A Corticotrophin-Releasing Factor: Partial Purification and Amino Acid Composition

By A. V. SCHALLY,* M. SAFFRAN AND BRIGETTE ZIMMERMANN
The Allan Memorial Institute of Psychiatry, McGill University, Montreal, Canada

(Received 13 January 1958)

Neurohypophyseal or hypothalamic tissue, in combination with a sympathetic amine, increases significantly the release of adrenocorticotrophic hormone (ACTH) from rat anterior-pituitary tissue *in vitro* (Saffran & Schally, 1955a). A corticotrophin-releasing factor (CRF) was detected in posterior-pituitary extracts and was shown to be distinct from oxytocin, vasopressin and histamine (Saffran, Schally & Benfey, 1955; Schally & Saffran, 1956). The active material has now been concentrated several thousand-fold by serial paper chromatography and the amino acids present in an acid hydrolysate of a concentrate have been identified.

MATERIAL AND METHODS

Tissue preparations

Dilute acetic acid extracts. Acetone-dried powders of neurohypophyseal, hypothalamic or brain tissue were extracted with hot aq. 0.5% acetic acid, as described by Stehle (1933).

Precipitation with methanol. To 50 ml. of a 0.5% acetic acid extract of 2.5 g. of an acetone-dried powder of posterior-pituitary tissue was added 200 ml. of methanol. The mixture was kept at -20° overnight and was centrifuged in lots of 5 ml. per tube at 5000 rev./min. in the high-speed head of the International refrigerated centrifuge, model PR-2, temperature 0° . Each lot of the sediment was washed twice with 0.8 ml. of a mixture of 4 parts of methanol and 1 part of 0.5% acetic acid. The sediment was dissolved in 0.5% acetic acid, the solutions were pooled and the volume was adjusted to 12.5 ml. This yellowish, slightly turbid solution was kept in the refrigerator.

Protopituitrin. Protopituitrin is the name given by Parke, Davis and Co. to fraction C in Waring & Landgrebe's (1950) summary of the procedure of Kamm, Aldrich, Grote, Rowe & Bugbee (1928) for the separation of the pressor and oxytocic principles from posterior-pituitary powders. Material of either ox or hog origin was kindly supplied by Parke, Davis and Co., Detroit, Mich., U.S.A., and contained 12–30 i.u. of pressor activity and 11 to 15 i.u. of oxytocic activity/mg. One such preparation contained 0.25 i.u. of corticotrophic activity when assayed by the method of Saffran & Schally (1955b). Similar material of porcine origin was also obtained from N.V. Organon, Oss, Holland. One batch of the latter preparation was treated by the supplier with oxycellulose, according to the method of

Astwood, Raben & Payne (1952), resulting in a reduction to one-tenth in the content of ACTH.

Brain and hypothalamic extracts. These products, of porcine origin, were supplied by Nordio Biochemicals Ltd., Montreal. An acetone-dried powder of whole brain was treated by the procedure of Kamm *et al.* (1928) for the preparation of Protopituitrin from posterior-pituitary powder to yield a small amount of residue designated as brain Protopituitrin. Acetone-dried powders of separated hypothalami were extracted with acetic acid and the extract was treated with oxycellulose, according to the method of Astwood *et al.* (1952). The unadsorbed fraction and the adsorbed material eluted with 0.1N-HCl were lyophilized to yield white powders. The hypothalamic material insoluble in acetic acid was extracted with hot aq. 0.5% acetic acid.

Bioassay

Adrenocorticotrophic hormone. The *in vitro* bioassay of Saffran & Schally (1955b) was used for the estimation of ACTH in the tissue preparations and in the CRF test outlined below.

