# Metabolism of *trans* Acids in the Rat: Influence of the Geometric Isomers of Linoleic Acid on the Structure of Liver Triglycerides and Lecithins [1]

O. S. PRIVETT, L. J. NUTTER AND F. S. LIGHTLY
*University of Minnesota, The Hormel Institute, Austin, Minnesota*

*ABSTRACT* Studies were made on the structures of the liver lecithins and triglycerides of essential fatty acid (EFA)-deficient male rats of the Sprague-Dawley strain fed 5% supplements of *cis*, *cis*-linoleate, *cis*-9, *trans*-12-linoleate or *trans*-9, *trans*-12-linoleate or various mixtures of each of these compounds with *cis*, *cis*-linoleate or linolenate for 18 to 20 days. Enzymatic hydrolysis of lecithins with phospholipase A and triglycerides with pancreatic lipase showed that the *cis*, *cis* and *cis*-9, *trans*-12 isomers of linoleic acid were esterified predominately in the secondary positions, and that the *trans*, *trans* isomer was esterified predominately in the primary positions in these compounds. The precise positional arrangement of the geometric isomers of linoleic acid, as well as of other fatty acids, in any single molecular species, was influenced not only by the position to which they were directed dominantly but also by the relative degree that the other fatty acids were directed into the same position. The saturated fatty acids were esterified predominately in the primary positions in all molecular species; the *trans*, *trans* isomer of linoleic acid was esterified predominately in the primary positions in all molecular species except those containing saturated fatty acids. The *cis*, *cis* isomer of linoleic acid was esterified predominately in the $\beta$-position in all molecular species, except when it was associated with arachidonic acid which was esterified predominately in the $\beta$-position in all molecular species in which it was a constituent. Oleic acid and *cis*-9, *trans*-12-linoleic acid were distributed in the $\beta$-position with saturated or *trans*, *trans*-linoleic acid, and in the $\alpha$-position with all other polyunsaturated fatty acids.

It is well-established that fatty acids containing *trans* double bonds fed to rats are readily absorbed (1–5) and deposited in the tissue lipids (6–17). The nutritional effects of *trans* acids have not been clearly delineated although it has been well-demonstrated that they have no essential fatty acid activity (6, 8, 18, 19). The metabolic pathways of *trans* acids likewise have not been completely defined. Studies in this laboratory (6, 20) showed that little if any linoelaidate was converted to tetraene in the rat and that the *cis*-9, *trans*-12 isomer of linoleic acid was converted, but less efficiently than the *cis*, *cis* isomer, to eicosatetraenoic acid. Recently Selinger and Holman (21) also concluded that little if any linoelaidic acid was converted to tetraene in the rat, although earlier Holman (18) observed an increase in tetraene on feeding this isomer of linoleate and had concluded that it was converted to eicosatetraenoic acid. Knipprath and Mead (22) reported that the *trans*, *trans* isomer of linoleic acid was converted to eicosatetraenoic acid at least as efficiently as that of the naturally occurring *cis*, *cis* isomer on the basis of radioactive tracer experiments, although some of their other experiments did not appear to support this conclusion. A number of studies (21, 23)[2,3] have shown that *trans* acids are preferentially esterified in the primary positions of lecithins and triglycerides. Raulin et al.[4] also showed that *trans* acids are preferentially esterified in the primary position of phosphatidylethanolamine.

In general, studies on the metabolism of *trans* acids have been made with par-

Received for publication February 24, 1966.

[1] Supported in part by Public Health Service Research Grant no. AM-04942 from the National Institute of Arthritis and Metabolic Diseases.

[2] Raulin, J., P. Dauvillier and D. Lapous 1965 Distribution of elaidinized fatty acids in hepatic phospholipids of rats and pigs. International Conference on the Biochemistry of Lipids (9th), Noordwijk, The Netherlands (abstract no. 21).

[3] Cubera-Robles, E. 1965 Positional distribution of *trans* unsaturated fatty acids in the liver lecithins of rats fed hydrogenated oil. International Conference on the Biochemistry of Lipids (9th), Noordwijk, The Netherlands (abstract no. 20).

[4] See footnote 2.

tially hydrogenated, or elaidinized fats, linoelaidic acid or elaidic acid. In a recent study, Dr. W. E. M. Lands and the authors [5] demonstrated that the configuration and location of the double bonds influenced the rate of acyltransferase-catalyzed esterification of the geometric isomers of linoleic acid with α- or β-lysolecithin. It was also shown that the *trans, trans* and *cis*-9, *trans*-12 isomers of linoleic acid were preferentially esterified in the primary and secondary positions, respectively, in liver triglycerides and lecithins of the rat in in vivo experiments.

Presented here are further studies on the structures of liver lecithins and triglycerides of male rats of the Sprague-Dawley strain fed *cis*-9, *cis*-12; *cis*-9, trans-12; and *trans*-9, *trans*-12 isomers of methyl linoleate alone, in various mixtures and with linolenate.

## MATERIALS

Highly purified > 99% methyl linoleate, linoelaidate (*trans, trans* isomer of linoleate), and all-*cis*-linolenate were obtained from The Hormel Institute. Methyl *cis*-9, *trans*-12-octadecadienoate (*cis*-9, *trans*-12 isomer of linoleate) was prepared from pure ricinoleic acid via dehydration and a combination of fractional distillation and crystallization of the methyl esters as described by Jackson et al. (24). This compound contained only traces of impurities of other positional isomers as shown by a study of its structure by a special ozonolysis technique.[6]

## METHODS

Nine groups of a minimum of 5 male rats of the Sprague-Dawley strain, maintained from weaning to 4 months of age with a fat-free diet to make them deficient in essential fatty acids, were used in the present experiments. They were fed for short periods (18 to 20 days), 5% by weight as supplements to a fat-free diet, the above compounds in various combinations as follows: 1) fat-free (F.F. group (no supplement); 2) *cis, cis*-linoleate (*cis,cis* group); 3) *cis*-9, *trans*-12-linoleate (*cis,trans* group); 4) *trans, trans*-linoleate (*trans, trans* group); 5) *trans, trans* and *cis, cis* isomers of linoleate (1:1) (*trans, trans* and *cis, cis* group); 6) *trans, trans*-linoleate, linolenate (1:1) (*trans, trans* and linolenate group); 7) *cis*-9, *trans*-12-linoleate and *cis, cis*-linoleate (1:1) (*cis, trans* and *cis, cis* group); 8) *cis*-9, *trans*-12-linoleate and linolenate (1:1) (*cis, trans*-linoleate and linolenate group); 9) linolenate (linolenate group).

The fat-free diet consisted of 17% vitamin-test casein, 73% sucrose, 4% non-nutritive cellulose,[7] 4% Wesson salt mixture (25), 1% of casein containing 10% choline chloride and 1% of casein containing 5.2% of the following vitamin mixture: (in per cent) vitamin $K_5$, 0.35; vitamin $B_1$, 5.25; vitamin $B_2$, 5.25; vitamin $B_6$, 1.4; vitamin $B_{12}$, 0.09; vitamin $D_2$, 0.70; Ca pantothenate, 17.5; niacin, 17.5; inositol, 38.57; *p*-aminobenzoic acid, 13.15; folic acid, 0.20; and biotin, 0.04. Also, added separately to diet in ether solution (per kg of diet): vitamin A acetate, 4.8 mg, and vitamin E (*dl*-alpha-tocopherol N.F.), 33.6. Fresh diet was made every two days and stored at −20°.

At the end of each experiment the animals were lightly anesthetized with diethyl ether and killed by withdrawal of blood from their aortas. The tissues of each group were excised, frozen on dry ice and then stored at −20° until used.

*Analysis of triglycerides and lecithins.* The lipids were extracted from the pooled livers of each group with chloroform-methanol, 2:1 (v/v). The extracts were evaporated to dryness under vacuum at room temperature and re-extracted with chloroform, leaving most of the non-lipid material as a residue.

The crude lipid extracts were then fractionated on a DEAE-cellulose column as described by Rouser et al. (30). The neutral lipids were separated first by elution with chloroform. Next a fraction containing the lecithin was eluted with chloroform-methanol, 9:1 (v/v). The triglycerides were isolated in pure form from the first fraction by preparative TLC with pre-

[5] Lands, W. E. M., M. L. Blank, L. J. Nutter and O. S. Privett, unpublished data, 1966 (a manuscript, "A Comparison of Acyltransferase Activities in vitro with Distribution of Fatty Acids in Lecithin and Triglycerides in vivo," has been submitted for publication).

[6] Privett, O. S., and E. C. Nickell 1966 Determination of the specific position of *cis* and *trans* double bonds in polyenes. Lipids, in press.

[7] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

washed plates coated with Silica Gel G with a solvent system of 15% diethyl ether in petroleum ether. The lecithin was isolated from the second fraction by chromatographic fractionation on a column of "ammonium silicate" as described by Rouser et al. (26). The first fraction to emerge from the column was eluted with chloroform-methanol, 70:30 (v/v) containing 0.4% water. It contained no lecithin. The lecithin was eluted next by increasing the water in this solvent to 1.5%. Virtually complete recovery of the lecithin in pure form was obtained by this procedure as determined by TLC and infrared spectral analysis.

Triglyceride species composition was determined by a combination of thin-layer chromatography with plates coated with silicic acid impregnated with silver nitrate and pancreatic lipase as described in detail in previous work from this laboratory (27). Species composition of lecithins was determined by the same procedure applied to the corresponding aceto-1, 2-diglycerides derived via hydrolysis with phospholipase C and acetylation. The general technique has been described by Renkonen (28).

The species composition of lecithins was expressed in some experiments only in terms of the positional arrangement of the saturated and unsaturated fatty acids as groups. These analyses were determined from the distribution of the fatty acids between the $\alpha$- and $\beta$-positions of the molecule and the amount of the disaturated lecithin as described [8] previously or calculated from data obtained from the complete structural analysis as described above (27, 28).

Gas-liquid chromatography (GLC) of methyl esters was carried out with an F & M Model 609 flame ionization instrument equipped with a 213 cm (7 foot) by 0.6 cm (¼ inch) column packed with 10% ethyleneglycol succinate polymer phase [9] on Chromosorb W, 100–120 mesh, at 185°. Fatty acid composition was determined directly from the proportionalities of the peak area; linear response of the detectors of the instrument was established with standards obtained from The Hormel Institute.

## RESULTS AND DISCUSSION

The analyses of the fatty acids of the liver triglycerides of each group of animals are shown in table 1. Comparison with the fatty acid composition of the EFA-deficient (fat-free) group showed that the supplements of the isomers of linoleate were incorporated into the liver triglycerides and the changes in the composition obviously were related to the composition of the supplements. Since the liver triglycerides in the fat-free group of animals were devoid of 18:2 and higher polyunsaturated fatty acids, the presence of these acids in the other groups obviously arose from the dietary fatty supplements that were fed. Thus the 18:2 acids detected in each group, except for the linolenate group, consisted of the fed acid. In a previous study (6) the deposition of *trans, trans*-linoleate was confirmed by direct chemical evidence. Thus the 18:2 in the *cis, cis* group was *cis, cis*-linoleate, in the *trans, trans* group *trans, trans*-lino-

[8] Blank, M. L., and L. J. Nutter 1966 Determination of lecithin structure. Lipids, in press.
[9] EGSS-X, Applied Science Laboratories.

TABLE 1

*Fatty acid composition of liver triglycerides of rats fed isomers of linoleate* [1] *(wt %)*

| Group (supplement) | F.F.[2] | *cis,cis* | *cis, trans* | *trans,trans* | *trans,trans* + *cis,cis* | *trans,trans* + linolenate | *cis, trans* + *cis,cis* | *cis, trans* + linolenate | Linolenate |
|---|---|---|---|---|---|---|---|---|---|
| 16:0 | 31.6 | 29.4 | 27.1 | 28.6 | 26.2 | 29.0 | 34.3 | 29.0 | 25.5 |
| 16:1 | 13.5 | 7.7 | 10.3 | 13.2 | 10.8 | 9.3 | 14.2 | 8.9 | 11.0 |
| 18:0 | 2.1 | 2.4 | 1.5 | 3.3 | 2.5 | 2.2 | 1.2 | 2.4 | 2.2 |
| 18:1 | 51.5 | 53.5 | 55.0 | 49.2 | 41.1 | 34.8 | 40.9 | 44.3 | 35.8 |
| 18:2 | | 5.4 | 5.3 | 5.7 | 17.1 | 9.2 | 8.1 | 7.9 | 2.7 |
| 18:3 | | | 0.8 | | | 14.0 | | 5.8 | 21.8 |

[1] Major fatty acids. Small amounts of 14:0, 14:1, 17:0, 17:1, and a number of unidentified minor and trace acids were also detected.
[2] F.F. = group maintained with a fat-free diet.

leate, and in the *cis, trans* group *cis*-9, *trans*-12-linoleate. The origin of the 18:2 in the linolenate group is not known.

Analysis of the triglycerides permitted an insight into the mode in which the isomers of linoleate were incorporated into these compounds. Table 2 shows the percentage of each of the fatty acids esterified in the β-position of the molecule. If the fatty acids are distributed randomly between all 3 positions in the triglyceride molecule a value of 33.3 should be obtained for the percentage in the β-position. A value greater than 33% is considered to indicate a predominance in this position. In order to have a preference for the β-position over a possibility of esterification in either one of the primary positions the value should have to be greater than 50%. The *trans, trans* isomer of linoleic, palmitic and stearic acids were obviously incorporated predominately into the primary positions of the molecule because less than 33% of these acids were noted in the β-position. Regardless of the selectivity that might exist between the 2 primary positions, *cis, cis*-linoleate, *cis, trans*-linoleate and oleic acids were esterified predominately in the β-position. The 16:1 and linolenic acids appeared to be distributed fairly evenly among the primary and secondary positions. Because the small amounts of *trans, trans* and *cis*-9, *trans*-12 isomers of linoleic acid incorporated into triglycerides were distributed among many molecular species, it was not possible to isolate sufficient amounts of these species to determine the precise positional arrangement of these isomers of linoleic acid. However, in a previous study (29) with animals that were fed corn oil the saturated fatty acids were esterified predominately in the primary positions in all molecular species. Oleic acid was distributed predominately in the β-position in the triglyceride containing saturated fatty acids, and in the primary position in those glycerides containing polyunsaturated fatty acids. Linoleic acid was distributed primarily in the β-position in all species except those containing arachidonic acid.

*Lecithin analysis.* The fatty acid composition of the liver lecithins of all groups are shown in table 3. The fatty supplements influenced the fatty acid composi-

TABLE 2

*Distribution of each fatty acid in the β-position of the liver triglycerides (wt %)*

| Group (supplement) | F.F.[1] | *cis,cis* | *cis, trans* | *trans,trans* | *trans,trans* + *cis,cis* | *trans,trans* + linolenate | *cis, trans* + *cis,cis* | *cis, trans* + linolenate | Linolenate |
|---|---|---|---|---|---|---|---|---|---|
| 16:0 | 7.2 | 6.8 | 5.8 | 11.1 | 12.7 | 13.5 | 10.3 | 15.5 | 14.9 |
| 16:1 | 28.0 | 34.6 | 35.8 | 40.5 | 34.8 | 41.2 | 36.6 | 39.0 | 32.7 |
| 18:0 | 2.4 | 20.8 | trace | 11.7 | 18.0 | 18.1 | trace | 19.5 | 15.0 |
| 18:1 | 51.7 | 44.5 | 47.0 | 47.5 | 39.5 | 52.5 | 48.0 | 44.5 | 44.8 |
| 18:2 | | 71.0 | 41.0 | 19.3 | 40.4 | 14.5 | 56.0 | 39.3 | 65.4 |
| 18:3 | | | | | | 38.0 | | 28.2 | 35.0 |

[1] F.F. = group maintained with the basic fatfree diet.

TABLE 3

*Fatty acid composition of liver lecithins*[1] *(wt %)*

| Group (supplement) | F.F.[2] | *cis,cis* | *cis, trans* | *trans,trans* | *trans,trans* + *cis,cis* | *trans,trans* + linolenate | *cis, trans* + *cis,cis* | *cis, trans* + linolenate | Linolenate |
|---|---|---|---|---|---|---|---|---|---|
| 16:0 | 24.1 | 20.9 | 21.9 | 18.5 | 16.1 | 19.1 | 18.2 | 24.8 | 26.1 |
| 16:1 | 8.5 | 1.5 | 5.0 | 10.2 | 3.2 | 3.5 | 3.8 | 3.2 | 3.4 |
| 18:0 | 15.6 | 19.2 | 15.7 | 11.6 | 14.0 | 17.0 | 17.8 | 22.4 | 19.9 |
| 18:1 | 29.1 | 9.2 | 29.3 | 35.7 | 13.3 | 13.9 | 14.9 | 15.3 | 12.7 |
| 18:2 | 1.7 | 14.6 | 8.7 | 13.3 | 36.9 | 15.2 | 18.4 | 7.0 | 5.6 |
| 18:3 | 0.5 | | | 3.9 | | 3.5 | 1.4 | 1.7 | 2.8 |
| 20:3 | 14.9 | 1.5 | 7.4 | 5.0 | 1.9 | 2.4 | 3.4 | 3.4 | 1.0 |
| 20:4 | 2.2 | 33.0 | 12.0 | 1.4 | 13.5 | 1.7 | 17.4 | 4.2 | 7.6 |
| 20.5 | | | | | | 22.4 | | 17.1 | 20.1 |

[1] Major fatty acids. Small amounts of 14:0, 14:1, 17:0, 17:1, and a number of unidentified minor and trace acids were also detected.
[2] F.F. = group maintained with the basic fat-free diet.

tion of the liver lecithins more than the liver triglycerides, and the changes were fairly consistent throughout all groups. The effect of feeding the *cis, cis* isomer of linoleic acid to EFA-deficient rats is well-documented (30–32). In general, dietary linoleic acid gives a lowering of monoenoic acids and 5, 8, 11-eicosatrienoic acid with a simultaneous increase in linoleic and arachidonic acids. The fatty acid composition of the livers of the *cis, cis*-linoleate group in this study follows precisely this pattern. Similar effects were produced by the *cis*-9, *trans*-12-linoleate but to a lesser degree, and this effect could be observed through all the groups to which it was fed in the mixtures. The effect of dietary linolenate has also been documented and the changes noted here follow those reported previously (33–35). The *trans, trans* isomer of linoleate appeared to provide an even more severe effect in some respects in the direction indicated by lipid starvation. For example, the 20:4 was not increased yet the 20:3 was decreased, as we had noted previously (5). Less oleic acid was apparently converted to 5, 8, 11-eicosatrienoic acid in the *trans, trans*-linoleate groups of animals. As a result the amount of oleic acid built up to approximately 36% in the liver lecithin in the animals receiving the *trans, trans*-linoleic acid, except where it was fed in mixtures with linolenic or *cis, cis*-linoleic acid. In these groups the effect of the all-*cis* acids prevailed.

Since appreciable amounts of the dietary fat supplements were incorporated into the lecithins, an insight could be obtained into the manner in which the isomers of linoleic acid were incorporated into the molecule. The distribution of the fatty acids between the α- and β-positions of the lecithins followed the same general pattern as in the triglycerides, except for oleic acid in the *cis, cis* group and linolenic acid (table 4). Linolenic acid, as well as the other unsaturated fatty acids were preferentially esterified in the β-position, except oleic acid in the *cis, cis* group and the *trans, trans* isomer of linoleic acid. This isomer, as well as palmitic and stearic acids, were esterified predominately in the primary position of the molecule.

TABLE 4

*Distribution of each fatty acid between the α- and β-positions of lecithins (% by wt)*

| Group (supplement) | F.F.[1] | | *cis,cis* | | *cis, trans* | | *trans,trans* | | *trans,trans* + *cis,cis* | | *cis, trans* + *cis,cis* | | *trans,trans* + linolenate | | *cis, trans* + linolenate | | Linolenate | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | α- | β- | α- | β- | α- | β- | α- | β- | α- | β- | α- | β- | α- | β- | α- | β- | α- | β- |
| 16:0 | 96.9 | 3.1 | 89.6 | 10.4 | 89.8 | 10.2 | 88.5 | 11.5 | 86.5 | 13.5 | 88.3 | 11.7 | 87.4 | 12.6 | 93.9 | 6.1 | 93.1 | 6.9 |
| 16:1 | 46.9 | 53.1 | 79.7 | 20.3 | 36.9 | 63.1 | 29.3 | 70.7 | 38.5 | 61.5 | 43.4 | 56.6 | 28.2 | 71.8 | 16.4 | 83.6 | 44.1 | 55.9 |
| 18:0 | 100 | 0 | 90.9 | 9.1 | 94.4 | 5.6 | 91.9 | 8.1 | 97.9 | 2.1 | 96.7 | 3.3 | 97.3 | 2.7 | 99.3 | 0.7 | 98.4 | 1.6 |
| 18:1 | 34.5 | 65.5 | 56.4 | 43.6 | 29.4 | 70.6 | 22.3 | 77.7 | 36.9 | 63.1 | 35.3 | 64.7 | 12.6 | 87.4 | 8.5 | 91.5 | 21.6 | 78.4 |
| 18:2 | 6.0 | 94.0 | 15.0 | 85.0 | 29.0 | 71.0 | 62.9 | 37.1 | 36.1 | 63.9 | 13.5 | 86.5 | 77.5 | 22.5 | 27.7 | 72.3 | 100 | 0 |
| 18:3 | | | | | | | | | | | | | 14.7 | 85.3 | 0 | 100 | 35.7 | 64.3 |
| 20:3 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 |
| 20:4 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 10.7 | 89.3 | 16.7 | 83.3 | 0 | 100 | 0 | 100 | 0 | 100 |
| 20:5 | | | | | | | | | | | | | 0 | 100 | 0 | 100 | 2.7 | 97.3 |

[1] F.F. = group maintained on the basic fat-free diet.

A further insight into the mode of incorporation of the fatty acids into lecithin was indicated from the analysis of the types formed in each group (table 5). These results show a well-defined pattern relating to the structure of the isomer of linoleic acid that was fed. Only trace amounts of disaturated and small amounts of α-unsaturated-β-saturated types were formed, in accord with the observation that the saturated and unsaturated fatty acids were esterified preferentially in the α- and β-position respectively. The α-saturated-β-unsaturated type was formed in largest amounts and it varied in the groups in decreasing amounts in the following order: *cis, cis*-linoleate > fat-free > *cis, trans* group > *trans, trans*. The relative amounts of the diunsaturated type decreased in the opposite order. It is apparent from these and the results of the other groups that, although certain patterns appear to exist, the processes involved in the synthesis of specific molecular species of lecithin are highly complex.

