 ion of the factor was consistent with the $(M^+ - 18)$ ion of isoprenaline, formed by loss of H_2O . Both the factor and isoprenaline showed a doublet at m/e 123/124, indicating the presence of the grouping (IV). The base peak of m/e 72 is consistent with the fragment (V) obtained from isoprenaline.

N-n-Propylnoradrenaline (VI) was also examined in case the $-C_3H_7$ moiety of the factor was *n*-propyl rather than isopropyl. Ions were formed, as expected from the spectrum of isoprenaline, at m/e 123, 124 and 193, but the *n*-propyl compound gave no ions at m/e 176, 178 or 211. The factor could therefore be isoprenaline but not *N-n*-propylnoradrenaline. Adrenaline (VII) and noradrenaline (VIII) were ruled out by the absence from the spectrum of the factor of the m/e 139 ion derived from these compounds (Reisch *et al.*, 1968).

G.l.c. of the silyl derivatives of the factor and the catecholamines provided further evidence for the identity of the factor and isoprenaline (Table 3). Each compound gave a single sharp peak. Although their retention times were slightly different when examined separately, isoprenaline and lipid-mobilizing factor gave a single peak when chromatographed together. Noradrenaline did not appear even after 40 min.

Quantitative assessment of lipid-mobilizing factor

A small unweighed quantity of the purified factor was dissolved in ethanol. The E_{282} of the solution was equivalent to that of $41.5 \mu\text{g}$ of isoprenaline hydrochloride in the same volume of ethanol.

The ethanolic solution of the factor was evaporated to dryness and taken up in water. A sample of the aqueous solution was examined at several dilutions

Fig. 1. Effects of various concentrations of lipid-mobilizing factor and isoprenaline hydrochloride on lipolysis in a single suspension of rat adipocytes

The adipocytes were isolated from the epididymal fat-pads of normal rats by digestion with collagenase. Adipocytes (equivalent to 50 mg of tissue) were incubated for 1 h at 37°C . Fatty acids liberated under the influence of isoprenaline hydrochloride ($\circ$) or lipid-mobilizing factor ($\bullet$) were measured by a copper diphenylcarbazide method as described in the Materials and Methods section.

Table 3. Gas-liquid chromatography of lipid-mobilizing factor and catecholamines

Retention times on 5% SE 30 (Celite support) are given. Temperature was programmed to rise from 150°C to 250°C at 4°C/min . The flow rate was 50 ml/h. Further experimental details are given in the text.

Compound	Retention time (min)
Adrenaline	17.4
Noradrenaline	17.4
Isoprenaline	18.8
Lipid-mobilizing factor	18.2
Isoprenaline + lipid-mobilizing factor	18.2
<i>N-n</i> -Propylnoradrenaline	20.2

for lipolytic effect on an isolated fat-cell suspension, in comparison with isoprenaline hydrochloride. As shown in Fig. 1, the points for the factor could be superimposed almost exactly on the isoprenaline curve by putting $5\mu\text{l}$ of the solution of the factor equivalent to $0.1\mu\text{g}$ of isoprenaline hydrochloride. This gave an estimate of $42.5\mu\text{g}$ of isoprenaline as the lipolytic equivalent of the total amount of the factor, in good agreement with the value obtained by u.v. measurement.

The catecholamine content of lipid-mobilizing factor was assessed in comparison with isoprenaline and noradrenaline by the fluorimetric method. The factor behaved either as $10\mu\text{g}$ of noradrenaline or as $50\mu\text{g}$ of isoprenaline. Clearly, only the latter value agreed with the u.v. and lipolytic determinations.

Discussion

The experiments described above indicate that one of the lipolytic substances in sheep midbrain is isoprenaline. Lipolytic activity was also found in pig and calf midbrain. Although we did not identify the factor in calf midbrain as isoprenaline, it seems possible that the lipolytic activity found in that tissue by Hollett (1969) was due to isoprenaline rather than, as she suggested, to a derivative of hypoxanthine.

