Characterization of Lipid Constituents of Human "Fibroblasts" Cultivated *in vitro*.* (28862)

GILES G. BOLE, JR. AND C. WILLIAM CASTOR (Introduced by Burton L. Baker)
Rackham Arthritis Research Unit, Department of Internal Medicine, University of Michigan, Ann Arbor

The lipids of connective tissue cells grown in primary culture have been studied to compare their composition with that of other cells(1,2,3), and tissues(4,5,6). In this communication the lipid composition of several primary cell lines obtained from different donors and anatomical locations is reported and compared with that of 2 "permanent cell lines" cultured *in vitro*. Cells included in this study were examined during the first 2 or 3 months of *in vitro* culture, in order to minimize deviations from the normal *in vivo* state which occur with prolonged serial subcultures (7,8). The primary cell lines form significant quantities of mucopolysaccharide(9), and quantitative determination of their phospholipid composition afforded an opportunity to obtain data that might correlate with isotope tracer studies which have shown rapid turnover of specific phospholipids during cell secretion(10), and phagocytosis(3). During neoformation of experimental granulation tissue it has been demonstrated that local synthesis of lipids(11) occurs with rapid alterations in content and composition of the lipid constituents(12). Like liver tissue, the experimental granuloma is 20% lipid on a dry weight basis. Yet, compared with parenchymal organs the granulomas are cell poor. Therefore, unless lipids occur in extracellular location, an unusually high intracellular concentration would be expected in the connective tissue cells themselves.

Methods. The procedures employed in this laboratory for propagation and harvest of connective cells grown in primary monolayer culture have been reported(8,9). The cell cultures were supported by a semisynthetic medium composed of 80% medium 1066,† 10% fetal calf serum, and 10% human serum supplemented before use with L-glutamine. One hundred μg each of penicillin and streptomycin sulfate were added to each ml of culture medium. Characteristics of growth pattern, morphology, mucopolysaccharide production, and gross chemical composition, and enzymatic constituents of these cells have been published(9,13). The cells from a specific cell line were washed with saline, centrifuged into pellets which were pooled seriatim, and stored at —70°C. When sufficient material was available, it was thawed, lyophilized, weight and stored at —20°C in an evacuated desiccator over phosphorus pentoxide. Aliquots of these cell solids (8-45 mg) were extracted twice with ethanol-diethyl ether 3:1 (v/v) for 1 hour at room temperature, 3.0 ml volumes of solvent being used per 10 mg of dried solids. The ethanol-ether extract was dried in an atmosphere of nitrogen at 50°C with a rotary evaporator. This residue was extracted 3 times with 5 ml portions of chloroform, and the soluble material taken as the total tissue lipid extract. Small aliquots were used for total phosphorus, cholesterol, ester, and nitrogen analysis(12). The remaining extract from each of 10 cell lines was fractionated on 5 g silicic acid columns (0.9 × 12 cm), and eluted stepwise with 60 ml of chloroform, 150 ml of chloroform-methanol 7:3 (v/v), and 200 ml of absolute methanol. Details of preparation of these columns have been reported(11). The eluates were collected in bulk, evaporated under nitrogen, and resuspended in a small volume of chloroform. Phosphorus(14), ninhydrin nitrogen, cholesterol, and ester analyses were repeated on these column fractions.

The concentrated column eluates were further studied by chromatography on silicic acid impregnated paper. The neutral lipids in the chloroform eluent (Fr. I) were de-

* These studies were supported by grants from Nat. Inst. of Health. The Rackham Arthritis Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies.

† Connaught Med. Research Laboratory.

TABLE I. Total Lipid, Phospholipid, Glyceride, and Cholesterol Concentration in Human Cells Cultured *in vitro*.

