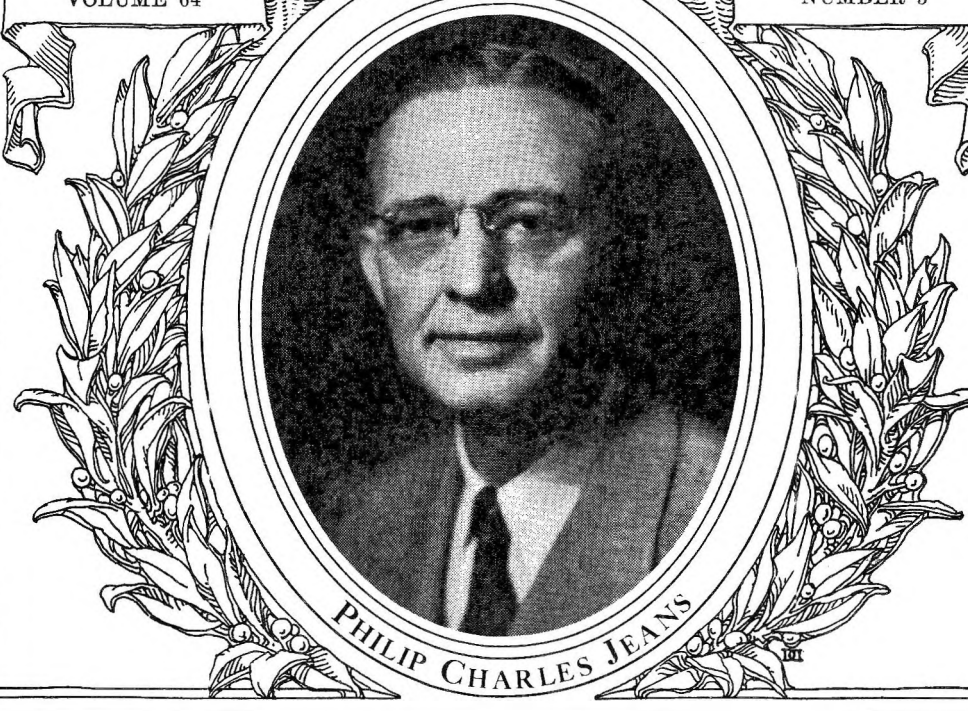
MARCH 10, 1958

THE JOURNAL OF NUTRITION

VOLUME 64 NUMBER 3



PHILIP CHARLES JEANS

Official organ of the American Institute of Nutrition

PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA 4, PA.

Price, $7.50 per volume, domestic; $8.00 per volume, foreign

Entered as second-class matter January 20, 1934, at the post office at Philadelphia, Pa., under Act of March 3, 1879.

Publications of The Wistar Institute

THE JOURNAL OF MORPHOLOGY

Devoted to the publication of original research on animal morphology, including cytology, protozoology, and the embryology of vertebrates and invertebrates. Articles do not usually exceed 50 pages in length.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF COMPARATIVE NEUROLOGY

Publishes the results of original investigations on the comparative anatomy and physiology of the nervous system.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE AMERICAN JOURNAL OF ANATOMY

Publishes the results of comprehensive investigations in vertebrate anatomy — descriptive, analytical, experimental.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE ANATOMICAL RECORD

Organ of the American Association of Anatomists and the American Society of Zoologists

For the prompt publication of concise original articles on vertebrate anatomy, preliminary reports; technical notes; critical notes of interest to anatomists and short reviews of noteworthy publications.

Issued monthly, 3 vols. annually: $30.00 Domestic, $32.00 Foreign, per year.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

Publishes papers embodying the results of original researches of an experimental or analytical nature in the field of zoology.

Issued 9 times a year, 3 vols. annually: $30.00 Domestic, $32.00 Foreign, per year.

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

Publishes original articles on comparative human morphology and physiology as well as on the history of this branch of science and the techniques used therein. In addition, it gives comprehensive reviews of books and papers, a bibliography of current publications, abstracts and proceedings of the American Association of Physical Anthropologists, and informal communications.

Issued quarterly, 1 vol. annually: $10.00 Domestic, $11.00 Foreign, per year.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Publishes papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects.

Issued bimonthly, 2 vols. annually: $20.00 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF NUTRITION

Organ of the American Institute of Nutrition

Publishes original researches in the field of nutrition and occasional reviews of literature on topics with which the journal is concerned.

Issued monthly, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

THE AMERICAN ANATOMICAL MEMOIRS

Publishes original monographs based on experimental or descriptive investigations in the field of anatomy which are too extensive to appear in the current periodicals. Each number contains only one monograph. List of monographs already published, with prices, sent on application.

ADVANCE ABSTRACT CARD SERVICE

Every paper accepted for publication in one of the above periodicals is accompanied by the author's abstract. The abstract and the complete bibliography reference to the paper as it will eventually appear is printed on the face of a standard library catalogue card. The Advance Abstract Card Service is issued promptly and in advance of the journal in which the paper is published.

Issued monthly (approximately 500 abstracts annually). Single set subscriptions, $5.00; two sets to a single subscriber $8.00, per year

These publications enjoy the largest circulation of any similar journals published.

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

THIRTY SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

Original first edition back volumes and numbers of

THE JOURNAL OF NUTRITION

Complete volumes	*Year*	*Price per volume*
1–7	1928–1929	$18.00
9–12	1935–1936	15.00
24	1942	12.00
26	1943	12.00
29–32	1945–1946	10.00
34–51	1947–1953	9.00
52–57	1954–1955	7.50
59–63	1956–1957	7.50
Index to volumes 1–15		.75
Index to volumes 16–36		2.25

Incomplete volumes	*Year*	*Price per single number*
16, nos. 1, 2, 4, 5, 6	1938	$4.00
18, nos. 3–6	1939	4.00
19, nos. 1, 2, 4	1940	3.00
20, nos. 2, 3, 6	1940	3.00
21, nos. 1, 4, 6	1941	3.00
22, nos. 5, 6	1941	3.00
23, nos. 2, 3, 5, 6	1942	3.00
25, nos. 2–6	1943	3.00
27, nos. 5, 6	1944	2.50
28, nos. 2–6	1944	2.50
33, nos. 2–6	1947	2.50
58, nos. 3–4	1956	2.50

Prices subject to change without notice. Availability depends upon prior sales

ALL UNLISTED VOLUMES AND NUMBERS PRIOR TO VOLUME 63 ARE OUT-OF-PRINT

Annual subscription $22.50 domestic; $24.00 foreign
Single copies $2.25 each

Send order with remittance to

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

for So Many Essential Nutrients..

Protein . . . of excellent quality

Energy . . . readily available

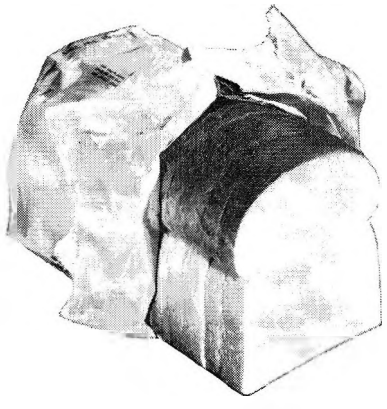
B vitamins . . . high in proportion to calories

Minerals . . . especially iron and calcium

Enriched Bread

is basic,

integral, compatible in most all diets in health and disease . . . regardless of dietary adjustment needed. Its fat content is minimal.



AMERICAN BAKERS ASSOCIATION
20 North Wacker Drive Chicago 6, Illinois

STUDIES ON THE TOXICITY OF *INDIGOFERA ENDECAPHYLLA*

I. TOXICITY FOR RABBITS

E. M. HUTTON, G. M. WINDRUM AND C. C. KRATZING

Commonwealth Scientific and Industrial Research Organization Plant and Soils Laboratory, Division of Plant Industry, Brisbane. Department of Pathology, Brisbane Hospital. Department of Physiology, University of Queensland, Brisbane, Australia

(Received for publication September 24, 1957)

INTRODUCTION

Indigofera endecaphylla Jacq. has been introduced or occurs naturally in Hawaii, Puerto Rico, Ceylon and other tropical countries, where it has been regarded with interest as a potential pasture legume. However, Emmel and Ritchey ('41) and Nordfeldt et al. ('52) have shown that it was toxic to rabbits, cows and sheep. The main effects observed were that liver degeneration occurred and that pregnant animals aborted. The work of Nordfeldt et al. ('52) indicated that the guinea pig was less susceptible to the toxin of *I. endecaphylla,* while Freyre and Warmke ('52) found that guinea pigs survived indefinitely although pregnant females aborted.

Rosenberg and Zoebisch ('52) designed a chick test for investigating toxicity in forage legumes and this was used by Morris, Pagán and Warmke ('54) who identified hiptagenic acid as the toxic component of *I. endecaphylla.* Previously Carter and McChesney ('49) had proved that hiptagenic acid was identical with β-nitropropionic acid. Further work by Cooke ('55) in Hawaii supported the view that the toxic constituent of *I. endecaphylla* was β-nitropropionic acid.

Apart from its toxicity, *I. endecaphylla* is the most promising summer pasture legume introduced by the C. S. I. R. O.

Plant and Soils Laboratory, Brisbane. It is eaten readily by grazing animals and has been shown[1] to have an average protein content of 25% on a dry matter basis and to produce 150 to 200 lbs. of nitrogen/acre in a season.

The work reported here was designed to find if a relationship existed between the β-nitropropionic acid content of diets containing *I. endecaphylla* and their toxicity. Toxicity was measured by the degree of liver damage produced in the rabbit.

MATERIALS AND METHODS

The main experiments have been done with a strain of *I. endecaphylla* from Ceylon and designated as 18557. Two other strains from Africa, 16069 and 16110, have also been investigated.

The β-nitropropionic acid content of these plants was estimated by the Cooke ('55) method. The pure synthetic β-nitropropionic acid was synthesized using the method of Hass, Feuer, and Pier ('51). The purified acid had a melting point of 70°C (corr.) and on analysis gave:— C, 30.83%; H, 4.38%; N, 11.4% and O, 52.2%. (The theoretical values for β-nitropropionic acid are:— C, 30.26%; H, 4.23%; N, 11.76% and O, 53.75%.) Thus the synthetic compound used in force-feeding was β-nitropropionic acid.

Locally-bred rabbits of various weights were housed in individual cages except for the experiment reported in table 2. Their food intakes were measured daily and their weights twice weekly. Water was provided ad libitum.

Diets contained either green leaf, cooked and dried green leaf, dried leaf, or seed. Diets containing synthetic β-nitropropionic acid were very unpalatable, so it was necessary to force-feed this compound daily with a stomach tube. The β-nitropropionic acid was administered in warm aqueous solution and the stomach tube flushed through with a sucrose solution before removal.

[1] Personal communication, W. W. Bryan and C. S. Andrew.

The livers were removed from the rabbits as soon as possible after they died or were killed and then fixed in 10% formol-saline. Paraffin sections were prepared and stained with haematoxylin and eosin, Van Gieson, and silver impregnation. Frozen sections were stained with Sudan IV to demonstrate fat.

RESULTS

β-*nitropropionic acid content of Indigofera endecaphylla*

The β-nitropropionic acid content of green leaf of strain 18557 was significantly less than that of either 16069 or 16110 which did not differ significantly from each other.

TABLE 1

β-Nitropropionic acid contents of three strains of I. endecaphylla

STRAIN	NO. PLANTS TESTED	MEAN	RANGE IN INDIVIDUAL PLANTS
		mg/gm fresh wt.	*mg/gm fresh wt.*
16069	363	13.2	5.0 – 26.0
16110	222	13.0	5.5 – 24.1
18557	190	6.5 [1]	1.1 – 14.4

[1] Strain 18557 significantly different ($P < 0.01$) from 16069 and 16110.

Cooke ('55) found that the β-nitropropionic acid content of immature and mature leaves of *I. endecaphylla* was 9.8 and 8.8 mg/gm fresh weight respectively, equivalent to 4.9 and 4.4% on a dry weight basis. These figures are comparable with those we have obtained with 18557 which is representative of the common lowland type.

No β-nitropropionic acid could be detected in the seeds by Cooke's ('55) test even after 4 days' germination, by which time the cotyledons had emerged from the seed coats. Digestion of 5 gm of finely ground seed with 150 mg of each of papain and takadiastase in 20 ml acetate buffer of pH 4 at 37°C for 16 hours failed to release any β-nitropropionic acid.

Animal experiments

(a) Green leaf. Rabbits were fed fresh green leaf supplemented with pellets made from a proprietary poultry meal.[2] This diet was quite palatable and the animals ate well initially.

The toxic effect of the diet became obvious clinically. Most of the animals showed decreased food intake, loss of weight, and became listless; many died and others were killed when moribund. The average survival time was approximately three weeks.

Table 2 shows the results of feeding green leaf from strains 18557, 16069, and 16110 to young rabbits of approximately 1.5 to 2.0 kg initial weight.

At death, the livers of these animals were firm in consistency and showed a finely granular surface. The exception was the liver of R7A which was pale, soft, and mottled.

Histologically, R7A showed gross degenerative swelling of parenchymal liver cells with some necrosis of cells. This necrosis was maximal in the periportal areas, where there was some evidence of early proliferation of bile ducts and some mononuclear cell infiltration. The other animals showed a progressive nodular cirrhosis. Irregular nodules of regenerating liver cells were separated by strands of loose connective tissue containing increased numbers of bile ducts. Many of the cells in the regeneration nodules were undergoing fatty degeneration or necrosis, and multinucleated regenerating cells were quite common. Figure 1A shows a representative area from the liver of R15. In some areas, regeneration nodules had undergone necrosis and showed organization by newly formed fibrous tissue. The fibrotic changes were not so pronounced in these animals as the parenchymal cell changes, but some more mature fibrous tissue was found in the animals surviving the longest, such as R18.

(b) Comparison between green leaf and seed. Four mature rabbits each approximately 3 kg in weight were offered a diet of 300 gm fresh *I. endecaphylla* per day with pellets, and

[2] Red Comb Special Mix no. 3, Poultry Farmers Co-op. Soc. Ltd., Brisbane. Contains 19.3% protein.

TABLE 2

Effect of feeding green I. endecaphylla leaf to rabbits

INDIGOFERA STRAIN	RABBIT NO.	SEX	SURVIVAL TIME	WEIGHT IN KG		MEAN DAILY LEAF INTAKE [1]	LIVER PATHOLOGY
				Initial	Final		
			days	*kg*	*kg*	*gm/rabbit*	
18557	R16	Male	22	2.23	1.42	46	Diffuse nodular cirrhosis
	R18	Male	32	2.04	1.28		Diffuse nodular cirrhosis
16069	R9	Male	16	1.59	0.91	34	Diffuse nodular cirrhosis
	R10	Male	20	1.68	1.29		Diffuse nodular cirrhosis
	R13	Male	22	1.94	1.36		Diffuse nodular cirrhosis
16069	R7A	Female	5	1.91	1.70	56	Acute liver degeneration and necrosis
	R15	Female	20	1.85	1.27		Diffuse nodular cirrhosis
	R14	Female	23	1.81	1.36		Diffuse nodular cirrhosis
16110	R12	Male	14	1.47	1.19	47	Diffuse nodular cirrhosis
	R11	Male	17	1.56	1.11		Diffuse nodular cirrhosis
	R17	Male	27	2.27	1.47		Diffuse nodular cirrhosis

[1] Leaf intake/rabbit calculated from total intake of groups containing two or three rabbits. The groups were offered 200 gm fresh leaf per day with pellets ad libitum.

two were fed pellets made up of 50% *I. endecaphylla* 18557 seed with meal. Of the two on seed pellets, R5 ate 90 gm over the first 24 hours and did not eat again till the 5th day, shortly before it became moribund at which time it was killed. R24 ate 88 gm seed pellets over a period of two days and became obviously ill. It refused to eat any further seed pellets but did eat green plant material (not *Indigofera*) daily until 24 hours before it became moribund on the 19th day. The clinical picture suggested an initial episode of severe toxic liver damage with anorexia followed by a period of partial recovery with reduced food intake before death.

Table 3 shows the results of this experiment. These diets were highly toxic and showed that the seed was at least as toxic as the leaf, though it contained no β-nitropropionic acid. The total intake of β-nitropropionic acid of the animals eating the green leaf is included in table 3.

Macroscopically the livers from this group were pale, soft, and mottled in appearance with the exception of the liver from R24 which was shrunken, granular, and firm in consistency.

Histologically, the rabbits on leaf (R1 to R4) showed widespread acute toxic changes in parenchymal liver cells. The cytoplasm was grossly swollen and granular, while the nuclei showed degenerative changes including eosinophilic inclusions. The changes were most severe in the periportal regions, where many cells were necrotic with deeply eosinophilic cytoplasm and pyknotic nuclei. Haemorrhages were present periportally and there was a variable proliferation of cells in these areas. These cells morphologically resembled bile duct cells. There was some mononuclear and polymorphonuclear cell infiltration of the portal tracts, but this was of relatively minor degree. The changes are illustrated in figures 1B and 1D. Figure 1B shows the severe degenerative changes with periportal hemorrhage and necrosis in R2, and figure 1C is from a normal rabbit for comparison. In figure 1D haemorrhage and early cell proliferation about the portal areas in R2 are shown.

TABLE 3

Results from feeding green tops and seed of I. endecaphylla to rabbits

RABBIT NO.	TREATMENT	SURVIVAL TIME	MEAN DAILY INTAKE/RABBIT	INTAKE OF β-NITROPROPIONIC ACID OVER FEEDING PERIOD	INITIAL WEIGHT	FINAL WEIGHT	LIVER PATHOLOGY
		days	*gm*	*gm*	*kg*	*kg*	
R1	300 gm/day green *I. endecaphylla* plus meal	6	144 green	6.05	2.36	2.78	Gross acute liver degeneration and necrosis
R2		5	145 green	5.08	3.01	3.12	Gross acute liver degeneration and necrosis
R3	pellets ad lib.	5	109 green	3.82	—	—	Gross acute liver degeneration and necrosis
R4		10	87 green	6.09	3.26	3.11	Gross acute liver degeneration and necrosis
R5	100 gm pellets comprising 50% seed and 50% meal	5	45 seed in 1 day	0.00	3.18	2.92	Gross acute liver degeneration and necrosis
R24		19	44 seed in 2 days	0.00	2.62	2.08	Diffuse nodular cirrhosis

R5, fed the seed, showed similar gross toxic changes to those seen in the animals fed the leaf, but of more severe degree (fig. 2A). R24 had diffuse nodular cirrhosis with areas of regeneration and was histologically similar to the livers of the animals fed the green leaf in the experiment summarized in table 2. This was in keeping with its longer period of survival than R5, and with the clinical state of the animal. The sequence of a severe toxic episode followed by partial recovery was consistent with the pathological changes of a diffuse nodular cirrhosis due to post-necrotic scarring.

(c) Dried leaf. Green *I. endecaphylla* 18557 herbage was treated in two ways. One batch was dried at 38°C, and the other was cooked for one hour at 121°C and 15 lbs. pressure before drying at 38°C. The material so obtained was made into pellets with equal parts of meal and fed to animals (table 4). The pellets were analysed for β-nitropropionic acid.

These pellets were unpalatable. The rabbits ate 20 to 25 gm daily for the first 5 to 6 days and then refused to eat more. Ordinary pellets were then offered and after a period of semi-starvation the consumption rose to about 90 gm daily. When the pellets containing *I. endecaphylla* were offered again the food intake fell to 14 to 20 gm daily. Feeding with normal pellets and those containing *I. endecaphylla* was continued alternately till the animals died.

The total intake of the pellets containing 50% dried leaf was only 220 and 562 gm respectively for R6A and R19, which gave a total intake of only 1.25 and 2.45 gm of β-nitropropionic acid respectively.

Macroscopically the livers of both animals were granular, yellow brown in colour, and reduced to approximately one third of normal size. Figure 2B shows the liver from R19.

Histologically both animals had a fine diffuse nodular cirrhosis (fig. 2C from R19). Areas of necrotic cells about portal triads were common in R6A. In R19 the liver cells in the regeneration nodules showed fatty infiltration and degenerative swelling, while the nodules were separated by more compact fibrous tissue than was seen in R6A. These differ-

TABLE 4

Effect on the rabbit liver of diets containing ordinary dried, and pressure-cooked and dried I. endecaphylla

RABBIT NO.	TREATMENT OF GREEN *I. endecaphylla* herbage	SURVIVAL TIME	TOTAL INTAKE OF		WEIGHT		LIVER PATHOLOGY
			Dried *I. endecaphylla*	β-nitropropionic acid	Initial	Final	
		days	*gm*	*gm*	*kg*	*kg*	
R6A	Dried at 38°C	50	110	1.25	3.23	3.00	Fine nodular cirrhosis
R19	Pressure cooked before drying at 38°C	84	281	2.45	3.94	3.21	Fine nodular cirrhosis

TABLE 5

Results from force-feeding two rabbits with synthetic β-nitropropionic acid

RABBIT NO.	FEEDING PERIOD	TOTAL INTAKE OF SYNTHETIC β-NITROPROPIONIC ACID	WEIGHT OF ANIMAL		CONDITION OF ANIMAL AT END OF EXPERIMENT	HISTOLOGICAL LIVER CHANGES
			At start	At end		
	days	*gm*	*kg*	*kg*		
R20	34	5.50	3.08	2.81	Lively	Nil
R22	22	6.50	3.60	3.50	Lively	Nil

ences are explained by the varying lengths of time the animals survived on the diets given. The result with the cooked and dried leaf indicates that the toxin is heat stable.

(d) Synthetic β-nitropropionic acid. This compound was force fed daily for 5 days per week with a stomach tube to ensure that the rabbits ingested quantities similar to those they would obtain from eating green *I. endecaphylla.* R20 was fed a daily dose of 0.25 gm and R22 was started on 0.25 gm, later increased to 0.5 gm daily, till both animals had received a dose comparable to the amount ingested from green *I. endecaphylla* leaf by the groups described above.

The rabbits were fed a normal diet ad libitum and this was readily eaten. The animals lost a little weight, but remained normally active till killed.

Details of this experiment are given in table 5. Livers from these animals were macroscopically normal. Histological sections (fig. 2D) showed no significant abnormality. It appears that β-nitropropionic acid in these dosages is not toxic to rabbit liver.

DISCUSSION

Our results show that a similar type of liver damage can be produced by feeding green leaf, dried leaf, or seed of *I. endecaphylla.* Leafage of the strains of *I. endecaphylla* used by us has a comparable toxicity to that of the strains fed to animals by previous workers. It is apparent from tables 3, 4, and 5 that toxicity of the different diets is not related to their β-nitropropionic acid contents. In addition, table 2 shows that with the comparable intakes of green leaf for the three strains of *I. endecaphylla,* the pattern of liver damage and survival time in the different rabbits is similar despite 16069 and 16110 each having almost twice the β-nitropropionic acid content of 18557 (table 1).

It can be seen from table 2 that all the animals lost weight. MacDonald and Thomas ('56) showed that rabbits maintained on a low-protein diet for long periods lost weight and developed a mild diffuse hepatic fibrosis. However, after 70 to 80 days on such a diet they found only diffuse fibrosis with no

evidence of a nodular fibrosis. The type of liver damage seen in our experiments was quite different, being a relatively rapid process of degeneration and necrosis with subsequent regeneration and fibrosis to give the appearance of a post-necrotic scarring type of cirrhosis. Our animals had a decreased food intake (which was almost certainly a symptom of the progressive liver disease) and this may have eventually placed the animals in a relatively suboptimal nutritional state and led to some exaggeration of the primary pathological changes.

The results reported in table 5 from force-feeding rabbits with synthetic β-nitropropionic acid is additional evidence that this compound is not primarily involved in the liver damage following ingestion of *I. endecaphylla* leaf. The amounts of the synthetic compound fed were comparable with the intakes of β-nitropropionic acid from eating green leaf (table 3), but were considerably in excess of those from feeding dried leaf (table 4).

The symptoms and pathological changes induced in the rabbits were essentially the same as those previously reported (Emmel and Ritchey, '41; Nordfeldt et al., '52). In addition we have been able to demonstrate that liver damage can range from acute necrosis with death in a few days to a progressive nodular cirrhosis. The development and severity of the liver damage apparently parallels the rate of ingestion of the toxin.

Although β-nitropropionic acid could not be detected in the seed by Cooke's ('55) method it is evident from table 3 that seed is a concentrated source of the liver toxin present in *I. endecaphylla.* McKay, Lalich, Schilling, and Strong ('54) found that seeds of *Lathyrus odoratus* were rich in a crystalline toxin identified later as an amino propionitrile. More recently Garbutt and Strong ('57) tested seeds of a range of leguminous species for amino propionitrile and found this substance only in *Lathyrus* species. We have found no trace of amino propionitrile in seeds of *I. endecaphylla* and no liver

changes have been reported in feeding experiments with species of *Lathyrus*.

The leaf of *I. endecaphylla* has a negligible alkaloid content,[3] although Gardner and Bennetts ('56) have reported three species of *Crotalaria* and two species of *Lupinus* which cause liver damage in stock due to their content of alkaloids. Gardner and Bennetts ('56) also described "Birdsville horse disease" caused by ingestion of *I. enneaphylla*, the toxin of which is unidentified. The possible role of pyrrolizidine alkaloids from species of *Crotalaria* and *Senecio* as aetiological factors in liver disease of man has been discussed recently (Bras, Berry and György, '57). We know of no reports of *Indigofera* species being implicated in human disease. Many cases of human cirrhosis are of doubtful aetiology, and the discovery of naturally occurring and potent liver toxins could be of significance in human nutrition.

SUMMARY

1. Rabbits fed green leaf, dried leaf or seed of *I. endecaphylla* develop severe liver damage.

2. β-Nitropropionic acid could not be detected in the seed though it is present in the leaf.

3. Leaf of two strains of *I. endecaphylla* each contained twice the amount of β-nitropropionic acid of the other strain used. This difference had no effect on the liver damage induced by feeding the three strains.

4. Synthetic β-nitropropionic acid is not toxic to rabbit liver when force fed in amounts comparable to that in the green leaf.

5. It appears that the toxin of *Indigofera endecaphylla* is not β-nitropropionic acid.

ACKNOWLEDGMENTS

We wish to thank Dr. J. Griffiths Davies, Associate Chief, C.S.I.R.O. Division of Plant Industry, for encouragement and

[3] Personal communication, J. R. Price.

advice throughout this project, and Dr. A. W. Pound, Director, Department of Pathology, Brisbane Hospital, for generous provision of facilities and for advice. Also we gratefully acknowledge the valuable technical assistance of Mr. L. B. Beall and the synthesis of the β-nitropropionic acid by Dr. M. P. Hegarty and Mr. G. F. Parker. Dr. M. P. Hegarty developed the analytical method used for estimating the β-nitropropionic acid content of single plants. Dr. K. W. Zimmerman of the C.S.I.R.O. Division of Industrial Chemistry did the microanalysis of the synthetic β-nitropropionic acid.

LITERATURE CITED

BRAS, G., D. M. BERRY AND P. GYÖRGY 1957 Plants as aetiological factor in veno-occlusive disease of the liver. The Lancet, *272:* 960.

CARTER, C. L., AND W. J. MCCHESNEY 1949 Hiptagenic acid identified as β-nitropropionic acid. Nature, *164:* 575.

COOKE, A. R. 1955 The toxic constituent of *Indigofera endecaphylla.* Arch. Biochem. Biophys., *55:* 114.

EMMEL, M. W., AND G. E. RITCHEY 1941 The toxicity of *Indigofera endecaphylla* Jacq. for rabbits. J. Amer. Soc. Agron., *33:* 675.

FREYRE, R. H., AND H. E. WARMKE 1952 Legume toxicity. Puerto Rico Fed. Expt. Sta. Ann. Report.

GARBUTT, J. T., AND F. M. STRONG 1957 Colorimetric determination of beta-aminopropionitrile in mature legume seeds. Agric. and Food Chem., *5*: 367.

GARDNER, C. A., AND H. W. BENNETTS 1956 The toxic plants of Western Australia. West Australian Newspapers Ltd., Periodicals Division, Perth.

HASS, H. B., H. FEUER AND S. M. PIER 1951 The preparation of 3-nitropropanoic acid. J. Am. Chem. Soc., *73:* 1858.

MACDONALD, I., AND G. A. THOMAS 1956 Investigations into the aetiology of an experimental nutritional hepatic fibrosis. Clin. Sci., *15:* 389.

MCKAY, G. F., J. J. LALICH, E. D. SCHILLING AND F. M. STRONG 1954 A crystalline *Lathyrus* factor from *Lathyrus odoratus.* Arch. Biochem. and Biophys., *52:* 313.

MORRIS, M. F., C. PAGÁN AND H. E. WARMKE 1954 Hiptagenic acid, a toxic component of *Indigofera endecaphylla.* Science, *119:* 322.

NORDFELDT, S., L. A. HENKE, K. MORITA, H. MATSUMOTO, M. TAKAHASHI, O. R. YOUNGE, E. H. WILLERS AND R. F. CROSS 1952 Feeding tests with *Indigofera endecaphylla* Jacq. (Creeping Indigo) and some observations on its poisonous effects on domestic animals. Tech. Bull. 15, Hawaii Agricultural Expt. Sta., College of Agric., Univers. Hawaii.

ROSENBERG, M. M., AND O. C. ZOEBISCH 1952 A chick test for toxicity in forage legumes. Agron. Jour., *44:* 315.

PLATE 1

EXPLANATION OF FIGURES

1A R15 Liver H & E × 30
Irregular regeneration nodules separated by loose connective tissue containing increased numbers of bile ductules.

1B R2 Liver H & E × 30
Gross parenchymal cell degeneration with haemorrhage and cell necrosis in periportal area.

1C Normal Rabbit Liver H & E × 30

1D R2 Liver H & E × 100
Proliferating cells morphologically resembling bile duct cells in portal area. Haemorrhage, parenchymal cell necrosis and gross degeneration can be seen.

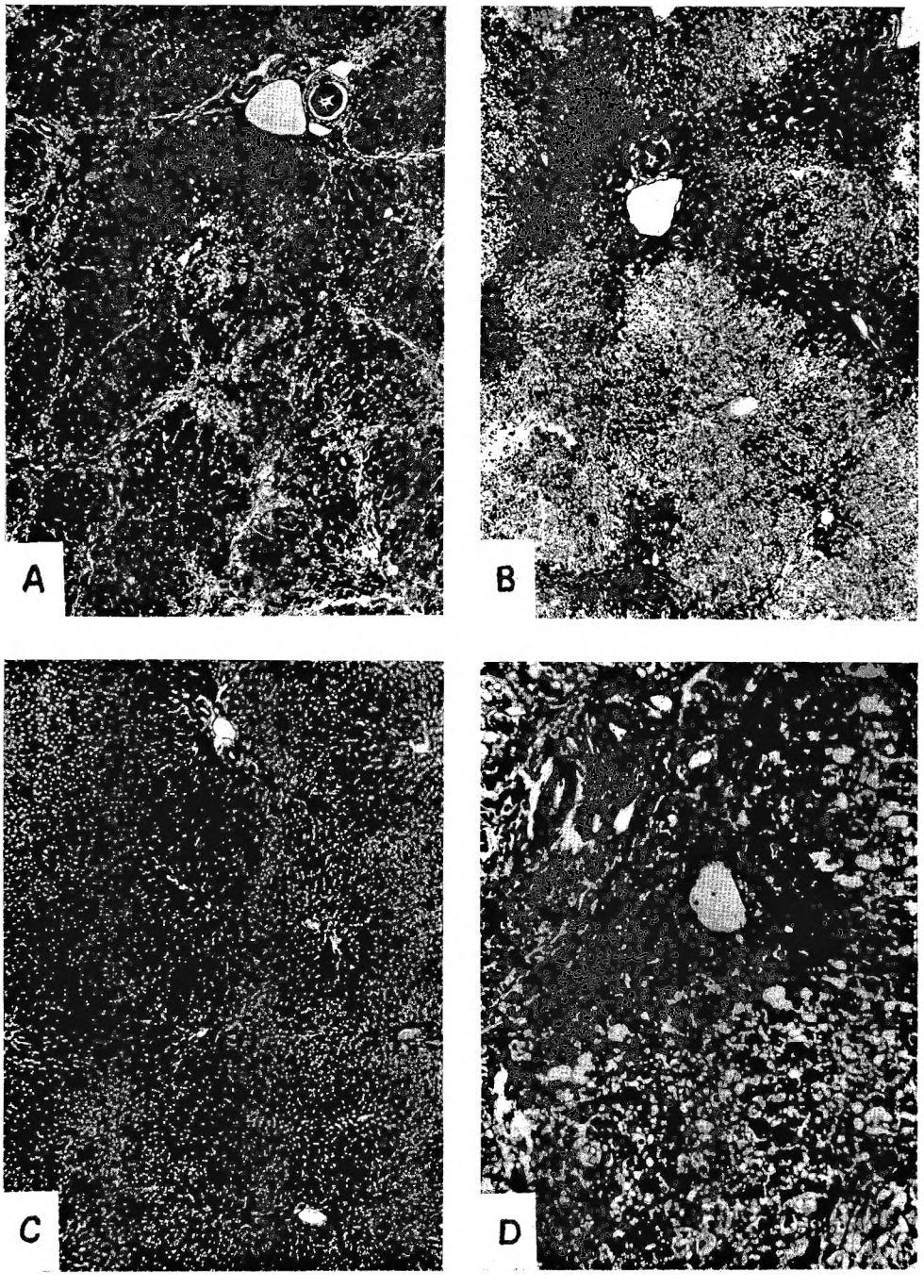

A
B
C
D

PLATE 2

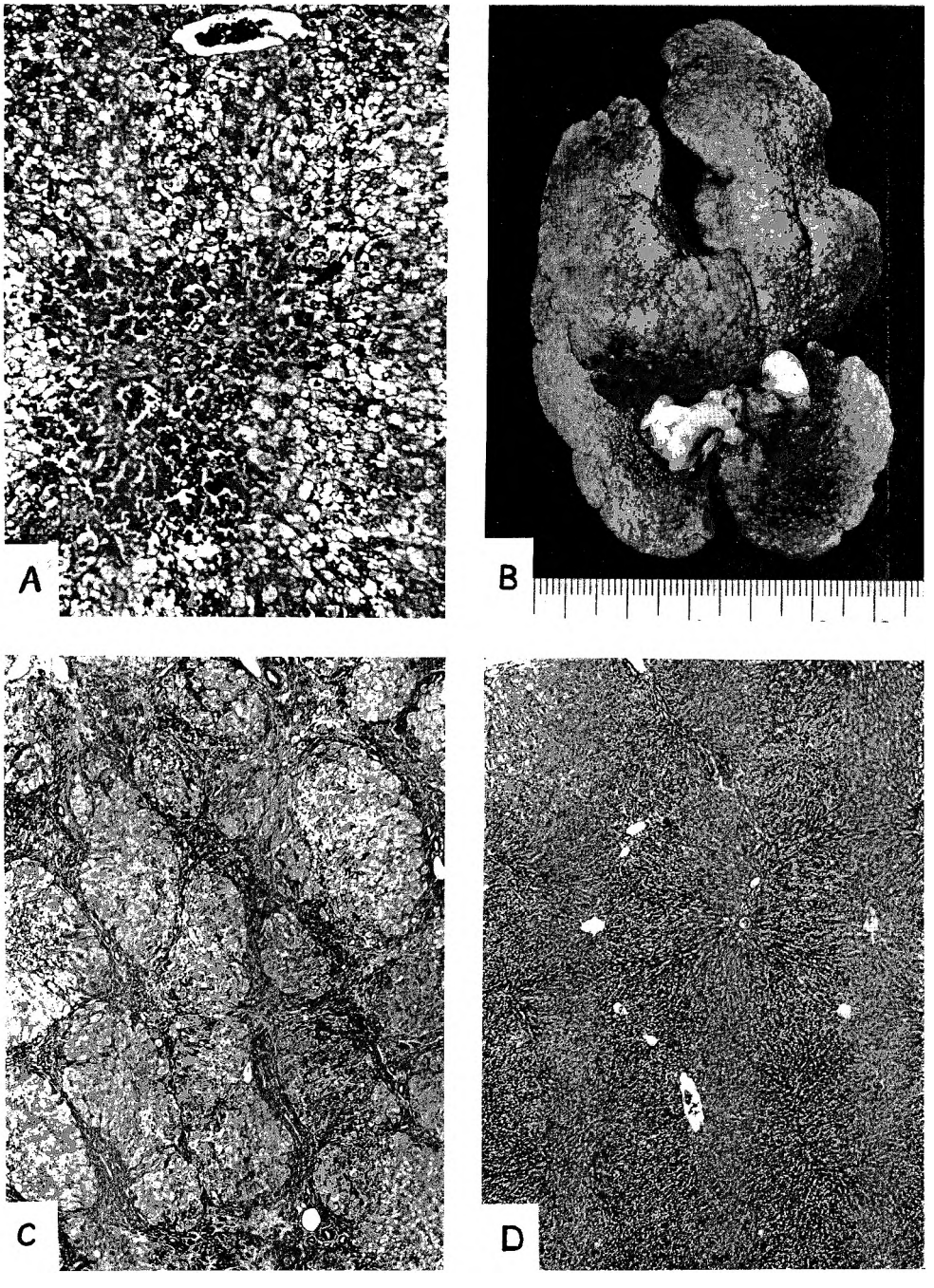
EXPLANATION OF FIGURES

2A R5 Liver H & E × 100
Similar to figure 1D. Gross parenchymal cell degeneration with necrosis, haemorrhage and some cellular proliferation in portal area.

2B R19
Macroscopic view of liver showing fine diffuse nodular cirrhosis.

2C R19 Liver H & E × 30
Fine nodular cirrhosis with nodules of parenchymal cells separated by connective tissue bands containing increased numbers of bile ductules and fibroblasts.

2D R20 Liver H & E × 30
No significant abnormality after force feeding synthetic β-nitropropionic acid. Compare with figure 1C from normal rabbit.


A
B
C
D

TRYPTOPHAN-NIACIN RELATIONSHIPS IN PREGNANCY [1]

ANNE W. WERTZ, MARY E. LOJKIN, BERYL S. BOUCHARD AND MARILYN B. DERBY

Nutrition Research Laboratory, School of Home Economics, University of Massachusetts, Amherst

(Received for publication August 26, 1957)

The increased excretion of certain metabolites of niacin during human pregnancy has been reported by several investigators (Moore et al., '47; Frazier et al., '49; Oldham et al., '49; and Lojkin et al., '52). The excretion of N^1-methylnicotinamide in the last trimester was found by Lojkin et al. ('52) to be as much as 200% of the niacin intake in some subjects. When the excretion of N^1-methyl-6-pyridone-3-carboxylamide was also considered, the total excretion of the two metabolites sometimes exceeded both the niacin intake and the amount that was thought to be obtainable from the dietary tryptophan. Subsequent investigation in this laboratory on the effect of hormonal administration on the niacin metabolism of intact and ovariectomized albino rats demonstrated that injections of a combination of progesterone and estrone increased the output of acid-hydrolyzable niacin and N^1-methylnicotinamide but did not affect the output of tryptophan (Lojkin, '56). These results suggested that the ovarian hormones increased the catabolism of protein, thereby liberating tryptophan for its conversion to niacin. It is also possible that the hormones present in the pregnant body may increase the efficiency of this conversion.

[1] Contribution 1111, University of Massachusetts Agricultural Experiment Station; supported in part by funds from Regional Project NE–16, Relationship of Nutrient Intake to Nutritional Status in Human Subjects.

This paper presents the results of a study of the excretion of tryptophan, niacin, N^1-methylnicotinamide (N^1-Me), N^1-methyl-6-pyridone-3-carboxylamide (pyridone), and quinolinic acid in a group of women who consumed a supplement of tryptophan for 14 days in the last trimester of pregnancy and again after the third month postpartum. The data indicate that the conversion of tryptophan to niacin is more efficient in the pregnant than nonpregnant state.

EXPERIMENTAL

Subjects. A group of 12 healthy women eating self-selected diets and living in their own homes cooperated in this study. They ranged in age from 21 to 36 years, and in weight from 105 to 155 pounds. There were 5 primiparae and 7 multiparae in the group; all had uncomplicated pregnancies and deliveries.

Plan of study. The excretion of the metabolites of niacin and tryptophan not only varies from day to day for the same individual but also changes throughout the months of pregnancy. In order to obtain reliable data for the output of these metabolites in the different experimental periods, the following plan was adopted, based on statistical evaluation of the analytical data obtained from a preliminary study on three subjects:[2]

Period 1: A 14-day period in the last trimester of pregnancy, during which at least 5 complete 24-hour urine samples and dietary records were obtained on nonconsecutive days.

Period 1S: A 14-day period immediately following period 1, during which each subject consumed a daily supplement of 500 mg of DL-tryptophan. Urine samples and dietary records were obtained as in period 1.

Period 2: A 14-day period immediately after the third month postpartum, during which urine samples and diet records were obtained as in period 1.

