in the choline-deficient rat.

Summary. Liver fat accumulation was studied in groups of young rats fed one of 3 low protein diets containing, respectively, 5%, 20% and 38% lard. Each diet furnished the same quantity of protein (casein and soy-a-protein). The rapidity and magnitude of fat accumulation was greatest in rats fed the 38% lard diet and least in those receiving the one containing 5% lard. In previous studies the same diets were fed for 15 weeks; the 38% lard diet produced the greatest incidence and the most severe degree of cirrhosis, whereas the group fed the 5% lard diet had the least severe degree of liver damage. The severity of the fatty cirrhosis that ultimately develops in choline deficient rats is chiefly determined by rate and magnitude of fat accumulation in the liver in the first 3 weeks of the experiment.

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Further Purification of TSH-Releasing Factor (TRF) from Sheep Hypothalamic Tissues, with Observations on the Amino Acid Composition.* (30063)

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We have reported previously(1) on the existence in crude extracts of hypothalamic tissues, of a polypeptidic substance (TRF) which specifically stimulates *in vivo* the secretion of adenohypophysial thyrotropin (TSH). We have subsequently described the early stages of purification of TRF(2,3) as well as its *in vitro* activity(4) on a system of isolated pituitary fragments. Evidence has been presented(1,2,4) to propose that TRF is a substance different from LRF (LH-Releasing Factor), CRF (Corticotropin-Releasing Factor), a- and β-Melanocyte Stimulating Hormones, bradykinin, and Von Euler's Substance P, all polypeptides known to be present in hypothalamic tissues. This paper reports the further purification of hypothalamic TRF.

Experimental procedures and results. a. *Starting materials, acetic acid extraction, gel filtration on Sephadex G25, bioassay.* Table I summarizes description of starting materials and of early stages of the purification procedure as described earlier(1,2). The 25 kg of hypothalamus without median eminence were lyophilized in industrial freeze-drying equipment prior to extraction with acetone (100 vol/1 g material). Two sizes of Sephadex G25 columns were used: 5.5 × 150 cm to which were applied 7.0 g of lyophilized acetic acid extract of the hypothalamic tissues; or 15 × 150 cm on which were applied 120-140 g of the same extract (Fig. 1). The bioassay for TRF used throughout this study is the *in vivo* method described earlier(5). Extraction and studies of biological activity through the Sephadex G25 filtration stage were conducted on aliquots of the 2 types of starting material used here, *i.e.*, median eminence-stalk fragments and hypothalamus fragments without median eminence (Table I). Because of the similarity of the results obtained (see *Discussion*), the bulk of the 2 types of material was pooled and processed further as in Table I.

b. *Counter current distribution.* The conditions as in(3) are summarized in Fig. 2,3; yields are reported in Table I.

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TABLE I. Summary of Steps in Purification of Hypothalamic TRF.

Other fractions	Description of starting material	Wt of material handled and/or obtain at each step of purification	Approximate minimal active dose ($p \leq 0.05$) in *in vivo* bioassay
	60,000 sheep median eminence and stalk 20,000 sheep hypothalamus without median eminence	30 kg 25 "	
	↓	↓	
Acetone extract inactive	← Acetone powder	→ 5 "	
	↓	↓	
15% w/w residue inactive	← 2N acetic acid extract	→ 1,200 g	
	↓	↓	
5% *Idem*	← 0.1M pyridine acetate extract	→ 1,000 "	
	↓	↓	
TSH, LRF, CRF, Vasopressin, Oxytocin, MSH, etc. (see 1, 3)	← Gel filtration on Sephadex G25 in 0.5 M pyridine acetate pH 5.6	→ 50 " TRF concentrate →	5.0 mg
	↓	↓	
LRF, Vasopressin, MSH (see 3)	← Counter current distribution 250 transfers n-butanol-pyridine-acetic acid	→ 3.8 g →	400 μg
	↓	↓	
	Counter current distribution 1000 transfers n-butanol-pyridine-acetic acid	→ 450 mg →	50 "
	↓	↓	
	Chromatography on IRC-50 $NH^4Ac \rightarrow HOAc$	→ 35.0 " →	10 "
	↓	↓	
8 other fractions inactive in TRF assay	← TLC on cellulose BuOH-HOAc-H^2O 4:1:5	→ .4 " →	.10 "
	Rechromatography TLC on cellulose BuOH-HOAc-H^2O 4:1:5	TRF, single spot at $\leq$ 100 μg/spot	