Corticotrophin-releasing factor. The detection of CRF activity was carried out by the method of Schally & Saffran (1956), which is a modification of the original method of Saffran & Schally (1955a). Anterior-pituitary tissue of the rat was cut into halves, one of which served as the control tissue to obtain the 'unstimulated' release of ACTH, and the other half was incubated for 1 hr. in the presence of the sample to be tested. A positive test for CRF was denoted by a significantly greater release of ACTH from the test tissue, indicated by a lower fiducial limit greater than 1. The potency of the CRF preparations was estimated from the lowest dose that caused a significant stimulation of the release of ACTH. In the earlier tests, 0.2 mm-(–)-noradrenaline was added to keep conditions the same as in the original method (Saffran & Schally, 1955a). Arterenol is, however, not required to demonstrate CRF activity in tissue extracts. In more recent tests, the pituitary tissue was pre-incubated for 1 hr. before the addition of CRF to the test tissue. The pre-incubation medium was discarded (cf. Saffran & Bayliss, 1953). Pre-incubation decreased and made more consistent the release of ACTH by the control tissue. In all cases the material under test was added to the experimental tissue at the start of the incubation period and to the control tissue at the end of the incubation period. In this way, all the adrenal tissue in the assay for ACTH was exposed to the materials added with the test sample. This precaution is important in view of the contamination of certain posterior-pituitary preparations by significant amounts of ACTH. In addition, any possible potentiation of the effect of ACTH at the adrenal level is eliminated.

* Present address: Department of Physiology, Baylor University College of Medicine, Texas Medical Center, Houston, Texas.

Colorimetric methods

Peptides. The method of Lowry, Rosebrough, Farr & Randall (1951) was used to estimate peptides.

Ninhydrin. Amino acids and related compounds were determined by the ninhydrin method of Moore & Stein (1954). The colour was developed in a total volume of 2 ml. The reagent blank was minimized by using 2-methoxy-ethanol redistilled over FeSO_4 , and recrystallized ninhydrin and hydrindantin.

Paper chromatography

All solvents, except the reagent-grade acetic acid, were redistilled before use. The water was glass-distilled.

For most chromatograms, Whatman no. 3 filter paper ($22\frac{1}{2}$ in. $\times$ $18\frac{1}{2}$ in.) was used. The paper used in the final stages of the separation of the active material was washed for several days by successive descending chromatography with 2*N*-acetic acid, water, ethanol and acetone. The material in 0.5% acetic acid solution was applied as a streak at the starting line.

Butan-1-ol-acetic acid-water (4:1:5, *by vol.*). This system (Partridge, 1948) was used for descending chromatography. The load at the starting line was 2.5–3 mg./cm. and the time of development was 40–48 hr. at 28°. As the developing solvent ran off the paper, the R_F of the zones could not be determined; therefore lysine, which has R_F 0.18 in this system, was run as a reference substance on every chromatogram.

***m*-Cresol saturated with water.** This solvent was used for ascending chromatography, with a load of 1.5–2 mg./cm. for 40–48 hr. at 28°. Occasional batches of *m*-cresol inactivated the CRF and could not be used even after redistillation.

Acetone-water (3:2, *v/v*). This system (Bentley & Whitehead, 1950) was used for ascending chromatography at 4°, with a load of 1–1.2 mg./cm. and a development period of 18–20 hr. Equally sharp separation of the zones resulted with or without urea under these conditions; therefore urea was omitted.

Propan-1-ol-water (4:1, *v/v*). Used for ascending chromatography, this system was run with a load of 0.5–1 mg./cm. at 28° for 20–24 hr.

Location of substances on paper. Amino acids and peptide material were detected by spraying narrow strips of the chromatograms with 0.2% ninhydrin in butanol, followed, after development of the ninhydrin colour, by a spray of 1% copper nitrate in ethanol. Vasopressin and oxytocin give a pink colour with this treatment. Ultraviolet-light-absorbing or fluorescing material, such as vasopressin and oxytocin, were located by examination in ultraviolet light. CRF and, in some cases, vasopressin, were located by biological tests on eluates of appropriate zones of the chromatogram.

Elution. Selected zones were cut horizontally from the chromatograms and were eluted by descending chromatography with 0.5% acetic acid for 8–20 hr., depending upon the area of the paper. The eluates, in 100 ml. beakers, were concentrated to small volumes or to dryness *in vacuo* over NaOH.

Amino acid analysis. The amino acids were determined as described by Peart (1956), except that the two-dimensional chromatographic method of Levy & Chung (1953)

was used to separate the amino acids. The chromatogram was sprayed with 1% ninhydrin in butan-1-ol containing 8% (*v/v*) acetic acid because of the alkaline buffer used in the chromatographic system. The ninhydrin colour was developed overnight in darkness. Proline was determined by the method of Chinard (1952) as modified by Bell, Howard, Shephard, Finn & Meisenhelder (1956).