[2] The authors are indebted to Dr. Walter D. Foster (formerly Regional Biometrician, West Virginia Agricultural Experiment Station, Morgantown, West Virginia) for the statistical interpretation.

Period 2S: A 14-day period immediately following period 2, during which each subject consumed a daily supplement of 500 mg of DL-tryptophan. Urine samples and diet records were obtained as in period 1.

During the week following periods 1S and 2S, three additional 24-hour urine samples were collected to determine whether the output of the metabolites was the same in the post- as in the pre-supplemented periods.

Dietary. Each subject weighed all solid food and weighed or measured all liquids consumed. The quantities were recorded in grams or fluid ounces. If mixed foods such as casseroles were eaten, the subjects recorded the recipes as well as the portions eaten.

Protein and niacin intakes were calculated from food value tables (Watt and Merrill, '50). The tryptophan content of the diet was obtained by analysis of many of the foods eaten and by calculation from published values [3] (Lyman and Kuiken, '49; Block and Bolling, '51; Wertz et al., '56).

The daily DL-tryptophan supplement of 500 mg for each subject was weighed, and approximately one-third the dose put into each of three capsules, one of which was taken at each meal on each day of the supplemented period.

Urine collection and analysis. Complete 24-hour urine samples were collected in brown glass bottles to which 10 ml of toluene had been added. The volumes of the urine samples were recorded, the urine filtered, and suitable aliquots either analyzed immediately or stored at -20°C until analyses were

[3] A much less time-consuming method of calculating tryptophan intake on a varied diet would be to estimate it as 1% of the protein intake. It was found in this laboratory from comparisons between the analyzed and calculated values for tryptophan in mixed diets that, 26 out of 36 times, the value obtained by analysis fell within ± 15% of the value for tryptophan calculated as 1% of the protein. The average value obtained by analysis for 36 individual 24-hour diets was 866 mg, and the value calculated as 1% of the protein was 852 mg, a difference of less than 2%. If food records for several days are obtained for an individual, it appears that a fairly good estimation of tryptophan intake can be obtained by estimating it as 1% of the protein. This procedure seems justified, as the tryptophan excretion appears to be independent of the tryptophan intake within wide limits.

made. All samples of urine in each period from each subject were analyzed for "free" and total tryptophan, N^1-Me, and H_2SO_4-hydrolyzable nicotinic acid. In each period representative samples from 10 subjects were analyzed for pyridone and from 6 subjects for quinolinic acid.

The microbiological method described by Steele et al. ('49) with *Leuconostoc mesenteroides* as the test organism was used for the determination of both "free" and total tryptophan. Hydrolysates for determination of total tryptophan were prepared by autoclaving 10 ml of urine in 2N NaOH at 15 lbs. for 5 hours. Nicotinic acid was determined by the method of Association of Vitamin Chemists ('51). The results obtained for nicotinic acid by this method include compounds such as nicotinuric acid, which stimulate the growth of *Lactobacillus arabinosus* but may not be normal metabolites of niacin (Lin and Johnson, '53; Reddi and Kodicek, '53). Since Henderson and Hirsch ('49) found that autoclaving for one half hour in 1 N H_2SO_4 resulted in 7 to 8% decarboxylation of quinolinic acid, the values obtained after hydrolysis of urine with 1 N H_2SO_4 probably included a small percentage of the quinolinic acid that is excreted in the urine. Quinolinic acid values were calculated as the difference between the values for "free" nicotinic acid obtained by microbiological assay of untreated urine samples and samples treated by autoclaving at 15 lbs. pressure for two hours with glacial acetic acid (Henderson, '49). The quinolinic acid value was obtained by multiplying the result by 1.36. N^1-methylnicotinamide was determined according to the method of Huff and Perlzweig ('47); pyridone, by the method of Holman ('54); and creatinine, by the alkaline picrate method described by Peters ('42).

The collection of urine was considered complete if the creatinine content of the sample fell within $\pm$ 10% of the average creatinine value obtained for the particular subject.

RESULTS AND DISCUSSION

The data on the protein and niacin intakes and the excretion of tryptophan and niacin metabolites are summarized in

table 1. There was considerable individual variation in the protein and niacin intakes and in excretion of the metabolites, which is illustrated by the magnitude of the standard deviations of the means.

The mean daily protein intake for all subjects was 68 and 66 gm in the pregnant and postpartum periods, respectively. This small difference in intake between the two periods was not statistically significant. The range in tryptophan intakes for the individual subjects was approximately 500 to 1100 mg per day. The mean daily niacin intake from the food was 13.8 mg in the pregnant period. However, three subjects were taking multi-vitamin supplements in the pregnant period, which raised the mean daily intake of the group to 19.4 mg. None of the women were taking vitamin supplements in the postpartum period in which the mean daily intake of niacin was 13.3 mg.

Tryptophan. The average daily excretions of "free" and total tryptophan were 32.7 and 50.5 mg, respectively, in period 1, and 10.7 and 14.4 mg, respectively, in period 2. The values for both "free" and total tryptophan were significantly higher in the pregnant than in the postpartum period.

That the excretions of certain amino acids increase during pregnancy has been reported by several investigators (Wallraff et al., '50; Miller et al., '54; Sheft and Oldham, '52). In the present study the excretion of tryptophan by subjects on the usual diet was approximately three times as great in late pregnancy as in the nonpregnant period. However, the ratio of total to "free" tryptophan, which was 1.3 in nonpregnancy and 1.5 in late pregnancy, was not greatly altered. In all periods studied, the excretion of tryptophan did not appear related to the protein intake. In the pregnant period, the greatest output of tryptophan, 102.6 mg, was made by a subject with an average daily protein intake of 51 gm. In contrast, a subject consuming 98 gm protein excreted 49.4 mg tryptophan, which was very close to the group mean. Frazier ('54) reported that there was no apparent relation-

TABLE 1

Average daily excretion of tryptophan and niacin metabolites

NO. OF CASES / PERIOD	12 Tryptophan "Free"	12 Tryptophan Total	12 Total niacin	12 N^1-Me [1]	10 Pyridone [2]	6 "Free" niacin	6 Quinolinic acid
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
				Late pregnancy			
1, Unsupplemented Protein, gm, 67.9 ± 13.3 Niacin, mg, 19.4 ± 10.9	32.7 ± 11.4 [3]	50.5 ± 21.5	2.56 ± 0.39	16.6 ± 3.1	24.8 ± 7.5	0.69 ± 0.11	3.85 ± 0.67
1S, 500 mg DL-tryptophan	28.1 ± 9.8 [3]	59.7 ± 19.9	2.95 ± 0.75	21.0 ± 5.0	41.6 ± 14.3	0.76 ± 0.08	4.72 ± 1.10
Changes from period 1 to 1S	−4.6 ± 1.0 [4]	9.1 ± 2.6	0.38 ± 0.07	4.4 ± 1.0	16.8 ± 3.0	0.07 ± 0.02	0.87 ± 0.30
Significance levels [5]	0.01	0.01	0.01	0.01	0.01	0.05	0.05
				Postpartum			
2, Unsupplemented Protein, gm, 65.7 ± 15.1 Niacin, mg, 13.3 ± 3.2	10.7 ± 3.1 [3]	14.4 ± 4.2	1.53 ± 0.71	5.4 ± 1.9	11.6 ± 2.9	0.48 ± 0.08	2.43 ± 0.47
2S, 500 mg DL-tryptophan	11.2 ± 3.3 [3]	26.1 ± 6.6	1.61 ± 0.64	6.2 ± 1.9	22.1 ± 5.7	0.46 ± 0.09	2.49 ± 0.32
Changes from period 2 to 2S	0.5 ± 0.05 [4]	11.7 ± 1.6	0.08 ± 0.06	0.8 ± 0.3	10.5 ± 1.5	−0.02 ± 0.04	0.06 ± 0.18
Significance levels [5]	n.s.	0.01	n.s.	0.02	0.01	n.s.	n.s.
Differences between unsupplemented periods 1 and 2	22.0 ± 3.4 [4]	36.1 ± 5.5	1.03 ± 0.20	11.2 ± 0.8	13.2 ± 2.2	0.21 ± 0.05	1.42 ± 0.20
Significance levels [5]	0.01	0.01	0.01	0.01	0.01	0.01	0.01

[1] N^1-methylnicotinamide.

[2] N^1-methyl-6-pyridone-3-carboxylamide.

[3] Standard deviations.

[4] Standard errors.

[5] Paired "t" test.

ship between the amounts of tryptophan excreted and the source or amount of dietary nitrogen.

In the pregnant period, during supplementation of the usual diets with 500 mg of DL-tryptophan, the mean output of "free" tryptophan decreased significantly from 32.7 to 28.1 mg. Only one subject maintained the initial excretion. The addition of tryptophan to the diet in the postpartum period did not cause a significant difference in the excretion of "free" tryptophan. The apparent increase in excretion of total tryptophan during tryptophan supplementation in both periods was probably caused by the excretion of D-tryptophan which does not stimulate the growth of the test organism *Leuconostoc mesenteroides* and was therefore measurable only after its racemization by alkaline hydrolysis. After the tryptophan had been removed from the diet, the average excretion of total tryptophan dropped to below the presupplemented value. The excretion of D-tryptophan by human subjects after administration of D-tryptophan has been reported by Langer and Berg ('55). The data reported by Sarrett and Goldsmith ('50) on the analysis by chemical means of the total tryptophan in the urine after the ingestion of DL-tryptophan indicated that a considerable portion of the D-tryptophan was excreted.

The percentage of the tryptophan intake that was excreted was higher in pregnancy than in the postpartum period. However, the addition of tryptophan to the diet did not greatly affect the percentage of the intake that was excreted. The tryptophan excretion was 7.4, 5.1, 2.2 and 2.3% of the total tryptophan intake in periods 1, 1S, 2 and 2S, respectively.

The results obtained in the present study for "free" tryptophan excretion of nonpregnant women agree with the results obtained by Frazier ('54) for college women and with those of Miller et al. ('54) and Wallraff et al. ('50) for nonpregnant women. The values for total tryptophan excretion are not in accord with those reported by Frazier ('54) but are in fairly good agreement with those reported by Wallraff et al. ('50). The values for "free" and total tryptophan for pregnant women are in the same range as those reported in the litera-

ture (Wallraff et al., '50; Miller et al., '54; and Sheft and Oldham, ('52).

Niacin. The mean values obtained for the "H_2SO_4-hydrolyzable" or total niacin were 2.56 and 1.53 mg in periods 1 and 2, respectively. The difference was statistically significant between the pregnant and postpartum periods. The excretion of total niacin was 13.2% of the intake in period 1 and 11.5% in period 2.

During supplementation with tryptophan, the increase in total niacin was significant in period 1S, but was not significant in period 2S. After the tryptophan was removed from the diet, the average excretion of total niacin returned to the presupplemented levels in the pregnant period.

The "free" niacin values, obtained from the microbiological assay of nonhydrolyzed urine samples for 6 subjects, were only one-third to one-fourth those obtained for total niacin. The average amount of "free" niacin excreted in the pregnant period was 0.69 mg daily, which was significantly higher than the mean of 0.48 mg in the postpartum period. The excretion of "free" niacin represented 4.5 and 3.9% of the niacin intake in the pregnant and postpartum periods, respectively. Supplementation with tryptophan did not significantly alter the excretion of "free" niacin in the postpartum period, but did increase slightly the excretion in the pregnant period.

Quinolinic acid. In late pregnancy, the mean daily excretion of quinolinic acid was 3.85 mg which was significantly higher than the mean excretion of 2.43 mg in the postpartum period. During tryptophan supplementation the average excretion of quinolinic acid increased 0.87 and 0.06 mg in periods 1S and 2S, respectively; this increase was significant in the pregnant period only. Although the excretion of quinolinic acid increased 22% in period 1S, the increase represented only 0.21% of the amount of quinolinic acid theoretically equivalent to the dose of DL-tryptophan. These findings indicate that, under the conditions of this experiment, quinolinic acid was not a major urinary product of the metabolism of tryptophan, especially in comparison with pyridone. Horwitt et al. ('56)

stated that quinolinic acid did not produce as good correlations with tryptophan ingestion as N^1-Me. Henderson et al. ('49) reported that the quinolinic acid excretion of 4 adult male subjects was 3.1 to 5.5 mg and rose to 10.6 to 11.9 mg when 5 gm of DL-tryptophan was ingested. The increase was highly significant although it represented only 0.18% conversion of the tryptophan. Sarrett ('51) presented data that showed an increase of approximately 2.0 to 3.0 mg in the daily excretion of quinolinic acid by three subjects ingesting daily supplements of 1 gm of L-tryptophan. By converting the nicotinic acid values "after acid autoclaving" found in previous studies to quinolinic acid, he obtained values which indicated that a daily supplement of 5 gm of DL-tryptophan led to an average increase of 9.3 mg in the daily quinolinic acid excretion of 11 subjects, and that ingestion of 5 gm of L-tryptophan increased the quinolinic acid excretion 9.9 mg for one subject. In the present study, the percentage of the dose of 500 mg of DL-tryptophan excreted as quinolinic acid by the women in late pregnancy appears to be comparable to the percentage of the dose excreted as quinolinic acid by the subjects cited above, who were ingesting larger amounts. However, the same women in the postpartum period did not follow this pattern.

N^1-Me and pyridone. In agreement with the findings of other workers, the results of this experiment show that the two major urinary metabolites of niacin are the methylated derivatives, N^1-Me and pyridone. The mean excretion of N^1-Me in pregnancy was 16.6 mg and in nonpregnancy 5.4 mg which is in good agreement with results previously reported from this laboratory. The difference between the excretions in the two periods is highly significant. The percentage of the niacin intake that was excreted as N^1-Me (calculated in terms of niacin by multiplying milligrams of N^1-Me by 0.90) was 76 and 36% for the pregnant and nonpregnant periods, respectively.

During supplementation with tryptophan there was a significant increase in the output of N^1-Me in both periods, but the

mean increase, 4.4 mg, in the pregnant period was 5 times that of the mean increase, 0.8 mg, in the nonpregnant period and represented a highly significant difference in response between the two periods. After the tryptophan was removed from the diet, the N^1-Me excretion returned to the presupplemented levels.

The increase in N^1-Me excretion for the individuals during tryptophan supplementation varied in the pregnant period from 0 to 10.0 mg daily and in the postpartum period from 0 to 3.2 mg. In pregnancy N^1-Me excretion increased during the first few days of supplementation. The urine samples were collected on alternate days, and the maximum excretion of N^1-Me was observed usually on the second or 4th day of the supplemented period. In the supplemented postpartum period, the increase was less marked, and the day of maximum excretion was not consistent for the group.

The mean daily pyridone excretion was 24.8 and 11.6 mg in periods 1 and 2, respectively. The difference between the two periods was highly significant. The average amount of pyridone excreted by the subjects in the postpartum period was approximately the same as the highest values reported for college women by Frazier et al. ('55) and lower than those reported for men by Walters et al. ('55). The pyridone excretion (calculated in terms of niacin by multiplying milligrams of pyridone by 0.81) was 104 and 71% of the niacin intake in periods 1 and 2, respectively. Rosenthal et al. ('53) found that, after graded doses of nicotinamide, the excretion of pyridone by human subjects increased more rapidly than did that of N^1-Me. The data in table 1 indicate that pyridone was also the metabolite most affected by the ingestion of tryptophan. The highly significant increases were 16.8 and 10.5 mg in periods 1S and 2S, respectively.

When the pyridone to N^1-Me ratios were calculated for the different periods, a change in the ratios was found which was consistent for all individuals and illustrated the disproportionate increase in pyridone excretion after tryptophan

supplementation. The ratios for pyridone to N^1-Me were 1.4, 1.8, 2.0 and 3.2 in periods 1, 1S, 2 and 2S, respectively.

The combined excretion of N^1-Me and pyridone was 109% of the niacin intake in the postpartum nonsupplemented period, which confirms the observation of Frazier et al. ('55) that the excretion of the methylated derviatives balances the niacin intake. In contrast, the combined excretion of N^1-Me and pyridone was 221% of the niacin intake in the pregnant nonsupplemented period.

Conversion of tryptophan to niacin. The increases in urinary excretion of N^1-Me and pyridone, expressed as niacin, for 10 women after supplementation of their usual diet with 500 mg of DL-tryptophan in late pregnancy and in the postpartum period, and the amount of tryptophan that appeared to be equivalent to 1 mg of niacin are shown in table 2. For the

TABLE 2

Increases in the combined excretion of N^1-methylnicotinamide (N^1-Me) and N^1-methyl-6-pyridone-3-carboxylamide (pyridone) (expressed as niacin) after the usual diet of 10 women was supplemented with 500 mg of DL-*tryptophan daily in the late-pregnant and postpartum periods*

SUBJECT NUMBER	PERIOD 1, LATE PREGNANCY		PERIOD 2, POSTPARTUM	
	Increase in combined N^1-Me and pyridone exoretion	Amount tryptophan equivalent to 1 mg niacin;[1] 250 ÷ niacin increase	Increase in combined N^1-Me and pyridone excretion	Amount tryptophan equivalent to 1 mg niacin [1] 250 ÷ niacin increase
	mg/24 hr.	*mg*	*mg/24 hr.*	*mg*
1	17.8	14	5.3	47
2	8.4	30	15.5	16
3	34.7	7	6.9	36
4	21.9	11	14.2	18
5	14.1	18	12.1	21
6	8.9	28	5.8	43
7	11.7	21	10.0	25
8	14.7	17	5.4	46
9	9.5	26	6.9	36
10	30.1	8	10.5	24
Average	17.2	18	9.3 [2]	31

[1] Calculation based on the assumption that only the L-tryptophan content (250 mg) of the supplement was converted to N^1-Me and pyridone.

[2] Difference between increases in excretions in periods 1 and 2 significant at the 5% level ($t = 2.48$).

calculation of the amount of tryptophan that was equivalent to 1 mg of niacin it was assumed that only L-tryptophan gave rise to the increased excretion of the methylated derivatives of niacin; the values were obtained by dividing 250 by the amount of the increase of the metabolites in the supplemented periods. Although Price and Brown ('56) found an increase in pyridone excretion when single oral doses of 2 gm of D-tryptophan were given to human subjects, they found that the apparent yields in pyridone obtained from 4 gm of DL-tryptophan could almost be accounted for by the L-tryptophan content. Sarrett and Goldsmith ('50), in a study of the metabolism of L- and DL-tryptophan in normal man and in pellagrins, obtained data which indicated that only the L-isomer of tryptophan was effective as a nicotinic acid precursor. In the present study, if the ingestion of the D-tryptophan influenced the excretion of N^1-Me and pyridone, the values obtained in the calculation of the tryptophan: niacin ratio would be somewhat erroneous, but the significance of the difference in response of the subjects in the two supplemented periods would not be altered.

The amount of tryptophan equivalent to 1 mg of niacin varied from 7 to 30 mg (mean 18 mg) in late pregnancy and from 16 to 47 mg (mean 31 mg) in the postpartum period. If the total supplement of 500 mg of DL-trytophan contributed to the increased excretion of N^1-Me and pyridone, the average tryptophan: niacin conversion ratio for the pregnant period would be 36:1 which is considerably less than the values reported for other subjects. Goldsmith et al. ('56) found that the amount of tryptophan that appeared to be equivalent to 1 mg of niacin varied from 31 to 87 mg with an average of 55 mg for 14 subjects. Horwitt et al. ('56) reported that "Comparisons of the levels of N^1-methylnicotamide excreted at different levels of niacin and tryptophan intake showed that approximately 60 mg of tryptophan are equivalent to 1 mg of niacin." In our laboratory, in a controlled dietary study, the amount of tryptophan equivalent to 1 mg of niacin

varied from 45 to 69 mg with an average of 56 mg for 4 female subjects (unpublished data).

The data in table 2 indicate that 9 of the 10 women (exception subject 2) appeared to exhibit greater efficiency in the conversion of tryptophan to niacin metabolites in the pregnant state, which is illustrated by the average increase, 17.2 mg, in the combined excretion of N^1-Me and pyridone during ingestion of the tryptophan supplement in this period compared to the average increase, 9.3 mg in the postpartum period. As mentioned above, the average excretion of niacin by these women in late pregnancy was equal to 221% of the average niacin intake. The increased efficiency in the ability of pregnant women to derive niacin from tryptophan suggests that dietary tryptophan could be the source of all the niacin metabolites that are excreted in excess of the niacin intake.

SUMMARY

The effect of tryptophan supplementation on the urinary excretion of "free" and total tryptophan, nicotinic acid, N^1-methylnicotinamide, N^1-methyl-6-pyridone-3-carboxylamide, and quinolinic acid have been reported for a group of women in the pregnant and in the nonpregnant state.

All the urinary metabolites studied were excreted in higher amounts in pregnancy than in the postpartum period.

During the 14-day period of daily supplementation with 500 mg of DL-tryptophan in late pregnancy, the excretion of all metabolites with the exception of "free" tryptophan increased. The excretion of "free" tryptophan decreased significantly. During supplementation in the nonpregnant period, there was an increase in the excretion of total tryptophan, N^1-methylnicotinamide, and N^1-methyl-6-pyridone-3-carboxylamide.

In the pregnant period, the increase in the excretion of niacin metabolites after the usual diet was supplemented with tryptophan was higher than in the postpartum period. The data support the view that the conversion of tryptophan to

niacin is more efficient in the pregnant than in the nonpregnant state.

LITERATURE CITED

ASSOCIATION OF VITAMIN CHEMISTS, INC. 1951 Methods of vitamin assay. 2nd ed. Interscience Pub., New York.

BLOCK, R. J., AND D. BOLLING 1951 The amino acid composition of proteins and foods. 2nd ed. C. C. Thomas, Springfield, Ill.

FRAZIER, E. I. 1954 The urinary excretion of tryptophan by human subjects on controlled diets varying in levels and sources of protein. J. Nutrition, *53:* 115.

FRAZIER, E. I., T. PORTER AND M. J. HUMPHREY 1949 The utilization of nicotinic acid by pregnant women. Ibid., *37:* 393.

FRAZIER, E. I., M. E. PRATHER AND E. HOENE 1955 Nicotinic acid metabolism in humans. I. The urinary excretion of nicotinic acid and its metabolic derivatives on four levels of dietary intake. Ibid., *56:* 501.

GOLDSMITH, G. A., O. N. MILLER AND W. G. UNGLAUB 1956 Efficiency of tryptophan as a niacin precursor. Federation Proc., *15:* 553.

HENDERSON, L. M. 1949 Quinolinic acid metabolism. II. Replacement of nicotinic acid for the growth of the rat and neurospora. J. Biol. Chem., *181:* 677.

HENDERSON, L. M., AND H. M. HIRSCH 1949 Quinolinic acid metabolism. I. Urinary excretion by the rat following tryptophan and 3-hydroxyanthranilic acid administration. Ibid., *181:* 667.

HENDERSON, L. M., G. B. RAMASARMA AND B. C. JOHNSON 1949 Quinolinic acid metabolism. IV. Urinary excretion by man and other mammals as affected by the ingestion of tryptophan. Ibid., *181:* 731.

HOLMAN, W. I. M. 1954 A colorimetric method for the determination of the principal metabolites of nicotinic acid in human urine. Biochem. J., *56:* 513.

HORWITT, M. K., C. C. HARVEY, W. S. ROTHWELL, J. L. CUTLER AND D. HAFFROM 1956 Tryptophan-niacin relationships in man. J. Nutrition, *60:* suppl. 1.

HUFF, J. W., AND W. A. PERLZWEIG 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. II. A sensitive method for the determination of N^1-methylnicotinamide in urine. J. Biol. Chem., *167:* 157.

LANGNER, R. R., AND C. P. BERG 1955 Metabolism of D- tryptophan in the normal human subject. Ibid., *214:* 699.

LIN, P. H., AND B. C. JOHNSON 1953 Nicotinic acid metabolism. II. The metabolism of radioactive nicotinic acid and nicotinamide in the rat. J. Am. Chem. Soc., *75:* 2974.

LOJKIN, M. E. 1956 Effect of ovariectomy and administration of ovarian hormones and testosterone on nicotinic acid metabolism of rats. J. Nutrition, *59:* 443.

LOJKIN, M. E., A. W. WERTZ AND C. G. DIETZ 1952 Metabolism of nicotinic acid in pregnancy. Ibid., *46:* 335

LYMAN, C. M., AND K. A. KUIKEN 1949 The amino acid composition of meat and some other foods. Bulletin 708, Texas Agricultural Experiment Station.

MILLER, S., V. RUTTINGER AND I. G. MACY 1954 Urinary excretion of ten amino acids by women during the reproductive cycle. J. Biol. Chem., *209:* 795.

MOORE, M. C., M. B. PURDY, E. J. GIBBENS, M. E. HOLLINGER AND G. GOLDSMITH 1947 Food habits of women during pregnancy. J. Am. Diet. Ass., *23:* 847.

OLDHAM, H., B. B. SHEFT AND T. PORTER 1949 Biochemical studies on primigravidae on self-selected diets differing greatly in nutritive value. Federation Proc., *8:* 391.

PETERS, J. H. 1942 The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., *146:* 179.

PRICE, J. M., AND R. R. BROWN 1956 Quantitative studies on human urinary metabolites of D-, DL-, acetyl-L-, and acetyl-D-tryptophan. Ibid., *222:* 835.

REDDI, K. K., AND E. KODICEK 1953 Metabolism of nicotinic acid and related compounds in man and rat. Biochem. J., *53:* 286.

ROSENTHAL, E. L., G. A. GOLDSMITH AND H. P. SARRETT 1953 Excretion of N^1-methylnicotinamide and the 6-pyridone of N^1-methylnicotinamide in urine of human subjects. Proc. Soc. Exp. Biol. Med., *84:* 208.

SARRETT, H. P. 1951 Quinolinic acid excretion and metabolism in man. J. Biol. Chem., *193:* 627.

SARRETT, H. P., AND G. A. GOLDSMITH 1950 Metabolism of L- and DL-tryptophan in normal man and in pellagrins. Ibid., *182:* 679.

SHEFT, B. B., AND H. OLDHAM 1952 Amino acid intakes and excretions during pregnancy. J. Am. Diet. Ass., *28:* 313.

STEELE, B. F., H. E. SAUBERLICH, M. S. REYNOLDS AND C. A. BAUMANN 1949 Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. J. Biol. Chem., *177:* 533.

WALLRAFF, E. B., E. C. BRODIE AND A. L. BORDEN 1950 Urinary excretion of amino acids in pregnancy. J. Clin. Invest., *29:* 1542.

WALTERS, C. J., R. R. BROWN, M. KAIHARA AND J. M. PRICE 1955 The excretion of N-methyl-2-pyridone-5-carboxamide by man following ingestion of several known or potential precursors. J. Biol. Chem., *217:* 439.

WATT, B. K., AND A. L. MERRILL 1950 Composition of foods — raw, processed, prepared. U.S.D.A. Agriculture Handbook No. 8.

WERTZ, A. W., P. K. RUTTENBERG, G. P. FRENCH, G. H. MURPHY AND L. P. GUILD 1953 Amino acid content of foods. J. Am. Diet. Ass., *32:* 926.

NUTRITION STATUS SURVEY OF THE SIXTH GRADE SCHOOL POPULATION OF CUBA

NORMAN JOLLIFFE, ROBERT S. GOODHART, MORTON ARCHER, HADY LOPEZ AND FLAVIO GALBAN DIAZ

Bureau of Nutrition, Department of Health, City of New York and Fundacion de Investigaciones Medicas, Havana, Cuba

(Received for publication September 20, 1957)

I. INTRODUCTION

A. Purpose

The present survey of the nutrition status of the 6th grade school children of Cuba was designed to examine a representative sample of the population for clinical and biochemical evidence of nutritional abnormality. The survey was conducted at the request of the Medical Committee of the Fundacion de Investigaciones Medicas (F.I.M.), Dr. Gustavo Cuervo Rubio, Chairman, to the Williams-Waterman Fund of the Research Corporation, and covered the period January 16 to February 8, 1956.

B. Background

Cuba is a long narrow island, averaging about 25 miles in width and stretching for about 720 miles in a generally east-wide direction. It is about 100 miles south of Florida and just below the Tropic of Cancer. It is, therefore, in about the same latitude as Mexico City, Manila, Canton, Calcutta and Cairo. The climate is sub-tropical, and frost-free, with the temperature normally ranging from 60 to 90°F. Rainfall averages about 55 inches annually, occurring mostly in May, June, September, October and November with winter the dry season.

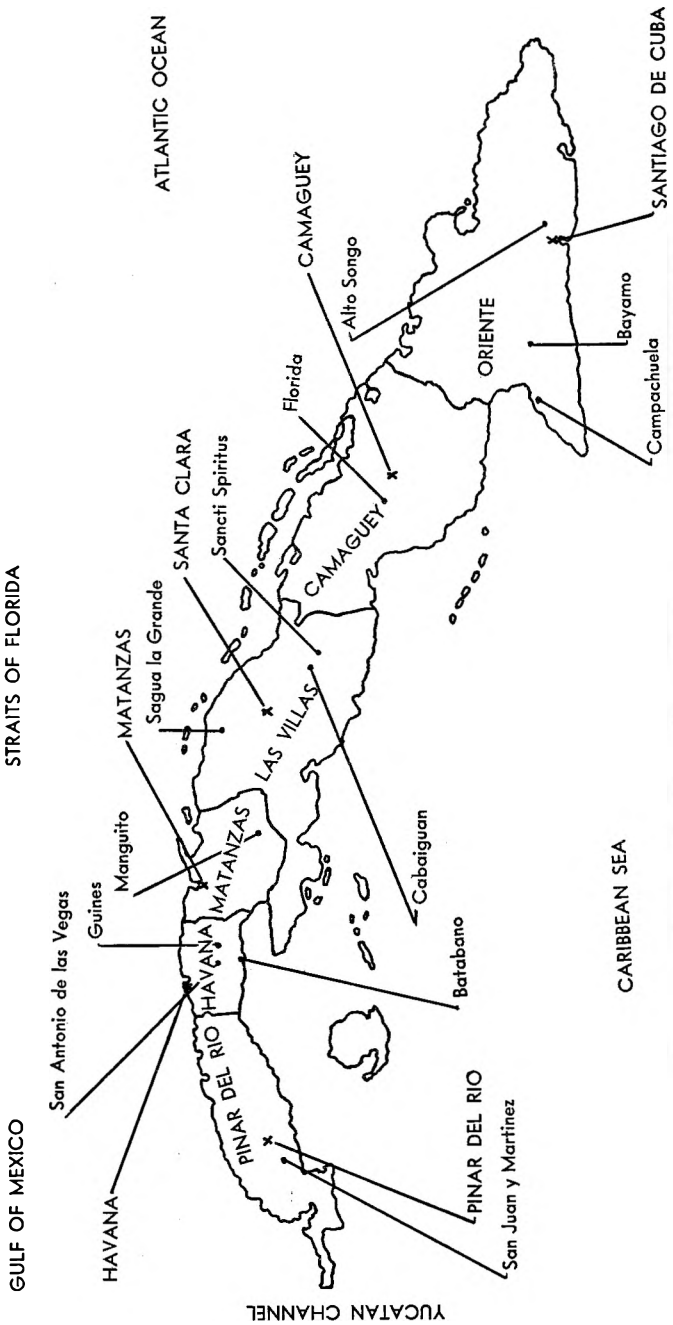


Fig. 1 Areas Surveyed in Cuba. Urban areas in CAPS. Rural areas in lower case.

The population of Cuba in 1956 was estimated to be 5,832,000 with a population density of 131.9 per square mile, higher than the 56.5 per square mile in the United States, but much lower than that of the other Greater Antilles.

About 74.4% of the population is classified as white, the balance composed of negro and mixed. About 55% is considered rural. The income per capita in 1955 was estimated [1] to be $347 in terms of 1953 prices.

Cuba contains 41,164 square miles or 28,000,000 acres. About two-thirds is flat or gently rolling with the remainder mountainous. About two-thirds is arable with about 5,000,000 acres, or 21%, under cultivation, the highest proportion of cultivated land in Latin America. Cuba supplies from 13 to 20% of the world's sugar supply and could produce more. In 1953, 3,890,000 acres (61%) of its cultivated land was in sugar cane, although about 1,500,000 acres were not harvested because of quota restrictions on sugar production. In no other country is so large a proportion of its cultivated land devoted to a single crop. Of Cuba's average annual exports (1948–53) of $650,000,000, almost $600,000,000 came from sugar or its by-products. Coffee is grown on 4.5% of its cultivated land and tobacco on another 3.4%. Of the remaining cultivated land, corn, yucca (manioca, cassava) malanga, plantain, potatoes, sweet potatoes, beans, rice, mangoes, pineapple and other fruits and vegetables are grown, almost all for domestic consumption. The balance of the arable land (43% of the total) is in pasture devoted primarily to beef and dairy cattle. In 1949, this produced 370,000,000 pounds of dressed beef and, in 1948, about 400,000,000 liters of milk. At present the production of beef does not satisfy the demand and some importation is necessary. In addition, Cuba slaughters more than 400,000 hogs each year, but must still import considerable amounts of pork products, chiefly lard. For example, in 1953 Cuba imported 146,400,000 pounds of lard.

[1] National Bank of Cuba.

C. Previous nutritional studies

Nutritional deficiencies in Cuba have been recognized for over 100 years since the first cases of beriberi were reported in 1853 (Hava, 1865). The Cuban diet was first evaluated in 1910 (Ferrer, '10) and was found to be low in calories, proteins, fats and carbohydrates when compared to the results of similar studies of European and North American diets. In 1935, infantile protein malnutrition, now known as kwashiorkor was described (Castellanos, '35) among the poorer classes, especially the negroes.

Since 1940, a series of studies has been reported in the Cuban medical literature and elsewhere dealing with many phases of the Cuban diet: vitamin C values (Valledor et al., '40a) and deficiencies (Valledor et al., '40b) thiamine values (Lopez, '43; Fernandez, '43), vitamin B deficiencies (Lopez, '43) and an extensive work on general nutritional deficiencies (Milanes, '44). The latter concluded that about 10% of the Cuban population suffered from some form of severe avitaminosis and that sub-clinical forms must greatly surpass this prevalence, particularly concerning the vitamin B complex.

Sellek et al. ('45) made plasma protein determinations in well children and in those with nutritional edema, vitamin B deficiency, parasitic anemia, malnutrition, nephrosis. cirrhosis and other diseases. Milanes, Spies and their associates, in a series of articles beginning in 1947 ('47, '48a,b,c, '49, '50) have contributed extensively to Cuban and English medical literature on sprue, pernicious anemia, nutritional anemias, folic acid and vitamin B_{12}. Alpha-tocopherol contents of foods (Johnson, '46) and levels in individuals were studied by Fernandez et al. ('47). These authors also studied the riboflavin content of the milk of cows ('48a) goats and humans ('48b) as well as the urinary excretion levels of middle class groups ('49).

In 1953, the Fundacion de Investigaciones Medicas (FIM) sponsored a pilot nutritional survey ('54) in a small rural

community near Havana and found deficiencies in almost all vitamins (especially riboflavin and ascorbic acid) and calcium but also found a good protein and calorie intake. In 1954, the FIM published a provisional table of values of 63 Cuban agricultural products (Navia et al., '54) which was supplemented in 1955 by an additional 37 items (Navia et al., '55).

II. METHODS

A. Selection of persons to be surveyed

In order to obtain a representative sample of the entire population the survey was conducted among the children 11 to 13 years of age attending school. At this age the special requirements of infants and young children (especially for protein) have passed, their lifetime dietary patterns have generally been established, and their nutritive requirements are near their maximum. At the same time, many of the complications often present in adults such as pregnancy and chronic disease are at a minimum. Also, a large proportion of this age group is still attending school and therefore conveniently available for examination. Moreover, children of this age are old enough not to fear examination and blood-sample letting and the veins are large enough for easy venous puncture. Many of these favorable factors diminish rapidly after this age, and samples of children in higher grades show progressively increasing proportions of the upper income groups.

All children 11 to 13 years of age attending public school were selected as well as all other 6th grade pupils irrespective of age. In Cuba, 80% of the 6th grade school children are 11 to 13 years old but considerable numbers do not start school until after 6 and, since advancement is governed by completion of each grade in succession, some 6th grade school children were over 13 years old. An attempt was made to have those children 11 to 13 years of age, not regularly attending school (because they had to work in the sugar cane fields), come to school on the day of the examination in order

to obtain as complete and as representative a sample of this age group as possible. It should be noted, however, that the actual cutting and grinding of sugar cane did not begin in most areas until February 6, and on the last three days of the survey (February 6 to 8) examinations were conducted in Alto Songo (a coffee area) and in urban areas. Therefore, only small numbers of these children were unavailable for examination at the time of the survey. It should be noted that children were not selected individually; entire classes or entire schools were selected up to the numbers needed to comprise about 5% of the 6th grade school population in each of the 6 provinces of Cuba. As shown in table 1, this number amounted to 37 persons per 100,000 population varying from 22 in Oriente to 55 in Havana province. The total number examined was actually 5.18% of the 6th grade school children in Cuba, varying from 4.80% in Camaguey province to 5.30% in Havana province.

The provincial capital cities — Pinar del Rio, Havana, Matanzas, Santa Clara, Camaguey and Santiago de Cuba — were selected to represent the urban areas. The numbers examined in each of these cities depend upon the number of urban 6th grade school children registered in the respective province in question. Under this system 1016 urban children were examined, 46.8% of the total of 2171. It may be recalled that 45% of the population is urban. The rural areas examined were selected by chance. Only two of the areas thus selected were subsequently changed. One (Cienfuegoes), because it possessed the highest rural income in the province and the other (Niquero), because a heavy rain the night before the scheduled examination had made the roads leading to it impassable. In the first instance a substitute area (Sagua la Grande) was selected by the FIM Medical Committee; in the latter instance the survey team went unannounced to two schools in Campechuela, a district as near to the area originally selected (Niquero) as road conditions permitted. Thus, 1155, or 53.2% of the total group surveyed were from rural areas.

TABLE 1

Distribution of sixth grade Cuban public school children

PROVINCE	TOTAL POPULATION (1953)	CHILDREN				
		Total 6th grade		Examined		
		Number	Per 100,000 population	Number	Per 100,000 population	Percent of 6th grade
Havana	1,540,829	15,864	1,030	841	55	5.30
Pinar del Rio	448,499	2,818	628	145	32	5.15
Matanzas	395,798	4,022	1,016	211	53	5.25
Las Villas	1,030,590	7,975	769	405	39	5.11
Camaguey	618,376	3,456	559	166	27	4.80
Oriente	1,798,185	7,801	434	403	22	5.17
Total	5,832,277	41,883	718	2171	37	5.18

By these means 18 areas, 6 urban and 12 rural, were selected for the survey. Figure 1 shows the geographic distribution of these areas. The areas in general excluded the wealthiest and the poorest but they did include the good, the bad, and the average. The rural communities examined represented not only sugar cane areas, which occurred in almost all rural districts, but also tobacco (San Juan y Martinez), coffee (Alto Songo), cattle (Sancti Spiritus), dairy farming (Bayamo), fishing (Batabano), and mixed farming (Guines). In each area, pupils from two to 6 schools were included. Private school pupils were examined only in the city of Havana where 163 were studied. This number represents 2.73% of the 6th grade private school registration of Cuba, a sample large enough to be valid considering the uniformity of the economic, racial and social groups involved.

B. Survey techniques

1. Clinical. The survey group first participated in a course of 10 one-hour lectures conducted by one of us (N.J.) directed toward the recognition of the clinical signs of malnutrition and emphasizing survey techniques. The individual physicians of the survey group were selected on the basis of their desire, interest, and ability to devote full time to the project for the duration of the study. Twelve physicians would have been the most efficient number. However, 18 qualified and were anxious for the training and experience and, since one important function of this type of survey is to train physicians in survey techniques, the larger number was accepted.

On arrival at a school, examining stations were set up, usually on a porch or patio. Chairs and small tables or writing boards were furnished each station which were arranged with the backs of the observers to the light and the subjects facing the light. The following stations were set up:

STATION NUMBER	ACTIVITY	
1.	*Identification.*	Local school teacher and team member recorded individual identification data.
2.	*Height and weight.*	Data recorded and apparent normality of weight for height evalauted clinically.
3.	*Skin fold thickness.*	Measured by means of skin ealipers.
4.	Examination of *hair, facial skin and neck.*	
5.	Examination of *eyes and lips.*	
6.	Examination of *tongue, gums and teeth.*	
7.	Examination of remainder of *skin, skeletal defects and edema,* if present.	
8.	*Neurologic examinations.*	
9.	*Complete checking of clinical findings* by senior team member.	
10.	Check of completeness of records and coding of data.	
11.	Laboratory. Blood and urine specimens.	

