EXPERIMENTAL ATHEROSCLEROSIS IN CEBUS MONKEYS*, ‡

By GEORGE V. MANN, M.D., STEPHEN B. ANDRUS,§ M.D., ANN McNALLY, AND FREDRICK J. STARE, M.D.

(From the Department of Nutrition, Harvard School of Public Health, and the Department of Pathology, Harvard Medical School, Boston)

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Productive study of atherosclerosis requires an appropriate animal species which will allow the necessary control of experimental conditions. Such a species must have a susceptibility to the development of arterial disease resembling that seen in human subjects. The necessary and crucial transfer of information to human affairs would also be facilitated if the dietary habits of the experimental species resembled those of man.

It is widely believed that atherosclerosis is rarely seen in subhuman primates as a naturally occurring disease and further that these animals are resistant to the experimental induction of atherosclerosis. Fox (1) has supplied some carefully collected information which supports the purported rarity of spontaneous atherosclerosis in primates from wild and zoological garden collections. However, the general belief that monkeys are resistant to experimental atherosclerosis is largely unfounded, for few concerted efforts have been made to produce the disease in these animals.

Kawamura (2) reported in 1927 that he was unable to induce atherosclerosis in *rhesus* monkeys fed cholesterol for periods up to 10 months. Sperry *et al.* (3) studied the serum cholesterol levels in *rhesus* monkeys, some of which were made hypothyroid by thyroidectomy. Cholesterol feeding led to a small but definite elevation of the serum levels. It is evident that neither the elevation of the serum levels obtained nor the duration of the experiments was sufficient to have produced vascular lesions. Hueper (4) reported an unsuccessful attempt to produce atherosclerosis in two young *rhesus* monkeys fed a diet containing 1 per cent cholesterol for 8 to 16 months.

Rinehart and Greenberg (5) have described vascular lesions in *rhesus* monkeys maintained on a pyridoxine deficient diet. They have reported atherosclerosis in such animals after 6 months on the regimen. It was also reported that cholesterol feeding

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§ Research Fellow, National Institutes of Health, Public Health Service.

induced a greater elevation of serum cholesterol in these pyridoxine-deficient animals than in controls (6). Similar experiments have been carried out in this laboratory with both *rhesus* and *Cebus* monkeys. Hypercholesterolemia has not been observed in pyridoxine-deficient or control animals of either species nor was it induced by prolonged cholesterol feeding at a 1 per cent level (7). Autopsy examination has revealed no gross arterial lesions of significance. In one *rhesus* monkey, after 9 months of pyridoxine deficiency, two minute lesions were seen in the gross after Sudan staining in the left subclavian artery and the abdominal aorta. These lesions microscopically appeared similar to those described by Rinehart.

The production of atherosclerosis in primates was thus an indecisive question because the recorded efforts were limited to very few animals, and the experiences with pyridoxine deficiency have not been adequately confirmed.

The following experiments have revealed methods for the production of atherosclerosis in *Cebus* monkeys. The necessary experimental conditions are described along with certain observations suggesting possible mechanisms of the phenomenon. This study reaffirms the hypothesis that atherosclerosis is fundamentally a metabolic disease subject to important dietary influences.

Methods

The New World primate, *Cebus fatuella*, has been used for this work. The methods of care and feeding for these animals have been described (8). The animals used were of both sexes and with a few exceptions were estimated to be adolescents 12 to 24 months of age. Commonly the deciduous teeth were shed during the experimental period. Animals were kept in single cages in air-conditioned rooms at 78° F. and were weighed weekly. In the present studies some animals were used repeatedly for assay of various dietary regimens. When this was done the animals were maintained between assays on the standard purified diet "840" (Table I) until the serum cholesterol levels had equilibrated at normal levels.

The periods for studying the various diets were 5 to 34 weeks in length. During these periods restrained but unanesthetized animals were bled at approximately 2 week intervals in the post-absorptive state. Blood was drawn from the antecubital vein into a dry syringe. A few drops were transferred to a small heparinized tube for hematologic measurements and the remainder allowed to clot for the recovery of serum.

Serum cholesterol was measured initially by the hemolytic method of White and Mann (9). When it was established that the method of Abell *et al.* (10) was more reliable, this method was introduced. Comparative studies of the two methods on 472 serum samples indicated that the Abell method gave values averaging 11.9 mg. per cent higher than those obtained with the hemolytic method. The data have been corrected accordingly. Serum lipoproteins were measured on alternate bleedings by the method of Gofman *et al.* (11). The indices of technical reliability of these various measurements are recorded in Table II as the standard errors of duplicate samples performed on human sera during the course of the present study. Because of the limited volume of monkey serum available, duplicate determinations were not done on this material. These human data may underestimate the technical variability with monkey material, especially for lipoproteins.

The diets used in these studies were modifications of the basal diet 840 in which the protein is furnished by casein (Table I). The adequacy of this diet for the *Cebus* monkey has been established with animals kept in good health for periods of 4 to 5 years on this food exclusively. Other animals have, during a similar period, been subjected to repeated deficiency states and

rehabilitated on diet 840. The counterpart of this diet with the protein furnished by the soy bean product αprotein[1] is designated 820α. Several additional dietary modifications are indicated in Table I. Dietary additions of cholesterol and corn oil were made at the expense of sucrose. Sucrose was used to replace protein on the low protein diets. The *dl*-methionine and *l*-cystine supplements were either incorporated in the diet at the expense of carbohydrate or weighed as powders and mixed in the daily diet portion. Diets were prepared at 2 weekly intervals and stored at 0–5° C. in covered, paper cartons. Rancidity was not observed with these precautions. The vitamin allotment was added as an alcoholic solution to the daily food portion and mixed.

TABLE I

The basal diet 840 and several modifications are identified according to the level and type of protein (C indicating casein, α indicating α protein) and level of fat (F indicating 15 per cent fat diets).

Diet	840	825CF	820α	820αF	810C	810CF	810α	810αF
Ingredients								
Casein (vitamin extract)	25	25	—	—	10	10	—	—
α Protein	—	—	20	20	—	—	10	10
Sucrose	60.3	48.3	60.3	53.3	70.3	63.3	70.3	63.3
Salt mixture*	4	4	4	4	4	4	4	4
Corn oil (mazola)	8	15	8	15	8	15	8	15
Cod liver oil U.S.P.	2	2	2	2	2	2	2	2
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
p-Aminobenzoic acid	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Inositol	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol	0	5	5	5	5	5	5	5

Each day 5 ml. of a 20 per cent ethanol solution containing the following vitamins were added to the food portion: Thiamine HCl 1 mg., riboflavin 1 mg., pyridoxine HCl 1 mg., niacin 4.9 mg., calcium pantothenate 3 mg., folacin 0.1 mg., biotin 0.02 mg., and ascorbic acid 25 mg.

* Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

The cholesterol fed was a crystalline product obtained by solvent extraction from beef central nervous system.[2] The cholesterol was incorporated into the diet in the following manner: The dietary fat (corn oil) was heated to 150° C. on a hot plate and the measured quantity of cholesterol added with stirring. A translucent solution was obtained which was then poured over the carbohydrate component of the diet. With stirring, the sugary paste formed was incorporated into the remaining elements of the diet.

Animals were fed once daily as much of the diet as they would consume. This daily food intake approximated 30 to 60 gm. It has not been feasible to obtain accurate food intake records because of scattering.

[1] Generous amounts of αprotein have been supplied for these studies by the Glidden Company, Chicago, through the courtesy of Mr. W. M. Bain.

[2] Large amounts of this material have been generously supplied by the Armour and Co. Laboratories, Chicago.

Animals were sacrificed by etherization and complete autopsies performed. The tissues were fixed in neutral buffered formalin and Zenker's solution. Formalin-fixed gross specimens of heart, aorta, and brain were stained ½ to 2 hours in a saturated ethanolic (70 per cent) solution of Sudan IV, which greatly facilitated examination, photography, and selection of tissue blocks. Paraffin-embedded tissues were stained with hematoxylin and eosin, phloxin-methylene blue, or Gomori's aldehyde fuchsin and trichrome stains (12). Frozen sections were used for Sudan IV and unstained polarized light preparations. Sections to be photographed were stained with Sudan black. In addition a number of selective stains were used and will be noted in the histologic descriptions.

TABLE II

Technical Variability of Methods Used for Measurement of Serum Cholesterol and Lipoproteins

Procedure	Duplicates No. of pairs	Standard error Duplicates*
		mg. per cent
Total cholesterol	533	9.80
Lipoprotein		
S_f12-20	643	4.85
S_f21-35	365	3.13
S_f35-100	365	7.47

* S.E. duplicates $= \sqrt{\frac{\Sigma \text{duplicate difference}^2}{2 \times \text{No. of pairs}}}$.