The positional arrangement of the fatty acids in the lecithins (table 6) also followed a pattern that related to composition in accordance with their relative tendencies to be esterified in the α- or

TABLE 5

*Species analysis of liver lecithins of rats fed isomers of linoleate* [1] *(% by wt)*

| | Group (supplement) | Species | | |
|---|---|---|---|---|
| | | α-saurated, β-unsaturated | α-unsaturated, β-saurated | α-unsaturated, β-unsaturated |
| 1 | F.F.[2] | 76.2 | 1.4 | 22.2 |
| 2 | *cis,cis* | 80.3 | 2.9 | 16.8 |
| 3 | *cis,trans* | 69.3 | 4.4 | 26.3 |
| 4 | *trans,trans* | 59.8 | 3.6 | 36.6 |
| 5 | *trans,trans* + *cis,cis* | 56.1 | 5.3 | 38.6 |
| 6 | *cis,trans* + *cis,cis* | 69.2 | 5.6 | 25.2 |
| 7 | *trans,trans* + linoleate | 67.1 | 6.3 | 26.6 |
| 8 | *cis,trans* + linolenate | 92.1 | 3.3 | 4.6 |
| 9 | Linolenate | 89.4 | 4.2 | 6.4 |

[1] Disaturated lecithins were present in only trace amounts.
[2] F.F. = group maintained with a fat-free diet.

TABLE 6

*Structural analysis of liver lecithins of rats fed isomers of linoleate (% by wt)*

| Group (supplement) | F.F.[1] | *cis,cis* | *cis, trans* | *trans,trans* |
|---|---|---|---|---|
| Lecithin species [2] | | | | |
| α-Sat, β-sat | tr | — | — | — |
| α-Sat, β-mono | 39.9 | 9.2 | 29.0 | 45.5 |
| α-Mono, β-sat | 0.8 | 1.8 | 0.9 | 3.0 |
| α-Sat, β-diene | 3.4 | 25.4 | 13.7 | 3.5 |
| α-Diene, β-sat | 0.1 | 0.2 | 0.4 | 0.1 |
| α-Sat, β-triene | 26.8 | 3.3 | 12.5 | 9.2 |
| α-Triene, β-sat | 0.2 | 0.2 | 2.2 | 0.4 |
| α-Sat, β-tetra | 6.1 | 42.4 | 14.1 | 1.6 |
| α-Tetra, β-sat | 0.3 | 0.7 | 0.9 | 0.1 |
| α-Mono, β-mono | 11.2 | 1.4 | 9.0 | 11.7 |
| α-Mono, β-diene | 1.8 | 4.7 | 3.4 | 3.7 |
| α-Diene, β-mono | 0.8 | 0.4 | 5.5 | 13.6 |
| α-Mono, β-triene | 8.0 | 0.2 | 2.4 | 4.4 |
| α-Triene, β-mono | 0.4 | — | 0.2 | 0.2 |
| α-Mono, β-tetra | — | 7.3 | 3.5 | — |
| α-Tetra, β-mono | — | — | 0.2 | — |
| α-Diene, β-tetra | — | 1.7 | 1.2 | — |
| α-Tetra, β-diene | — | — | 0.1 | — |

[1] F.F. = group maintained with the basic fat-free diet.
[2] Sat = saturated; mono = monounsaturated; diene = diunsaturated; triene = triunsaturated; and tetra = tetraunsaturated fatty acids.

β-position. The saturated fatty acids were esterified predominately in the α-position in all molecular species. Phospholipase A hydrolysis showed that the *trans, trans* isomer of linoleic acid was distributed predominately in the α-position also, but in those molecular species in which it was a constitutent with saturated fatty acids it was predominately in the β-position. Oleic acid was also distributed predominately in the β-position, as indicated by phospholipase A hydrolysis, but it was in the α-position predominately in those species in which it was associated with *cis, cis*-linoleic acid, trienoic or tetraenoic fatty acids. The tendency of *cis, cis*-linoleic acid to force oleic acid into the α-position was indicated by the results of the phospholipase A hydrolysis of the lecithin of the *cis, cis* group. Relative composition of the 18:2 in the group fed mixtures of these isomers was not determined. It would be expected that the 18:2 in these groups of animals in the α- and β-positions would correspond generally to the *cis, cis* and *trans* isomer composition of this fraction. Since *cis, cis*-linoleic acid and arachidonic acid exhibited strong preferences for esterification in the β-position, this position may be important in the interconversion of essential fatty acids. Thus, it appears that the positional arrangement of the fatty acids in lecithin depends not only on the position that they are directed dominantly, but the relative degree that the other constituent fatty acids are also directed into the same positions. The main factors involved in the structure of the fatty acids that accounts for these characteristics is the degree of unsaturation and the configuration of the double bond. The chain length and positions of the double bonds within these structural variations may also influence the structure of lecithins and triglycerides but no data are presently available on this aspect of the biosynthesis of lecithins and triglycerides.

## LITERATURE CITED


1. Ono, K., and D. S. Frederickson 1964 The metabolism of $^{14}$C-labeled *cis* and *trans* isomers of octadecenoic and octadecadienoic acids. J. Biol. Chem., *239:* 2482.
2. Coots, R. H. 1964 A comparison of the metabolism of elaidic, oleic, palmitic, and stearic acids in the rat. J. Lipid Res., *5:* 468.
3. Kennedy, E. P., and A. L. Lehninger 1950 The products of oxidation of fatty acids by isolated rat liver mitochondria. J. Biol. Chem., *185:* 275.
4. Loriette, C., G. Clément, and J. Raulin 1962 Effect of prolonged ingestion of peanut oil containing *trans* polyunsaturated fatty acids on the structure of depot fat triglycerides in the white rat. C. R. Acad. Sci., *255:* 2204.
5. Loriette, C., A. Rerat, G. Clément and J. Raulin 1963 Preferential incorporation of elaidinized fatty acids in the α-positions of the reserve triglycerides of pigs. C. R. Acad. Sci., *257:* 3679.
6. Privett, O. S., and M. L. Blank 1964 Studies on the metabolism of linoelaidic acid in the essential fatty acid deficient rat. J. Am. Oil Chemists' Soc., *41:* 292.
7. Aaes-Jørgensen, E. 1961 Essential fatty acids. Physiol. Rev., *41:* 1.
8. Holman, R. T., and E. Aaes-Jørgensen 1956 Effect of *trans* fatty acid isomers upon essential fatty acid deficiency in rats. Proc. Soc. Exp. Biol. Med., *93:* 175.
9. Johnston, P. V., O. C. Johnson and F. A. Kummerow 1957 Non-transfer of *trans* fatty acids from mother to young. Proc. Soc. Exp. Biol. Med., *96:* 760.
10. Johnston, P. V., O. C. Johnson and F. A. Kummerow 1958 Deposition in tissues and fecal excretion of *trans* fatty acids in the rat. J. Nutrition, *65:* 13.
11. Barbour, A. D. 1933 The deposition and utilization of hydrogenation isooleic acid in the animal body. J. Biol. Chem., *101:* 63.
12. Kaufmann, H. P., and G. Menkel 1964 The occurrence of *trans* fatty acids. Fette: Seifen: Anstrichmittel, *66:* 6.
13. Sinclair, R. G. 1935 The metabolism of the phospholipids. VIII. The passage of elaidic acid into tissue phospholipids. Evidence of the intermediary role of liver phospholipid in fat metabolism. J. Biol. Chem., *111:* 515.
14. Sinclair, R. G. 1936 Blood phospholipid as a transport mechanism. J. Biol. Chem., *115:* 211.
15. McConnell, K. P., and R. G. Sinclair 1937 Evidence of selection in the building up of brain lecithins and cephalins. J. Biol. Chem., *118:* 131.
16. Kohl, M. F. F. 1938 A balance sheet of fat absorption. I. The storage of elaidic acid by the rat over a one-day period. J. Biol. Chem., *126:* 709.
17. Carroll, K. K. 1965 Dietary fat and the fatty acid composition of tissue lipids. J. Am. Oil Chemists' Soc., *42:* 516.
18. Holman, R. T. 1951 Metabolism of isomers of linoleic and linolenic acids. Proc. Soc. Exp. Biol. Med., *76:* 100.
19. Privett, O. S., F. J. Pusch and R. T. Holman 1955 Polyethenoid fatty acid metabolism. VIII. Nonpotency of *cis*-9, *trans*-12-linoleate

as essential fatty acid. Arch. Biochem. Biophys., 57: 156.
20. Blank, M. L., and O. S. Privett 1963 Studies on the metabolism of *cis, trans* isomers of methyl linoleate and methyl linolenate. J. Lipid Res., 4: 470.
21. Selinger, Z., and R. T. Holman 1965 The effects of *trans, trans*-linoleate upon the metabolism of linoleate and linolenate and the positional distribution of linoleate isomers in liver lecithin. Biochim. Biophys. Acta, 106: 56.
22. Knipprath, W. G., and J. F. Mead 1964 The metabolism of *trans, trans*-octadecadienoic acid. Incorporation of *trans, trans*-octadecadienoic acid into the $C_{20}$ polyunsaturated acids of the rat. J. Am. Oil Chemists' Soc., 41: 437.
23. Raulin, J., C. Loriette and G. Clément 1963 Conditions d'incorporation des acides gras élaïdisés aux triglycérides de réserve du rat blanc. Biochim. Biophys. Acta, 70: 642.
24. Jackson, J. E., R. F. Paschke, W. Tolberg, H. M. Boyd and D. H. Wheeler 1952 Isomers of linoleic acid. Infrared and ultraviolet properties of methyl esters. J. Am. Oil Chemists' Soc., 29: 229.
25. Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.
26. Rouser, G., A. J. Bauman, G. Kritchevsky, D. Heller and J. S. O'Brien 1961 Quantitative chromatographic fractionation of complex lipid mixtures: brain lipids. J. Am. Oil Chemists' Soc., 38: 544.
27. Blank, M. L., B. Verdino and O. S. Privett 1965 Determination of triglyceride structure via silver nitrate-TLC. J. Am. Oil Chemists' Soc., 42: 87.
28. Renkonen, O. 1965 Individual molecular species of different phospholipid classes. II. A method of analysis. J. Am. Oil Chemists' Soc., 42: 298.
29. Privett, O. S., M. L. Blank and B. Verdino 1965 Influence of dietary fat on triglyceride structure in the rat. J. Nutrition, 85: 187.
30. Holman, R. T., and H. Mohrhauer 1963 The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. J. Lipid Res., 4: 151.
31. Holman, R. T. 1960 The ratio of trienoic: tetraenoic acids in tissue lipids as a measure of essential fatty acid requirements. J. Nutrition, 70: 405.
32. Caster, W. O., and R. T. Holman 1961 Statistical study of the relationship between dietary linoleate and the fatty acids of heart and blood lipids. J. Nutrition, 73: 337.
33. Holman, R. T. 1964 Nutritional and metabolic interrelationships between fatty acids. Federation Proc., 23: 1062.
34. Rahm, J. J., and R. T. Holman 1964 Effect of linoleic acid upon the metabolism of linolenic acid. J. Nutrition, 84: 15.
35. Rahm, J. J., and R. T. Holman 1964 Studies of the metabolism of polyunsaturated acids by short-term experiments. J. Nutrition, 84: 149.

# Metabolism of Physiological Doses of Thiazole-2-$^{14}$C-labeled Thiamine by the Rat [1]

MESBAHEDDIN BALAGHI [2,3] AND WILLIAM N. PEARSON
*Division of Nutrition, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee*

*ABSTRACT* The metabolism of thiazole-2-$^{14}$C-labeled thiamine was studied in the rat in order to compare its metabolism to that of pyrimidine-$^{14}$C-labeled thiamine. The catabolic products of the thiazole-2-$^{14}$C-labeled thiamine in the urine were separated by column and paper chromatographic techniques. More than 20 urinary metabolites were detected. The generation of $^{14}CO_2$ indicated breakdown of the thiazole moiety of thiamine. The marked similarity in the urinary chromatographic pattern found in this study to that obtained previously with pyrimidine-$^{14}$C-labeled thiamine suggests that most of the urinary metabolites are derivatives of the entire thiamine molecule.

Recent studies of the catabolism of pyrimidine-$^{14}$C-labeled thiamine have demonstrated the presence of a minimum of 22 different metabolites in rat urine (1). Of these metabolites only thiamine and 2-methyl-4-amino-5-pyrimidinecarboxylic acid have been identified (2). In an attempt further to elucidate the nature of the remaining unidentified thiamine metabolites, studies of the overall catabolism of thiazole-$^{14}$C-labeled thiamine have been carried out and are presented in this report.

## MATERIALS AND METHODS

Adult female rats of the Sprague-Dawley strain were used throughout the experiment. Housing of the animals, collection of urine and feces, and the composition of the thiamine-deficient diet have been described previously (3). Thiazole-2-$^{14}$C-labeled thiamine was purchased from the Nuclear Chicago Corporation (specific activity 5600 cpm/μg). The purity of the compound was checked by column chromatography, paper chromatography, radioautography, thiochrome analysis and treatment of thiamine deficiency in rats.

The rats were depleted for 3 weeks with a thiamine-free diet and then intraperitoneal injections of radioactive thiamine were started. They were maintained for 4 weeks at each thiamine dosage level so that equilibrium was attained and the excretion of radioactivity in the urine, feces, and the respiratory $CO_2$ was studied. Daily thiamine intake levels of 30 μg, 50 μg and 100 μg were studied.

At the end of each 24-hour collection period, the urine was pooled and an aliquot was taken for liquid scintillation counting. Feces samples were homogenized with 10 volumes of 0.2 N sulfuric acid in a Waring Blendor. The homogenate was steamed in an autoclave for 30 minutes and centrifuged for 45 minutes at 10,000 rev/min. The precipitate was resuspended in another volume of acid and re-extracted in a similar manner. The combined supernatant solutions were stirred for 30 minutes and duplicate samples were taken for scintillation counting. For respiratory studies, the rats were placed in a respiration chamber and the expired carbon dioxide was collected by drawing the expired air first through a column of Drierite and then through 2 successive cylinders, each containing 300 ml of a mixture of ethanolamine and ethylene glycol monomethyl ether (1:3 by volume) as suggested by Jeffay and Alvarez (4). At the end of each experiment, the ethanolamine trapping mixtures were combined and stirred for 30 minutes, and

Received for publication February 1, 1966.
[1] Supported in part by Public Health Service Research Grant no. AM-07709 and no. AM-5441 from the National Institute of Arthritis and Metabolic Diseases.
[2] Present address: Imperial Iranian Army Nutrition Committee, Army Medical Department, Aziz Khan Crossroad, Hafez Avenue, Tehran, Iran.
[3] A portion of these data was taken from a thesis submitted by Mesbaheddin Balaghi to the faculty of the graduate school of Vanderbilt University in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry.

duplicate samples were taken for scintillation counting.

The radioactive metabolites in urine were purified by adsorption upon acid-washed charcoal and elution with a mixture of pyridine/ethanol/water (10/45/45 by volume). Eighty-five per cent of the radioactivity was adsorbed on charcoal and 75% of the adsorbed radioactivity was eluted by this method. The eluate was then subjected to chromatography on Amberlite CG-50 resin. Each peak obtained from this column was further resolved by ascending paper chromatography and the radioactive bands were localized by radioautography. The details of these procedures have been described by Neal and Pearson (1).

For determination of radioactivity in the fractions collected from ion exchange chromatography, 0.5 ml of each fraction was placed on an aluminum planchet, and 1 ml of ethanol was added. The planchets were then dried and counted in a Nuclear Chicago D-47 gas-flow counter equipped with a Model M5 sample changer. The radioactivity of the urine samples, feces extracts, and the pooled peaks obtained from column chromatography of urine were determined by liquid scintillation counting. The scintillation fluid used consisted of a mixture of: naphthalene, 100 g; PPO (2,5-diphenyloxazole) 7 g; POPOP (1, 4-bis-2-(5-phenyloxazolyl)-benzene) 0.3 g; and sufficient *p*-dioxane to make the volume up to one liter. The scintillation fluid used for counting the ethanolamine $CO_2$ trapping mixture was a 2:1 mixture of toluene and ethylene glycol monomethyl ether which contained 5.5 g of PPO/liter. In each case 10 ml of scintillation fluid were used per ml of sample and the internal recovery technique was used for correction of quenching.

Bioautography with the medium of Deibel et al. (5) was used to determine the biological activity of radioactive compounds found in urine. For this purpose 200 ml of single strength *Lactobacillus viridescens* thiamine assay medium [4] containing 2% agar was autoclaved, cooled to 45°, seeded with a washed suspension of a 24-hour-old culture of *L. viridescens*, and poured into a rectangular glass dish. When the medium solidified, the entire paper chromatograph or the excised bands containing the radioactive metabolites were put on the surface of the plate for 1 to 5 minutes. The paper was then removed and a layer of 400 ml of sterile 2% agar containing 25 ml of a 2% aqueous solution of 2,3,5-triphenyltetrazolium chloride was poured on the plate. When the second layer solidified, the plate was covered and incubated at 30° for 24 hours. The growth areas appeared as diffuse red spots against a yellow background. It was possible to detect the biological activity of 0.1 mμg of thiamine by this method.

## RESULTS

When the injection of thiazole-$^{14}$C-labeled thiamine at a level of 30 μg daily was initiated in the thiamine-depleted rats, only 3.1% of the injected radioactivity appeared in the urine during the first 24 hours. This percentage increased slowly day by day and reached 43.7% on the twelfth day of injection. As the dosage of thiamine increased, a larger percentage of the radioactivity appeared in the urine. In table 1 it is shown that the urinary excretion of radioactivity accounts for 29 to 42% of that injected when the thiamine intake is between 30 and 100 μg/day. The rather large standard deviations observed were due in part to the difficulty of obtaining complete urine collections.

Analysis of the feces collected during a 9-week period from 16 rats maintained with 30 μg of $^{14}$C-thiamine/day yielded an average daily fecal excretion of 17.5% of the dose. As in the case of urine, the radioactivity of the feces increased grad-

TABLE 1

*Urinary excretion of $^{14}$C-labeled thiamine compounds, expressed as percentage of the dose of radioactive thiamine* [1]

| Dose | Excreted in urine |
|---|---|
| *μg* | *%* |
| 30 | 29.18 ± 6.70 [2] |
| 50 | 38.29 ± 5.45 |
| 100 | 42.00 ± 10.08 |

[1] Each figure represents the mean daily excretion of 16 rats for 4-week periods (30 and 50 μg) or one-week period (100 μg).
[2] SD.

[4] Difco Laboratories, Detroit, Michigan.

TABLE 2

*Excretion of radioactive $CO_2$ by rats receiving various dosage levels of thiazole-$^{14}C$-labeled thiamine*

| Dosage levels | | | | | |
|---|---|---|---|---|---|
| 30 μg/day | | 50 μg/day | | 100 μg/day | |
| Total $^{14}CO_2$[1] | | Total $^{14}CO_2$[1] | | Total $^{14}CO_2$[1] | |
| | *% of dose* | | *% of dose* | | *% of dose* |
| 7.0 | 23.5 | 7.3 | 14.6 | 7.2 | 7.2 |
| 5.4 | 18.3 | 6.8 | 13.6 | 7.8 | 7.8 |
| 8.1 | 27.0 | 6.6 | 13.2 | | |
| 8.1 | 27.0 | 6.5 | 13.0 | | |
| 7.2 | 24.2 | | | | |
| 7.1 ± 1.1 [2] | 24.0 ± 3.61 | 6.8 ± 0.35 | 13.6 ± 1.24 | 7.5 ± 0.42 | 7.5 ± 0.42 |

[1] Each value represents the $^{14}CO_2$ excreted in a 24-hour period by a single rat expressed as micrograms of injected thiamine or as percentage of the dose. The values at the 30- and 50-μg dosage levels represent 24-hour collections but those at the 100-μg dosage level are extrapolations of 8-hour collections.

[2] Mean ± SD.

ually after initiation of the injections and only 11.8% of the intake appeared in the feces during the first week.

Table 2 shows the results of the respiratory studies. These were carried out at various times during the fifth and sixth weeks after initiation of the particular dosage in question. With thiamine intakes of 30, 50 and 100 μg/day, 24.0, 13.6 and 7.5% of the injected dose is excreted as radioactive carbon dioxide, respectively. The absolute excretion of $^{14}CO_2$ was relatively constant at the 3 levels of intake.

To study the rate of turnover of the urinary thiamine compounds, 6 rats were depleted for a period of 3 weeks by feeding a thiamine-deficient diet. The animals then received daily injections of 30 μg thiazole-$^{14}C$-labeled thiamine for 3 weeks. At the end of this period the injection of $^{14}C$-thiamine was stopped and daily injections of 30 μg of unlabeled thiamine were begun. The excretion of radioactivity in the urine was studied over the 32-day period starting with the last injection of the $^{14}C$-thiamine. Figure 1 shows that the rate of loss is a hyperbolic function with a half-life of about 9 days. The effect of thiamine deficiency on the urinary excretion of these compounds was studied in another group of rats whose body thiamine stores were labeled by daily injections of 30 μg of thiazole-$^{14}C$-labeled thiamine. Thiamine deficiency was then induced by discontinuing the thiamine injections. Measurement of the radioactivity in the urine of these animals gave a curve similar to that in figure 1, but the early part of the curve was steeper and the half-life of the radioactive components was about 6 days, 33% shorter than in the case of thiamine-sufficient rats.

A typical Amberlite CG-50 elution pattern of the radioactive metabolites of thiazole-$^{14}C$-labeled thiamine is shown in figure 2. In most experiments between 75 to 90% of the radioactivity placed on

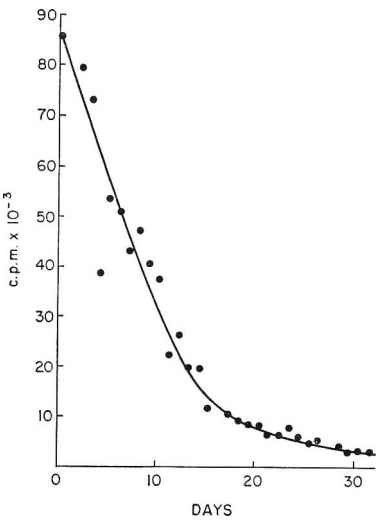


Fig. 1 The daily urinary radioactivity of rats receiving 30 μg of thiazole-$^{14}C$-labeled thiamine/day for 3 weeks when transferred to 30 μg of unlabeled thiamine/day. Each point represents the total radioactivity excreted in the 24-hour urine samples of 6 rats.

the column was recovered by liquid scintillation counting. The radioactive metabolites were resolved into 5 peaks. Table 3 shows the percentage of total radioactivity residing in each of the 5 peaks at the 30- and 50-μg levels of intake. More than 50% of the total radioactivity appears in the third peak. Peak IV (which contains thiamine) was found to contain an increasing percentage of the total radioactivity as the intake is increased.