At station 9 each person was completely re-examined by the two senior members, each assisted by one Cuban member of the team in rotation, for accuracy and consistency of the earlier findings. When discrepancies were found, the original examiner was called for consultation. At first, as is usual in such team nutrition status surveys, the tendency was to over-diagnose and upgrade, but this tendency disappeared by the end of the first week. Each medical station was attended by two physicians with one moving on to the next station in rotation each day. After the first day, therefore, each station was manned by a person with a day's experience, and by a new examiner. By the end of the survey each station was manned by two relatively experienced persons, each of whom had spent, in addition, a day with each of the senior members of the team checking the results. As a result of the course of instruction, a practice run, the division of the clinical examinations into 8 stations and the rotation of the medical personnel, several Cuban members of the team gained and demonstrated the ability and the leadership necessary to conduct nutrition status surveys on their own responsibility.

The first 15 to 20% of each sex examined at each locality were sent to the laboratory station for non-fasting blood and

casual urine samples. A second 15 to 20% were then sent to the laboratory for blood samples for the pyridoxine phosphate determination until 115 samples were obtained. These samples were immediately centrifuged, the buffy white cell layer withdrawn, placed in dry ice, and shipped by air express to the laboratories of Merck and Co. The results of the pyridoxine phosphate determinations will be presented in a separate report.[2]

2. Laboratory. The laboratory procedures were as follows: Sodium versenate was used as the anticoagulant. Blood hemoglobin content was measured by the direct colorimeter procedure using the Sahli clinical hemoglobinometer using blood from the vein and plasma protein was measured by the copper sulfate method. (Phillips et al., '43).

For the determination of plasma vitamin A and carotene, the dichlorohydrin method was applied by using 1 ml of plasma and the micro-colorimeter attachment of the Evelyn Colorimeter (Sobel and Snow, '47). Plasma ascorbic acid was determined by the Roe method using 1 ml of plasma (Roe and Kuether, '43).

Oxalic acid (500 mg) was used as the urine stabilizing agent. For the determination of urinary thiamine, the fluorometric method of assay with thiochrome was used (Hennesy and Cerecedo, '39), with the correction of calculating suggested by Najjar and Ketron ('44).

Fluorometric methods were used for urinary riboflavin (Connor and Strub, '41) and for urinary excretion of N-methylnicotinamide (Coulson et al., '44); creatinine was determined by the alkaline picrate method (Peters, '42).

III. FOOD CONSUMPTION DATA

Data for the disappearance of food at the retail level (as collected by the U.S. Department of Agriculture for the United States) is not available in Cuba. Therefore, food consumption data on a per capita basis in Cuba are gross estimates and uncertain at best.

[2] Boxer, Goodhart and Pruss, in preparation.

Table 2 presents our best estimates of the nutrients available in Cuba per capita per day. It is based on estimates of the various foods available for human consumption compiled by the Instituto Nacional de Reforma Economica ('55) and is similar to the data presented by the FAO in the results of their Second World Food Survey ('52). These components were gathered by this Instituto from official records for various years between 1945 and 1953.

Most calculations of the nutrients were based on analyses of the nutrient composition of Cuban foods as determined by the FIM Laboratories (Navia et al., '55). Values not yet determined specifically for Cuban foods were given the values found in the tables of Bowes and Church ('48).

According to these data rice constitutes the largest single source of calories, accounting for 22.2% of the total, followed by sugar (17.5%), lard and oils (13.7%) animal flesh (11.5%), flour (8.8%) and beans (5.6%).

When contrasted with similar data for the United States, it appears that the Cuban diet is comparable (90 to 110%) only in ascorbic acid, carbohydrates and iron. All the other essential nutrients are substantially lower with the greatest differences appearing in calcium, vitamin A and riboflavin, each substantially less than 50% of the United States intake.

Comparing these figures with the National Research Council weighted allowances, we find that the Cuban diet is materially lower in calcium, vitamin A, thiamine and riboflavin. The National Research Council weighted allowances generally provide about a 50% margin of safety excluding waste or cooking losses. Cooking losses will substantially reduce the intake of thiamine from that available as given in table 2. Moreover, averages as presented in the table, ordinarily mean that about 50% of the cases have values below the average figure. Since we are interested in individuals rather than in averages, the levels of nutrients available to approximately half of the Cuban individuals must be below the already low average value whether compared with similar data for the

TABLE 2

Nutrients available for civilian consumption per capita per day — Cuba, 1953

	AMOUNT PER DAY	CALORIES	PROTEIN	FAT	CARBO-HYDRATE	CALCIUM	IRON	VITAMIN A	ASCORBIC ACID	THIAMINE	RIBO-FLAVIN	NIACIN
	oz.		*gm*	*gm*	*gm*	*mg*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*
Milk	6.31	132	6.7	7.48	9.4	225.8	0.2	307	1.9	0.08	0.33	0.2
Meat	2.10	213	13.6	14.15	—	5.9	1.8	—	—	0.04	0.11	2.5
Pork	0.18	20	0.8	1.53	—	0.4	1.1	—	—	0.04	0.01	0.2
Chicken	0.11	7	0.6	0.11	—	0.4	0.1	—	—	—[7]	0.01	0.2
Fish [1]	0.75	78	16.8	0.55	—	9.4	0.6	—	—	0.01	0.08	1.9
Eggs	0.04	10	0.1	0.63	—[7]	3.0	0.1	62	—	—[7]	0.02	—[7]
Oil	0.24	60	—	6.63	—	—	—	—	—	—	—	—
Lard	1.10	278	—	30.82	—	—	—	—	—	—	—	—
Sugar	3.99	449	—	—	111.6	—	—	—	—	—	—	—
Beans	1.53	147	8.6	0.56	25.9	63.9	4.4	—	0.9	0.71	0.09	1.0
Corn	1.40	143	3.6	1.38	29.6	2.4	0.7	174	—	0.12	0.03	0.8
Green vegetables [2]	0.49	4	0.2	0.04	0.7	13.2	0.2	548	7.7	—[7]	0.01	0.1
Tomatoes	0.73	5	0.2	0.06	0.8	1.8	0.2	225	6.0	0.02	0.01	0.2
Garlic	0.18	2	0.1	0.01	0.4	1.4	0.1	7	0.4	0.01	—[7]	—[7]
Onion	0.37	27	0.1	0.02	0.7	4.0	0.1	5	0.8	—[7]	—[7]	—[7]
Potatoes	0.75	20	0.4	0.02	4.0	2.3	0.1	4	3.2	0.02	0.01	0.3
Other root vegetables [3]	1.88	88	0.5	0.11	21.2	18.0	0.5	80	18.0	0.04	0.02	0.4
Bananas	3.07	85	0.1	0.17	22.1	0.7	0.5	371	8.6	0.04	0.04	0.6
Fruits — domestic [4]	1.87	5	0.3	0.13	7.3	7.0	0.2	599	57.6	0.03	0.02	0.2
citrus	0.61	7	0.1	0.03	1.9	2.1	0.1	9	6.4	—[7]	0.01	—[7]
imported [5]	0.14	2	—[7]	0.02	0.6	0.4	—[7]	2	0.2	—	—	—[7]
Rice	5.56	572	11.0	0.47	125.3	23.5	3.6	—	—	0.17	0.06	3.1
Flour, white meat	2.10	226	7.1	0.77	43.8	14.2	0.8	—	—	0.15	0.04	1.2
Coffee	0.05	—[7]	—[7]	—	—[7]	0.1	0.1	—	—	—	—	—
Total Cuba		2580	70.9	65.7	405.3	400.0	15.5	2393	111.7	1.48	0.9	12.9
Total United States [6]		3190	96	144.0	389.0	1050.0	17.0	7700	115.0	1.85	2.35	19.7
Cuba as percentage of U.S.		77.3	73.9	45.6	104.2	38.1	91.2	31.1	97.1	80.0	38.3	65.5
Weighted NRC allowance		2600	65	—	—	80.1	11.7	4560	70	1.28	1.76	12.8
Cuba as percentage of NRC		99.3	109.2	—	—	50.0	132.3	52.4	159.5	115.8	51.2	100.0

[1] Cod, red snapper. [2] Chard, cabbage. [3] Yuca, malanga. [4] Weighted average of pineapple (¼), papaya (⅓), mango (¼) and guava (⅙).
[5] Apples, pears. [6] N.F.S.–78, U.S. Dept. of Agriculture, Table 4, 1954. [7] Trace.

United States or with the National Research Council allowances.

IV. FINDINGS

A. Clinical

The results of the clinical examination are summarized in tables 3 to 6. In these tables the totals for the private schools, all from the city of Havana, are not included in the totals for the province of Havana because the findings in the private schools are quite different from those of the public schools of Havana and elsewhere. They are, however, included in the grand totals for all of Cuba.

1. Weight.[3]

The clinical estimate of the apparent normality of the weight for height shows that the distribution of normal, obese and underweight subjects in the private schools is 70.6, 19.0 and 10.4% respectively, compared with 50.7, 5.6 and 43.7% respectively, in the public schools (table 3). The figures for underweight, representing insufficient calorie intakes from any cause including deficiencies in certain vitamins are especially interesting. If the 10.4% underweight in the private school group is representative of all those Cuban children with an adequate available supply of food, then the 43.7% underweight in the public school group contains an excess of 33.3% who may be presumed underweight primarily because sufficient nutrients in food are not available to them.

If the relative frequencies of underweight in the urban and rural areas are compared, it appears that underweight

[3] The figures for height and weight in relation to age and sex, the skin fold measurements and their correlation with height, weight and sex, await a detailed analysis of these figures by Dr. Charles Garcia Ribiou, director, Museu Antropogica Montane of the University of Havana, and by Dr. Alvarez of the survey team.

TABLE 3

Distribution of clinical findings in sixth grade school children

CONDITION	SCHOOL LOCALE	TOTAL			PRIVATE	PUBLIC		
		Total	Urban	Rural	Urban	Total	Urban	Rural
	NUMBER	2171	1016	1155	163	2008	853	1155
		%	%	%	%	%	%	%
Weight — normal		52.2	54.0	50.6	70.6	50.7	50.9	50.6
obese		6.6	9.8	3.8	19.0	5.6	8.1	3.8
underweight		41.2	36.2	45.6	10.4	43.7	41.0	45.6
Acne		15.1	18.3	12.2	23.9	14.3	17.2	12.2
Gross conjunctival spots		17.1	12.8	21.0	8.0	17.9	13.7	21.0
Follicular hyperkeratosis		36.6	32.4	40.3	19.6	37.9	34.8	40.3
Xerosis — White [1]		61.3	56.7	64.5	55.8	61.9	57.0	64.5
Mixed [2]		78.3	75.0	83.2	0	78.3	75.0	83.2
Negro [3]		91.6	92.0	91.0	0	91.6	92.0	91.0
Active angular stomatitis		20.9	6.7	33.3	2.5	22.4	7.5	33.3
Angular scars only		22.4	21.8	22.9	1.2	24.3	25.7	22.9
Active scars		43.4	28.4	56.6	3.7	46.7	33.2	56.6
Nasolabial seborrhea		36.2	40.9	32.0	19.6	37.5	45.0	32.0
Circumcorneal injection		0.3	0.3	0.4	0.6	0.3	0.2	0.4
Magenta tongue		0.3	0.4	0.3	0.6	0.3	0.4	0.3
Reddened tongue		0.1	0	0.1	0	0.1	0	0.1
Papillary lesions		7.0	7.9	6.1	11.0	6.6	7.3	6.1
Gingivitis		43.2	37.3	48.3	12.3	45.7	42.1	48.3
Gingivitis with bleeding		21.0	16.1	25.2	2.5	22.5	18.8	25.2
Perifolliculosis		0.6	0.3	1.0	0	0.7	0.4	1.0
Purpura		0.1	0.2	0.1	0	0.2	0.2	0.1
Absent ankle jerks		0.2	0.1	0.3	0	0.2	0.1	0.3
Edema of ankles		0	0	0	0	0	0	0
Skeletal abnormalities		9.3	12.5	6.4	9.2	9.3	13.1	6.4

[1] As percentage of total White.

[2] As percentage of total Mixed.

[3] As percentage of total Negro.

is somewhat more frequent (but not significantly so)[4] in the rural areas, 41.8 to 47.2% while obesity is significantly more frequent in the urban areas, 8.1 to 3.8%.

It is of interest to note that there is a significant difference between the sexes. In the public schools, a significantly higher proportion of the girls are obese and a significantly lower proportion are underweight (table 5).

The province of Matanzas and Oriente are the only ones which showed figures significantly different from the averages of all of the public school group. If the proportion of children registered in the sixth grade is used as a rough economic index of the various provinces, one can see from table 1 that Matanzas is second best and Oriente the worst. In Matanzas, where 1016 children per 100,000 population are registered in the sixth grade, the relative incidence of normal, obese and underweight is 59.0, 6.6 and 34.4%. In Oriente, where only 434 children per 100,000 population are registered in the sixth grade, the relative proportions are 48.4, 2.0, and 49.6%. However, it should be noted that Havana, where 1030 children are registered per 100,000 population, and Las Villas, where 769 children per 100,000 population are registered in the sixth grade, showed figures not materially different from each other or from Cuba as a whole. It may well be that school facilities and availability as well as economic level and the number of children available influence the number of children attending the sixth grade.

In addition to the girls, other groups that show significantly high proportions of obesity are the private schools (over the

[4] In the course of this report, the following terminology has been employed along with the associated confidence limits as determined from the standard distributions of "t" and chi-square:

Term	*P*
Highly significant	0.990 and over
Significant or significantly	0.950 to 0.989
Probably significant	0.900 to 0.949
Tendency somewhat, not significant	less than 0.900

Where group comparisons employ the terms "low," "lower," "high" or "higher," these are to be understood as statistically significant unless otherwise qualified.

public schools) and urban areas (over rural). Among the provinces, Oriente is significantly lower in proportion of obese individuals than the others while the urban portion of Las Villas shows a tendency to be high in this category.

With respect to underweight, the private schools are lower than the public schools but there does not appear to be any difference between the urban and rural areas. In the provinces, Camaguey and Oriente appear to be somewhat higher than the remainder (table 4).

2. Adolescent acne.

The presence of acne vulgaris was recorded not as a nutritional defect but as an index of puberty which develops in most children about this time. In general agreement with the well-known fact that undernutrition often delays the onset of puberty, the data show that every group which exhibited significantly high proportions of obese also showed significantly high proportion with acne, i.e. private schools, girls and urban areas. The proportion with these various conditions and symptoms are presented in tables 3 and 5.

Several provinces are distinctly different from the others. Havana is low, with the difference confined to its urban areas, particularly the urban boys. On the other hand, Matanzas and Las Villas are high and here again, the difference is exhibited mainly by the urban and male populations (tables 4 and 6).

3. Deficiencies.

a. Signs suggesting vitamin A deficiency. (1) Gross conjunctival spots: These are areas of visible elevated conjunctival thickening that occur at the meridian of the eyes adjacent to the cornea at three and 9 o'clock. These elevated spots are usually designated as penguecula when the eye is moist and as Bitot spots when the eyes are dry. While the former are often associated with atmospheric dust, exposure and non-specific association with malnutrition, the latter are found in areas of extremely severe vitamin A

TABLE 4

Distribution of clinical findings in sixth grade public school children by province

CONDITION	PROVINCE	TOTAL			HAVANA			PINAR DEL RIO		
	LOCALE	Total	Urban	Rural	Total	Urban	Rural	Total	Urban	Rural
	NUMBER	2008	853	1155	678	309	369	145	63	82
		%	%	%	%	%	%	%	%	%
Weight — normal		50.7	50.9	50.6	51.6	50.2	52.8	53.8	55.6	52.4
obese		5.6	8.1	3.8	6.5	7.1	6.0	4.8	7.9	2.4
underweight		43.7	41.0	45.6	41.9	42.7	41.2	41.4	36.5	45.2
Acne		14.3	17.2	12.2	11.1	10.4	11.7	12.4	9.5	14.6
Gross conjunctival spots		17.9	13.7	21.0	15.9	9.1	21.7	17.9	11.1	23.2
Follicular hyperkeratosis		37.9	34.8	40.3	20.4	21.0	19.8	31.0	15.9	42.7
Xerosis — White [1]		61.9	57.0	64.5	59.3	53.3	62.9	75.0	61.2	84.5
Mixed [2]		78.3	75.0	83.2	78.0	77.8	78.6	85.7	77.8	100.0
Negro [3]		91.6	92.0	91.0	89.3	87.9	100.0	81.8	100.0	66.7
Active angular stomatitis		22.4	7.5	33.3	17.4	4.2	28.5	4.8	1.6	7.3
Angular scars only		24.3	25.7	22.9	12.2	11.0	13.3	26.2	15.9	34.1
Active scars		46.7	33.2	56.6	29.6	15.2	41.7	31.0	17.5	41.5
Nasolabial seborrhea		37.5	45.0	32.0	27.0	29.1	25.2	49.7	57.1	43.9
Circumcorneal injection		0.3	0.2	0.4	0.2	0	0.3	0	0	0
Magenta tongue		0.3	0.4	0.3	0	0	0	0	0	0
Reddened tongue		0.1	0	0.1	0	0	0	0	0	0
Papillary lesions		6.6	7.3	6.1	6.9	6.5	7.3	6.9	6.3	7.3
Gingivitis		45.7	42.1	48.3	35.0	28.8	40.1	40.7	39.7	41.5
Gingivitis with bleeding		22.5	18.8	25.2	12.8	9.4	15.7	18.6	11.1	24.4
Perifolliculosis		0.7	0.4	1.0	0.1	0.3	0	0	0	0
Purpura		0.2	0.2	0.1	0.1	0.3	0	0	0	0
Absent ankle jerks		0.2	0.1	0.3	0.1	0.3	0	0	0	0
Edema of ankles		0	0	0	0	0	0	0	0	0
Skeletal abnormalities		9.3	13.1	6.4	10.9	19.1	4.1	2.8	1.6	3.7

TABLE 4 *(continued)*

Distribution of clinical findings in sixth grade public school children by province

CONDITION	PROVINCE	MATANZAS			LAS VILLAS			CAMAGUEY			ORIENTE		
	LOCALE	Total	Urban	Rural	Total	Urban	Rural	Total		Rural	Total	Urban	Rural
	NUMBER	211	103	108	405	172	233	166	80	86	403	126	277
		%	%	%	%	%	%	%	%	%	%	%	%
Weight — normal		59.0	66.0	51.9	48.6	45.3	51.1	45.2	40.0	50.0	48.4	52.4	46.6
obese		6.6	7.8	5.6	6.9	13.4	2.1	6.6	8.8	4.7	2.0	3.2	1.4
underweight		34.4	26.2	42.6	44.4	41.3	46.8	48.2	51.2	45.3	49.6	44.4	52.0
Acne		21.8	30.1	13.9	15.8	24.4	9.4	14.5	17.5	11.6	15.1	17.5	14.1
Gross conjunctival spots		22.7	19.4	25.9	24.0	23.8	24.0	16.3	13.8	18.6	13.2	7.9	15.5
Follicular hyperkeratosis		45.0	37.9	51.8	53.8	50.6	56.2	58.4	61.2	55.8	41.9	37.3	44.0
Xerosis — White [1]		61.3	50.0	71.6	58.7	57.7	59.3	71.0	86.0	60.8	60.4	42.3	63.8
Mixed [2]		84.6	66.7	100.0	78.8	91.7	44.4	93.8	90.9	100.0	73.2	60.0	84.6
Negro [3]		96.7	94.1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	88.7	89.1	88.5
Active angular stomatitis		28.9	8.7	48.1	38.5	16.9	54.5	41.6	8.8	72.1	9.4	4.0	11.9
Angular scars only		42.7	41.7	43.5	31.1	47.1	19.3	10.2	12.5	8.1	33.3	32.5	33.6
Active scars		71.6	50.5	91.7	69.6	64.0	73.8	51.8	21.2	80.2	42.1	36.5	45.5
Nasolabial seborrhea		53.1	59.2	47.2	42.2	60.5	28.8	48.2	55.0	41.9	33.7	38.9	31.4
Circumcorneal injection		0	0	0	0.2	0.6	0	1.8	1.2	2.3	0.2	0	0.4
Magenta tongue		0	0	0	1.2	1.7	0.9	0	0	0	0.2	0	0.4
Reddened tongue		0	0	0	0	0	0	0.6	0	1.2	0	0	0
Papillary lesions		7.6	8.7	6.5	6.2	5.8	6.4	5.4	6.2	4.7	6.5	11.1	4.3
Gingivitis		39.8	40.8	38.9	50.6	46.5	53.6	56.0	56.2	55.8	59.3	61.9	58.1
Gingivitis with bleeding		11.8	3.9	19.4	26.4	21.5	30.0	28.3	35.0	22.1	39.2	43.7	37.2
Perifolliculosis		0.9	0	1.9	1.7	1.2	2.1	1.2	0	2.3	0.5	0	0.7
Purpura		0	0	0	0.2	0.6	0	0	0	0	0.2	0	0.4
Absent ankle jerks		0.5	0	0.9	0.5	0	0.9	0	0	0	0	0	0
Edema of ankles		0	0	0	0	0	0	0	0	0	0	0	0
Skeletal abnormalities		5.2	4.9	5.6	5.9	7.6	4.7	10.2	21.2	0	13.9	13.5	14.1

[1] As percentage of total White.

[2] As percentage of total Mixed.

[3] As percentage of total Negro.

TABLE 5

Distribution of clinical findings in sixth grade school children by sex

CONDITION	SCHOOL	TOTAL			PRIVATE			PUBLIC		
	SEX	Total	Male	Female	Total	Male	Female	Total	Male	Female
	NUMBER	2171	1132	1039	163	103	60	2008	1029	979
		%	%	%	%	%	%	%	%	%
Weight — normal		52.2	46.6	58.4	70.6	68.0	75.0	50.7	44.4	57.4
obese		6.6	5.5	7.8	19.0	20.4	16.7	5.6	4.0	7.3
underweight		41.2	48.1	33.7	10.4	11.7	8.3	43.7	51.7	35.2
Acne		15.1	11.4	19.1	23.9	19.4	31.7	14.3	10.6	18.3
Gross conjunctival spots		17.1	21.8	12.0	8.0	11.7	1.7	17.9	22.8	12.7
Follicular hyperkeratosis		36.6	37.5	35.5	19.6	21.4	16.7	37.9	39.2	36.7
Xerosis		68.4	62.3	75.2	55.8	49.5	66.7	69.5	63.6	75.7
Active angular stomatitis		20.9	23.1	18.4	2.5	2.9	1.7	22.4	25.2	19.4
Angular scars only		22.6	21.6	23.7	1.2	1.9	0	24.3	23.5	25.1
Active scars		43.4	44.7	42.1	3.7	4.9	1.7	46.7	48.7	44.5
Nasolabial seborrhea		36.2	32.5	40.2	19.6	20.4	18.3	37.5	33.7	41.6
Circumcorneal injection		0.3	0.4	0.2	0.6	1.0	0	0.3	0.3	0.2
Magenta tongue		0.3	0.4	0.2	0.6	0	1.7	0.3	0.5	0.1
Reddened tongue		0.1	0.1	0	0	0	0	0.1	0.1	0
Papillary lesions		7.0	6.7	7.2	11.0	7.8	16.7	6.6	6.6	6.6
Gingivitis		43.2	46.6	39.4	12.3	11.7	13.3	45.7	50.1	41.0
Gingivitis with bleeding		21.0	23.6	17.9	2.5	1.0	5.0	22.5	25.9	18.7
Perifolliculosis		0.6	0.5	0.8	0	0	0	0.7	0.6	0.8
Purpura		0.1	0.2	0.1	0	0	0	0.2	0.2	0.1
Absent ankle jerks		0.2	0.1	0.3	0	0	0	0.2	0.1	0.3
Edema of ankles		0	0	0	0	0	0	0	0	0
Skeletal abnormalities		9.3	12.5	5.7	9.2	13.6	1.7	9.3	12.4	5.9

TABLE 6

Distribution of clinical findings in sixth grade public school children by sex by province

CONDITION	PROVINCE	TOTAL			HAVANA			PINAR DEL RIO		
	SEX	Total	Male	Female	Total	Male	Female	Total	Male	Female
	NUMBER	2008	1029	979	678	355	323	145	72	73
		%	%	%	%	%	%	%	%	%
Weight — normal		50.7	44.4	57.4	51.6	44.2	59.8	53.8	48.6	58.9
obese		5.6	4.0	7.3	6.5	4.8	8.4	4.8	2.8	6.8
underweight		43.7	51.7	35.2	41.9	51.0	31.9	41.4	50.0	32.9
Acne		14.3	10.6	18.3	11.1	7.0	15.5	12.4	12.5	12.3
Gross conjunctival spots		17.9	22.8	12.7	15.9	18.6	13.0	17.9	26.4	9.6
Follicular hyperkeratosis		37.9	39.2	36.7	20.4	19.4	21.4	31.0	30.6	31.5
Xerosis		69.5	63.6	75.7	66.4	63.7	69.3	76.6	70.8	82.2
Active angular stomatitis		22.4	25.2	19.4	17.4	21.1	13.3	4.8	6.9	2.7
Angular scars only		24.3	23.5	25.1	12.2	11.3	13.3	26.2	30.6	21.9
Active scars		46.7	48.7	44.5	29.6	32.4	26.6	31.0	37.5	24.7
Nasolabial seborrhea		37.5	33.7	41.6	27.0	26.2	27.9	49.7	47.2	52.1
Circumcorneal injection		0.3	0.3	0.2	0	0	0	0	0	0
Magenta tongue		0.3	0.5	0.1	0	0	0	0	0	0
Reddened tongue		0.1	0.1	0	0	0	0	0	0	0
Papillary lesions		6.6	6.6	6.6	6.9	7.0	6.8	6.9	8.3	5.5
Gingivitis		45.7	50.1	41.0	35.0	39.4	30.0	40.7	48.6	32.9
Gingivitis with bleeding		22.5	25.9	18.7	12.8	14.6	10.2	18.6	20.8	16.4
Perifolliculosis		0.7	0.6	0.8	0.1	0	0.3	0	0	0
Purpura		0.2	0.2	0.1	0.1	0.3	0	0	0	0
Absent ankle jerks		0.2	0.1	0.3	0.1	0	0.3	0	0	0
Edema of ankles		0	0	0	0	0	0	0	0	0
Skeletal abnormalities		9.3	12.4	5.9	10.9	13.2	8.4	2.8	5.6	0

CONDITION	PROVINCE	MATANZAS		
	SEX	Total	Male	Female
	NUMBER	211	108	103
		%	%	%
Weight — normal		58.8	55.6	62.1
obese		6.6	5.6	7.8
underweight		34.6	38.9	30.1
Acne		21.8	16.7	27.2
Gross conjunctival spots		22.7	31.5	13.6
Follicular hyperkeratosis		45.0	45.4	44.7
Xerosis		69.2	59.3	79.6
Active angular stomatitis		28.9	26.9	31.1
Angular scars only		42.7	43.5	41.7
Active scars		71.6	70.4	72.8
Nasolabial seborrhea		53.1	49.1	57.3
Circumcorneal injection		0	0	0
Magenta tongue		0	0	0
Reddened tongue		0	0	0
Papillary lesions		7.6	6.5	8.7
Gingivitis		39.8	38.0	41.7
Gingivitis with bleeding		11.8	9.3	14.6
Perifolliculosis		0.9	0	1.9
Purpura		0	0	0
Absent ankle jerks		0.5	0.9	0
Edema of ankles		0	0	0
Skeletal abnormalities		5.2	8.3	1.9

TABLE 6 *(continued)*

LAS VILLAS			CAMAGUEY			ORIENTE		
Total	Male	Female	Total	Male	Female	Total	Male	Female
405	218	187	166	85	81	403	191	212
%	%	%	%	%	%	%	%	%
48.6	43.6	54.5	45.2	47.1	43.2	48.4	36.6	59.0
6.9	5.0	9.1	6.6	3.5	9.9	2.0	1.0	2.8
44.4	51.4	36.4	48.2	49.4	46.9	49.6	62.3	38.2
15.8	15.6	16.0	14.5	5.9	23.5	15.1	9.4	20.3
24.0	31.2	15.5	16.3	17.6	14.8	13.2	17.3	9.4
53.8	54.1	53.5	58.4	57.6	59.3	41.9	50.3	34.4
64.9	52.3	79.7	77.7	71.8	84.0	73.4	72.3	74.5
38.5	45.0	31.0	41.6	36.5	46.9	9.4	11.0	8.0
31.1	28.4	34.2	10.2	10.6	9.9	33.3	32.5	34.0
69.6	73.4	65.2	51.8	47.1	56.8	42.7	43.5	42.0
42.2	34.9	50.8	48.2	40.0	56.8	33.7	29.8	37.3
0.2	0.5	0	1.8	2.4	1.2	0.2	0	0.5
1.2	1.8	0.5	0	0	0	0.2	0.5	0
0	0	0	0.6	1.2	0	0	0	0
6.2	6.9	5.3	5.4	2.4	8.6	6.5	6.8	6.1
50.6	57.3	42.8	56.0	57.6	54.3	59.3	66.0	53.3
26.4	29.8	22.5	28.3	30.6	25.9	39.2	51.3	28.3
1.7	1.8	1.6	1.2	2.4	0	0.5	0	0.9
0.2	0.5	0	0	0	0	0.2	0	0.5
0.5	0	1.1	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
5.9	6.9	4.8	10.2	16.5	3.7	13.9	20.4	8.0

deficiency such as occur in certain areas of Africa, India and Southeast Asia. Not a single Bitot spot was observed.

Several observers have related pengueculae to vitamin A deficiency, but this hypothesis has not been generally accepted. They are, however, highly frequent in malnourished population groups and less frequent in the better nourished groups. Their prevalence increases with age.

The occurrence of gross conjunctival spots is relatively high in public schools, males and rural areas (tables 3 and 5). In this survey, they were found in 8.0% of the private school group and in 17.9% of the public school pupils. However, the private schools are not significantly different from the urban Havana public schools. This difference may thus be a result of location in an urban area rather than a true difference between the children attending the different types of schools. They were more common in rural areas (21.0%) than in the urban areas (13.7%). The occurrence of these spots among the boys (21.8%) is significantly higher than among the girls (12.0%). Inasmuch as only one case showed a blood level of vitamin A under 20.0 μg per 100 ml blood, some other cause must be sought for the prevalence of this condition. In the provinces, Oriente is significantly lower and Las Villas higher than the others. In Havana, the urban boys are so low that they also make the entire urban group significantly low (table 4).

(2) Follicular hyperkeratosis: Follicular hyperkeratosis occurred in 19.6% of the private school children and in 37.9% of the public school children (table 3). Its highest prevalence was in Camaguey, 58.4% and its lowest in Havana, 20.4% where it is not significantly different from that in the private schools. Comparison of these findings with the proportion of children registered in the 6th grade in the various provinces shows no obvious correlations.

As indicated above, the various provinces vary widely in the incidence of follicular hyperkeratosis. All of Havana and the urban portion of Pinar del Rio were significantly low with the remainder fairly high (table 4).

(3) Xerosis: Excluding the colored population (mixed and negro) who are known to have a higher prevalence of xerosis unrelated to vitamin A deficiency than the white population, this condition occurred in 55.3% of the white private school children and 61.9% of those in the public schools, a difference which is not significant. Neither are the urban areas significantly different from the rural (table 3). The prevalence of xerosis in the mixed and negro groups was at its peak in Camaguey, an area also noted for follicular hyperkeratosis. It may be recalled that gross conjunctival spots were not usually prevalent in Camaguey, occurring in 16.3% of the subjects, a figure not different from the 17.9% of the 6th grade public school children in all of Cuba.

In previous surveys, the senior author has used the prevalence of hyperkeratosis of the arms as an index of the prevalence of vitamin A deficiency in population groups provided there were other clinical supporting signs of vitamin A deficiency, e.g., following hyperkeratosis, gross conjunctival spots or Bitot spots. In Cuba, only 8 cases had extensive follicular hyperkeratosis involving back or buttocks and not a single Bitot spot was observed. From the clinical data alone, one cannot therefore use follicular hyperkeratosis of the arms as an index of vitamin A deficiency in this population group.

b. Signs suggestive of riboflavin deficiency. (1) Active angular stomatitis: Active angular stomatitis was found in 2.5% of the private school children and 22.4% of those in public school. In the public school group, the highest frequency occurred in Camaguey (41.6%) and in Las Villas (38.5%). Its lowest frequency was in Oriente and Pinar del Rio. "Angular scars only" occurred in an additional 1.2% of the private school children, and in 24.3% of the public school children (table 3). Together, active angular stomatitis and "angular scars only" occurred in 3.7% of the private school children and in 46.7% of the public school children, a difference highly significant.

(2) Nasolabial seborrhea, circumcorneal injection and magenta tongue: Supporting clinical signs of riboflavin de-

ficiency are nasolabial seborrhea, circumcorneal injection and magenta tongue. Nasolabial seborrhea occurred in 19.6% of the private school children and 37.5% of the public school children. It is noteworthy that among those with nasolabial seborrhea, those who also showed acne manifested no difference in level of riboflavin from those without acne. Circumcorneal injection and magenta tongue each occurred very infrequently, affecting only very small proportions of the population. These are signs, however, of rather severe riboflavin deficiency.

The high prevalence of clinical findings suggestive of riboflavin deficiency accompanied by a high prevalence of low excretion of riboflavin in the urine indicates a real deficiency of riboflavin in this population. The occurrence of the latter finding in the private school children is negligible.

c. Signs suggestive of niacin deficiency. Papillary lesions of the tongue: The findings of significant lesions of the papillae of the tongue, that is, lesions more advanced than involvement only of the tip and lateral margins, is a key clinical sign of niacin deficiency. It also occurs in cobalamin, folic acid, pyridoxine, and possibly other deficiencies. In the past, sprue was frequent in Cuba, but at the present time its frequency in the Calixto Garcia Hospital compared with 10 years ago has markedly diminished [5] (Galban, '56). Cobalamin deficiency is not frequent in Cuba. At the present time both mycrocytic and hypochronic anemia, except from blood loss, are also rare. These significant tongue lesions occurred in 11.0% of the private school children, and 6.6% of the public school children. No public school group showed as high a prevalence of those lesions as the children in the private schools.

Comparing private and public schools with respect to urine levels of N-methyl nicotinamide, we discover that the boys in urban Havana public schools have significantly higher levels than the private school boys while the girls show the same tendency but at a level which is not quite significant.

[5] Personal observation of one of us (F. G. D.).

The difference in occurrence of tongue lesions may thus be at least partly attributable to this strong tendency of the private schools toward a lower level of N-methyl nicotinamide excretion.

Confirmatory signs of niacin deficiency are pellagra skin lesions and a scarlet red tongue. As shown in tables 3 and 4, a reddened tongue was practically non-existent among the group surveyed and no skin lesions suggestive of pellagra were observed. From these clinical observations it is concluded that clinical niacin deficiency is negligible in Cuba.

Nevertheless, the laboratory analysis reveals that over half the school children, both private and public, possess levels of N-methyl nicotinamide below 2 mg/gm creatinine (table 8). It is difficult to explain this discrepancy between nicotinamide levels and the small number exhibiting significant lesions of the tongue papillae unless the intake of protein (70.9 gm per capita per day, table 2) supplies sufficient tryptophan to protect against clinical niacin deficiency, or the level of excretion of N-methyl nicotinamide is not a reliable measure of niacin deficiency.

d. Signs suggestive of ascorbic acid deficiency. Gingivitis: Gingivitis with bleeding of the gums has been used as a key sign of ascorbic acid deficiency when accompanied by a high prevalence of confirmatory signs. These confirmatory signs include perifolliculosis and purpura. Gingivitis with bleeding on pressure occurred in 2.5% of the private school children and 22.5% of the public school children, but this high frequency was associated with only 0.7% of perifolliculosis and only 0.015% of purpura. The clinical signs therefore suggest that there is little ascorbic acid deficiency in Cuba and that the high incidence of gingivitis is based on other causes. The laboratory findings confirm this deduction since less than 1% of the children in the study had blood levels of ascorbic acid under 0.3 mg/100 ml of blood.

Private school children have a definitely lower prevalence of gingivitis and gingivitis with bleeding than those in the public schools, even the urban Havana public schools. This

difference appears to be a real difference between the children attending each type of school and between their economic status. In the rural areas, males are significantly higher than the females while in the urban areas, the males are also higher but not significantly so. These two combined cause total public school males to be significantly higher than total public school females. In the private schools, there is no difference between male and female for gingivitis. Surprisingly, for gingivitis with bleeding, the boys are significantly lower than the girls. This is counter to all other trends where the males are higher than the females.

As expected, the prevalence in urban areas is lower than that in rural areas, both in total and by sex. In the provinces, Havana and Oriente are significantly different from the other provinces, Havana being lower and Oriente higher than would ordinarily be expected. The differences exhibited in these two provinces are due directly to the difference in location since all components (urban and rural; male and female) show the same trend.

e. Signs suggestive of thiamine deficiency. Absent ankle jerks: The diagnosis of a bilateral symmetrical polyneuritis due to thiamine deficiency cannot be made with certainty unless the ankle jerks are absent. This finding was made in none of the private school children and in only 0.2% of the public school children. It should be noted, however, that in this age group, absent ankle jerks are infrequent even with a sizeable prevalence of beriberi in adults. In Formosa, (Jolliffe and Tung, '56) absent Achilles tendon reflexes was encountered only rarely in the 6th grade school children even though 59.0% of them excreted less than 50 μg of thiamine per gram of creatinine in the urine. At this level of urinary thiamine excretion in the Formosan 6th grade school children, there was clinical evidence of beriberi in about 5% of the adult population screened.

Laboratory findings with respect to the urinary levels of thiamine excretion indicate that the Cuban experience parallels that of Formosa. Subclinical thiamine deficiency (below

40 μg/gm creatinine) was discovered in 42.4% of the private school children and in 13.4% of those in public schools.

f. Signs suggestive of protein deficiency. Edema: No cases of peripheral edema were noted among the Cuban school children. Total serum proteins in these 6th grade school children were practically all in the normal range with only 1.4% below 6.0 gm/100 ml of blood and 3.1% below 6.2 gm. This may be related to the estimated total daily protein intake of 70.9 gm of which 32.6 gm are of vegetable and 38.3 gm of animal origin. Although growth failure may be a sign of protein and vitamin deficiencies, as well as of calories, this study was not designed to differentiate between these various causes. Accordingly, we are unable to draw any conclusions as to the role of the lower protein intake in relation to the smaller size of the public school children of Cuba as compared with such children in the United States.

4. Skeletal deformities

Skeletal deformities suggestive of healed rickets were found in 9.2% of the private school children and in 9.3% of the public school children. However, those in the private schools are significantly lower than the 19.1% discovered in the urban Havana public school children. This indicated a true difference exists between children attending the two types of school. This contrast probably arises from diverse economic circumstances, from differing intakes of vitamin D supplements and particularly from different color composition of the pupils and racial factors. In both public and private schools, the boys exhibit a significantly higher prevalence of skeletal deformities than the girls.

In general urbanized colored people show the highest prevalence of rickets. In Pinar del Rio, Matanzas, and Las Villas, there were few colored people and the occurrence of these skeletal defects was low. In urban Havana, urban Camaguey, and urban and rural Oriente where there are large numbers of colored people, the prevalence of these deformities was the greatest.

In Havana and Camaguey provinces, the urban girls show a decidedly higher proportion of skeletal deformities than the rural girls whereas in the other provinces, these two groups are comparable. In both provinces, the urban areas are significantly high compared to the urban areas of the other provinces and their rural areas are significantly low compared to other rural areas. Between themselves, the two provinces differ in that the high prevalence of skeletal abnormalities in urban Havana is exhibited equally by the boys and the girls whereas, in urban Camaguey, the boys alone make the total prevalence significantly high. The low prevalence in both rural Havana and rural Camaguey is caused by the low prevalence among the boys inasmuch as the girls show average levels.

The only other area noteworthy for this condition is rural Oriente which exhibits an extremely high prevalence, particularly for the boys.

5. *Anemia*

Laboratory findings revealed infrequent occurrence of low hemoglobins, with only 4.3% of the determinations among the public school children below 11 gm/100 ml of blood. None of the private school children was below this level. These levels may be compared with those in the Formosa survey where 34.8% of the 6th grade school children were below this same level of 11 gm/100 ml. In Formosa, the per capita intake per day of iron in 1953 was established at 8.8 mg compared with 15.5 mg in Cuba (table 2).

It may be concluded that anemia is not a major problem among 6th grade school children in Cuba. However, in certain sections of the island, the prevalence of anemia is substantially higher than for the country as a whole. For example, urban Matanzas, 11.1%; rural Pinar del Rio, 9.1%; rural Havana, 8.2%. This may indicate the need for some local inquiry as to the reasons for these differences.

6. Group comparisons

The presentation so far has described the prevalence of specific clinical signs in various segments of the 6th grade school population of Cuba. An alternate presentation would entail the clinical characteristics of entire groups such as male and female, private and public school pupils, urban and rural areas and individual provinces. The following summaries will give these group descriptions and comparisons and set forth the significant differences resulting from the diversity of location, sex, type of school or degree of urbanization. Where a condition or symptom is not mentioned, it may be assumed that no significant difference was found.

a. Private schools vs. public schools. Private school children were compared to urban Havana public school children in order to eliminate the effects of any variations resulting from differing locations or degree of urbanization.