c. *Ion exchange chromatography on IRC-50.* Following the 1000-transfer distributions, the zone containing TRF (K = 0.41) as determined by the bioassay was separated, evaporated to dryness, redissolved in dilute acetic acid and lyophilized. Aliquots of this material were chromatographed on IRC-50; after several pilot experiments, the following conditions were adopted: the resin was prepared in the NH_4^+ form, according to Hirs *et al*(6) and equilibrated with ammonium acetate 0.02 M, pH 5.0; 100 mg to 750 mg of the TRF concentrate following the 1000 transfers step (Table I) were applied on a column 1 × 30 cm, flow rate 2-3 ml/hr, 2.0 ml fractions being collected. After about 12 hold-up volumes, the ammonium acetate buffer was changed to 0.5 N acetic acid, and after about 20 hold-up volumes to 5.0 N acetic acid. Peptide concentration was followed by measuring the O.D. of the effluent at 275 mμ. After completion of the chromatography, the effluent was separated in several fractions (Fig. 4) which were lyophilized, redissolved in 0.01 N acetic acid and assayed for TRF activity. TRF activity was consistently and solely located in a zone of the effluent appearing after 2½ hold-up volumes following application of the 0.5 M acetic acid eluent. From the results of assays on single tubes rather than on lyophilized fractions, it appears that the peak of TRF activity does not correspond to the peaks of optical density at 275 mμ on the basis of which fractions 9 and 10 were cut in the experiment reported in Fig. 4.

d. *Thin-layer chromatography on cellulose.* An aliquot of the lyophilized material from the fraction containing TRF activity after IRC-50 was applied to a thin-layer chroma-

tography plate with a 0.25 mm layer of MN-Cellulose N300 (Brinkmann Instr. Co.). The chromatogram was developed with butanol-acetic acid-water (4:1:5) upper phase. Reaction with ninhydrin in butanol-collidine showed 9 clearly identified spots (Fig. 5a). Four preparative separations were then performed on the material with TRF activity recovered after the IRC-50 stage; fractions were eluted in 0.5 N acetic acid and assayed for TRF activity. Only one fraction (Fraction 5, R_f : 0.35) showed TSH-releasing activity. Rechromatography of an aliquot of this material showed it to give a single spot with ninhydrin; at twice the amount a trace of a component with a lower R_f value appeared (Fig. 5b), which suggests that in sampling the preparative TLC plate, the cut was not completely accurate and a trace of Fraction 4 (Fig. 5a) was included. The TRF spot gave a strongly positive reaction with Pauly's reagent (diazotized sulfanilic acid) (Fig. 5c) and a reddish color with ninhydrin in collidine-butanol.

e. *Amino acid composition.* Hydrolyses were performed in 6 N HCl, at 110°C for 24