RESULTS

The normal level of serum cholesterol in this species has been established by 103 determinations on 69 individuals. These measurements indicate a mean of 142 mg. per cent with a S.D. of 51 mg. per cent. Serial measurements on animals maintained for months and up to 4 years on the basal diet 840 have revealed no trend of these serum levels with time.

In Table III are shown the data obtained from animals maintained on the basal diet 840 with 0.5, 1.0, and 2.0 per cent of added cholesterol and also from animals on diet 810CF with 5 per cent cholesterol. In the same table are shown the responses to cholesterol feeding of two monkeys made diabetic with repeated intravenous alloxan treatments. Monkey 1-4 was mildly diabetic (0.1 gm. urine glucose per 24 hours) and monkey 1-5 was severely diabetic with 5 to 8 gm. of urine glucose per 24 hours. The latter occasionally required insulin treatment for acidosis and on one occasion was treated for diabetic coma. These levels of cholesterol feeding in the presence of diabetes did not induce hypercholesterolemia and were in fact associated with low serum cholesterol levels. This effect of severe diabetes on the serum cholesterol level agrees with our published hypothesis (13) relating stress to serum cholesterol level control. The moderate elevations of serum cholesterol observed with the high fat, high cholesterol diet 810CF were not then considered sig-

nificant. Further observations relating such levels of cholesterol to atherosclerosis will be discussed later.

During a study of the effect of choline deficiency on *Cebus* monkeys a diet low in available methionine was devised (unpublished observations). In this diet the soy bean product known as αprotein, which has been shown to have a low level of available methionine, was used as the source of protein (14). It was observed that inclusion of cholesterol at a 5 per cent level in such a

TABLE III

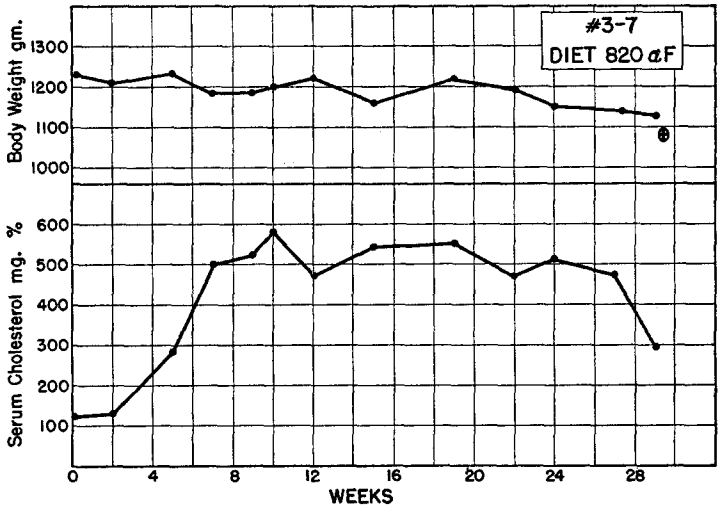
Response of Normal and Diabetic Monkeys to Several Levels of Dietary Fat and Cholesterol

Monkey No.	Clinical status	Diet	Cholesterol	Duration	Serum total cholesterol		
					Initial	Maximal	Final
			per cent	*wks.*	*mg. per cent*	*mg. per cent*	*mg. per cent*
4	Normal	840	0.5	13	141	165	163
2-8	Normal	840	0.5	13	180	192	123
1-4	Mild diabetes	840	1.0	20	112	162	66
1-5	Severe diabetes	840	1.0	20	108	130	76
1-7	Normal	840	1.0	20	180	186	85
1-4	Mild diabetes	840	2.0	26	70	152	154
1-5	Severe diabetes	840	2.0	26	76	121	114
1-7	Normal	840	2.0	26	185	221	191
3-0	Normal	810CF	5.0	31	144	344	276
3-2	Normal	810CF	5.0	31	112	306	182
4-7	Normal	810CF	5.0	8	184	257	191
4-8	Normal	810CF	5.0	8	101	217	174
1-6	Normal	810CF	0	25	112	125	125

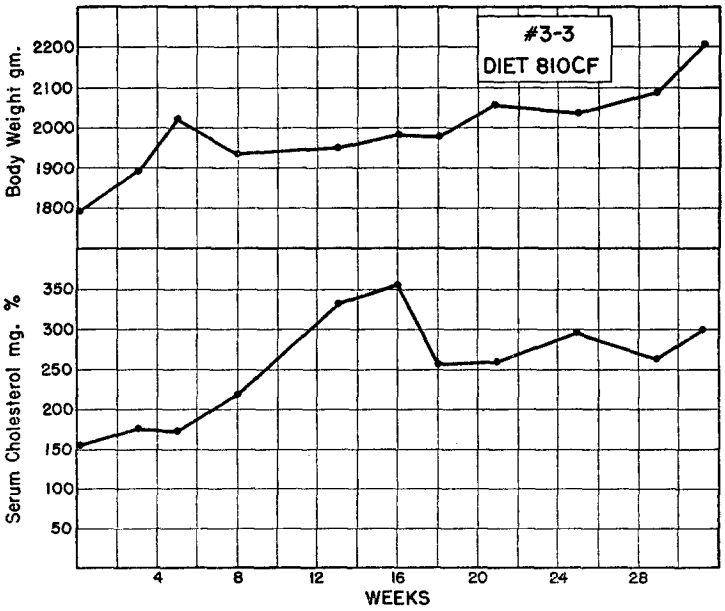
diet led to hypercholesterolemia but only in the choline-fed control animals. Further investigation of this finding has led to a procedure for the production of hypercholesterolemia and atherosclerosis in monkeys.

One of the first observations of this striking response to a diet low in methionine, yet containing adequate choline, (820αF) is shown in Text-fig. 1 in which it may be observed that the serum cholesterol level rose to 500 mg. per cent at the 7th week of the experiment and remained at a high level. Diets made with αprotein at a 20 per cent level do not permit further growth and in young animals may lead to loss of weight.

Text-fig. 2 illustrates the small but definite increase of serum cholesterol in an animal fed a 10 per cent casein diet high in fat and with 5 per cent added



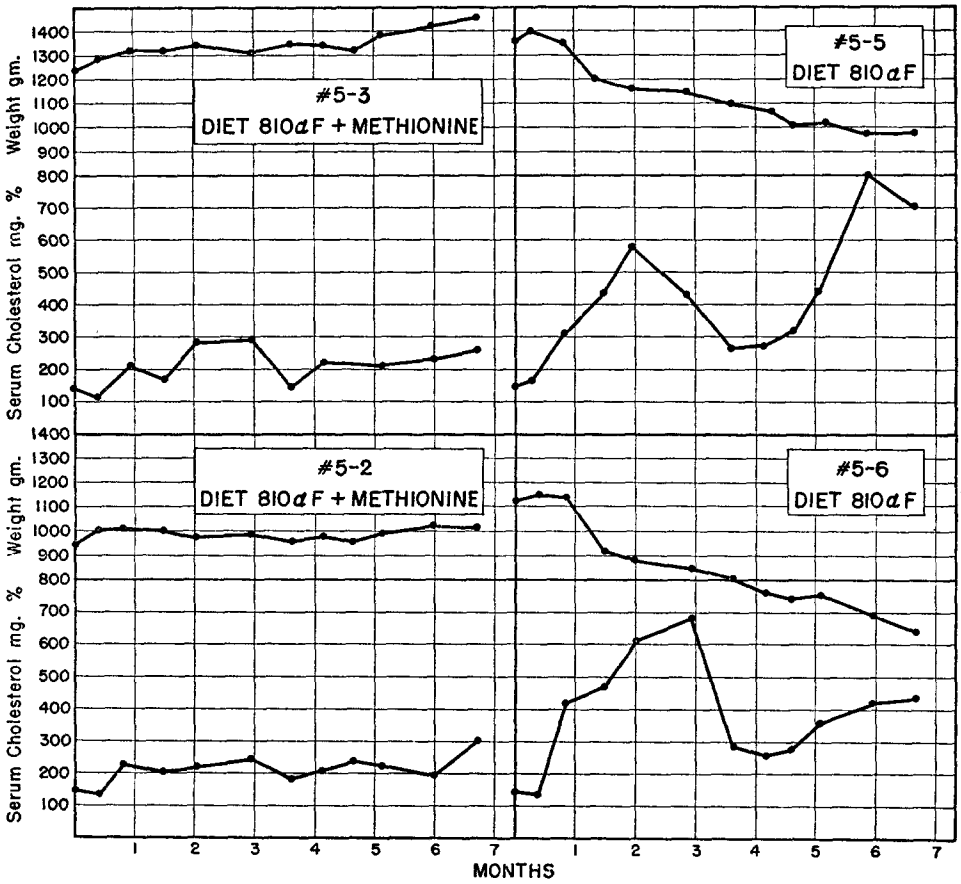
TEXT-FIG. 1. The response of a *Cebus* monkey fed a 20 per cent αprotein diet containing 15 per cent fat and 5 per cent cholesterol.