Analysis of these comparisons is presented in tabular form in table 7. In addition to these differences, which were com-

TABLE 7

Significant clinical differences between groups

GROUP	PROPORTION SIGNIFICANTLY	
	Higher	Lower
Private (vs. public schools)	Obesity Acne	Underweight Angular scars Active angular stomatitis Gingivitis
Male (vs. female)	Underweight	Acne
In public schools	Gross conjunctival spots Active angular stomatitis Gingivitis with bleeding Skeletal abnormalities	Xerosis Nasolabial seborrhea
In private schools	Gross conjunctival spots Skeletal abnormalities	Gingivitis with bleeding
Urban (vs. rural)	Nasolabial seborrhea Skeletal abnormalities	Gross conjunctival spots Active angular stomatitis Total active scars Gingivitis with bleeding

mon to both sexes, private school males have less gingivitis with bleeding than the urban Havana public school males, and private school females show a larger prevalence of skeletal deformities than their public school counterparts.

b. Male vs. female. In the public schools, a large number of true differences (table 7) between the sexes occur (i.e. attributable only to the difference in sex rather than location, type of school or degree of urbanization). In addition to these differences common to both urban and rural areas, rural boys are less obese and have more gingivitis than the rural girls.

By sharp contrast, table 7 shows only a small number of differences between the boys and girls attending private school.

It should be noted that the difference in gingivitis with bleeding is in opposite directions in the private and the public schools. However, since a total of only 4 cases was noted in the private schools, conclusions should only be drawn with caution.

c. Urban vs. rural. Distinct differences appear between the urban and rural areas. The true differences between these groups are presented in table 7. In addition, there were significantly more obese among the urban boys than among the rural boys, while the urban girls exhibit a significantly higher prevalence of acne than the rural girls.

B. Laboratory

1. General findings. Biochemical determinations were made on about one person in every 6 who were examined clinically. The exact number varied from 329 (15.2%) for vitamin A and carotene to 350 (16.1%) for hemoglobin, hematocrit and protein. In the course of the statistical analysis, it was determined that such extremely high correlations existed between hemoglobin and hematocrit and between vitamin A and carotene that the latter determination of each pair could be safely ignored.

Tables 8 and 9 show the percentage distributions of the biochemical findings. These tables indicate the number of determinations in each group, the geographical breaks, type of school and the proportions falling below specified limits. Some reference to the biochemical data has already been made in attempting to explain some of the clinical manifestations. These involve the lower ranges of the biochemical determinations in search of a link between the laboratory and clinical findings. Another type of analysis of the chemical data has been made to reveal significant differences which arise from diversity in location, sex, type of school or degree of urbanization.

2. *Group Comparisons*

a. Private schools vs. public schools

Inasmuch as all the examinations of private school children were conducted in urban Havana, comparison with the urban Havana public school children served to eliminate any differences which were a result of differing degrees of urbanization or different location on the island. As a result, differences were discovered between private and public school children which would be attributed solely to the contrast in pupils attending each type of school. This analysis indicates that hemoglobin and riboflavin levels in the private school children are higher than those in the public school boys. Though the differences between urinary levels of thiamine in private and in urban Havana public school children are large, they are not statistically significant.

b. Males vs. females

Only two differences were discovered in the total data which could be attributed to difference in sex. In the urban areas, the males showed higher blood levels of carotene than the females. In the rural areas, the females possessed higher levels of N-methyl nicotinamide than the males. Within the

TABLE 8

Distribution of biochemical findings in sixth grade school children

BIOCHEMICAL	SCHOOL LOCALE		TOTAL			PRIVATE	PUBLIC		
			Total	Urban	Rural	Urban	Total	Urban	Rural
Hemoglobin, gm/100 ml		No.	350	169	181	30	320	139	181
< 11.0		%	4.0	3.0	6.0	0	4.3	3.6	5.0
< 12.4		%	50.3	47.9	52.5	30.0	52.2	51.8	52.5
Protein, gm/100 ml		No.	350	169	181	30	320	139	181
< 6.0		%	1.4	3.0	0	3.3	1.3	2.9	0
< 6.2		%	3.1	6.5	0	6.7	2.8	6.5	0
Vitamin A, μg/100 ml		No.	329	156	173	28	301	128	173
< 20.0		%	0.3	0	0.6	0	0.3	0	0.6
< 30.0		%	0.9	0.6	1.2	0	1.0	0.8	1.2
Carotene, μg/100 ml		No.	329	156	173	28	301	128	173
< 100.0		%	0.9	0	1.7	0	0.9	0	1.7
< 200.0		%	7.0	3.8	9.8	0	7.6	4.7	9.8
Ascorbic acid, mg/100 ml		No.	335	154	181	23	312	131	181
< 0.3		%	0.9	1.3	0.6	0	1.0	1.5	0.6
< 0.6		%	4.8	6.5	3.3	8.7	4.5	6.1	3.3
Thiamine, μg/gm creatinine		No.	344	163	181	31	313	132	181
< 40		%	18.9	28.6	10.2	42.4	13.4	25.2	10.2
< 50		%	24.6	35.1	15.1	48.5	22.1	31.9	15.1
Riboflavin, μg/gm creatinine		No.	336	161	175	28	308	133	175
< 100		%	30.8	33.3	28.5	12.1	32.7	38.5	28.5
< 200		%	61.9	61.9	61.8	36.4	64.5	68.2	61.8
N-methyl nicotinamide, mg/gm creatinine		No.	334	161	173	31	303	130	173
< 2.0		%	56.4	57.6	55.2	51.5	56.9	59.0	55.2
< 4.0		%	83.8	87.3	80.7	87.9	83.1	87.1	80.7

TABLE 9

Distribution of biochemical findings in sixth grade public school children by province

BIOCHEMICAL	PROVINCE		TOTAL			HAVANA			PINAR DEL RIO		
	LOCALE		Total	Urban	Rural	Total	Urban	Rural	Total	Urban	Rural
Hemoglobin, gm/100 ml		No.	320	139	181	108	47	61	26	15	11
< 11.0		%	4.3	3.6	5.0	7.1	6.4	8.2	3.8	0	9.1
< 12.4		%	52.2	51.8	52.5	38.0	51.1	27.9	65.4	53.3	81.8
Protein, gm/100 ml		No.	320	139	181	108	47	61	26	15	11
< 6.0		%	1.3	2.9	0	3.6	8.5	0	0	0	0
< 6.2		%	2.8	6.5	0	8.3	19.1	0	0	0	0
Vitamin A, μg/100 ml		No.	301	128	173	95	37	58	23	15	8
< 20.0		%	0.3	0	0.6	0	0	0	0	0	0
< 30.0		%	1.0	0.8	1.2	0	0	0	0	0	0
Carotene, μg/100 ml		No.	301	128	173	95	37	58	23	15	8
< 100.0		%	0.9	0	1.7	0.3	0	1.7	0	0	0
< 200.0		%	7.6	4.7	9.8	3.3	0	4.7	0	0	0
Ascorbic acid, mg/100 ml		No.	312	131	181	98	39	59	26	15	11
< 0.3		%	1.0	1.5	0.6	2.5	5.1	1.7	0	0	0
< 0.6		%	4.5	6.1	3.3	13.3	17.9	10.2	0	0	0
Thiamine, μg/gm creatinine		No.	313	132	181	99	43	56	22	11	11
< 40		%	13.4	25.2	10.2	23.1	24.4	10.7	13.0	0	27.3
< 50		%	22.1	31.9	15.1	19.8	28.9	12.5	17.4	8.3	27.3
Riboflavin, μg/gm creatinine		No.	308	133	175	95	44	51	23	12	11
< 100		%	32.7	38.5	28.5	34.3	37.8	44.7	39.1	33.3	45.5
< 200		%	64.5	68.2	61.8	66.3	73.3	60.7	82.6	66.7	100.0
N-methyl nicotinamide, mg/gm creatinine		No.	303	130	173	94	41	53	21	12	9
< 2.0		%	56.9	59.0	55.2	40.9	44.2	32.1	47.6	25.0	77.8
< 4.0		%	83.1	87.1	80.7	68.7	81.4	58.9	81.0	66.7	100.0

TABLE 9 *(continued)*

Distribution of biochemical findings in sixth grade public school children by province

BIOCHEMICAL	PROVINCE		MATANZAS			LAS VILLAS			CAMAGUEY			ORIENTE		
	LOCALE		Total	Urban	Rural	Total	Urban	Rural	Total	Urban	Rural	Total	Urban	Rural
Hemoglobin, gm/100 ml		No.	35	18	17	66	27	39	25	13	12	60	19	41
< 11.0		%	8.6	11.1	5.9	3.0	0	5.1	0	0	0	0	0	0
< 12.4		%	77.1	72.2	82.4	62.1	59.3	64.1	48.0	23.1	75.0	48.3	42.1	51.2
Protein, gm/100 ml		No.	35	18	17	66	27	39	25	13	12	60	19	41
< 6.0		%	0	0	0	0	0	0	0	0	0	0	0	0
< 6.2		%	0	0	0	0	0	0	0	0	0	0	0	0
Vitamin A, μg/100 ml		No.	35	18	17	65	27	38	23	12	11	60	19	41
< 20.0		%	2.8	0	5.9	0	0	0	0	0	0	0	0	0
< 30.0		%	5.7	0	11.8	1.5	0	3.7	0	0	0	0	0	0
Carotene, μg/100 ml		No.	35	18	17	65	27	38	23	12	11	60	19	41
< 100.0		%	0	0	0	3.1	0	5.3	0	0	0	0	0	0
< 200.0		%	14.3	5.6	23.5	16.9	18.5	15.8	4.3	0	9.1	3.3	0	4.9
Ascorbic acid, mg/100 ml		No.	35	18	17	66	27	39	25	13	12	62	19	43
< 0.3		%	0	0	0	0	0	0	0	0	0	0	0	0
< 0.6		%	0	0	0	0	0	0	0	0	0	1.6	5.2	0
Thiamine, μg/gm creatinine		No.	34	18	16	64	28	36	28	13	15	66	19	47
< 40		%	25.0	44.4	5.6	25.8	35.7	18.4	3.4	0	6.2	9.1	26.3	2.1
< 50		%	25.0	44.4	5.6	37.9	50.0	28.9	6.9	0	12.5	16.7	36.8	8.5
Riboflavin, μg/gm creatinine		No.	35	18	17	62	27	35	29	13	16	64	19	45
< 100		%	33.3	33.3	33.3	25.8	46.4	10.5	31.0	23.1	37.5	24.2	47.4	14.5
< 200		%	58.3	50.0	66.7	60.6	78.6	47.4	62.1	38.5	81.3	63.6	78.9	57.4
N-methyl nicotinamide, mg/gm creatinine		No.	32	17	15	65	28	37	29	13	16	62	19	43
< 2.0		%	54.3	82.4	27.8	56.1	60.7	52.6	75.9	69.2	81.3	84.1	84.2	84.1
< 4.0		%	74.3	94.1	55.6	92.4	97.9	92.1	100.0	100.0	100.0	95.2	89.5	97.7

individual provinces several other examples of sex differences are in evidence. For example, in Pinar del Rio, girls show significantly higher levels of plasma proteins than the boys; in Matanzas males were higher than females with respect to ascorbic acid.

c. Urban vs. rural

Several true differences exist between the urban and rural areas. The rural areas show levels of protein and thiamine distinctly higher than those of the urban areas. In particular, the urban males are different from the rural males. In addition to being lower in protein and thiamine, as stated above, they are also higher in hemoglobin and carotene than the rural males. Only the urban females, on the other hand, show higher levels of vitamin A than the rural females (in addition to the difference in protein and thiamine).

V. DISCUSSION AND RECOMMENDATIONS

A. Discussion

1. Previous studies — the experience of previous nutrition surveys in Newfoundland in 1944 (Adamson et al., '45) and in 1948 (Aykroyd et al., '49) and in Formosa (Jolliffe and Tung, '56; Pollack, '56) indicated a good correlation of clinical signs with the biochemical findings. Despite the fact that clinical signs by themselves are non-specific for given nutritional deficiencies, surprisingly accurate predictions of the laboratory findings were made based on the prevalence of certain key clinical signs when accompanied by other supporting clinical signs. This type of evidence was considered sufficiently adequate to lead the senior author to recommend that biochemicacl methods for vitamin A and carotene, ascorbic acid, riboflavin and possibly niacin could all be safely omitted in dietary surveys. Table 10 indicates the clinical findings which were the bases for the predictions concerning the laboratory findings.

2. This study — The current survey of Cuba has tended to substantiate the ability of clinical signs to predict roughly laboratory findings with respect to riboflavin, vitamin A and ascorbic acid. They still leave niacin in doubt.

a. Vitamin A — Table 11 clearly shows that the prevalence of gross conjunctival spots and follicular hyperkeratosis of the arms without a considerable frequency of similar lesions of the back and without Bitot spots do not afford sufficient

TABLE 10

Clinical indicators of vitamin deficiency

NUTRIENT	CLINICAL SIGN
Riboflavin	Active angular stomatitis [1]
	Magenta tongue
	Nasolabial seborrhea
Vitamin A	Follicular hyperkeratosis [1]
	Other folliculosis
	Gross conjunctival spots
Ascorbic acid	Hyperemia of gums
	Perifolliculosis
	Purpura
Niacin	Significant papillary tongue lesion [1]
	Scarlet red tongue
	Skin lesions (pellagra-like)

[1] Key clinical sign.

clinical evidence of vitamin A deficiency to predict a high frequency of low levels of serum vitamin A. The prevalence of either gross conjunctival spots or follicular hyperkeratosis alone without folliculosis of the back and buttocks cannot be used as indicating the prevalence of vitamin A deficiency as reflected by the very low prevalence of blood vitamin A levels below 20 mg/100 ml. It is believed that the estimated per capita dietary intake of 2393 units (table 2) must be in error. An error in the dietary intake of vitamin A could be made very easily because of erroneous estimation of the intake of mangoes, a fruit exceptionally rich in vitamin A and widely consumed in season. Certainly, the high blood levels of

vitamin A do not support the consumption of only about 2400 IU per capita per day.

b. Niacin — In the case of niacin, clinical signs failed to predict the surprisingly high prevalence of very low nicotinamide excretion levels. The clinical manifestations presented in table 11 would indicate that niacin deficiency is negligible in Cuba whereas the laboratory determinations disclose that well over half of the survey subjects excreted very low levels of N-methyl nicotinamide.

TABLE 11

Comparison of clinical and biochemical findings by school type

PERCENTAGES	PRIVATE SCHOOLS	PUBLIC SCHOOLS		
		Total	Urban	Rural
Vitamin A				
Low in vitamin A	0	0.3	0	0.6
With gross conjunctival spots	8.0	17.9	13.7	21.0
With fol. hyperkeratosis	19.6	37.9	34.8	40.3
With Bitot spots	0	0	0	0
N-methyl nicotinamide				
Low in nicotinamide	51.5	56.4	59.0	55.2
With papillary tongue lesions	11.0	6.6	7.3	6.1
With scarlet tongue	0	0.1	0	0.1
With pellagrous skin lesions	0	0	0	0
Riboflavin				
Low in riboflavin	12.1	30.8	38.5	28.5
With active angular stomatitis	2.5	22.4	7.5	33.3
With nasolabial seborrhea	19.6	37.5	45.0	32.0
With magenta tongue	0.6	0.3	0.4	0.3
Ascorbic acid				
Low in ascorbic acid	0	1.0	1.5	0.6
With gingivitis with bleeding	2.5	22.5	18.8	25.2
With perifolliculosis	0	0.7	0.4	1.0
With purpura	0	0.2	0.2	0.1
Thiamine				
Low in thiamine	42.4	18.9	25.2	10.2
With absent ankle jerks	0	0.2	0.1	0.3

It is possible that 2 mg of N-methyl nicotinamide per gram of creatinine is a level above which niacin deficiency is not apt to occur; it may well be, however, that the per capita intake of 70.9 gm of protein (table 2) of which 38.6 gm are derived from animal sources — represents a sufficiently high intake of tryptophan to prevent clinical signs of pellagra, even with low intakes of niacin and low levels of N-methyl nicotinamide in the urine. This would seem to be a reasonable explanation in view of the daily per capita intake of 12.9 mg of niacin, a level at which considerable numbers should have low excretion rates of the end product of niacin metabolism. On the other hand, as previously noted, the excretion of N-methyl nicotinamide may not be a reliable index of niacin deficiency.

c. Riboflavin — The key clinical sign of active angular stomatitis was supported by an even higher prevalence of nasolabial seborrhea and by a very small prevalence of magenta tongue. This latter lesion, even in the most marked riboflavin-deficient subjects, is a late manifestation. The clinical signs therefore correctly predicted a high prevalence of riboflavin deficiency in the Cuban 6th grade school population but consistently underestimated the low excretion rates in every group except the rural public schools where they were of about the same order. It would seem from these data that in Cuba, nasolabial seborrhea may be a better key clinical sign than angular stomatitis. However, the significance of this sign may be complicated by the simultaneous presence of acne.

d. Ascorbic acid — Although 22.5% of the public school children were found to have gingivitis with bleeding gums, the low prevalence rates of perifolliculosis and purpura afforded no supporting evidence of scurvy. The cause of the gingivitis must be other than ascorbic acid deficiency. This clinical prediction is supported by the laboratory findings which indicate that less than 1% of these children possess levels of ascorbic acid under 0.3 mg/100 ml of blood.

e. Thiamine — Although the results of the previous studies had indicated that laboratory procedures would still be necessary to detect causes of subclinical thiamine deficiency, we have used the bilateral absence of ankle jerks as a screening device for beriberi for it cannot be diagnosed in an adult when they are present. Table 11 reveals that the absence of ankle jerks, although a good screen for beriberi, is a poor screen for subclinical thiamine deficiency. In fact, absent ankle jerks were practically non-existent compared to the substantial proportions with the low thiamine levels.

B. Recommendations

If the rice were enriched to the level required in the United States for enriched flour and rice and all other foods were consumed at the same levels as indicated in table 2, there would be a significant increase in the consumption of all enrichment nutrients; riboflavin by 60%, thiamine by 45%, niacin by 30% and iron by 13%, bringing the daily per capita intake of those 4 micronutrients up to 1.44, 2.15, 16.6 and 17.5 mg respectively. Riboflavin, even after enrichment, would still be 18% below the National Research Council levels, but thiamine and niacin, without considering the lower calorie intakes, would exceed the National Research Council levels by 30% for niacin and 68% for thiamine, amounts not in excess of a satisfactory safety factor considering the high cooking losses particularly for thiamine.

VI. SUMMARY AND CONCLUSIONS

A study of the nutritional status of the population of Cuba was made by examining 2171 6th grade school children in all parts of the island, selected so as to constitute a representative sample of this age group. The entire group received clinical examination with about one in 6 being subjected to biochemical examination for hemoglobin, hematocrit, protein, vitamin A, carotene, ascorbic acid, thiamine, riboflavin, N-methyl nicotinamide and pyridoxine phosphate.

From the clinical and biochemical findings, it is concluded that deficiencies of protein, vitamin A and ascorbic acid are practically non-existent in Cuba. Only 4.0% of the population exhibited hemoglobin levels under 11 gm/100 ml of blood which indicates that iron, folic acid or vitamin B_{12} deficiencies are of minor significance on the island. Underweight occurs in 43.7% of the public school children and in 10.4% of the private school children. Since the private school children probably represent the irreducible minimum of underweight in a population group having adequate food supply, the proportion underweight in this group would seem to indicate that about a third of the public school group is not receiving sufficient nutrients to maintain normal weight.

Riboflavin deficiency, expressing itself as either active anangular stomatitis or nasolabial seborrhea, is the most common clinically manifest specific nutrient deficiency on the island, occurring in a 5th to a third of the children. This high prevalence of clinical riboflavin deficiency is supported by the low excretion of riboflavin in the urine. Although there is a high prevalence of subclinical thiamine and niacin deficiency in Cuba, as measured by urinary excretion, there is no clinical evidence of either beriberi or pellagra. With these dangerously high levels of subclinical thiamine and niacin deficiency and the high prevalence of riboflavin deficiency, it appears that vitamin enrichment of a staple article of food such as rice would be an extremely valuable contribution to the public health.

VII. ACKNOWLEDGMENTS

The stimulus for this investigation was provided by the Medical Committee of the Fundacion de Investigaciones Medicas (F.I.M.), Chairman Dr. Gustavo Cuervo Rubio, and Doctors Angel Vieta, Laureano Falla, Octavio Montoro, Angeal Reand and Gustavo Cardelle, their efficient directress Mrs. Louise Smith and by Dr. R. R. Williams of the Williams-Waterman Fund.

The Finlay Institute and their Director, Dr. Enrique Saladrigas, provided the laboratory facilities and at all times were helpful in coordinating the work with other sections of the government.

The National Vitamin Foundation contributed the time and expenses of Dr. Robert S. Goodhart, the I.C.A. or Point Four contributed the expenses of Dr. Norman Jolliffe and Dr. Kenneth Shull. The Williams-Waterman Fund contributed the expenses of Mrs. Norman Jolliffe, the Cuban members of the survey team, and the F.I.M. Laboratories of Nutrition. The time and expenses of Dr. George E. Boxer and the special laboratory materials required for the determination of pyridoxine phosphate were contributed by Merck & Co., Inc., Rahway, New Jersey. The Ministry of Education, and the Army, through the courtesy of General Eulogio Cantillo, and the F.I.M. furnished transportation for the team. The Ministry of Education, with the help of the Cuban Red Cross in one locality, furnished the transportation for the pupils from their own schools to central schools for examination. The United States Information Service furnished a portable power unit on a truck for electricity at Alto Songo where electric power was unavailable.

The Nutrition Clinics Fund of New York contributed the time and services of Mr. Morton Archer, who made the statistical analysis, and the expenses of a second visit of Dr. and Mrs. Norman Jolliffe to Cuba in connection with the survey.

The biochemical portion of this survey was handled by the F.I.M. Laboratories of Nutrition, Dr. Robert S. Harris, Scientific Director and Dra. Hady Lopez, Director, and their coworkers Dras. Margarita Cimadevilla, Catalina Durruthy, and Edelmira Fernandez, and Dr. Kenneth Shull. Mr. Rogelio Lee, technician from the Calixto Garcia Hospital assisted the laboratory in the field. Dr. George E. Boxer of Merck & Co. supervised the preparation of the blood specimens for the pyridoxine phosphate determinations which were then sent in dry ice by plane to his laboratory at Merck & Co.

The organization of the field trips was directed by Dr. Vieta, with the one-the-spot assistance of Dr. Flavio Galban. Assisting in the field were the following physicians from the University (Calixto Garcia) Hospital:

Drs. Flavio Galban, Ernesto E. Juncosa Sicre, Crestes Rodondo Cordoves, Roberto A. Pornoy Madariago, Armando Pena Pereiro, Modesto Pinon Hernandez, Gerardo E. Espinosa Sanches, Luis Charbonier Garcia, Julio C. Escobar Goenago; and from the School of Hygiene, Department of the Ministry of Education were Dr. Gonzalo Garcia Gonzalez, Director, and Drs. Hector Coronel, Gustavo Carro Suarez, Esmeralda Torras Blanco, Armando Alpendre, Leopoldina L. Roque Romero, Mario Garcini Verona, Zilda Tabares Ventura, and Hortensia Rojo Leon. Assisting in the tabulation of the data were Drs. Juncosa, Pinon and Galban. Joining the group as observers during the last week of the survey were Dr. Amara Chandrapananda of the School of Public Health of Bankok, Thailand, and Dr. Theodore B. Van Itallie of the School of Public Health of Harvard University. Dra. America Perez Cisneros of the Ministry of Education accompanied the team and supervised the recording of the identification data.

LITERATURE CITED

ADAMSON, J. D., N. JOLLIFFE, H. D. KRUSE, O. H. LOWRY, P. E. MOORE, B. S. PLATT, W. H. SEBRELL, J. W. TICE, F. F. TISDALL, R. M. WILDER AND P. C. ZAMECNIK 1945 Medical Survey of Nutrition in Newfoundland. Can. Med. Assoc. J., *52:* 227.

AYKROYD, W. R., N. JOLLIFFE, O. H. LOWRY, P. E. MOORE, W. H. SEBRELL, R. E. SHANK, F. F. TISDALL, R. M. WILDER AND P. C. ZAMECNIK 1949 Medical Resurvey of Nutrition in Newfoundland. Ibid., *60:* 329.

BOWES, A. DE P., AND C. F. CHURCH 1948 Food Values of Portions Commonly Used. College Offset Press.

CASTELLANOS, O. 1935 Contribucion al estudio clinico de las avitaminosis B en Cuba; el sindrome pelagroide-beri-berico. Bol. Soc. Cubana de Pediatria, *7:* 5.

CONNER, R. T., AND G. J. STRAUB 1941 Combined determination of riboflavin and thiamine in food products. Ind. and Eng. Chem., (Anal. Ed.), *13:* 385.

COULSON, R. A., P. ELLINGER AND M. HOLDEN 1944 A method for the estimation of nicotinamide methochloride in urine. Biochem. J., *38:* 150.

FERNANDEZ, F. E. 1943 Determinacion quimica de la tiamina en algunos productos alimenticos cubanos, y una prueba biologica de la guyaba. Bol. Soc. Cubana de Pediatria, *15:* 141.

FERNANDEZ, F. E., M. JOHNSON AND H. SOCARRAS 1947 Estudio cuantitativo de la riboflavina (vitamin B-2) en leches de vaca: fresca y pasteurizada; leches en polvo, condensada, evaporada, y leche fresca de cabra que existen en el mercado. Revista Cubana de Pediatria, *19:* 382.

———— 1948a Riboflavina, vitamina B-2, libre y combinada en leche de mujer. Ibid., *20:* 39.

———— 1948b Determinacion de la vitamina B-2 en la orina. Ibid., *20:* 580.

———— 1949 Determinacion del alfa-tocoferol (vitamin E) en suero humano. Ibid., *21:* 222.

FERRER, H. 1910 Apuntes sobre la racion alimenticia del obrero cubano. Anales de la Academia de Ciencias de la Habana, *47:* 41.

FOOD AND AGRICULTURE ORGANIZATION, UNITED NATIONS 1952 Second World Food Survey. P. 44.

FUNDACION DE INVESTIGACIONES MEDICAS 1954 Survey Nutricional. Bol. Col. Med. de la Habana, *5:* 1.

HAVA, J. 1865 Anales de la Academia de Ciencias de la Habana, *2:* 158.

HENNESSY, D. J., AND L. R. CERECEDO 1939 The determination of free and phosphorylated thiamine by a modified thiochrome assay. J. Am. Chem. Soc., *61:* 179.

INSTITUTO NACIONALE DE REFORMA ECONOMICA 1955 El Cubano — Un Pueblo Defectuosamente Alimentado, Costa no. 4, Oct. 15.

JOHNSON, M. 1946 Determinacion del alfa-tocoferol en aceites y vegetales. Revista Cubana de Pediatria, *28:* 92.

JOLLIFFE, N., AND T. C. TUNG 1956 Nutrition Status Survey of the Civilian Population of Formosa. Metabolism, *5:* 309.

LOPEZ, H. 1943 Estudio de la vitamina B-1 en algunos alimentos cubanos. Boletin oficial del Ministerio de Salubridad y Asistencia Social, *46:* 139.

MILANES, F. 1944 Importancia y alcance de las enfermedades por deficiencia nutricional en Cuba. Vida Nueva, *53:* 91.

MILANES, F., T. D. SPIES, G. G. LOPEZ AND T. ARAMBURU 1947 Synthetic Folic Acid. J. Am. Med. Assoc., *134:* 18.

———— 1948a Observations on the antianemic properties of vitamin B_{12}. South. Med. J., *41:* 522.

———— 1948b Observations on the hemopoietic response of persons with tropical sprue to vitamin B_{12}. Ibid., *71:* 6.

———— 1948c Thyamine, folic acid and vitamin B_{12} in nutritional macrocytic anemia, tropical sprue and pernicious anemia. Lancet, *2:* 519.

———— 1949 Tentative appraisal of vitamin B_{12} as a therapeutic agent. J. Am. Med. Assoc., *139:* 521.

———— 1950 Antianemic properties of a reaction product of vitamin B_{12} and the intrinsic factor. South. Med. J., *43:* 206.

NAJJAR, V. A., AND K. C. KETRON 1944 An improved thiochrome method for the determination of thiamine in urine. J. Biol. Chem., *152:* 579.

NAVIA, J. M., H. LOPEZ, I. D. CLEMENT, M. CIMADEVILLA, E. FERNANDEZ, A. VALIENTE AND R. S. HARRIS 1954 Tabla provisional de la composicion, nutritiva de los alimentos cubanos. Bol. Col. Med. de la Habana, *5:* 87.

———— 1955 Tabla provisional de la composicion nutritiva de los alimentos cubanos. Ibid., *6:* 145.

PETERS, J. H. 1942 The determination of creatinine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., *146:* 179.

PHILLIPS, R. A., D. D. VAN SLYKE, V. P. DOLE, K. EMERSON, P. B. HAMILTON, AND R. M. ARCHIBALD 1943 Copper sulfate method for measuring specific gravities of whole blood and plasma. Bul. U. S. Army Med. Dept. (no. *71*): 68.

POLLACK, H. 1956 Clinical Indicator Signs of Nutritional Insufficiencies Before and After Enrichment of Rice with Synthetic Vitamins. Metabolism, *5:* 231.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2-4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399.

SELLEK, A., J. C. MAS MARTIN AND E. DE CASTRO 1945 Proteinemia normal y patologica en clinica infantil. Revista Cubana de Pediatria, *17:* 71.

SOBEL, A. E., AND S. D. SNOW 1947 The estimation of serum vitamin A with activated glycerol dichlorohydrin. J. Biol. Chem., *171:* 617.

VALLEDOR, T., F. E. FERNANDEZ AND B. A. FERNANDEZ 1940a Determinacion de las cifras "standard" en vitamin C de diversos tipos de leche en nuestro medio. Bol. Soc. Cubana de Pediatria, *12:* 532.

———— 1940b Determinacion de las vitamina C en las frutas de Cuba. Ibid., *12:* 543.

DIGESTIBILITY OF INDIVIDUAL FATTY ACIDS IN THE RAT

K. K. CARROLL

The Collip Medical Research Laboratory, University of Western Ontario, London, Ontario, Canada

(Received for publication August 2, 1957)

Earlier work in this laboratory has shown that feeding the mono-unsaturated fatty acids, erucic (C_{22}) and nervonic (C_{24}) to rats as 10% by weight of the diet raised the concentration of adrenal cholesterol (Carroll, '53) and increased the fecal excretion of cholesterol (Carroll and Noble, '56). Because of the altered appearance and increased bulk of feces excreted on these synthetic experimental diets, it was apparent that at this level the fatty acids were incompletely digested, and it therefore seemed desirable to undertake a study of the digestibility of these and other fatty acids in comparing their relative effects on cholesterol metabolism.

The method described by Augur, Rollman and Deuel ('47) for measuring the coefficient of digestibility of ingested fat was used, with some modifications, in most of our early experiments. However, the fecal fat extracted by their procedure was found to contain appreciable quantities of non-lipid material and alternative methods for extracting fecal lipids were investigated.

METHODS AND RESULTS

Digestibility has been defined by Deuel ('55) as an index of the completeness with which a foodstuff is removed from the intestine during its passage through the body. It is normally expressed in terms of a Coefficient of Digestibility, which is the percentage of the ingested foodstuff that gains entrance into the body and hence is not lost in the feces.

For our experiments this was calculated as follows:

$$\text{Coefficient of Digestibility} = \frac{[\text{fatty acid ingested}] - [\text{fecal fat} - \text{metabolic fat}]}{\text{fatty acid ingested}} \times 100$$

The term "metabolic fat" is introduced to allow for the fecal lipid which arises endogenously rather than from ingested fatty acid. This was determined in separate experiments (table 1) by measuring the amount of fecal fat excreted when the fatty acid in the diet was replaced by glucose. It was expressed in terms of milligrams per rat per day so that the same correction could be applied for each experimental diet. Augur et al. ('47) expressed their correction in terms of milligrams per gram of dried stool and it therefore varied with the weight of feces excreted by rats on the different test diets.

The following method was used in measuring the coefficient of digestibility. Male rats of the Sprague-Dawley strain, weighing 80 to 90 gm, were placed on the experimental diets, allowed a two-day orientation period and the food intake and fecal output were measured for the following 7- or 8-day period. Then, in order to obtain a duplicate result, similar records were kept for a second 7- or 8-day period. The rats were caged in groups of three unless otherwise stated but the results have been expressed in terms of milligrams per rat per day.

Synthetic diets were used in these experiments. Their composition, expressed as percentage by weight was as follows: casein 22, glucose 63, fatty acid 10, salt mixture 5. To these were added 5 gm per 100 gm of cellu flour and a supplement of water-soluble vitamins. In a few experiments, where indicated, a fat-soluble vitamin mixture was also added. The fat-free diet used for determining "metabolic fat" consisted of: casein 19%, glucose 77%, salt mixture 4%, and the cellu flour and vitamin supplements. The source of the materials and the composition of the vitamin mixtures have been described previously (Carroll and Noble, '56). The salt mixture was formulated according to Steenbock and Nelson ('23)

except that the amount of calcium phosphate in the mixture was doubled.

In the first series of experiments, the pooled feces were dried, first in air and then in a vacuum desiccator and 10-gm aliquots were ground and extracted with ether in a Soxhlet overnight to remove neutral fat and free fatty acids. The extracted residue was then removed, acidified by grinding in a mortar with 3 ml of concentrated hydrochloric acid and re-extracted with ether to obtain the fatty acids present as soaps. The ether was removed and the extracts were dried to constant weight in a vacuum over phosphorus pentoxide.

The results of these experiments are presented in table 1. It can be seen that the short-chain fatty acids up to C_{10} were completely digested. Further increase in chain length resulted in a progressive drop in digestibility so that relatively little of the C_{18} fatty acid (stearic) was absorbed.

With mono-unsaturated fatty acids, the coefficient of digestibility was still high at C_{18} but decreased with increasing chain length at about the same rate as in the saturated series. From these results it appears that the presence of a double bond near the middle of the chain is equivalent in terms of digestibility to shortening the chain length by about 6 carbon atoms.

With most fatty acids tested, there seemed to be no consistent difference between the coefficient of digestibility determined for the first collection period and that determined for the second collection period. With erucic acid, however, the coefficient of digestibility seemed to be nearly always higher during the second collection period. The average values obtained during the first and second collection periods respectively were, for erucic acid, 47 and 61, for methyl erucate 57 and 67 and for ethyl erucate 52 and 66.

Alternative methods for extraction of fecal fat

In the method of Augur et al. ('47), the initial extraction of feces with ether yields predominately lipid material which can nearly all be redissolved in petroleum ether. However,

TABLE 1

Coefficient of digestibility of fatty acids using ether-hydrochloric acid method for extracting fecal fat

(Results are expressed as mg/rat/day)

FATTY ACID FED	NO. OF DETERMINATIONS	FAT INGESTED	WEIGHT OF DRY FECES	FAT EXCRETED			FAT ABSORBED	COEFFICIENT [1] OF DIGESTIBILITY
				Neutral fraction	Soap fraction	Total (corrected for metabolic fat)		
None [2]	4	—	940	38	83	—	—	—
None	8	—	850	17	81	—	—	—
			Saturated fatty acids					
Butyric (C_4) [3]	2	1240	950	19	62	0	1240	100
Caproic (C_6) [3]	2	1050	720	16	41	0	1050	100
Caprylic (C_8) [3]	2	1060	750	19	39	0	1060	100
Caprylic (C_8)	2	960	650	15	53	0	960	100
Capric (C_{10})	2	720	550	15	54	0	720	100
Lauric (C_{12})	2	790	630	35	175	110	680	86 (85–87)
Palmitic (C_{16})	4	1230	1490	130	610	640	590	48 (45–53)
Stearic (C_{18})	4	1310	2000	450	810	1160	150	12 (0–22)
Behenic (C_{22})	2	1370	2210	760	610	1270	100	7 (7)
α-Hydroxybehenic (C_{22})	2	1410	2350	1030	360	1290	120	9 (6–12)
			Mono-unsaturated fatty acids [1]					
Oleic (C_{18})	4	1180 (1040–1290)	940 (830–1050)	190 (150–240)	100 (90–120)	190	990	84 (82–87)
Oleic, methyl ester	4	1190 (1080–1300)	830 (760–910)	60 (56–70)	90 (66–120)	50	1140	96 (93–98)
Petroselenic (C_{18}) [4] methyl ester	2	1060 (960–1160)	940 (880–1000)	170 (160–200)	140 (125–150)	210	850	80 (75–85)
Erucic (C_{22}) [2]	6	1140 (910–1370)	1320 (1060–1490)	280 (250–340)	360 (290–540)	540	600	53 (37–61)
Erucic, methyl ester	8	1150 (1000–1360)	1160 (910–1460)	240 (180–310)	310 (180–560)	440	710	62 (37–75)
Erucic, ethyl ester	4	1180 (1050–1290)	1250 (1190–1350)	280 (230–340)	300 (240–360)	480	700	59 (50–68)
Nervonic (C_{24}) [2]	6	1160 (960–1440)	2150 (1490–3300)	340 (230–490)	310 (540–1150)	1000	160	14 (0–24)

[1] The range of values is given by the figures within parentheses.

[2] Some of the diets used in these experiments contained fat-soluble vitamins, in which case a different factor was applied to correct for metabolic fat.

[3] Fatty acids fed as their sodium salts.

[4] Prepared from carrot seed oil by the method of Christian and Hilditch ('29). The ester had an iodine value of 75.

the residue obtained by ether extraction following acidification either with 50% sulfuric acid or with hydrochloric acid is usually a black oil or gum which may in some cases be largely insoluble in petroleum ether.

An alternative procedure has been described by Hopkins, Murray and Campbell ('55) who used a Soxhlet extraction with petroleum ether to remove neutral fat and free fatty acids. They then acidified the residual feces in the Soxhlet cup by covering them with a solution containing 5 volumes of glacial acetic acid in 100 volumes of petroleum ether and allowed the mixture to stand for two hours to convert soaps to free acids. The free acids were recovered by continuing the extraction with the acidified petroleum ether.

This procedure was applied in our experiments to feces from rats on a fat-free and on a 10% erucic acid diet and the results were compared with those obtained in our earlier work. In the case of rats on a fat-free diet, the crude extract obtained after acidification with hydrochloric or sulfuric acids amounted to 100 to 200 mg/gm of feces, of which only 20 mg or less could be dissolved in petroleum ether. The extract obtained after acidification with acetic acid weighed 20 to 25 mg/gm of feces and was completely soluble in petroleum ether. With feces from animals fed the erucic acid diet, the extract obtained after acidification with acetic acid yielded a white solid residue which had values for melting point and neutral equivalent approximating those of erucic acid. In this case, and with other fatty acids having low coefficients of digestibility, it was generally not possible to obtain all of the fatty acid from the soap fraction by a single extraction although the acetic acid was increased from 5 to 10 volumes per cent in the solution used for acidification. Therefore the acidification and extraction were repeated two or three times if necessary until only negligible quantities of lipid were extracted. When this was done, the total weight of fecal lipid compared very well with the weight of petroleum ether-soluble material extracted by the older methods.

The initial extraction with petroleum ether in the new method yielded somewhat smaller amounts of lipid than extraction with ether and it was noted that the sterol fraction was incompletely extracted. An alternative method was therefore tried in which the feces were first extracted with ether and then acidified with 10% acetic acid in ether and re-extracted. Neutral ether appeared to extract nearly all of the sterol fraction and the combined neutral and acid lipid extract weighed about the same when either ether or petroleum ether was used. It also appeared that the lipid was completely extracted because further extraction of the insoluble fecal residue with alcohol and chloroform in the Soxhlet yielded only very small amounts of material. Since we were interested in the fecal sterols, the ether-acetic acid method was used in most of our subsequent work. It should be noted, however, that the calcium soaps of fatty acids such as oleic and linoleic are appreciably soluble in neutral ether and would therefore mainly appear in the neutral extract. A better approximation of the ratio of soap to free acid might therefore be obtained by using petroleum ether.

Some results obtained with this method are shown in table 2. The coefficients of digestibility of oleic and stearic acids appear to differ significantly from those obtained in our earlier work but the variation from experiment to experiment is so great that it is difficult to be certain that these changes have resulted from the use of a different extraction procedure. New values for eicosenoic and myristic acids are in the range that would be predicted from a consideration of the results with related fatty acids.

