of only one day, the treated cell cultures invariably showed increased growth in comparison with control cultures, as determined by cell counts. In nearly all series, the effect on growth reached its maximum after 4 or 5 days of incubation, and in most cases, interestingly, this difference was reduced by the 7th day. Some series exhibited nearly a 2-fold increase in the cell count at the 4 to 5 day incubation period. These increases are statistically significant at levels shown in the table.

Investigation of HeLa cells revealed a similar but less remarkable increase in cell population induced by Dilantin Sodium (Table III). The fibroblasts isolated from the fibrosarcoma evidenced no reaction to the Dilantin Sodium; in fact, growth was retarded for several days.

The explanation for this apparent *in vitro* stimulation of fibroblast-like cells is not known. It is apparently caused by the diphenylhydantoin structure (or some metabolic product) rather than to the additional sodium ions present because addition of gram-equivalent amounts of sodium did not significantly stimulate growth.

Summary. Addition of sodium diphenylhydantoinate (Dilantin Sodium) to tissue culture of human fibroblast-like cells resulted in stimulation of their proliferation. A similar but more limited stimulation to proliferation occurred with HeLa cells but there was no detectable effect on cells isolated from rat fibrosarcoma.

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Gas-Liquid Chromatography of Methyl Esters of Fatty Acid from Human and Chicken Brain Lipids.* (25779)

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Purification of individual lipid fractions followed by analysis of component fatty acids involves laborious procedures while constituent highly unsaturated acids may suffer oxidative degradation(1-5). It appears beneficial if fatty acid composition patterns of total brain lipids could be surveyed simply and rapidly by gas chromatography before more detailed analyses were attempted. In the present study analyses for a number of normal saturated, unsaturated and α hydroxy fatty acids from samples of human and chicken brain lipids are described.

Methods. Preparation of fatty acids from brain lipid. Samples of human cerebrum obtained on autopsy† and pooled whole chicken brains were extracted with 2:1 chloroform-methanol solution according to procedure of Folch *et al.*(6). The lipid-containing solution was concentrated by evaporation of solvent under vacuum. The concentrate was poured into 4 times its volume of ice cold acetone containing 1 ml of 5% $MgCl_2$ in 94% ethanol/100 ml of acetone(7). The precipitated lipid was then filtered off under N_2, washed several times with acetone-magnesium chloride solution, dried under vacuum, and weighed. This precipitation procedure removes acetone-soluble cholesterol which, with certain exceptions, occurs in brain as free sterol only. Lipid thus obtained had a negative reaction to Liebermann-Burchard test for β-ol sterols. Dried lipid was subjected to methanolysis by refluxing 24 hours with super-dry half-saturated methanolic HCl. Methyl esters were removed from the solution by 4 extractions with diethyl ether. The combined ether ex-

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† Male aged 65, cause of death cerebral hemorrhage. Involved area of brain was not used for analysis.