Chemical treatment of corticotrophin-releasing factor

Partial acid hydrolysis. A sample was heated in 2.2*N*-HCl for 90 min. at 110° in a sealed ampoule.

Total acid hydrolysis. Amounts (0.1–1.0 mg.) were heated with 0.2–2.0 ml. of 6*N*-HCl for 20 hr. at 110° in a sealed tube.

Reduction. Reduction with thioglycollate was carried out according to Ames, Moore & Van Dyke (1950), except that the concentration of thioglycollic acid was increased to 0.05*M* and the time of reaction to 24 hr.

Oxidation. Performic acid oxidation was carried out by the method of Sanger (1949) or Popenoe & du Vigneaud (1953). This procedure destroys the pressor and ACTH activities (Dedman, Farmer & Morris, 1955).

RESULTS

Activity of neural-tissue extracts

Several preparations of neural origin were explored for CRF activity in the search for a suitable starting material for the concentration of CRF. Of the preparations examined (Table 1), hypothalamic extracts and Prototuitrin exhibited consistent activity at a dose of 10 μg . Prototuitrin was chosen for further study because it is available in relatively large amounts as a stage in the preparation of vasopressin and oxytocin. The apparent inhibition of release of ACTH by higher doses of some preparations is partly explained by the presence of ACTH in these preparations. The addition of external ACTH to the test system masks the stimulated release of ACTH by the isolated pituitary tissue. Table 2 lists the ACTH content of two posterior-lobe preparations and shows how treatment of Prototuitrin with oxycellulose decreases markedly the contamination by ACTH.

Earlier experiments (Saffran *et al.* 1955) had suggested that CRF had the properties of a peptide. Accordingly paper-chromatographic methods used for the separation of peptides were applied to the concentration of CRF from Prototuitrin. Prototuitrin separated into a large number of ninhydrin-staining components in each of the solvent systems, and the zone containing CRF activity in each system was located by testing eluates of portions of the paper. Both ninhydrin and biological-activity patterns were reproducible. The systems were then applied serially to concentrate the CRF.

Table 1. Corticotrophin-releasing activity of extracts of neural tissue

For details of preparations see text.

Preparation	Dose (μ g./flask)	No. of tests	No. of tests with lower fiducial limit >1	Mean ratio of ACTH release (experimental control)
Brain extract	1	1*	0	1.2
Brain extract	10	1*	0	0.5
Brain extract	100	1*	0	1.0
Brain extract	1000	1*	0	0.7
Brain Protopituitrin	10	1	0	0.9
Brain Protopituitrin	100	1	0	1.3
Brain Protopituitrin	500	1	0	2.3
Hypothalamic extract, unabsorbed by oxycellulose	10	2	2	2.9
Hypothalamic extract, absorbed by oxycellulose	10	1	1	2.4
Hypothalamic extract, insoluble in acetic acid	10	1	1	2.4
Posterior-pituitary extract, hog	10	4*	1	1.5
Posterior-pituitary extract, hog	14.5	7*	1	1.3
Posterior-pituitary extract, hog	48	3*	2	1.3
Posterior-pituitary extract, hog	72	2*	0	0.9
Methanol precipitate	1	4*	1	1.5
Posterior-pituitary extract, ox	10	4*	1	1.4
Posterior-pituitary extract, ox	100	2*	0	0.9
Protopituitrin	1	4	1	1.3
Protopituitrin	10	11	8	2.2
Protopituitrin	100	2	0	1.4
Protopituitrin, oxycellulose-treated	1	1	1	1.8
Protopituitrin, oxycellulose-treated	10	1	1	2.6

* ($\pm$)-Noradrenaline 0.4 mM. The remainder of the tests were with pre-incubated tissue.

Table 2. Content of adrenocorticotrophic hormone in posterior-pituitary preparations

Figures in parentheses are the 95% confidence limits.