Since it was known that the main component of peak I is a pyrimidine carboxylic acid (3) and since urinary pigments render studies of this peak difficult, this peak was not studied further. Peaks II, III, IV, and V were reduced in volume under vacuum at 40°, lyophylized, and dissolved in 0.5 ml of water. Ascending chromatography was then carried out for 18 hours on Whatman no. 40 filter paper in *n*-propanol/water/1 M acetate buffer, pH 5.0 (70/20/10 by volume). The dried paper was exposed to Eastman No-Screen x-ray film for 5 to 30 days depending upon the radioactivity of the sample. Figure 3 shows a schematic representation of the radioautograms with the 5 compounds of quantitative importance being accented with thick lines. The mean $R_F$ values of the 10 radioactive metabolites found by this method are also shown. These values varied considerably from one chromatogram to another because of the variable amounts of brown urinary pigments that were present. The latter appeared in rather large amounts principally in peaks II and III.

After the compounds were located on the paper by radioautography, their bio-

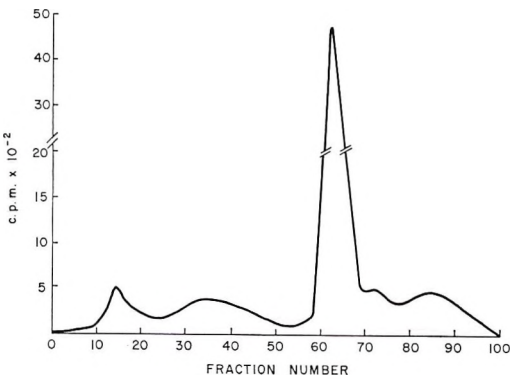


Fig. 2 Column chromatography of desalted rat urine on Amberlite CG-50 (200–400 mesh; $H^+$ form, 1 × 40 cm column). Flow rate 60 ml/hour, fraction size, 5 ml. The activity in counts per minute as determined by gas flow counting is that of 0.50-ml aliquots of the 5-ml fractions.

TABLE 3

*Distribution of radioactivity among the 5 peaks obtained from Amberlite CG-50 chromatography of desalted urine from rats receiving 30 or 50 μg of thiazole-$^{14}$C-labeled thiamine/day*

| Peak no. | Daily intake of thiamine [1] | |
|---|---|---|
| | 30 μg/day | 50 μg/day |
| | *% of total urinary radioactivity* | |
| I | 7.0 ± 1.2 [2] | 9.3 ± 3.3 |
| II | 12.9 ± 1.0 | 10.7 ± 1.2 |
| III | 63.2 ± 1.7 | 53.2 ± 4.9 |
| IV | 5.6 ± 0.9 | 19.9 ± 5.9 |
| V | 11.0 ± 1.7 | 6.5 ± 5.9 |

[1] The values at the 30 μg intake level are the average of 5 experiments, each experiment representing the metabolites from a pooled one-week urine collected from 16 rats. The values at the 50-μg intake levels are the average of 6 such experiments.
[2] SD.

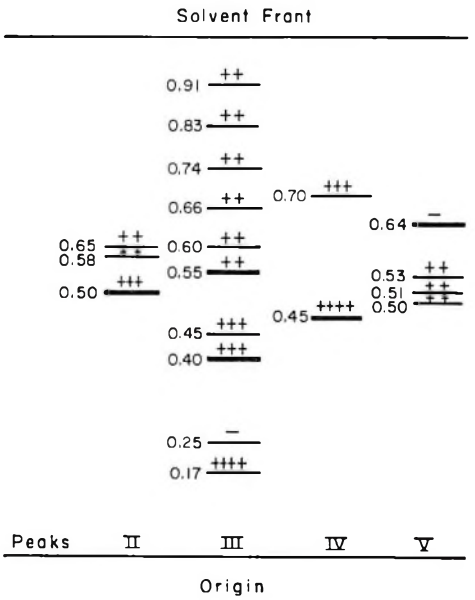


Fig. 3 Schematic drawing of radioautograms of radioactive peaks seen in figure 2. Solvent *n*-propanol/water/1 M acetate buffer pH 5.0 (70/20/10 by volume). Ascending technique for 18 hours. Chromatograms were exposed to the x-ray film for 10 days. The thick lines represent the main components of each peak. The mean $R_F$ values are shown to the right of each band and the biological activity for *L. viridescens* is shown on the left. (++++ extensive growth; +++ good growth; ++ slight growth, − no growth.)

logical activity was checked by bioautography. The results of these studies are also shown in arbitrary growth units in figure 3. All of the 10 metabolites except the metabolite with the $R_F$ of 0.25 in peak III and the metabolite of $R_F$ 0.64 in peak V showed traces of biological activity for *L. viridescens*. Tube assay of the urine, however, indicated that the percentage of microbiological activity was no more than 2% of that found by measurement of radioactivity.

## DISCUSSION

As the dosage of thiamine was increased, the amount of radioactivity appearing in the urine also increased (table 1). For example, when the daily thiamine intake was increased from 30 to 100 μg the amount of the radioactivity appearing in urine increased from 29 to 42% of intake. Under these conditions the metabolite in urine that increased disproportionately with the dosage was thiamine itself (table 3), i.e., when the daily thiamine intake was increased from 30 to 50 μg, the percentage of total urinary radioactivity in peak 4 (which contains thiamine) increased 3 times (from 5.6 to 19.9%). This observation agrees with other data reported from this laboratory (6) and is consistent with the concept that urinary free thiamine represents an amount in excess of the minimal physiological needs of the animal.

Iacono and Johnson (8) reported that 16 urinary metabolites of thiazole-$^{14}C$-labeled thiamine were excreted by the rat. In this study using a more elaborate separation procedure we have detected 19 in addition to those present in peak I of the Amberlite CG-50 column. Had the latter been considered, our total would have exceeded 20, i.e., comparable to the number found in our previous studies with pyrimidine $^{14}C$-labeled thiamine (1). The data of Iacono and Johnson (8) also indicate that three of their radioactive bands contained the bulk of the radioactivity. This is in reasonable agreement with our observation of 5 major radioactive metabolites because it is likely that their major radioactive bands contained more than one compound.

The breakdown of the thiazole ring as evidenced by the generation of $^{14}CO_2$ is in accord with other reports in the literature. Borsook et al. (7) studied the metabolism of $^{35}S$-labeled thiamine in man and found up to 25% of the intake as inorganic sulfate in the urine. Iacono and Johnson (8) recovered 0.2 to 3.7% of a 1-mg dose of thiazole-$^{14}C$-labeled thiamine as respiratory $^{14}CO_2$. The quantitative aspects of the data reported by these 2 groups of workers cannot, however, be compared with those recorded here since their data were obtained in acute studies, whereas ours were derived from long-term studies at physiological levels of thiamine intake. In these studies, with daily thiamine intakes of 30, 50 and 100 μg, 24%, 13.6% and 7.5% of the radioactivity was excreted as $^{14}CO_2$ and the absolute values for radioactive $CO_2$ excreted were equivalent to 7.1, 6.8 and 7.5 μg of thiamine, respectively.

These data indicate that the thiazole ring of thiamine is considerably more labile than when it exists as the free thiazole moiety. The latter is quite stabile metabolically as shown by the experiments of Imai et al. (9). These workers injected $^{35}S$-labeled 4-methyl-5-(2 hydroxyethyl) thiazole into rats and recovered more than 95% of the dose as 4-methylthiazole-5-acetic acid, a derivative of the intact thiazole ring. This stability difference can be demonstrated in vitro as well, i.e., a pH of 8.2 is sufficient to open the thiazole ring of thiamine to give a thiol form which readily oxidizes to the disulfide in air at room temperature. Under similar conditions, the free thiazole molecule remains intact.

According to currently accepted theory, the carbon in the 2-position of the thiazole ring is the active metabolic site of the thiamine molecule. Because, in our studies, the absolute amount of thiamine destroyed by oxidation of this carbon atom to $^{14}CO_2$ approximates the minimum thiamine requirement of the rat (10) (7–8 μg) it is tempting to suggest that this oxidative loss may be a measure of the "endogenous" thiamine utilization (minimal requirement?) of the rat. Our data suggest further that this oxidation may be independent of thiamine intake. Whether it is also independent of body stores cannot be

decided since no estimates of the latter were made.

The pattern of urinary radioactivity obtained by column chromatography on CG-50 (fig. 2) strongly resembles that reported by Neal and Pearson (1) in similar studies with pyrimidine-$^{14}$C-labeled thiamine. Since the pyrimidine ring is not broken down to form $CO_2$ it would be anticipated that a higher percentage of radioactivity from thiamine containing $^{14}$C in the pyrimidine ring would appear in urine than from thiazole-$^{14}$C-labeled thiamine. This has actually been observed by Neal and Pearson (unpublished data) who recovered 76 to 81% of the injected radioactivity from the urine of rats that had received 100 μg of pyrimidine-labeled thiamine daily for a long period. In these studies which were carried out with thiazole-$^{14}$C-labeled thiamine under essentially equivalent experimental conditions the recovery of radioactivity from urine was only about 40 to 45%.

The marked similarities of the urinary chromatographic pattern obtained with both thiazole-$^{14}$C- and pyrimidine-$^{14}$C-labeled thiamine suggest that the large number of thiamine metabolites found in urine by Neal and Pearson (1) may be derivatives of the entire thiamine molecule rather than metabolic products of its pyrimidine or thiazole moieties. Furthermore, the marked lability of the thiazole ring observed in this study makes it highly unlikely that free thiazole or its derivatives occur in any quantity in rat urine.

## LITERATURE CITED


1. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. I. Metabolic products found in urine. J. Nutrition, *83:* 343.
2. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. II. Isolation and identification of 2-methyl-4-amino-5-pyrimidinecarboxylic acid as a metabolite of thiamine in rat urine. J. Nutrition, *83:* 351.
3. Balaghi, M., and W. N. Pearson 1966 Tissue and intracellular distribution of radioactive thiamine in normal and thiamine-deficient rats. J. Nutrition, *89:* 127.
4. Jeffay, H., and J. Alvarez 1961 Ethanolamine as $C^{14}O_2$ absorbant. Analyt. Chem., *33:* 612.
5. Deibel, R. H., J. B. Evans and C. F. Niven, Jr. 1957 Microbiological assay for thiamine using *Lactobacillus viridescens*. J. Bacteriol., *74:* 818.
6. Pearson, W. N., E. Hung, W. J. Darby, Jr., M. Balaghi and R. A. Neal 1966 Excretion of metabolites of $^{14}$C-pyrimidine-labeled thiamine by the rat at different levels of thiamine intake. J. Nutrition, *89:* 133.
7. Borsook, H., E. R. Buchman, J. B. Hatcher, D. M. Yost and E. McMillan 1940 The course of thiamine metabolism in man as indicated by the use of radioactive sulfur. Proc. Nat. Acad. Sci., *26:* 412.
8. Iacono, J. M., and B. C. Johnson 1957 Thiamine metabolism. I. The metabolism of thiazole-2-$C^{14}$ thiamine in rat. J. Am. Chem. Soc., *79:* 6321.
9. Imai, Y., Z. Suzuoki and A. Kobata 1960 Metabolism of 4-methyl-5-β-hydroxyethylthiazole-$S^{35}$ in rats. J. Biochem., *48:* 341.
10. Scott, E. M., and I. V. Griffith 1957 A comparative study of thiamine-sparing agents in the rat. J. Nutrition, *61:* 421.

# Importance of an Accurate Reference Diet in the Evaluation of Proteins for Chick Growth using Plasma Amino Acid Titers [1,2]

R. E. SMITH [3]
*Canada Department of Agriculture, Research Branch, Nappan, Nova Scotia*

*ABSTRACT* Chick growth trials were conducted which established that lysine was the only limiting amino acid in sunflower meal. Plasma amino acid titers were determined on chicks fed sunflower meal and on chicks fed 2 crystalline amino acid mixtures which were shown to support equal rates of growth. One of the amino acid mixtures had 5 essential amino acids at levels greater than the other and hence could be considered in excess of the chicks' requirement for growth. A lower amino acid titer in the plasma of chicks fed the sunflower meal than in chicks fed the reference diet was taken as an indication of a deficiency of this amino acid in the intact protein. Using the amino acid mixture containing the excess amino acids as a reference, sunflower meal appeared to be limiting in several amino acids. When the other mixture was used as a reference, only lysine appeared to be limiting. The use of plasma titers to evaluate proteins by this method therefore depends on a reference diet which contains neither deficient nor excessive levels of amino acids.

Previous reports in this series described a method of using free amino acid levels in blood plasma to predict the adequacy of intact proteins for chick growth (1, 2). The method accurately predicted those amino acid deficiencies in the test protein which could be readily revealed in growth trials, but also indicated the presence of certain amino acid deficiencies which could not be so demonstrated.

It was suggested that these false predictions could be attributed to the use of a reference diet (3) which contained several amino acids at levels in excess of the chick's requirement. A revised reference diet has since been formulated (4) which contains five of the essential amino acids at lower levels. The present study was conducted to compare the 2 reference diets, using sunflower meal as the test protein.

## EXPERIMENTAL

A series of growth trials was conducted to determine the limiting amino acids in sunflower meal (exps. 1–3). Sunflower meal with and without supplemental lysine was then compared with the 2 reference amino acid mixtures in a growth trial (exp. 4) and in a plasma amino acid study (exp. 5).

TABLE 1
*Composition of the basal diets*

| | % |
|---|---|
| Basal diet A | |
| Glucose [1] | 50.74 |
| Sunflower meal [2] | 42.79 |
| Salts 59A [3] | 5.27 |
| Corn oil | 1.00 |
| Choline chloride | 0.20 |
| Vitamins (2.0 g/kg) [4] | + |
| Penicillin (11 mg/kg) | + |
| Total | 100.00 |
| Basal diet B | |
| Cornstarch | var. |
| Nitrogen source | var. |
| Corn oil | 15.00 |
| Salt mixture [5] | 5.37 |
| Cellulose [6] | 3.00 |
| $NaHCO_3$ | 1.00 |
| Choline chloride | 0.20 |
| Vitamins (2.0 g/kg) [4] | + |
| Total | 100.00 |

[1] Cerelose, Corn Products Company, New York.
[2] Equivalent to 18.00% crude protein in the diet.
[3] Salt mixture as a percentage of the total diet: $CaCO_3$, 2.166; $KH_2PO_4$, 1.05; $CaHPO_4 \cdot 2H_2O$, 0.94; NaCl, 0.8; $MgSO_4$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.02; $ZnCO_3$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.002; KI, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.001. Total, 5.27.
[4] Klain et al. (6).
[5] Klain et al. (6) ($ZnCO_3$ substituted for $ZnCl_2$ in salt mixture).
[6] Solka Floc, Brown Company, Chicago.

Received for publication January 8, 1966.

[1] Paper no. 3 on the use of free amino acid concentrations in blood plasma in evaluating the amino acid adequacy of intact proteins for chick growth.
[2] Contribution no. 193, Department of Animal and Poultry Science.
[3] Present address: Animal Research Institute, Central Experimental Farm, Ottawa, Ontario.

TABLE 2

*The supplementation with lysine of sunflower meal when fed as the sole source of protein to growing chicks*

| Exp. no. | Diet no. | Supplement to sunflower meal-glucose basal diet A [1] | Gain | Gain Feed |
|---|---|---|---|---|
| | | | *g* | |
| 1 | 1 | None | 147 [2] | 0.44 |
| | 2 | 0.20% L-lysine | 183 | 0.53 |
| | 3 | 0.40% L-lysine | 174 | 0.50 |
| | 4 | 0.60% L-lysine | 183 | 0.53 |
| | 5 | 0.80% L-lysine | 179 | 0.53 |
| | 6 | 1.00% L-lysine | 179 | 0.53 |
| 2 | 1 | 0.25% L-lysine | 199 [2] | 0.54 |
| | 2 | As 1 + 0.10% DL-methionine | 203 | 0.55 |
| | 3 | As 1 + 0.20% DL-methionine | 195 | 0.55 |
| | 4 | As 1 + 0.30% DL-methionine | 197 | 0.56 |
| | 5 | As 1 + 0.40% DL-methionine | 201 | 0.54 |
| | 6 | As 1 + 0.50% DL-methionine | 199 | 0.56 |
| 3 | 1 | 0.25% L-lysine + 0.10% DL-methionine | 243 [3] | 0.55 |
| | 2 | As 1 + 0.05% L-histidine | 239 | 0.55 |
| | 3 | As 1 + 0.10% L-histidine | 238 | 0.55 |
| | 4 | As 1 + 0.15% L-histidine | 241 | 0.56 |
| | 5 | As 1 + 0.20% L-histidine | 238 | 0.53 |
| | 6 | As 1 + 0.25% L-histidine | 251 | 0.56 |

[1] Table 1.
[2] Average gain of 10 chicks in each of 3 replicates from 7 to 19 days of age.
[3] Average gain of 10 chicks in each of 3 replicates from 7 to 21 days of age.

*Experiments 1–3.* The basal diet used in experiments 1–3 is shown in table 1 as basal diet A. In experiment 1, this basal diet was supplemented with graded levels of lysine, added at the expense of glucose. Each diet was fed to 30 chicks in 3 replicates of 10 chicks each for a period of 12 days commencing when the chicks were 7 days of age.

Maximal growth occurred at the 0.20% level of added lysine (table 2) in this experiment. There was no further growth response when methionine was added on top of 0.25% lysine (exp. 2), or when histidine was added in addition to both lysine and methionine (exp. 3).

Thus lysine appears to be the only amino acid in sunflower meal which can be shown as limiting for chick growth by growth trials.

*Experiment 4.* Experiment 4 was designed to test the growth-promoting potential of sunflower meal and sunflower meal plus lysine as compared with that of the 2 reference amino acid mixtures shown in table 3. Each amino acid source was incorporated into basal diet B (table 1) and fed to 9 chicks in 3 replicates of 3 chicks for the period from 8 to 14 days of age (table 4).

The sunflower meal provided the same level of crude protein (18.34%) as the original reference amino acid mixture. The revised amino acid mixture provided only 17.61% calculated crude protein (N × 6.25).

TABLE 3

*Composition of reference amino acid mixtures*

| | Amino acid mixtures | |
|---|---|---|
| | Original | Revised |
| | % | % |
| L-Arginine·HCl | 1.33 | 1.33 |
| L-Histidine·HCl·$H_2O$ | 0.62 | 0.41 |
| L-Lysine·HCl | 1.40 | 1.19 |
| L-Tyrosine | 0.63 | 0.63 |
| L-Tryptophan | 0.225 | 0.225 |
| L-Phenylalanine | 0.68 | 0.68 |
| DL-Methionine | 0.55 | 0.35 |
| L-Cystine | 0.35 | 0.35 |
| L-Threonine | 0.85 | 0.65 |
| L-Leucine | 1.20 | 1.20 |
| L-Isoleucine | 0.80 | 0.80 |
| L-Valine | 1.04 | 0.82 |
| Glycine | 1.60 | 1.60 |
| L-Glutamic acid | 12.00 | 12.00 |
| L-Proline | 1.00 | 1.00 |
| Total | 24.275 | 23.235 |

Chick growth with the revised amino acid mixture was equivalent to that with the original amino acid mixture, indicating that the latter did in fact contain excess levels of at least the 5 amino acids which were altered (table 4). The poor growth with the sunflower meal and the marked response to lysine supplementation, confirmed the results of experiment 1.

*Experiment* 5. In experiment 5, plasma amino acid titers were determined on chicks fed diets similar to those fed in experiment 4. For this purpose, each diet was fed to 8 individually caged chicks by offering 0.8 g diet/chick every 30 minutes for a period of 6 hours. Thirty minutes after the final feeding, blood samples were taken and pooled for analysis. Pretreatment training of the chicks and details of the feeding, blood sampling and analytical procedures are described in a previous publication (1).

Chicks fed the revised reference mixture had lower plasma levels of nearly all amino acids than chicks fed the original reference mixture (table 5). As might be expected, the greatest differences in plasma pattern occurred with those amino acids present at lower levels in the revised diet. The reduction in all amino acid levels of the plasma implies a

TABLE 4

*Growth of chicks fed 4 diets differing in amino acid sources and composition (exp. 4)*

| Diet no. | Description of diet | Gain/chick/day [1] | Gain/Feed |
|---|---|---|---|
| | | *g* | |
| 1 | Original reference diet [2] | 14.4 [a] | 0.70 |
| 2 | Revised reference diet [3] | 14.0 [a] | 0.63 |
| 3 | Sunflower meal [4] | 12.6 [b] | 0.54 |
| 4 | Sunflower meal + 0.25% L-lysine [5] | 17.3 [c] | 0.65 |

[1] Means are not significantly different ($P < 0.05$) if followed by the same superscript letter.
[2] Basal diet B (table 1) + original amino acid mixture (table 3); provides 18.34% crude protein.
[3] Basal diet B (table 1) + revised amino acid mixture (table 3); provides 17.61% crude protein.
[4] Basal diet B (table 1) + sunflower meal to provide the equivalent of 18.34% crude protein.
[5] As footnote 4 plus lysine.