tracts were washed free of mineral acid, dried over anhydrous sodium sulfate, and the solvent removed under vacuum. Samples of the methyl esters were hydrogenated at room temperature and atmospheric pressure using platinum oxide as catalyst. Hydrogenation was continued until there was no further absorption of hydrogen. *Oxidation of α hydroxy fatty acids.* Samples of hydrogenated free fatty acids were prepared by refluxing the hydrogenated methyl esters for 3 hours with 1N KOH, acidifying the soaps and extracting free acids with ether. The washed, dried fatty acids were subjected to oxidation procedure described by Kishmoto and Radin(5). The α hydroxy acids are selectively oxidized and thus shortened by 1 carbon atom. After oxidation, methyl esters were again prepared by refluxing fatty acids for 3 hours with half saturated methanolic HCl. The method was verified by oxidation of α hydroxystearic, α hydroxylignoceric (cerebronic acid); mixtures of α hydroxystearic, stearic, and palmitic acids and of cerebronic, lignoceric, and behenic acids. Cerebronic acid was prepared by the method of Müller(8). Gas chromatographic analysis indicated that normal acids were unaffected by oxidation; 92% of α hydroxy acids were shortened by one carbon atom. Due to overoxidation the remaining 8% of hydroxy acids were shortened by 2 carbon atoms. *Gas chromatographic analysis.*‡ Identification peaks of the unknowns were made by comparison of retention times with known compounds and by use of internal standards. Standards available were all of even-numbered, saturated fatty acids from C^{12} through C^{24}, oleic, linoleic, arachidonic, C^{19} and C^{21} saturated fatty acids. Tentative identifications of other peaks were made by determination of the "carbon-number" as recently suggested by Woodford and van Gent(9). The carbon-numbers were obtained by plotting retention times of positively-identified straight-chain esters against their chain length on semi-logarithmic paper (and a straight line joining them is drawn). Values corresponding to retention time of any other peak can be read off the graph indicating a hypothetical chain length of a hypothetical straight-chain ester which would be eluted at this point. This figure was termed the carbon-number. As pointed out by Woodford and van Gent, this number is characteristic of a particular ester on a specific type of stationary phase and is sufficiently constant to allow comparison with runs by other authors. Carbon-numbers calculated in our study were compared with those reported by Woodford and van Gent. Our values obtained on polar DEGS column were compared with values obtained on polar maleic adipate ethylene glycol (MAE) column. From this information, together with observations made on disappearance of certain peaks after hydrogenation, we tentatively identified unknown peaks. Quantitative determinations were made on the basis of peak areas measured with a planimeter. Chromatograms of hydrogenated samples were used to determine total amounts of fatty acids of each chain length from C^{14} through C^{24}. The time for complete elution through C^{24} was 58 minutes. When untreated samples were run there was considerable shifting of retention times of major saturated fatty acids. Degree of shifting depended on number and amount of compounds eluted between 2 major saturated peaks; the shift became large between C^{20}-C^{22} and C^{22}-C^{24} peaks; this prolonged elution time through C^{24} saturated to 71 minutes. Several peaks were observed after the C^{24} saturated, but only 4 of these were used in calculations, since after this point peaks became ill-defined. Methyl esters of α hydroxystearic and α hydroxylignoceric acid were not eluted from the column under our conditions. Therefore, amounts of α hydroxy fatty acids present in samples were determined by running hydrogenated methyl esters, which had been subjected to oxidation procedure as de-

‡ Instrument employed was a model 90C Aerograph with thermal conductivity cells as detectors, using a Daystrom-Weston Recorder with 1 m.v. full scale sensitivity. Carrier gas was helium. Bridge current was 200 m.a. Various polar polyester-type column packings were considered for use and diethylene glycol succinate (DEGS) was chosen as giving better resolution between several of the saturated and unsaturated fatty acids of the same chain length. A 10-foot column with ¼ inch O.D. was employed, since shorter columns gave poor resolution of the longer-chain methyl esters. Temperature for all runs was 240°C.

TABLE I. Percentage Composition of Human and Chicken Brain Lipid Fatty Acids as Determined by Gas Chromatographic Analysis of Hydrogenated Methyl Esters.

	% fatty acid			
	Human*		Chicken	
Fatty acid	1	2	Lot 1 (7 chicks 4 wk old)	Lot 2 (7 chicks 6 wk old)
C^{10}-C^{14}	3.4	1.66	1.89	2.68
C^{16}	17.25	20.01	23.35	23.17
C^{18}	45.58	47.53	44.19	42.90
C^{20}	9.21	9.54	8.94	8.21
C^{22}	11.88	10.04	15.17	13.88
C^{24}	7.94	7.51	4.98	5.87
Total odd & branched chain acids	4.74	3.71	1.48	3.29

* No. 1 and 2 refer to 2 different parts of cerebrum from the same human brain.

scribed above, and measuring the increase in odd-chain acid peaks. Since these analyses were made on hydrogenated samples, this procedure gives amounts of α hydroxy acids according to their chain length only. The odd-chain α hydroxy acids which have been reported as occurring in small amounts in brain cerebrosides(4) were not considered.

Results. Chromatography of hydrogenated methyl esters gave well-defined peaks of all even-numbered esters, from C^{14} through C^{24}, and odd-numbered esters from C^{17} through C^{23}. Peaks with retention times corresponding to C^{16} and C^{18} branched chain esters were also observed, but in amounts of less than 0.5% only. In agreement with observations of others(4) further peaks corresponding to C^{25} and C^{26} esters appeared after the well-eluted C^{24} peak. However, these peaks were ill-defined and were not included in the calculations. Approximations indicate that these longer-chain acids occur in amounts between 2 and 4% in the human and less than 3% in chicken brain fatty acids. Composition of fatty acids according to their chain length is presented in Table I.