Donor	Sex	Age	Tissue	mg lipid/100 mg tissue solids	mg/100 mg lipid		
					Phospholipid	Glyceride	Cholesterol
M.H.	♀	50	synovium	17.8	77.1	9.6	13.2
H.H.	♂	70	"	12.0	78.9	8.5	12.5
W.L.	♂	63	"		*	+†	+
J.M.	♂	44	"	16.0	77.7	10.2	11.9
I.H.	♀	41	"		*	+	+
I.H.	♀	41	periosteum	17.5	73.3	15.9	10.6
I.H.	♀	41	skin	14.0	84.1	5.1	10.7
D.S.	♀	9	"		*	+	+
D.S.	♀	9	pleura		*		
JP_p‡	♂	43	synovium	12.6	82.6	6.6	10.6
JP_t	♂	43	"	10.4	80.3	4.4	15.2
J111	♀		monocytic leukemia	9.1	64.0	12.9	22.9
Chang			liver	13.5	69.0	18.7	12.1
Chang △			"	9.3	83.3	10.3	6.3

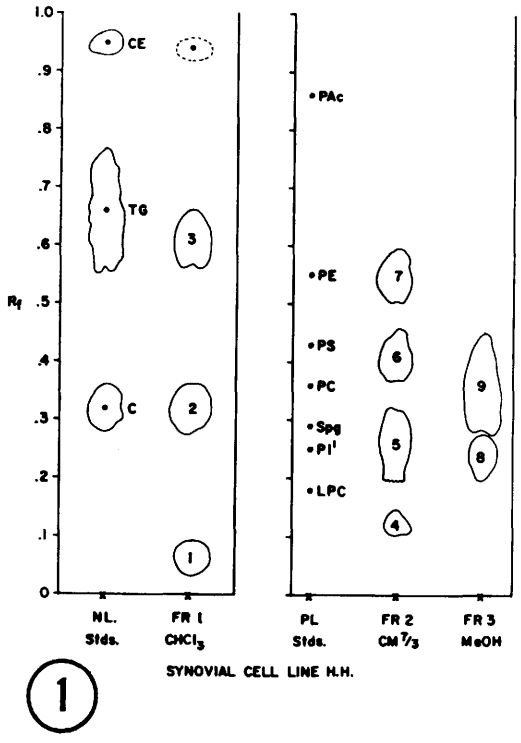
* Quantitative determination of phospholipids only.
† Qualitative detection by chromatography.
‡ Chronic inflammation of synovium (JP_p), "epithelial transformation" during culture *in vitro*, (JP_t).

veloped in n-heptane:2,6-dimethyl-4-heptanone,(96:3), and n-heptane: benzene: glacial acetic acid,(91:9:1). Lipids were identified using chromatographically pure tripalmitin, cholesterol, and cholesterol esters from plasma as reference compounds, and by their staining reactions with rhodamine 6G as described by Marinetti(15). Quantitative silicic acid paper chromatography(16) of the phospholipids in the chloroform-methanol (Fr. II), and methanol (Fr. III) column eluates was performed using 2,6-dimethyl-4-heptanone: acetic acid: water, (40:20:3) as a solvent system. Aliquots from each column fraction were studied in quadruplicate and identified by staining with rhodamine 6G, ninhydrin, 2,4-dinitrophenylhydrazine, and by using the choline spot test(16). Standards from commercial and natural sources(11), further purified by chromatography and solvent fractionation were applied simultaneously to each chromatogram. At least 2 of the chromatogram strips of each unknown were used for extraction and quantitative determination of phosphorus content(16) in each phospholipid component.

Four of the lipid extracts were subjected to the microhydrolysis procedure of Dawson(5), and the hydrolysates chromatographed in phenol:acetic acid:ethanol, and the alkali-labile component developed in a second dimension with methanol: formic acid: water prior to phosphorus analysis. Aliquots of these lipid extracts were hydrolyzed in 6N HCl for 18 hours and chromatographed for qualitative identification of amine-containing constituents and inositol as described by Phillips(17).

Results. The major lipid constituents of the various cell lines are recorded in Table I. The phospholipids were studied in all cell lines, neutral lipids in 10 lines, while in 5 instances the amount of lipid available did not permit quantitative determination of the neutral lipid constituents. Connective tissue cells were derived from synovium in 6 cases, from other anatomical sites in 2 cases, and were compared with duplicate studies on lipid extracts from cell solids obtained by culture of the malignant monocyte J-111(18), and Chang liver cell(19) maintained at 37° and 40°C (Chang△). The lipids of synovial cell line JP were studied before and after "epithelial transformation" *in vitro*, (JP_p and JP_t).