Preparation	ACTH (i.u./mg.)
Posterior-pituitary extract, ox	0.19 (0.09-0.44)
Protopituitrin	0.25 (0.11-0.54)
Protopituitrin, oxycellulose-treated	0.03 (0.025-0.035)

Stages of purification

- (1) *Chromatography in butanol-acetic acid-water.* The CRF activity was recovered in the zone with R_F 0.05-0.2 (lysine=0.18). This zone contained one-half to one-quarter of the weight of the Protopituitrin applied to the starting line, so that the active material eluted from this zone was concentrated two to four times.
- (2) *Chromatography in m-cresol.* The active material from step 1 was chromatographed in the m-cresol system. After development, the paper was dried overnight in the fume hood at room temperature, with an extra fan to increase the circulation of air within the hood. The area with R_F 0.46-0.64 consistently contained the activity. Eluates of this zone were taken to dryness several times *in vacuo* over NaOH to remove traces of m-cresol. The material contained one-twenty-fifth to one-fortieth of the weight of Protopituitrin.
- (3) *Rechromatography in m-cresol.* The eluate from step 2 was rechromatographed on washed

- filter paper in the m-cresol system to separate completely from CRF the faster-running peptides, including vasopressin (R_F 0.85), which tended to tail at the relatively high loads used in step 2, and thus contaminate the CRF. The zone with R_F 0.46-0.64 again contained the activity in about $\frac{1}{170}$ of the starting weight of Protopituitrin. Washed paper was used in this and in the subsequent two steps to minimize contamination of the preparation with substances such as metals which might hasten inactivation.
- (4) *Chromatography in acetone-water.* The material concentrated in step 3 was chromatographed in the acetone-water system, in which the activity was located in the zone with R_F 0.35-0.46. This zone contained $\frac{1}{2200}$ - $\frac{1}{2600}$ of the weight of Protopituitrin. The weight was estimated by the method of Lowry *et al.* (1951) and by weighing, when larger-scale separations were run.
- (5) *Chromatography in propanol-water.* The material from step 4 was chromatographed as two spots in the propanol-water system. At this stage of purification the ninhydrin-staining spots coincided with ultraviolet-light-absorbing or fluorescing areas. Examination in ultraviolet light revealed two faintly fluorescing spots with R_F 0.07-0.22 and 0.33-0.43, and a strongly absorbing spot with R_F 0.22-0.33. Activity was consistently located in the fluorescing zones. The absorbing zone was not active in limited tests at low-dose levels. The zone with R_F 0.07-0.22 contained $\frac{1}{4600}$ - $\frac{1}{5000}$ of the

weight of Protopituitrin, as estimated by the method of Lowry *et al.* (1951). The other active zone contained even less material.

The steps in the purification of CRF are outlined in Table 3. At each step, a portion corresponding to 10 μ g. of Protopituitrin was used in the biological test for CRF (Table 4). This dose is consistently active, suggesting that there were no serious losses in activity during the course of purification. In contrast, similar doses of material eluted from other zones in the first four steps were inactive.

The facilities of our Laboratory limited the capacity of each run to 3 g. of Protopituitrin. Table 3 illustrates the enormous amount of labour involved in the spotting, running and elution of approximately 50 chromatograms needed to concentrate 600 μ g. of CRF.

Table 5 lists the R_F of CRF, the vasopressins, oxytocin and α -corticotrophin in the four solvent systems to demonstrate the ability of the combination of these systems to separate CRF from the other known hormones in the starting material.

Amino acid composition

Table 6 lists the amino acids found in acid hydrolysates of two preparations of CRF with R_F 0.07–0.22 in the propanol–water system, one made from Protopituitrin, provided by Parke, Davis and Co., and the other from similar material, treated with oxycellulose, supplied by N. V. Organon. The two preparations differ significantly in their content of glycine, tyrosine and alanine. Both consistently stimulated the release of ACTH at a dose of 0.5 μ mg., but the preparation made from Protopituitrin was decidedly more potent. Traces of threonine and of an unknown ninhydrin-reacting material (R_F 0.6 in butanol–acetic acid–water and R_F 0.18 in phenol–*m*-cresol, pH 9.3) were found in the preparation made from Organon starting material.