TEXT-FIG. 2. The response of a monkey to a 10 per cent casein diet containing 15 per cent fat and 5 per cent cholesterol.

cholesterol (diet 810CF). A similar upward trend has been shown in the animals described in Table III.

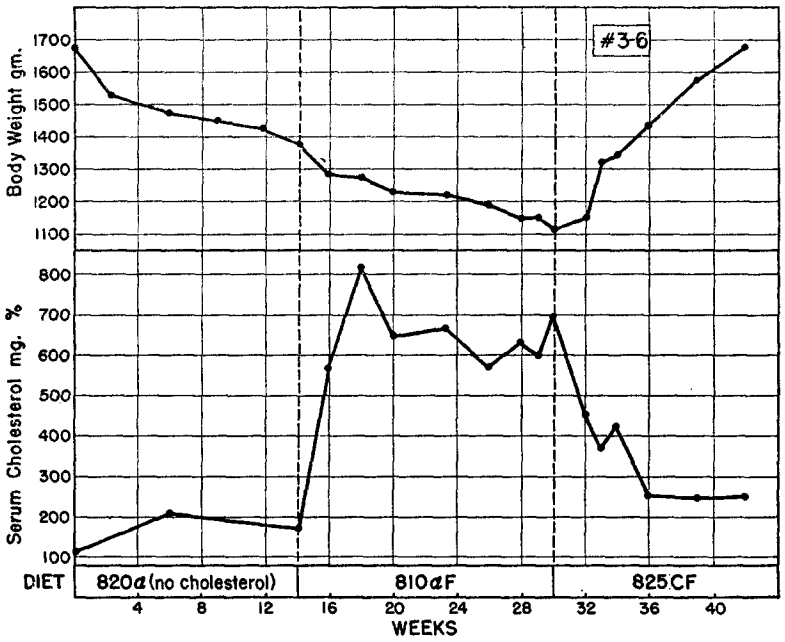
Interpretation of these responses of serum cholesterol to various dietaries requires some definition of an "abnormal" concentration or rate of increase



TEXT-FIG. 3. The effect of inclusion of *dl*-methionine at a 1 per cent level in the diets of two monkeys fed a 10 per cent αprotein, 15 per cent fat, 5 per cent cholesterol ration compared with unsupplemented controls.

of serum cholesterol. For the present work an arbitrary level of 300 mg. per cent has been selected as the upper limit of normality. Since this level is approximately 3 standard deviations above the mean of normal animals, there is a very small likelihood ($p = 0.001$) of such an elevation appearing by chance. This criterion also eliminates from consideration many equivocal effects of feeding regimens. As will be shown, serum cholesterol levels of

300 mg. per cent or more will, when maintained for 18 weeks or longer, lead to atherosclerosis in these monkeys. While long continuation of serum levels somewhat less than 300 mg. per cent may produce the same result, this has not been sufficiently studied for consideration here. Such an expectation is plausible and is of the utmost importance when such observations as these are transferred to human affairs in which typically, less pronounced hypercholesterolemia is observed over periods of years rather than weeks.



TEXT-FIG. 4. The response of a monkey to three successive rations illustrating the lack of hypercholesterolemia unless cholesterol was fed and the curative effect of a casein diet.

The role of dietary methionine in this phenomenon is illustrated in Text-fig. 3. Four monkeys were fed a 15 per cent fat, high cholesterol diet made with 10 per cent αprotein (810αF). Two of the monkeys, Nos. 5-2 and 5-3, received a daily supplement of 1 gm. of crystalline *dl*-methionine. At this low and inadequate protein intake the unsupplemented animals lost weight steadily and became hypercholesterolemic in 2 to 3 weeks. The serum cholesterol remained elevated until the 29th week when the animals were autopsied. The two animals whose diets were supplemented with methionine showed no significant changes in serum cholesterol and their body weights were constant or increased.

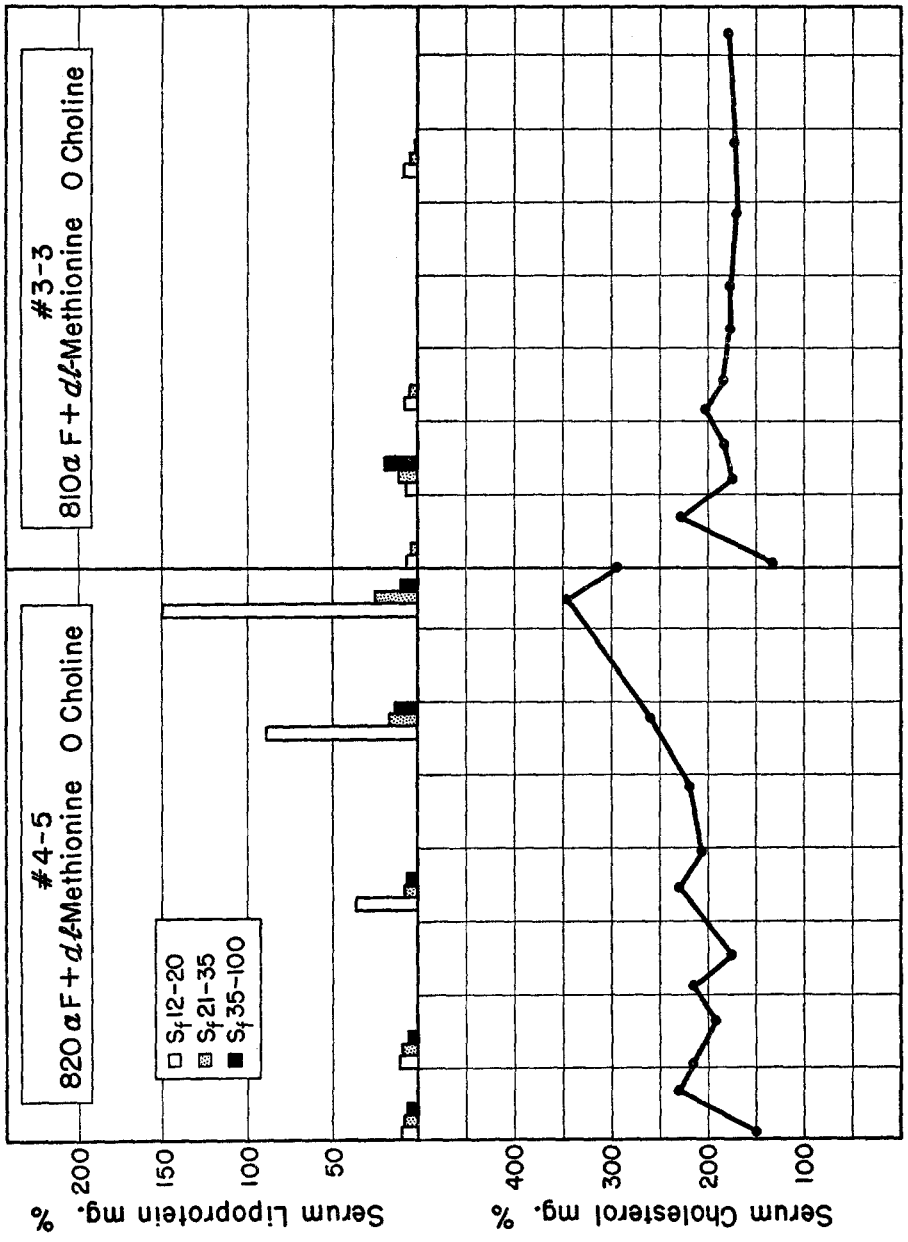
The curative effect of a casein diet is illustrated in Text-fig. 4. In this study

animal 3-6 was fed a diet containing 20 per cent of the methionine-deficient αprotein with no cholesterol (diet 820α without added cholesterol) for a period of 14 weeks. During this time there was a loss of body weight but no hypercholesterolemia. At the end of this period the diet was altered to contain only 10 per cent of the methionine-deficient protein, 15 per cent of fat, and 5 per cent of cholesterol (diet 810αF). Body weight continued to decrease but a very marked hypercholesterolemia promptly developed. After a period of 16 weeks the diet was changed to one containing 25 per cent casein but the high fat and 5 per cent cholesterol of the preceding diet were maintained (diet 825CF). The serum cholesterol levels were promptly restored to near normal levels and remained there despite the increased appetite and food consumption associated with the more adequate casein containing diet.