TABLE 5

*Free amino acid levels in chick blood plasma resulting from the feeding of 2 crystalline amino acid reference diets and diets containing sunflower meal with and without supplemental* L-*lysine* [1]

| | | Original reference diet (A) | Revised reference diet (B) | Sunflower meal (C) | Sunflower meal + 0.25% L-lysine (D) |
|---|---|---|---|---|---|
| | | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* |
| 1 | Threonine | 139.1 | 90.0 | 95.5 | 91.8 |
| 2 | Valine | 42.5 | 9.9 | 36.3 | 26.1 |
| 3 | Cystine | 18.9 | 14.3 | 16.3 | 12.0 |
| 4 | Methionine [2] | 23.0 | 13.8 | 13.7 | 12.5 |
| 5 | Isoleucine | 9.0 | 7.2 | 16.9 | 12.0 |
| 6 | Leucine | 10.2 | 8.5 | 16.2 | 9.6 |
| 7 | Tyrosine | 11.8 | 12.7 | 18.2 | 13.2 |
| 8 | Phenylalanine | 7.1 | 7.4 | 15.7 | 14.1 |
| 9 | Lysine | 33.5 | 24.6 | 10.5 | 24.6 |
| 10 | Histidine [3] | 21.0 | 13.0 | 22.9 | 12.8 |
| 11 | Arginine | 28.5 | 22.0 | 71.0 | 58.6 |
| 12 | Serine | 54.0 | 57.3 | 40.7 | 43.5 |
| 13 | Proline | 38.6 | 32.6 | 23.0 | 16.7 |
| 14 | Glutamic acid | 45.5 | 49.2 | 22.8 | 16.8 |
| 15 | Glycine | 74.6 | 71.6 | 34.4 | 27.6 |
| 16 | Alanine | 188.3 | 177.2 | 46.1 | 37.7 |

[1] See footnotes table 4 for description of diets.
[2] Corrected for methionine sulfoxides.
[3] Corrected for 1-methyl- and 3-methyl-histidine.

better utilization of all amino acids when none are present in excess.

Plasma amino acid levels with the sunflower meal diets have been expressed as a percentage of those with each reference diet in table 6. Compared with the original reference diet, sunflower meal gave lower plasma levels of a number of amino acids, suggesting that sunflower meal is deficient in these amino acids. However, when compared with the revised reference diet the only apparent deficiency in sunflower meal was lysine. This points up the importance of the reference diet in evaluating proteins by this method.

When the sunflower meal was supplemented with lysine, there was a general lowering of the plasma levels of most of the other amino acids so that there were more "apparent" deficiencies after supplementation than before. Thus, correction of a deficiency has the same effect in promoting better utilization of the plasma amino acids as does removal of excess amino acids. As would be expected, comparison with the original reference mixture showed more apparent deficiencies than comparison of the supplemented sunflower meal with the revised mixture. In the latter case, only the sulphur amino acids appeared to be deficient.

## DISCUSSION

Changing the composition of the reference diet markedly improved the accuracy of the prediction of amino acid deficiencies in the intact protein of sunflower meal. It is clear that the presence of excess amino acids in the reference diet can give rise to false predictions of deficiencies in the test protein as suggested earlier (1, 2).

The fact that neither reference diet supported chick growth equal to that with the supplemented sunflower meal diet suggests that more information is needed on the utilization of intact protein diets versus crystalline amino acid diets for chick growth.

The plasma amino acid technique was originally conceived as a means of evaluating the amino acid adequacy of intact proteins. It is now apparent that it may be just as valuable in assessing the amino acid requirements of the chick. When the plasma amino acid titers of chicks fed a test protein indicate deficiencies in the

TABLE 6

*Comparisons of the data from table 5 expressing the free amino acid levels of the chick blood plasma as a percentage of each of the reference standards*

| | | Original reference diet [1] | | Revised reference diet [2] | |
|---|---|---|---|---|---|
| | | Sunflower meal [3] | Sunflower meal [3] + 0.25% L-lysine | Sunflower meal [3] | Sunflower meal [3] + 0.25% L-lysine |
| | | C/A × 100 | D/A × 100 | C/B × 100 | D/B × 100 |
| 1 | Threonine | 69 | 66 | 106 | 102 |
| 2 | Valine | 85 | 61 | 367 | 264 |
| 3 | Cystine | 86 | 63 | 114 | 84 |
| 4 | Methionine [4] | 60 | 54 | 99 | 91 |
| 5 | Isoleucine | 188 | 133 | 235 | 167 |
| 6 | Leucine | 159 | 94 | 191 | 113 |
| 7 | Tyrosine | 154 | 112 | 143 | 104 |
| 8 | Phenylalanine | 221 | 199 | 212 | 191 |
| 9 | Lysine | 31 | 73 | 43 | 100 |
| 10 | Histidine [5] | 109 | 61 | 176 | 98 |
| 11 | Arginine | 249 | 206 | 323 | 266 |
| 12 | Serine | 75 | 81 | 71 | 76 |
| 13 | Proline | 60 | 43 | 71 | 51 |
| 14 | Glutamic acid | 50 | 37 | 46 | 34 |
| 15 | Glycine | 46 | 37 | 48 | 39 |
| 16 | Alanine | 24 | 20 | 26 | 21 |

[1] Calculated using the original reference diet as a standard.
[2] Calculated using the revised reference diet as a standard.
[3] Sunflower meal equivalent to 18.34% crude protein.
[4] Corrected for methionine sulfoxides.
[5] Corrected for 1-methyl- and 3-methyl-histidine.

protein which are known not to exist, it indicates that the reference amino acid mixture is at fault. This use of plasma amino acids as a measure of amino acid requirements was suggested by Almquist (5) some years ago.

LITERATURE CITED

1. Smith, R. E., and H. M. Scott 1965 Use of free amino acid concentrations in blood plasma in evaluating the amino acid adequacy of intact proteins for chick growth. I. Free amino acid patterns of blood plasma of chicks fed unheated and heated fishmeal proteins. J. Nutrition, 86: 37.
2. Smith, R. E., and H. M. Scott 1965 Use of free amino acid concentrations in blood plasma in evaluating the amino acid adequacy of intact proteins for chick growth. II. Free amino acid patterns of blood plasma of chicks fed sesame and raw, heated, and overheated soybean meals. J. Nutrition, 86: 45.
3. Dean, W. F., and H. M. Scott 1962 The development of an amino acid standard for the early growth of chicks. Poultry Sci., *41:* 1640.
4. Dean, W. F., and H. M. Scott 1965 The development of an amino acid reference diet for the early growth of chicks. Poultry Sci., *44:* 803.
5. Almquist, H. J. 1956 Amino Acid Handbook, eds., R. J. Block and K. W. Weiss. Charles C Thomas, Springfield, Illinois, pp. 152–155.
6. Klain, G. J., H. M. Scott and B. C. Johnson 1958 The amino acid requirement of the growing chick fed crystalline amino acids. Poultry Sci., 37: 976.

# Use of α-Aminoisobutyric Acid as an Internal Indicator in Chick Plasma Amino Acid Studies [1,2]

R. E. SMITH [3]
*Canada Department of Agriculture, Research Branch, Nappan, Nova Scotia*

*ABSTRACT* α-Aminoisobutyric acid (AIBA), a non-metabolizable amino acid, was incorporated into the diets of one-week-old chicks to serve as an indicator of amino acid absorption. The levels of AIBA and of the metabolizable amino acids in the plasma were subsequently determined. Plasma concentrations of AIBA were found to be proportional to dietary levels fed. In general the indicator revealed that imbalanced diets were not as rapidly absorbed as balanced diets. Relating the metabolizable amino acid uptake to that of the indicator improved the precision with which known amino acid deficiencies in both crystalline amino acid and intact protein diets could be predicted by the plasma amino acid method described.

In our original communication on plasma amino acid titers as indicators of protein quality (1) it was emphasized that valid comparisons between the reference and test diets could be achieved only under conditions of equal feed intake and after absorption had reached a steady state. To ensure these conditions, the chicks were trained to consume 0.8 g of feed every 30 minutes for a period of 6 hours prior to blood sampling.

However, in subsequent investigations (2, 3) it was observed that diets with amino acid deficiencies tended to accumulate in the crop in greater quantities than balanced diets. This was taken as an indication that the diets were not reaching the absorptive areas of the intestinal tract at equal rates.

It was reasoned that if an indicator were added to each diet, its level in the plasma would provide a relative measure of the amounts of feed reaching the small intestine. The indicator selected was α-aminoisobutyric acid (AIBA), which has been shown to be a non-metabolizable amino acid (4, 5 and 6).

The experiments reported here were conducted to determine whether the use of an indicator in this manner would improve the precision of the plasma amino acid method of evaluating proteins, and whether the indicator selected was suitable for the purpose.

## EXPERIMENTAL

Three of the four experiments described below were plasma amino acid studies, and one was a growth trial. In the growth trial, each diet was fed ad libitum to 3 replicates of 3 chicks each for the 6-day period from 8 to 14 days of age. For the plasma amino acid studies, the diets were fed individually to 8 chicks in equal allotments every 30 minutes for 6 hours. Blood samples were taken 30 minutes after the final feeding. Pre-trial training of the chicks and details of the feeding, blood sampling and analytical procedures are described in a previous publication (1).

*Experiment 1.* To establish the suitability of AIBA as an indicator, it was necessary first to determine whether its level in the plasma reflected the level present in the diet, and second, whether it interfered with the absorption of other amino acids. These points were checked in the first experiment, in which graded levels of AIBA were added to the reference diet. Plasma amino acid titers on these diets are shown in table 1.

α-Aminoisobutyric acid appeared in the plasma in amounts which were propor-

Received for publication January 8, 1966.

[1] Paper no. 4 on the use of free amino acid concentrations in blood plasma in evaluating the amino acid adequacy of intact proteins for chick growth.

[2] Contribution no. 194, Division of Animal and Poultry Science.

[3] Present address: Animal Research Institute, Central Experimental Farm, Ottawa, Ontario.

TABLE 1

*Free amino acid levels in blood plasma of chicks fed a crystalline amino acid reference diet* [1] *containing various levels of α-aminoisobutyric acid (exp. 1)*

| | | Level of α-aminoisobutyric acid, % | | | |
|---|---|---|---|---|---|
| | | 0.0 | 0.5 | 1.0 | 1.5 |
| | | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* |
| 1 | Threonine | 165.8 | 180.2 | 219.8 | 205.5 |
| 2 | Valine | 47.5 | 47.8 | 41.0 | 56.7 |
| 3 | Cystine | 20.2 | 26.1 | 21.6 | 23.6 |
| 4 | Methionine [2] | 31.6 | 29.1 | 24.9 | 36.6 |
| 5 | Isoleucine | 19.9 | 19.9 | 19.8 | 19.5 |
| 6 | Leucine | 11.7 | 14.7 | 9.0 | 18.8 |
| 7 | Tyrosine | 14.5 | 14.2 | 14.7 | 15.0 |
| 8 | Phenylalanine | 10.5 | 13.3 | 12.0 | 13.7 |
| 9 | Lysine | 53.6 | 81.1 | 60.8 | 65.7 |
| 10 | Histidine [3] | 24.5 | 34.7 | 37.3 | 44.0 |
| 11 | Arginine | 39.1 | 45.9 | 33.6 | 50.2 |
| 12 | Serine | 62.4 | 68.5 | 67.3 | 71.7 |
| 13 | Proline | 47.2 | 57.8 | 50.9 | 87.7 |
| 14 | Glutamic acid | 46.2 | 31.3 | 25.6 | 52.4 |
| 15 | Glycine | 88.6 | 89.6 | 84.5 | 117.1 |
| 16 | Alanine | 176.5 | 158.3 | 151.4 | 215.3 |
| 17 | AIBA [4] | 0.0 | 23.0 | 52.4 | 80.7 |

[1] Smith, R. E. (3).
[2] Corrected for methionine sulfoxides.
[3] Corrected for 1-methyl- and 3-methyl-histidine.
[4] AIBA denotes α-aminoisobutyric acid.

tional to the levels present in the diet. Even though plasma levels varied widely in the case of a few amino acids, the variation was not related to the level of AIBA in the diet. There was, therefore, no indication that AIBA interfered with absorption of other amino acids. Thus it appeared that AIBA met the stipulated requirements of a suitable indicator.

*Experiment 2.* Experiment 2 was designed to show whether the use of AIBA as an indicator would improve the precision of the plasma amino acid method in evaluating proteins with single amino acid deficiencies. Sesame meal and soybean meal are two such proteins known to be deficient in lysine and methionine, respectively.

Diets were prepared in which sesame meal and soybean meal replaced the amino acid mixtures of the reference diets. The test diets were fed with and without the appropriate limiting amino acid. Both the original and the revised reference diets were used in this experiment (3), and 0.50% AIBA was added to all diets.

Compared with the original reference diet, sesame meal appeared to be deficient in 4 essential amino acids in addition to lysine (table 2). Similarly soybean meal appeared to be deficient in a number of amino acids in addition to methionine. Supplementation of the proteins with their respective limiting amino acids increased rather than decreased the number of "apparent" deficiencies.

Using the revised reference diet as the standard for comparison, there were fewer apparent deficiencies in all cases, but a number remained which cannot be demonstrated in growth trials. A possible explanation for these anomalous indications of amino acid deficiencies may lie in the low AIBA titers for the test diets as compared with the reference diets. This was interpreted to mean that absorption of the test diets was occurring at a slower rate.

In table 3 the amino acid titers of each treatment in table 2 were converted to a per 100 μg/ml AIBA-basis and then expressed as a percentage of those of the revised reference diet. Expressed in this way, only lysine appears to be deficient in the sesame meal diet, and only the sulfur amino acids in the soybean meal

diet. In both diets the deficiencies disappear with adequate amino acid supplementation.

*Experiment 3.* The proteins tested in the previous experiment were limited to those with a single amino acid deficiency. A more stringent test of the plasma amino acid method of evaluating proteins is posed by diets with more than one amino acid deficiency. With this in mind a growth trial was conducted with diets having single and multiple amino acid

TABLE 2

*Free amino acid levels in blood plasma of chicks fed reference and test diets (exp. 2)*

| | | Original reference diet [1] | Revised reference diet [1] | Sesame meal [2] | Sesame + 0.55% L-lysine | Soybean meal [2] | Soybean + 0.24% DL-methionine |
|---|---|---|---|---|---|---|---|
| | | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* | *μg/ml plasma* |
| 1 | Threonine | 165.6 | 111.3 | 104.2 | 57.1 | 81.7 | 67.2 |
| 2 | Valine | 55.0 | 20.3 | 29.2 | 22.4 | 22.6 | 15.2 |
| 3 | Cystine | 24.8 | 18.5 | 26.8 | 14.7 | 4.2 | 10.4 |
| 4 | Methionine [3] | 36.4 | 32.8 | 25.8 | 22.3 | 6.8 | 14.5 |
| 5 | Isoleucine | 17.7 | 19.4 | 13.2 | 9.4 | 14.4 | 10.6 |
| 6 | Leucine | 15.1 | 16.5 | 18.2 | 13.0 | 17.8 | 12.9 |
| 7 | Tyrosine | 9.7 | 12.7 | 22.2 | 18.9 | 9.9 | 13.6 |
| 8 | Phenylalanine | 10.4 | 11.0 | 14.8 | 13.6 | 16.1 | 13.1 |
| 9 | Lysine | 61.6 | 43.5 | 10.8 | 20.1 | 46.1 | 43.3 |
| 10 | Histidine [4] | 28.0 | 14.4 | 30.8 | 20.1 | 18.4 | 21.2 |
| 11 | Arginine | 43.7 | 46.3 | 102.8 | 114.3 | 58.7 | 51.5 |
| 12 | Serine | 69.2 | 66.7 | 61.2 | 47.9 | 84.3 | 58.5 |
| 13 | Proline | 55.1 | 55.2 | 37.8 | 30.8 | 29.3 | 24.0 |
| 14 | Glutamic acid | 74.4 | 67.9 | 14.8 | 14.9 | 19.6 | 15.0 |
| 15 | Glycine | 114.9 | 123.4 | 34.8 | 26.2 | 31.0 | 26.3 |
| 16 | Alanine | 185.8 | 208.1 | 44.8 | 37.3 | 53.3 | 39.9 |
| 17 | AIBA [5] | 29.5 | 33.4 | 14.8 | 11.6 | 17.0 | 14.2 |

[1] Smith, R. E. (3).
[2] Equivalent to 18.34% C.P.
[3] Corrected for methionine sulfoxides.
[4] Corrected for 1-methyl- and 3-methyl-histidine.
[5] α-Aminoisobutyric acid (0.50%) added to all diets.

TABLE 3

*Amino acid levels in test diets corrected for absorption rate and expressed as a percentage of the revised reference diet [1] (exp. 2)*

| | | Sesame meal | Sesame + 0.55% L-lysine | Soybean meal | Soybean + 0.24% DL-methionine |
|---|---|---|---|---|---|
| 1 | Threonine | 211 | 148 | 144 | 142 |
| 2 | Valine | 325 | 318 | 219 | 176 |
| 3 | Cystine | 327 | 229 | 45 | 132 |
| 4 | Methionine | 178 | 196 | 41 | 104 |
| 5 | Isoleucine | 154 | 139 | 146 | 128 |
| 6 | Leucine | 249 | 227 | 212 | 184 |
| 7 | Tyrosine | 395 | 429 | 153 | 252 |
| 8 | Phenylalanine | 304 | 356 | 288 | 281 |
| 9 | Lysine | 56 | 133 | 208 | 234 |
| 10 | Histidine | 482 | 401 | 251 | 345 |
| 11 | Arginine | 501 | 711 | 249 | 262 |
| 12 | Serine | 207 | 207 | 248 | 206 |
| 13 | Proline | 155 | 161 | 104 | 102 |
| 14 | Glutamic acid | 49 | 63 | 57 | 52 |
| 15 | Glycine | 64 | 61 | 49 | 50 |
| 16 | Alanine | 49 | 52 | 50 | 45 |
| 17 | AIBA | — | — | — | — |

[1] The absolute values of each treatment in table 2 were converted to a per 100 μg/ml AIBA basis, then were expressed as a percentage of those values of the revised reference diet.

deficiencies. A description of the diets and the growth data are shown in table 4.

The revised reference diet served as a control, and was modified to produce diet 2, which was deficient in lysine, diet 3, which was limiting first in lysine and second in valine, and diet 4 which was deficient in valine alone. Casein isonitrogenously replaced the amino acid mixture of the reference diet to give diet 5. Casein has been shown to be limiting first in arginine, then glycine, and then methionine (7). In diet 6 the casein was supplemented with these amino acids as well as with histidine. The last 2 diets were included in the trial to determine the effect of AIBA on growth rate when added at levels of 0.25% and 0.50%.

Growth rates with diets 2, 3, 4 and 5 were all below the rate with the reference diet, indicating deficiencies of one or more amino acids in each case. Lysine, as planned, proved to be more deficient than valine in the crystalline amino acid diets. Growth rate with the casein diet was particularly poor because of its deficiencies, but these were largely corrected by amino acid supplementation in diet 6. Growth with the last 2 diets indicated that AIBA has no influence on growth at the 0.25% level. There may have been some growth depression when 0.50% was added.

*Experiment 4.* In this experiment the first 6 diets of the preceding growth trial were supplemented with 0.50% AIBA and fed to chicks for the purpose of determining plasma amino acid titers. The resultant plasma amino acid levels are shown in table 5.

Those amino acids known to be deficient in the test diets were in every case present at low levels in the plasma, i.e., less than the level for the corresponding amino acid on the reference diet. Where there was more than one deficient amino acid (diets 3 and 5) the plasma titers indicated the order in which they were limiting. However, in all cases except diet 5, plasma titers indicated deficiencies in addition to those known to be present.

Plasma levels of AIBA were lower for all test diets than for the reference diet, but did not differ greatly among the test diets. This suggested that the test diets were again being absorbed at a slower rate than the reference diet.

In an attempt to correct for these presumed differences in rate of absorption, all plasma amino acid levels were converted to a per 100 unit AIBA-basis and then expressed as a percentage of the reference levels in table 6.

Examination of the data of table 6 shows that when the amino acid levels in the plasma are expressed in terms of the level of AIBA present, anomalous deficiencies disappeared in diets 2, 3, 4 and 5. Plasma amino acid comparisons for casein alone suggest that there is ample histidine in casein. It may be that supplementing the casein with histidine created an imbalance rather than correcting one. This might account in part for the low levels of threonine, isoleucine and arginine after supplementation. However,

TABLE 4

*Growth of chicks fed the revised reference diet and various test diets (exp. 3)*

| Diet no. | Description of diets | Gain/ chick/ day [1] | Gain / Feed |
|---|---|---|---|
| | | *g* | |
| 1 | Revised reference diet | 12.7 | 0.58 |
| 2 | As 1 with lysine at 0.65% (50% of requirement) | 5.7 | 0.38 |
| 3 | As 2 with valine at 0.62% (75% of requirement) | 5.6 | 0.36 |
| 4 | As 1 with valine at 0.62% (75% of requirement) | 10.0 | 0.53 |
| 5 | Casein [2] | 3.6 | 0.22 |
| 6 | As 5 + amino acid supplement [3] | 11.6 | 0.52 |
| 7 | As 1 + 0.25% AIBA | 12.7 | 0.58 |
| 8 | As 1 + 0.50% AIBA | 11.7 | 0.56 |

[1] Mean daily gain of 3 chicks in 3 replicates from 8 to 14 days of age.
[2] Casein incorporated to provide 17.61% crude protein in the diet.
[3] As a percentage of the diet: L-arginine·HCl, 0.681; glycine, 2.093; L-histidine·HCl·$H_2O$, 0.384; DL-methionine, 0.384.

TABLE 5

*Plasma amino acid levels in chicks fed reference and test diets (exp. 4)*

| | | Revised reference diet (1) | As (1) with lysine at 50% of requirement (2) | As (2) with valine at 75% of requirement (3) | As (1) with valine at 75% of requirement (4) | Casein [1] (5) | As (5) + supplemental amino acids [2] (6) |
|---|---|---|---|---|---|---|---|
| | | *μg/ml plasma* | | *μg/ml plasma* | | *μg/ml plasma* | |
| 1 | Threonine | 84.6 | 112.5 | 107.7 | 83.9 | 106.6 | 53.8 |
| 2 | Valine | 22.7 | 35.0 | 19.6 | 9.6 | 58.8 | 30.5 |
| 3 | Cystine | 22.5 | 20.6 | 20.3 | 19.2 | 15.6 | 13.1 |
| 4 | Methionine | 25.7 | 25.0 | 22.0 | 25.1 | 16.9 | 49.5 |
| 5 | Isoleucine | 17.8 | 26.8 | 25.0 | 23.5 | 25.4 | 14.2 |
| 6 | Leucine | 16.7 | 24.6 | 23.2 | 23.6 | 34.9 | 18.6 |
| 7 | Tyrosine | 14.5 | 21.2 | 18.9 | 17.4 | 29.0 | 27.2 |
| 8 | Phenylalanine | 10.0 | 12.2 | 11.0 | 12.1 | 20.7 | 13.8 |
| 9 | Lysine | 64.4 | 6.1 | 4.6 | 58.1 | 112.1 | 62.5 |
| 10 | Histidine | 12.4 | 9.8 | 14.7 | 12.3 | 22.9 | 22.6 |
| 11 | Arginine | 44.8 | 62.4 | 58.4 | 57.2 | 8.4 | 35.1 |
| 12 | Serine | 64.9 | 64.1 | 59.8 | 67.5 | 88.5 | 105.1 |
| 13 | Proline | 58.9 | 56.9 | 53.0 | 66.3 | 148.7 | 126.1 |
| 14 | Glutamic acid | 40.6 | 19.1 | 26.6 | 37.6 | 20.2 | 16.0 |
| 15 | Glycine | 99.7 | 76.3 | 75.5 | 108.4 | 24.8 | 135.1 |
| 16 | Alanine | 148.9 | 115.0 | 109.5 | 155.3 | 55.6 | 55.9 |
| 17 | AIBA [3] | 32.1 | 24.3 | 25.9 | 27.4 | 26.6 | 27.5 |

[1] Casein to provide 17.61% crude protein.
[2] As a percentage of the diet: L-arginine·HCl, 0.681; glycine, 2.093; L-histidine·HCl·$H_2O$, 0.384; DL-methionine, 0.384.
[3] α-Aminoisobutyric acid (0.50%) added to all diets.