Chemical treatment

The biological activity in one of two preparations was abolished by reduction with thioglycolic acid, oxidation with performic acid and extensive acid hydrolysis (Table 7). However, treatment with

Table 3. Steps in the concentration of corticotrophin-releasing factor

Solvent system	Wt. applied (mg.)	No. of chromatograms	Average concentration factor
Butanol–acetic acid–water	3000	30	1
<i>m</i> -Cresol	900–1800	10–20	3
<i>m</i> -Cresol rechromatography	90–140	2–3	33
Acetone–water	20	1	170
Propanol–water	1.2–1.5	1	2400
Final product	0.6	44–55	4800

Table 4. Typical release of adrenocorticotrophic hormone by corticotrophin-releasing factor at various stages of preparation

Stage	Dose (μ mg.)	% of control	95% fiducial limits
<i>m</i> -Cresol, rechromatographed	60	630	330–1200
<i>m</i> -Cresol, rechromatographed	6	350	190–670
Acetone–water	4	380	150–960
Propanol–water	2	300	150–600
Propanol–water	1.1	270	190–390
Propanol–water	0.9	400	240–690

Table 5. R_F values of oxytocin, vasopressin, adrenocorticotrophic hormone and corticotrophin-releasing factor

	Butanol–acetic acid–water	R_F in <i>m</i> -cresol–water	Acetone–water	Propanol–water
Oxytocin	0.37	0.85	0.71	0.22
Arginine vasopressin	0.16	0.85	0.65	0.18
Lysine vasopressin	—	0.85	0.69	—
α -Corticotrophin*	0	1.0	—	—
CRF (zone)	0.05–0.20	0.46–0.64	0.35–0.46	0.07–0.22 0.33–0.43

* Personal communication from Professor C. H. Li.

2·2N-HCl at 110° for 90 min. does not apparently affect the activity of a CRF preparation at the third step of the purification procedure.

DISCUSSION

At present, the greatest weight of evidence supports the hypothesis that the release of ACTH from the adenohypophysis is governed by a material elaborated in the hypothalamic area of the brain (Harris, 1951). Proof of this hypothesis requires the isolation of material, from the hypothalamus, which activates the release of ACTH from the pituitary in a system in which the response to stress has been abolished by some means. The existence of a special, short vascular route (the hypophyseal-portal system) for this material suggests that its biological activity is relatively short-lived. The anatomical relationship between the hypothalamus and the neurohypophysis further implies that the substance may be found in the posterior lobe of the pituitary body as well (Zuckerman, 1952), in a manner analogous to the hormones of the posterior lobe, oxytocin and vasopressin (Scharrer & Scharrer, 1954). As a rule, the

hormones elaborated by any one endocrine organ are members of the same chemical family. If the hypothalamus-neurohypophysis system obeys this rule, then the hypothetical substance suggested by Harris (1951) and others should be chemically similar to the peptide hormones, vasopressin and oxytocin. However, lipid, lipoprotein and protein, as well as peptide preparations, have been claimed to have the properties of the hypothetical substance.

Slusher & Roberts (1954) reported the concentration of a lipoprotein and a lipid from cow posterior hypothalamus that caused eosinopenia and depletion of adrenal ascorbic acid in intact rats. Tests in normal animals are of dubious value. Roberts (1955) added that these fractions also showed activity in rats rendered unresponsive to stress by lesions in the hypothalamus, but a paper describing this work has not yet appeared.

Recently, Porter & Rumsfeld (1956) reported that plasma-protein fractions prepared from 'hypophyseal portal-vessel blood' caused significant falls in the adrenal ascorbic acid of intact rats treated with hydrocortisone, whereas similar fractions from systemic blood were inactive. Because only about 0·5 µmg. of our peptide material is active in our test, perhaps these lipoprotein and protein fractions, which were administered in relatively large doses, contained some adsorbed CRF, accounting for their activity.

Other authors (e.g. Martini & Morpurgo, 1955) suggested that oxytocin and vasopressin themselves were able to stimulate the release of ACTH and other hormones from the adenohypophysis. However, evidence from our Laboratory (Saffran *et al.* 1955; Schally & Saffran, 1956) and from Guillemin's laboratory (Guillemin & Rosenberg, 1955; Guillemin, Hearn, Cheek & Housholder, 1957) pointed to the presence in commercial preparations of vasopressin of another peptide component which stimulated the release of ACTH by adenohypophyseal tissue *in vitro*. This paper shows the active material to be chemically related to the other hormones of the hypothalamus-neurohypophysis system, vasopressin and oxytocin.

