evidence against the direct action of estrogen on the anterior pituitary and suggests an indirect effect, probably through the hypothalamus.

Summary. Addition of estradiol 17-B to the medium of anterior pituitaries cultured *in vitro* for 3 days resulted in a decrease in lactogen production. Estradiol levels of 0.1, 0.5, and 5.0 μg/ml of medium lowered lactogen production 16.7, 23.9, and 27.3%, respectively, below that noted for controls. The administration of 1 and 10 μg of estradiol/day for 10 days *in vivo,* and subsequent culturing of pituitaries from these animals *in vitro,* resulted in a lactogen production 31.6 and 27.6% higher, respectively, than that of the control. Co-cultures of anterior pituitaries and hypothalami with estradiol in the medium (0.5 μg/ml) did not significantly alter lactogen production from that noted for the control. Electrophoretic separation of the proteins present in lyophilized control medium revealed the presence of 4 components, the most prominent of which was the second. In cultures where estradiol was added to the medium, electrophoretic separation indicated that the second component was now equal in intensity to that of the third. Lyophilized medium from cultures in which pituitaries were estrogenized *in vivo* resulted, when separated electrophoretically, in the second component being more intense than the second component of control medium. The intensity of the second protein component appeared to be related to the lactogen content of the medium. It is believed that lactogen stimulating effect of estrogen *in vivo* is not directly on the anterior pituitary but acts indirectly, probably through the hypothalamus.

1. Reece, R. P., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1936, v34, 402.
2. ———, *Mo. Agr. Exp. Sta. Res. Bull.* 1937, 266.
3. Meites, J., Turner, C. W., *ibid.,* 1948, 415,
4. ———, PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 190.
5. Victor, J., Anderson, D. H., *Am. J. Physiol.,* 1937, v120, 154.
6. Gaul, G., Villee, C. A., *J. Clin. Endocrinol. Metab.,* 1959, v19, 1357.
7. Nicoll, C. S., Meites, J., *Endocrinology,* 1962, v70, 272.
8. Ratner, A., Talwalker, P. K., Meites, J., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 12.
9. Meites, J., Kahn, R. H., Nicoll, C. S., *ibid.,* 1961, v108, 440.
10. Gala, R. R., Reece, R. P., *ibid.,* 1963, v114, 422.
11. Kanematsu, S., Sawyer, C. H., *Anat. Rec.,* 1962, v139, 244.

Received December 6, 1963. P.S.E.B.M., 1964, v115.

Experimental Hypertensions Treated with CCl_4: Measurements of Adrenal Function, Vascular Responsiveness, Angiotensinase and Converting Enzyme.* (29110)

HUBERT F. LOYKE (Introduced by A. C. Corcoran)
Department of Clinical Investigation, Research Division, St. Vincent Charity Hospital, Cleveland, Ohio

Elevated blood pressure of experimental renal (ER)(1) and desoxycorticosterone (DCA) hypertensions(2) has been lowered to normal by chronic administration of CCl_4 in rats. A likely cause of this is deficiency of renin-substrate (hepatogenous angiotensinogen (ATgen)(3), but serum concentrations of this protein were not altered by CCl_4 in ER hypertensive rats(4). Other possible causes of the blood pressure reduction considered in this report are: 1, adrenal insufficiency, 2, decreased vascular responsiveness, 3, increased "angiotensinase" (ATase) and 4, impairment of "converting enzyme" (CE). This last transforms inactive deca-peptide, angiotensin I (AT I), to the active octa-peptide, angiotensin II (AT II).

Methods. Male rats weighing 120 to 140 g were divided into groups. Renal hypertension was produced in 42 animals by ligating both

* This study was supported by USPHS grant.

poles of one kidney, with contralateral nephrectomy one week later. Pellets containing 20 mg of DCA were inserted subcutaneously in 24 animals. These were divided into various sub-groups.

All rats were fed Purina rat chow and given tap water *ad lib*. The rats were weighed twice weekly and warmed to approximately 40°C for pressure measurements by the tail microphone method(5) (no anesthesia). When hypertension developed and persisted for 3 months, injections of CCl_4 (0.15 ml/kg undiluted analytic reagent) were given subcutaneously twice weekly for 15 consecutive doses. Two rats with renal hypertension were not given CCl_4. Tail blood was sampled at intervals and 24-hour urine samples collected before bleeding.

