other species. This paper describes the isolation and identification of 2-amino-3-hydroxyacetophenone from the urine of rats. The biosynthetic pathway by which the compound is formed from tryptophan was discussed by DALGLIESH[1].

Isolation and Identification of 2-amino-3-hydroxyacetophenone. Male Wistar albino rats, weighing 150 to 200 g, received daily for 10 days 600 mg of L-tryptophan per kg of body weight by i.p. injection. Ten 24-hour urine collections from 6 rats (1000 ml) were pooled, centrifuged and filtered. The filtrate was acidified to pH 3 with acetic acid and shaken with ethyl ether-ethanol (3:1). The organic phase was evaporated to dryness under nitrogen. The residue was dissolved in 100 ml of 1*N* HCl and hydrolyzed at 80 °C for 1 h under nitrogen gas. The solution was adjusted to pH 3–4 with sodium hydroxide and treated with ethyl ether. The ether extract was washed with 0.1% Na_2CO_3 solution, dried over sodium sulfate, anhydrous, and concentrated in vacuo. The sticky dark brown residue was dissolved in a small volume of dried ether and applied to a column of silicic acid, 1.5 cm in diameter and 9 cm long, which was then eluted by 30 ml of dried ether free from peroxide. The eluate was evaporated to dryness under nitrogen. The residue was dissolved in a small volume of methanol and partially purified by ascending paper chromatography. Whatman No. 3 MM papers were used with the solvent system of MASON and BERG[2] containing 1 ml of glacial acetic acid per 100 ml of solvent. *n*-Butanol-acetic acid-water (4:1:1) was also used. The fluorescent area corresponding to 2-amino-3-hydroxyacetophenone of the chromatograms was cut out and treated with methanol : ether (1:2) to extract the fluorescent material. The extract from paper strips was concentrated in vacuo. The residue was further purified by thin-layer chromatography. Thin layers of silica gel G (Merck, Darmstadt, Germany) were prepared on glass plates. The solvent systems of ethylacetate-isopropanol-28% ammonia water (9:6:4) and chloroform-ethylacetate-formic acid (60:40:1) were used. The details of thin-layer chromatography have already been described[3,4]. The isolated material was pure on both thin-layer and paper-chromatograms.

The comparison of the new compound from rat's urine with authentic sample. The isolated compound had Rf values identical with synthetic 2-amino-3-hydroxyacetophenone in the various solvent systems used for paper and thin-layer chromatography (Table). When spots of natural and synthetic 2-amino-3-hydroxyacetophenone were subjected to the color reactions of Ekman's reagent[5], diazotized sulfanilic acid (DSA)[6] and Ehrlich's reagent[5], identical color development was obtained with both natural and synthetic samples, with absorption maxima at 233, 270 and 378 nm in methanol. When the isolated compound was converted to a volatile derivative with diazomethane and trifluoroacetic anhydride and analyzed by gasliquid chromatography according to the method described before[7], the retention time of the volatile derivative of the isolated compound was indistinguishable with that of the volatile derivative (2-trifluoroacethylamino-3-methoxyacetophenone) of authentic sample.

Results of paper and thin layer chromatography of the product with authentic sample

	Product	Synthesized
Paper chromatography[a]		
Mason-Berg with 1% acetic acid, Rf	0.91	0.91
n-Butanol-acetic acid-water (4:1:1), Rf	0.88	0.88
Thin layer chromatography		
Ethylacetate-isopropanol-28% ammonia water (9:6:4), Rf	0.88	0.88
Chloroform-ethylacetate-formic acid (60:40:1), Rf	0.72	0.72
Fluorescence at 3650 Å	Greenish blue	Greenish blue
Diazotized sulfanilic acid	Pink orange	Pink orange
Ekman's reagent	Purple	Purple
Ehrlich's reagent	Pink orange	Pink orange
Absorption maxima at ph 7.0 (nm)	233	233
	270	270
	378	378

[a] Whatman No. 3 MM filter paper.