Total lipids varied from 9.1 to 17.8 mg per 100 mg of dry cell solids. Phospholipids constituted the major constituent in each instance and with 3 exceptions cholesterol concentration exceeded that of glycerides (mono-,



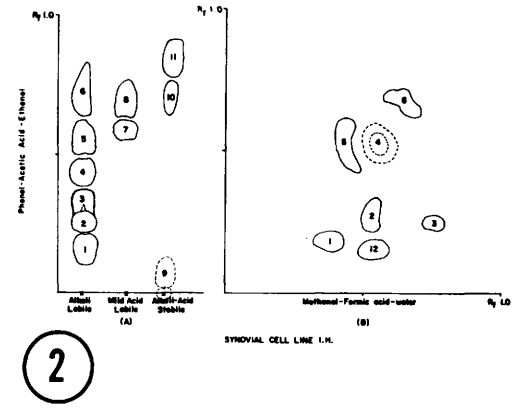


FIG. 1. Silicic acid paper chromatography of column aliquots for synovial cell H.H., $CHCl_3$ eluate contains: 1, mono-, diglycerides; 2, cholesterol (C); 3, triglycerides (TG); and a trace of cholesterol ester (CE). The mobility of phospholipid standards is compared with those eluted from columns by $CHCl_3$/methanol; 4, "diphosphoinositide"; 5, phosphatidyl myoinositol (PI'); 6, phosphatidyl serine, (PS); 7, phosphatidyl ethanolamine, (PE); and by methanol: 8, sphingomyelin, (Spg); 9, phosphatidyl choline (PC). LPC and PA_c identify lysophosphatidyl choline and "phosphatidic acid," respectively.

FIG. 2. A. Unidimensional paper chromatography of phospholipid hydrolysates from synovial cell line I.H. B. Two-dimensional paper chromatography of the phosphorus containing products of mild alkaline hydrolysis from this cell line. Identity of components with parent phospholipid abbreviated in parentheses: 1, glycerylphosphorylinositol, phosphorylinositol (PI'); 2,3, glycerylphosphorylserine (PS), and glycerophosphoric acid (PA_c); 4, cyclic products of alkaline hydrolysis; 5, glycerylphosphorylethanolamine (PE); 6, glycerylphosphorylcholine (PC); 7, glycerylphosphorylethanolamine, (ethanolamine plasmalogen); 8, glycerylphosphorylcholine (choline plasmalogen); 9, 10, unidentified; 11, phosphorylcholine, sphingophosphorylcholine (Spg.); 12, hydrolysis products of polyphosphoinositides, tentative.

di-, and triglyceride). The highest intracellular cholesterol concentration was found in the malignant monocyte. The Liebermann-Burchard reaction was shown to be due to cholesterol alone, by study of the neutral lipids of this cell and of J. M. by gas liquid chromatography.‡ Paper chromatography revealed that free cholesterol, mono-, diglycerides, and triglycerides occurred in all cell lines. Trace amounts of esterified cholesterol were demonstrated in all cell lines, except HH, JP_t, and J-111. A tracing of the neutral lipid chromatogram of cell line H.H. is shown in Fig. 1, (left panel). Conditions of *in vitro* incubation appeared to influence the lipid composition of the Chang cell. The 2 permanent cell lines, and JP_t were used to compare the efficiency of lipid extraction by ethanol-ether with that of chloroform-methanol 2:1 as described by Folch(21). As reported by Phillips(17) comparable results were obtained by both procedures.

Data on the phospholipid constituents of these cell lines are presented in Table II. Phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine, and phosphatidyl myoinositol were present in each cell extract. Phosphatidyl serine was not detected qualitatively or quantitatively in I.H. derived from periosteum, although it was present in connective cells from this donor obtained from skin. Polyglycerylphosphatide or "phosphatidic acid"(16) was identified in 7 instances with the highest concentration found in cells from donor D. S., age 3, JP_t, and the malignant monocyte. Plasmalogens of phospha-

‡ These studies(20) were kindly carried out by Dr. Edward L. Napier, Gastroenterology Research Unit, Univ. of Michigan Med. School.

TABLE II. Percentage Composition of the Major Phospholipid Constituents from Human Connective Tissue Cells Cultivated *in vitro*.