TABLE 6

*Data of table 5 corrected for absorption rate and expressed as a percentage of the revised reference diet [1] (exp. 4)*

| | | Lysine at 50% of requirement | Lysine at 50% and valine at 75% of requirement | Valine at 75% of requirement | Casein | Casein + supplemental amino acids |
|---|---|---|---|---|---|---|
| 1 | Threonine | 176 | 158 | 116 | 152 | 74 |
| 2 | Valine | 204 | 107 | 50 | 313 | 157 |
| 3 | Cystine | 121 | 112 | 100 | 84 | 68 |
| 4 | Methionine | 128 | 106 | 114 | 79 | 225 |
| 5 | Isoleucine | 199 | 174 | 155 | 172 | 93 |
| 6 | Leucine | 195 | 172 | 166 | 252 | 130 |
| 7 | Tyrosine | 193 | 162 | 140 | 241 | 219 |
| 8 | Phenylalanine | 161 | 136 | 142 | 249 | 161 |
| 9 | Lysine | 13 | 9 | 106 | 210 | 113 |
| 10 | Histidine | 104 | 147 | 116 | 223 | 213 |
| 11 | Arginine | 184 | 162 | 150 | 23 | 91 |
| 12 | Serine | 130 | 114 | 122 | 165 | 19 |
| 13 | Proline | 128 | 111 | 132 | 305 | 250 |
| 14 | Glutamic acid | 62 | 81 | 108 | 60 | 46 |
| 15 | Glycine | 101 | 94 | 127 | 30 | 160 |
| 16 | Alanine | 102 | 91 | 122 | 45 | 44 |
| 17 | AIBA | — | — | — | — | — |

[1] The absolute values of each treatment in table 5 were converted to a per 100 μg/ml AIBA basis, then were expressed as a percentage of those values of the revised reference diet.

threonine and isoleucine could be the fourth and fifth limiting amino acids in casein.

## DISCUSSION

The experiments in this report were conducted to evaluate the usefulness of non-metabolizable AIBA as a marker in plasma amino acid techniques. The results of the first experiment demonstrate that AIBA is absorbed into the blood stream at a rate which is proportional to dietary levels, and at the same time does not materially affect the plasma levels of the metabolizable amino acids. In all of this work it has been assumed that AIBA is absorbed at a rate proportional to that of all other amino acids, and that AIBA travels down the gastrointestinal tract at the same rate as all other components of the diet. This is a particularly important assumption when comparing crystalline amino acid diets with intact protein diets.

The reason for using AIBA in the diet was that although diets may be fed to chicks in equal portions, and even though the chicks are able to ingest equal amounts, there apparently is no guarantee that the components of such diets will be digested and absorbed at equal rates. That such a difference exists has been demonstrated where the more deficient (imbalanced) diets and the natural diets (possibly also imbalanced) exhibit lower AIBA levels in the plasma than the complete reference crystalline diet.

The different rates of uptake have been shown to result in erroneous interpretation of plasma data, but conversion of the plasma titers to an equi-AIBA ratio helps to reduce the number of "apparent" deficiencies by yielding plasma amino acid titers which are more or less proportional to dietary uptake. The data presented prove that plasma amino acid titers are useful for predicting the limiting amino acids in proteins exhibiting one limiting amino acid. In order to achieve reliable results, however, the technique used must embody the refinements of the revised reference diet, precise feeding practices and the incorporation of an indicator (AIBA) into the diet under present conditions.

Where multiple amino acid deficiencies exist in proteins they can also be demonstrated by this technique. In general, the first-limiting amino acid will exhibit a lower titer than the reference, but because of the accumulation in the plasma of the remaining amino acids as a result of this deficiency, second- and third-limiting amino acids may have higher levels than the reference and thus appear adequate. Supplementation with the first-limiting amino acid then allows amino acid deficiencies of a lower order to become apparent.

Many problems remain to be solved before this technique attains the precision desired. Of prime importance is the continual improvement of the reference diet, not only with respect to the essential amino acid components but also to the so-called nonessential group. As mentioned earlier, an essential amino acid deficiency results in the accumulation of the other essential amino acids in the plasma. However, certain of the nonessential amino acids frequently decrease rather than increase under these circumstances. This suggests that the nonessential amino acids, in addition to their role in protein synthesis, may function in the elimination of amino acid excesses in the plasma, and in such a way, possibly through their contribution to uric acid formation, be required for the maintenance of a homeostatic plasma environment. Not only will the optimal levels of the nonessential amino acids have to be determined in the reference diet but it is perhaps time that the heretofore nonessential amino acids be regarded as essential for maximal efficiency in protein metabolism.

## LITERATURE CITED

1. Smith, R. E., and H. M. Scott 1965 Use of free amino acid concentrations in blood plasma in evaluating the amino acid adequacy of intact proteins for chick growth. I. Free amino acid patterns of blood plasma of chicks fed unheated and heated fishmeal proteins. J. Nutrition, 86: 37.
2. Smith, R. E., and H. M. Scott 1965 Use of free amino acid concentrations in blood plasma in evaluating the amino acid adequacy of intact proteins for chick growth. II. Free amino acid patterns of blood plasma of chicks fed sesame and raw, heated and overheated soybean meals. J. Nutrition, 86: 45.

3. Smith, R. E. 1966 Importance of an accurate reference diet in the evaluation of proteins for chick growth using plasma amino acid titers. J. Nutrition, 89: 271.
4. Christensen, H. N., A. J. Aspen and E. G. Rice 1956 Metabolism in the rat of three amino acids lacking α-hydrogen. J. Biol. Chem., 220: 287.
5. Segal, S. 1964 Hormones, amino-acid transport and protein synthesis. Nature, 203: 17.
6. Bavetta, L. A., and M. E. Nimi 1964 Tissue distribution of α-aminoisobutyric acid and nitrogen metabolism in the rat. II. Effects of environmental temperature and dietary imbalance. J. Nutrition, 82: 379.
7. Waterhouse, H. N., and H. M. Scott 1961 Glycine need of the chick fed casein diets and the glycine, arginine, methionine and creatine interrelationships. J. Nutrition, 73: 266.

# Comparative Absorption of Calcium from Calcium Gluconate and Calcium Lactate in Man [1]

HERTA SPENCER, JOSEPHINE SCHECK, ISAAC LEWIN AND JOSEPH SAMACHSON
*Metabolic Section, Veterans Administration Hospital, Hines, Illinois*

*ABSTRACT* Calcium balance and radiocalcium ($^{47}Ca$) studies were performed under controlled metabolic conditions in 8 patients during the intake of calcium gluconate and of calcium lactate in order to determine the absorption of calcium from these 2 salts. The calcium balances were somewhat more positive in all but one of the patients during the intake of calcium lactate than that of calcium gluconate, the average improvement in calcium balance being statistically significant at a level of $P < 0.05$. This improvement was due mainly to a slight but consistent decrease in fecal calcium excretions. The average net absorption was increased by 96 mg in the lactate study, significant at a level of $P < 0.001$. $^{47}Ca$ studies also indicated greater absorption of calcium from calcium lactate than from calcium gluconate, as evidenced by the increase in the average $^{47}Ca$ plasma level and the decrease in average fecal $^{47}Ca$ excretions during the intake of calcium lactate. The increased absorption of calcium on calcium lactate intake was also observed at different intake levels of protein and calcium in a patient receiving long-term corticosteroid therapy.

The availability of calcium from different sources has been the subject of investigation for many years (1–3). The utilization of different calcium salts has been reported to be similar (2, 3) as well as dissimilar (4, 5) in experimental animals. In studies performed in man the absorption of calcium from different calcium salts has been reported to be similar (6,7).

Supplemental calcium is indicated in certain conditions of calcium deficiency and this type of treatment has been suggested for patients with osteoporosis (8–10). The present studies were performed to investigate the intestinal absorption of calcium from 2 calcium salts, calcium gluconate and calcium lactate, under controlled dietary conditions in man, using tracer techniques and metabolic balances.

## EXPERIMENTAL

The comparative absorption of calcium, administered as the gluconate and as the lactate was studied under controlled dietary conditions in 8 male patients on the Metabolic Research Ward. The age and diagnoses of the patients and the type and duration of the studies are listed in table 1. All patients were fully ambulatory and in good physical condition despite the diagnoses indicating minor chronic abnormalities; the renal and intestinal function and the clinical laboratory tests of all patients were normal. All patients received the same constant, analyzed low calcium diet, which contained an average of 200 mg calcium and 820 mg phosphorus/day. The composition of the basal diet is listed in table 2. The patients received this diet from 30 to 60 days before the studies were performed. The energy intake was not the same for all patients and adjustments in calories and in nitrogen intake were made according to the ideal weight, the previous dietary habits, the activity and the age of the patients. Although the same food lots were used, there was some variability in nitrogen content of the 6-day food aliquots, especially due to the differences in nitrogen content of the meats. Supplements of calcium in equivalent amounts were added to this diet as the gluconate or as the lactate, all other constituents of the diet remaining unchanged. During supplementation with calcium gluconate, the calcium intake averaged 1,657 mg/day; during supplementation with calcium lactate, 1,672 mg/day. The average intake of phosphorus in the 2 study phases was 865 mg and 870 mg/day, respectively.

Received for publication December 27, 1965.

[1] Supported in part by Public Health Service Research Grant no. A-5572 from the Natonal Institute of Arthritis and Metabolic Diseases, and in part by the National Dairy Council.

TABLE 1

*List of patients studied*

| Patient | Age | Diagnosis | Study | |
|---|---|---|---|---|
| | | | Type | Days |
| 1 | 61 | Pulmonary calcifications | calcium gluconate | 66 |
| | | | calcium lactate | 30 |
| 2 | 51 | Osteoporosis, mild | calcium gluconate | 36 |
| | | | calcium lactate | 38 |
| 3 | 51 | Osteoarthritis | calcium gluconate | 40 |
| | | | calcium lactate | 14 |
| 4 | 40 | Peripheral neuropathy | calcium gluconate | 50 |
| | | | calcium lactate | 24 |
| 5 | 43 | Peripheral neuropathy | calcium gluconate | 24 |
| | | | calcium lactate | 32 |
| 6 | 47 | Osteoporosis | calcium gluconate | 48 |
| | | | calcium lactate | 38 |
| 7 | 42 | Osteoporosis | calcium gluconate | 48 |
| | | | calcium lactate | 18 |
| 8 | 48 | Cortisone osteoporosis | calcium gluconate [1] | 30 |
| | | | calcium lactate [1] | 30 |
| | | | calcium gluconate [1] | 30 |
| | | | calcium gluconate [2] | 42 |
| | | | calcium lactate [2] | 32 |
| | | | calcium gluconate [3] | 54 |
| | | | calcium lactate [3] | 24 |

[1] During high protein-high calcium intake.
[2] During normal protein-high calcium intake.
[3] During high protein-medium calcium intake.

TABLE 2

*Composition of metabolic low calcium diet (2223 kcal) (basal diet)*

| | Protein | Carbohydrate | Fat |
|---|---|---|---|
| | *g/day* | *g/day* | *g/day* |
| Fruit juice, 200 ml | | 24 | |
| Rice, [1] 30 g | 22 | 24 | |
| Bread, 150 g | | 78 | |
| Spaghetti, [1] 20 g | | 15 | |
| Jelly, 120 g | | 78 | |
| Potatoes, [2] 100 g | 5 | 19 | |
| Canned vegetables, 175 g | | 15 | |
| Sweetened canned fruit, 225 g | | 44 | |
| Sugar, 28 g | | 28 | |
| Meat (beef and turkey), 200 g | 44 | | 28 |
| Butterfat, 50 g | | | 41 |
| Cream, 20 g | | | 2 |
| Total | 71 | 325 | 71 |

[1] Weight of raw food.
[2] Weight of cooked food.

The average duration of the calcium gluconate studies was 45 days and of the calcium lactate studies, 28 days. The patients did not receive any medications, except for patient 8, who received 20 mg prednisone/day throughout all study phases. In this latter patient, the comparative effect of calcium gluconate and calcium lactate was studied during 2 intake levels of both calcium and protein.

The calcium lactate studies followed the calcium gluconate studies immediately in 4 patients (patients 4, 5, 6, and 8) and were performed from 2 weeks to 2 months

after the calcium gluconate study in 3 patients (patients 1–3). In patient 7, the time interval between the calcium gluconate and calcium lactate study was 7 months. The studies were performed from October to April in 5 patients (patients 1, 3–6), from January to April in patient 2 and continuously from February to July in patient 8. In patient 7, the calcium gluconate studies were performed in November and December and the calcium lactate studies in June and July.

Complete collections of urine and stool were obtained throughout the studies. Demineralized water containing 4 to 5 mg calcium/liter, was used for drinking and food preparation. The body weight, the fluid intake, the urine output and the urinary excretions of creatinine, calcium and phosphorus were determined daily. Metabolic balances of calcium, phosphorus and nitrogen were determined on aliquots of 6-day pools of urine and stool and on aliquots of the diet in each 6-day metabolic period.

Radioisotope studies were carried out during the intake of both calcium gluconate and calcium lactate. A single tracer dose of $^{47}Ca$ as the chloride (30 to 50 μCi) was administered orally with breakfast in each study. The plasma levels of $^{47}Ca$ were determined at 1, 4, 8 and 24 hours after the oral administration of $^{47}Ca$, daily in the first week of the study and at less frequent intervals thereafter. The urinary $^{47}Ca$ excretions were determined daily on aliquots of each 24-hour urine collection and the fecal $^{47}Ca$ excretions on each stool specimen. The studies averaged 24 days during the intake of calcium gluconate and 20 days during the intake of calcium lactate.

The radioassays of $^{47}Ca$ in plasma, urine and stool were performed in a well-type NaI crystal γ-scintillation counter equipped with a single channel pulse height analyzer. Standards equivalent to the administered dose were always counted along with the samples of plasma, urine and stool. The $^{47}Ca$ level in plasma was expressed as percentage of the administered dose per liter, the urinary $^{47}Ca$ excretion as percentage of dose per total urine volume per day and the fecal $^{47}Ca$ as percentage of dose per stool specimen.

The net absorption of $^{47}Ca$, as percentage of the administered dose, was determined by subtracting the fecal $^{47}Ca$ excretion (in per cent) from 100. The true absorption was calculated by correcting for endogenous fecal $^{47}Ca$ excretion, determined from excretion values following the intravenous injection of $^{47}Ca$ to the same patient. For patients who did not receive $^{47}Ca$ intravenously, the endogenous fecal $^{47}Ca$ excretion was estimated to be 15% of the absorbed dose. This estimate is usually very close to the true value, and the error introduced is small (11).

Urinary calcium was determined by a modification of the method of Shohl and Pedley (12), serum calcium by a modification of the Kramer-Tisdall method (13), nitrogen by the Kjeldahl method (14) and phosphorus by the method of Fiske and Subbarow (15). Stool calcium and phosphorus were determined on dry-ashed aliquots of 6-day stool pools which were dissolved in hydrochloric acid.

## RESULTS

The results obtained in the calcium gluconate and calcium lactate studies are shown in table 3. The slight variations in nitrogen and calcium intake from patient to patient and also in the same patient in the 2 study phases are due to differences in food intake, to inherent differences in the composition of the different food lots and to rejection of small amounts of food. The lower protein and calcium intake of patient 5 was due to caloric adjustments which had to be made in accordance with the previous dietary habits of this patient. In patients 1 to 5 the average urinary calcium excretion was similar during the intake of calcium gluconate and of lactate; in patients 6 and 7, the calciuria was approximately 50 mg and 100 mg higher per day during the intake of calcium lactate, respectively. The fecal calcium excretion decreased in 6 of the 7 patients (patients 1, 3–7), during the intake of calcium lactate but was little changed in patient 2. The calcium intake of patients 2 and 4 was slightly greater during the intake of calcium lactate than during the intake of calcium gluconate. The calcium balances became more positive in patients 1 to 7 during the intake of calcium lactate, the

TABLE 3

*Calcium balances of patients receiving calcium gluconate and calcium lactate supplements*

| Patient | Study days | Study | Calcium | | | | | Phosphorus | | Nitrogen | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Intake | Urine | Stool | Balance | Change in balance | Intake | Balance | Intake | Balance |
| | | | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* |
| 1 | 66 | Calcium gluconate | 1894 | 163 | 1727 | + 4 | | 874 | + 82 | 14,893 | +1750 |
| | 30 | Calcium lactate | 1864 | 180 | 1558 | +126 | +122 | 938 | + 93 | 14,876 | +1622 |
| 2 | 36 | Calcium gluconate | 2022 | 45 | 1757 | +220 | | 924 | +225 | 15,552 | +4025 |
| | 38 | Calcium lactate | 2107 | 43 | 1792 | +272 | + 52 | 892 | +161 | 15,035 | +4200 |
| 3 | 40 | Calcium gluconate | 1813 | 64 | 1568 | +181 | | 882 | +183 | 14,838 | +3052 |
| | 14 | Calcium lactate | 1800 | 67 | 1455 | +278 | + 97 | 816 | +156 | 13,826 | +2923 |
| 4 | 50 | Calcium gluconate | 1065 | 76 | 892 | + 97 | | 835 | +198 | 14,852 | +3796 |
| | 24 | Calcium lactate | 1113 | 69 | 863 | +181 | + 84 | 859 | +169 | 14,468 | +3388 |
| 5[1] | 24 | Calcium gluconate | 853 | 111 | 808 | − 66 | | 802 | + 64 | 11,346 | +1494 |
| | 32 | Calcium lactate | 796 | 108 | 598 | + 90 | +156 | 794 | +132 | 12,761 | +1967 |
| 6 | 48 | Calcium gluconate | 2201 | 321 | 1650 | +230 | | 929 | + 83 | 15,177 | +2922 |
| | 30 | Calcium lactate | 2260 | 376 | 1575 | +309 | + 79 | 949 | +153 | 15,396 | +2649 |
| 7 | 48 | Calcium gluconate | 1752 | 140 | 1500 | +112 | | 780 | + 93 | 13,421 | +1762 |
| | 18 | Calcium lactate | 1766 | 246 | 1351 | +169 | + 57 | 837 | +131 | 13,326 | +1275 |

[1] The lower nitrogen and calcium intake of patient 5 was due to adjustments in food intake (see text).

improvement in balance ranging from 52 to 156 mg/day. The average improvement in calcium balance for all patients including patient 8 (table 4), during the intake of calcium lactate as compared with calcium gluconate, was 71.6 mg/day, which is significant at a level of $P < 0.05$. The net absorption of calcium which is the difference between intake and fecal excretion was greater in the calcium lactate phase by 96 mg/day with a significance of $P < 0.001$. The changes in the phosphorus balances were consistent with the changes in calcium and nitrogen balances in most patients. The nitrogen balances of all patients were positive and showed fluctuations during the two study phases.

Although the average urinary calcium excretion did not differ markedly in the calcium gluconate and calcium lactate studies, in some of the patients the urinary calcium excretion increased distinctly during the initial phase of calcium lactate intake. Figure 1 shows that the urinary calcium excretion of patient 6 was about 300 mg/day in a 36-day study, during the intake of calcium gluconate. When calcium lactate was substituted in equivalent amounts for calcium gluconate, the uri-

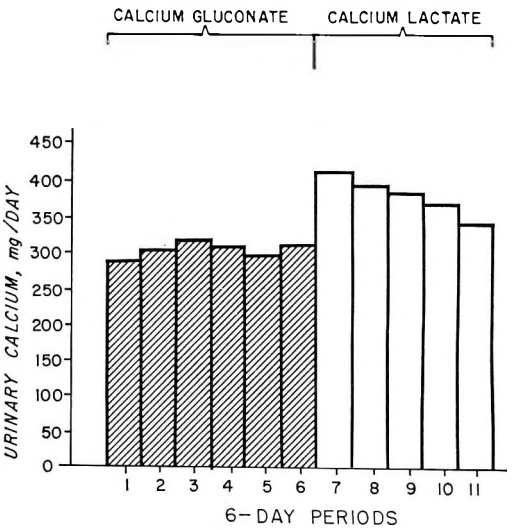


Fig. 1 Urinary calcium excretion during intake of calcium gluconate and calcium lactate. Each bar represents the average urinary calcium excretion per day for each 6-day metabolic period. Equivalent amounts of calcium were given as calcium gluconate and as calcium lactate. Patient 6.