Table 6. *Molar proportions of amino acids found after total acid hydrolysis of corticotrophin-releasing factor*

Amino acid	CRF prepared from starting material supplied by	
	Parke, Davis and Co.	Organon
Cystine	0·89	1·06
Aspartic acid	0·89	1·27
Glutamic acid	0·89	1·18
Lysine	0·54	0·96
Phenylalanine	0·83	0·90
Proline	1·10	1·26
Tyrosine	1·00	0
Glycine	1·00	2·00
Serine*	0·98	1·11
Histidine	0·64	1·10
Alanine	0·22	0·73
Arginine	0·33	0·36

* Corrected for 10% destruction, cf. Levy, Geshwind & Li (1955).

Table 7. *Chemical treatment and activity of corticotrophin-releasing factor*

Preparation	Treatment	Dose (µg.)	Release of ACTH (% of control)	95% limits (%)
80% Methanol precipitate	0·1M-Sodium phosphate buffer, pH 7·4 (control)	1	290	160-520
	Thioglycollate in phosphate buffer	1	98	50-190
	Formic acid (control)	5	245	130-464
	Performic acid	1	86	54-137
	Performic acid	5	90	68-120
	Performic acid	5	142	62-322
<i>m</i> -Cresol, rechromatographed (Table 3, step 3)	2·2N-HCl, 90 min.	0·33	320	210-480
	6N-HCl, 22 hr.	0·33	110	80-140

That CRF is distinct from oxytocin and vasopressin was first shown in early experiments in which the activity was separated from vasopressin by paper chromatography in one system (Saffran *et al.* 1955). The present work utilizes the high resolving power of four successive chromatographic systems to isolate CRF. As depicted in Table 4, chromatography in butanol-acetic acid separates CRF (R_F 0.05–0.2) from oxytocin (R_F 0.37) as well as from a large number of small peptides (high R_F) and of very basic peptides ($R_F \leq 0.05$). This system was used previously by Benfey (1953) for the separation of vasopressin and oxytocin. The *m*-cresol system separates CRF (R_F 0.46–0.64) from ox and hog vasopressins (R_F 0.85) and from α -corticotrophin (R_F approx. 1.0). A further separation of CRF from these hormones is brought about by the acetone-water step. This separation, as well as the chemical analysis, ensures that the final product is neither vasopressin nor oxytocin. The final, propanol-water, step resolves the active material into two or more equally-potent spots, whose relationship will be the subject of further investigation. The sequence in which the solvent systems are used is critical, because the last two systems are more sensitive to overloading and to the presence of gummy contaminants in Protopituitrin. The final product is relatively unstable, but insufficient quantities were available to attempt systematically to stabilize it, although precautions were taken to avoid contamination with heavy metals or contact with peroxides in the solvents.

The material prepared by Guillemín *et al.* (1957) from hypothalamic and neurohypophyseal extracts, and called by them fraction D, resembles in its properties the CRF prepared in our Laboratory. Fraction D is chromatographically distinct from vasopressin, promotes the release of ACTH from isolated anterior-lobe tissue, is most unstable in the purified form (fraction ΔD), and is apparently a small peptide. According to Guillemín *et al.* (1957) fraction D may also be obtained, although by more drastic extraction procedures, from extra-neural sources as well. Fraction D exerts activity at doses ranging upward from 0.5 μ g., whereas our most highly purified material has detectable activity, when freshly prepared, at 1/1000 of that dose. Probably variations in assay procedures, as well as a different degree of purification, contribute to the difference in the minimal effective dose.

Lysine vasopressin and CRF have seven or eight amino acids in common; in addition, the CRF preparations consistently contain major amounts of serine and histidine, and alanine and arginine are found in smaller, variable proportions. This indicates that the CRF preparation is not yet chemically pure, although it is possible that a family of similarly active peptides may exist. The composi-

tion and specificity of CRF from various species will have to be studied in view of the differences between hog and ox vasopressin (Van Dyke, Adamsons & Engel, 1955).