Zusammenfassung. 2-Amino-3-hydroxyacetophenon wurde aus Rattenharn nach Verabreichung von L-Tryptophan isoliert und identifiziert.

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Effects of Estradiol on the in vitro Incorporation of Acetate-1-^{14}C and Choline-1,2-^{14}C into the Phospholipids of Human Peripheral Arteries

Synthesis of total phospholipids by the human arterial wall has been demonstrated in vivo from phosphate-^{32}P[1] and in vitro from acetate-^{14}C[2,3]; recently the synthesis of the individual phospholipid classes in human arteries has been investigated[4].

Estrogenic hormones have been shown to increase phospholipid synthesis in liver[5], uterus[6] and human and canine arterial intimas[7]. Estrogen treatment in humans specifically increases serum lecithin levels[8], and in rats specifically increases the lecithin species containing stearic and arachidonic acids[9].

The present study is a preliminary investigation into the effects of estradiol on the incorporation of precursors into the individual phospholipids and phospholipid fatty acids of human peripheral arteries.

Material and methods. Above the knee amputated legs were obtained immediately after surgery, and the distal femoral artery and popliteal and tibial arteries dissected

free. Ten specimens were obtained, all of which were from male patients, ages 60–80 and all of which showed advanced occlusive atherosclerotic changes. The arteries were freed of adventitia and incized longitudinally down the middle; one-half was placed in an isotonic Krebs-Ringer phosphate buffer solution with glucose, and the other half in the same solution containing in addition estradiol, 10^{-5} *M*. After addition of 20 µc of either acetate-1-^{14}C or choline-1,2-^{14}C (Nuclear Chicago, both adjusted to specific activity of 1.03 mc/m*M*) to each, the flasks were incubated for 4 h in an air atmosphere at 37.5 °C in a Dubnoff metabolic shaking incubator. 5 incubations were done per catagory (acetate-^{14}C control, acetate-^{14}C + estradiol, choline-^{14}C control, choline-^{14}C + estradiol). Carbon dioxide was collected in a center well containing 20% NaOH, and the $^{14}CO_2$ radioactivity determined as previously described[10].

Washing the tissues free of radioactive precursor, extraction of the lipids, and isolation of the major phospholipids by thin layer chromatography, phosphorous analysis and radioactivity determinations were all done as previously described[10–12].

Percentages of the major fatty acids and specific radioactivities of the phosphatidyl choline fatty acids after incubation of the arteries with the acetate-^{14}C were determined by gas chromatographic analysis and collection of fatty acids, and liquid scintillation counting, all as previously described[10]. Of the fatty acids, only palmitic, stearic and oleic acids had sufficiently high activity for accurate collection and counting.

Probabilities that observed differences in the data were due to chance were calculated by the *t*-test, and only those differences where $P < 0.05$ have been considered significant.

Results and discussion. The amounts of the major individual phospholipids in mg/g dry weight of arteries ± S.D. were as follows: sphingomyelin 2.4 ± 0.5, phosphatidyl choline 3.5 ± 0.8, phosphatidyl serine 1.3 ± 0.2, phosphatidyl ethanolamine 1.9 ± 0.4; incubation with estradiol had no significant effect on these amounts.

The amounts of $^{14}CO_2$ evolved during the incubations in DPM/mg dry wt. of artery were as follows: acetate-^{14}C + no estradiol 1750 ± 162; acetate-14C + estradiol 1670 ± 144; choline-^{14}C + no estradiol 106 ± 13; choline-^{14}C + estradiol 95 ± 11.

The specific activities of the major arterial phospholipid classes after incubation with acetate-^{14}C or choline-^{14}C, with or without estradiol added, are indicated in Table I. Choline-^{14}C appeared to be incorporated to a greater extent than the acetate-^{14}C. This may have been partially due to the lesser availability of substrate due to the more rapid oxidation of acetate to CO_2, to the greater utilization of acetate by other pathways as compared to the more selective utilization of choline for phospholipid synthesis, or to the utilization of pre-formed glycerides rather than newly synthesized fatty acids for phospholipid synthesis.