DISCUSSION

The coefficients of digestibility obtained in our experiments for saturated fatty acids are in reasonable agreement with those reported in the literature (Deuel, '55) but our value for oleic acid is somewhat lower than that reported by Paul and McCay ('42). The low coefficient of digestibility obtained for erucic acid bears out the suggestion of Deuel, Cheng and

TABLE 2

Coefficient of digestibility of fatty acids using ether-acetic acid method of extracting fecal fat

(Results are expressed as mg/rat/day)

FATTY ACID FED	NO. OF DETERMINATIONS	FAT INGESTED	WEIGHT OF DRY FECES	FAT EXCRETED			FAT ABSORBED	COEFFICIENT [1] OF DIGESTIBILITY
				Neutral fraction	Soap fraction	Total (corrected for metabolic fat)		
None	6	—	750	19	13	—	—	—
				Saturated fatty acids				
Caprylic (C_8)	2	1000	860	15	9	0	1000	100
Myristic (C_{14})	2	1040	1090	40	360	370	670	64 (64–65)
Palmitic (C_{16})	4	1340	1780	240	610	820	520	39 (27–55)
Stearic (C_{18})	2	1410	2180	330	780	1080	330	23 (14–33)
				Mono-unsaturated fatty acids				
Oleic (C_{18})	4	1180	1140	260	75	310	870	74 (72–79)
Eicosenoic (C_{20})	2	1410	1330	470	50	490	920	65 (64–66)
Erucic (C_{22})	6	1190	1340	275	340	580	610	51 (45–62)
Nervonic (C_{24})	2	1560	2340	435	840	1240	320	20 (19–21)

[1] The range of values is given by the figures within parentheses.

Morehouse ('48) that the presence of this fatty acid is mainly responsible for the poor digestibility of rape-seed oil. There is some indication from our results (table 1) that esters are better digested than free fatty acids, particularly in the case of oleic acid and methyl oleate.

Cheng, Morehouse and Deuel ('49) have suggested a relationship between the melting point of fats and their digestibility but this concept is not supported by our data. Erucic and nervonic acid melt at 32 and 39° respectively and are probably both in the liquid state after ingestion but they have low digestibilities of the same order as those of palmitic and stearic acids which melt at 64 and 69° respectively. Results from other laboratories (Mattil and Higgins, '45; Scribante and Favarger, '54; Buensod and Favarger, '56) have also indicated that melting point does not play a major role in determining the digestibility of fats.

The method of Augur et al. ('47) for measuring digestibility seems to give results which in most cases are comparable to those obtained when ether-acetic acid was used for the fecal extractions. However, the correction for metabolic fat in the method of Augur et al. is fairly large and failure to apply it leads to a difference of 8 to 10 units in the coefficient of digestibility. The correction is much smaller in the ether-acetic acid method and amounts to only about two units. This difference is very small in comparison with the variations from experiment to experiment observed for individual fatty acids in our studies and for all practical purposes it could probably be neglected.

It is very desirable that the correction for metabolic fat should be reduced, if possible, to the point of being insignificant because the method by which it is normally measured (i.e. as the amount of fecal lipid excreted on a fat-free diet) is entirely unsatisfactory. It is recognized that the amount of endogenous lipid may be altered by the nature of the diet and several instances of this may be cited. For example, in our earlier experiments (Carroll and Noble, '56) the amount of endogenous cholesterol appearing in the feces was in-

creased appreciably by feeding certain fatty acids. It has also been observed in the present study that the amount of fecal lipid excreted on diets containing short-chain fatty acids was less than that excreted on a fat-free diet (table 1). Norcia and Lundberg ('54) concluded that the endogenous fecal lipid was not significantly affected by the presence of tripalmitin, triolein or a mixture of tripalmitin and trilinolein in the diet, while Buensod and Favarger ('56) were able to obtain a direct measure of endogenous fecal lipid by using deuterated fatty acids in their feeding experiments and found that endogenous excretion was increased appreciably by feeding palmitic acid or stearic acid but not by feeding tripalmitin or tristearin. From these results it appears that the excretion of endogenous lipid may be affected by dietary fat but it is not possible at present to predict the magnitude of this effect.

The ether-acetic acid method of extraction of feces appears to offer certain advantages over older methods, particularly in that it yields a much cleaner extract. It has the disadvantages that it is often not possible to obtain all of the soap fraction by means of a single acidification and extraction and that the method tends to be rather time-consuming. Ether was used as the solvent in our extractions because we were primarily interested in the sterol fraction of the feces and this was more completely extracted with neutral ether than with low-boiling petroleum ether. However, the use of petroleum ether gave similar coefficients of digestibility in any particular case and it is possible that a better approximation of the ratio of soaps to free fatty acids could be obtained by using petroleum ether since the calcium soaps of some fatty acids were found to have an appreciable solubility in neutral ether. Attempts to obtain further improvement in the extraction procedure by using formic acid rather than acetic acid and by using other solvents such as alcohol, chloroform and higher-boiling petroleum ether seemed in each case to give less satisfactory results.

The use of the term "coefficient of digestibility" may seem unsuitable for studies with free fatty acids where one is

essentially measuring degree of absorption. However, "coefficient of digestibility" has been widely used as a measure of the difference between the amount of a foodstuff ingested and the amount excreted in the feces, whereas "coefficient of absorption" has been used to define the rate of absorption as measured by the amount of administered substance remaining in the gut several hours after a single dose has been given by mouth. (Deuel, '55, Favarger, '56.) Further, although digestion is normally referred to as the splitting of foodstuffs in the gastrointestinal tract, it is also used in a wider sense to cover breakdown and absorption, since a substance has to be absorbed in order to be considered digestible. In many instances it is also impossible in practice to measure breakdown and absorption separately because the two processes go on simultaneously. From these various considerations it has seemed to us preferable to continue to use the term "coefficient of digestibility" for any value which measures the percentage of a substance removed by the body during its passage through the gastrointestinal tract.

SUMMARY

The digestibility of straight-chain saturated fatty acids from C_4 to C_{22} and of mono-unsaturated fatty acids from C_{18} to C_{24} has been measured in the rat. Short-chain saturated fatty acids up to C_{10} were completely digested. From C_{10} to C_{18} the digestibility decreased progressively and very small amounts of the C_{18} and higher fatty acids were absorbed. The digestibilities of the mono-unsaturated fatty acids were approximately the same as those of saturated fatty acids with 6 less carbon atoms. These results do not support the concept of an inverse relationship between the digestibility of fatty acids and their melting points.

Different methods of extracting fecal fat have been investigated and acidification of the feces with acetic acid to obtain fatty acids present as soaps appears to be preferable to acidification with hydrochloric or sulfuric acids.

ACKNOWLEDGMENTS

This work was begun during the tenure of a Fellowship from the Canadian Life Insurance Officers Association and was completed with the assistance of a grant from the Life Insurance Medical Research Fund. Financial assistance was also provided by the National Research Council of Canada. The author wishes to acknowledge the technical assistance of Miss Shirley Barrett and Mr. E. A. Andersen.

LITERATURE CITED

AUGUR, V., H. S. ROLLMAN AND H. J. DEUEL, JR. 1947 The effect of crude lecithin on the coefficient of digestibility and the rate of absorption of fat. J. Nutrition, *33:* 177.

BUENSOD, M., AND P. FAVARGER 1956 Observations sur la digestibilité des acides palmitique et stéarique et de leurs esters glycériques chez le rat. Helv. Physiol. et Pharmacol. Acta, *14:* 299.

CARROLL, K. K. 1953 Erucic acid as the factor in rape oil affecting adrenal cholesterol in the rat. J. Biol. Chem., *200:* 287.

CARROLL, K. K., AND R. L. NOBLE 1956 Erucic acid and cholesterol excretion in the rat. Can. J. Physiol. and Biochem., *34:* 981.

CHENG, A. L. S., M. G. MOREHOUSE AND H. J. DEUEL, JR. 1949 The effect of the level of dietary calcium and magnesium on the digestibility of fatty acids, simple triglycerides, and some natural and hydrogenated fats. J. Nutrition, *37:* 237.

CHRISTIAN, B. C., AND T. P. HILDITCH 1929 Seed fats of the Umbelliferae II. The seed fats of some cultivated species. Biochem. J., *23:* 327.

DEUEL, H. J. JR. 1955 The Lipids, Vol. II Biochemistry. Interscience Publishers Inc., N. Y., Chap. III, 195.

DEUEL, H. J. JR., A. L. S. CHENG AND M. G. MOREHOUSE 1948 The digestibility of rape-seed oil in the rat. J. Nutrition, *35:* 295.

FAVARGER, P. 1956 Données récentes sur l'absorption intestinale des graisses et du cholesterol. Annales de la Nutrition et de l'Alimentation, *10:* 211.

HOPKINS, C. Y., T. K. MURRAY AND J. A. CAMPBELL 1955 Optimum ratio of saturated to mono-unsaturated fatty acids in rat diets. Can. J. Biochem. and Physiol., *33:* 1047.

MATTIL, K. F., AND J. W. HIGGINS 1945 The relation of glyceride structure to fat digestibility. I. Synthetic glycerides of stearic and oleic acids. J. Nutrition, *29:* 255.

NORCIA, L. N., AND W. O. LUNDBERG 1954 Fat excretion. The influence of dietary fat on fecal fat excretion. Ibid., *54:* 491.

PAUL, H., AND C. M. MCCAY 1943 The utilization of fats by herbivora. Arch. Biochem., *1:* 247.

SCRIBANTE, P., AND P. FAVARGER 1954 Etude de la digestibilité de l'acide stéarique et de ses esters glycérique chez le rat. Helv. Physiol. et Pharmacol. Acta, *12:* 74.

STEENBOCK, H., AND E. M. NELSON 1923 Fat-soluble vitamins. XIII. Light in its relation to ophthalmia and growth. J. Biol. Chem., *56:* 355.

FACTORS AFFECTING DIGESTIBILITY OF FATTY ACIDS IN THE RAT

K. K. CARROLL AND J. F. RICHARDS [1]
The Collip Medical Research Laboratory, University of Western Ontario, London, Ontario, Canada

(Received for publication August 2, 1957)

In the experiments described in the preceding paper (Carroll, '58), a number of saturated and mono-unsaturated fatty acids were fed to rats in synthetic diets and their coefficients of digestibility were measured. In most of those experiments, the fatty acids were fed in the non-esterified form, whereas normally they are ingested in the form of triglycerides. The present study has therefore been concerned with differences between the digestibility of non-esterified fatty acids and those fed as triglycerides. In addition, the effect of a number of other variables on digestibility has been investigated, such as the level of protein in the diet, the type of salt mixture used, and the age of the animals.

METHODS

The general procedure employed in these experiments and the diets used were the same as those described in the preceding paper. Some of the experiments were carried out before our investigation of the methods for extraction of fecal lipid, and in these the method of Augur, Rollman and Deuel ('47) for measuring digestibility was used, with the modifications previously indicated. Otherwise the fecal lipids were extracted by the ether-acetic acid method (Carroll, '58). The amount of free fatty acid in the neutral ether extract was

[1] Holder of a Studentship from the National Research Council of Canada (1956–57).

determined by titrating an aliquot with N/50 potassium hydroxide in ethanol, using phenolphthalein as indicator. The neutral equivalent was taken to be that of the dietary fatty acid. The total glycerol content was determined on another aliquot following hydrolysis with approximately 10 volumes of 15% potassium hydroxide in 60% ethanol on a steam bath for 40 minutes. The hydrolysis mixture was diluted with water, acidified and filtered. The filtrate was made to volume and an aliquot used for determination of glycerol by the method of Lambert and Neish ('50) as modified by Korn ('55). A further aliquot of the original ether extract was taken to dryness and the residue was oxidized with 60% perchloric acid. It was then taken up in water and aliquots were analyzed for phosphorus (King, '32) and calcium (Wang, '35).

RESULTS

Digestibility of triglycerides versus non-esterified fatty acids

The results of these experiments are presented in table 1. It can be seen that triolein and trilinolein were almost completely digested in contrast to the rather low digestibilities of oleic acid and linoleic acid. Trierucin was also better digested than erucic acid, but the triglycerides of the saturated fatty acids appeared to be digested to a lesser extent than the free fatty acids. The improved digestibility of triolein and trilinolein over that of the corresponding free acids was due mainly to a reduction in the amount of neutral lipid excreted in the feces whereas the improved digestibility of trierucin resulted from a decrease in the fatty acids excreted as soaps. When stearic acid and palmitic acid were fed as triglycerides, most of the fecal lipid was in the neutral fraction but when they were fed as free acids, most of the fecal lipid was in the soap fraction.

Further examination of the neutral fecal lipid fraction from the various experiments helped to explain the observed differences in digestibility (table 2). There appeared to be little or

TABLE 1

Digestibility of triglycerides versus non-esterified fatty acids [1]

(Results are expressed as mg/rat/day)

FAT OR FATTY ACID FED [2]	BODY WEIGHT [3]		FAT INGESTED	WEIGHT OF DRY FECES	FAT EXCRETED			FAT ABSORBED	COEFFICIENT OF DIGESTIBILITY
	Initial	Final			Neutral fraction	Soap fraction	Total (corrected for metabolic fat)		
	gm	*gm*							
Triolein	86	163	1230	850	25	10	10	1220	99
Oleic acid	85	162	1240	1240	290	80	340	900	73
Trilinolein	73	135	990	810	40	20	30	960	97
Linoleic acid	78	156	1160	1090	140	75	190	970	84
Trierucin	85	123	970	990	230	160	360	610	63
Erucic acid	83	139	1410	1660	290	470	740	670	48
Tristearin	85	149	1370	2200	1110	100	1180	190	14
Stearic acid	83	132	1410	2180	330	780	1080	330	24
Tripalmitin	84	159	1420	2230	920	220	1110	310	22
Palmitic acid	87	143	1340	1780	240	610	820	520	40

[1] Purified casein (Nutritional Biochemicals, Cleveland, Ohio) was used in these diets, and the salt mixture was formulated according to Steenbock and Nelson ('23) except that double the amount of calcium phosphate was used.

[2] Triolein, trilinolein, tristearin and tripalmitin were obtained from the Hormel Institute, Austin, Minnesota. Trierucin, oleic acid, linoleic acid and erucic acid were provided through the courtesy of Dr. B. M. Craig, Prairie Regional Laboratory, National Research Council, Saskatoon, Sask. Stearic acid and palmitic acid were obtained from Nutritional Biochemicals, Cleveland, Ohio. All fats were fed as 10% by weight of the diet.

[3] Diets were fed for 16 days.

no glyceride in this fraction except when tripalmitin or tristearin was fed. These two triglycerides seemed to be less readily hydrolyzed during their passage through the gastrointestinal tract and this accounts for the large amount of neutral lipid in the feces when they are fed.

TABLE 2

Composition of neutral fecal extracts
(Results expressed as mg/rat/day)

FAT OR FATTY ACID	WEIGHT OF NEUTRAL FRACTION	FREE FATTY ACID	GLYCERIDE [1]	CALCIUM [2] PHOSPHORUS COMPLEX	CALCIUM [3] SOAP
Triolein	25	8	3	2	0
Oleic acid	290	70	3	105	105
Trilinolein	40	7	3	3	0
Linoleic acid	140	75	5	45	30
Trierucin	230	40	5	60	45
Erucic acid	260	100	3	75	30
Tristearin	1110	230	930	10	5
Stearic acid	440	225	0	0 [4]	0
Tripalmitin	920	215	735	0 [4]	0
Palmitic acid	340	155	2	0 [4]	0

[1] Calculated as triglyceride from the amount of glycerol in the hydrolyzed extract, but no distinction was made between mono-, di- and triglyeeride.

[2] Calculated from the amount of phosphorus in the extract according to the formula of Swell et al. ('56b), substituting the dietary fatty acid for oleic acid where necessary.

[3] Calculated from the excess of calcium over that required for complex formation.

[4] A small amount of phosphorus was present in these extracts but there was no detectable calcium.

The neutral lipid fraction from the feces of rats fed oleic acid, linoleic acid, erucic acid or trierucin contained relatively large amounts of calcium and phosphorus. This appeared to be present in the form of a complex similar to that described by Swell, Trout, Field and Treadwell ('56 a, b) as occurring in the feces of rats fed oleic acid. In agreement with their results and those of Pihl ('55) and Kim and Ivy ('52), much less lipid-soluble phosphorus was found in the feces of rats

fed saturated fatty acids or triglycerides of either saturated or unsaturated fatty acids, with the exception of trierucin. Our experiments have also included feeding tests with the monounsaturated fatty acids eicosenoic and nervonic and the results have shown that eicosenoic acid promotes an excretion of calcium and phosphorus comparable with that of oleic acid while nervonic acid is no more effective than the saturated fatty acids.

In table 2 the weight of the calcium and phosphorus complex has been calculated from the weight of phosphorus excreted, on the basis of the formula suggested by Swell et al. ('56b), and assuming that the fatty acid portion of the molecule was derived from the dietary fat. The crude fecal extracts in the experiments with oleic acid, linoleic acid, erucic acid and trierucin contained an excess of calicum over that required for complex formation and this has been shown in the table as calcium soap of the dietary fatty acid. These figures for complex and soap excretion are intended only to give a rough measure of the amounts that could be present and should not be taken to imply that all of the phosphorus and calcium in the extracts is combined in this manner. However, further experiments, particularly with the fecal extract of rats fed erucic acid, have shown the presence of a substance containing calcium, phosphorus and erucic acid which was soluble in ether and petroleum ether, could be precipitated by acetone or methanol and which after several precipitations gave analytical figures similar to those of Swell et al. ('56b). It has also been demonstrated that the calcium salts of oleic, linoleic and erucic acid can be partially extracted by neutral ether in a Sohxlet so it is possible for these soaps to be present in the neutral ether extract.

Effect of salt mixtures on digestibility

The salt mixture used in our earlier synthetic diets was formulated according to Steenbock and Nelson ('23) except that double the recommended amount of calcium phosphate

was used. In later experiments this was replaced by the Phillips and Hart ('35) salt mixture and it appeared that the change was associated in some cases with a reduction in the digestibility of individual fatty acids. It was suspected that the reduced digestibility might have resulted from the increased amount of calcium in the Phillips and Hart mixture since Cheng, Morehouse and Deuel ('49) showed clearly that increasing the amount of calcium and magnesium in the diet lowered the digestibility of higher melting simple triglycerides and hydrogenated fats.

A survey of a number of salt mixtures recommended for use in synthetic diets revealed wide variations in calcium content and in the calcium-phosphorus ratio. The magnesium varied less and was present in much smaller quantities than the calcium in all mixtures. The salt mixtures could be classified into three main groups on the basis of their calcium and phosphorus content. Those formulated by McCollum and Simmonds ('18) and by Steenbock and Nelson ('23) were low in calcium (7% by weight) and relatively high in phosphorus (Ca: P ratio 0.60 to 0.64). The Wesson ('32) modification of the Osborne-Mendel mixture, the U.S.P. XIV, the Jones-Foster ('42) and the Phillips-Hart ('35) mixtures contained about twice as much calcium and somewhat less phosphorus (Ca: P ratio 1.39 to 1.94). Finally, the Hubbell-Mendel-Wakeman ('37) mixture contained three times as much calcium and had the lowest phosphorus content (Ca: P ratio 4.25). This latter mixture was designed to be added to diets at the 2% level rather than the more usual 4 to 5% but it has frequently been used at the 4 to 5% level (Norcia and Lundberg, '54; Scribante and Favarger, '54, Swell, Flick, Field and Treadwell, '55; Okey and Lyman, '56).

Because most of these salt mixtures are being used in various laboratories for the preparation of synthetic diets it seemed desirable to carry out a few experiments in order to assess the magnitude of the effects that they might have on the digestibility of fatty acids. When oleic and erucic acid were fed in a diet containing the Phillips and Hart salt mix-

ture at the 5% level or the Hubbell-Mendel-Wakeman mixture at the 2% level, their digestibilities were the same or slightly lower than those observed previously with the modified Steenbock salts (table 3). When the Hubbell-Mendel-Wakeman mixture was raised to the 5% level, much lower coefficients of digestibility were obtained. On the other hand, both fatty acids were more than 90% digested when a salt mixture containing no calcium was used.

In these experiments, decreased digestibility of oleic acid was associated mainly with an increase in weight of the neutral fecal lipid fraction, while decreased digestibility of erucic acid was associated with a greater increase in the soap fraction. This difference may be due partly to the method of extraction since calcium oleate was found to have a greater solubility in neutral ether than calcium erucate. On the oleic acid diet, the calcium content of the neutral ether extract of feces increased from 0.24 mg/rat/day with the calcium-free salt mixture to 64 mg/rat/day with the 5% Hubbell-Mendel-Wakeman mixture. Corresponding figures for the erucic acid diet were 0.60 and 7.0 mg/rat/day respectively. The phosphorus content of the extract also was increased more on the oleic acid diet (0.11 to 6.8 mg/rat/day) than on the erucic acid diet (0.22 to 1.6 mg/rat/day), but the calcium phosphorus complex formed with either fatty acid appeared to readily soluble in ether.

It seems possible that the effects of different salt mixtures on digestibility may be greater for non-esterified fatty acids than for triglycerides. In one experiment, however, a coefficient of digestibility of 35 was obtained for trierucin fed in a diet containing 5% of the Hubbell-Mendel-Wakeman salt mixture. This is considerably lower than that obtained for trierucin fed with the modified Steenbock salt mixture (table 1), but triglycerides like triolein and trilinolein would perhaps be almost completely digested regardless of the salt mixture.

TABLE 3

Effect of different salt mixtures on digestibility
(Results are expressed as mg/rat/day)

SALT [1] MIXTURE	PERCENT IN DIET	FATTY ACID FED	BODY WEIGHT [2]		FAT INGESTED	WEIGHT OF DRY FECES	FAT EXCRETED			FAT ABSORBED	COEFFICIENT OF DIGESTIBILITY
			Initial	Final			Neutral fraction	Soap fraction	Total (corrected for metabolic fat)		
			gm	*gm*							
Calcium free	5	Oleic	86	120	880	570	25	5	5	875	99
Phillips-Hart	5	Oleic	84	169	1570	1570	220	150	340	1230	78
Hubbell-Mendel-Wakeman	2	Oleic	84	150	1160	930	220	45	240	920	79
Hubbell-Mendel-Wakeman	5	Oleic	88	165	1400	1930	740	100	810	590	42
Calcium free	5	Erucic	85	120	940	540	90	20	80	860	92
Phillips-Hart	5	Erucic	82	158	1630	2160	370	750	1090	540	33
Hubbell-Mendel-Wakeman	2	Erucic	86	148	1260	1430	175	505	650	610	48
Hubbell-Mendel-Wakeman	5	Erucic	84	165	1590	2600	220	1070	1260	330	21

[1] The salt mixtures and casein used in these diets were obtained from Nutritional Biochemicals, Cleveland, Ohio. Analysis of the casein showed 0.77% phosphorus and little or no calcium. The calcium-free mixture consisted of magnesium chloride 47.5%, potassium citrate 20.7%, potassium chloride 19.7%, sodium chloride 8.2%, potassium sulfate 2.1%, ferric citrate 1.6%, potassium iodide 0.04%, sodium fluoride 0.04%, manganous sulfate 0.02% and potassium alum 0.008%. It contained 12.1% of magnesium.

[2] Diets were fed for 16 days.

Effect of other factors on digestibility

The effect of other dietary alterations and of age of the animals on the digestibility of erucic acid have also been examined and the results are presented in table 4. Digestibility appeared to be increased by a high-protein and decreased by a low-protein diet. It should be noted, however, that the animals on the low-protein diet lost weight during the experiment. The addition of desiccated thyroid or of thiouracil had relatively little effect on digestibility although the amount of fatty acid ingested was much greater on the diet containing thyroid. Experiments with rats of different ages suggested that the digestibility of erucic acid was somewhat decreased in older rats.

DISCUSSION

The results obtained in these experiments indicate that the triglycerides of unsaturated fatty acids are better digested than the corresponding non-esterified fatty acids. In fact, one would expect to find that triolein and trilinolein are almost completely digested since most naturally-occurring fats and oils have coefficients of digestibility of 95 or above (Deuel, '55).

Conversely, the triglycerides of palmitic and stearic acid appeared to be digested to a lesser extent than the free fatty acids. Low digestibility values for saturated triglycerides have also been obtained by Cheng et al. ('49), Mattson, Alexander, Baur and Reller ('56) and Karvinen, Lin and Ivy ('57), but considerably higher values were reported by Norcia and Lundberg ('54) and Scribante and Favarger ('54). The results of Norcia and Lundberg were influenced by the type of conditioning to which the animals were subjected, but those of Scribante and Favarger are not readily explained, particularly since Buensod and Favarger ('56) reported values for palmitic acid and stearic acid which are in reasonable agreement with ours. It should be noted, however, that rather wide variations in digestibility have been observed for any individual fatty acid in our experiments (Carroll, '58).

TABLE 4

Digestibility of erucic acid under various conditions [1]

	NO. OF DETERMINATIONS	FAT INGESTED	FAT EXCRETED			FAT ABSORBED	COEFFICIENT [3] OF DIGESTIBILITY
			Neutral fraction	Soap fraction	Total [2] (corrected for metabolic fat)		
High protein	2	850	200	200	310	540	63 (54–72)
Low protein	2	650	140	400	440	210	33 (29–37)
0.1% thyroid	2	1310	390	410	640	670	51 (42–60)
0.3% thiouracil	2	900	250	300	450	450	50 (49–51)
Young rats (84 gm)	6	1140	280	360	540	600	53 (37–61)
Adult rats (221 gm)	4	1760	360	740	970	790	45 (35–56)
Old rats (386 gm)	4	1700	390	830	1040	660	39 (35–43)

[1] The modified method of Augur et al. ('47) was used in these experiments. The standard diet was the same as that used in earlier experiments. (Carroll, '58.) Additions of thyroid (desiccated thyroid — Burroughs Wellcome) and of thiouracil were made to this diet. For the high- and low-protein diets, the casein was increased to 65% and decreased to 5.5% by weight respectively at the expense of the glucose.

[2] The metabolic fat was determined in each experiment by feeding a fat-free diet having the appropriate additions or alterations.

[3] The figures within parentheses give the range of values.

The presence of large amounts of glyceride in the feces of rats fed tristearin and tripalmitin indicates that these triglycerides are not readily hydrolyzed during their passage through the gastrointestinal tract. Ivy, Karvinen and Lin ('57) obtained similar results with tripalmitin but found that trielaidin was readily hydrolyzed.

The poor digestibility of oleic acid, linoleic acid and erucic acid relative to their corresponding triglycerides is associated with increased fecal excretion of calcium and phosphorus in the lipid fraction extractable with neutral ether. It seems probable that the fatty acids are poorly digested because they form calcium soaps and calcium-phosphorus containing complexes which are not readily absorbed, particularly since the digestibility of oleic acid and erucic acid was decreased still further by increasing the calcium content of the diet (table 3).

Relatively large amounts of free fatty acid are also liberated in the intestine during the digestion of triglycerides (Borgstrom, '52; Mattson, Benedict and Beck '54) but much larger amounts would be present when the fatty acids are administered in the non-esterified form. There is good evidence that a large proportion of triglyceride fat is absorbed without being completely hydrolyzed (Reiser, '55) and it has been reported by Buensod and Favarger ('56) that monoglycerides are better digested than either triglycerides or free fatty acids.

It is interesting that calcium and phosphorus were present in quantity in the fecal extracts of rats fed erucic acid either as the triglyceride or as the free fatty acid while this was not true for oleic or linoleic acids. The difference may be related to the slow rate of absorption which we have observed for erucic acid and which has also been reported for rapeseed oil (Thomasson, '56).

Our experiments with different salt mixtures confirm the findings of Cheng et al. ('49) that the amount of calcium in the diet may play an important role in the digestibility of fatty acids and this should be borne in mind when choosing a

salt mixture for synthetic diets. It would appear that calcium is more important than magnesium in this respect.

Our studies on the effect of a low protein diet in decreasing digestibility also confirm the results of other workers (Deuel, '55). We are not aware of previous studies on the relation of age or thyroid activity of rats to their ability to digest fat.

SUMMARY

Triolein, trilinolein and trierucin were more completely digested than the corresponding non-esterified fatty acids. Palmitic and stearic acids were very poorly digested either as triglycerides or as free acids. When diets containing oleic, linoleic, eicosenoic or erucic acids were fed, the neutral ether extract of the feces contained large amounts of calcium and phosphorus. This also occurred with trierucin but not with other triglycerides, saturated fatty acids or nervonic acid.

The calcium content of the diet was found to play an important role in determining the digestibility of fatty acids. This should be considered in preparing synthetic diets because commonly-used salt mixtures cover a wide range of calcium concentrations.

The digestibility of erucic acid appeared to improve as the protein level in the diet was increased. The addition of desiccated thyroid or of thiouracil did not affect the digestibility although the total amount of fatty acid ingested was much greater in the former case. The digestibility of erucic acid seemed to be lower in old than in young rats.

ACKNOWLEDGMENT

Financial assistance for this work has been provided by the Life Insurance Medical Research Fund and by the National Research Council of Canada. The authors wish to acknowledge the valuable technical assistance of Mr. E. A. Andersen.

LITERATURE CITED

AUGUR, V., H. S. ROLLMAN AND H. J. DEUEL, JR. 1947 The effect of crude lecithin on the coefficient of digestibility and the rate of absorption of fat. J. Nutrition, *33:* 177.

BORGSTROM, B. 1952 On the action of pancreatic lipase on triglycerides in vivo and in vitro. Acta Physiol. Scand., *25:* 328.

BUENSOD, M., AND P. FAVARGER 1956 Observations sur la digestibilité des acides palmitique et stéarique et de leurs esters glycérque chez le rat. Helv. Physiol. et Pharmacol. Acta, *14:* 299.

CARROLL, K. K. 1958 Digestibility of individual fatty acids in the rat. J. Nutrition, *64:* 399.

CHENG, A. L. S., M. G. MOREHOUSE AND H. J. DEUEL, JR. 1949 The effect of the level of dietary calcium and magnesium on the digestibility of fatty acids, simple triglycerides, and some natural and hydrogenated fats. J. Nutrition, *37:* 237.

DEUEL, H. J., JR. 1955 The Lipids Vol. II. Biochemistry. Interscience Publishers, Inc. New York, Chap. III, 195.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

IVY, A. C., E. KARVINEN AND T. M. LIN 1957 Composition of fecal fat in the absence of pancreatic juice in the rat. Acta Physiol. Scand., *38:* 207.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, *24:* 245.

KARVINEN, E., T. M. LIN AND A. C. IVY 1957 Impairment of triglyceride absorption by the exclusion of pancreatic juice in the rat. Amer. J. Physiol., *188:* 61.

KIM, K. S., AND A. C. IVY 1952 Factors influencing cholesterol absorption. Ibid., *171:* 302.

KING, E. J. 1932 The colorimetric determination of phosphorus. Biochem. J., *26:* 292.

KORN, E. D. 1955 Clearing factor, a heparin-activated lipoprotein lipase. I. Isolation and characterization of the enzyme from normal rat heart. J. Biol. Chem., *215:* 1.

LAMBERT, M., AND A. C. NEISH 1950 Rapid method for estimation of glycerol in fermentation solutions. Can. J. Research, *28B:* 83.

MATTSON, F. H., J. C. ALEXANDER, F. J. BAUR AND H. H. RELLER 1956 Short-term feeding studies on acetin fats. J. Nutrition, *59:* 277.

MATTSON, F. H., J. H. BENEDICT AND L. W. BECK 1954 Composition of intestinal lumen lipides following the feeding of triglycerides, partial glycerides or free fatty acids. Ibid., *52:* 575.

MCCOLLUM, E. V., AND N. SIMMONDS 1918 A study of the dietary essential, water soluble B. in relation to its solubility and stability towards reagents. J. Biol. Chem., *33:* 55.

NORCIA, L. N., AND W. O. LUNDBERG 1954 Fat excretion. The influence of dietary fat on fecal fat excretion. J. Nutrition, *54:* 491.

OKEY, R., AND M. M. LYMAN 1956 Protein intake and liver cholestrol: effects of age and growth of the test animal. Ibid., *58:* 471.

PHILLIPS, P. H., AND E. B. HART 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

PIHL, A. 1955 On the mode of action of fatty acids in cholesterol absorption. Acta Physiol. Scand., *34:* 197.

REISER, R. 1955 Recent studies of fat digestion and absorption. Clin. Chem., *1:* 93.

SCRIBANTE, P., AND P. FAVARGER 1954 Étude de la digestibilité de l'acide stéarique et de ses esters glycériques chez le rat. Helv. Physiol. et Pharmacol. Acta, *12:* 74.

STEENBOCK, H., AND E. M. NELSON 1923 Fat-soluble vitamins. XIII. Light in relation to ophthalmia and growth. J. Biol. Chem., *56:* 355.

SWELL, L., D. F. FLICK, H. FIELD. JR. AND C. R. TREADWELL 1955 Role of fat and fatty acid in absorption of dietary cholesterol. Amer. J. Physiol., *180:* 124.

SWELL, L., E. C. TROUT, JR., H. FIELD, JR. AND C. R. TREADWELL 1956a Effect of dietary fat and fatty acid on fecal excretion of a calcium oleate phosphate complex. Proc. Soc. Exp. Biol. Med., *92:* 613.

———— 1956b Occurrence and nature of a fecal phosphorus-containing lipide. J. Biol. Chem., *223:* 743.

THOMASSON, H. J. 1956 The biological value of oils and fats. IV. The rate of intestinal absorption. J. Nutrition, *59:* 343.

WANG, C. C. 1935 Improvements in the method for calcium determination in biological material. J. Biol. Chem., *111:* 443.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339.

EFFECTS OF FEEDING WOOL-FAT STEROLS ON THE STEROL CONTENT OF SERUM AND LIVER OF THE RAT [1]

CHARLES H. DUNCAN AND MAURICE M. BEST
WITH THE TECHNICAL ASSISTANCE OF JOAN D. WATHEN
Department of Medicine, University of Louisville School of Medicine, Louisville, Kentucky

(Received for publication August 29, 1957)

The effects on serum and liver cholesterol of the administration of "isocholesterol," a mixture of 30-carbon sterols occurring in wool fat, to the rat have recently been reported (Best and Duncan, '57). The addition of 5% of "isocholesterol" to a low-cholesterol diet had no significant effect on liver and serum cholesterol levels. The addition of 5% of "isocholesterol" to a 1% cholesterol diet did, however, exert a marked inhibitory effect on the accumulation of cholesterol in the liver which otherwise occurs on such a high cholesterol diet. It also resulted in a lower level of serum cholesterol although the difference was significant in only one of two experiments performed.

The major components of "isocholesterol" are lanosterol, dihydrolanosterol, agnosterol and dihydroagnosterol (Clayton and Bloch, '56). In the studies utilizing the mixture no conclusions could be drawn as regards the effect of the individual components. Limited quantities of lanosterol, dihydrolanosterol and agnosterol have since become available and their effect on serum and liver cholesterol levels of the cholesterol-fed rat were studied. The absorbability of "isocholesterol" and the three available constituents has also been studied.

[1] This investigation was supported by research grants from the National Heart Institute, U. S. Public Health Service (H-1946), and from Eli Lilly and Company.

METHODS

Six experimental diets were utilized: *basic diet* — rabbit pellets [2] plus 5% of cottonseed oil; *high cholesterol* — basic diet plus 1% of cholesterol; *cholesterol- "isocholesterol," cholesterol-lanosterol, cholesterol-dihydrolanosterol* and *cholesterol-agnosterol,* each consisting of basic diet plus 1% of

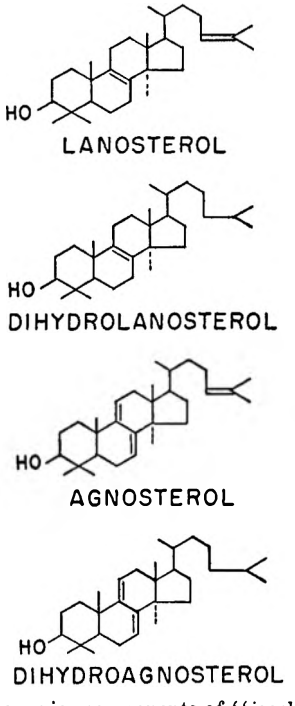


Fig. 1 The major components of "isocholesterol."

cholesterol and 2% of the appropriate wool-fat sterol. The rabbit pellets were impregnated with the wool-fat sterols dissolved in chloroform and the chloroform evaporated prior to the addition of the cholesterol in warm cottonseed oil.

Male white rats,[3] of approximately 300 gm, were employed. Groups of 6 animals of similar mean weight were fed each

[2] Purina.
[3] Holtzman.

of the experimental diets ad libitum for a period of two weeks, at which time they were anesthetized with intraperitoneal amobarbital sodium, blood obtained by cardiotomy and the liver removed. The livers were digested overnight in 20% potassium hydroxide in alcohol, and cholesterol in liver and serum determined by the method of Abell and associates ('52).

The optical density of the final Liebermann-Burchard color reaction was read in two Coleman Junior spectrophotometers. At 30 minutes the sample was read at 620 mμ, the approximate maximum absorption of the color produced by cholesterol, and immediately thereafter it was read at 460 mμ, the approximate maximum absorption of the color produced by the 30-carbon wool-fat sterols. The effect on the ratio of the optical density at 460 to that at 620 mμ of the *in vitro* addition to cholesterol of 1, 2 and 5% of each of the wool fat sterols was also determined.

RESULTS

The mean liver cholesterol concentrations of the 6 groups of rats are given in table 1. The relative differences are unchanged if the cholesterol content is related to body weight rather than liver weight. The addition of 1% of cholesterol to the basic diet for two weeks resulted in an approximately 4-fold increase in liver cholesterol. The further addition of

TABLE 1

Effects of wool fat sterols on the serum and liver cholesterol of the cholesterol-fed rat

DIET	WEIGHT GAIN	SERUM CHOLESTEROL	LIVER CHOLESTEROL
	%	*mg/100 ml*	*mg/100 gm*
Basic	13	89 ± 7[1]	335 ± 35
High cholesterol	16	92 ± 16	1333 ± 520
Cholesterol-"isocholesterol"	15	85 ± 9	990 ± 358
Cholesterol-lanosterol	16	94 ± 8	1190 ± 273
Cholesterol-dihydrolanosterol	15	84 ± 9	609 ± 245
Cholesterol-agnosterol	14	80 ± 7	840 ± 175

[1] Values given are the mean and standard deviation (estimated, using n − 1) for groups of 6 animals.

2% of "isocholesterol" resulted in a slightly lower mean cholesterol concentration, but the difference is not statistically significant ($0.2 < P < 0.3$). As previously reported, 5% of added "isocholesterol" does significantly inhibit deposition of cholesterol in the liver, resulting in a mean value of 342 ± 32 mg/100 gm (Best and Duncan, '57).

Of the three components of "isocholesterol" available for study only the dihydrolanosterol significantly inhibited cholesterol accumulation ($P < 0.01$). Differences in the mean serum cholesterol levels of the 6 groups of rats were small and not statistically significant.

TABLE 2

Comparison of the Liebermann-Burchard reaction given by the wool-fat sterols with that given by cholesterol

STEROL	O.D. 620	O.D. 460	RATIO $\frac{\text{O.D. 460}}{\text{O.D. 620}}$
Cholesterol	0.111 [1]	0.061	0.550
"Isocholesterol"	0.032	0.722	22.6
Lanosterol	0.038	0.902	23.7
Dihydrolanosterol	0.028	0.968	34.6
Agnosterol	0.026	0.889	34.1

[1] Values tabulated are the mean of 4 determinations, and are expressed as optical density per 0.1 mg of sterol. Measurements were made in 19 × 105 mm round cuvettes.

Although Schonheimer reported that he was unable to detect any absorption of "isocholesterol," it was decided to restudy this point (Schonheimer et al., '30). Lanosterol, dihydrolanosterol, agnosterol and "isocholesterol" resemble cholesterol in that they result in color development when added to a mixture of acetic anhydride, acetic and sulfuric acids, a modified Liebermann-Burchard reaction. Cholesterol gives a blue-green color with maximum absorption at approximately 620 mμ while the wool-fat sterols give a yellow-green color with maximum absorption at approximately 460 (table 2). The effects on the Liebermann-Burchard reaction of the *in vitro* addition to cholesterol of various proportions of the wool-fat sterols is summarized in table 3. It will be noted that as little as 1%

of wool-fat sterol significantly increases the ratio of the optical density at 460 mμ to that at 620.

In table 4 are the results of the attempt to detect any absorbed wool-fat sterol in the sera or livers of the rats to which they were fed. Tabulated are the mean ratios of the optical density at 460 to that at 620 mμ of the Liebermann-Burchard reaction given by the non-saponifiable petroleum ether extractable material obtained from the sera and livers of the various groups of animals. In the rats fed the *cholesterol-"isocholesterol"* and *cholesterol-agnosterol* diets both serum and liver gave a significantly increased optical density at 460

TABLE 3

Effect on the Liebermann-Burchard reaction of cholesterol of the addition of small amounts of wool-fat sterol

	RATIO $\frac{\text{O.D. 460}}{\text{O.D. 620}}$ of cholesterol plus			
	0%	1%	2%	5%
"Isocholesterol"	0.550 [1]	0.602	0.670	0.861
Lanosterol	0.550	0.626	0.697	0.954
Dihydrolanosterol	0.550	0.643	0.727	1.000
Agnosterol	0.550	0.642	0.722	0.954

[1] Values tabulated are the mean of 4 determinations. A constant amount of cholesterol, 0.3 mg, was employed. To this was added 0, 0.003, 0.006 and 0.015 mg of each of the wool-fat sterols. Color was developed by the Abell modification of the Liebermann-Burchard reaction and read in 19 × 105 mm round cuvettes.