Adrenal function was estimated from 24-hour urine outputs of Na, K, 17-ketosteroids (17KS) and aldosterone (Ald) by each group and in experimental groups, at the end of both hypertensive and CCl_4 phases. Urinary Ald was determined by the double isotope dilution technique.† Pressor tests were done during amobarbital anesthesia after intraperitoneal injections of heparin (500 U) and 3 mg/kg of pentolinium chloride. Responses were calibrated against standard Val 5 AT II amide (Hypertensin, Ciba #E-7141) and expressed as μg equivalents thereof.

To detect changed vascular responsiveness, certain rats from each group were studied during graded injections of AT and tests were repeated after injection of Ald (d-aldosterone in water 0.1 mg/ml).

Angiotensinase determinations were done by adding 0.5 ml serum samples to 8 ml of a standard solution(6) containing 0.5 mg, i.e., 10 Goldblatt units (GU) of AT, 119 ml H_2O, 5 ml 0.5 M PO_4 buffer pH 7.4, 2 ml 0.1% thiomersal and distilled water to make 100 ml. This was incubated for 2 hours at 37°C, then boiled for 10 minutes to stop the reaction. The supernatant from centrifugation was frozen for assay.

Converting enzyme (CE) activity was demonstrated in sera of each group by chromatographic separation of decapeptide, AT I and presence of octapeptide, AT II. This separation has been described(7) by a procedure other than that used in this study.

Two or three 0.5 ml serum samples were pooled from each group of rats. ATase was destroyed by bringing pH of the plasma to pH 4.0 with H_3PO_4 for 30 minutes at 25°C and then readjusting to pH 7.0 with NaOH. This mixture was incubated with 2 ml of ATase-free, salt-free hog renin (0.3 ml = 1 GU) for 10 minutes at 37°C and pH brought to 5.5 with HCl, followed by boiling for 10 minutes. The supernatant from centrifugation was frozen and stored for assay.

Supernatant (0.2 ml) of each sample was streaked on Whatman #1 paper, 7½″ × 22½″ long. The chromatogram was developed in a descending fashion in an enclosed glass cylinder at 25°C in 1 M NaCl, 0.02 N HCl mixture. When the solvent front reached the end (6 hours), the paper was cut horizontally while wet into 25 strips each 2 cm in width. Each cut was made into a wick eluted by allowing distilled water to flow by gravity through the paper into a trough containing 0.5% bacitracin. Synthetic AT II in graded doses (.005, .010 and .015 μg) was then given to an assay rat followed by 0.6 ml

TABLE I. Angiotensinase and 17-Ketosteroid Determinations in Experimental Renal and DCA Hypertensive Rats Before and Following CCl_4 Treatment. Weights, blood pressure and serum values are average results.

	Control period	Hypertensive period	CCl_4 period
	Systolic blood pressure, mm Hg		
Renal	116	190	144
DCA	108	168	148
Control	110	140	138
	Serum angiotensinase, units/ml		
Renal		1.45	1.37
DCA		1.58	1.50
Control		1.50	1.53
	17-ketosteroids, 24/hour urine		
Renal		.3	.2
DCA		.2	.2
Control		.5	.3
	Number of animals		
Renal	12	12	8
DCA	7	17	7
Control	10	10	7
	Weight in grams		
Renal	127	280	272
DCA	118	326	292
Control	118	272	313

† Aldosterone determinations performed by Bio-Science Laboratories, Los Angeles, Calif.

TABLE II. Twenty-four Hour Urine Means for Aldosterone, Sodium and Potassium, in Both Renal and DCA Treated Rats Compared with Their Controls.

	No. animals	Urine vol (ml/24-hr)	Specific gravity	Urinary aldosterone (μg/24-hr)	Urinary sodium (meq/l/24-hr)	Urinary potassium (meq/l/24-hr)
DCA hypertensive	12	35	1.440	1.12	3.2	8.8
Renal hypertensive and CCl_4	11	52	1.320	.13	3.4	8.5
Control	15	60	1.187	.09	5.0	3.2

of each of the 25 tubes of eluate and again by test doses of standard all in duplicate assay.

Results. Blood pressure (BP). Systolic BP of more than 170 mm Hg (mean, 175) were found in all ER and DCA hypertensive animals. BP in controls ranged from 130 to 145 mm Hg (Table I). After CCl_4, blood pressure was reduced to an average of 144 in ER and 148 in DCA groups. The 2 untreated ERs remained hypertensive (mean 190 mm Hg).