Guillemín *et al.* (1957) have discussed the physiological significance of CRF, whose biological activity has thus far been demonstrated in a test *in vitro* on isolated tissues.

SUMMARY

1. Neurohypophyseal and hypothalamic extracts contain a corticotrophin-releasing factor (CRF) that stimulates the release of adrenocorticotrophic hormone (ACTH) from isolated anterior-pituitary tissue.

2. Serial paper chromatography in four different solvent systems has been applied to the concentration of the CRF in Protopituitrin, a posterior-pituitary concentrate prepared as a stage in the purification of oxytocin and vasopressin.

3. Purified CRF of neurohypophyseal origin significantly increases the release of ACTH at doses as low as 1 μ mg.

4. Purified CRF has the chemical and chromatographic properties of a peptide, distinct from oxytocin and vasopressin.

5. After acid hydrolysis of purified CRF, the following amino acids were detected: cystine, aspartic acid, glutamic acid, glycine, proline, lysine, phenylalanine, alanine, serine and histidine.

We are grateful to Dr D. A. McGinty, of Parke, Davis and Co., Detroit, Mich., U.S.A., Mr K. Antoft, of Nordic Biochemicals Ltd., Montreal and to N.V. Organon, Oss, Holland, for generous gifts of tissue extracts. This investigation was supported by a Federal-Provincial Mental Health Grant (No. 604–5–425) and by grants from the Foundations' Fund for Research in Psychiatry, Canada Packers Ltd., and N.V. Organon. We should like to thank Dr R. A. Cleghorn for his encouragement.

REFERENCES

- Ames, R. G., Moore, D. H. & Van Dyke, H. B. (1950). *Endocrinology*, **46**, 215.
- Astwood, E. B., Raben, M. S. & Payne, P. W. (1952). *Recent Progr. Hormone Res.* **7**, 1.
- Bell, P. H., Howard, K. S., Shephard, R. G., Finn, B. M. & Meisenhelder, J. H. (1956). *J. Amer. chem. Soc.* **78**, 5059.
- Benfey, B. G. (1953). *Brit. J. Pharmacol.* **8**, 435.
- Bentley, H. R. & Whitehead, J. K. (1950). *Biochem. J.* **46**, 341.
- Chinard, F. P. (1952). *J. biol. Chem.* **199**, 91.
- Dedman, M. L., Farmer, T. H. & Morris, C. J. O. R. (1955). *Biochem. J.* **59**, xii.
- Guillemín, R., Hearn, W. R., Cheek, W. R. & Housholder, D. E. (1957). *Endocrinology*, **60**, 488.
- Guillemín, R. & Rosenberg, B. (1955). *Endocrinology*, **57**, 599.
- Harris, G. W. (1951). *Brit. med. J.* **2**, 627.

- Kamm, O., Aldrich, T. B., Grote, K. W., Rowe, L. W. & Bugbee, E. P. (1928). *J. Amer. chem. Soc.* **50**, 573.
- Levy, A. L. & Chung, D. (1953). *Analyt. Chem.* **25**, 396.
- Levy, A. L., Geshwind, I. I. & Li, C. H. (1955). *J. biol. Chem.* **213**, 187.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Martini, L. & Morpurgo, C. (1955). *Nature, Lond.*, **175**, 1127.
- Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Peart, W. S. (1956). *Biochem. J.* **62**, 520.
- Popenoe, E. A. & du Vigneaud, V. (1953). *J. biol. Chem.* **205**, 133.
- Porter, J. C. & Rumsfeld, H. W. jun. (1956). *Endocrinology*, **58**, 359.
- Roberts, S. (1955). *Recent Progr. Hormone Res.* **11**, 108.
- Saffran, M. & Bayliss, M. J. (1953). *Endocrinology*, **50**, 639.
- Saffran, M. & Schally, A. V. (1955a). *Canad. J. Biochem. Physiol.* **33**, 408.
- Saffran, M. & Schally, A. V. (1955b). *Endocrinology*, **56**, 523.
- Saffran, M., Schally, A. V. & Benfey, B. G. (1955). *Endocrinology*, **57**, 439.
- Sanger, F. (1949). *Biochem. J.* **44**, 126.
- Schally, A. V. & Saffran, M. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 636.
- Scharrer, E. & Scharrer, B. (1954). *Recent Progr. Hormone Res.* **10**, 183.
- Slusher, M. & Roberts, S. (1954). *Endocrinology*, **55**, 245.
- Stehle, R. L. (1933). *J. biol. Chem.* **102**, 573.
- Van Dyke, H. B., Adamsons, K. jun. & Engel, S. L. (1955). *Recent Progr. Hormone Res.* **11**, 1.
- Waring, H. & Landgrebe, F. W. (1950). In *The Hormones*, vol. 2. Ed. by Pincus, G. and Thimann, K. V. New York: Academic Press Inc.
- Zuckerman, S. (1952). *Ciba Foundation Symp., Endocrinology*, vol. 4, p. 213. London: Churchill.