TABLE 4

*Calcium, phosphorus and nitrogen balances of patient 8 [1] (long-term corticosteroid therapy)*

| Calcium intake | Protein intake | Study days | Study | Calcium | | | | Phosphorus balance | Nitrogen | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Intake | Urine | Stool | Balance | Change in balance | | Intake | Balance |
| | | | | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* | *mg/day* |
| High | high | 30 | calcium gluconate | 1901 | 551 | 1533 | −183 | | + 6 | 18,044 | +1829 |
| | | 30 | calcium lactate | 1923 | 582 | 1428 | − 87 | + 96 | −49 | 19,001 | +1391 |
| | | 30 | calcium gluconate | 1990 | 541 | 1593 | −144 | | −45 | 18,919 | +2051 |
| High | intermediate | 42 | calcium gluconate | 1979 | 547 | 1484 | − 52 | | −67 | 11,141 | + 86 |
| | | 32 | calcium lactate | 1978 | 560 | 1364 | + 54 | +106 | −29 | 10,594 | − 71 |
| Intermediate | high | 54 | calcium gluconate | 922 | 411 | 682 | −171 | | +53 | 19,631 | +2529 |
| | | 24 | calcium lactate | 960 | 384 | 640 | − 64 | +107 | −29 | 19,303 | +1595 |

[1] The patient received 20 mg of prednisone/day in all study phases.

nary calcium excretion increased promptly by an average of about 100 mg/day in the first 6 days of calcium lactate administration and decreased gradually in the subsequent 6-day periods. However, the urinary calcium excretion was still higher after 30 days of calcium lactate intake than during the intake of calcium gluconate. A prompt increase in calciuria was also observed during the intake of calcium lactate in patient 7 (fig. 2). The urinary calcium excretion of this patient ranged from 173 mg/day to 132 mg/day in the six 6-day periods of calcium gluconate intake. When an equivalent amount of calcium was administered as the lactate 7 months later, the urinary calcium excretion was considerably higher. The average urinary calcium excretion per 6-day period ranged from 260 mg/day to 236 mg/day. When calcium lactate was then replaced by equivalent amounts of calcium as the gluconate, the daily urinary calcium excretion decreased promptly (fig. 2).

The mineral and protein balances of patient 8 are listed in table 4. This patient had received corticosteroid therapy for 8 years and continued to receive 20 mg prednisone/day during the present study. Two intake levels of protein and of calcium were used. During the high protein-high calcium intake, the urinary calcium excretion was high, approximately 500 mg/day, with an intake of either calcium gluconate or calcium lactate. The average calciuria was, however, slightly higher (by an average of 31 mg/day) in the calcium lactate study and decreased promptly by an average of 41 mg/day when calcium lactate was again replaced by calcium gluconate. The average fecal calcium excretion was lower by approximately 100 mg/day during the intake of calcium lactate than during the intake of calcium gluconate and the calcium balance improved by approximately 100 mg/day in the calcium lactate study. On re-administration of calcium gluconate, the fecal calcium increased to about the same level as in the first calcium gluconate study, and the calcium balance became somewhat more negative. During the intake of the intermediate amount of protein, the calcium intake remaining unchanged, similar observations were made: the fecal calcium de-

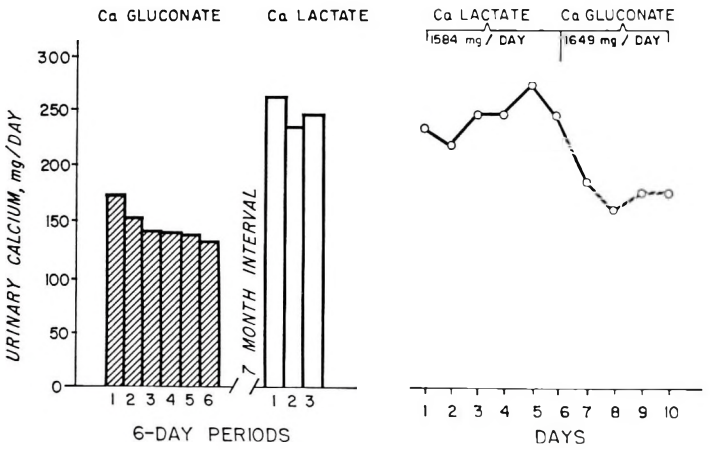


Fig. 2 Urinary calcium excretion during intake of calcium gluconate and calcium lactate. Each bar represents the average urinary calcium excretion per day for each 6-day metabolic period. The daily urinary calcium excretions during the intake of calcium lactate and calcium gluconate are those of the second study phase. Patient 7.

creased by 120 mg/day during the administration of calcium lactate and the urinary calcium excretion increased very slightly, resulting in an improvement of the calcium balance by 106 mg/day. When the calcium intake was decreased from an average of 1979 mg to 922 mg/day, the protein intake remaining high, the urinary calcium excretion was lower than during high calcium intake and averaged 411 mg/day in the calcium gluconate phase. When calcium gluconate was replaced by calcium lactate during this high protein-intermediate calcium intake, both the urinary and the fecal calcium excretions decreased slightly and the average calcium balance improved by 107 mg/day. The phosphorus balances of this patient varied with the changes in calcium and nitrogen balances, similar to the changes observed in the remaining patients. The nitrogen balances were positive during high protein intake; they became markedly less positive and were even slightly negative when the nitrogen intake was decreased to approximately 11,000 mg/day.

Table 5 lists the cumulative fecal $^{47}Ca$ excretions in the calcium gluconate and calcium lactate studies. The fecal $^{47}Ca$ excretions were consistently lower during the intake of calcium lactate than during the intake of calcium gluconate, although the differences were not great in most patients. The average difference of 7.9% was significant at a level of $P < 0.05$.

Figure 3 shows that the plasma levels of $^{47}Ca$ of patient 1 were higher after the oral administration of this tracer during the intake of calcium lactate than during the intake of calcium gluconate. Similar results were obtained in five other patients (patients 1, 2, 4, 6 and 7), while they remained in the same range in both studies in 2 patients (patients 5 and 8), and were slightly lower in the calcium lactate study in patient 3 (table 6). The

TABLE 5

*Fecal $^{47}Ca$ excretion in patients receiving calcium gluconate and calcium lactate supplements*

| Patient | Cumulative $^{47}Ca$ excretion [1,2] | | Difference [3] |
|---|---|---|---|
| | Calcium gluconate | Calcium lactate | |
| | *% of dose* | *% of dose* | |
| 1 | 78.9 | 68.4 | 10.5 |
| 2 | 78.6 | 73.7 | 4.9 |
| 3 | 78.5 | 71.9 | 6.6 |
| 4 | 72.3 | 70.6 | 1.7 |
| 5 | 63.0 | 60.5 | 2.5 |
| 6 | 65.5 | 61.4 | 4.1 |
| 7 | 85.9 | 61.4 | 24.5 |
| 8 | 64.3 [4] | 56.1 [4] | 8.2 |

[1] A single tracer dose of $^{47}Ca$ was given orally on the first day of the study.

[2] The excretions represent the cumulative fecal $^{47}Ca$ excretions up to the time when less than 1% of the dose was excreted daily in stool.

[3] Difference between fecal $^{47}Ca$ excretion during the intake of calcium gluconate and calcium lactate.

[4] With high protein diet. This patient received 20 mg prednisone per day in both study phases.

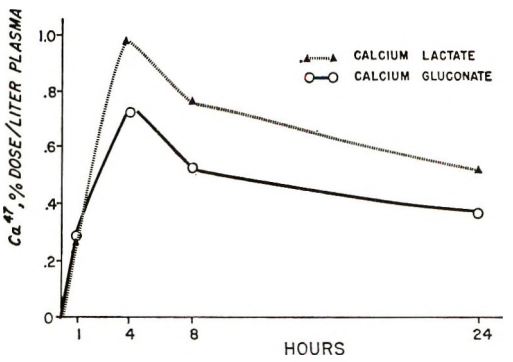


Fig. 3 Plasma levels during intake of calcium gluconate and calcium lactate (oral $^{47}Ca$). A single tracer dose of $^{47}CaCl_2$ was given orally during the intake of calcium gluconate and of calcium lactate.

$^{47}Ca$ plasma levels at 4, 8 and 24 hours, during the intake of calcium lactate, were compared with those of the calcium gluconate study and a ratio was obtained which indicated the relative absorption during the intake of calcium lactate and calcium gluconate. The average value of this ratio for the 8 patients was 1.19, significantly higher than 1.00, at a level of $P < 0.05$, indicating that the absorption of $^{47}Ca$ was higher during the ingestion of calcium lactate than of calcium gluconate.

## DISCUSSION

Several reports in the literature have indicated that calcium lactate is a poor source of calcium (2). Most studies, however, were performed in young animals or in young adults in whom the difference in utilization of calcium from various calcium salts may not be appreciable.

In experiments continued over 3 generations in rats, the addition of calcium lactate and of disodium phosphate to a low calcium-low phosphorus diet was reported to be as effective as milk in promoting growth in the first generation of rats but was less effective in maintaining this growth rate in succeeding generations (4). Calcium acetate, chloride, levulinate and gluconate have been used in calcium absorption studies with isolated loops of the upper part of the small intestine of rats (16). The absorption of calcium from the isolated loops was found to be higher from

TABLE 6

*$^{47}Ca$ Plasma levels during the intake of calcium gluconate and calcium lactate (oral administration of $^{47}Ca$)*

| Patient | Study | $^{47}Ca$ plasma levels, Hours [1] | | | | $^{47}Ca$ plasma ratio: [2] Ca lactate / Ca gluconate |
|---|---|---|---|---|---|---|
| | | 1 | 4 | 8 | 24 | |
| | | % of dose/liter of plasma | | | | |
| 1 | Ca gluconate | 0.28 | 0.73 | 0.53 | 0.36 | 1.40 |
| | Ca lactate | 0.27 | 0.98 | 0.76 | 0.52 | |
| 2 | Ca gluconate | 0.54 | 0.54 | 0.48 | 0.26 | 1.38 |
| | Ca lactate | 0.51 | 0.71 | 0.63 | 0.43 | |
| 3 | Ca gluconate | 0.62 | 1.87 | 1.53 | 1.02 | 0.88 |
| | Ca lactate | 0.45 | 1.60 | 1.30 | 1.01 | |
| 4 | Ca gluconate | 0.38 | 0.78 | 0.64 | 0.39 | 1.20 |
| | Ca lactate | 0.42 | 0.84 | 0.74 | 0.60 | |
| 5 | Ca gluconate | 0.58 | 1.01 | 0.78 | 0.55 | 0.99 |
| | Ca lactate | 0.39 | 0.93 | 0.83 | 0.56 | |
| 6 | Ca gluconate | 0.65 | 0.96 | 0.78 | 0.50 | 1.24 |
| | Ca lactate | 0.52 | 1.22 | 0.96 | 0.59 | |
| 7 | Ca gluconate | 0.21 | 1.02 | 0.78 | 0.51 | 1.41 |
| | Ca lactate | 0.38 | 1.41 | 1.08 | 0.76 | |
| 8 | Ca gluconate | 0.82 | 1.50 | 1.08 | 0.74 | 1.02 |
| | Ca lactate | 0.59 | 1.48 | 1.13 | 0.76 | |

[1] Hours after the oral administration of $^{47}Ca$.
[2] Values are averages. Ratios were determined by relating the sum of $^{47}Ca$ plasma levels at 4, 8 and 24 hours in the 2 study phases.

calcium gluconate than from other salts. However, no differences in absorption of calcium from the various salts were observed in intact rats (16). In another study, calcium from calcium lactate was found to be absorbed to a lesser extent than from calcium chloride when introduced into Thiry-Vella fistulas in dogs (5). More recently, studies of the absorption of calcium in dogs with Thiry-Vella fistulas have been reported (17). In a limited series of experiments the absorption of calcium from the chloride, lactate and gluconate in distilled water or in a hypotonic solution has been found to be similar.[2]

In studies performed in man, Patton and Sutton (6) found no significant difference in the utilization of calcium from the lactate, gluconate, sulfate or carbonate in young college women. However, the order in which the calcium salts were administered was found to be a significant factor and the salt taken first was utilized to a greater extent, regardless of which salt was used. In a subsequent study in college women Patton (7) found that calcium in milk, calcium carbonate, and calcium gluconate were utilized to the same extent. In a study in children comparisons were made on the effect of calcium lactate with that of milk, and the criteria used were those of height and weight increments (18). Since milk supplies many nutrients, in addition to calcium, that favorably influence growth, the effect of a calcium salt alone cannot be used for comparison.

The results obtained in the present investigation indicate that calcium is absorbed better from calcium lactate than from calcium gluconate in man as judged by three separate sets of analytical data, i.e., the changes in stool calcium analyses and calcium balances, in fecal $^{47}Ca$ excretions and in $^{47}Ca$ plasma levels. The differences in the results obtained with the 2 calcium salts for the group of patients are statistically significant for each of these criteria. It cannot be predicted whether the fecal excretions of calcium and of $^{47}Ca$ would have increased if calcium lactate had been administered for a prolonged period of time. The increase in calcium balance in patient 2 in the calcium lactate phase cannot be interpreted with certainty because the calcium intake was 85 mg higher in this study than in the calcium gluconate study.

All patients were studied with calcium gluconate before they were studied with calcium lactate. Since the sequence of administration of the calcium salts was reported to influence the absorption of calcium (6), the order of administration of the calcium salts in the present study should have favored the absorption of calcium from the gluconate. The greater absorption of calcium lactate than of calcium gluconate in the present study would, therefore, be even more significant.

The difference in the results reported here and those obtained by other investigators (5, 6) may be due in part to the fact that the amount of calcium added to the diet in the present study was high in comparison with the dietary calcium intake. The amount of calcium contained in the calcium supplements constituted from 87% to 90% of the total calcium intake in this study. In investigations reported in the literature the total calcium intake was often relatively low and the amounts of calcium given in the form of calcium salts represented 50% or less of the total calcium intake. The estimation of the effects of the calcium supplements may be difficult under these circumstances. For instance, in the studies of Patton and Sutton (6), the amounts of calcium added as gluconate, lactate and in forms of other salts was low, 400 mg/day, and the diet contained a similar amount of calcium, 347 mg calcium/day. In a study of the comparative utilization of calcium in milk and of calcium contained in carrots (19), the dietary calcium averaged 270 mg/day, and the additional calcium supplied by 700 g carrots/day was 202 mg. In the latter study, the lower absorption of calcium from carrots than from milk may have been due to the low concentration of calcium in a relatively large mass of unabsorbable vegetable material.

In most of the patients in the present study the urinary calcium excretions were similar during the intake of calcium gluconate and of calcium lactate except in patients 6 and 7. The increased calciuria

[2] Cramer, C. F., personal communication.

was probably the result of greater intestinal absorption of calcium from calcium lactate than from calcium gluconate. The increase in urinary calcium excretion in patient 7, during the intake of calcium lactate, must be interpreted with caution, since the calcium lactate study was performed 7 months after the calcium gluconate study and the possibility of a spontaneous increase in urinary calcium excretion must be considered. However, the prompt decrease in urinary calcium excretion upon replacing calcium lactate by calcium gluconate in this patient (fig. 2), indicated that the increase in calciuria most probably resulted from the higher absorption of calcium lactate; the lower excretion of fecal calcium during the intake of calcium lactate was also indicative of greater absorption of calcium.

It is of interest that the results in patient 8 were similar to those of the other patients despite prolonged corticosteroid therapy. Also, the effect of calcium lactate in decreasing fecal calcium excretion was independent of the intake level of protein and of calcium. The slightly higher urinary calcium excretions during supplementation with calcium lactate on intermediate as well as on high protein-high calcium intake, also indicated better absorption of calcium lactate than of calcium gluconate. The decrease in urinary calcium excretion during the intake of the intermediate level of calcium as the lactate in patient 8 may have been a consequence of lowering the intake of calcium.

It has been claimed that calcium gluconate causes diarrhea in man (20). Both calcium gluconate and calcium lactate in the amounts given were well tolerated by the patients reported here and neither of the 2 calcium salts caused untoward side reaction.

Gluconate salts have been used as complexing agents, but the binding constant, Kp, for calcium gluconate, is low, 1.21, and is almost the same as the value of 1.07 given for calcium lactate (21). As complexing agents, neither lactate nor gluconate ions would be expected to bind calcium more strongly than the proteins and carbohydrates of food. The reason for the better absorption of calcium from calcium lactate than from calcium gluconate in man remains conjectural.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Vernice Vankinscott, R.N., Head Nurse, Metabolic Research Unit, for valuable assistance in this study.

LITERATURE CITED

1. McCollum, E. V. 1957 A History of Nutrition. Houghton Mifflin Company, Boston, p. 269.
2. Steenbock, H., E. B. Hart, M. T. Sell and J. H. Jones 1923 The availability of calcium salts. J. Biol. Chem., *56:* 375.
3. Gaunt, W. E., J. T. Irving and W. Thomson 1939 A long-term experiment with rats on a human dietary. II. Calcium and phosphorus depletion after replacement. J. Hygiene, *39:* 91.
4. Givens, M. H., and L. B. Mendel 1917 Studies in calcium and magnesium metabolism. I. The effects of base and acid. J. Biol. Chem., *31:* 421.
5. Robinson, C. S., D. E. Stewart and F. Luckey 1941 The changes in composition of solutions of calcium chloride and calcium lactate in the intestine. J. Biol. Chem., *137:* 283.
6. Patton, M. B., and T. S. Sutton 1952 The utilization of calcium from lactate, gluconate, sulfate and carbonate salts by young college women. J. Nutrition, *48:* 443.
7. Patton, M. B. 1955 Further experiments on the utilization of calcium from salts by college women. J. Nutrition, *55:* 519.
8. Whedon, G. D. 1959 Effects of high calcium intake on bone, blood and soft tissues; relationship of calcium intake to balance in osteoporosis. Federation Proc., *18:* 1112.
9. Nordin, B. E. C. 1960 Osteomalacia, osteoporosis and calcium deficiency. Clin. Orthopaed., *17:* 235.
10. Schwartz, E. C., V. William and V. A. Panariello 1964 Metabolic balance studies of high calcium intake in osteoporosis. Am. J. Med., *36:* 233.
11. Spencer, H., M. Li, J. Samachson and D. Laszlo 1960 Metabolism of strontium-85 and calcium-45 in man. Metabolism, *9:* 916.
12. Shohl, A. T., and F. G. Pedley 1922 A rapid and accurate method for calcium in urine. J. Biol. Chem., *50:* 537.
13. Clark, E. P., and J. B. Collip 1925 A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., *63:* 461.
14. Hawk, P. B., B. L. Oser and W. H. Summerson 1947 Practical Physiological Chemistry. The Blakiston Company, Philadelphia, p. 814.

15. Fiske, C. H., and Y. T. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., 66: 375.
16. Nicolaysen, R. 1951 Influence of vitamin D on absorption of calcium from intestine of rats. Acta Physiol. Scand., 22: 260.
17. Cramer, C. F. 1963 Quantitative studies on the absorption and excretion of calcium from Thiry-Vella intestinal loops in the dog. In: The Transfer of Calcium and Strontium Across Biological Membranes, ed., R. H. Wasserman. Academic Press, New York, p. 75.
18. Aykroyd, W. R., and B. G. Krishman 1939 A further experiment on the value of calcium lactate for Indian children. Ind. J. Med. Res., 27: 409.
19. Breiter, H., R. Mills, E. Rutherford, W. Armstrong and J. Outhouse 1942 The utilization of the calcium of carrots by adults. J. Nutrition, 23: 1.
20. Nicolaysen, R. 1963 Discussion II. In: The Transfer of Calcium and Strontium Across Biological Membranes, ed., R. H. Wasserman. Academic Press, New York, p. 109.
21. Chaberek, S., and A. E. Martell 1959 Organic Sequestering Agents. John Wiley and Sons, New York, p. 512.

# Growth Response of Turkey Poults to Fractions of Soybean Meal [1]

MELVIN GRIFFITH [2] AND ROBERT J. YOUNG
*Department of Poultry Science and Graduate School of Nutrition, Cornell University, Ithaca, New York*

*ABSTRACT* A study was conducted to concentrate and characterize the component of soybean meal which has been reported to improve the growth of turkey poults. A water extract of raw soybean meal increased the growth of turkey poults when added to purified diets based on isolated soybean protein or casein-gelatin. The factor could be extracted from heated soybean meal with methanol. This factor appeared to be partially dialyzable, and insoluble in phenol. Extraction of the water extract with methanol brought about a concentration of the growth factor in the methanol-soluble portion. The factor appeared to be organic in nature, although a small response to the ash was also obtained. Extraction of the methanol-soluble fraction with chloroform-methanol inactivated or destroyed the factor. The growth factor could be made unavailable to the poult by reacting it with acetic anhydride. It was then reactivated by KOH hydrolysis.

Several investigators (1–6) have shown that soybean meal contains a factor which increases the growth of turkey poults fed an isolated soybean protein diet adequate in all known nutrients. Kratzer et al. (1, 2) reported that the factor could be extracted from 44% soybean meal with methanol, and could then be partially extracted from the methanol extract by benzene. Wilcox et al. (3–5) and Carlson et al. (7) reported the concentration of a growth factor in a water extract of 50% soybean meal, either raw or heated. The factor could not be extracted from soybean meal by acetone or ethanol. Griffith et al. (6) noted that the factor could be extracted with water from unheated solvent-extracted soybean flakes, but little was extracted with water from heated 50% protein soybean meal.

The present report describes experiments conducted to concentrate and characterize the component of soybean meal responsible for the improved growth of turkey poults.

## MATERIALS AND METHODS

The compositions of the purified basal diet and the positive control diet containing soybean meal are shown in table 1. In addition, a casein-gelatin basal diet was used in one experiment. It consisted of 30.9% casein, 10% gelatin, 31% sucrose, 7.1% cellulose, 5% soybean oil, 0.5% arginine, 0.25% methionine and the vitamins and minerals shown in table 1. The fractions prepared from soybean meal were substituted for sucrose in the basal diet. Diets were fed ad libitum from one day to 3 weeks of age to either Broad Breasted Empire White or Wrolstad Medium White turkey poults. Triplicate pens of poults were housed in thermostatically controlled battery brooders with wire-mesh floors. Poults were group-weighed and feed consumption was determined weekly. All poults were weighed individually at the end of the experiment.

Fractions were prepared from raw, solvent-extracted soybean flakes (brewer's flakes) by the following procedures.

*Water extract (WE).* Raw soybean flakes were mixed with 8 liters of water/kg of flakes and the pH was adjusted to 4.7 with acetic acid. The mixture was stirred for one hour at a temperature of 60°. The extract was squeezed from the insoluble residue in a screw press. The residue was extracted a second time, the extracts were combined and held in a tank until the fine particles settled out. The supernatant was decanted and placed in

Received for publication January 24, 1966

[1] This work was supported in part by grants-in-aid from Agway, Inc. and the Distillers Feed Research Council. The authors are also grateful to Dr. W. W. Cravens, Central Soya Company, Inc., Decatur, Indiana, for the supply of unheated solvent extracted soybean flakes used in this work.

[2] Present address: Department of Poultry Science, Louisiana State University, Baton Rouge.