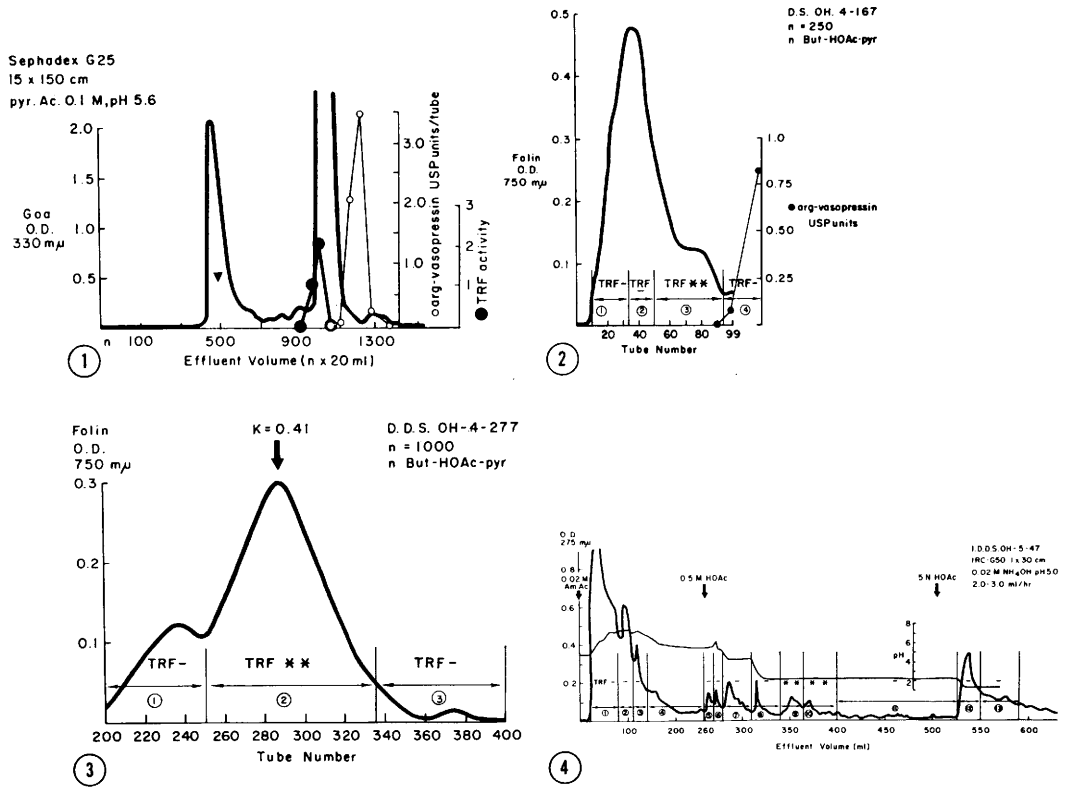

FIG. 1. Gel filtration of 146.0 g of 2N acetic acid extract of ovine hypothalamus without median eminence-stalk (ca 15000 fragments)—Sephadex G25, medium grade, pyridine acetate 0.1M pH 5.6, flow rate 400.0 ml/1 hr, column size: 15 × 150 cm. ●: TRF activity, arbitrary scale, relative activity in the assay, when aliquots of the effluent are assayed simultaneously (see text, a). ▲ in non-retarded fraction (tubes 400-600) refers to TSH-like activity observed in this area of effluent. Vasopressin, measured by recording blood pressure of pithed rat, using USP reference standard for posterior pituitary.

FIG. 2. Counter current distribution for 250 transfers of 2.0 g of TRF-concentrate following Sephadex G25. Fractions combined as indicated by circled numbers. Fraction 4 is the effluent upper phase from transfers 100 to 250. Statistical analysis of bioassays by Dunnett's t test as in (11): (—), not significant; (**), $p = 0.01$.

FIG. 3. Counter current distribution for 1000 transfers of 1.7 g of the TRF fractions pooled from 10 distributions as in Fig. 2. Designations as in Fig. 2.

FIG. 4. Ion exchange chromatography on IRC-50, of 750 mg of the TRF fraction from one 1000 transfer distribution as in Fig. 3. 2.0 ml fractions. Heavy line, O.D. at 275 mμ; thin line, pH. Other designations as in Fig. 2.

hours, under nitrogen; amino acid analyses were run on the Beckman Spinco model 120B accelerated system analyzer. An aliquot of the material with TRF activity after IRC-50 (Fig. 4) gave the following molar ratios of amino acids: Lys_3, His_4, Arg_3, Asp, Thr, Ser_2, Glu_2, Pro, Gly_2, Ala, Val, Leu_5, Tyr, Phe_2. After preparative thin-layer chromatography, the highly purified TRF (Fig. 5b) yielded the following molar ratios of amino acids: Lys, His_4, Thr, Ser, Glu_3, Pro_3, Gly, Ala, Met, Leu, Tyr. Hydrolysis of the fraction 4 from the preparative thin-layer chromatography (Fig. 5a) gave the following molar ratios of amino acids: Lys, His_4, Asp, Thr, Ser_3, Glu, Gly, Ala, Val_2, Leu_2, Tyr_3; this material, which has no TRF activity, was analyzed for comparison with the TRF because it is closely related to TRF in terms of physical behavior in various systems (Sephadex, CCD, IRC-50, TLC partition chromatography) and also because it was still detectable as a minor contaminant in this preparation of TRF (Fig. 5b). The level of this contamination was insufficient, however, to contribute significant quantities of aspartic acid to the TRF fraction. The molar ratios of amino acids reported above and the behavior of TRF during molecular sieving as compared to vasopressin and MSH(1,7, also Fig. 1) *suggest* an octadecapeptide for hypothalamic TRF. A final quantitative analysis for TRF cannot now be claimed, however, on account of the small quantities of peptide available for the level of sensitivity of our current method of analysis.