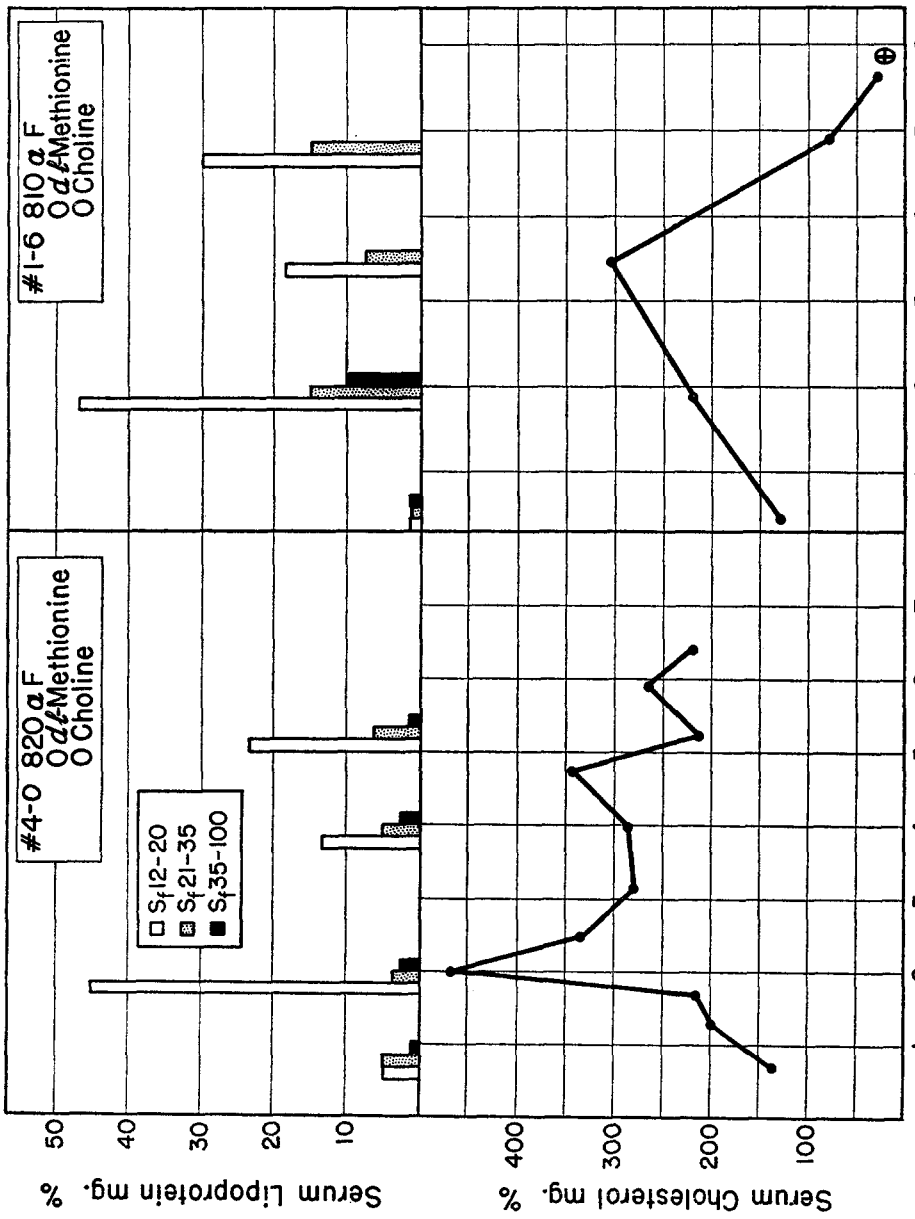
The hypercholesterolemic response of monkeys fed a high fat, cholesterol-containing diet made with the methionine-deficient αprotein was found dependent upon the presence of choline in the diet. This requirement was suggested by our first observations in the choline deficiency experiments (unpublished) when only the choline-fed control animals showed the serum cholesterol change. Animals have been maintained on choline-deficient diets 820αF and 810αF with *dl*-methionine added at a 1 per cent level. A hypercholesterolemia usually is not observed (Text-fig. 5). An occasional animal, such as monkey 4-5 in this figure, has shown a slow elevation of the serum cholesterol. The lack of response shown in monkey 3-3, in this instance on a lower protein level, was more typical.

When similar diets were fed, omitting *dl*-methionine as well as choline (Text-fig. 6), the animals soon became sick and developed grossly fatty livers. Changes in the serum lipid were erratic but generally the values remained within normal limits. Such animals are sicker than those with only a methionine deficiency, and since they do not eat as well, the decreased cholesterol intake may account in part for the failure to develop hypercholesterolemia. The fact that dietary choline is necessary for the production of this hypercholesterolemic response to dietary cholesterol, while methionine prevents it, indicates that hypercholesterolemia is related to a sulfur amino acid deficiency and not to a so called "methyl group" deficiency. This relationship was further emphasized by the observations (Text-fig. 7) that daily supplementation with *l*-cystine orally would partially or completely prevent the hypercholesterolemia associated with diet 820αF. The curative effect of either *l*-cystine or *dl*-methionine is shown in Table IV.

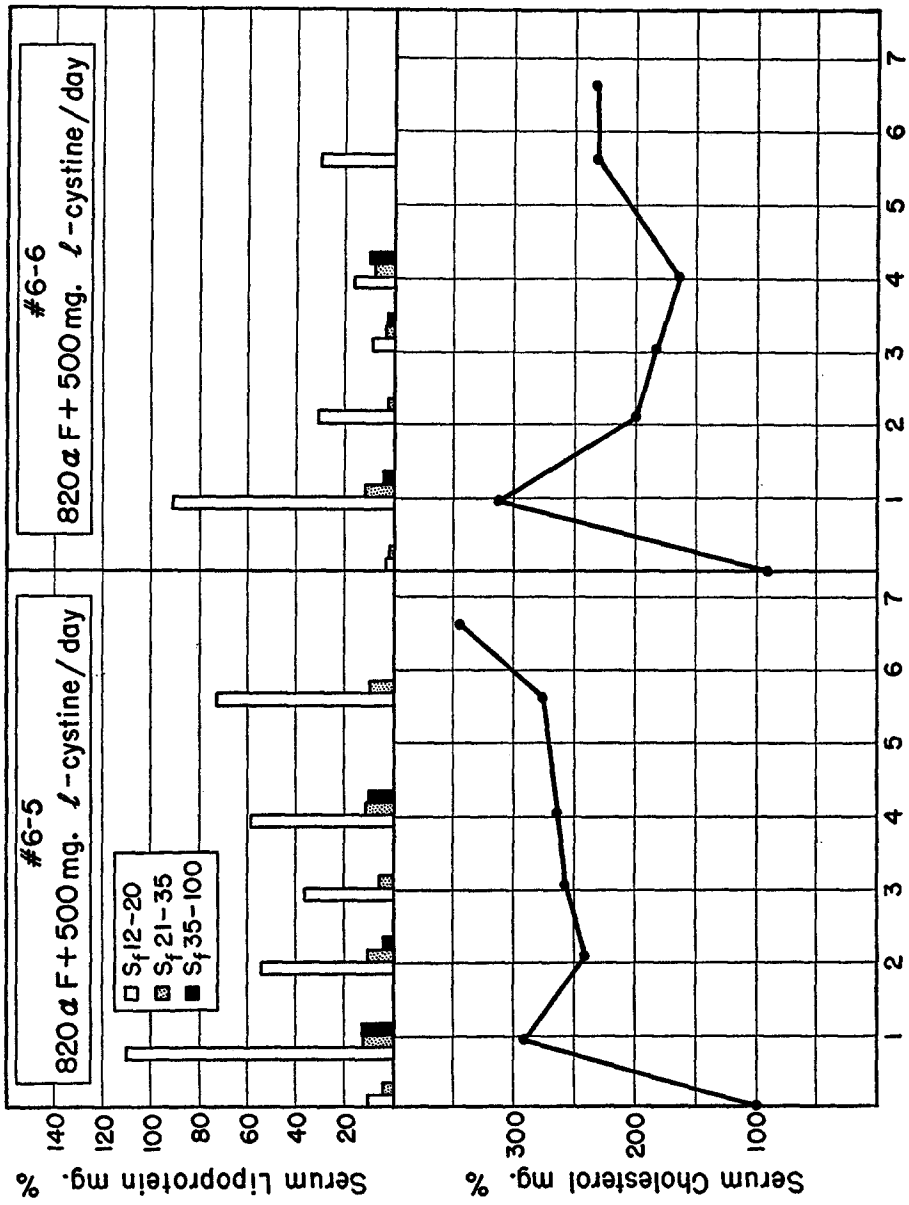
Measurements of serum lipoproteins of the S_f 12–20, 21–35, and 35–100 classes have generally been done on alternate bleedings of the animals used in this work. The normal lipoprotein pattern in this species is illustrated in Text-fig. 8 A. The sequential patterns observed in a monkey (No. 5-7) fed the standard atherogenic diet (820αF) are shown in the same figure. These



TEXT-FIG. 5. The responses of two monkeys fed *a*protein diets supplemented with 1 per cent methionine but without choline. Athough containing 15 per cent fat and 5 per cent cholesterol, the serum lipid alterations were variable and less extensive than in animals receiving choline.