TABLE 4

Effect of feeding wool-fat sterols on the Liebermann-Burchard reaction given by rat serum and liver

DIET	RATIO $\frac{\text{O.D. 460}}{\text{O.D. 620}}$	
	Serum	Liver
High cholesterol	0.552 ± 0.029 [1]	0.556 ± 0.035
Cholesterol-"isocholesterol"	0.636 ± 0.039	0.637 ± 0.043
Cholesterol-lanosterol	0.567 ± 0.028	0.566 ± 0.008
Cholesterol-dihydrolanosterol	0.562 ± 0.023	0.559 ± 0.027
Cholesterol-agnosterol	0.685 ± 0.023	0.670 ± 0.028

[1] Values given are the mean and standard deviation (estimated using n − 1) for groups of 6 animals.

($P < 0.01$). The ratio is that obtained by the *in vitro* addition of 1% to 2% of agnosterol to cholesterol.

DISCUSSION

"Isocholesterol," a mixture of 30-carbon sterols derived from wool fat, has been reported previously to inhibit the accumulation of cholesterol in the liver of the cholesterol-fed rat when added to the diet in 5% concentration. In this study it was added in only 2% concentration, and although the mean liver cholesterol was somewhat lower than that of the cholesterol-fed controls the difference was not significant. It would seem that "isocholesterol" is a much less effective inhibitor of cholesterol absorption than the plant sterol, β-sitosterol, which at the 2% level almost completely prevents the increase in liver cholesterol which otherwise results from 1% cholesterol feeding (mean liver cholesterol 303 ± 42 as compared to 1283 ± 281 mg/100 gm in the cholesterol-fed control group).[4]

Of the three constituents of "isocholesterol" available for study, only dihydrolanosterol at a 2% concentration significantly inhibited cholesterol accumulation in the liver. That lanosterol and agnosterol, like "isocholesterol," had no significant effect at the 2% concentration is in accord with the report that they are the major constituents of "isocholesterol." Limited supplies of lanosterol, dihydrolanosterol and agnosterol precluded their administration at higher concentrations.

Although the constituents of "isocholesterol" are not precipitated by digitonin, the determination of cholesterol by the method of Abell and associates is not dependent upon the formation of an insoluble digitonide, and any wool-fat sterol present would contribute to the final Liebermann-Burchard color development. No absorption of lanosterol or its dihydro derivative was detected by the technique employed, but the results suggest that agnosterol was absorbed and was present in small amounts in serum and liver.

[4] Unpublished observations.

SUMMARY

Sterols derived from wool fat have been added to a 1% cholesterol diet and their effects on serum and liver sterol content of the rat determined. So studied were the mixture of 30-carbon sterols designated "isocholesterol" and three of its purified components, lanosterol, dihydrolanosterol and agnosterol.

At a 2% concentration in the diet all the wool-fat sterols exerted some inhibitory effect on cholesterol accumulation in the liver. Dihydrolanosterol was most effective in this regard, followed in order by agnosterol, "isocholesterol" and lanosterol.

No absorption of lanosterol or dihydrolanosterol was detected, but the results indicate that agnosterol was absorbed and was present in small amounts in serum and liver of rats to which it was fed.

LITERATURE CITED

ABELL, L. L., B. B. LEVY, B. B. BRODIE AND F. E. KENDALL 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

BEST, M. M., AND C. H. DUNCAN 1957 Inhibitory effect of "isocholesterol" on the absorption of cholesterol. Circulation Research, *5:* 401.

CLAYTON, R. B., AND K. BLOCH 1956 Biological synthesis of lanosterol and agnosterol. J. Biol. Chem., *218:* 305.

SCHONHEIMER, R., H. BEHRING AND R. HUMMEL 1930 Über die spezifitat der resorption von sterinen abhangig von ihrer konstitution. Z. Physiol. Chem., *192:* 117.

EFFECT OF ALTITUDE AND DIET ON HEMATOPOIESIS AND SERUM CHOLESTEROL [1]

IRENE R. PAYNE

Home Economics Division, University of Wyoming, Laramie [2]

(Received for publication September 3, 1957)

This study was instigated for the purpose of obtaining biochemical and physical data on women residing at an altitude of 7200 feet and in a geographical area different from that of California, at sea level, where a similar study was made (Gillum and Morgan, '55; Gillum et al., '55). It was felt that data gathered by the same methods in both studies would provide good comparative information to determine if altitude and geographical conditions have any effect on serum-cholesterol levels.

METHODS AND PROCEDURE

A group of 70 healthy women 60 years of age and older, who had lived in Albany County at least two years, served as subjects. The women kept 7-day diet records and were given physical examinations after that period. On the morning after the termination of the record, the patients were taken to Ivinson Memorial Hospital, where blood samples were

[1] Published with the approval of the Director, Wyoming Agricultural Experiment Station, as Journal Paper no. 96.

[2] This study was a phase of the Western Regional Research Project W-44 on Cholesterol and W-4 on Nutritional Status of the Aging, financed in part by the U. S. Department of Agriculture through funds appropriated under the Research and Marketing Act of 1946. It was done with the cooperation of the Albany County Medical Society and Ivinson Memorial Hospital.

drawn by venipuncture. The technical staff at the hospital analyzed the samples for hemoglobin and made red- and white-cell counts. Hemoglobin was measured by the method of Drabkin ('49).

Sedimentation rates and packed-cell volumes were measured on the fresh, oxalated blood samples in this laboratory. The method of Wintrobe and Landesberg ('35) was used for sedimentation rates. The volume of packed cells was obtained after the sedimentation rate was determined by placing the tube in a centrifuge and spinning it at 3000 r.p.m. for 20 minutes.

The fresh blood samples were centrifuged at the time they were taken; the serum was taken off and frozen until analysis for total serum cholesterol and free cholesterol by the method of Sperry and Webb ('50).

The dietary records were evaluated for composition of cholesterol, protein, animal fat, and vegetable fat, using Okey's ('45) tables for cholesterol and the United States Department of Agriculture Handbook (Watt and Merrill, '50).

The principles of statistical analysis were obtained from Snedecor ('56).

RESULTS AND DISCUSSION

For the sake of easy comparison, we adopted the same methods of presenting these data as did Gillum et al. ('55). The values in table 1 show that the means for the cholesterol values are higher than those obtained in the California study in each age group, except the 80+ group.

A total mean value of 287 mg/100 ml for serum cholesterol was found in the Wyoming study as compared to that of 270 in the California survey. A part of the increase is no doubt due to the exclusion of the 50 to 60 age group which appeared in the California study. Although a larger proportion of the total fat intakes of the Wyoming women is in the form of animal fat, no correlation is found between serum cholesterol and any of the fat data. The fact that 55.8% of the women in the Wyoming study are overweight as compared to 24% of

the California women does not explain the higher cholesterol levels in view of the fact that no correlation can be calculated between these two measurements.

Partial correlation coefficients determined for all other factors with total serum cholesterol and with free serum cholesterol reveal that age and hemoglobin have the two highest coefficients with both forms of cholesterol. Hemoglobin has more influence in the data than any other factor. Apparently age

TABLE 1

Mean serum cholesterol of women over 60 years of age

AGE GROUP	NO. OF CASES	TOTAL SERUM CHOLESTEROL			FREE SERUM CHOLESTEROL			FREE CHOLESTEROL
		Mean	Range	Std. error	Mean	Range	Std. error	
		mg/100 ml			*mg/100 ml*			%
60–64	25	282	160–378	12	83	51–109	3.7	29.4
65–69	20	304	216–403	11	83	56–145	4.5	27.3
70–74	17	282	192–450	17	85	53–128	4.7	30.1
75–79	3	331	278–384	31	85	70–105	10.5	25.7
	8	280			81			29.9
80 +	5	229	174–290	21	78	68– 85	3.0	34.1
Total	70	287	160–450	7	83	51–145	2.2	29.2

is an influential factor also, but consideration of the two together with serum cholesterol complicates the picture. It is felt that the appearance of peak values of the two measurements at different age levels partially masks the true relationship of each to serum cholesterol.

It is known that hemoglobin, hematocrit, and red cell count in residents is increased with increase in altitude (Spector, '56; Hurtado et al., '45). Atland and Highman ('51) proved that the increase in oxygen-carrying capacity of blood is accomplished, at least in part, by an increase in the actual number of red blood cells.

Since the membrane of red blood cells contains measurable amounts of cholesterol and the average cell has a surface of

approximately 136 μ^2, it is readily seen that the cholesterol content is greatly increased with each cell. Degenerate "ghost" cells are common in the blood and are no doubt increased in proportion to the increased number of erythrocytes. The fatty and lipid content of the stroma which composes the "ghost" cells would tend to keep the cells afloat in the plasma, in this way causing the serum to have increased amounts of cholesterol.

The California study showed no increase in hemoglobin with increase in age. An increase in red cell volumes is not necessarily accompanied by increase in hemoglobin, as shown by Olbrich ('47). A different hematopoietic response occurs in adaptation to high altitudes than that which occurs from a dysfunction at sea level or perhaps that which occurs with age. Serum cholesterol derived from "ghost" cells would increase in either case and would be dependent on the number and fragility of the cells.

Effect of age

The serum cholesterol levels which have been reported for women in comparable age groups show some variation. Whereas Swanson et al. ('55) give mean values of 260 mg of cholesterol per 100 ml serum for the 60 to 69 age group and 251 mg for the 70 to 79 age group, Butler et al. ('56) report mean values of 302 mg of cholesterol for the 60 to 69 age group and 239 for the 70+ group, and Gillum et al. ('55) show average mean values of 274.5 mg for the 60 to 69 age group and 265 mg for the 70 to 79 age group.

The values found in the present study are higher at every age level, except the 80+ group, than those in the California study. The trend toward a peak level in the 60 to 69 decade reported by several investigators appears to be present in this study also. The highest serum cholesterol values occur in the 60 to 69 age group and appear to be quite accentuated.

A positive simple correlation of 0.284, significant at the 5% level, was found in this study between the percentage of

free cholesterol and age. The partial correlation coefficient of age with percentage of free cholesterol of 0.333 is significant at the 1% level. The absolute values for free serum cholesterol shown in table 1 indicate that the level of free serum cholesterol remains essentially the same throughout all the age ranges. If the percentage of free cholesterol were positively correlated with age, this would mean that the total serum cholesterol would have to decrease while the free serum cholesterol remained the same. (The partial correlation coefficient of age to total serum cholesterol was —0.295.) Perhaps a study of individuals beyond the age of 70 would prove this correlation to be even stronger, but the figures in table 1 do not uphold the correlation shown by the statistical analysis.

Effect of cholesterol intake

Keys et al. ('56) concluded that serum cholesterol is independent of cholesterol intake, as did Wilkinson et al. ('50). Gillum et al. ('55) found a correlation significant at the 5% level. A positive correlation of 0.292, significant at the 5% level, was found in this study. Demands on dietary cholesterol could conceivably be subject to hematopoietic factors discussed later.

Serum cholesterol levels were divided into three groups so that approximately one-third of the total number of cases occurred in each group: less than 260 mg per 100 ml, 260 to 320 mg, and more than 320 mg. The percentage of the total number which fell into each group was 37.1, 32.9 and 30.0% respectively. The graphical presentation of the distribution of the serum cholesterol among 4 ranges of daily cholesterol intake (fig. 1) does not show a trend throughout all the ranges. However, the ranges above 300 mg of cholesterol intake indicate a trend. The lowest percentage of those with 300 to 449 mg of cholesterol intake have the highest serum cholesterol, and that percentage increases so that at the highest range of cholesterol intake it is the highest percentage. The highest percentage of those with an intake of 300 to 449 mg

of cholesterol daily have the lowest serum cholesterol, and that percentage becomes the lowest in both of the higher ranges of daily cholesterol intake.

Effect of fat intake

Most of the evidence available shows that serum cholesterol levels are positively correlated with fat intakes. The evidence in regard to animal fat versus vegetable fat remains controversial. Brozek, Buzina and Mikic ('57) and Beveridge et al. ('55) found that the source of fat, whether animal or

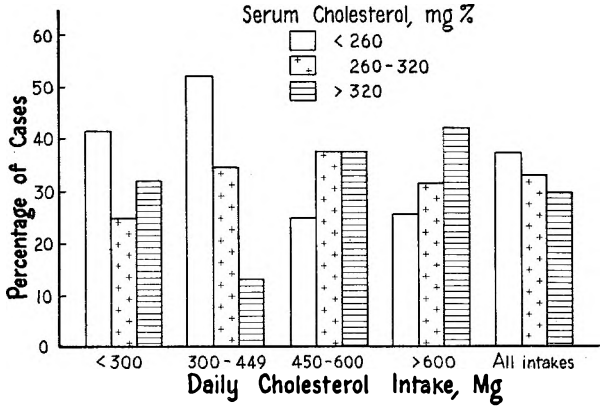


Fig. 1 Distribution of serum cholesterol among 4 ranges of daily cholesterol intake.

vegetable, made a difference in the total serum cholesterol. Subjects with higher animal fat intakes had higher serum cholesterol. Studies by Keys et al. ('57) and Gillum et al. ('55) indicate the source of fat makes no difference in levels of serum cholesterol.

Although the means of total daily fat intake for the California study and the Wyoming study are 72.4 and 72.6 respectively, the quantities of animal and vegetable fat differ. Whereas, the California women ingested an average of 50.6 gm of animal fat and 21.7 gm of vegetable fat, the Wyoming subjects ingested 61.3 and 11.3 gm of the two fats, respectively.

This variation in the amounts of animal fat and vegetable fat is the only dietary variation. However, no significant correlation was found between the intake of animal fat or vegetable fat and serum cholesterol.

Figure 2 shows the distribution of serum cholesterol values among three ranges of daily fat intake: i.e., less than 60 gm, 60 to 90 gm, and more than 90 gm. There is no evidence of a trend for serum cholesterol values to increase with a higher intake of fat. In fact, the percentage of those with the highest serum cholesterol is lower than the values with the lowest serum cholesterol in each range of daily fat intake.

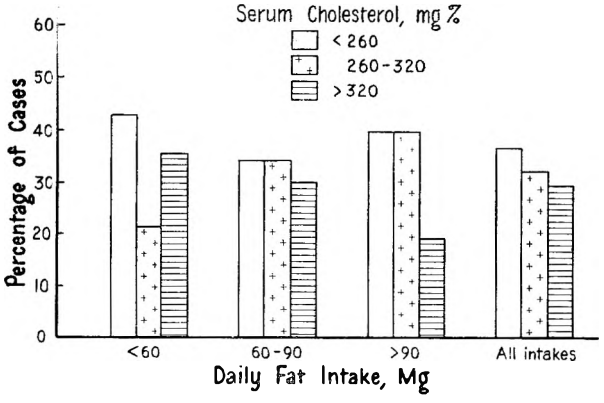


Fig. 2 Distribution of serum cholesterol among three ranges of daily fat intake.

It would appear that the higher level of serum cholesterol in the Wyoming women is not explained by the dietary intake of total fat, animal fat, or vegetable fat.

Effect of protein intake

Gillum et al. ('55) found no significant correlation between serum cholesterol and protein intake. We used (fig. 3) the same ranges of daily protein intake as they did: i.e., less than 50 gm, 50 to 70 gm, and more than 70 gm. Although the percentage of those with the highest serum cholesterol decreases with the increase in protein intake, the variation in the percentage

of those with the lowest serum cholesterol in the three ranges of protein intake eliminates the possibility of a trend. An insignificant correlation exists between serum cholesterol and protein intake.

Body weight

Obesity has been found to be associated with the increase in frequency of angina pectoris, coronary disease, and hypertension in men but not in women (Master et al., '53). Hobson et al. ('53) found a significant correlation between obesity and

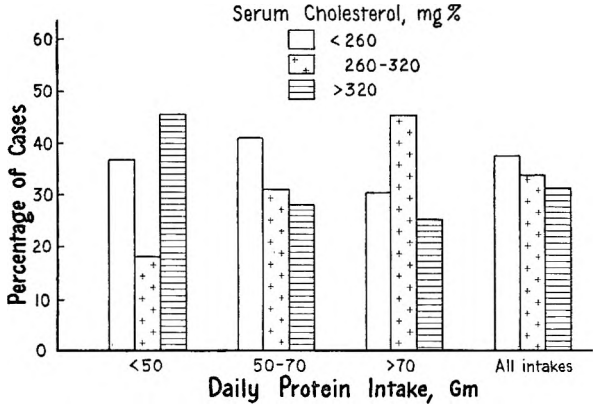


Fig. 3 Distribution of serum cholesterol among three ranges of daily protein intake.

serum cholesterol in women, and Gofman and Jones ('52) found an association between certain lipoprotein classes and obesity, which they state is obscured by measurement of total serum cholesterol.

The body weights and serum cholesterol levels of these subjects were compared. The underweight or overweight values were calculated from ideal weights in the Metropolitan Life Insurance tables (Metropolitan Life Insurance Co., '52).

According to figure 4, no trend is obvious in the relationship of body weight to serum cholesterol. Nor did we find any association of obesity with age. Although the average serum

cholesterol levels of the Wyoming women is higher than that of the California women and there are 55.8% of the Wyoming women overweight as compared to 24% of the California women, the data do not indicate any correlation between obesity and serum cholesterol levels. The obesity classifications of the women for Wyoming and California respectively, based on percentage overweight were as follows: <-20 to -10%, 7.1 and 29%; -10 to $+10\%$, 37.1 and 48%; and $+10$ to $>+20\%$, 55.8 and 24%.

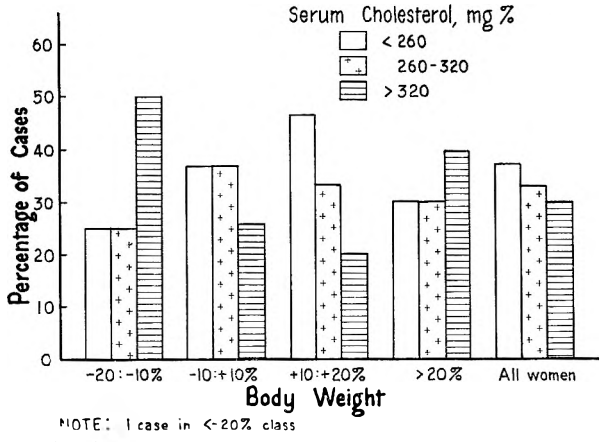


Fig. 4 Distribution of serum cholesterol among various degrees of overweight and underweight.

Packed cell volumes

The mean value for hematocrit for the women in this study is 46.1%. Gillum and Morgan ('55) found a mean value of 44.6%. When an analysis of variance was calculated on the hematocrit values of the two studies, it was found that $F = 10.36$, significant at the 1% level. This is to be expected, since there is evidence that increase in altitude is accompanied by a rise in volume of packed cells in residents at various altitudes.

The positive correlation of hematocrit with hemoglobin, $r = 0.453$, significant at the 1% level, only substantiates the expected close relationship between these two measurements.

Hurtado et al. ('45) found that a definite rise in serum proteins occurred at high altitudes. Since the plasma volume remains the same or decreases slightly at high altitudes (Hurtado et al., '45), the increase in serum proteins and cholesterol would be attributable, in part, to an increase in concentration due to a decreased volume of plasma. Such a change indicates an increase in the specific gravity of the plasma which more nearly approaches the specific gravity of the erythrocytes, thus leading to a decrease in sedimentation rate. This theory gains support from the negative correlation factor of $r = -0.280$ calculated for hematocrit with sedimentation rate.

The mean value of the hematocrits in the Wyoming study (46%) showed an increase of 3% over the mean value in the California study (44.6%), indicating that there is a decrease of 3% in volume of plasma. This does not account for the entire increase of 6% in serum cholesterol of the Wyoming women over California women.

Sedimentation rates

The sedimentation rates are found to be negatively correlated with hematocrit ($r = -0.280$) and with hemoglobin ($r = -0.324$).

Although Gillum and Morgan ('55) found an upward trend in sedimentation rates with age in women, such a trend is not apparent in the present study. The mean value of sedimentation rates for women in Wyoming was found to be 21.4 mm/hr. as compared to that for women in California of 20.8 mm/hr. This represents an increase of 3%, which coincides closely with the increase in the mean value of the hematocrits.

Hemoglobin

The most outstanding results of this study are to be seen in the correlation of hemoglobin with other measurements. Significant correlations of hemoglobin with 5 of the 13 other factors considered in the investigation have been found in this study. The most significant of these was the partial cor-

relation coefficient (0.393) of hemoglobin to total serum cholesterol. Hemoglobin also had a partial correlation coefficient of —0.323 to free serum cholesterol.

In addition, the mean value of hemoglobin for all women in Wyoming is 14.6 gm per 100 ml of blood, compared to 13.4 gm per 100 ml for women in California. This represents a difference of 9%, which is in the same range as the 6% increase of total serum cholesterol in Wyoming women over California women. Table 2 shows a trend for hemoglobin values to in-

TABLE 2

Mean blood values of women over 60 years of age

AGE GROUP	NO. OF CASES	HEMOGLOBIN		HEMATOCRIT		SEDIMENTATION RATES	
		Mean	S.E.[1]	Mean	S.E.	Mean	S.E.
		gm/100 ml		*%*		*mm/hr.*	
60–64	25	14.0	0.24	44	0.8	23	1.7
65–69	20	14.6	0.23	46	1.1	20	2.8
70–74	17	15.5	0.34	49	1.6	20	2.9
75–79	3	14.9	0.80	47	2.8	26	6.3
		14.8		46		24	
80+	5	14.8	0.70	46	1.3	22	2.4
Total	70	14.6	0.48	46	0.6	21	1.2

[1] Standard error.

crease with increase in age up to the 70 to 74 age group and then to decrease. This is in agreement with results reported by Olbrich ('47). The highest average value appears in that age group immediately above the age group in which the highest total serum cholesterol values have been reported by several investigators and also that found in this study. A correlation coefficient of 0.270 between hemoglobin and age was found significant at the 5% level. Gillum and Morgan ('55) did not find much variation in hemoglobin at the different age levels, except a drop in the values for the 80+ age group.

Altitude appears to be an influencing factor on serum cholesterol levels and the over-all blood picture. However, other factors are known to influence hemoglobin. A positive correlation of cholesterol in the diet and hemoglobin has been calculated from our data. The value of $r = 0.233$, just barely significant at 5%, approximates that of $r = 0.292$ found between total serum cholesterol and cholesterol in the diet.

SUMMARY

Data were collected on dietary, physical, and blood factors of 70 healthy women over 60 years of age who had lived in Albany County, Wyoming, for at least two years. The county is 7200 feet above sea level and is in a geographical area different from San Mateo County, California, at sea level. The study paralleled a similar one conducted in San Mateo County, California, in 1949 by Gillum and Morgan ('55) and by Gillum et al., ('55).

It was found that the total mean value for serum cholesterol was higher in the Wyoming women than in the California women. The women in the Wyoming study consumed a larger percentage of total fat in the form of animal fat. More than twice the percentage of women in the Wyoming study were overweight than in the California survey. The hematocrits, sedimentation rates, and hemoglobins of the Wyoming women were greater than those of the California women.

Significant partial correlation coefficients are reported between age and total serum cholesterol, hemoglobin and total serum cholesterol, and hemoglobin and percentage of free serum cholesterol. A significant simple correlation coefficient was found between daily cholesterol intake and total serum cholesterol; hemoglobin was found to be significantly correlated with hematocrit, sedimentation rate, age, daily cholesterol intake, and total serum cholesterol.

The results of the study indicate that altitude does have an effect upon the factors considered. The theory is proposed that increase in hemoglobin, coinciding with increase in red cell count, which occurs at high altitude, is accompanied by

an increase in degenerate "ghost" cells which contribute cholesterol to the serum. It is suggested that the increase in serum cholesterol which occurs with increase in age may be due to hematopoietic changes which occur with age and which resemble those changes found with increase in altitude.

LITERATURE CITED

ATLAND, P. D., AND B. HIGHMAN 1951 Acclimatization response of rats to discontinuous exposures to simulated high altitudes. Am. J. Physiol., *167:* 261.

BEVERIDGE, J. M. R., W. F. CONNELL, G. A. MAYER, J. B. FIRSTBROOK AND M. S. DEWOLFE 1955 The effects of certain vegetable and animal fats on the plasma lipids of humans. J. Nutrition, *56:* 311.

BROZEK, J., R. BUZINA AND F. MIKIC 1957 Population studies on serum cholesterol and dietary fat in Yugoslavia. Am. J. Clin. Nutrition, *5:* 279.

BUTLER, L. C., M. T. CHILDS AND A. J. FORSYTHE 1956 The relation of serum cholesterol to the physical measurements and diet of women. J. Nutrition, *59:* 469.

DRABKIN, D. L. 1949 The standardization of hemoglobin measurement. Am. J. Med., *217:* 710.

GILLUM, H. L., AND A. F. MORGAN 1955 Nutritional status of the aging. I. Hemoglobin levels, packed cell volumes and sedimentation rates of 577 normal men and women over 50 years of age. J. Nutrition, *55:* 265.

GILLUM, H. L., A. F. MORGAN AND D. W. JEROME 1955 Nutritional status of the aging. IV. Serum cholesterol and diet. Ibid., *55:* 449.

GOFMAN, J. W., AND H. B. JONES 1952 Obesity, fat metabolism and cardiovascular disease. Circulation, *5:* 514.

HOBSON, W., A. JORDAN AND C. ROSEMAN 1953 Serum cholesterol levels in elderly people living at home. Lancet, *265:* 961.

HURTADO, A., C. MERINO AND E. DELGADO 1945 Influence of anoxemia on the hemopoietic activity. Arch. Int. Med., *75:* 284.

KEYS, A., J. T. ANDERSON, O. MICKELSEN, S. F. ADELSON AND F. FIDANZA 1956 Diet and serum cholesterol in man: Lack of effect of dietary cholesterol. J. Nutrition, *59:* 39.

KEYS, A., N. KIMURA, A. KUSUKAWA AND M. YOSHITOMI 1957 Serum cholesterol in Japanese coal miners. Am. J. Clin. Nutrition, *5:* 245.

MASTER, A. M., H. L. JAFFE AND K. CHESKY 1953 Relationship of obesity to coronary disease and hypertension. J. Am. Med. Assoc., *153:* 1499.

METROPOLITAN LIFE INSURANCE COMPANY 1942 Ideal weights for women. Statistical Bull., *23:* 6.

OLBRICH, O. 1947 Blood changes in the aged. Edinburgh Med. J., *54:* 306.

OKEY, R. 1945 Cholesterol content of foods. J. Am. Diet. Assoc., *21:* 341.

SNEDECOR, G. W. 1956 Statistical Methods.

SPECTOR, W. S. 1956 Handbook of Biological Data, 273.

SPEERY, W. M., AND M. WEBB 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 107.

SWANSON, P., R. LEVERTON, M. R. GRAM, H. ROBERTS AND I. PESEK 1955 Blood values of women: cholesterol. J. Gerontol., *10:* 41.

WATT, B. K., AND A. L. MERRILL 1950 Agriculture Handbook No. 8 Composition of Foods: Raw, Processed, Prepared. U. S. Department of Agriculture, Washington, D. C.

WILKINSON, C. F., E. BLECHA AND A. REIMER 1950 Is there a relation between diet and blood cholesterol? Arch. Int. Med., *85:* 389.

WINTROBE, M. M., AND J. W. LANDESBERG 1935 A standardized technique for the blood sedimentation test. Am. J. Med. Sci., *189:* 102.

OBSERVATIONS ON PROTEIN DIGESTION IN VIVO [1]

I. RATE OF DISAPPEARANCE OF INGESTED PROTEIN FROM THE GASTROINTESTINAL TRACT

J. D. GUPTA, A. M. DAKROURY AND A. E. HARPER
Department of Biochemistry, University of Wisconsin, Madison

(Received for publication September 16, 1957)

The unsatisfactory state of our knowledge of protein digestion *in vivo* has been emphasized by Geiger ('51), Fisher ('54) and Nasset ('57) who have pointed out that much of our information about this subject has been obtained from *in vitro* experiments. Fisher ('54) has drawn attention to evidence, presented many years ago (Janney, '15), that amino acids from intact protein are absorbed very little slower than those from an acid hydrolyzate of protein and to the large difference between the rate of protein digestion *in vivo* and that *in vitro*. Clearly, many factors which affect protein digestion and the absorption of the products of digestion in the animal body are not taken into account in *in vitro* investigations. It would seem, therefore, that an integrated picture of these processes can be obtained only through experiments on intact animals.

The overall digestibility and the nutritive value of many food and feed proteins have been determined (Mitchell, '48) but there have been relatively few direct studies of the rates

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the National Livestock and Meat Board, Chicago, Illinois, and Campbell Soup Company, Camden, N. J. Some of the crystalline vitamins were kindly provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

of digestion of different proteins or of the factors that affect these rates. Geiger ('51) and Geiger, Courtney and Geiger ('52) studied gastrointestinal digestion and absorption of a few proteins by determining the amount of nitrogen in the stomach and in the small intestine of adult rats at regular intervals after they had consumed about 600 mg of protein. The percentage of ingested nitrogen which could not be recovered from the gastrointestinal tract was taken as absorbed. For each interval of time studied they observed that casein disappeared from the gastrointestinal tract more rapidly than zein but less rapidly than meat and fish proteins. Similar experiments were reported by Nasset, Schwartz and Weiss ('55) who fed dogs albumin, zein or a protein-free diet, and measured the amounts of amino acids in the gastrointestinal contents one and one-half hours later.

The observations reported below represent the initial results of an investigation of protein digestion *in vivo* and of factors that may affect the rate of protein digestion. In these experiments, in contrast to those of Geiger and associates ('51, '52) and Nasset and associates ('54, '57), the measurements of nitrogen disappearance [2] from the gastrointestinal tract were made on animals that had consumed a complete diet. Dreisbach and Nasset ('54), during the course of a study of carbohydrate absorption, did measure the disappearance of casein under similar conditions but obtained results that were difficult to interpret.

EXPERIMENTAL PROCEDURES

Adult male rats weighing between 150 and 200 gm were used throughout. The rats were transferred from a stock cage to individual cages with raised screen bottoms and were offered food for only a single two-hour period each day. This practice was adopted in order to train the rats to eat a definite and fairly large amount of the experimental diet quickly when it was offered on the day of experiment. The entire feeding

[2] Nitrogen disappearance is a gross measurement influenced by both the rate of digestion and the rate of absorption of the end products of digestion.

procedure was as follows: after transfer to the individual cages the rats were fed ad libitum for one or two days to allow them to become accustomed to the purified diet. Thereafter they were offered the diet for only two hours daily at the same time each day. After an initial weight loss the rats gradually gained weight, until, in about two weeks, they had regained their starting weight and were suitable for experiment.

The proteins studied were defatted beef, alcohol-extracted casein and zein. All these proteins were fed at a level of 15% in the diet. A protein-free diet and a diet in which an amino acid mixture served as the source of nitrogen were also studied. The latter contained each of the essential amino acids at levels equivalent to 1.25 times the stated requirements (Rose, '57). The addition of 1% of glycine and 3.5% of glutamic acid brought the nitrogen content to the same level as that in the diets containing the test proteins. Other ingredients of all the diets were salts 4 (Hegsted et al., '41), 4%; corn oil, 5%; vitamin mixture (Harper and Benton, '56), 0.25%; choline chloride, 0.15%; and sucrose, which was the dietary carbohydrate in all cases, to make 100%.

After the rats had been trained they were offered 6 gm of the experimental diet in petri dishes. The dishes were removed after 10 to 15 minutes when it was estimated that about 4 to 5 gm of the diet had been eaten. By putting paper towels under the cages any spilled food could be recovered. The amount of food consumed was determined by difference. Since the amount of food eaten may affect the rate of digestion only rats that consumed 4 to 5 gm were used for experiment. The rats did not eat the amino acid diet readily upon the first offering and had to be trained to eat it for three to 4 days before they could be used for experiment.

Rats were killed at regular intervals after they had eaten. The contents of the stomach and of the small intestine (except for a few centimeters just above the ileocecal junction where there was some accumulation of solid material even in rats that had been starved for 24 hours) were washed out sep-

arately with water, made up to known volume and homogenized in a Waring blendor. Nitrogen was determined on aliquots of the homogenates using a micro-Kjeldahl method. Owing to the pronounced insolubility of zein, the entire washings of the stomach or the small intestine of the rats offered the zein diet were transferred to large Kjeldahl flasks and nitrogen was determined directly on the total contents. Four to 6 rats were used for each interval of time studied.

RESULTS

The results, expressed as percentages of the total nitrogen ingested, are presented in figures 1, 2 and 3. The values for "endogenous" nitrogen, determined as nitrogen recovered after feeding a protein-free diet, have not been subtracted but are shown separately in the figures as percentages of the average nitrogen intake of all the experimental groups.

As can be seen from figure 1, which gives a measure of the rate of stomach emptying, beef proteins, casein, zein and an amino acid mixture passed from the stomach about equally rapidly during the first two hours after ingestion. After two hours zein was emptied less rapidly than the others and at the end of 6 hours was the only nitrogen source present in an amount greater than 20% of that originally ingested. The values for casein and beef at zero time [3] were somewhat higher than those for zein and the amino acid mixture which might indicate either a somewhat greater flow of gastric secretion when these proteins are ingested or a somewhat slower initial rate of emptying. Taken all together the curves show that the rate of stomach emptying in animals subjected to this regimen was quite uniform and rapid up to two hours after eating, by which time about 60% of the ingested nitrogen had been emptied. The rate of passage of nitrogen from the stomach then fell off somewhat after two hours but continued gradually, the beef proteins being emptied a little more quickly than the others.

[3] This refers to values taken immediately after the feeding period.

The values for recovery of nitrogen from the small intestine (fig. 2) show that except in the case of the alcohol-soluble protein, zein, relatively little nitrogen above the "endogenous" value accumulated in the small intestine. The values were somewhat higher in each case up to two hours, the values for beef and the amino acids being a little above those for casein, but even without a correction for "endogenous" nitrogen, they represented less than the equivalent of 10% of the ingested nitrogen.

The value for total recovery of nitrogen from the gastro-intestinal tract (fig. 3) show that almost 100% of the ingested nitrogen could be recovered at zero time. The somewhat

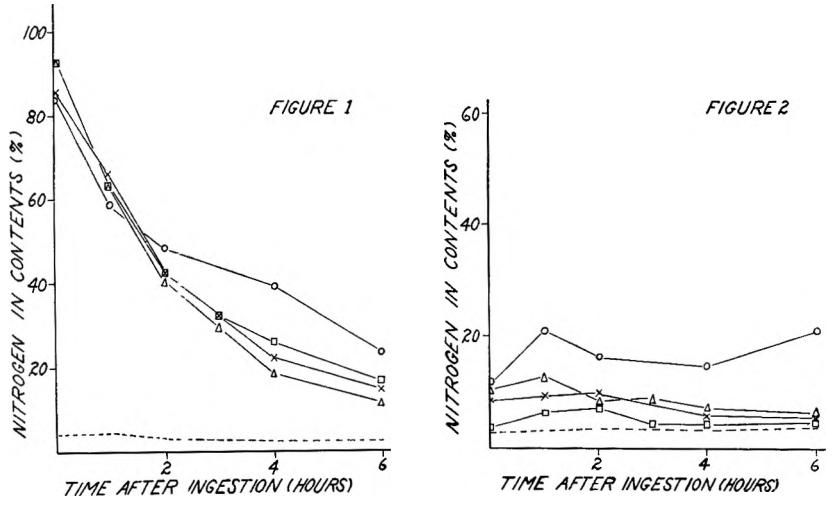


Fig. 1 Percentage of ingested nitrogen recovered from the stomachs of rats at intervals after the ingestion of 4 to 5 gm of a complete diet containing 15% of protein.

× ——— × amino acid mixture ○ ——— ○ zein
△ ——— △ beef proteins ------- protein-free diet
□ ——— □ casein

Fig. 2 Percentage of ingested nitrogen recovered from the small intestine of rats at intervals after the ingestion of 4 to 5 gm of a complete diet containing 15% of protein.

× ——— × amino acid mixture ○ ——— ○ zein
△ ——— △ beef proteins ------- protein-free diet
□ ——— □ casein

higher value for the recovery of the nitrogen of beef proteins again suggests that beef may stimulate the flow of digestive juices. The curves, except for that for zein, follow closely those for stomach emptying; a reflection of the very small accumulation of nitrogen in the small intestine. The curve for zein, which is much above the others, reflects the slower disappearance of this protein from the small intestine.

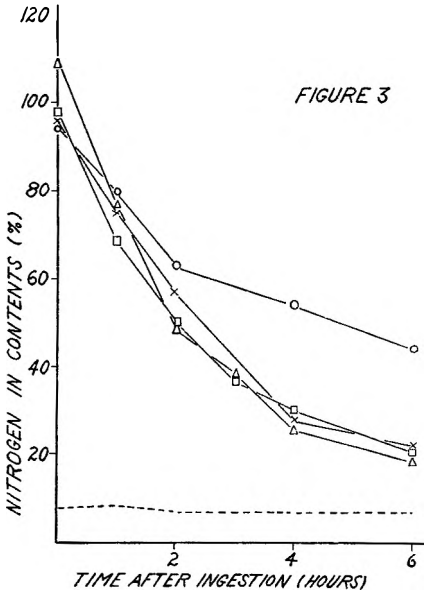


Fig. 3 Percentage of ingested nitrogen recovered from the entire gastrointestinal tract at intervals after the ingestion of 4 to 5 gm of a complete diet containing 15% of protein.

× ———— × amino acid mixture ○ ———— ○ zein
△ ———— △ beef proteins ------- protein-free diet
□ ———— □ casein

DISCUSSION

The curve for stomach-emptying time (fig. 1) is similar to that presented by Lepkovsky and associates ('57) and by Harper and Spivey ('57) who determined the rate of disappearance of total solids from the stomachs of rats fed a single test meal. Lepkovsky et al. ('57) also found very little

accumulation of solids in the small intestine at any time after feeding, as was the case in the present study. In contrast, in experiments with rats fed a single test meal, Dreisbach and Nasset ('54) recovered from the gastrointestinal tract, up to 5 hours after feeding, more protein than was ingested. Nevertheless, their values corrected for the protein found after feeding a protein-free diet, although not as consistent as those of the present study or that of Lepkovsky et al. ('57), do follow the same trend. Dreisbach and Nasset ('54) used untrained rats that had been starved for 48 hours and fed small quantities of diet. Therefore, since most of their values are low, the relative contribution of "endogenous" nitrogen would be much greater and would increase the margin of error.

The results of the present study cannot be compared directly with those of Geiger ('51). In his experiments protein (containing 100 mg of nitrogen) was fed alone or, in some cases, together with from 400 to 800 mg of carbohydrate or fat, in contrast to the 4 to 5 gm of complete diet (containing about the same amount of nitrogen) consumed by the rats used in the present study. Nevertheless, the observations on the relative rates of disappearance of the nitrogen of casein, meat proteins and zein are in good agreement with those of Geiger. He observed that the presence of carbohydrate or fat with the protein slowed the rates both of stomach emptying and of disappearance of nitrogen; therefore, the absolute values obtained in the two studies would not be expected to agree. The major discrepancy is in the values for the distribution of the nitrogen-containing residue between the intestine and the stomach. After feeding zein alone Geiger found that within two hours almost the entire residue (60% of ingested zein) was present in the intestine, whereas, in the present study, when zein was fed as a component of a complete diet 40% of the ingested nitrogen was still present in the stomach 4 hours later. Nasset et al. ('54) also found in their experiments with dogs that much of the zein remained in the stomach after one and one-half hours. They fed a meal containing 80% protein, 10% lard and 10% sucrose.

These observations indicate, as Geiger has pointed out, "that gastric emptying time is dependent on some characteristics of the protein." It is also evident from a comparison of the results of the two investigations that other dietary components affect both stomach emptying and nitrogen disappearance. The results of Dreisbach and Nasset ('54) indicate that the type of dietary carbohydrate affects the rate of disappearance of nitrogen, more complex carbohydrates causing the rate to be slower. A comprehensive study of the effects of total food intake, of the levels of various dietary components and of the types of these dietary components is required in order to ascertain the significance of the effects of these factors on the rate of disappearance of ingested introgen.