Acetate-^{14}C incorporation was greatest into the phosphatidyl choline fraction, and estradiol added to the media significantly increased incorporation only into phosphatidyl choline. Incorporation into phosphatidyl ethanolamine was slightly reduced by estradiol.

In Table II are shown the specific activities of the fatty acids of phosphatidyl choline which incorporated sufficient acetate-^{14}C for accurate counting. Palmitic acid incorporated by far the highest amount of precursor in both groups. Estradiol added to the media appeared to significantly increase incorporation of acetate-^{14}C into palmitic acid only. The percentages of the individual phosphatidyl choline fatty acids were: palmitic 35.2 ± 3.6, stearic 6.5 ± 0.9, oleic 19.6 ± 2.3, linoleic 12.0 ± 1.8, and arachidonic 16.7 ± 3.0. These proportions were not significantly affected by incubation with estradiol.

The demonstrations in these experiments of specific increases in incorporation of acetate-^{14}C into the phosphatidyl choline fraction of human peripheral arteries, and also increased choline-^{14}C incorporation into these arteries induced by estradiol are similar to the observed effects of estradiol on uterine phospholipid and fatty acid synthesis previously reported[5, 6, 13]. The enhanced incorporations induced by estradiol in the present experiments may have been an additive result of increased de novo fatty acid synthesis and a stimulation by estradiol of incorporation of the fatty acid, choline and/or phos-

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Table I. Specific activities of human arterial phospholipids after incubation with acetate-1-^{14}C or choline-1,2-^{14}C, with and without estradiol[a]

	Acetate-1-^{14}C		Choline-1,2-^{14}C	
	No estradiol	Estradiol	No estradiol	Estradiol
Sphingomyelin	360 ± 64	440 ± 77	820 ± 67	1,260 ± 140
Phosphatidyl choline	1,450 ± 130	2,310 ± 250	18,250 ± 1,100	27,900 ± 1,580
Phosphatidyl serine	1,710 ± 182	1,530 ± 125	1,150 ± 99	1,040 ± 124
Phosphatidyl ethanolamine	1,480 ± 131	1,020 ± 104	1,230 ± 85	1,180 ± 130

[a] DPM/mg phospholipid ± S.D.

Table II. Specific activities of human arterial phosphatidyl choline palmitic, stearic and oleic acids after incubation with acetate-1-^{14}C, with and without estradiol[a]

	No estradiol	Estradiol
16:0	2,450 ± 260	4,300 ± 382
18:0	620 ± 103	560 ± 116
18:1	270 ± 54	225 ± 70

[a] DPM/mg fatty acid ± S.D.

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phate precursors into phosphatidyl choline. The determination of the exact stage of phosphatidyl choline synthesis which is affected by estradiol will require further study[14].

Zusammenfassung. Bei Bebrütung von Arterien in Gegenwart von Östradiol wurde der Einbau von ^{14}C-Azetat und ^{14}C-Cholin in Phosphatidylcholin deutlich vermehrt.

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Inhibition of Phospholipase A by a Naturally Occurring Peptide in *Bothrops* Venoms

Compared with other snake venoms, the activity of phospholipase A (EC 3.1.1.4.) in the venoms of *Bothrops* species (e.g. *B. neuwiedii* (Argentine and Brazilian), *B. jararaca, B. jararacussu, B. atrox*) is relatively weak[1]. Furthermore, with these venoms the enzyme activity shows a characteristic lag-period (15–18 min), which can be observed either with egg-yolk lipoprotein[1,2] or with pure sonicated phosphatidylcholine[3] as substrate. The results reported here show that both the apparent inactivity of the enzyme and the lag-effect are due to a peptide inhibitor, normally present in *Bothrops* venoms which, under well defined medium conditions, is highly effective on phospholipase A. It must be recalled that Habermann[4] had already postulated the role of a natural inhibitor as cause of the initially low activity of phospholipase A in the venom of *Crotalus d. terrificus*.