Donor	Tissue	Phosphatidyl choline	Sphingomyelin	Phosphatidyl ethanolamine	Phosphatidyl serine	Phosphatidyl myoinositol	"Phosphatidic" acid	Plasmalogen PC/PE‡	Other§	Unidentified
M.H.	syn	43.0 ± 2.0*	16.2 ± 1.8	16.0 ± 0	8.2 ± 2.3	10.0 ± 3.0	0	+†	3.7 ± .8	0
H.H.	"	52.0 ± .5	7.5 ± .3	26.0 ± 4.5	5.0 ± 1.1	5.7 ± 2.8	0	+	.8 ± .7	0
W.L.	"	60.1 ± 1.5	9.6 ± 2.4	13.9 ± 1.2	5.2 ± 2.4	5.9 ± 1.6	2.1 ± 1.3		2.7	0
J.M.	"	48.2 ± .3	12.3 ± .2	16.2 ± 1.3	5.5	9.5 ± 1.0	0	+	2.5	2.7 ± .3
I.H.	"	47.5 ± 6.2	17.3 ± 4.0	12.4 ± 4.1	2.5 ± 1.4	3.4 ± .4	1.6 ± .6	.3 ± .1/.5 ± .1	9.8 ± 9.4‖	3.9 ± 3.8¶
I.H.	perio	52.4 ± .6	14.5 ± 1.0	16.5	0	11.5	0		.5	1.0
I.H.	skin	52.5 ± 1.5	12.0 ± 1.5	18.2 ± .3	3.7 ± 1.3	7.2 ± 1.3	0		1.0 ± .5	2.0 ± .5
D.S.	"	52.5 ± 1.7	11.1 ± .6	17.7 ± .7	5.6 ± 1.2	5.2 ± .3	4.3 ± .7		3.3 ± 1.5	0
D.S.	pleura	56.7 ± 6.5	14.9 ± 1.6	10.3 ± 5.5	4.1 ± .1	6.0 ± 1.4	4.2 ± 1.5		3.4 ± .7	0
JP_p	syn	49.0 ± 2.5	9.5 ± 2.5	12.5 ± 4.5	6.0 ± 1.5	8.3 ± 2.3	0		9.3 ± 3.2	2.2 ± 1.8
JP_t	"	52.4 ± 2.1	7.8 ± 1.12	15.1 ± 1.2	8.9 ± .7	7.2 ± 2.1	4.5 ± .1	7.5/+	0	3.9 ± .7
J111	monocyte	55.1 ± 2.1	9.1 ± .9	16.5 ± 1.1	6.7 ± .3	7.1 ± 1.7	4.3 ± 0	3.0/3.6	0	.8 ± .8
Chang	liver	38.6 ± 9.4	22.9	16.8 ± 6.5	2.1 ± 1.1	3.0 ± .9	.9 ± .4	1.4/3.3	2.5 ± .8‖	7.6 ¶
Chang △	"	43.0 ± 4.5	12.7 ± 4.3	12.3 ± 2.2	11.3 ± 3.1	17.0 ± 3.5	0		2.5 ± 1.5	0

* Mean and observed range, mean alone represents value for duplicate determinations. † Positive qualitative plasmalogen reaction(16). ‡ Phosphatidal choline/phosphatidal ethanolamine. § Other indicates polyphosphoinositide (diphosphoinositide) except for (‖) where value represents cyclic products of alkaline hydrolysis according to Dawson(5). ¶ Indicates material unidentified in strong acid hydrolysate of these two cell lines.

tidyl choline and ethanolamine were detected qualitatively in M. H., H. H., J. M., and JP_t, and quantitatively in JP_t, J-111, I.H.$_{syn}$, and Chang. The values for the last two were determined independently as the mild acid labile components according to Dawson(5). For JP_t, and J-111 the values represent a fraction of the diester value found in Table II, since on silicic acid paper chromatography these 2 compounds are not resolved from their diesters(16). Included under other constituents are products only tentatively identified. In 10 of 12 cell lines examined by silicic acid chromatography a component eluted from columns by chloroform-methanol 7/3 had a mobility on paper, and staining reactions indicating it was a polyphosphoinositide, ("diphosphoinositide")(16,22). The value for I.H.$_{syn}$, and Chang represents the cyclic products of alkaline hydrolysis(5). In these 2 cell lines unidentified material was found in addition to sphingomyelin in the strong acid hydrolysate. In the other cells unidentified material represents phosphorus detected at the origin or solvent on the silicic acid paper chromatograms. Recovery of phospholipids studied by silicic acid chromatography was 85-95%, and using Dawson's hydrolysis procedures it was 80-85%.