When untreated methyl esters were chromatographed, over 25 well-resolved peaks were observed. On the basis of retention data, use of standards, collection and rechromatography, many of these peaks were positively identified. Other peaks were assigned a tentative identity on the basis of their carbon numbers. Although a higher temperature and a different, but polar, column was used in our study, carbon numbers of positively identified peaks agreed well with those of Woodford and van Gent(9). Not all peaks observed represented a single component since a number of esters had coincident retention time; retention time of C^{24} tetraenoic arachidonic) was coincident with that of C^{22} saturated (behenic) acid (Table II). With exception of 4 resolved peaks beyond C^{24} saturated, which appeared to any degree on human brain ester chromatograms only, no further peaks were resolved sufficiently to justify their use in calculations. The percentage composition (Table II) therefore represents percent of total components eluted and not absolute amounts in brain fatty acids, although in most cases the values are probably close to actual quantities present. Horwitt *et al.*(1) reported over 30% palmitic acid in chick cerebella fatty acids as determined by gas chromatography. While in our study, whole brain, not cerebellum alone, was analyzed, it appears that 30% does not represent the absolute amount of palmitic acid in chick brain lipid. These investigators did not separate any acids beyond C^{22} under their conditions, while our results and those of others (4,5) indicate that there are considerable quantities of C^{24} as well as small amounts of C^{25} and C^{26} (saturated and unsaturated) fatty acids in brain tissue. Elution of these longer acids is undoubtedly one of the bigger problems involved in gas chromatographic analysis. Temperatures above 230°C were necessary for their elution from polar stationary phase columns. Furthermore, when complex mixtures of untreated esters were chromatographed, satisfactory resolutions of most esters could not be achieved with a column much less than 10 feet in length. The separating efficiencies on the 10 foot column were determined by dividing one retention time by another. Values for separation of C^{18} saturated:C^{18} monoenoic, C^{18} monoenoic:C^{18} dienoic, C^{18} dienoic:C^{18} trienoic, C^{18} trienoic:C^{20} saturated were 1.15, 1.18, 1.13, 1.17 respectively.

Percentages of α hydroxy fatty acids as determined by increase in odd-chain acids after

TABLE II. Fatty Acid Composition of Human and Chicken Brain Lipids Expressed as % of Total Fatty Acids Eluted from DEGS Column at 240°C.

Fatty acid	Carbon No.†	% composition Human 1	Human 2	Chicken Lot 1	Chicken Lot 2
Below C^{14}		.22	.27	1.47	1.7
C^{14} myristic	14.0 14.0	.44	.54	.42	.40
C^{16} branched	15.75 15.6	.44	.27	1.27	1.89
" palmitic	16.0 16.0	18.87	20.15	27.24	24.77
" monoenoic	16.55 16.4	.88	1.09	1.48	1.42
*C^{17} branched	16.72 16.7	.22	.27		
* " monoenoic	17.32 17.3	.44	.27	2.32	1.42
C^{18} stearic	18.0 18.0	25.09	25.05	27.45	28.31
" monoenoic	18.32 18.3	28.86	29.41	22.81	21.23
" dienoic	19.02 18.9	.60	.31	.85	.59
" trienoic	19.6 19.65	.53	.31	.37	.83
C^{20} arachidic	20.0 20.0	2.85	2.96	1.0	1.12
* " dienoic	20.65 20.7	.86	.75	.63	1.12
*C^{21} saturated	21.0 21.0	.25	.14		.18
*C^{20} trienoic, C^{21} monoenoic	21.42	1.11	.53	1.97	2.48
C^{22} behenic, C^{20} tetraenoic	22.0 22.0	3.69	3.40	2.93	4.19
*C^{20} pentaenoic	22.42 22.2	.56	.51	.13	.41
*C^{23} saturated	23.0 23.0	.33	.26	.13	.34
*C^{22} tetraenoic	23.35	1.78	1.96	1.41	1.92
C^{24} lignoceric, *C^{22} pentaenoic	24.0 24.0	8.71	8.61	5.89	4.89
* " monoenoic, C^{22} hexaenoic	24.32 24.3	.62	.30	.20	.77
" unsaturated	24.75	1.21	.82		
	25.15	.46	1.00		
	25.72	1.04	.87		

* For these fatty acids, identification is tentative only.