f. *Biological activity.* Table I shows doses of materials used in the bioassay at various steps of the purification. It can be seen by calculating the total number of minimal active doses available in the yields (in dry weight) at each of the purification steps that no loss of biological activity was evident up to the chromatography on IRC-50. Even though this last procedure introduces a considerable purification factor, it may have led to irreversible adsorption or inactivation of some of the material. At the doses tested, purified TRF has no LRF or CRF activity: at the first counter current distribution step the doses of material active in the TRF assay (Table I) were inactive in the assay for LRF (measurement of plasma LH in castrated-estradiol-progesterone blocked rat [see

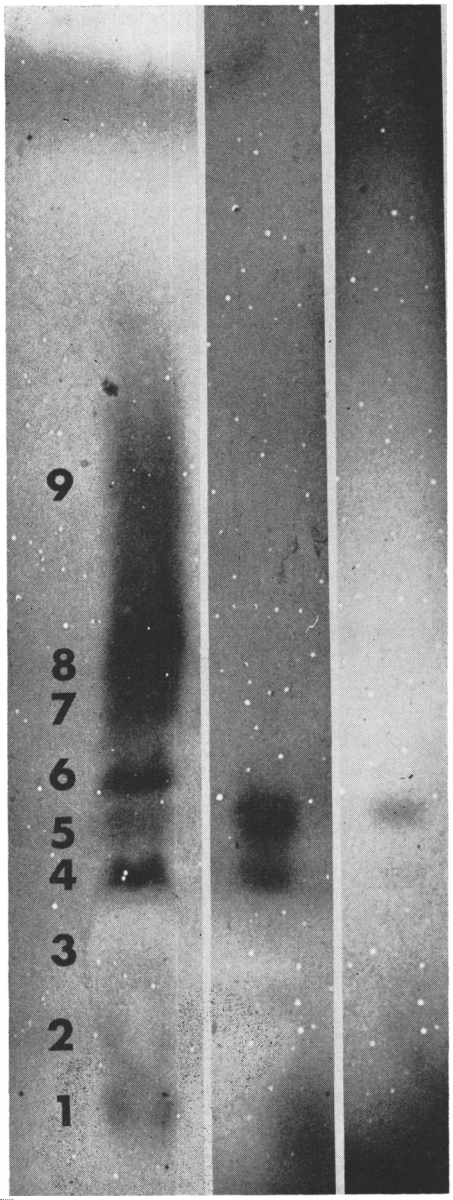

FIG. 5. a: Thin layer chromatography of an aliquot of fractions 9 + 10 following IRC-50 as in Fig. 4; TRF-activity only in fraction 5. b: Rechromatography (TLC) of fraction 5 from a preparative scale TLC as in Fig. *5a*; note the concentration of the TRF active fraction (fraction 5) and diminution of the contaminant (fraction 4), when compared to Fig. *5a*; ninhydrin staining. c: Pauly reaction on the plate of Fig. 5b; the peptide spot does not photograph well because of the reddish background.

9]) and in the assay for CRF (measurement of plasma corticosterone in Dexamethasone-Nembutal blocked rat [see 10]).

Discussion. Schreiber *et al*(8) have previously claimed isolation of a hypothalamic material of bovine origin with TSH-releasing activity; no evidence of homogeneity of the material discussed was presented. The composition reported by these authors (Lys, Val, Ser, Gly, Ala, Asp, Glu, Leu, Ileu) for this material differs considerably from that of the preparation described here.

Some of the data obtained in the experiments reported here bear on the relative concentration of TRF in various areas of the hypothalamus, *i.e.*, the median eminence-stalk region *vs* the remaining part of the hypothalamus. Equal weights of these 2 tissues give approximately similar quantities (in terms of biological activity and also in terms of dry weight) of the TRF fraction at the Sephadex G25 stage. Thus we have here no evidence that would be in favor of a gradient of concentration of TRF towards median eminence, as it has been reported to be the case for LRF(9). Following these observations, it was decided to use indifferently for the purification procedure of TRF (Table I) starting material made of median eminence-stalk or hypothalamus free of median eminence. In recent studies we have found it even simpler to use fragments containing the total hypothalamic area with the attached pituitary stalk which ensures presence of the median eminence.

On the basis of the results shown here, our earlier report(1) of the presence of TSH activity in crude extracts of hypothalamic tissues is confirmed; it is further extended to show the presence of TSH activity in the non-retarded fraction of extracts of hypothalamic fragments *without* median eminence and pituitary stalk (Fig. 1). This observation does not answer the question of the origin of adenohypophysial (-like) hormonal activities in hypothalamic tissues; it cannot, however, be explained simply in terms of contaminating pituitary tissues of distalis or tuberalis origin. The terminology used above in referring to *TSH activity* is in keeping with the caution that must be exercised here as we should not imply that adenohypophysial hormone TSH is necessarily involved in these observations.