We are unaware of any previous report of the presence of isoprenaline in brain. An isoprenaline-like substance was found in cat, rabbit, monkey and human adrenal by Lockett (1954) and in cat plasma by Lockett (1957) and Eakins & Lockett (1961). Their evidence rested on the lowering of blood pressure and the inhibition of uterine motility.

A number of artifacts have been noted during studies on the catecholamines and their relevance to lipid-mobilizing factor has been considered. Roberts (1964) found that two substances, phenol and NaH_2PO_4 , used in the chromatography of catecholamines could depress blood pressure and also inhibit uterine motility. We did not use phenol and the NaH_2PO_4 in the buffers would have been removed during the later stages of chromatography. In any case, Roberts (1964) suggested that the phosphate could not account for the isoprenaline-like substance reported by Lockett (1954, 1957) and Eakins & Lockett (1961), because the effects were 'qualitatively and quantitatively different from those of isoprenaline'.

Our identification of isoprenaline is based qualitatively on the mass spectrum of purified brain ex-

tracts, and is supported by evidence from t.l.c. and g.l.c. and the depressor effects on blood pressure. Quantitative evidence of the identity of the factor and isoprenaline has been obtained from fluorescence measurements of trihydroxyindole derivatives and from lipolytic potency.

We thank Dr. W. M. Scott of the Physico-chemical Measurements Unit, Harwell, Berks., U.K., for measuring the mass spectra, Mr. B. Bashford for the g.l.c., Dr. R. H. Poyser of our Medicinal Research Centre, Harlow, for the cardiovascular studies and Miss J. M. Westwater for technical assistance.

References

- Best, C. H. & Campbell, J. (1936) *J. Physiol. (London)* **86**, 190-203
- Caldwell, I. C. (1969) *J. Chromatogr.* **44**, 331-341
- Černhorský, M., Čepelík, J., Lincová, D. & Wenke, M. (1966) *J. Pharm. Pharmacol.* **18**, 188-189
- Cohen, P. P. (1957) in *Manometric Techniques and Tissue Metabolism* (Umbreit, W. W., Burris, R. H. & Stauffer, J. F., eds.), pp. 147-149, Burgess Publishing Co., Minneapolis
- Crout, R. (1961) *Stand. Methods Clin. Chem.* **3**, 62-80
- Desbals, B., Desbals, P. & Agid, R. (1970) in *Adipose Tissue, Regulation and Metabolic Function* (Jeanrenaud, B. & Hepp, D., eds.), pp. 28-31, Academic Press, London and New York
- Eakins, K. E. & Lockett, M. F. (1961) *Brit. J. Pharmacol. Chemother.* **16**, 108-115
- Hollett, C. R. (1968a) *Biochem. Biophys. Res. Commun.* **30**, 600-606
- Hollett, C. R. (1968b) *Biochem. Biophys. Res. Commun.* **32**, 48-54
- Hollett, C. R. (1969) *Biochim. Biophys. Acta* **176**, 511-523
- Kawazu, M. (1960) *Jap. Patent* 13923
- Keith, R. W., Le Tourneau, D. & Mahlum, D. (1958) *J. Chromatogr.* **1**, 534-536
- Larsson, S. (1967) *Acta Physiol. Scand. Suppl.* **294**, 9
- Laurell, S. & Tibbling, G. (1967) *Clin. Chim. Acta* **16**, 57-62
- Lockett, M. F. (1954) *Brit. J. Pharmacol. Chemother.* **9**, 498-505
- Lockett, M. F. (1957) *Brit. J. Pharmacol. Chemother.* **12**, 86-96
- Popov, N., Pohle, W., Roestler, W. & Matties, H. (1967) *Acta Biol. Med. Ger.* **18**, 695-702
- Reisch, J., Pagnucco, R., Alfes, H., Jantos, J. & Möllmann, H. (1968) *J. Pharm. Pharmacol.* **20**, 81-86
- Roberts, D. J. (1964) *J. Pharm. Pharmacol.* **16**, 313-322
- Roberts, D. J. & Broadley, K. J. (1967) *J. Chromatogr.* **27**, 407-412
- Rodbell, M. (1964) *J. Biol. Chem.* **239**, 375-380