TEXT-FIG. 6. The response of monkeys to a protein diets with 15 per cent fat and 5 per cent cholesterol but without either methionine or choline supplements.



TEXT-FIG. 7. The partial prevention of serum lipid abnormalities by the inclusion of 500 mg. of *l*-cystine in the daily diet is illustrated.

progressive changes are similar to those described by Jones *et al.* (15) in the cholesterol-fed rabbit. The patterns are in most respects similar to those seen in human subjects of varying ages and clinical states and associated with various degrees of hypercholesterolemia, except that little or no material with a flotation rate greater than S_f30 was found. In monkeys with these dietary treatments, there is first an increase of the material moving at rates of S_f3–8 under the conditions of the present measurements (Text-fig. 8 B); with continuation of the dietary treatment there are successively larger amounts of material in the S_f8–12 class and then increases in the S_f12–20 class with only minimal increases in the S_f21–100 classes of lipoproteins (Text-fig. 8 C). Finally, associated with cholesterol levels above 400 mg. per cent, there is a

TABLE IV

Response of Hypercholesterolemic Monkeys to Treatment with Sulfur Amino Acids

Monkey No.	Diet	Treatment	Duration	Initial serum cholesterol	Final serum cholesterol
			days	*mg. per cent*	*mg. per cent*
4-7	820αF	1 gm. *dl*-methionine per day	10	285	195
4-7	820αF	*dl*-Methionine as 1 per cent diet	52	213	178
6-7	820αF	0.5 gm. *dl*-methionine per day	30	480	238
4-4	820αF	1 gm. *l*-cystine per day	10	315	261
4-3	820αF	1 gm. *l*-cystine per day	10	332	174
6-9	820αF	0.5 gm. *l*-cystine per day	30	532	650

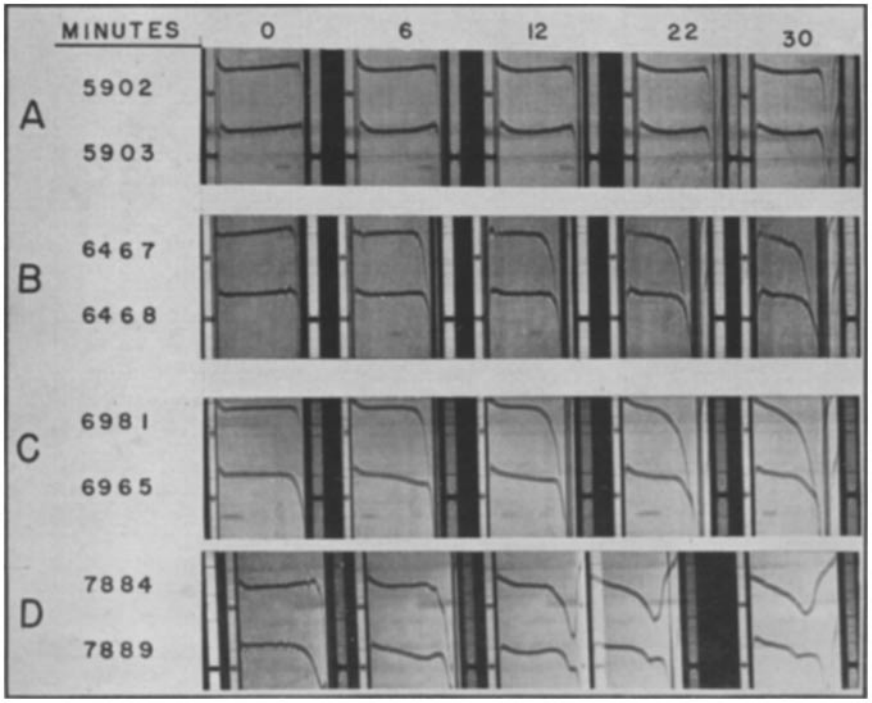
diminution of the main peak at S_f3–8 position and a tendency for the major component or main peak to move as S_f10–16 material (Text-fig. 8 D).

Since, as will be seen, the persistent hypercholesterolemia produced often is associated with atherosclerosis in this species, it is apparent that an unusual opportunity is offered to compare the relationship between these two serum lipid measurements and of each of these respectively to the morphologic changes in the vessels. At the present time we have adequate data to consider only the cholesterol-lipoprotein interrelationship.

When the data obtained with all monkeys on diet 820αF are arranged in scatter diagram in which cholesterol level is plotted in relation to the S_f12–20 level, positive correlation is apparent (Text-fig. 9). Computation reveals a product-moment correlation coefficient of $r = 0.735$ which is significant at the 1 per cent level. Similar treatment of individual data obtained from animals receiving several dietary treatments reveal the values of r indicated in Table V.

A summary of the observations of the response of the serum cholesterol

and lipoprotein to several diets indicates the following: an increase of the level of fat in the diet from 8 to 15 per cent increased the daily increment



TEXT-FIG. 8. The lipoprotein patterns of blood serum obtained from *Cebus* monkeys at several stages of the development of atherosclerosis. Measurements are made with the ultracentrifuge using the method of Gofman *et al.* (11). Photographs are made from left to right at the indicated time intervals. Each film strip (labelled by letter) describes two samples identified by number.

A, samples 5902 and 5903 are those of normal monkeys 6-6 and 6-7 respectively, measured with a threefold concentration of lipoprotein.

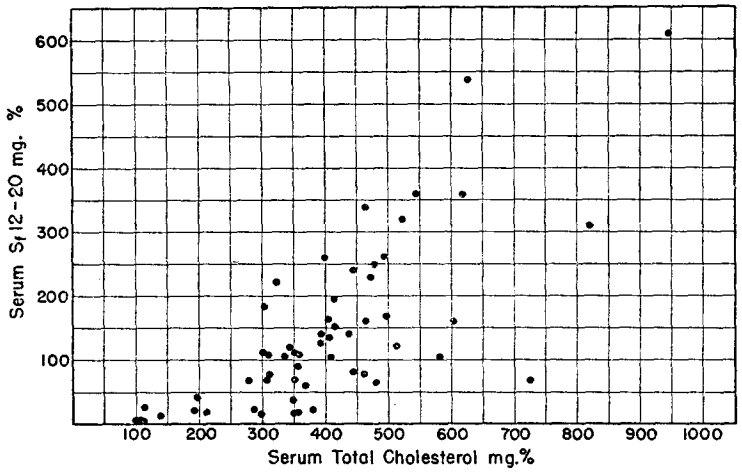
B, samples 6467 and 6468 were obtained from monkeys 5-7 and 5-8 respectively which had been 28 days on diet 820αF. The lipoprotein has been concentrated fourfold for this measurement.

C, sample 6981 was obtained from monkey 5-7 after 62 days on diet 820αF. The lipoproteins are concentrated threefold for the measurement. Sample 6965 is that of a 79 year normal male human subject measured with a fivefold concentration of lipoprotein. The similarity of human and monkey patterns is apparent.

D, sample 7884 was obtained from monkey 5-7 after 165 days on diet 820αF measured without concentration of the lipoproteins. Sample 7889 is the serum of a 60 year old normal female human subject with a fivefold concentration of lipoprotein.

of serum cholesterol. Casein at a 10 per cent level in the diet led to a greater serum cholesterol increment than did a diet with 25 per cent casein. The addition of 1 gm. of *dl*-methionine to the daily ration containing αprotein

prevented the hypercholesterolemia with a dietary fat level of 8 per cent and minimized it with fat at the 15 per cent level. Supplementation with *l*-cystine was less effective in either preventing or curing the abnormality. In general the hypercholesterolemia was facilitated by high fat diets and minimized by



TEXT-FIG. 9. The relation of total serum cholesterol to S_f12–20 class of lipoprotein in monkeys on the atherogenic diet 820αF. A total of 57 observations made on 15 monkeys is included.

TABLE V

Correlation between Total Serum Cholesterol and Lipoprotein of the S_f12-20 Class in Groups of Monkeys Receiving Various Dietary Treatments

Diet	No. of monkeys	No. of observations	r	Significance probability p
820αF	15	57	0.735	<0.01
810αF	7	25	0.793	<0.01
820αF + *dl**	7	14	0.488	>0.05
810αF + *dl*	2	7	0.985	<0.01
820αF 0 choline 810αF 0 choline	3	16	0.505	>0.01–<0.05
820αF + *dl* 0 choline 810αF + *dl* 0 choline	3	11	0.841	<0.01

* *dl* refers to *dl*-methionine.

high protein diets. These data indicated that the dietary protein effect on the response of the monkeys to cholesterol feeding was related to the content of the essential amino acid, methionine. The effect of *l*-cystine in partially preventing and reversing the serum cholesterol abnormality further emphasizes the importance of the sulfur amino acids.