Venom enzyme and inhibitor can be separated by a fractionation procedure which, briefly, consists of the following steps: a) two gel-filtrations on Sephadex G-50 at pH 4.5; b) chromatography on SE-Sephadex C-25 at pH 4.5 with concave gradient elution and c) gel-filtration on Sephadex G-25 at pH 7.4. Figure 1 presents a typical elution pattern for Step 3. Peak F_e, eluted with the void volume, contained enzyme activity but did not show lag. The total phospholipase activity recovered in peak F_e was 275% of that measured in the original venom sample. The second, included peak F_i (K_{av}-value = 0.252) was enzymatically inactive but its addition to F_e provoked both a strong inhibition of the enzyme and a lag which resembled that observed[5] with the crude venom (Figure 1; upper-right corner). Further purification of F_e on DEAE-cellulose at pH 7.6, with linear or concave gradient elution (step 4 of phospholipase A purification) yielded 2 electrophoretically homogeneous fractions with specific activities 62- and 54-fold higher, respectively, than the crude venom. The pure phospholipase fractions were also strongly inhibited by F_i.

Purification of F_i by chromatography on SE-Sephadex at pH 4.5 (concave gradient elution), by paper electrophoresis or by paper chromatography yielded a ninhydrin-reacting material. F_i lost its inhibitory activity after treatment with trypsin or acid hydrolysis (10 N HCl). Paper chromatography of the hydrolysate yielded several amino acids, which is consistent with the peptide nature of F_i. After this work was completed a similar peptide inhibitor has been reported by Braganza et al.[6] in *Naja naja* venom (fam. Elapidae).

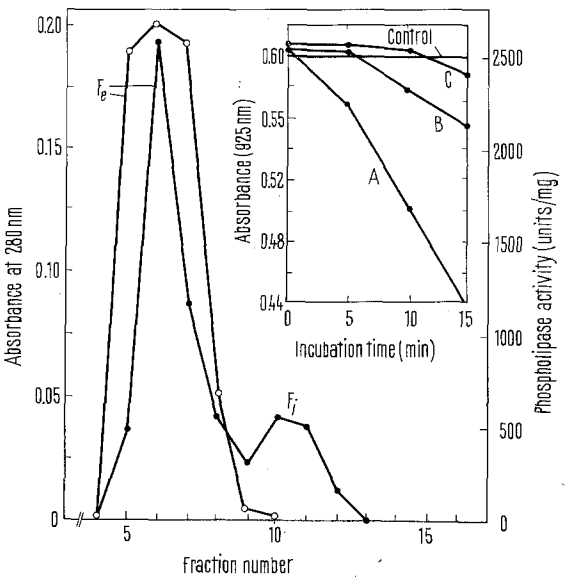

Fig. 1. Separation of phospholipase A and its inhibitor by gel-filtration on Sephadex G-25 (step 3 of the purification procedure). The column (20.5 × 1.8 cm; gel-bed volume, 53.5 ml) was equilibrated with 1 mM EDTA, 5 mM phosphate-*Tris* buffer pH 7.4, at 0–2 °C. The sample (2.2 ml, 248 m-units of A_{280} per ml) was applied to the column and eluted with the equilibration buffer. 2.7 ml fractions were collected at a flow rate of 2.3 ml/h. ●, absorbance at 280 nm; △, phospholipase specific activity measured with the turbidimetric method[1]. At the right, upper corner, inhibition of F_e by F_i. The curves represent the phospholipase activity of 0.14 mg of F_e preincubated 5 min at 0 °C with buffer (curve A) or with 0.56 (curve B) and 1.13 (curve C m-units A_{280} of F_i.

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