The relatively high values for phosphoinositide found in most of the cell lines compared well with those for non-cephalin phospholipids eluted from columns by chloroform-methanol 7/3. This value was determined by difference of the μmoles of phosphorus minus μmoles of ninhydrin nitrogen found in the eluate for 7 of the cell extracts. The phospholipids of cell line H.H. resolved by silicic acid paper chromatography following column chromatography are shown in Fig. 1, (right panel). In Fig. 2 tracings of chromatograms of the water soluble deacylation products of phospholipids from cell line I.H.$_{syn}$ are presented. The Dawson hydrolysis procedure was carried out on extracts from 4 of the cell lines studied. The results were confirmed in 2 of the extracts where silicic acid chromatography was also employed.

Serine, ethanolamine, and 3 to 5 amino acids were demonstrated by ninhydrin re-

action following paper chromatography of aliquots from the total lipid extract subjected to hydrolysis in 6N HCl. Inositol, glucose, and phosphoinositol were identified by silver reaction(17) on chromatography of other aliquots from the acid hydrolysate.

Discussion. The lipids of human connective tissue cells, "fibroblasts," cultivated *in vitro* have been found to be similar in total amount and percentage composition to those of a variety of other cells and tissues studied by comparable techniques(1,2,5,6). Modest differences in concentration of phosphatidic acid, absence of phosphatidyl serine in cells derived from periosteum, and variations in total phospholipid, cholesterol and glyceride must be interpreted with caution.

Critical evaluation of technical limitations on the methods employed in this study have recently been reviewed(16) and applies particularly to identification of several of the minor phospholipid constituents. Within these limitations, the similarity of lipid composition for cells from different donors, anatomical sites, and age is one of the most striking features of this study. Although high concentrations of individual lipids, *e.g.*, heart cardiolipin, and ethanolamine plasmalogen of brain, distinguish these two organs from several others examined by the same methods (5), no such unique pattern has been found in this study of human fibroblasts. The relatively high content of phosphoinositide in several of the cell lines has been reported in other cells(2,5).

Although a correlation was sought between mucopolysaccharide production and quantitative phospholipid composition, no relationship could be established with phosphatidic acid or phosphoinositide content. Of the cells derived from synovium those with the longest generation times and highest mucopolysaccharide production had the greatest total cephalin content (phosphatidyl serine + phosphatidyl ethanolamine). This relationship did not exist for the other primary cell lines or the "permanent cell lines" examined. The high intracellular content of cholesterol observed in the malignant monocyte was shown by gas-liquid chromatography to be due to free cholesterol. Other cells have been found to have similar high concentrations of cholesterol(1,6). No major differences in the lipid composition of the cell JP were found after "epithelial transformation" had occurred *in vitro*.

An additional observation of importance to investigators working with cells in tissue culture is the apparent stability of the lipid components after varying periods of cultivation *in vitro*. Modulation or "dedifferentiation" of this component of cell structure would appear to be minimal. However, environmental conditions appear to be of importance as indicated by the changes recorded for the Chang cell cultured at 37° and 40°C.

One important purpose of this investigation was to determine whether connective tissue cells, free of extracellular components had an unusually high lipid content. Since these data do not support such an observation, they lend further indirect support to evidence(11,12) suggesting that in experimental granulation tissue some of the lipid must exist in extracellular locations, *i.e.*, in connective tissue "ground substance."

Summary. Lipids from connective tissue cells cultivated *in vitro* were identified by silicic acid column and paper chromatography, and by Dawson's hydrolysis procedures. Phospholipids constituted 64-84% of the total lipid with the remainder approximately equally distributed between cholesterol and the glycerides. Total lipid concentration ranged from 9.1 to 17.2 mg per 100 mg of dry cell solids. Phosphatidyl choline,-ethanolamine,-myoinositol, sphingomyelin, and plasmalogens were identified in all cells examined. Phosphatidyl serine was undetected in one, and "phosphatidic acid" undetected in several of the cell lines. No unusual intracellular concentration of lipid or definite correlation with mucopolysaccharide production was demonstrated.