The amino acid deficiency necessary for the production of this experimental disease did not produce a significant alteration of the plasma proteins. The total proteins, albumin, and globulin concentrations of the representative animals described in Table VI are not abnormal. These were measured by the method of Pillemer and Hutchinson (16).

Since the isolated soy bean protein, αprotein, is high in residual sulfite content, control experiments were done to study the effects of equivalent sodium

TABLE VI

Serum Proteins in Monkeys with Hypercholesterolemia and Atherosclerosis Produced by Dietary Means

Monkey No.	Diet	Duration of treatment	Total protein	Albumin	Globulin	Fibrinogen	A/G
		wks.	*gm. per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*	
5-4	810αF	9	6.83	—	—	0.44	—
		26	6.00	3.10	2.51	0.39	1.23
5-5	810αF	9	7.00	—	—	0.46	—
		26	6.66	3.09	3.14	0.43	0.99
5-6	810αF	9	6.50	—	—	0.42	—
5-7	820αF	8	7.78	4.19	3.08	0.51	1.36
5-8	820αF	8	7.98	3.56	3.90	0.52	0.92
6-1	820αF	8	8.04	4.32	3.20	0.52	1.35
6-3	820αF	8	7.55	3.81	3.25	0.49	1.17
6-5	820αF + *l*-cystine	8	8.00	4.16	3.32	0.52	1.25
6-6	820αF + *l*-cystine	8	7.30	3.46	3.36	0.48	1.03

Normal Values for Cebus Monkeys (7)

No. animals	No. observations	Total protein	Albumin	Globulin	A/G
		gm. per cent	*gm. per cent*	*gm. per cent*	
5	67	7.66 ± 0.47*	3.64 ± 0.47	3.50 ± 0.67	1.10 ± 0.37

* ± standard deviation of observations.

bisulfite supplements given to monkeys on high fat, high cholesterol diets made with casein. These experiments revealed no ensuing hypercholesterolemia. Parenteral supplementation with thiamine of animals maintained on αprotein diets had no effect, indicating that the sulfite had not destroyed the dietary thiamine to create a deficiency.

It was of great interest to determine whether the hypercholesterolemia produced would eventually lead to atherosclerosis. It has been reported that in dogs and rats persistent elevation of serum cholesterol levels have not led to atherosclerosis (17, 18). However, Steiner and Kendall (19) have reported

atherosclerosis in hypothyroid, cholesterol-fed dogs. It appears that production of this disease is dependent upon additional unknown factors. The monkey described in Text-fig. 1 was sacrificed after a period of 23 weeks with serum cholesterol levels above 400 mg. per cent and with proportionate elevations of the serum lipoproteins. The autopsy of this animal revealed for the first time gross plaques of the proximal aorta and great vessels.

Autopsies have been performed on seventeen animals fed high cholesterol, αprotein diets made with adequate choline. These animals maintained serum cholesterol levels of 300 mg. per cent or more for periods up to 31 weeks. Three additional animals have been autopsied which were fed similar diets but deficient in choline. Seven control animals have been autopsied.

Of the seventeen animals fed the αprotein diet with added cholesterol and adequate choline, nine have maintained hypercholesterolemia for 18 or more weeks. Four of these animals showed aortic lesions which were marked, four showed minimal lesions, and one animal was negative both in the gross and microscopically. Of the eight animals on this diet with hypercholesterolemia of less than 18 weeks, there was moderate aortic involvement in one, minimal in two, and five animals showed no vessel disease.

Two of the three animals fed a similar diet but also deficient in choline showed minimal aortic lesions, the remaining animal being negative. None of the seven control animals showed demonstrable aortic changes.

The aortic lesions consisted of smooth, non-calcified, pale yellow plaques ranging in size from pin-point flecks to 1 cm. in diameter (Figs. 1 and 2). The margins of the large lesions were raised and irregular. Though generally only slightly elevated, an occasional plaque measured 1 to 2 mm. in thickness. The lesions, while present throughout the aorta, were concentrated in the ascending portion and the arch and were often seen at the orifices of the great vessels. In two instances these lesions partially occluded the lumena of the innominate ostia. In the same animals lesions were present about the orifices of the coronary arteries. Similar lesions were seen about the orifices of the intercostal and renal arteries. Involvement of the aortic leaflet of the mitral valve was common. Minute plaques in the large branches of the aortic arch and occasionally in the femoral arteries were seen, but generally only after Sudan staining of the gross specimen.

The aortic lesions of one animal, No. 4-7, were distinctly paler than most, gray in color, and surrounded by a yellow border. With Sudan treatment only the latter areas were stained. This was exceptional in that the plaques from other animals showed brilliant red staining throughout the areas involved. No gross lesions were seen in the coronary, renal, or cerebral arteries.

Microscopically, aortic lesions consisted of sharply circumscribed thickenings of the intima. They were composed chiefly of large "foam cells" the nuclei of which resembled those of histiocytes, small numbers of lymphocytes,

occasional fibroblasts, and rare polymorphonuclear leukocytes (Figs. 3 and 5). Occasionally a large needle-shaped crystal cleft was seen. No convincing increase in ground substance or metachromasia has been seen. Moderate numbers of fine elastic fibrils could be demonstrated with Verhoeff's stain or Gomori's aldehyde fuchsin and fine collagen fibrils by a number of techniques, including Mallory's aniline blue, Van Gieson's stain, Gomori's trichrome, and Lillie's allochrome (periodic acid–Schiff–picromethyl blue (20)). No fibrillae have been demonstrated by Lillie's modification of the Bielschowsky-Maresch silver technique or by Mallory's phosphotungstic acid–hematoxylin stain. Using the allochrome method, it has not been possible to demonstrate an increase in periodic acid–Schiff-positive substances. Generally the underlying, internal elastic membrane has shown no change, although in monkey 4-7, there was a striking reduplication of this structure. In another animal there were distinct breaks in this membrane and the underlying lamellae and "foam cells" appeared to be streaming down into the media (Figs. 3 and 4). No other significant medial changes were seen in the paraffin-embedded tissues.

Sudan staining of frozen sections revealed large amounts of fat in the intimal lesions, chiefly but not always intracellular. No fat was seen in endothelial cells. Large amounts of fine, globular Sudanophilic material were also seen in the media—occasionally quite deep and even approaching the adventitia. These globules were frequently clustered along elastic fibers. The latter in addition frequently showed diffuse Sudan staining properties just below the intimal plaques. With polarized light birefringent material was seen. In some instances this was predominately intimal and in others medial. Large needle-shaped crystals unaffected by heating were seen, as well as small needles and amorphous material, some of which after heating showed the Maltese cross pattern of polarization characteristic of fluid crystals. Thus by the criteria of Leary (21) and Lison (22) there was both free and esterified cholesterol present in addition to neutral fat.

Monkey 4-7, mentioned above, showed the most fibrotic lesions of the series. Peripherally, the lesions resembled the intimal lesions described above, but in the central portion they were relatively acellular, free of demonstrable fat, and showed a marked increase of coarse elastic, and collagen fibers.

On only one occasion has a cellular, fat-containing intimal plaque been seen in the coronary arteries. In 9 animals, however, frozen sections have revealed focal fat globules deep in the walls of small intramural coronary arteries without other changes. Similar findings have been seen in small arteries in the spleen, liver, kidney, adrenal, gastrointestinal tract, and testes. Only rarely has birefringent material been demonstrated. Occasionally a myocardial vein showed similar fat. The significance of these fatty changes in small vessels is not known. They were not seen in control animals.

Occasional minute subcapsular scars and hyalinized glomeruli have been observed in the kidneys, although these lesions were also seen in the controls.

Monkey 4-7 showed in addition a number of ischemic glomeruli with marked axial thickening of the basement membrane. With Lillie's allochrome technique this tissue was seen to be Schiff-positive and in addition a number of these glomeruli showed an increase in collagen. These changes were not, however, generalized. The majority of the glomeruli appeared normal. No arteriolar or muscular arterial lesions have been seen. No gross or microscopic lesions were seen in the cerebral arteries.