TABLE 1
*Experimental diets*

| | Basal | 20% soybean meal |
|---|---|---|
| | % | % |
| Soybean oil | 7.00 | 9.00 |
| Isolated soybean protein [1] | 41.00 | 29.43 |
| Soybean meal, 50% | — | 20.00 |
| Sucrose | 38.665 | 29.235 |
| Cellulose [2] | 3.00 | 2.00 |
| Glycine | 0.50 | 0.50 |
| Methionine | 0.75 | 0.75 |
| Mineral mix [3] | 2.00 | 2.00 |
| Vitamin mix [4] | 0.666 | 0.666 |
| Choline Cl, 70% | 0.354 | 0.354 |
| $CaHPO_4 \cdot 2H_2O$, NF | 5.00 | 5.00 |
| $CaCO_3$, USP | 1.065 | 1.065 |
| Total | 100 | 100 |
| Protein, % | 37.25 | 37.25 |
| Energy, kcal/kg (metabolizable energy) | 3496 | 3400 |
| Metabolizable energy-to-protein ratio | 94 | 91 |

[1] ADM C-1 Assay protein, Archer-Daniels-Midland Company, Minneapolis.
[2] Solka-Floc, Brown Company, Berlin, New Hampshire.
[3] Mineral mix provided the following in g/100 g diet: NaCl, 0.7; KCl, 0.7; $MgSO_4$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.02; (in mg/100 g diet) $MnSO_4 \cdot H_2O$, 25; KI, 2; $CuSO_4 \cdot 5H_2O$, 2; $ZnCo_3$, 15; $NaMoO_4 \cdot 2H_2O$, 0.4; $Na_2SeO_3$, 0.4; and $CoCl_2 \cdot 6H_2O$, 0.4.
[4] Vitamin mix provided the following/kg of diet: vitamin A, 13,200 IU; vitamin $D_3$, 1980 IU; *d*-α-tocopheryl acetate, 20 IU; (in mg/kg) nicotinic acid, 88; Ca pantothenate, 22; riboflavin, 10; pyridoxine·HCl, 10; thiamine·HCl, 10; folic acid, 1; menadione sodium bisulfite, 2; vitamin $B_{12}$, 0.02; biotin, 0.22; and ethoxyquin, 130.

a steam kettle, stirred and heated until most of the water evaporated. The thick slurry remaining was placed in pans and evaporated to a thick paste in an oven at 60°. The final material was approximately 66% dry matter.

*Methanol-soluble portion of water extract (ME).* Batches of about one kilogram of water extract paste were placed in a pan containing 4 liters of warm methanol. The water extract was manually separated into small pieces with the aid of a spatula and rubber gloves. The mixture was placed in an oven at 45° for one hour and allowed to settle. It was filtered through Whatman no. 12 filter paper while still inside the 45° oven. The residue from this filtration was stored under methanol and extracted a second time. This extraction was carried out in a 5-liter Waring Blendor in which one-kilogram batches of the residue were ground with 3 liters of warm methanol, at slow speed, for 2 minutes. The material from the blender was poured through a 0.32-cm mesh screen. The material which passed through the screen was allowed to settle and filtered inside an oven as described above. The extracts were combined and the methanol evaporated. Analysis of the resulting molasses-like material showed that it contained 12% protein (N × 6.25), 7% fat (chloroform-methanol, 2:1) and 11% ash. It also showed a strong Benedict's test for reducing sugars.

*Acetic anhydride treatment of the methanol extract (ME-Ac).* A 600-g sample of ME was refluxed on a steam bath for 2 hours with 2 liters of acetic anhydride. A violent reaction was observed to occur for a period of several minutes after the mixture had been refluxed for some time. At the end of the treatment, water was added to the flask, and the solution was neutralized with NaOH. A portion of the material which had become water-insoluble was allowed to settle out and the water solution was decanted. The water was partially evaporated from the water-soluble fraction leaving a paste which was added to the diet. The water-insoluble material was washed out of the containers with acetone, the acetone evaporated, and the material dissolved in ethanol for addition to the diet.

*Hydrolyses of the acetone-soluble fraction after acetic anhydride treatment.* The acetone-soluble fraction (ME-Ac) resulting from the treatment described above was placed in a flask and the acetone evaporated. A liter of 5 N KOH was added and the solution was refluxed for 6 hours. The material was then cooled, neutralized with sulfuric acid and the water was partially evaporated. The material added to the diet included the potassium salts resulting from the neutralization procedure.

*Chloroform-methanol extraction of the methanol extract.* A 300-g sample of ME was refluxed with 1.5 liters of chloroform-methanol, 2:1 (v/v), for one hour. The liquid was decanted, and the extraction of the insoluble material was repeated. The extracts were combined and the solvents evaporated. The chloroform-methanol-soluble and -insoluble materials were added to separate diets.

*Ash of the methanol extract.* The sample of ME was charred on a hotplate and then ashed in a muffle furnace for 24 hours at 500°.

*Phenol-soluble and -insoluble fractions of the water extract.* One hundred grams of soybean water extract (WE) in 330 g of water were combined with 215 g of phenol. The mixture was heated until the phenol dissolved at about 80° and was then allowed to cool in a separatory funnel. The water layer containing the phenol-insoluble material was removed and washed 5 times with diethyl ether. The water was then evaporated.

Diethyl ether (400 ml) was added to the phenol layer and the phenol-ether solution was washed 5 times with water. The water washings of the phenol-ether solution were washed 5 times with diethyl ether to remove all the phenol. The water was evaporated and the residue was added to the diet.

*Dialyzed water extract.* A sample of water extract equivalent to 7% of the diet was dialyzed in 28-mm-diameter dialysis tubing against running water for 36 hours. The non-dialyzable fraction was dried and added to the diet.

*NaOH treatment of methanol extract.* Six hundred grams of ME in 9 liters of methanol were adjusted to pH 12 with NaOH, allowed to stand in a cooler over night and filtered. The precipitate was dissolved in water and neutralized. The methanol was evaporated from the filtrate prior to neutralization. Most of the water was evaporated from both fractions before they were added to the diet.

*Separation of liquid and solid portions of concentrated water extract.* After most of the water was evaporated from the water extract, some material clumped together to form soft lumps. This solid material was separated from the liquid by filtration in one experiment and by centrifugation in another.

*Methanol extract of heated soybean meal.* Commercial 44% soybean meal was extracted with methanol in a Soxhlet-type extractor for 48 hours. The methanol was evaporated and the methanol-soluble material was added to the diet.

*Charcoal adsorption procedure.* Six hundred grams of ME in 8 liters of methanol were stirred with 250 g of charcoal and filtered. The procedure was repeated, using 450 g of charcoal.

*General treatment.* All fractions of raw soybean flakes and the intact flakes which were ground and used in the positive control diet were autoclaved for 30 minutes at 107° before addition to the diet.

Statistical analyses of the data were performed for analysis of variance and Duncan's new multiple range test (8) at the 5% level of probability.

## RESULTS

The results of feeding graded levels of water extract are presented in table 2. Water extract of raw soybean flakes added to the diet at levels equivalent to 20%, 30% or 40% soybean meal was equally effective, showing that a level of extract equivalent to 20% soybean meal was sufficient for maximal growth response. Recombined extract and residue promoted

TABLE 2

*Effect of different levels of soybean water extract on growth response of poults*

| Supplement | Water extract in diet [1] | Avg wt 3 weeks | Feed/gain |
|---|---|---|---|
| | % | g | |
| Basal | — | 412 [a] | 1.37 |
| Water extract ~ 20% of soy flakes | 7.2 | 486 [b] | 1.26 |
| Water extract ~ 30% soy flakes | 10.8 | 477 [b 2] | 1.37 |
| Water extract ~ 40% soy flakes | 14.5 | 491 [b 2] | 1.40 |
| Water extract + 12.8% residue ~ 20% soy flakes | 7.2 | 497 [b] | 1.26 |
| 20% soybean flakes, heated | — | 492 [b] | 1.26 |

[1] Includes about 30% water remaining in finished preparation.
[2] Average of triplicate lots of 8 female Empire White poults each. Other values in this experiment are averages of triplicate lots of 11 poults each. Values followed by the same letter are not significantly different.

growth to the same extent as the intact soybean meal.

The effects of dialyzing the water extract or extracting it with phenol are shown in table 3. Approximately one-half of the growth-promoting activity was retained after dialysis. The growth factor did not appear to be soluble in phenol, although some activity may have been retained in the phenol and not recovered.

Extraction of heated soybean meal with methanol resulted in a growth response from the methanol extract (table 3). However, extraction of raw soybean flakes with methanol and heated soybean meal with water failed to remove the growth factor.[3]

The data summarized in table 4 show that either the water extract or the intact soybean meal gave a growth response when included in a casein-gelatin diet, although this basal diet did not support as good growth as the isolated soybean protein basal diet. No effect on growth was observed when soybean oil in the basal diet was replaced with lard and safflower oil.

In experiments A and B (table 5) further fractionations of water extract were performed. Filtration of the water extract to separate the liquid and suspended material was not effective in concentrating the growth factor in either fraction. When a more efficient separation of the solid material was made by centrifugation, the growth factor was observed only in the liquid portion of the extract. A fractionation in experiment A using 2:1 methanol-water, and the results of using graded concentrations of methanol in experiment B both show that the growth factor was soluble in both methanol and water.

The methanol-soluble fraction of the water extract (ME) was subjected to several different treatments (experiment C,

[3] Griffith, M. 1965 Investigation of the growth factor and phosphorus availability factor of soybeans with turkey poults. Ph.D. Thesis, Cornell University, Ithaca, New York.

TABLE 3

*Growth response to some fractions of the water extract of soybean*

| Supplement | Supplement in diet [1] | Avg wt, [2] 3 weeks | Feed/gain |
|---|---|---|---|
| | % | g | |
| Basal | — | 400 [a] | 1.32 |
| Water extract ~ 20% raw soybean flakes | 7.0 | 482 [bcd] | 1.28 |
| Nondialyzable water extract | 0.8 | 439 [abc] | 1.30 |
| Phenol-soluble water extract | 2.4 | 416 [ab] | 1.36 |
| Phenol-insoluble water extract | 2.5 | 442 [abc] | 1.31 |
| Methanol extract of 44% soybean meal | 6.2 | 490 [cd] | 1.27 |
| 20% soybean flakes, heated | — | 546 [d] | 1.17 |

[1] Includes approximately 30% water in completed supplement. All fractions of water extract were added at levels equivalent to the level of water extract fed.
[2] Average of triplicate pens of 10 male Empire White poults each. Weights followed by the same letter are not significantly different.

TABLE 4

*Effect of changes in the basal diet on growth of turkey poults*

| Supplement | Avg wt, 3 weeks [1] | Feed/gain |
|---|---|---|
| | g | |
| Basal (soybean protein) | 412 [b] | 1.37 |
| +7.4% water extract ~ 20% raw soybean flakes [2] | 474 [d] | 1.35 |
| +20% soybean flakes, heated | 492 [d] | 1.26 |
| +4% lard+3% safflower oil (substituted for 7% soybean oil) | 424 [bc] | 1.35 |
| Basal (casein-gelatin) | 351 [a] | 1.28 |
| +7.4% water extract ~ 20% raw soybean flakes [2] | 463 [cd] | 1.38 |
| +20% soybean flakes, heated | 484 [d] | 1.15 |

[1] Average of triplicate pens of 11 female Empire White poults each. Weights followed by the same letter are not significantly different.
[2] Includes approximately 30% water in supplement.

TABLE 5

*Growth-promoting activity of various fractions of the water extract of soybeans (WE)*

| Supplement | Supplement in diet [1] | Avg wt, 3 weeks | Feed/gain |
|---|---|---|---|
| | % | g | |
| Experiment A [2] | | | |
| Basal | — | 423 [a] | 1.31 |
| Water extract (WE) ~ 40% raw soybean flakes | 11.5 | 465 [b] | 1.38 |
| Filtrate of WE | 9.2 | 465 [b] | 1.41 |
| Insoluble fraction of WE | 4.0 | 459 [b] | 1.37 |
| WE-insoluble in 2:1 methanol-water | 4.8 | 444 [ab] | 1.32 |
| WE-soluble in 2:1 methanol-water | 6.2 | 461 [b] | 1.34 |
| 20% soybean flakes, heated | — | 508 [c] | 1.29 |
| Experiment B [3] | | | |
| Basal | — | 433 [ab] | 1.27 |
| Water extract (WE) ~ 20% raw soybean flakes | 6.5 | 467 [abc] | 1.35 |
| WE-insoluble in 1:2 methanol-water | 1.4 | 472 [bc] | 1.24 |
| WE-soluble in 1:2 methanol-water | 3.9 | 470 [bc] | 1.30 |
| WE-insoluble in 2:1 methanol-water | 1.8 | 416 [a] | 1.34 |
| WE-soluble in 2:1 methanol-water | 3.5 | 466 [abc] | 1.34 |
| WE-insoluble in pure methanol | 3.4 | 439 [abc] | 1.34 |
| WE-soluble in pure methanol | 1.8 | 470 [bc] | 1.31 |
| Precipitate from centrifuged WE | 0.5 | 439 [abc] | 1.30 |
| Supernatant from centrifuged WE | 5.0 | 481 [bc] | 1.28 |
| 20% soybean flakes, heated | — | 490 [c] | 1.19 |

[1] Includes approximately 30% water in completed supplement.
[2] Average of triplicate lots of 4 male and 4 female Wrolstad poults each.
[3] Average of triplicate lots of 10 male Empire White poults each. Weights followed by the same letter are not significantly different.

table 6). The growth factor was present after a sample had been refluxed for 6 hours with 6 N $H_2SO_4$ and neutralized with NaOH. However, when a sample of the extract was refluxed with chloroform-methanol, 2:1, to separate the lipid and non-lipid fractions, no activity was noted in either fraction. The growth factor appeared to have been destroyed or made unavailable by this treatment. Some growth-promoting activity was present in the ash of the methanol extract, but this response did not duplicate the response to the intact extract, indicating that the primary factor responsible for the improved growth of turkey poults was organic in nature.

The results of 3 experiments in which additional fractionations of the methanol-soluble fraction (ME) were carried out are presented in table 6 (experiments D, E and F). The ME which was stirred with charcoal and filtered appeared to have been reduced in growth-promoting activity to about the same extent as the reduction in total material. Adjusting the pH of the methanol solution to 12 appeared to divide the growth factor between the 2 fractions or partially destroy it.

When the ME was treated with acetic anhydride (experiment D) there was no growth response to either the water-soluble fraction or the water-insoluble fraction which was soluble in acetone. Growth-promoting activity could be restored to the acetone-soluble fraction by refluxing it with 5 N KOH (exp. E). In experiment F, a growth response was again obtained to the hydrolyzed acetone-soluble material, whereas there was no response to the unhydrolyzed acetone-soluble material, thus confirming the results of the 2 previous experiments.

## DISCUSSION

The results of the present study suggest that there may be a change in the solubility properties of the growth factor in soybeans when it is subjected to heat. Previous attempts to extract the factor from raw soybean meal with methanol, and from heated soybean meal with water were unsuccessful.[4] However, in this study the growth factor was extracted from raw soybean meal by water and from heated soybean meal with methanol. It was also

[4] See footnote 3.

TABLE 6

*Influence of various treatments of the methanol-soluble fraction on the growth of poults*

| Supplement to basal diet | Supplement to diet [1] | Avg wt, 3 weeks [2] | Feed/ gain |
|---|---|---|---|
| | % | g | |
| Experiment C [3] | | | |
| None | — | 310 [a] | 1.46 |
| Methanol-soluble fraction of the water extract of raw soybean flakes (ME) | 1.8 | 373 [bc] | 1.38 |
| ME refluxed with 6 N $H_2SO_4$ | 1.8 | 362 [abc] | 1.48 |
| Ash of ME | 0.02 | 344 [abc] | 1.29 |
| ME-insoluble in 2:1 chloroform-methanol | 1.6 | 318 [ab] | 1.38 |
| ME-soluble in 2:1 chloroform-methanol | 0.2 | 319 [ab] | 1.42 |
| 20% soybean meal | | 383 [c] | 1.31 |
| Experiment D [4] | | | |
| None | — | 313 [ab] | 1.38 |
| ME | 3.7 | 377 [ef] | 1.37 |
| Charcoal filtrate of ME | 2.4 | 355 [cdef] | 1.37 |
| ME water-soluble after acetic anhydride treatment | 3.9 | 295 [a] | 1.39 |
| ME acetone-soluble after acetic anhydride treatment | 2.5 | 315 [abc] | 1.49 |
| ME precipitated by NaOH (pH 12) | 1.6 | 340 [bcde] | 1.35 |
| Me not precipitated by NaOH | 3.9 | 329 [abcd] | 1.45 |
| 20% soybean meal | — | 384 [f] | 1.26 |
| Experiment E [3] | | | |
| None | — | 324 [a] | 1.47 |
| ME | 2.3 | 384 [b] | 1.39 |
| ME acetone-soluble fraction hydrolyzed after acetic anhydride treatment | 4.4 | 383 [b] | 1.41 |
| 20% soybean meal | — | 405 [b] | 1.33 |
| Experiment F [5] | | | |
| None | — | 424 [ab] | 1.38 |
| ME | 2.2 | 445 [ab] | 1.41 |
| Me acetone-soluble after acetic anhydride treatment | 1.4 | 406 [a] | 1.36 |
| ME acetone-soluble fraction hydrolyzed after acetic anhydride treatment | — | 473 [b] | 1.40 |
| 20% soybean meal | — | 482 [b] | 1.34 |

[1] Values shown include 30 to 50% water in supplement.
[2] Values within the same experiment followed by the same letter are not significantly different.
[3] Fractions added at a level equivalent to 20% soybean meal. Weights are averages of triplicate lots of 9 mixed sex Wrolstad poults each.
[4] All fractions in this experiment added at a level equivalent to 40% soybean meal. Weights are averages of triplicate lots of 5 male and 5 female Wrolstad poults each.
[5] Fractions added at a level equivalent to 20% soybean meal. Weights are averages of triplicate lots of 9 female Empire White poults each.

observed that the growth factor was soluble in methanol after water extraction from the raw soybean meal. However, Wilcox et al. (3) extracted the factor from heated soybean meal with water.

It appears that the growth factor is quite thermostable, since there was no loss of activity when the water extract and the residue after extraction were recombined and compared with the intact soybean meal. The factor was found to be dialyzable in agreement with the previous reports (3).

Previous experiments (6)[5] established that the basal diet was adequate in all known nutrients. The response from soybean meal or fractions thereof did not appear to be due to the correction of deleterious properties of the isolated soybean protein diet, since the growth response to soybean meal or the water extract was also obtained when it was fed in a casein-gelatin diet (table 4). Poults fed the casein-gelatin basal diet grew significantly less well than those fed the isolated soybean protein diet. Since all poults grew at the same rate when the 2 diets were supplemented with either the heated soybean flakes or the water extract, it appears that the casein-gelatin basal may be more deficient in the growth factor than the

[5] See footnote 3.

isolated soybean protein basal diet. This possibility is supported by the observations of Westerfeld and Hermans (9) who reported the presence of an unidentified growth factor in isolated soybean protein.

The factor was found to be stable to refluxing with acid but was destroyed or made unavailable by refluxing with chloroform-methanol. Since no activity was observed in either fraction after the latter treatment, it was not possible to determine whether the factor was lipid or nonlipid in nature. Purified soybean oil did not appear to contain the factor, unless the other fats used also contained it. Several attempts were made to concentrate the factor in benzene. Variable and inconclusive results were obtained in 4 experiments in contrast with Kratzer et al. (2) who were able to extract a portion of the growth activity from a methanol-soluble extract with benzene.

The ash of the extract caused a small growth response. This observation agrees with previous work (6) in which the ash of the intact soybean meal also resulted in a small growth response. Since no growth response was obtained when the amounts of known essential minerals were increased,[6] the small response from the ash raised the possibility that some mineral not now recognized as essential may promote the growth of poults. These results also indicate that the organic factor may contain an inorganic element which either separately, or as part of the molecule of the unknown factor, causes a growth response in turkey poults.

The growth factor can be reacted with acetic anhydride and rendered unavailable to the poult (table 6). The growth-promoting activity can be restored by hydrolyzing with 5 N KOH. These results suggest the presence of an amine or hydroxyl group in the growth factor whis is essential for its biological activity.

## LITERATURE CITED


1. Kratzer, F. H., P. Vohra, R. L. Atkinson, P. N. Davis, B. J. Marshall and J. B. Allred 1959 Fractionation of soybean oil meal for growth and antiperotic factors. Poultry Sci., *38:* 1049.
2. Kratzer, F. H., B. Starcher and E. W. Martin 1964 Fractionation of soybean meal for growth and antiperotic factors. 3. Growth promoting activity in benzene soluble fraction. Poultry Sci., *43:* 663.
3. Wilcox, R. A., C. W. Carlson and W. Kohlmeyer 1961 Separation by dialysis of a water soluble growth factor(s) found in soybean oil meal. Poultry Sci., *40:* 1766.
4. Wilcox, R. A., C. W. Carlson, W. Kohlmeyer and G. F. Gastler 1961 Evidence for a water soluble factor(s) in soybean oil meal. Poultry Sci., *40:* 94.
5. Wilcox, R. A., C. W. Carlson, W. Kohlmeyer and G. F. Gastler 1961 The growth response of turkey poults to a water-extract of soybean oil meal as influenced by different sources of isolated soybean protein. Poultry Sci., *40:* 1353.
6. Griffith, M., R. J. Young and M. L. Scott 1966 Influence of soybean meal on growth and phosphorus availability in turkey poults. Poultry Sci., *45:* 189.
7. Carlson, C. W., J. McGinnis and L. S. Jensen 1964 Antirachitic effects of soybean preparations for turkey poults. J. Nutrition, *82:* 366.
8. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.
9. Westerfeld, W. W., and A. C. Hermans 1962 Studies on the soy and fish solubles growth factors for chicks. J. Nutrition, *76:* 503.


[6] See footnote 3.