Perhaps the most obvious comment suggested by the results reported here relates to the large number of brain fragments (hypothalamus) that were necessary for purification of a small quantity of the hypothalamic neurohumor. Obviously a much larger number of brains will be necessary to provide enough of the polypeptide to approach its amino acid sequence. In our earlier work on *hypothalamic* CRF or in that of others (references in 10), the corticotropin releasing factor was never isolated in a homogeneous form: indeed the quantities of hypothalamic tissues used as starting material were much too small, in view of our current knowledge, to provide meaningful quantities of a hypothalamic neurohumor. Regarding our earlier isolation of α-CRF and β-CRF of *neurohypophysial* origin, one of us has commented elsewhere(10) about the possible significance of these substances. Thus, the problem of availability of large quantities of hypothalamic fragments collected in adequate conditions (sampling completed in less than 30 minutes after slaughtering) remains the absolute prerequisite for a meaningful program on isolation of hypothalamic releasing factors. In contradistinction to the erratic results encountered along the purification of CRF using neurohypophysial extracts as starting materials (see [10] and Saffran's discussion in the same reference, p. 126), the frozen hypothalamic fragments kept in freezers at $-20°C$ or the lyophilized fragments kept at $+4°C$ have proved, in the studies reported here, to be stable starting materials from which reproducible results could be expected.

Summary. Eighty thousand hypothalamus fragments of sheep brains (wet weight 55 kg) were extracted with 2 N acetic acid. The extract was filtered on Sephadex G25 in pyridine acetate 0.1 M, pH 5.6. The TRF-active zone was located by bioassay and further purified by CCD (250 transfers followed by 1,000 transfers) using the system n-butanol-pyridine-acetic acid (5:3:11), ion exchange on IRC-50 equilibrated with ammonium acetate 0.02 M, pH 5.0, and finally thin-layer chromatography on cellulose (butanol-acetic

acid-water, 4:1:5). The material obtained (ca 400 μg) at the last stage of purification was active *in vivo* to stimulate release of TSH at ≤0.1 μg dry weight. Highly purified TRF has no LH- or ACTH-releasing activity. Amino acids present in this preparation are reported.

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Antigenicity of the Peptide Chains Isolated from Human Hemoglobins A and F.* (30064)

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The conflicting reports concerning the antigenicity of the various human hemoglobins led to a study of the antigenicity of the isolated polypeptide components of hemoglobin. Herein is reported the first phase of this investigation.

Employing the isolated polypeptide subunits of the weakly antigenic human hemoglobins(1,2,3,4,5,6), we have observed the production in rabbits of antibodies specific for the α and β and γ polypeptide chains from hemoglobins A and F. These antibodies form insoluble complexes with their specific antigens and with the hemoglobin containing the peptide antigens.

Materials and methods. Each peptide chain emulsified in Freund's adjuvant, was injected subcutaneously into 2 rabbits at 4 sites. An initial injection of 20 mg (total) was followed for 10 weeks by weekly injections of 10 mg amounts. Two months later, the animals were reinjected at monthly intervals with 50 mg of peptide. The animals were bled biweekly.

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The α and β peptides of hemoglobin A were isolated by countercurrent distribution and the amino acid composition determined (7). Each peptide contained less than 1% of one residue of isoleucine. The β peptide was free of α peptide contaminant. The α peptide was associated with 10 to 15% of β peptide. The γ peptide, isolated from purified hemoglobin F by differential solubility, included approximately 25% α peptide. The amount of contamination of the antigen preparation was estimated using simultaneous equations formulated on the basis of the amino acid residues present in the α, β and γ peptides. The peptide content of each antigen was confirmed, using 7 M urea-starch gel electrophoretic fractionation in 0.1 M veronal buffer, pH 8.0, 350 volts for 24 hours. The antigen-antibody reactions were visualized by the Ouchterlony micro-double-diffusion method(8). The isolated peptide antigens were solubilized in rabbit serum. This serum from control (non-injected) rabbits had been previously tested to show that it did not react with the antiserum. All hemoglobins were purified by starch gel electrophoresis and the hemoglobin (as an antigen)