TABLE VII

Liver Lipids in Monkeys Fed Atherogenic Diets Compared with Those of Monkeys Supplemented with Methionine or Deficient in Choline

Diet	Monkey No.	Duration of treatment	Total lipid	Phospholipid	Cholesterol			
					Total	Free	Ester	Per cent free
		wks.	*gm. per cent*	*gm. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
820αF − *dl** + choline	5-7	25	7.65	1.66	304	199	105	66
	3-7	26	3.48	1.62	338	194	144	57
	4-1	25	5.38	1.90	649	282	367	44
810αF + *dl* + choline	5-2	27	6.05	3.03	277	142	135	51
	5-3	28	2.31	1.16	188	79.7	108	42
810αF − *dl* + choline	1-9	16	2.21	0.99	270	126	144	47
	5-4	28	4.29	2.52	155	79.2	75.8	51
	5-5	27	9.45	5.52	453	245	208	54
	5-6	28	9.90	5.34	393	236	258	48
810αF − *dl* 0 choline	1-6	7	14.04	2.06	292	195	97	67
	3-0	3	21.79	3.57	401	279	122	70
	3-2	11	10.70	5.05	485	349	136	72
820αF − *dl* 0 choline	3-8	26	10.20	3.18	480	262	218	55
	3-9	26	8.05	1.06	390	228	162	59

* *dl* refers to *dl*-methionine.

Only exceptionally were abnormal amounts of fat and smaller amounts of birefringent fluid crystals demonstrated in the livers of animals receiving adequate dietary choline. While most of the fat seen was present in the liver parenchymal cells, Kupffer cell fat was often present and the relative amounts of fat in the two cell types were usually parallel. Correlation of histologically demonstrable lipid with that determined chemically was poor except in those livers with very large amounts of fat. Minimal to moderate and occasionally marked, amounts of fat were found in splenic histocytes and in the renal tubular epithelium.

A striking feature of the vascular changes produced by cholesterol feeding

in rabbits is the associated visceral cholesterolosis (23). This is in contrast to the usual finding in human atherosclerosis and is an objection to equating the rabbit and human diseases. Autopsy observations in the present work with monkeys have not indicated gross infiltration of visceral organs with lipids in animals kept on the αprotein diets containing choline. Chemical measurements of the liver lipids by previously published methods (24) confirm these observations (Table VII). The animals were able to tolerate the high lipid, 20 per cent αprotein diets whether with or without supplementary *dl*-methionine without developing grossly fatty livers. With diet 810αF and without added methionine there was a moderate elevation of all the liver lipid components measured in two of four animals. By contrast, omission of choline from similar diets, in other monkeys, led to gross elevations of total liver lipids, especially when associated with low levels of dietary protein.

DISCUSSION

The development of a primate species for the experimental study of atherosclerosis is important for two reasons. First, there is a considerable amount of evidence, well summarized by Duff (23), which indicates that the cholesterolosis seen in rabbits following cholesterol feeding is peculiar to that species and is quite distinct from human atherosclerosis. Second, it is quite clear that solution of the problems of the cause and prevention of human atherosclerosis will be greatly delayed if they cannot be studied in a species allowing complete laboratory control and observation. Numerous experiments with a variety of species have revealed that the common laboratory animals, other than rabbits and chickens, do not develop hypercholesterolemia or atherosclerosis when fed large amounts of cholesterol. Even in the presence of marked hypercholesterolemia, produced by protein depletions (17) in dogs or by hypothyroidism in rats (18) and dogs (19), the development of atherosclerosis is inconstant. The present experience with monkeys indicates that incorporation of cholesterol into an otherwise normal diet does not lead to immediate hypercholesterolemia, although a measurable slow increase does occur (*cf.*, Table III, Text-fig. 3). This response may be of importance; it is possible, but unproven, that atherosclerosis would develop slowly in such animals, since the appearance of lesions seems to be determined by both the concentration of serum cholesterol and the time of exposure. If this proves to be the case, then the experimental model would approach the natural human disease more closely.

The experience with diabetic monkeys fed cholesterol showed that uncontrolled diabetes and the associated polyphagia do not cause hypercholesterolemia, but on the contrary caused reduction of cholesterol values. This accords with the hypothesis that reduced serum cholesterol levels are associated with physiologic stress (13).

The demonstration that hypercholesterolemia and consequent atherosclerosis in these monkeys were dependent upon a deficiency of the sulfur amino acids, methionine and cystine, was based upon the use of the soy bean product designated αprotein. Although there are significant amounts of sulfur amino acids in this isolated soy bean protein, it has been demonstrated that these amino acids are relatively less available than are those of casein and most other non-leguminous proteins (25). The non-availability appears to be due to the presence in unheated soy bean of an antitrypsin which interferes with the hydrolysis and absorption of the sulfur amino acids. In addition, αprotein contains some heat-resistant substance which inhibits growth when fed to rats (26). The present data have shown that supplementary *dl*-methionine can prevent or reverse the alimentary hypercholesterolemia. *l*-Cystine was only partially effective in preventing the rise and was relatively less effective than methionine when given later. The unequal actions of *dl*-methionine and *l*-cystine are consistent with the hypothesis that sulfur amino acids were deficient, since it is known that *l*-cystine can substitute only partially for *dl*-methionine in meeting the growth requirements of rats receiving an abundance of dietary methylating material (27).

The presence of choline in these diets appeared essential for the development of sustained hypercholesterolemia. In the absence of the choline the animals had poor appetites, grew poorly, and died with fatty livers. Some choline-deficient animals showed a temporary increase of serum cholesterol (Text-fig. 6, monkey 4-0); this suggests that choline had an indirect action, such as the support of general liver function, rather than a specific role in the disturbance of cholesterol metabolism observed.

The present material does not resolve the complex problem of the true relationship of serum cholesterol and serum lipoprotein measurements respectively to one another and to atherosclerosis. The present indications are that the two measurements are better correlated with one another in monkeys with experimental atherosclerosis than they are in human beings (monkey $r = 0.680$, human $r = 0.54$ (28)). However, the correlation is not as good in monkeys as in cholesterol-fed rabbits ($r = 0.95$) (28). The quantitative relation of these measurements to the degree of vascular involvement will be considered when more anatomic material is available.

The data shown indicate some degree of variability in serum lipid response between individuals on a similar regimen. This is relatively smaller than that seen in rabbits where occasionally completely resistant animals are found (29) along with many degrees of response (30). In the present work no sex differences were observable although a sex ratio of 1:4 (M:F) and the use of adolescent animals may have obscured differences.

A few studies have revealed no great associated elevation of the serum neutral fat and phospholipids. The sera of these hypercholesterolemic animals

are clear or only slightly lipemic. The ratio of free to total cholesterol in the serum is not disturbed on the sulfur amino acid–deficient diets. The charts indicate that treatment of animals with either a casein-containing diet or with a pure amino acid, methionine, leads to a prompt recovery of the serum cholesterol and lipoproteins. The potential reversibility of the lesions has not been sufficiently studied for discussion.

The chemical and histologic observations have revealed no extensive lipid infiltration of the visceral organs. This is in contrast to the cholesterolosis observed in cholesterol-fed rabbits.

Several dietary conditions are of importance in this phenomenon. Moderately high fat diets with high levels of cholesterol are essential as is the sulfur amino acid deficiency. This amino acid deficiency is effective short of gross changes in the serum protein levels. This response of serum cholesterol appears unrelated to that observed in nephrotic patients or that produced by Li and Freeman (17) in protein-depleted dogs.

The observations described here cannot justifiably be used for inferences applicable to the human disease. To attribute human atherosclerosis to deficiency of an amino acid would be both naive and premature at this time. Of more importance is the recognition of a primate species and a dietary procedure for further study of atherosclerosis.

SUMMARY

Atherosclerosis has been produced in *Cebus* monkeys by dietary means. This disease has been produced by feeding diets high in cholesterol and low in sulfur amino acids over periods of 18 to 30 weeks. Within 2 to 8 weeks this regimen caused the concentration of cholesterol in the serum to rise to 300 to 800 mg. per cent. The hypercholesterolemia could be largely prevented by feeding 1 gm. per day of *dl*-methionine or *l*-cystine as supplements to the diet. After the serum concentration had become elevated, it could be restored to normal by feeding 1 gm. of *dl*-methionine but only partially restored by 0.5 gm. of *l*-cystine daily.

The vascular lesions were in the ascending aorta but extended from the valves of the left ventricle to the proximal portions of the carotid and femoral arteries. Minimal lesions have been observed in the coronary arteries. The aortic lesions were chiefly characterized by the presence of lipid-laden phagocytes and increase in collagen and elastic fibers. The lipids were in part cholesterol derivatives. Visceral cholesterolosis was not associated with this disease.