Adrenal factors. Urinary 17 KS 24-hour levels in hypertensive and the CCl_4 periods were somewhat lower (Table I) in hypertensive groups than in controls. Urinary 24-hour K rose and Na decreased during CCl_4 periods. Urinary Ald outputs rose during CCl_4 phases in both ER and to a greater degree in DCA, as compared with control group (Table II).

Vasopressor reactions. Pressor responsiveness to AT (Table III) was not altered by CCl_4 treatment nor affected by immediately preceding intravenous injections of Ald, in any group.

Angiotensinase values were alike (Table I) for both hypertensive groups. During CCl_4 periods in both the ER and DCA groups, levels fell insignificantly (0.08 unit/ml) and were unchanged in controls.

Converting enzyme. Sera of untreated, normal rats (Table IV), tested concurrently with

TABLE III. Response to Graded Doses of Angiotensin and d-aldosterone in Renal, DCA Treated Hypertensive Rats with Their Untreated Normotensive Controls. Angiotensin given (1) prior to and (2) after aldosterone.

Animal			Arterial pressures		Pressure responses mm Hg to graded doses of test agents									
			Pre	Post	Angiotensin (1) (μg)			Aldosterone (μg)				Angiotensin (2) (μg)		
			Anesthesia											
Group	No.	Wt (g)	mm Hg	mm Hg	.005	.010	.015	1	2	3	4	.005	.010	.015
Control	22	310	140	44	26	40	48	2	0	0	3			
	26	350	140	40	20	26	30	0	0	−4	0	16	25	28
	29	315	145	84	12	24	32							
	30	310	145	69	20	30	38	5	−4	0	0	26	28	44
	25	325	140	62	43	59	78	8	10					
	28	335	140	32	40	58	68	6	8	22				
	24	250	140	28	28	30	38	0	0	4	2	22	32	29
	23	310	145	100	14	16	16							
Avg		313	142	57.4	25.4	35.4	43.5	3.5	3	4.3	1	21.3	28.3	33.7
Renal	5	390	170	76	50	52	48	0	0	10	5	39	52	58
	14	430	170	62	30	32	39			2	6	27	40	40
Hypertensive untreated avg		410	170	69	40	42	43.5	0	0	6	5.5	33	46	49
CCl_4	8	280	145	50	16	18	25	0	0	0	0			
Treated	15	310	160	76	18	34	36	4	0	2	3	22	32	32
Renal	13	270	145	49	26	34	42	6	0	0	0	16	18	24
Hypertensive avg		286	150	58.3	20	28.6	34.3	3.3	0	.66	1	19	25	28
CCl_4	19	310	180	128	12	18	26	0	0	8	4	14	30	36
Treated	17	310	150	94	36	44	44	0	0	0	0	22	43	46
	16	315	150	66	40	55	65	6	5	7	8	20	27	42
DCA	20	310	145	39	14	25	32	0	6	0	3	23	30	36
Hypertensive avg		311	156	81.7	25.5	35.5	41.7	1.5	2.75	3.75	3.75	19.75	32.5	40

TABLE IV. Identification of Angiotensin I is in the Area of Tubes 3-4-5-6-7 and Angiotensin II in Tubes 15-16-17-18. Eluate values listed in mm Hg rise for each tube in duplicate assay. Controls were sera of untreated normal rats, listed as above because tests were concurrent with indicated phases in experimental groups.

Type group	Animal No.	Blood pressure (mm Hg)	Eluate tube number with pressure rise in mm Hg																								
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Hypertensive phase																											
Renal hypt			6															6	4						6		
	1–5–6	203	8														7	9					6	9			
			6	6													3		3								
	8–9–10	170	6	4											5	5											17
																	2		2								
	13–14–15	168						9									6	2								8	26
DCA hypt	16–17–18	170	3													4	7	5	2	2	6	5					8
			12		2	10	2									3	3										8
	19–20	165			5	11	6																				
Control	22–23–24	143		5										6	6	7	4		4	8	8		11				
			10														14	18	15								
	25–26–27	140													4	6	9										12
																	2										6
	28–29–30	140															2	3									9
Carbon tetrachloride phase																											
Renal	5–6–14	163														12	16	2								5	17
	13–15	153						5	10																		8
								18	8																	3	4
DCA	16–17	150						15	17																	4	7
			6	2			12	14	6																	7	7
	18–20	143					15	15	2																		10
																	2									4	2
Control	24–25–26	140															2	3								6	6
														14												15	14
	28–29–30	147																								10	9