The Component Acids and Glycerides of the Depot Fat of an Indian Panther (*Panthera pardus fusca*)

BY S. P. PATHAK AND B. N. TRIVEDI

Department of Industrial Chemistry, Banaras Hindu University, Banaras-5, India

(Received 26 November 1957)

Little is known about the fatty acid composition and glyceride structure of depot fats of land animals, particularly of those of the Carnivora, and quantitative data for the panther (*Panthera pardus fusca*) fat are not available. A study was therefore undertaken of the fatty acid composition and the nature of the glycerides of the depot fat of a panther of Indian origin.

EXPERIMENTAL

A light-cream-coloured fat was obtained from the abdominal depots of a freshly killed male panther from the forest of Indore (Central India). The saponification equivalent (s.e.) of the fat was 285.6 and iodine value (i.v.) was 62.4 with 3.2% of free fatty acid (as oleic). It was purified by washing an ethereal solution with dilute aqueous alkali, when the s.e. and i.v. of the neutral fat were 284.8 and 62.8 respectively.

Determination of component acids. A sample (140 g.) of the fat was saponified and the mixed fatty acids (130 g.; i.v. 65.5) were recovered. The latter were then resolved into two groups (1 and 2) of simpler mixtures of fatty acids by the lead salt-ethanol method (Hilditch, 1956). Each group of fatty acids was separately converted into the methyl esters. The esters were then subjected to fractional distillation under high vacuum (0.1 mm.) through an efficient electrically heated and packed column (Longenecker, 1937). The composition of each subfraction was calculated from their respective saponification equivalents and iodine values according to the method recommended by Hilditch

(1956). The final composition of the fat was then computed from these figures.

Determination of component glycerides. A portion (195 g.) of the neutral fat (i.v. 62.7) was dissolved in acetone (1.95 l.) and the solution kept at 0°, when 20.6 g. (i.v. 40.8) of glycerides crystallized out. The soluble portion, 174.4 g. (i.v. 63.5), was again crystallized from acetone (1.74 l.) at -12°. The soluble portion (93 g.; i.v. 65.7) and the insoluble portion (81.4 g.; i.v. 59.4) were further crystallized separately from acetone (20 g./100 ml.) at -12°; the former gave an insoluble portion (11 g.) with i.v. 60.3 and a soluble portion (82 g.) with i.v. 66.4. The latter gave an insoluble portion (35.5 g.) with i.v. 51.4 and a soluble portion (45.9 g.) with i.v. 62.6. Attempts were made to crystallize all the above fractions from ether at various temperatures, but were unsuccessful.

Different fractions thus obtained were combined according to similarity of iodine values. The procedure gave three fractions of 56.1 g. (i.v. 47.5), 56.9 g. (i.v. 62.1) and 82.0 g. (i.v. 66.4) respectively which were named *A*, *B* and *C*. The mixed acids obtained by the usual method from these fractions were further subjected to the lead salt-ethanol method of separation. These acid fractions were called *AA*, *AB*, *BA*, *BB*, *CA* and *CB* respectively. Each fraction was converted into the methyl ester and fractionated by distillation. Lastly, the possible component glycerides were calculated from the composition of the individual group in the manner described by Hilditch & Meara (1942).

A separate determination of the fully saturated glycerides by the method of Hilditch & Lea (1927) was done to check the computed values.