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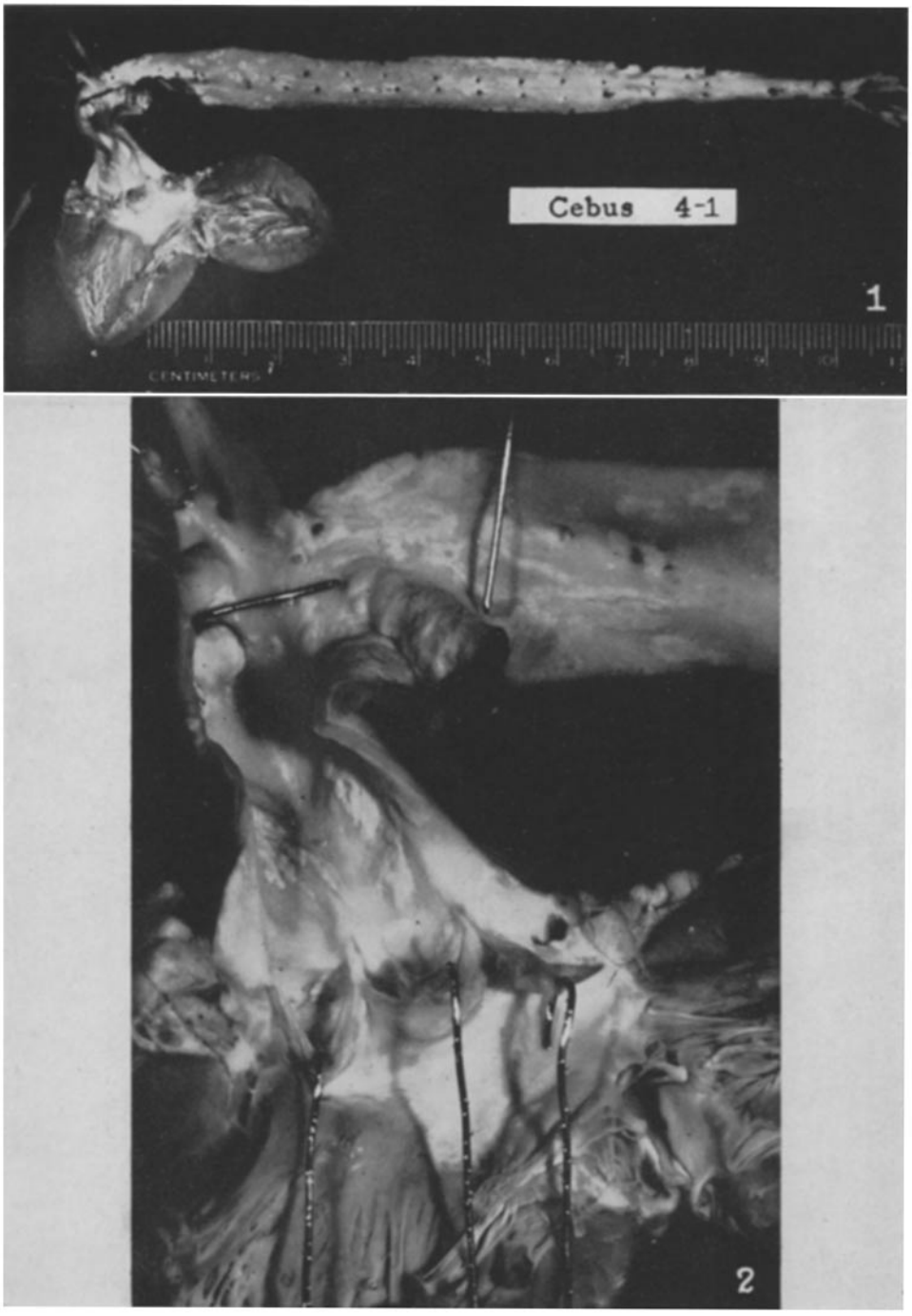
EXPLANATION OF PLATES

PLATE 26

FIG. 1. Monkey 4-1. Heart and aorta, unstained, showing numerous intimal lesions × 1.

FIG. 2. Monkey 4-1. Detail from Fig. 1 of mitral and aortic valves and aortic arch × 4. Note the lesions on mitral valve, in sinuses of Valsalva, and at the orifice of innominate artery. The probes lie in the innominate artery and at the level of obliterated ductus arteriosus.



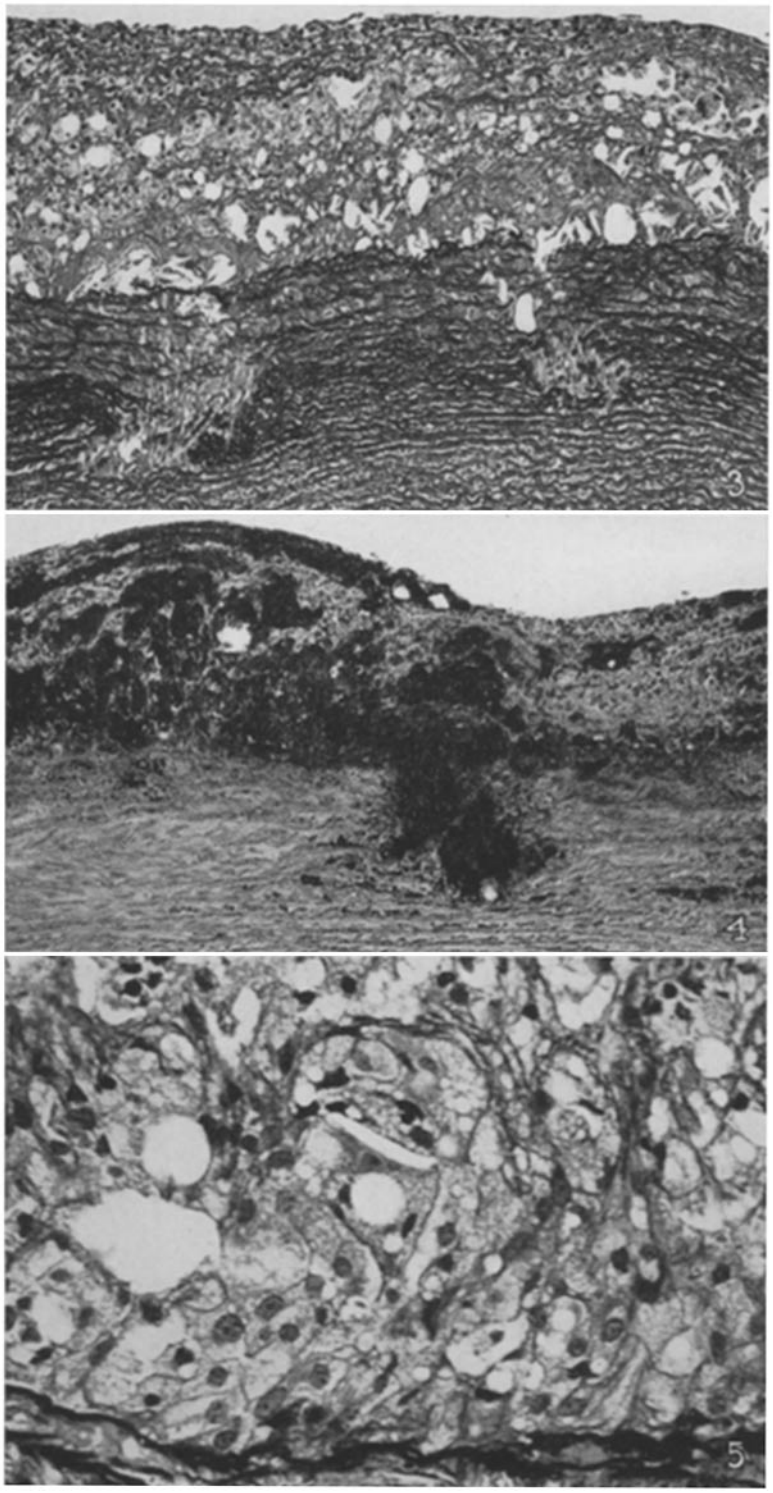
(Mann *et al.*: Atheroslcerosis in *Cebus* monkeys)

PLATE 27

FIG. 3. Monkey 4-1. Aldehyde fuchsin, hematoxylin, and trichrome stain. Aortic lesion. The thickened intima shows "foam cells," large vacuoles, crystal clefts, and elastic fibers. The elastic lamellae of the underlying media are interrupted in two areas with an increase in cellularity. × 133.

FIG. 4. Monkey 4-1. Frozen section, Sudan black stain, hematoxylin eosin. A tissue block adjacent to that of Fig. 3. There is marked intimal Sudanophilia. Note the prominent medial staining. In this plane of the section this is related to an interruption not only of the elastic lamellae but of the internal elastic membrane as well. × 133.

FIG. 5. Monkey 4-1. Weigert's iron chloride hematoxylin and eosin. A detail of the same tissue block shown in Fig. 3. The internal elastic membrane can be seen along the lower edge of the field. × 533.



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