those of ER and DCA rats during hypertensive and CCl_4 phases of the study, consistently yielded only activity like that of AT II. The same was true of sera of ER hypertensive rats. One pool of sera from DCA hypertensive rats yielded only AT II and the other AT I and II activities. During CCl_4-induced normotension, the only identified product of incubation of sera from ER and DCA rats was AT I, with the exception of a group of ER rats that yielded only AT II, and had not shown a definite fall in pressure.

Post-CCl_4 sera from rats 13 and 15, and in a second experiment, 16 and 17 were mixed with sera of controls and incubated. These yielded as products both ATs I and II whereas control sera repeatedly yielded only AT II (Table IV).

Comment. Blood pressure. As previously reported(1,2,4), CCl_4 administration usually decreased pressures of ER and DCA hypertensive rats while hypertension persisted in untreated ERs.

Adrenal. Increased urinary Ald in DCA and ER CCl_4-treated groups, the absence of an immediate pressor response to injected Ald in controls and hypertensives and lack of post-Ald augmentation of response to AT, seem to exclude deficiency of this hormone as the mechanism of decreased arterial pressure. Further, urinary 17-KS did not differ between test and control groups and adrenals were histologically normal.

Pressor responsiveness to epinephrine and norepinephrine had previously(4) been found similar in pre- and post-CCl_4 groups. CCl_4-treatment had previously somewhat enhanced renin responsiveness, but the present data show that decreased responsiveness to angiotensin is not a mechanism of CCl_4-induced normotension.

Serum angiotensinase values did not differ from normal in the 2 hypertensive groups and were unchanged by CCl_4. Serum ATase has been found variable in ER dogs in which hypertension could develop and persist without alterations in this factor(8). In man, ATase is increased in renal arterial hypertension, chronic glomerulonephritis with hypertension, complicated essential hypertension and pheochromocytoma but reduced in secondary aldosteronism(9). In human cirrhosis, ATase has been found to be increased(10).

Qualitative change in renal pressor system. Serum of ER rats, when incubated with renin is well known to yield AT II. However, proportions of AT I and II present in sera of various types of experimental hypertension have not been evaluated previously. ER and DCA rats showed no consistent differences in characteristics of AT formed, which was substantially AT II. Following CCl_4 treatment that restored pressure to control levels (mean 148 mm Hg), the major reaction product changed from AT II to AT I although serum from the CCl_4-treated ER rats with no fall in pressure (mean systolic pressure 163 mm Hg), resembled untreated hypertensives and controls in yielding primarily AT II. This suggests that CCl_4-induced pressure fall may be associated with circulation of myotropically inactive(11) AT I and defective formation of AT II because of deficit in CE, which is presumed to be formed in liver(12). Since CCl_4 is a hepatotoxin the same mechanism might account for restoration of normotension during cirrhosis with hypergammaglobulinemia(13) in previously hypertensive patients. Were there a deficit in CE, post-CCl_4 sera from ER or metacorticoid DCA CCl_4-treated rats that had shown large falls in pressure should yield AT II when mixed and incubated with CE-containing normal serum. This was not the case in 2 tests. Hence, formation of a CE-inhibitor during CCl_4-induced liver injury seems a likely basic mechanism of the BP-lowering effect of CCl_4.

Summary. During restoration of normotension by giving CCl_4 to experimental renal and post-DCA hypertensive rats, serum incubated with renin yields predominantly angiotensin I. This suggests a deficit in hepatogenous converting enzyme. However, incubation of post-CCl_4 normotensive serum with normal serum containing this enzyme still yielded angiotensins I and II. This suggests that CCl_4 liver injury is associated with formation of a substance that inhibits circulating converting enzyme. Adrenal failure, angiotensinase excess and decreased pressor responsiveness to angiotensin do not participate in the pressure lowering effect of CCl_4.