Since all of the essential amino acids must be present simultaneously at the site of protein synthesis for efficient utilization of dietary protein (Elman, '39; Geiger, '47 and '48) information about the relative rates of digestion of different proteins and the availability of the individual amino acids from proteins should make it possible to assess more accurately their nutritive value. An appreciable difference between the rates of digestion of two proteins might limit their value as mutual supplements (Mitchell, '48). Also differences in the rates at which the individual amino acids of a single protein become available as, for example, threonine in rice (Pecora and Hundley, '51) might give rise to discrepancies between the calculated and the actual nutritive value of that protein.

The similarity of the results obtained using casein, beef or an amino acid mixture as the source of nitrogen, together with Fisher's ('54) comments about the rapidity of protein digestion *in vivo* points up the need for more detailed information about the absorption of the products of protein digestion. The failure of free amino acids to disappear from the gastrointestinal tract more rapidly than intact casein or beef proteins suggests that the rate of protein digestion becomes a limiting factor in protein utilization only when the protein consumed is relatively insoluble as is the case with zein.

SUMMARY

The rate of disappearance of nitrogen from the stomach and the small intestine of rats fed on diets containing 15% of casein, zein or beef proteins has been determined. A protein-free diet and a diet containing an amino acid mixture which provided the same level of nitrogen as the proteins were also studied.

Nitrogen disappeared from the entire gastrointestinal tract at nearly the same rate when the diet contained casein, beef or an amino acid mixture and much more slowly when it contained zein. Only small quantities of nitrogen were recovered from the digestive tract after feeding a protein-free diet.

Zein was emptied from the stomach more slowly than were casein, a mixture of amino acids or the proteins of beef. Zein also tended to accumulate in the intestine. Although 60% of the nitrogen of casein, beef and the amino acid mixture had passed from the stomach within two hours, there was little accumulation of nitrogen in the small intestine. This emphasizes the great efficiency of the digestive process and demonstrates that amino acids fed as intact protein may pass from the digestive tract into the body as rapidly as those fed in the free form.

LITERATURE CITED

DREISBACH, L. S., AND E. S. NASSET 1954 Absorption of carbohydrate and protein as affected by feeding cornstarch, bana and glucose. J. Nutrition, *53:* 523.

EIMAN, R. 1939 Time factor in retention of nitrogen after intravenous injection of a mixture of amino acids. Proc. Soc. Exp. Biol. Med., *40:* 484.

FISHER, R. B. 1954 Protein Metabolism. London: Methuen and Co., Ltd., Chapter 1.

GEIGER, E. 1947 Experiments with delayed supplementation of incomplete amino acid mixtures. J. Nutrition, *56:* 83.

———— 1948 The role of time factor in feeding supplementary proteins. Ibid., *36:* 813.

———— 1951 Extra caloric function of dietary components in relation to protein utilization. Federation Proc., *10:* 670.

GEIGER, E., G. W. COURTNEY AND L. E. GEIGER 1952 The physiological availability of some essential amino acids present in zein, in the acid and in the enzymatic hydrolyzates of zein. Arch. Biochem. Biophys., *41:* 74.

HARPER, A. E., AND D. A. BENTON 1956 Observations on some nutritional factors that influence the lipotropic activity of methionine. Biochem. J., *62:* 440.

HARPER, A. E., AND H. E. SPIVEY 1957 Effect of type of carbohydrate on protein utilization. Federation Proc., *16:* 387.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

JANNEY, N. W. 1915 A note on the rate of metabolism of proteins and amino acids. J. Biol. Chem., *22:* 191.

LEPKOVSKY, S., R. LYMAN, D. FLEMING, M. NAGUMO AND M. M. DIMICK 1957 Gastrointestinal regulation of water and its effect on food intake and rate of digestion. Am. J. Physiol., *188:* 327.

MITCHELL, H. H. 1948 The biological utilization of proteins and protein requirements. In Proteins and Amino Acids in Nutrition Ed., M. Sayhun, Reinhold Publishing Corp., New York, pp. 46–81.

NASSET, E. S. 1957 Role of the digestive tract in the utilization of protein and amino acids. J. Am. Med. Assoc., *164:* 172.

NASSET, E. S., P. SCHWARTZ AND H. V. WEISS 1955 Digestion of proteins *in vivo.* J. Nutrition, *56:* 83.

PECORA, L. J., AND J. M. HUNDLEY 1951 Nutritional improvement of white polished rice by the addition of lysine and threonine. Ibid., *44:* 101.

ROSE, W. C. 1938 Nutritive significance of the amino acids. Physiol. Rev., *18:* 109.

HUMAN UTILIZATION OF DEHYDROASCORBIC ACID [1,2]

JEAN H. SABRY,[3] KATHERINE H. FISHER AND MARY L. DODDS
College of Home Economics and Agricultural Experiment Station, The Pennsylvania State University, University Park

(Received for publication September 21, 1957)

Since dehydroascorbic acid may represent a significant portion of the total ascorbic acid content of some foods, especially those subjected to storage or processing, knowledge of the extent to which this form of ascorbic acid can be utilized by humans is important for accurate assessment of the vitamin C activity of these foods.

DeRitter, Cohen and Rubin ('51) reported that crystalline dehydro-L-ascorbic acid and dehydro-L-ascorbic acid methanolate were completely available to humans, and Clayton, McSwiney and Prunty ('54) have stated that orally administered dehydroascorbic acid is quantitatively equal to ascorbic acid for humans. The criterion of availability in these studies was urinary excretion of ascorbic acid following consumption of relatively large amounts, 300 to 600 mg, of ascorbic and dehydroascorbic acid. Todhunter, McMillan and Ehmke ('50) found that both blood levels and urinary excretions of ascorbic acid by young women subjects were comparable whether 65 mg per day of ascorbic acid from orange juice or 65 mg per day of dehydroascorbic acid from norite-treated orange juice was consumed.

[1] Paper 2193, Journal Series, Pennsylvania Agricultural Epxeriment Station. Authorized September 6, 1957.

[2] Research Contribution no. 152, College of Home Economics.

[3] Presented from a dissertation submitted to the Graduate School of the Pennsylvania State University in partial fulfillment of the requirement for the Doctor of Philosophy degree.

In a preliminary experiment in this laboratory with 4 young men, it was found that urinary excretions of ascorbic acid were significantly less when 83% of the total daily intake of 110 mg of ascorbic acid was in the dehydro form than when 91% was in the reduced form (Sabry, '57). It was felt worth while to continue the investigation of the utilization of dehydroascorbic acid under more precisely defined conditions of comparison with ascorbic acid. Two experiments were conducted, and are herein reported, in which the utilization by young men of dehydroascorbic acid and ascorbic acid were compared at two levels of intake. The physiological responses used to evaluate utilization were blood plasma levels and urinary excretions of ascorbic acid and total ascorbic acid.

EXPERIMENTAL

The two experiments were similar in plan. Each was conducted with 6 subjects, young men 18 to 28 years of age. Five of the same subjects served on both experiments.

The experiments commenced with a period of adjustment to the level of ascorbic acid intake. The adjustment period in experiment 1 was 13 days and that in experiment 2, 16 days. Following this, the two intake forms of the vitamin were fed alternately as supplements to a low-ascorbic acid diet in 4 8-day test periods. A cross-over design of subjects and supplements was used whereby three subjects were given ascorbic acid during the first and third test periods and dehydroascorbic acid in the second and 4th test periods, while for the other three subjects the sequence was reversed and dehydroascorbic acid given for the first and third test periods and ascorbic acid for the second and 4th test periods. Periods within each experiment followed consecutively, with no interval between.

The diets were planned to contain 25 mg total ascorbic acid per day during the adjustment period and 10 mg per day during test periods (Sabry, '57). The calculated intake of all other nutrients met or surpassed the Recommended Dietary Allowances of the National Research Council ('53). Foods

containing ascorbic acid were analyzed daily for total ascorbic acid by the method of Roe and Oesterling ('44), and for ascorbic acid by the method of Loeffler and Ponting ('42), with modification for high-protein foods. The dehydroascorbic acid content of food was calculated as the difference between total and ascorbic acid concentrations.

Supplements of ascorbic and dehydroascorbic acid were taken with the noon meal. Crystalline reduced ascorbic acid was used throughout and crystalline dehydroascorbic acid was used for experiment 1 and the first two test periods of experiment 2. For the last two test periods of experiment 2 dehydroascorbic acid was prepared by norite oxidation of ascorbic acid. This dehydroascorbic acid solution was prepared fresh daily not more than one and one-half hours before administration to the subjects. The average concentration of dehydroascorbic acid of the preparation was 99.3 mg per 100 ml. The ascorbic acid content was never greater than 0.3 mg per 100 ml.

The intake of total ascorbic acid was planned as 110 mg per day in experiment 1, and as 75 mg per day in experiment 2. The actual intakes of ascorbic, dehydro, and total ascorbic acid from food and supplement in the two experiments are shown in table 1.

Fasting blood samples and 24-hour urine collections were taken the last 4 days of each test period. Blood plasma was analyzed for total ascorbic acid by the method of Bessey ('50) and for ascorbic acid by the micromethod of Mindlin and Butler ('38), adapted for use with the Beckman spectrophotometer. Plasma samples of 0.1 ml were used for both determinations. Urine was preserved with oxalic acid which permitted the determinations of ascorbic acid by the method of Loeffler and Ponting ('42). The method of Roe and Kuether ('43) was used for analysis of total ascorbic acid in urine.

For the analysis of variance of blood and urine values the average values of each subject for each test period were used. Due to minor infections and medication it was inadvisable to collect data from subject 3 for the first two test periods of

TABLE 1

Daily intakes of ascorbic, dehydro and total ascorbic acid from supplement and diet (means and standard deviations)

	SOURCE AND FORM OF INTAKE				
	Supplement		Diet		Both
	Ascorbic acid	Dehydroascorbic acid	Ascorbic acid	Dehydroascorbic acid	Total ascorbic acid
	mg	*mg*	*mg*	*mg*	*mg*
Experiment 1					
Adjustment period	85		11.6 ± 5.5	14.6 ± 6.3	111.2 ± 4.1
Test periods					
Ascorbic acid	100		8.0 ± 1.7	4.2 ± 1.5	111.2 ± 1.3
Dehydroascorbic acid		100	8.0 ± 1.7	4.2 ± 1.5	111.2 ± 1.3
Experiment 2					
Adjustment period	50		7.6 ± 2.5	15.3 ± 6.2	72.9 ± 5.0
Test periods					
Ascorbic acid	65		6.3 ± 1.6	5.1 ± 2.0	76.4 ± 1.8
Dehydroascorbic acid		65	6.3 ± 1.6	5.7 ± 3.0	77.0 ± 2.8

experiment 1 and from subject 1 for the last two test periods of experiment 2. To complete the statistical analysis missing values were calculated for these subjects (Bliss, '51).

RESULTS AND DISCUSSION

Blood plasma levels and urinary excretions of ascorbic and total ascorbic acid in experiment 1 are shown in table 2 and

TABLE 2

Mean blood plasma levels and urinary excretions of ascorbic acid and total ascorbic acid for subjects receiving 110 mg total ascorbic acid per day

SUBJECT	TEST PERIOD	FORM OF SUPPLEMENT	PLASMA LEVEL		URINARY EXCRETION	
			AA [1]	Total AA	AA	Total AA
			mg/100 ml	*mg/100 ml*	*mg/day*	*mg/day*
1	I	AA	0.82	1.10	25.4	28.4
	II	Dehydro AA	0.93	1.14	16.1	24.4
	III	AA	0.82	1.10	36.2	43.7
	IV	Dehydro AA	0.80	1.17	19.0	23.0
2	I	Dehydro AA	0.66	0.95	11.7	12.2
	II	AA	0.80	1.10	16.0	28.0
	III	Dehydro AA	0.73	0.96	6.9	15.1
	IV	AA	0.88	1.20	24.0	37.3
3	I	Dehydro AA	0.58 [2]	0.89 [2]	20.4 [2]	20.2 [2]
	II	AA	0.76 [2]	1.02 [2]	25.9 [2]	34.2 [2]
	III	Dehydro AA	0.70	0.99	21.1	27.2
	IV	AA	0.84	1.17	31.7	37.4
4	I	AA	0.67	1.05	42.1	43.2
	II	Dehydro AA	0.76	0.99	15.6	22.4
	III	AA	0.70	1.03	38.4	46.5
	IV	Dehydro AA	0.74	1.06	15.2	21.6
5	I	AA	0.32	0.48	7.2	8.9
	II	Dehydro AA	0.40	0.58	7.2	11.8
	III	AA	0.48	0.68	8.4	11.8
	IV	Dehydro AA	0.70	0.86	9.4	10.3
6	I	Dehydro AA	0.36	0.73	9.2	11.1
	II	AA	0.67	0.86	12.4	22.4
	III	Dehydro AA	0.70	0.94	13.6	20.4
	IV	AA	0.89	1.19	25.2	34.6
	Mean	AA	0.72	0.99	24.4	31.4
		Dehydro AA	0.67	0.94	13.8	18.3

[1] AA = ascorbic acid; Dehydro AA = dehydroascorbic acid.

[2] Calculated missing values for statistical analysis.

F values from analyses of variance of these data are presented in table 3. It is apparent from the blood values that the 13 days of adjustment were not sufficient for subjects 5 and 6 to come into equilibrium at this level of intake, 110 mg per day. Mean plasma levels of ascorbic and total ascorbic acid were slightly higher for the ascorbic acid than for the dehydro form of intake. The differences are not statistically significant. Mean urinary excretions of ascorbic and total ascorbic acid were higher on ascorbic acid intake than those on dehydroascorbic acid intake. The differences shown are statistically highly significant.

TABLE 3

F values for plasma levels and urinary excretions of ascorbic acid and total ascorbic acid for subjects receiving 110 mg total ascorbic acid per day

SOURCE OF VARIATION	DEGREES OF FREEDOM	F VALUES			
		Plasma		Urine	
		AA[1]	Total AA	AA	Total AA
Subjects	5	6.79[2]	18.03[2]	6.03[2]	7.01[2]
Test periods	3	6.42[2]	9.68[2]	0.94	1.66
Form of ascorbic acid intake	1	1.58	3.42	17.48[2]	26.91[2]
Residual	12				

[1] AA = ascorbic acid.
[2] Significant at 1% level.

The results of experiment 2 are shown in tables 4 and 5. The lower intake of ascorbic acid in this experiment is reflected in the lower plasma levels and markedly lower urinary returns of ascorbic acid. All subjects appear to have been in, or close to, equilibrium at the ascorbic acid intake provided, 75 mg per day. Mean blood plasma levels of ascorbic acid and of total ascorbic acid were higher for the ascorbic acid form of intake than for the dehydro form. Neither of these differences is statistically significant. Mean urinary excretions of ascorbic and total ascorbic acid were again higher on the intake of ascorbic acid than on dehydroascorbic acid intake. The differences, though small, are both highly significant statistically.

From intake and urine data, the utilization of dehydroascorbic acid relative to that of ascorbic acid was calculated on the basis of total ascorbic acid retention during supplementation with each form of the vitamin. Based on the urinary data on the high intake of ascorbic acid in experiment 1,

TABLE 4

Mean blood plasma levels and urinary excretions of ascorbic acid and total ascorbic acid for subjects receiving 75 mg total ascorbic acid per day

SUBJECT	TEST PERIOD	FORM OF SUPPLEMENT	PLASMA LEVEL		URINARY EXCRETION	
			AA [1]	Total AA	AA	Total AA
			mg/100 ml	*mg/100 ml*	*mg/day*	*mg/day*
1	I	Dehydro AA	0.54	0.73	6.5	8.5
	II	AA	0.52	0.72	5.4	8.1
	III	Dehydro AA	0.49 [2]	0.74 [2]	6.8 [2]	10.2 [2]
	IV	AA	0.49 [2]	0.73 [2]	6.2 [2]	10.7 [2]
2	I	AA	0.51	0.77	3.9	9.3
	II	Dehydro AA	0.49	0.70	2.8	8.6
	III	AA	0.56	0.79	6.5	12.1
	IV	Dehydro AA	0.62	0.87	4.1	11.8
3	I	AA	0.54	0.79	7.8	12.1
	II	Dehydro AA	0.60	0.73	3.8	11.0
	III	AA	0.52	0.80	8.3	13.7
	IV	Dehydro AA	0.50	0.68	3.6	11.0
4	I	AA	0.69	0.99	8.2	12.6
	II	Dehydro AA	0.47	0.79	4.6	9.5
	III	AA	0.62	0.92	9.4	14.2
	IV	Dehydro AA	0.37	0.78	3.8	11.6
6	I	Dehydro AA	0.52	0.71	4.4	10.2
	II	AA	0.48	0.67	4.3	10.1
	III	Dehydro AA	0.45	0.72	3.6	11.8
	IV	AA	0.49	0.67	3.6	13.4
7	I	Dehydro AA	0.44	0.65	3.8	8.9
	II	AA	0.53	0.69	3.4	8.2
	III	Dehydro AA	0.39	0.74	4.6	10.1
	IV	AA	0.37	0.72	5.1	11.3
	Mean	AA	0.53	0.77	6.0	11.3
		Dehydro AA	0.49	0.74	4.4	10.3

[1] AA = ascorbic acid; dehydro AA = dehydroascorbic acid.

[2] Calculated missing values for statistical analysis.

the utilization of dehydroascorbic acid was 85% that of ascorbic acid, while on the low intake in experiment 2, the utilization of dehydroascorbic acid was 98% that of ascorbic acid. The results of these experiments would suggest that the lower intake was utilized more economically than the larger one. Penney and Zilva ('43) have suggested this to be true of guinea pigs.

Blood plasma levels of ascorbic acid did not indicate a difference in the extent of utilization of the two intake forms such as was evidenced by the urinary data, especially in experiment 1. The effective ascorbic acid intake, at the levels

TABLE 5

F values for plasma levels and urinary excretions of ascorbic acid and total ascorbic acid for subjects receiving 75 mg total ascorbic acid per day

SOURCE OF VARIATION	DEGREES OF FREEDOM	F VALUES			
		Plasma		Urine	
		AA [1]	Total AA	AA	Total AA
Subjects	5	1.19	5.55 [2]	5.40 [2]	8.20 [2]
Test periods	3	0.72	1.87	8.55 [2]	15.19 [2]
Form of ascorbic acid intake	1	1.25	2.39	17.02 [2]	10.34 [2]
Residual	12				

[1] AA = ascorbic acid.
[2] Significant at 1% level.

of dehydroascorbic acid provided during these studies, may have been sufficiently great to maintain plasma content of the vitamin. Or, the 8-day test periods may not have afforded sufficient time for the blood plasma levels to respond to slight differences in effective ascorbic acid intake. Fisher and Dodds ('54) have shown that the excretion of ascorbic acid adjusts much more quickly to changes in intake levels of the vitamin than does the plasma ascorbic acid content.

Differences did exist, in both plasma and urine, between ascorbic and total ascorbic acid measurements, but the magnitude of these differences was not influenced by the form of intake of ascorbic acid. The response to dehydroascorbic acid was the same whether ascorbic or total ascorbic acid measure-

ments were considered. Todhunter et al. ('50) also observed that the plasma and urinary effects of feeding dehydroascorbic acid were the same for both analyzed forms.

SUMMARY AND CONCLUSIONS

In two experiments, designed to compare the utilization of dehydroascorbic acid with that of ascorbic acid, 12 young men consumed from 92 to 96% of their total daily ascorbic acid intake alternately as ascorbic acid and as dehydroascorbic acid in 4 8-day test periods.

By the criterion of blood plasma content of ascorbic and total ascorbic acid, dehydroascorbic acid was as well utilized as ascorbic acid at two levels of intake, 75 and 110 mg per day.

Urinary excretions of both ascorbic and total ascorbic acid were less on dehydroascorbic acid intake than on ascorbic acid intake. On the basis of total ascorbic acid retention, the utilization of dehydroascorbic acid was 85% that of ascorbic acid on daily intakes of 110 mg and 98% on daily intakes of 75 mg.

Although it was concluded on the basis of urinary returns that dehydroascorbic acid was less well utilized by these subjects than was ascorbic acid, the extent of utilization of dehydroascorbic acid sufficiently approached that of ascorbic acid at both levels of intake for the maintenance of comparable blood plasma levels of ascorbic acid on both forms of intake.

LITERATURE CITED

BESSEY, O. A. 1950 Microchemical methods. VI. Ascorbic acid. Vitamin Methods, ed. by P. Gyorgy. Academic Press, Inc., New York. Vol. I: 303–308.

BLISS, C. I. 1951 1951 Statistical methods in vitamin research. Vitamin Methods, ed. by P. Gyorgy. Academic Press, Inc. New York. Vol. II: 481.

CLAYTON, B. E., R. R. MCSWINEY AND R. T. G. PRUNTY 1954 Metabolism of dehydroascorbic acid and the effect of adrenocorticotrophic hormone and cortisone. Biochem. J. *58:* 542–552

DERITTER, E., N. COHEN AND S. H. RUBIN 1951 Physiological availability of dehydro-L-ascorbic acid and palmitoyl-L-ascorbic acid. Science, *113:* 628–631.

FISHER, K. H., AND M. L. DODDS 1954 Variability in the measure of total ascorbic acid utilization by the human. J. Nutrition, *54:* 389–396.

LOEFFLER, H. J., AND J. D. PONTING 1942 Ascorbic acid. Rapid determination in fresh, frozen, or dehydrated fruits and vegetables. Ind. Eng. Chem., Anal. Ed., *14:* 846–849.

MINDLIN, R. L., AND A. M. BUTLER 1938 The determination of ascorbic acid in plasma; a macromethod and micromethod. J. Biol. Chem., *122:* 673–686.

NATIONAL RESEARCH COUNCIL 1953 Recommended dietary allowances. National Academy of Sciences — National Research Council Publication 302. Washington.

PENNEY, J. R., AND S. S. ZILVA 1943 The chemical behaviour of dehydro-l-ascorbic acid *in vitro* and *in vivo*. Biochem. J., *37:* 403–417.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399–407.

ROE, J. H., AND M. J. OESTERLING 1944 The determination of dehydroascorbic acid and ascorbic acid in plant tissues by the 2, 4-dinitrophenylhydrazine method. Ibid., *152:* 511–517.

SABRY, J. H. 1957 Human utilization of dehydroascorbic acid. The Pennsylvania State University. Ph.D. thesis.

TODHUNTER, E. N., T. MCMILLAN AND D. A. EHMKE 1950 Utilization of dehydroascorbic acid by human subjects. J. Nutrition, *42:* 297–308.

COMPARATIVE MEASUREMENTS OF ASCORBIC ACID AND TOTAL ASCORBIC ACID OF BLOOD PLASMA [1,2]

JEAN H. SABRY [3] AND MARY L. DODDS
College of Home Economics and Agricultural Experiment Station, The Pennsylvania State University, University Park

(Received for publication September 21, 1957)

Micromethods are in general use for the determination of blood plasma or serum ascorbic acid in utilization studies and in surveys. At the present time the Bessey-Lowry micro-adaptation (Bessey, '50) of the Roe and Kuether ('43) method for the determination of total ascorbic acid is widely used. In ascorbic acid studies done prior to the publication of the Bessey-Lowry method (Lowry, Lopez and Bessey, '45), the most commonly used measure was that of plasma or serum ascorbic acid by reduction of 2, 6-dichlorophenolindophenol. Some workers have reported that the two measurements of ascorbic acid give comparable results (Lowry, Lopez and Bessey, '45; Todhunter, McMillan and Ehmke, '50). Others have obtained consistently higher values for the measurement of total ascorbic acid than for ascorbic acid (Davey, Wu and Storvick, '52; Linkswiler, '54; Stewart, Horn and Robson, '53a, b). The differences between the two measurements reported by Stewart et al. ('53a, b) were approximately 0.20 mg per 100 ml of plasma, and those found by Linkswiler ('54) were equal to about 20% of the total ascorbic acid content of blood.

[1] Paper 2194, Journal Series, Pennsylvania Agricultural Experiment Station. Authorized September 6, 1957.

[2] Research Contribution no. 153, College of Home Economics.

[3] Presented from a dissertation submitted to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirement for the Doctor of Philosophy degree.

An established relationship between the two determinations should be of help in the interpretation of earlier work, using ascorbic acid levels in blood, in terms of current methodology. Data have been collected and analyzed to provide information on the variation within each method and difference between these two measurements of plasma ascorbic acid.

EXPERIMENTAL

Blood samples were taken by fingertip puncture, collected in oxalated vials, centrifuged immediately, and determinations made in duplicate or triplicate.

The method of Bessey and Lowry (Bessey, '50) was used for the determination of total ascorbic acid in blood plasma. The micromethod of Mindlin and Butler ('38) adapted for use with the Beckman spectrophotometer was used to determine ascorbic acid. The turbidity correction suggested by Bessey was used ('38). Both methods were scaled for use with plasma samples of 0.1 ml.

RESULTS AND DISCUSSION

Variation within methods. Using data from samples on which two or more determinations of ascorbic or total ascorbic acid were made, the standard deviation of chemical determinations by each method was calculated for ascorbic acid standard solutions and for blood plasma (table 1). The standard deviation of chemical determinations of 0.112 mg per 100 ml for ascorbic acid in plasma by the Mindlin and Butler micromethod is in the lower region of the range of 0.08 to 0.2 mg per 100 ml reported by Beebe ('42) for the micromethod using the photoelectric colorimeter. The standard deviation of determinations by the Bessey-Lowry micromethod for total ascorbic acid of 0.036 mg per 100 ml is intermediate between the standard deviations of 0.03 and 0.52 mg per 100 ml reported in the literature for serum samples of one-tenth the volume of plasma samples used in this study (Bessey, '50; Clayton et al., '54).

When values for the standard deviation of chemical determinations were calculated for plasma samples grouped according to ascorbic acid concentration, there was no indication that the standard deviation of either determination increased with increasing ascorbic acid concentration. The absolute error of either method is thus apparently independent of the ascorbic acid content of the plasma, although the percentage error would be greater for samples of low ascorbic acid concentration.

TABLE 1

Standard deviations of multiple determinations of ascorbic acid and of total ascorbic acid

	NO. OF SAMPLES	NO. OF DETERMINATIONS	MEAN CONTENT OF ASCORBIC ACID	S OF CHEMICAL DETERMINATIONS [1]
			mg/100 ml	*mg/100 ml*
Ascorbic acid				
Ascorbic acid standards	33	149	1.00	0.050
Blood plasma	119	333	0.68	0.112
Total ascorbic acid				
Ascorbic acid standards	33	101	1.00	0.026
Blood plasma	98	241	0.87	0.036

[1] Standard deviation, $S = \frac{(x - \bar{x})^2}{(n - 1)}$

Comparison of methods. Determinations of total and ascorbic acid were made on 61 non-hemolyzed plasma samples from 7 subjects. For comparison of the two measurements two statistical procedures were applied; the t-test for paired variates (Goulden, '39) and analysis of variance with correction for disproportion among subclasses to provide an unbiased estimate of the mean difference (Snedecor, '46). Both procedures are valid. The t-test is based on comparison of the mean values of each sample for ascorbic and total ascorbic acid. The analysis of variance takes into consideration the values of the individual determinations going into each sample mean. The results of these comparisons are reported in table 2.

The mean difference between the total and ascorbic acid measurements of the 61 plasma samples was 0.18 mg per 100 ml and the unbiased estimate of the mean difference was also 0.18 mg per 100 ml (table 2). By either statistical test these differences are highly significant.

The difference found between the measurements of total and ascorbic acid in blood plasma cannot be ascribed to a combination of errors of chemical determination. The error term for the analyses of variance procedure is derived from the differences among the multiple determinations within individual

TABLE 2

Mean of differences between ascorbic and total ascorbic acid measurements of non-hemolyzed plasma and statistical ratios of significance

Number of plasma samples for both determinations	61
Number of individual determinations of ascorbic acid	149
Number of individual determinations of total ascorbic acid	108
Mean content of ascorbic acid	0.75 mg/100 ml
Mean content of total ascorbic acid	0.93 mg/100 ml
Mean difference, total ascorbic acid minus ascorbic acid	0.18 mg/100 ml, $t = 9.47$ [1]
Unbiased estimate of mean difference, total ascorbic acid minus ascorbic acid	0.18 mg/100 ml, $F = 207.33$ [1]

[1] Significant at 1% level.

samples. In each series the significance of the difference between the two measurements is considerably higher when it is determined by analysis of variance than when measured by the t-test.

It was considered possible that at least part of the difference observed between ascorbic and total ascorbic acid contents of blood plasma might be due to oxidation of ascorbic acid in the time between collection of the blood and precipitation of protein. Ascorbic acid of plasma to which metaphosphoric acid has been added to precipitate the protein has been

shown to be stable for 24 hours (Cushman and Butler, '38; Golden and Garfinkel, '42). As a check on the effect of delay in analysis before precipation of protein, portions of 10 plasma samples were precipitated for analysis immediately after collection and centrifugation, other portions were precipitated 60 to 90 minutes later. The ascorbic acid content of the early-precipitated samples was still lower than that of the total ascorbic acid by 0.12 mg per 100 ml. For the same plasma precipitated 60 to 90 minutes later the ascorbic acid content was 0.20 mg per 100 ml less than the total ascorbic acid. This indicates that some oxidation of ascorbic acid to dehydroascorbic acid before analysis of the sample was probably a common occurrence, and one that would be encountered in any situation when more than one or two samples are analyzed at one time. However, this does not appear to be the sole explanation of the differences observed between the two measurements, although the possibility of oxidative changes in blood from the time it leaves the capillaries until after centrifugation was not eliminated.

Differences between ascorbic and total ascorbic acid contents of blood plasma and serum of a similar magnitude to those found in this study were observed by Linkswiler ('54) and by Stewart et al. ('53a, b). They have attributed the difference to the presence of dehydroascorbic acid in blood. However, this interpretation is subject to question. Aside from the possibility of oxidative changes in blood after leaving the capillaries, the two methods of analyses are empirical in nature and based on different chemical reactions. A difference between them may be an artifact due to the influence of different interfering substances.

These data on identical plasma samples scaled to the same size and using the same instrumentation add to the evidence that a difference exists between the two measures, and with a degree of constancy in magnitude. This demonstrated relationship should aid in the interpretation of findings pertaining to ascorbic acid nutriture accumulated by the two methods.

SUMMARY AND CONCLUSIONS

A comparison was made of micro determinations of ascorbic and total ascorbic acid contents of 61 non-hemolyzed samples of plasma. The measurement of total ascorbic acid averaged 0.18 mg per 100 ml higher than that of ascorbic acid in the same plasma. The difference between the two analyzed forms in plasma was statistically highly significant. While the higher values for total than for ascorbic acid are not considered to be conclusive evidence for the presence of dehydroascorbic acid in circulating blood, they do indicate that a correction should be applied if direct comparisons are desired between values for plasma ascorbic acid and levels of the vitamin reported as total ascorbic acid.

LITERATURE CITED

BEEBE, G. W. 1942 Medical evaluation of nutritional status. XIII. The experimental error of determinations of ascorbic acid in plasma by micromethod of Mindlin and Butler. The Milbank Memorial Fund Quarterly, *20:* 389–415.

BESSEY, O. A. 1938 A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem., *126:* 771–784.

———— 1950 Microchemical methods. VI. Ascorbic acid. Vitamin Methods, ed. by P. Gyorgy. Academic Press, Inc., New York. Vol. I: 303–308.

CLAYTON, M. M., M. J. BABCOCK, W. D. FOSTER, S. STREGEVSKY, R. E. TUCKER, A. W. WERTZ AND H. H. WILLIAMS 1954 A referee blood experiment involving the use of microchemical methods. J. Nutrition, *52:* 383–394.

CUSHMAN, M., AND A. M. BUTLER 1938 Use of cyanide in the determination of ascorbic acid. Proc. Soc. Exp. Biol. Med., *39:* 534–539.

DAVEY, B. L., M. L. WU AND C. A. STORVICK 1952 Daily determination of plasma, serum and white cell-platelet ascorbic acid in relation to the excretion of ascorbic and homogentistic acids by adults maintained on a controlled diet. J. Nutrition, *47:* 341–351.

GOLDEN, W. R. C., AND L. GARFINKEL 1942 Medical evaluation of nutritional status. XII. The stability of ascorbic acid in whole blood, plasma, and plasma filtrates. J. Biol. Chem., *144:* 447–452.

GOULDEN, C. H. 1939 Methods of Statistical Analysis. John Wiley and Sons, Inc., New York.

LINKSWILER, H. 1954 Ascorbic acid in blood following ingestion of dehydro and reduced ascorbic acid. Federation Proc., *13:* 464.

LOWRY, O. H., J. A. LOPEZ AND O. A. BESSEY 1945 The determination of ascorbic acid in small amounts of blood serum. J. Biol. Chem., *160:* 609–615.

MINDLIN, R. L. AND A. M. BUTLER 1938 The determination of ascorbic acid in plasma; a macromethod and micromethod. Ibid., *122:* 673–686.

ROE, J. H., AND C. A. KUETHER 1943 The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., *147:* 399–407.

SNEDECOR, G. W. 1946 Statistical Methods. Fourth Edition. The Iowa State College Press, Ames.

STEWART, C. P. D. B. HORN AND J. S. ROBSON 1953a The effect of cortisone and adrenocorticotropic hormone on the dehydroascorbic acid of human plasma. Biochem. J., *53:* 254–261.

———— 1953b Dehydroascorbic acid in human blood plasma. Proc. Nutrition Soc., *12:* 300–305.

TODHUNTER, E. N., T. MCMILLAN AND D. A. EHMKE 1950 Utilization of dehydroascorbic acid by human subjects. J. Nutrition, *42:* 297–308.

STUDIES OF AMINO ACID DIETS FOR THE CHICK

M. R. SPIVEY FOX, GEORGE M. BRIGGS AND LIGIA O. ORTIZ

Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland

(Received for publication September 16, 1957)

There are few reports of experiments with chicks in which amino acid mixtures have been used to replace dietary protein. In general, growth of chicks with this type of purified diet has been poor; however, it has been possible to establish which amino acids are essential for the chick and to estimate a requirement of each of these (Committee on Animal Nutrition, National Research Council, '54).

Hegsted ('44) fed chicks a diet in which nitrogen was supplied by 12 amino acids (19.6% of the diet). He used 10-day-old chicks that had received a commercial starting ration for one week followed by a protein-free diet for three days. The growth rate of chicks fed the amino acid diet was about half that of those receiving the same diet with casein, arginine, glycine and cystine substituted for the amino acid mixture. Almquist and Grau ('44) and Grau and Peterson ('46) fed a stock diet during a preliminary period of 10 to 14 days. They also observed poor growth with their amino acid diets. Luckey et al. ('47) reported that growth with 6 different amino acid diets was not as good as that obtained with purified casein-type diets. In these experiments day-old chicks were either fed a protein-free basal diet for three to 4 days or were placed on the amino acid diet immediately. Benton et al. ('55) fed the essential amino acids at a level one and one-

half times that recommended by Almquist ('52) plus supplemental non-essential amino acids. They found that during a 13-day test period growth of chicks fed the amino acid diet was always appreciably improved by supplemental protein (5 to 10% of the diet).

Fisher and Johnson ('56) showed that normal egg production could be maintained in hens fed an amino acid diet. More recently Fisher and Johnson ('57) reported that 5 chicks in a period of one week grew as well on a modification of their earlier amino acid diet as controls fed a practical diet. Unfortunately, results of longer periods of growth were not reported.

In this study chicks were fed highly purified diets containing the amino acid mixture of Fisher and Johnson ('56) as the sole source of protein nitrogen for 4-week periods. The effect upon growth of altering various constituents in the amino acid diets was also studied.

EXPERIMENTAL

Day-old female New Hampshire chicks from a commercial hatchery were distributed into groups of 4 chicks each for amino acid diets and 6 chicks each for the control diet. In each experiment the chicks had the same initial weight. The chicks were maintained in electrically heated batteries with screen wire floors; diet and tap water were available at all times. The chicks were weighed at weekly intervals and the total diet consumption of each group during the 4-week experiment was recorded. At the end of most experiments the chicks were autopsied and inspected grossly for deviations from normal; the degree of gizzard erosion was noted for each chick.

The control diet C12 contained 20% vitamin-free casein, 8% gelatin and 0.3% methionine; otherwise it was similar to the amino acid diet used. Diet C12 was identical with diet C2 (Fox, Ortiz and Briggs, '55) except that in diet C12 Salts

B[1] (reagent grade) replaced Salts A (C. P. or U.S.P. grades) used in diet C2.

The amino acid diet C42 had the following percentage composition: glucose [2] 42.9, starch 20, amino acid mix 7 [3] 18.9, corn oil 8, hydrogenated vegetable oil [4] 4, Chick Salts B 6, choline chloride 0.2. Vitamins [5] were incorporated at levels in excess of the chick's requirement. Supplements to diets replaced an equivalent weight of glucose. In one experiment the amino acid mixture previously used by Briggs and Fox ('57) was substituted for amino acid mix 7 in diet C42 (diet C42A).

RESULTS

In figure 1 are presented the mean weekly weights of typical groups of chicks receiving diets C12, C42, and C42A. The growth rates with diets C12 and C42 were the same at one and two weeks of age; however, the growth of chicks receiving the amino acid diet C42 fell considerably below that of the diet C12 group between two and 4 weeks. The growth of chicks fed the amino acid mix of Briggs and Fox ('57) in diet C42A was poor from the beginning. This rate of growth was similar

[1] Sixty grams of Chick Salts B supplied the following in grams per kilogram of diet: $CaCO_3$ 15, K_2HPO_4 9, Na_2HPO_4 7.3, $Ca_3(PO_4)_2$ 14, $MgSO_4$ 2.44, NaCl 8.9, $Fe_2(SO_4)_3 \cdot 6H_2O$ 0.28, $MnSO_4 \cdot H_2O$ 0.42, KI 0.04, $ZnSO_4 \cdot 7H_2O$ 0.04, $CuSO_4 \cdot 5H_2O$ 0.02, glucose 2.56. The glucose replaced the water of hydration in $MgSO_4 \cdot 7H_2O$ when the change to the anhydrous salt was made.

[2] Cerelose, from Corn Products Refining Company, Argo, Illinois.

[3] Amino acid mix 7 (Fisher and Johnson, '56) contributed the following percentage of amino acids to the diet: DL-alanine 1.0, L-arginine·HCl 1.3, L-aspartic acid 0.5, L-cystine 0.3, L-glutamic acid 3.5, glycine 1.0, L-histidine·HCl 0.6, DL-isoleucine 2.0, L-leucine 1.4, L-lysine·HCl 1.2, DL-methionine 0.4, DL-phenylalanine 1.0, L-proline 0.5, DL-serine 1.0, DL-threonine 1.0, DL-tryptophan 0.4, L-tyrosine 0.6, DL-valine 1.2. Lysine was purchased from E. I. duPont de Nemours Co., Inc., Wilmington, Delaware; glycine from Dow Chemical Co., Midland, Michigan; alanine from California Foundation for Biochemical Research, Los Angeles, California; all other amino acids from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] Crisco, from Procter and Gamble, Cincinnati, Ohio.

[5] The following vitamins were added in milligrams per kilogram of diet: vitamin A acetate 6, vitamin D_3 0.02, α-tocopherol acetate 50, 2-methyl-1, 4-naphthoquinone 1, thiamine·HCl 16, riboflavin 16, calcium pantothenate 40, nicotinic acid 200, pyridoxine·HCl 16, *d*-biotin 0.6, folacin 6, vitamin B_{12} 0.04.

to that reported earlier with this and other amino acid mixtures.

Data are presented in table 1 from three typical experiments in which the amino acid diet C42, with various modifications, was fed. When the fat level of diet C42 was reduced from 12 to 4%, growth was significantly depressed in 4 weeks. Increasing the amount of amino acid mix 7 to 25% of the diet did not alter growth. The addition of 10% of vitamin-free

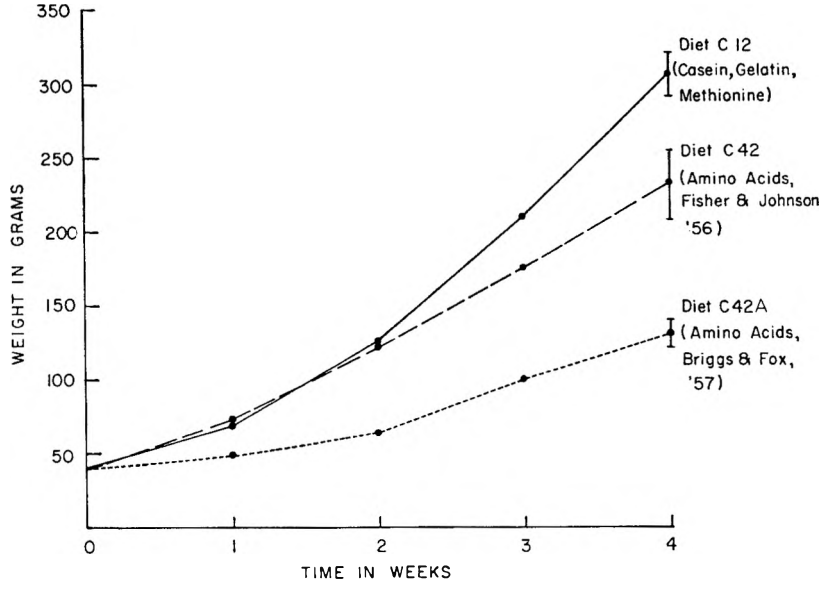


Fig. 1 Growth curves (with 4-week standard errors) of chicks fed the purified protein and amino acid diets as indicated.

casein to diet C42, however, resulted in growth equal to that obtained with the casein-gelatin-methionine diet (C12). The addition of 11% of amino acid mixture 5, which simulated the composition of casein, resulted in a higher mean weight by 4 weeks in series 153. Although this was not statistically significant, the same trend has been seen in other experiments [6] suggesting that the increase was real.