1. Reed, C. F., Swisher, S. N., Marinetti, G. V., Eden, E. G., *J. Lab. Clin. Med.*, 1960, v56, 281.

2. Blomstrand, R., Nakayama, F., Nilsson, I. M., *ibid.*, 1962, v59, 771.

3. Karnovsky, M. L., Wallach, D. F. H., *J. Biol. Chem.*, 1961, v236, 1895.

4. Yamakawa, T., Ueta, N., Irie, R., *Japan J. Exp. Med.*, 1962, v32, 289.
5. Dawson, R. M. C., *Biochem. J.*, 1960, v75, 45.
6. Speer, R. J., Ridgway, H., Hill, J. M., *Am. J. Clin. Path.*, 1962, v38, 297.
7. Berman, L., Stulberg, C. S., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 730.
8. Castor, C. W., Prince, R. K., Dorstewitz, E. L., ibid., 1961, v108, 574.
9. ———, *Lab. Invest.*, 1962, v11, 703.
10. Hokin, L. E., Hokin, M. R., *J. Gen. Physiol.*, 1961, v44, 61.
11. Bole, G. G., *J. Clin. Invest.*, 1963, v42, 787.
12. Bole, G. G., Roseman, S., Lands, W. E. M., *J. Lab. Clin. Med.*, 1962, v59, 730.
13. Castor, C. W., Prince, R. K., *Lab. Invest.*, 1963, v12, 38.
14. Chen, P. S., Toribara, T. Y., Warner, H., *Anal. Chem.*, 1956, v28, 1756.
15. Marinetti, G. V., Stotz, E., *Biochim. et Biophys. Acta*, 1960, v37, 570.
16. Marinetti, G. V., *J. Lipid Res.*, 1962, v3, 1.
17. Phillips, G. B., *Biochim. et Biophys. Acta*, 1958, v29, 594.
18. Osgood, E. E., Brooke, J. H., *Blood*, 1955, v10, 1010.
19. Chang, R. S. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 440.
20. Vanden Heuvel, W. J. A., Sweeley, C. C., Horning, E. C., *J. Am. Chem. Soc.*, 1960, v82, 3481.
21. Folch, J., Lees, M., Sloane Stanley, G. H., *J. Biol. Chem.*, 1957, v226, 497.
22. Tomlinson, R. V., Ballou, C. E., *ibid.*, 1961, v236, 1902.

Received September 13, 1963. P.S.E.B.M., 1964, v115.

Hyperresponsiveness of Renal Hypertensive Rats to Intravenous Angiotensin II.* (28863)

J. R. GASKELL, R. K. JONES AND A. P. SHAPIRO (Introduced by Melvin Hess)
Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

Although bioassay techniques have demonstrated increased circulating blood levels of angiotensin in patients with malignant hypertension and with renal artery obstruction, investigators have not been able to correlate circulating angiotensin levels with essential hypertension(1). Thus, attention has been directed toward the problem of intrinsic vascular sensitivity to angiotensin and to the question of angiotensin inactivators and their effect on rate of degradation.

Studies on angiotensin inactivating mechanisms have yielded conflicting results, with controversy arising between *in vivo* and *in vitro* observations. Thus, *in vivo* studies with I^{131}-labeled angiotensin have indicated a prolonged half-life in hypertensive plasma(2), yet *in vitro* studies with this compound have shown an increased rate of destruction in hypertensive plasma(3). The validity of these data have been questioned recently by studies of Khairallah *et al* using tritiated angiotensin II which indicate that the iodine labeled compound may not give a true picture of the half-life of the polypeptide(4). However, Hickler, using a bioassay technique in normotensive rats, has estimated the *in vitro* biological half-life of angiotensin by incubation with human hypertensive and normotensive plasma and demonstrated that angiotensin inactivation occurs more quickly in the hypertensive plasma(5). He suggests that this may represent an enzymatic adaptation to chronic angiotensinemia. Wood, on the other hand, found an increased pressor response to angiotensin in the offspring of hypertensive individuals when he reinfused their blood after it had been incubated with angiotensin (6); from these data, he suggested an impaired angiotensin degradation in these subjects.

Using bioassay techniques in rats, Blaquier has found that extracts of kidneys from normotensive and hypertensive rats show no difference in angiotensinase activity(7), while Bing actually describes decreased inactivation of angiotensin by hypertensive renal tissue

* Supported by funds from Health Research and Services Foundation of United Fund of Allegheny County and U.S.P.H.S.