I am indebted to Dr. S. W. Hoobler (Univ. of Michigan) for encouragement and constructive criticism, to Dr. A. C. Corcoran and Dr. M. N. Levy (this hospital) for editorial assistance, to Dr. W. Wagner (Ciba, Inc.) for supplies of d-aldosterone and hypertensin, to Dr. L. Skeggs for generous advice and particularly to Dr. Paul E. Wisenbaugh (both of Crile V. A. Hospital, Cleveland) for a description of his procedures used for chromatographic separation of angiotensins I and II.

1. Loyke, H. F., Plucinsky, J. J., Crawford, T. L., *Circulation Res.,* 1960, v8, 535.

2. Loyke, H. F., Mackrell, J. S., *J. Lab. Clin. Med.,* 1961, v58, 871.

3. Page, I. H., McSwain, B., Knapp, G. M., Andrus, W. K., *Am. J. Physiol.,* 1941, v135, 214.

4. Loyke, H. F., *Am. J. Med. Sci.,* in press.

5. Friedman, M., Freed, S. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 670.

6. Braun-Menendez, E., Fasciolo, J. C., LeLoir, L. F., Munoz, J. M., Taquini, A. C., *Renal Hypertension,* Charles C Thomas, Springfield, Ill., 1946.

7. Schwyzer, R., Iselin, B., Kappeler, H., Riniker, B., Rittel, W., Zuber, H., *Helv. Chem. Acta,* 1958, v41, 1287.

8. Harakal, M. D., Collins, D. A., *Circulation Res.,* 1956, v4, 612.

9. Hickler, R. B., Lauler, D. P., Thorn, G. W., *J. Clin. Invest.,* 1963, v42, 635.

10. Klaus, D., Kaffarnik, H., Pfeil, H., *Klin. Wschr.,* 1963, v41, 380.

11. Skeggs, L. T., Kahn, J. R., Shumway, N. P., *J. Exp. Med.,* 1956, v103, 295.

12. Page, I. H., Bumpus, F. M., *Physiol. Rev.,* 1961, v41, 331.

13. Loyke, H. F., *Arch. Intern. Med.,* 1962, v110, 45.

Received December 9, 1963. P.S.E.B.M., 1964, v115.

Isolation of Ribonucleotide-Peptide Complexes from *Pasteurella multocida.** (29111)

A. S. ISSALY† AND A. O. M. STOPPANI

Institute of Biochemistry, School of Medicine, University of Buenos Aires, and Laboratory for Cell Metabolism, National Commission for Atomic Energy

The presence of amino acids (or peptides) in ribonucleic acid preparations has been reported with RNA‡ from yeast(1-3), several microorganisms(4), pancreas(5), brain(6), liver(7), tobacco mosaic virus(7), etc. Nucleotide-peptide complexes have been detected in the TCA or ethanol extracts from *Chlorella*(8), yeast(9,10), etc. Similar observations are reported now with *Past. multocida.*

Materials and methods unless stated otherwise were the same as used previously(11). Dowex 1, 2% (X_2) 200-400 mesh and Dowex 50, 2% (X_2) 200-400 mesh were obtained from Bio-Rad Labs., Richmond, Calif., and Zeo-Karb 225 from Permutit Co. Ltd. L-*iso*-leucine-U-C^{14} (1.24 mc/mmole) and Na_2S^{35} (0.36 mC/mmole) were from the Radiochemical Centre, Amersham, Great Britain. The ninhydrin reaction was performed as described by Troll and Cannan(12).

Isolation and hydrolysis of RNA. The methods described in (13-15) were applied according to the following procedure. In steps 1-2 the amount of solvent used was 20 ml/g of cell residue. *Step 1 (removal of TCA-soluble fraction).* Acetone-dried *Past. multocida* (strain Beaufort No. 28 from Pasteur Inst., Paris) was suspended in distilled water and extracted with 5% (w/v) TCA at 4°. After centrifugation the residue was washed in the centrifuge with water, and then with ethanol (twice), ethanol-ether mixture (1:1; v/v) and ether. *Step 2 (removal of phospholipids).* The dried residue was extracted successively with *a*) ethanol-ether mixture (3:1; v/v) at 60° for 15 minutes, *b*) with methanol-chloroform mixture (1:1, v/v) at 65° for

* This investigation was supported by grants from Rockefeller Foundation and Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

† On leave from Inst. Nacional de Microbiología.

‡ Abbreviations used are: RNA, ribonucleic acid; TCA, trichloracetic acid; AMP, adenylic acid; CMP, cytidylic acid; GMP, guanylic acid and UMP, uridylic acid.