[6] Unpublished data.

TABLE 1

Modification of chick growth by changes in the amino acid diets [1]

DIET	SERIES 142		SERIES 146		SERIES 153		AVERAGE		
	4 wk. wt. ± S. E.		4 wk. wt. ± S. E.		4 wk. wt. ± S. E.		4 wk. wt. ± S. E.		Feed Effic.[2]
	gm		*gm*		*gm*		*gm*		
C42 (amino acid diet)	252	12	231	24	220	18	234	8	0.43
C42 minus 4% corn oil and 4% hydrogenated vegetable oil [3]	180	12	..		..		180	12	0.43
C42 + 6.1% amino acid mix 7 [4]	..		240	36	..		240	36	0.38
C42 + 10% vitamin-free casein	..		315	10	323	29	319	14	0.58
C42 + 11.1% amino acid mix 5 [5]	..		..		258	14	258	14	0.58
C12 (purified casein diet)	309	28	320	14	295	24	308	13	0.58

[1] Four chicks per group receiving amino acid diets; 6 chicks per group receiving casein-gelatin-methionine diet.

[2] Grams of gain per gram of diet consumed.

[3] Crisco.

[4] Amino acid mix of Fisher and Johnson ('56).

[5] Amino acid composition formulated to simulate casein. One hundred and eleven grams of this mixture contained the following amino acids (in grams): DL-alanine 3.12, L-arginine·HCl 3.22, DL-aspartic acid 5.14, L-cystine 0.44, L-glutamic acid 19.30, glycine 1.76, L-histidine·HCl 2.52, DL-isoleucine 10.90, DL-leucine 8.32, L-lysine·HCl 6.90, DL-methionine 3.00, DL-phenylalanine 4.12, L-proline 10.00, DL-serine 4.62, DL-threonine 8.06, L-tryptophan 1.18, L-tyrosine 5.80, and DL-valine 11.60.

The amino acid diet was utilized less efficiently than the intact protein diet. Supplementary casein or amino acids equivalent to casein increased the feed efficiency of diet C42 to equal that of diet C12.

Mortality was low in all experiments. Slight gizzard erosion was seen in all chicks; it was unaffected by the source of dietary nitrogen. One per cent of an antiacid adsorbent, aluminum hydroxide-magnesium trisilicate,[7] and 1% sodium bicarbonate (used by Fisher and Johnson, '56) were fed with diet C42.[8] Since these two constituents affected neither growth nor gizzard erosion, the data have been omitted.

DISCUSSION

Diet C42 used in these studies offers advantages over the diets used by Fisher and Johnson ('56, '57) because diet C42 contained certain more highly purified constituents. Diet C42 is suited to studies on requirements for unidentified factors and trace minerals such as molybdenum and selenium. The Chick Salts B used in the present experiments was composed entirely of reagent grade chemicals, whereas Fisher and Johnson used 2.5% limestone as a partial source of mineral elements. Chick Salts B has supported optimal growth of chicks fed a casein-type diet in 15 experiments.[9] The antiacid adsorbent used by Fisher in this type of diet was unnecessary here, probably as a consequence of the use of this salt mix. Salts B contained more magnesium than was present in the diet of Fisher and Johnson, which had only about half of the level suggested by the NRC ('54).

The failure of chicks fed amino acid diet C42 to grow at an optimum rate during the third and 4th weeks of the experiment may be due to several factors. Amino acid balance is undoubtedly important, as suggested by the better growth with a supplement of amino acid mix 5 (simulating casein) and the lack of response when the amino acid mixture of

[7] Gelusil, from Warner-Chilcott Laboratories, New York, New York.

[8] See footnote 6.

[9] See footnote 6.

diet C42 was increased. These data suggest that an unidentified factor is required for normal growth and development of the chick. The newly hatched chick might conceivably have a limited supply of such an essential nutrient sufficient for the first two weeks of life. The good growth obtained with the casein supplement also suggests: (1) a need for intact protein or peptides, (2) a need for slower release and absorption of amino acids presumably associated with digestion of protein, (3) beneficial changes in physical properties of a diet containing protein. These latter factors would seem to be less important, because they would be expected to affect growth from the very beginning of the experiment.

An amino acid diet offers numerous advantages over a purified protein diet since the amino acid diet may be formulated to exclude all nutrients not known to be required by the chick. At the present time, the amino acid mixture of Fisher and Johnson may be used during a two-week assay to extend our knowledge of amino acid requirements of the chick, and to compare the utilization of D- and L- forms of amino acids. Under the conditions described above, the segment of the experimental period from the second week to the 4th affords an opportunity to assess the value of unidentified factors.

SUMMARY

Day-old female New Hampshire chicks fed a diet containing the amino acid mixture of Fisher and Johnson ('56) as the sole source of protein grew as well up to two weeks of age as chicks receiving a similar complete diet containing casein, gelatin, and methionine. Between two and 4 weeks the growth of chicks fed the amino acid diet fell considerably below that of the chicks fed the purified protein diet. The addition of 10% casein to the amino acid diet resulted in growth equal to that of the controls; part of the activity of casein could be attributed to its amino acid content. When the amino acid diet contained 12% of fat, growth was superior to that obtained when the diet contained 4% of fat.

This highly improved amino acid diet provides an opportunity for study of unrecognized factors and for determining amino acid requirements and interrelationships.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Helen M. Hood and Esther M. Hurley.

LITERATURE CITED

ALMQUIST, H. J. 1952 Amino acid requirements of chickens and turkeys — a review. Poultry Sci., *31:* 966.

ALMQUIST, H. J., AND C. R. GRAU 1944 The amino acid requirements of the chick. J. Nutrition, *28:* 325.

BENTON, D. A., H. E. SPIVEY, A. E. HARPER AND C. A. ELVEHJEM 1955 Factors affecting the growth of chicks on amino acid diets. Arch. Biochem. Biophys., *57:* 262.

BRIGGS, G. M., AND M. R. S. FOX 1957 Thioctic acid in complete and deficient diets for chicks. Poultry Sci., *36:* 657.

COMMITTEE ON ANIMAL NUTRITION, NATIONAL RESEARCH COUNCIL 1954 Nutrient requirements for poultry, Pub. 301.

FISHER, H., AND D. JOHNSON, JR. 1956 The amino acid requirement of the laying hen. I. The development of a free amino acid diet for maintenance of egg production. J. Nutrition, *60:* 261.

———— 1957 An improved free amino acid diet for growing chicks. Poultry Sci., *36:* 444.

FOX, M. R. S., L. O. ORTIZ AND G. M. BRIGGS 1955 Toxicity of ethionine in the young chick. J. Agr. Food. Chem., *3:* 436.

GRAU, C. R., AND D. W. PETERSON 1946 The isoleucine, leucine, and valine requirements of the chick. J. Nutrition, *32:* 181.

HEGSTED, D. M. 1944 Growth in chicks fed amino acids. J. Biol. Chem., *156:* 247.

LUCKEY, T. D., P. R. MOORE, C. A. ELVEHJEM AND E. B. HART 1947 Growth of chicks on purified and synthetic diets containing amino acids. Proc. Soc. Exp. Biol. Med., *64:* 348.

THE INFLUENCE OF CARBOHYDRATE ON THE UTILIZATION OF RATIONS CONTAINING SOYBEAN ALPHA PROTEIN

U. D. REGISTER AND E. W. PETERSON
Department of Biochemistry, College of Medical Evangelists
Loma Linda, California

(Received for publication September 23, 1957)

During the development of vegetable protein rations for producing vitamin B_{12}-deficient rats, it was noted that better growth was obtained when sucrose was replaced by starch in the rations. Earlier work (Lewis et al., '50) with hyperthyroid animals also showed a greater response to vitamin B_{12} with starch rations than with sucrose rations. This effect of starch, however, was attributed primarily to its possible antithyrotoxic activity. A growth-stimulating effect of starch has been reported in animals fed rations deficient in B vitamins (Elvehjem, '48). Harper et al. ('53) and Monson et al. ('54) reported increased growth with 9% casein rations when starch or dextrin was used rather than sucrose. However, when the casein level was raised to 18%, no difference in growth response with different carbohydrates was observed.

Preliminary studies in this laboratory indicated that this growth effect of starch still occurred at a 28.5% level of unheated soybean alpha protein. Melnick et al. ('46) suggested that the decreased growth rate of rats on unheated soybean protein rations, as contrasted to those on heated soybean protein rations, was due to a delayed release of amino acids in the intestine. Monson et al. ('50) observed that rations with dextrin pass more slowly through the intestinal tract than those with sucrose. Geiger ('51) reported that proteins which remain longer in the digestive tract promote better growth. In view of these observations and the con-

trasting results with casein and alpha protein rations, work was undertaken to investigate further these relationships.

EXPERIMENTAL

Male Sprague-Dawley rats weighing from 40 to 60 gm were maintained in individual metal cages with raised screen bottoms and were fed the experimental rations ad libitum. The animals were weighed weekly. The basal ration contained the following in grams: sucrose or corn starch 75.6, alpha protein or casein 14.3, corn oil 5, salts mixture (Hegsted et al., '41) 4, choline chloride 0.1, and vitamin mix 1.0. The vitamin mix provided in milligrams per 100 gm of ration: thiamine chloride 0.3, riboflavin 0.4, pyridoxine hydrochloride 0.3, calcium pantothenate 2.0, nicotinic acid 2.0, folic acid 0.025, biotin 0.01, menadione 0.1, inositol 10.0, and *p*-amino benzoic acid 15. In addition, all rats received two drops of Haliver oil weekly. When indicated, methionine was added at a 0.5% level, and vitamin B_{12} was added to provide 5 μg per 100 gm of ration. Supplements were added, or alterations in the levels of protein were made at the expense of the carbohydrate. The 14.3% of alpha protein or casein provides approximately 12.5% of protein as determined by Kjeldahl analysis ($N \times 6.25$).

Apparent digestibility coefficients were determined using Cr_2O_3 as the indicator substance, which was included in the ration at the 1% level. The analysis for Cr_2O_3 was done by the method of Bolin et al. ('52) and the calculation according the Lucas ('52). Individual analyses were done on each rat in the group. During the 5th week of growth, food consumption records were kept and protein efficiency ratios grams gain per gram of protein eaten were calculated on the rations with the 12.5% protein level.

RESULTS

The rations used with a 25% level of protein and the growth response of the animals fed these rations are recorded in table 1. The animals fed the rations with alpha protein with

TABLE 1

Effect of different carbohydrates on growth response of rats fed rations containing casein or soybean alpha protein

RATION	AVERAGE GAIN PER WEEK (5 WEEK PERIOD)		
	12.5% protein [1]	25% protein	15.5% protein
	gm	*gm*	*gm*
Alpha protein [2] + sucrose	0.6 ± 0.8 (5)	5.0 ± 0.7[3] (5)[4]	
Alpha protein + sucrose + 0.5% methionine	11.5 ± 1.2 (10)	18.0 ± 1.7 (5)	
Alpha protein + sucrose + 0.5% methionine + vitamin B_{12}	15.1 ± 1.5 (10)	30.9 ± 1.1 (10)	14.0 ± 0.9 (10)
Alpha protein + sucrose + 0.5% methionine + vitamin B_{12} (double level of vitamins)	12.0 ± 1.0 (5)		
Alpha protein + starch	1.6 ± 0.5 (5)	8.6 ± 0.7 (10)	
Alpha protein + starch + 0.5% methionine	19.5 ± 1.3 (10)	28.3 ± 1.1 (15)	
Alpha protein + starch + 0.5% methionine + vitamin B_{12}	27.0 ± 1.4 (5)	39.1 ± 1.2 (15)	31.3 ± 0.9 (10)
Alpha protein + starch + sucrose [5] + 0.5% methionine		28.8 ± 2.4 (5)	
Alpha protein + starch + sucrose + 0.5% methionine + vitamin B_{12}		37.6 ± 1.8 (5)	
Casein + sucrose	27.6 ± 2.0 (5)	37.4 ± 1.4 (5)	33.6 ± 0.5 (5)
Casein + starch	26.9 ± 2.0 (5)	36.0 ± 2.1 (5)	31.4 ± 0.9 (5)

[1] Casein or alpha protein at a level of 14.3% provided approximately 12.5% protein.
[2] Soybean alpha protein, Glidden Co., Chicago, Illinois.
[3] Mean ± standard error of the mean.
[4] Numbers within parentheses represent the numbers of animals per group.
[5] Equal parts starch and sucrose to make up carbohydrate.

out methionine supplementation showed very little growth with either sucrose or starch as the carbohydrate. This would be the expected result as methionine is the limiting factor in unheated soy protein.

Vitamin B_{12} produced an increased growth response in the animals receiving the alpha protein rations. In rations using sucrose or starch the differences were highly significant ($P < 0.001$); with the sucrose-starch mixture the difference was less significant ($P < 0.05$).

With the rations supplemented with methionine and methionine plus vitamin B_{12}, the difference in growth between animals fed sucrose and starch was highly significant ($P < 0.001$). Rations containing both sucrose and starch were as effective in supporting growth as those with starch alone. In rations containing casein at this level, no difference in growth was noted between the groups of animals fed starch and those fed sucrose. In this laboratory, no significant difference in growth has been observed between sucrose and starch rations containing heated soybean protein at a 25% level. In one experiment when lactose was used as the source of carbohydrate, the unsupplemented animals lost weight and the vitamin B_{12}-supplemented animals gained 4 to 6 gm per week.

The growth of the animals fed the rations that contained 14.3% alpha protein or casein (12.5% protein) is also shown in table 1. At this level of protein, the results of supplementation with methionine and vitamin B_{12} were similar to those found with the higher level of protein. The animals fed the alpha protein rations containing a double level of vitamins showed no increase in growth over those fed the corresponding ration with usual level of vitamins. Increased growth was observed with animals fed alpha protein rations in which the sucrose had been replaced by starch ($P < 0.001$). At this level of casein, no increase in growth was obtained with animals fed rations containing starch over the rate of growth obtained with sucrose rations. No difference in growth was observed

in animals fed starch rations containing casein or alpha protein supplemented with methionine and vitamin B_{12}.

In table 1 are recorded the growth response of animals fed rations containing 18% casein or alpha protein (15.5% protein). At this level of protein, animals fed diets with alpha protein showed better growth with starch than with sucrose ($P < 0.001$). Rations containing casein at this level supported growth equally well with sucrose or starch.

From table 2, it can be seen that no significant difference was found in apparent digestibility of the rations by altering the carbohydrate ($P < 0.5$).

TABLE 2

Apparent digestibility coefficients of sucrose and starch rations

RATION	DIGESTIBILITY COEFFICIENT [1]
18% alpha protein + sucrose + 0.5% methionine + vitamin B_{12}	90.4 ± 0.8
18% alpha protein + starch + 0.5% methionine + vitamin B_{12}	90.9 ± 0.5
18% casein + sucrose	92.8 ± 0.3
18% casein + starch	92.3 ± 0.5
14.3% alpha protein + sucrose + 0.5% methionine + vitamin B_{12}	90.6 ± 0.3
14.3% alpha protein + starch + 0.5% methionine + vitamin B_{12}	89.7 ± 0.6

[1] Mean ± standard error of the mean.

The protein efficiency ratios (PER) or grams gain per gram of protein eaten, as calculated for the 12.5% protein level during the 5th week, were 1.35 for the alpha protein ration containing sucrose and methionine, 1.98 for the alpha protein ration containing starch and methionine, 1.93 for the casein and sucrose, and 1.96 for the casein and starch rations. Thus the PER of the starch-alpha protein ration was approximately 50% greater than that of the sucrose-alpha protein ration.

DISCUSSION

In the rations used in these experiments with alpha protein, starch as the source of carbohydrate supported better growth and food utilization than did sucrose. This growth effect was still seen when half the starch was replaced by sucrose.

Harper and Katayama ('53) observed similar effects with a 9% casein ration or a ration containing an amino acid mixture equivalent to 9% casein, but these effects were not evident with higher levels of casein. Similar results were obtained in this laboratory with the levels of casein used; however, the growth-stimulating effect of starch was still found when the level of alpha protein was increased to 18 or 28.5%. In one experiment, the decreased ability of animals to utilize unheated alpha protein-sucrose rations was removed by using heated soybean protein.

The structure of the protein apparently was not a factor in the carbohydrate effect observed by Harper et al. ('53) with the 9% casein rations, since an amino acid mixture equivalent to 9% casein produced the same growth differences. The fact that the increased growth with starch was still seen with higher levels of unheated alpha protein, and not with casein or heated soybean protein, would suggest an additional mechanism for the unheated alpha protein rations.

Munro ('51) reported that feeding carbohydrate and protein at different times produced a greater loss of nitrogen in the urine than if they had been fed together. The decreased utilization of nitrogen of the sucrose-alpha protein ration may have been partially due to a time difference, since sucrose is hydrolyzed and absorbed rapidly as compared with unheated alpha protein.

Coefficients of digestibility do not account for these differences, because the digestibility was similar with the sucrose and starch rations. Melnick et al. ('46) found no difference in digestibility between heated and unheated soy protein. They suggested that the amino acids were released too late *in vivo* from unheated soybean protein to be available for growth. Monson et al. ('50) reported that rations containing sucrose pass through the intestinal tract more rapidly than rations utilizing starch. Geiger ('51) stated that proteins which remain in the intestinal tract longer promote better growth and amino acid utilization. The alpha protein used in our experiments was unheated. The increased time required

for passage of the starch ration through the intestinal tract would tend to decrease the effect of delayed release of essential amino acids. Therefore, an adequate amount of amino acids would be available at a level in the digestive tract for efficient absorption and synthesis into protein. With the sucrose rations, some amino acids might not be released until they reached the lower intestine where absorption apparently occurs, but utilization for protein synthesis is less efficient, either because of a delay in availability or because the amino acids have been converted to some other nitrogenous compounds.

Many reports (Elvehjem, '48) have shown that the intestinal flora in animals fed starch rations differs from that in those fed rations containing sucrose. The type of flora produced with starch rations favors an increased synthesis of nutrients such as vitamins and possibly amino acids; however, the decreased growth on sucrose rations in our experiments was not due to a lack of known vitamins, since a double level of vitamins had no effect on growth when added to the sucrose diet.

Antibiotics which have a growth-stimulating effect also modify the intestinal flora. The flora resulting from a ration containing starch may stimulate growth by one of the mechanisms for growth stimulation by antibiotics given by Stokstad ('55). More work must be done before a definite mechanism can be established by which increased growth is obtained from starch rations.

SUMMARY

The influence of carbohydrate upon the growth of rats fed casein and soybean alpha protein rations at levels from 12.5 to 25% has been studied.

At the protein levels studied, alpha protein rations with starch supported better growth of rats than those with sucrose. This effect was not seen with casein or heated soybean protein. No differences in coefficient of digestibility were observed; however, increased protein efficiency ratios were

noted with unheated alpha protein when sucrose was replaced by starch.

Differences in rate of release of amino acids from protein, time of passage through the intestinal tract, and altered intestinal flora have been discussed as possible mechanisms.

ACKNOWLEDGMENT

The author is indebted to Merck and Company, Inc., Rahway, New Jersey, for crystalline vitamin B_{12} and to Parke, Davis and Company, Detroit, Michigan, for Haliver Oil with Viosterol.

This research was supported in part by the Williams-Waterman Fund of the Research Corporation of New York.

LITERATURE CITED

BOLIN, D. W., R. P. KING AND E. W. KLOSTERMAN 1952 A simplified method for the determination of chromic oxide (Cr_2O_3) when used as an index substance. Science, *116:* 634.

ELVEHJEM, C. A. 1948 Nutritional significance of the intestinal flora. Federation Proc., *7:* 410.

GEIGER, E. 1951 Extra caloric function of dietary components in relation to protein utilization. Ibid., *10:* 670.

HARPER, A. E., AND M. C. KATAYAMA 1953 The influence of various carbohydrates on the utilization of low protein rations by the white rat. J. Nutrition, *49:* 261.

HARPER, A. E., W. S. MONSON, D. A. ARATA, D. A. BENTON AND C. A. ELVEHJEM 1953 Influence of various carbohydrates on the utilization of low protein rations by the white rat. Ibid., *51:* 523.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

LEWIS, U. J., D. V. TAPPAN, U. D. REGISTER AND C. A. ELVEHJEM 1950 Effect of carbohydrate on growth responses to vitamin B_{12} in the hyperthyroid rat. Proc. Soc. Exp. Biol. Med., *74:* 568.

LUCAS, H. L. 1952 Algebraic relationships between digestion coefficients determined by the conventional method and by indicator methods. Science, *116:* 301.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymatic digestion of protein as a factor in nutrition. Science, *103:* 326.

MONSON, W. J., L. S. DIETRICH AND C. A. ELVEHJEM 1950 Studies on the effect of different carbohydrates on chick growth. Proc. Soc. Exp. Biol. Med., *75:* 256.

MONSON, W. J., A. E. HARPER, D. A. BENTON AND C. A. ELVEHJEM 1954 The effect of level of dietary protein on the growth of chicks fed purified diets containing sucrose or dextrin. J. Nutrition, *53:* 563.

MUNRO, H. N. 1951 Carbohydrate and fat as factors in protein utilization and metabolism. Physiol. Rev., *31:* 449.

STOKSTAD, E. L. R. 1955 Antibiotics in animal nutrition. Food Tech., *9:* 405.

THE DIETARY NITROGEN REQUIREMENTS OF THE CAT[1]

S. A. MILLER AND J. B. ALLISON
Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey

(Received for publication September 30, 1957)

Recent studies involving the nutrition of the cat have emphasized the relatively high requirements of this animal for protein and certain vitamins (daSilva et al., '50, '52, '55; Krehl, Cowgill and Whedon, '55; Dickenson and Scott, '56, a, b; Allison et al., '56). These studies demonstrate the need for continued development of purified and natural diets for the more exact determination of nitrogen and other requirements of the cat. The following experiments were planned, therefore, to develop further an experimental diet to establish more accurately the nitrogen requirements for maintenance and growth and to determine the caloric intake associated with these dietary needs. These experiments were considered as the first step in the determination of the amino acid requirements and metabolism in the cat.

EXPERIMENTAL

The diet used in these experiments was developed over a period of several years, the amounts of protein, carbohydrate, fat and vitamins being varied while feeding kittens or adult cats (Allison et al., '56). This diet, recorded in table 1, should not be considered to represent absolute requirements for each constituent but it does have sufficient quantities of each to support excellent growth of kittens and appears adequate for maintenance of the adult. The proportion of nitrogen to

[1] The studies were supported by a grant-in-aid from the Mark L. Morris Animal Foundation.

calories seemed to be satisfactory for the growth of a muscular body without excess fat. Exact studies need to be made, however, to determine the effect of the diet on body composition.

The diet was prepared as follows: one-half of the agar was dissolved in the water in which all of the lard was melted. The remainder of the agar was added and the mixture heated further. Water was added to replace the amount lost through vaporization. The hot agar solution and fat emulsion was

TABLE 1

Composition of the diet for cats

INGREDIENTS	AMOUNT	VITAMINS	AMOUNT
	gm		*mg/1000 gm dry diet*
Protein	250	Thiamine	20
Sucrose	10	Riboflavin	20
Dextrose	202	Pyridoxine	20
Dextrin	140	Menadione	20
Lard	231	Niacin	80
Cod liver oil [1]	20	Ca Pantothenate	80
Corn oil	50	Inositol	200
Liver powder	30	Folic acid	1
Agar	66	PABA	80
Salt mixture [2]	40	Biotin	1
Vitamin E in hydrogenated vegetable fat [3]	10	Choline	2000
Distilled water	750		

[1] Cod liver oil contains 40,000 units of Vitamin A and 4,000 units of Vitamin D.
[2] Wesson ('32).
[3] Vegetable fat contains 40 units of Vitamin E.

poured into a vessel containing the dry ingredients, the whole being thoroughly mixed by a mechanical stirrer. After a slight cooling, the corn oil and hydrogenated shortening containing the alpha tocopherol were added and mixed. The preparation was cooled further and then the cod liver oil and finally, all of the vitamins were added. Mixing was continued to blend the constituents. The cooled diet was transferred to pans to gel. It was kept under refrigeration and was prepared twice weekly. When the protein content of the diet was altered, the carbohydrate components were changed

proportionately so that all of the diets were isocaloric, containing about 2.9 Cal./gm.

The diet was cut into one-quarter inch cubes for feeding. Cats develop definite food habits so that if the cubes were not prepared at approximately the above size or if the consistency of the diet was too soft, the animals reduced their food intake. It is important, therefore, in experiments with cats to standardize as much as possible the preparation of the diet and procedures in feeding.

Each 7-day period was divided into two parts, the first three days being used as an adjustment period. During the subsequent 4 days, urine and feces were collected and food intakes measured. The nitrogen excreted and that present in the diet was determined by the Kjeldahl method.

RESULTS

Although the diet used in these experiments will undoubtedly be improved, it does support good growth of kittens and maintenance of adults. The rate of gain of male kittens was approximately 160 gm/week while the rate for females was 135 gm/week during the fast-growing period. These rates are equal to or greater than those reported for growth in kittens fed other diets (Dickenson and Scott, '56a). The rate of growth and overall appearance of the kittens seemed best when the diet contained from 25 to 35% of casein on a dry basis. Lowering the casein concentration much below 25% decreased the protein intake sufficiently to retard development of the animal.

An estimate of nitrogen needs can be made by measuring the nitrogen balance while feeding an optimum diet. The balances obtained while feeding the diet recorded in table 1 are plotted in the upper part of figure 1. Each point in this figure is an average of data obtained while feeding 4 different groups of littermates with from three to 4 kittens in each group. These data illustrate the amount of nitrogen retained for growth in these kittens fed the purified diet. Although this amount of nitrogen may not be the optimum for growth, it was

associated, as pointed out above, with a good rate of gain and good overall clinical appearance of the kittens. The amount of nitrogen retained for growth was approximately 1.7gm/day/kg of body weight at 5 weeks of age. This retention decreased to approximately 0.5 gm/day/kg of body weight at 25 weeks. Nitrogen equilibrium was reached at about 55

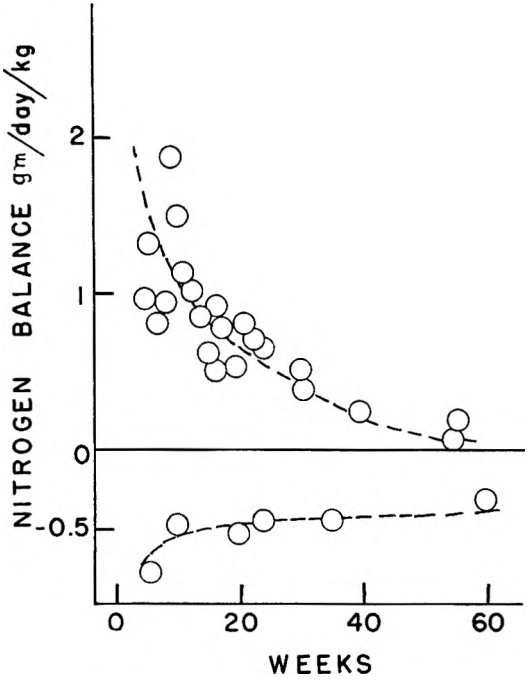


Fig. 1 Average positive nitrogen balances produced in litters of kittens fed diet recorded in table 1. Average negative balances produced when the kittens were fed a protein-free diet.

weeks. The lower curve in figure 1, in the region of negative balance, is a measure of the amount of nitrogen lost during a short period of feeding a protein-free diet, an estimate of the amount of nitrogen necessary to maintain equilibrium in the cat at different ages. Thus, the adult cat, with full protein stores, required approximately 0.5 gm of nitrogen/day/kg of body weight to maintain its protein stores. In the young cat of less than 10 weeks, the maintenance requirement, estimated

in this way, increased to 0.7 or more gm/day/kg of body weight. This type of increase in maintenance requirement in the young is consistent with their higher basal metabolic rate.

The caloric intake also decreased with increasing age (fig. 2). During the early phases of growth, when the energy requirements are greatest, the intake was about 250 Cal./day/kg of body weight. This intake decreased rapidly to approximately 134 Cal./day/kg of body weight at 30 weeks. The

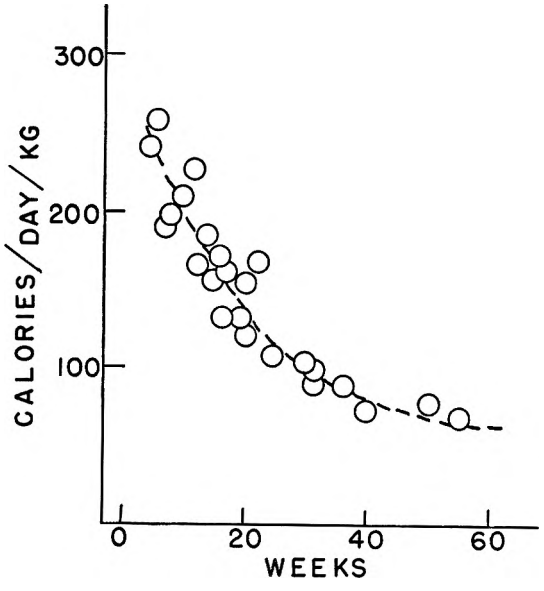


Fig. 2 Average caloric intakes when litters of kittens were fed diet recorded in table 1.

energy need became constant, in animals kept in metabolism cages, at about 60 Cal./day/kg of body weight at 50 weeks. Cats allowed to exercise in runs increased their caloric intakes to about 80 to 90 Cal./day/kg of body weight.

These data on nitrogen and caloric requirements of the cat demonstrate that this animal needs relatively the same number of calories as a beagle dog for maintenance and growth but the cat requires more nitrogen. A beagle puppy retained approximately 1 gm of nitrogen/day/kg of body weight at wean-

ing. At 10 weeks the nitrogen retention had dropped to 0.5 gm and then decreased gradually to 0.2 gm/day/kg of body weight at adulthood (Allison, '55b). Thus, the cat has a higher nitrogen to calorie ratio than the dog for maintenance and growth, a fact that may be correlated with a higher metabolic activity of proteins in the cat. The adult cat, however, adapts, as do other animals, to reduced intakes so that nitrogen equilibrium can be established by feeding as low as 0.2 gm of nitrogen/day/kg of body weight and still have the animal appear clinically normal. The optimum level of the protein reserves is an unknown variable in cats as it is, indeed, in all animals. The question is whether these reserves should be maximal with a high catabolic activity or be at some lower level. The determination of the optimum level of these reserves is one of the important considerations in nutrition today.

The ability of various dietary proteins to supply the need for nitrogen varies, of course, with the nutritive value of the protein. One measure of nutritive value is the nitrogen balance index which has been defined as the rate of change of nitrogen balance with respect to nitrogen intake at any given level of intake (Allison, '55a). It is a measure of the rate of filling of the protein stores. If the relationship between nitrogen balance and intake is linear in the region of negative and low positive balance, the index is constant over that range and can be calculated according to the following equation:

$$\text{Index (K)} = \frac{B - Bo}{I} \qquad (1)$$

where B is the nitrogen balance produced while feeding nitrogen intake (I) and Bo is the nitrogen balance resulting from feeding a protein-free diet. A linear relationship between nitrogen balance and intake was demonstrated over the above range of nitrogen balance while feeding cats a purified diet (Allison et al., '56). Experiments were done, therefore, to determine the indexes in the adult cat for egg albumin, casein and wheat gluten. Relatively low nitrogen intakes were

fed to the animals to be sure that the protein reserves would remain essentially constant during the determination and that the data would fall within the linear portion of the nitrogen balance-intake curve. Under these experimental conditions, the cats adapted to low intakes by a reduction in excretion of urea nitrogen so that they came into equilibrium at lower nitrogen intakes than 0.5 gm/day/kg of body weight. This latter value was established for adults with full reserves with high catabolic activity.

TABLE 2

Average values (5 to 8 cats) for nitrogen intakes (I), *nitrogen balances produced by these intakes* (B), *nitrogen balances produced while feeding a protein-free diet* (Bo) *and nitrogen balance index* (K) *for various dietary protein sources*

PROTEIN SOURCE	NITROGEN INTAKE I	NITROGEN BALANCE DURING NITROGEN FEEDING B	NITROGEN BALANCE PROTEIN-FREE DIET Bo [1]	INDEX K
	gm/day/kg	*gm/day/kg*	*gm/day/kg*	
Egg albumin	0.301	0.010	— 0.294	1.01
Egg albumin	0.473	0.142	— 0.329	1.00
Casein	0.394	0.144	— 0.212	0.90
Casein	0.531	0.169	— 0.331	0.94
Casein	0.379	0.065	— 0.291	0.92
Wheat gluten	0.432	0.100	— 0.214	0.73
Wheat gluten	0.588	0.200	— 0.254	0.77

[1] In experiments involving casein and wheat gluten, Bo was determined by feeding egg albumin diets rather than protein-free diets.

The nitrogen balance indexes for the various dietary proteins as determined in the adult cat are recorded in table 2. Since the index for egg albumin was essentially unity, the egg diet was fed to the animals to determine Bo in equation (1) during determinations of indexes for the other two proteins, thereby eliminating the depleting effect of the protein-free feeding period. The data in this table demonstrate that the indexes for casein (0.92) and for wheat gluten (0.75) are higher than those obtained in similar experiments involving dogs. The indexes were 0.75 for casein and 0.45 for wheat gluten when these proteins were fed to adult dogs (Allison,

'55a). The high value for wheat gluten may represent a need for less lysine in the diet of the adult cat than in the dog. Possibly the cat, like the rat (Mitchell, '47), can synthesize sufficient lysine for the maintenance of labile protein stores of the adult. It is possible that the sulfur amino acid requirement is less in the adult cat than in the rat or dog since the index for casein is so much higher in the cat than in these other fur-bearing animals. It should be emphasized that these indexes are for maintenance in the adult and may not apply directly to the growth of new tissue protein in the kitten nor to the repletion of depleted protein reserves in the adult. Further studies are under way to determine the sulfur amino acid and lysine requirements for maintenance and growth in both kittens and adults.

SUMMARY

A purified diet, relatively high in vitamin B complex and protein, was found to be satisfactory for the growth and maintenance of the cat. Using this diet, the nitrogen retention for maintenance plus growth and caloric intakes were estimated at different ages from weanling to adulthood. The ability of the adult cat to adapt to different nitrogen intakes emphasizes the need to determine the magnitude of optimum protein reserves. The nitrogen balance indexes for egg albumin, casein and wheat gluten were determined for maintenance of protein reserves in adult cats, these indexes being approximately 1.0, 0.92, and 0.75 respectively.

LITERATURE CITED

ALLISON, J. B. 1955a Biological evaluation of proteins. Physiol. Rev., *35:* 664.

———— 1955b Proteins in health and sickness. Gaines Veterinary Symposium monograph, *5:* 9.

ALLISON, J. B., S. A. MILLER, J. R. MCCOY AND M. K. BRUSH 1956 Studies on the nutrition of the cat. The North Am. Vet., *37:* 38.

DASILVA, A. C. 1950 The domestic cat as a laboratory animal for experimental nutritional studies. 1. General considerations. Acta Physiol. Latinoamericana, *1:* 20.

DASILVA, A. C., R. FRIED AND R. C. DEANGELIS 1952 The domestic cat as a laboratory animal for experimental nutritional studies. 3. Niacin requirements and tryptophane metabolism. J. Nutrition, *46:* 399.

DASILVA, A. C., R. C. DEANGELIS, M. A. PONTES AND M. F. MANSUR GUERIOS 1955 The domestic cat as a laboratory animal for experimental nutritional studies. 4. Folic acid deficiency. J. Nutrition, *56:* 199.

DICKENSON, C. D., AND P. P. SCOTT 1956a Nutrition of the cat. 1. A practical stock diet supporting growth and reproduction. Brit. J. Nutrition, *10:* 304.

———— 1956b Nutrition of the cat. 2. Protein requirements for growth of weanling kittens and young cats maintained on a mixed diet. Ibid., *10:* 311.

KREHL, W. A., G. R. COWGILL AND A. P. WHEDON 1955 Nondeleterious effects of polyoxyethylene esters in the nutrition of rats and cats. J. Nutrition, *55:* 35.

MITCHELL, H. H. 1947 Protein utilization by the adult rat: the lysine requirements. Arch. of Biochem., *12:* 293.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339.

NOTICE TO CONTRIBUTORS

THE JOURNAL OF NUTRITION, a copyrighted periodical, appears monthly for the publication of original research bearing on the subject of nutrition and occasional reviews of the literature dealing with this subject.

THE JOURNAL OF NUTRITION is the official organ of the American Institute of Nutrition. The officers of the Institute for 1957–58 are: R. R. Williams, President; William J. Darby, Vice-President; George M. Briggs, Secretary; J. B. Brown, Treasurer; R. W. Engel, Paul Gyorgy, L. A. Maynard, E. W. McHenry, Councillors.

Preliminary notices, or papers already published or in press elsewhere, will not be accepted. Unusually long papers that would take a disproportionate part of a single issue can be considered only if published as a supplement, the entire cost of which is assumed by the author.

The paper must be accompanied by an author's abstract not to exceed 225 words, which will be published in The Wistar Institute Advance Abstract Card Service.

Manuscripts and drawings should be sent by express prepaid or by registered mail to the Editor, Dr. George R. Cowgill, Yale University Nutrition Laboratory, 333 Cedar Street, New Haven 11, Conn. If two complete copies of the paper are submitted, consideration by the Journal can be expedited.

Manuscripts and drawings should be submitted in complete and finished form with the author's complete address. All drawings should be marked with the author's name. The Wistar Institute reserves the right to return to the author for revision material which is not in proper form for the printer. When the amount of tabular or illustrative material or both is judged to be excessive, or unusually expensive, authors may be requested to pay the excess cost.

Manuscripts should be typed in double spacing on one side of bond or heavy-bodied paper 8½ × 11 inches and should be sent flat. Page 1 should include, in the following order: complete title, author's name, institution from which the paper came, with city and state, total number of figures, shortened form of title (not more than 35 letters and spaces), address to which the proof is to be sent. The text starts on page 2.

Tables, quotations (extracts of over 5 lines), and all other material usually set in type smaller than the text, should be typed each on a separate sheet. Footnotes to the text should be numbered consecutively (including those on page 1) and typed in order on a separate sheet. Explanations of figures should be treated in the same manner. Footnotes to a table should be made a part of the table, and should be typed directly beneath it. Citations of literature in the text should be made by author and by numerals to indicate the year of publication. Authors' names (followed by year, title of paper, etc.) should be arranged alphabetically in a list at the end of the text.

The original drawings, not photographs of drawings, should accompany the manuscript. When photographs are used for halftone reproduction, glossy prints should be sent. Authors should indicate on the manuscript the approximate position of text figures. If there are illustrations so large as to require mailing separately, reduced copies of them that can be mailed with the manuscript should be provided in order to expedite consideration of the paper.

Figures should be drawn for reproduction as line or halftone engravings, unless the author is prepared to defray the cost of a more expensive form of illustration. Letters and figures should be uniform and large enough so that no character will be less than 2 mm high after reduction to at least the width of the page (4¼ inches). Double plates will not be accepted. Figures should be numbered consecutively beginning with the text figures and continuing through the plates. The reduction desired should be clearly indicated on the margin of the drawing.

Drawings intended for photographic reproduction should be made on white or blue-white paper or bristol board — not on cream-white or yellow-tone. Photographs intended for halftone reproduction should be securely mounted with colorless paste — never with glue, which discolors the photograph.

Galley proofs and engraver's proofs of figures are sent to the author. All corrections should be clearly marked thereon. For further details important for the preparation of a satisfactory manuscript, ask The Wistar Institute for a copy of its leaflet entitled: A GUIDE FOR AUTHORS.

THE JOURNAL OF NUTRITION is a copyrighted scientific periodical and reprints are intended primarily for the author's use. Authors are furnished 50 reprints, with covers, gratis. Additional copies may be obtained according to the rates which will be sent the authors as soon as the manuscript has been examined at The Wistar Institute, after acceptance. Other parties ordering reprints must have their orders approved by the authors, and must give written assurance that distribution will be restricted to professional personnel and others interested in the science of nutrition, and that no advertising or imprinting will be attached to the reprints thus furnished.

THE JOURNAL OF NUTRITION

VOL. 64 MARCH 10, 1958 No. 3

CONTENTS

PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America