† Figures under No. are carbon-numbers reported by Woodford and van Gent for MAE column (see text).

oxidation of hydrogenated fatty acids are presented in Table III. Kishimoto and Radin (5) reported that the cerebroside from adult rat brain contains about 66% α hydroxy acids. Since most of brain hydroxy acids are probably located in the cerebrosides and this fraction constitutes 10-12% of total brain lipid, 4-6.8% hydroxy acids found in our study fall within the expected range. In agreement with these investigators for rat brain cerebrosides we found that in total fatty acids from human brain C^{24} α hydroxy fatty acid predominated. However, amounts of C^{24} acids in chicken brain were somewhat smaller (Table III). This finding may be peculiar to that species, but since the chickens in this study were still in the growing stage, more analyses on older chicks are essential before this can be assumed. In the present study more C^{18} α hydroxy acids were found in all samples than would be expected from results of Kishimoto and Radin. However, these authors reported that some of their less pure cerebrosides contained more α hydroxypalmitic and hydroxystearic acids. This observation appears to explain our findings and indicates that these shorter chain hydroxy acids occur in fractions other than cerebrosides. Amounts of α hydroxypalmitic acid we observed were very small as indicated by increases of only 0.5-0.1% in C^{15} acids after oxidation. Variations in the hydroxy acids between the 2 samples of human cerebrum are explained by the fact that sample 1 was considerably richer in white matter than was sample 2. It is

TABLE III. α-Hydroxy Fatty Acids in Human and Chicken Brain Fatty Acids.

α-Hydroxy fatty acid	% in brain fatty acids* Human 1	Human 2	Chicken Lot 1	Chicken Lot 2
C^{18}	3.05	1.11	1.73	1.24
C^{20}	.15	.16	.44	.29
C^{22}	.46	.45	.56	1.16
C^{24}	2.98	4.27	1.09	1.94
Total	6.64	5.99	3.82	4.63

* Corrected for over oxidation by 8%.

planned to analyze white and gray matter separately and from different brain areas. It is the major advantage of our procedures that very small amounts of esters are required for analysis, 3 μl being sufficient for a single chromatogram, and as little as 10 μl for total analyses including hydrogenation and oxidation. Hence it is possible to analyze gray and white matter from several different brain areas from a small laboratory animal.

Summary. Gas chromatographic analysis of normal fatty acids according to chain length from C^{10} through C^{24} and even-numbered α hydroxy acids from C^{18} through C^{24} was reported for samples from human and chicken brain. Separation of 25 resolved peaks representing saturated and unsaturated normal fatty acids was achieved by chromatography of esters on a 10 foot diethylene glycol polyester succinate column. Over half of these peaks were identified, tentative identifications were given for remainder and their relative amounts in human and chicken brain fatty acids were reported.

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Fatty Acid Pattern of Human Bile under Normal and Pathological Conditions.* (25780)

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It was found(1) with the aid of gas-liquid chromatography (GLC) that total fatty acids of human bile constitute a complex mixture of several compounds. It was also shown that purified bile lecithin contains palmitic, stearic, oleic, linoleic, and arachidonic acids, and small amounts of several other fatty acids. Fatty acids of the bile lecithin are asymmetrically distributed on the molecule and represent about 90% of total fatty acids of human bile. The lecithin of the bile may play an important role together with bile acids in holding cholesterol in solution(2,3). If lecithin has a pronounced physiological action, differences in fatty acid composition of the bile lecithin might influence such a balanced system. The purpose of this study was to investigate which fatty acids are normally present in human bile and to see how these may vary in biliary disease.

Methods. Material. Specimens of gallbladder bile and hepatic bile were collected from different cases. Table I gives clinical data. Three cases with duodenal ulcer had no signs of biliary disease. The bile was collected into methanol and stored in a refrigerator until analyzed. Fractionation and Gas-Liquid Chromatography: Total fat was extracted with chloroform:methanol according to Folch *et al.*(4). Total fatty acids were isolated after saponification and acidification, then methylated and submitted to analysis by gas-liquid chromatography as described previously(1). The lecithin from 3 different bile

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