# Cellular Response in Rats during Malnutrition at Various Ages [1,2]

MYRON WINICK AND ADELE NOBLE
*Department of Pediatrics, Cornell University Medical College, New York, New York*

*ABSTRACT* Malnutrition retards growth in animals and children. Recovery of normal stature on refeeding depends, in part, on age at onset of deprivation. To investigate the cellular events underlying this time-dependence, rats were exposed to 21 days of caloric restriction at birth, at weaning and at age 65 days and then refed normally until adulthood. Total organ weight, protein, RNA, and DNA were measured during the periods of caloric restriction and subsequent refeeding. Total organ DNA and weight/DNA, protein/DNA, and RNA/DNA ratios in test and control animals reared in the usual manner served as indexes of changes in organ cell size and number. Malnutrition from birth to weaning resulted in a proportional decrease in weight, protein, RNA, and DNA, indicating a reduction in cell number without alteration in cell size. These animals did not recover normal growth when adequately refed. Malnutrition from weaning to 42 days of age resulted in a proportional reduction in weight, protein, RNA, and DNA in all organs except brain and lung. Although weight, protein, and RNA were reduced in these 2 organs, DNA was unaffected. Refeeding was accompanied by recovery in weight of these 2 organs only, resulting in an animal retarded in overall growth with normal-size brain and lung. Finally, malnutrition from 65 to 86 days of age resulted in maintenance of DNA values in all organs except spleen and thymus, whereas weight, protein, and RNA were reduced. The reduced ratios coupled with normal DNA suggest decrease in cell size with retention of cell number. All organs in these animals except thymus recovered normal size on refeeding and all ratios returned to normal. These data suggest that cellular effects of malnutrition depend on the phase of growth in the animal at the time of malnutrition. Early malnutrition impeded cell division and the animal did not recover. Malnutrition at a later stage of growth resulted in reduction of cell size from which the animal could recover.

Severe growth retardation often accompanies malnutrition in young animals and children (1). Recovery after adequate refeeding depends in part on the age of onset of the malnutrition (2). The earlier the animal or child becomes the victim of severe malnutrition the greater is the likelihood of permanent stunting. The mechanism by which the same stimulus, malnutrition, early in development causes permanent stunting and later in development only temporary stunting with recovery, has not been elucidated.

Recent studies on normal growth suggest a new approach to the study of this problem. Since DNA is constant within a single diploid cell in any species (3), it is possible to calculate the number of diploid cells in a given organ at any time by analyzing for total organ DNA and dividing by DNA per cell (6.2 μμg in the rat) (4). Once this number is ascertained, weight, protein, or RNA per cell can be calculated after total organ analysis for each of these components. Weight and protein per cell can be expressed either in absolute figures or as a weight-to-DNA or protein-to-DNA ratio. Both estimates indicate cell mass or size.

Studies in the rat using these methods have suggested the partition of normal growth into 3 periods: cell division alone; cell division with concomitant cell enlargement; and cell enlargement alone with no further increase in the number of cells (5). Although the age span of these periods varies for individual organs, the evidence suggests that early caloric restriction affecting primarily cell division may render recovery impossible, whereas later restriction affecting the size of cells may be

Received for publication February 28, 1966.
[1] Supported in part by a grant from the Ross Laboratories, no. 15-0532082, and the American Cancer Institutional Research Grant IN-73D no. 2.
[2] Presented in part at the Annual Meeting of the Society for Pediatric Research, Atlantic City, April 30, 1965.

compatible with subsequent recovery. The following experiments were undertaken to test this possibility.

## MATERIALS AND METHODS

*Biochemistry.* Analytic methods used in biochemical determinations have been described previously (5). In brief, animals were killed, and tissues were weighed and immediately processed or quick-frozen. DNA, RNA, and protein were separated by a modified Schmidt-Thannhauser procedure (6) and individual fractions were assayed for their respective substances. DNA was determined by Burton's modification of the diphenylamine reaction (7), RNA by the orcinol reaction (8), and protein by the method of Lowry (9). Analyses were carried out in triplicate with less than one per cent differences between matched samples.

*Experimental design.* Rats of the Sprague-Dawley strain were used in all experiments. After weaning they were reared in separate cages. Only male animals were killed for tissue analysis. Animals were divided into 3 groups and compared with littermate controls reared on routine feeding regimens.

Group 1, caloric restriction at birth: Litters of newborn rats were combined so that a total of 18 animals nursed from a

TABLE 1

*Organ weight and protein content after caloric restriction* [1]

| | Group 1 [2] | | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| | Weight | Protein | Weight | Protein | Weight | Protein |
| | *g* | *mg* | *g* | *mg* | *g* | *mg* |
| Whole animal | | | | | | |
| Control | 59.1 | 5871 | 119.4 | — | 266.8 | — |
| Experimental | 28.6 | 2862 | 62.0 | — | 188.1 | — |
| Brain | | | | | | |
| Control | 1.49 | 96.14 | 1.59 | 307 | 1.80 | 335.6 |
| Experimental | 1.23 | 84.26 | 1.40 | 263 | 1.67 | 289 |
| Heart | | | | | | |
| Control | 0.36 | 58.08 | 0.65 | 122 | 1.01 | 142.5 |
| Experimental | 0.19 | 27.21 | 0.55 | 72.9 | 0.75 | 118.8 |
| Lung | | | | | | |
| Control | 0.39 | 41.3 | 1.11 | 171.0 | 1.50 | 213 |
| Experimental | 0.14 | 15.6 | 0.64 | 142.1 | 1.16 | 127 |
| Liver | | | | | | |
| Control | 3.14 | 437.5 | 7.20 | 1835 | 10.58 | 1920 |
| Experimental | 1.39 | 200.1 | 4.06 | 909 | 8.56 | 1619 |
| Kidney | | | | | | |
| Control | 0.36 | 73.3 | 1.27 | 226 | 1.42 | 267 |
| Experimental | 0.20 | 41.8 | 0.77 | 178 | 0.97 | 198 |
| Thymus | | | | | | |
| Control | 0.33 | 45.3 | 0.38 | 105 | 0.75 | 207 |
| Experimental | 0.07 | 12.1 | 0.15 | 59.1 | 0.43 | 102 |
| Spleen | | | | | | |
| Control | 0.29 | 37.5 | 0.48 | 104 | 0.77 | 175 |
| Experimental | 0.06 | 10.9 | 0.17 | 31.1 | 0.30 | 73 |
| Submaxillary gland | | | | | | |
| Control | 0.17 | 17.5 | 0.26 | 66.3 | 0.59 | 55.4 |
| Experimental | 0.13 | 13.2 | 0.16 | 28.5 | 0.50 | 47.0 |
| Gastrocnemius | | | | | | |
| Control | 0.08 | 7.3 | 0.13 | 23.0 | 0.57 | 86.1 |
| Experimental | 0.03 | 2.2 | 0.07 | 13.1 | 0.24 | 11.9 |

[1] Each figure represents the mean of at least 5 animals. The maximal variation was 10% in whole animal and no more than 7% in individual organs.

[2] Group 1: caloric restriction from birth to 21 days; group 2: caloric restriction from 21 to 42 days; and group 3: caloric restriction from 65 to 86 days.

single mother. Controls were kept in normal-size litters (9 to 12 animals per mother). At weaning (21 days), all animals were fed in the usual way for the remaining experimental period.

Group 2, caloric restriction at weaning: All animals were nursed normally for 21 days; test animals thereafter received a restricted diet as described below for 21 days and then were fed routinely. Controls were fed normally throughout the experimental period to 133 days of age.

Group 3, caloric restriction at 65 days: Animals were nursed normally and weaned to normal diets until 65 days old. At this time the test animals were fed the restricted diet for 21 days and were then returned to normal diets. Controls were nursed, weaned, and maintained with normal diets throughout.

At the end of each period of caloric restriction 5 animals were killed. The rest were refed to age 133 days at which time five more were killed. At this age adult weight had been reached and maintained for at least 2 weeks.

All animals were fed a commercial laboratory chow.[3] Control animals were always exposed to a surplus of food. Restricted diets consisted of the same food given in measured amounts, calculated per gram of rat weight to be about one-half

[3] Rockland Rat Mouse Diet, Animal Feeds Inc., 3255 Park Avenue, Bronx 51, New York.

TABLE 2

*Organ weight and protein content after refeeding* [1]

| | Group 1 | | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| | Weight | Protein | Weight | Protein | Weight | Protein |
| | *g* | *mg* | *g* | *mg* | *g* | *mg* |
| Whole animal | | | | | | |
| Control | 376.4 | — | 382.4 | — | 374.4 | — |
| Experimental | 297.1 | — | 314.6 | — | 379.2 | — |
| Brain | | | | | | |
| Control | 1.88 | 306 | 1.92 | 302 | 1.87 | 299 |
| Experimental | 1.60 | 256 | 1.88 | 291 | 1.91 | 317 |
| Heart | | | | | | |
| Control | 1.42 | 272 | 1.31 | 261 | 1.28 | 257 |
| Experimental | 0.94 | 201 | 1.01 | 217 | 1.34 | 254 |
| Lung | | | | | | |
| Control | 2.21 | 557 | 2.19 | 561 | 2.16 | 564 |
| Experimental | 1.87 | 482 | 2.20 | 559 | 2.10 | 548 |
| Liver | | | | | | |
| Control | 12.63 | 4067 | 13.2 | 4226 | 12.42 | 4019 |
| Experimental | 8.86 | 3014 | 10.1 | 3764 | 13.10 | 4328 |
| Kidney | | | | | | |
| Control | 1.41 | 220 | 1.30 | 216 | 1.30 | 212 |
| Experimental | 0.92 | 164 | 1.01 | 173 | 1.21 | 193 |
| Thymus | | | | | | |
| Control | 0.72 | 127 | 0.65 | 119 | 0.65 | 114 |
| Experimental | 0.40 | 84 | 0.29 | 59 | 0.33 | 64 |
| Spleen | | | | | | |
| Control | 0.56 | 196 | 0.60 | 227 | 0.58 | 202 |
| Experimental | 0.37 | 112 | 0.48 | 168 | 0.59 | 198 |
| Submaxillary gland | | | | | | |
| Control | 0.60 | 60 | 0.62 | 63.1 | 0.59 | 55.8 |
| Experimental | 0.40 | 39 | 0.42 | 40.0 | 0.62 | 59.8 |
| Gastrocnemius | | | | | | |
| Control | 1.68 | 161 | 1.70 | 169 | 1.61 | 157 |
| Experimental | 1.41 | 142 | 1.20 | 122 | 1.50 | 164 |

[1] Each figure represents the mean of at least 5 animals. The maximal variation was 10% in whole animal and no more than 7% in individual organs.

the required caloric intake for adequate growth (10).

## RESULTS

*1. Weight.* The experimental animals fed restricted diets in all 3 groups lagged behind controls in total body weight gain and in weight gain for all organs studied (table 1).

Following refeeding, none of the animals in group 1 recovered normal body or organ weights. In group 2 only brain and lung regained normal weight, whereas in group 3 body and all organs except thymus reached normal weight (table 2).

These data demonstrate that the earlier the caloric restriction, the less likely is attainment of normal body and organ weight after adequate refeeding.

*2. Protein.* The increase in total protein observed during normal growth in all organs is curtailed approximately equally in all 3 groups during caloric restriction (table 1). Refeeding was accompanied by qualitative and quantitative effects on total organ protein similar to those on weight. The protein content of all organs of animals in group 1 remained low; in group 2 brain and lung recovered; and in group 3 all organs except thymus recovered (table 2). The effect on total organ protein is thus comparable to the effect on total organ weight: caloric restriction prevents normal increments of increase and the earlier the

TABLE 3

*Organ RNA and DNA content after caloric restriction* [1]

| | Group 1 | | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| | RNA | DNA | RNA | DNA | RNA | DNA |
| | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* |
| Whole animal | | | | | | |
| Control | 109.3 | 97.81 | — | — | — | — |
| Experimental | 67.25 | 42.21 | — | — | — | — |
| Brain | | | | | | |
| Control | 3.46 | 2.18 | 4.27 | 2.94 | 4.32 | 3.28 |
| Experimental | 2.18 | 1.48 | 3.40 | 2.81 | 4.02 | 3.39 |
| Heart | | | | | | |
| Control | 0.92 | 0.622 | 1.57 | 0.798 | 2.02 | 1.39 |
| Experimental | 0.71 | 0.377 | 0.555 | 0.678 | 1.57 | 1.33 |
| Lung | | | | | | |
| Control | 2.21 | 1.96 | 3.36 | 3.17 | 4.87 | 3.56 |
| Experimental | 1.32 | 1.06 | 1.84 | 3.48 | 4.06 | 3.61 |
| Liver | | | | | | |
| Control | 19.26 | 4.96 | 43.3 | 9.77 | 97.34 | 25.02 |
| Experimental | 13.17 | 3.28 | 23.83 | 6.89 | 75.91 | 24.91 |
| Kidney | | | | | | |
| Control | 1.44 | 1.65 | 4.42 | 3.93 | 5.50 | 3.80 |
| Experimental | 0.89 | 0.99 | 3.30 | 3.07 | 4.15 | 3.69 |
| Thymus | | | | | | |
| Control | 1.07 | 2.74 | 3.06 | 10.3 | 1.41 | 1.06 |
| Experimental | 0.74 | 0.99 | 0.874 | 2.85 | 0.81 | 0.76 |
| Spleen | | | | | | |
| Control | 1.40 | 2.30 | 2.45 | 5.29 | 4.41 | 10.62 |
| Experimental | 0.68 | 0.76 | 1.09 | 2.07 | 1.63 | 2.67 |
| Submaxillary gland | | | | | | |
| Control | 2.54 | 1.17 | 3.23 | 1.78 | 4.77 | 1.92 |
| Experimental | 1.18 | 0.59 | 2.09 | 1.07 | 4.14 | 2.16 |
| Gastrocnemius | | | | | | |
| Control | 0.18 | 0.060 | 0.516 | 0.128 | 0.75 | 0.52 |
| Experimental | 0.11 | 0.022 | 0.311 | 0.089 | 0.16 | 0.54 |

[1] Each figure represents the mean of 5 separate animals with a maximal variation in either direction of less than 8%.

TABLE 4

*Organ RNA and DNA content after refeeding* [1]

| | Group 1 | | Group 2 | | Group 3 | |
|---|---|---|---|---|---|---|
| | RNA | DNA | RNA | DNA | RNA | DNA |
| | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* |
| Whole animal | | | | | | |
| Control | — | — | — | — | — | — |
| Experimental | — | — | — | — | — | — |
| Brain | | | | | | |
| Control | 4.16 | 3.10 | 4.29 | 2.97 | 4.37 | 3.02 |
| Experimental | 3.30 | 2.42 | 4.47 | 2.92 | 4.61 | 3.13 |
| Heart | | | | | | |
| Control | 2.14 | 1.43 | 2.02 | 1.40 | 1.99 | 1.47 |
| Experimental | 1.67 | 1.12 | 1.78 | 1.09 | 2.05 | 1.45 |
| Lung | | | | | | |
| Control | 4.53 | 3.34 | 4.41 | 3.28 | 4.87 | 3.18 |
| Experimental | 2.47 | 1.82 | 4.30 | 3.20 | 4.44 | 3.22 |
| Liver | | | | | | |
| Control | 95.72 | 25.68 | 93.0 | 25.26 | 93.6 | 25.09 |
| Experimental | 71.31 | 19.82 | 76.2 | 20.1 | 120.2 | 24.02 |
| Kidney | | | | | | |
| Control | 4.67 | 3.79 | 4.53 | 3.40 | 4.48 | 3.84 |
| Experimental | 3.12 | 2.08 | 4.01 | 2.81 | 4.07 | 3.82 |
| Thymus | | | | | | |
| Control | 1.44 | 1.47 | 1.42 | 1.30 | 1.41 | 1.32 |
| Experimental | 0.97 | 0.82 | 0.99 | 0.96 | 1.06 | 1.07 |
| Spleen | | | | | | |
| Control | 4.02 | 9.68 | 4.52 | 10.58 | 4.41 | 10.06 |
| Experimental | 2.87 | 6.94 | 3.06 | 7.63 | 4.53 | 9.94 |
| Submaxillary gland | | | | | | |
| Control | 4.47 | 1.94 | 4.68 | 2.01 | 4.68 | 1.98 |
| Experimental | 3.32 | 1.32 | 3.85 | 1.48 | 4.79 | 1.99 |
| Gastrocnemius | | | | | | |
| Control | 0.76 | 0.51 | 0.80 | 0.57 | 0.78 | 0.50 |
| Experimental | 0.62 | 0.40 | 0.61 | 0.32 | 0.81 | 0.49 |

[1] Each figure represents the mean of 5 separate animals with a maximal variation in either direction of less than 8%.

caloric restriction, the less likely is recovery on refeeding.

3. *RNA.* Total RNA was reduced in all organs in all groups following restricted feedings (table 3). The reduction again parallels the decrease observed in weight and protein. Reductions in RNA are not as great in some instances, and in one organ, brain in group 3, there is overlap between control and experimental values. Refeeding resulted in complete recovery in those organs which show recovery in weight and protein (table 4).

4. *DNA.* In group 1 the experimental animals showed less DNA in total body and in all organs at the end of the experimental period (table 3) and at the end of the period of refeeding (table 4). Caloric restriction caused a persistent reduction in the number of cells of all organs. In group 2 only brain and lung were unaffected. All other organs showed reduced amounts of DNA which persist despite refeeding (tables 3 and 4). In group 3 there was no effect on DNA except in thymus and spleen (table 3). Thus, although organ weight, protein, and RNA were reduced in all groups at the end of caloric restriction, DNA was reduced only when the restriction was carried out in postnatal life and only those organs in which normal DNA increase has been curtailed failed to

recover — all organs in group 1, all organs except brain and lung in group 2, and only thymus in group 3.

5. *Ratios.* Weight-to-DNA and protein-to-DNA ratios are normal in all organs in group 1; thus the reduction in weight and total protein parallels the decrease in DNA. The effect of dietary restriction in early life, as indicated above, is solely on cell number, cell size remaining constant.

The ratios are similar in the group 2 animals for all organs except brain and lung. The normal ratios again indicate a reduction in cell number and not in cell size. In brain and lung, however, the maintenance of normal values for DNA with a concurrent lowering of organ weight and protein results in lower ratios and confirms the decrease in cell size in these 2 organs. In lung the weight-to-DNA ratio is decreased more than the protein-to-DNA ratio. During refeeding only brain and lung recovered as indicated by a return to normal ratios. In group 3 all organs except lymphoid tissue contain normal quantities of DNA and hence their weght-to-DNA and protein-to-DNA ratios are reduced. Only cell size was affected. Refeeding resulted in complete correction of these ratios and recovery of normal size in all organs except thymus.

The lymphoid organs demonstrate reduced DNA values at the end of the period of caloric restriction in all groups (table 3). During refeeding, the failure of DNA synthesis in spleen in groups 1 and 2 persisted, whereas in group 3 complete recovery occurred.

## DISCUSSION

Normal growth both in the whole rat and in the individual organs is due initially to the acquisition of new cells with the size of individual cells remaining constant. Later, growth is associated with both cellular hyperplasia and hypertrophy. And finally growth is by hyperplasia alone (5). These stages merge gradually into one another and vary in duration with each organ. However, before weaning, all organs grow primarily by cell division. Between weaning and about 65 days of age beginning in brain and lung the pattern shifts. After 65 days growth in all organs is due primarily to cell enlargement. The effect of any stimulus on growth inhibition may, therefore, be time-dependent. Studies by other investigators suggest that retardation in body growth of animals and children is less likely to be reversible the younger the animal or child at the onset of malnutrition (2) (11). To investigate this time-dependence, 3 periods were chosen with prior knowledge of the growth phases of the various organs — 0 to 21 days, cellular hyperplasia of all organs; weaning to 42 days, continuing cellular hyperplasia in all organs except brain and lung; 65 to 86 days, cellular hypertrophy of all organs except lymphoid tissue (5). The severity of the malnutrition was kept relatively constant by controlling the duration and providing a diet that maintained weight but did not allow any weight gain.

The results indicate that caloric restriction prevents the expected increase in weight, total protein, and RNA in all groups of animals regardless of the time of onset of the malnutrition. The lack of weight gain, then, is due not to the state of hydration but to the curtailment of normal increases in cytoplasmic constituents. DNA, however, is affected by malnutrition only before it has ceased to increase. It is the interference with cell division, therefore, which is time-dependent. There is no recovery after refeeding only in those organs where cell division has been curtailed. Thus caloric restriction results in curtailment of normal growth no matter when its onset but this effect is reversible by refeeding as long as cell division has not been affected.

Cell division may be interrupted by malnutrition at any time. In spleen, where cell division is always in progress, DNA values were reduced at the end of caloric restriction even in group 3. However, during the period of actual organ growth by cell division (increase in total organ DNA) caloric restriction was not followed by recovery (groups 1 and 2). Later, when final size was attained and cell division and replacement had equilibrated, recovery was possible (group 3). This is the only regenerating organ that has been studied but similar effects could be anticipated in intestinal mucosa and bone marrow. In the thymus, which is the only organ in which cell number actually declines, cal-

oric restriction at any time appears to hasten this decline.

Since the duration of cell division varies in different organs, differential effects on organ recovery are possible following refeeding. In the test animals in group 2, although all organ growth was retarded after caloric restriction, brain and lung recovered normal weight after refeeding. The adult animal in this group is small but has a normal-size brain and lung.

Although these data are entirely descriptive, they suggest that growth failure observed with malnutrition may be of 2 types, that these types depend on age of onset, and that the ability to recover may be dependent on the cellular type of growth failure produced. Reduction in cell number results in permanent stunting whereas reduction in cell size results in recovery of normal stature after refeeding. Although similar data are not available in human malnutrition, the clinical evidence suggests that recovery in the human is also time-dependent. It is possible that permanent stunting in younger malnourished children and growth recovery in older malnourished children can be similarly explained by differential effects on cell division and cell size.

## LITERATURE CITED

1. Dunn, M. S., E. A. Murphy and L. B. Rockland 1947 Optimal growth of the rat. Physiol. Rev., 27: 72.
2. McCance, R. A., and E. M. Widdowson 1962 Nutrition and growth. Proc. Roy. Soc. Lond., *156:* 326.
3. Boivin, A., R. Vendrely and C. Vendrely 1948 L'acide désoxyribonucléique du noyau cellulaire, dépositaire des caractères héréditaires; arguments d'ordre analytique. C. R. Acad. Sci., 226: 1061.
4. Enesco, M., and C. P. Leblond 1962 Increase in cell number as a factor in the growth of the young male rat. J. Embryol. Exp. Morphol., *10:* 530.
5. Winick, M., and A. Noble 1965 Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in the rat. Develop. Biol., *12:* 451.
6. Schmidt, G., and S. J. Thannhauser 1945 A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissue. J. Biol. Chem., *161:* 83.
7. Burton, K. 1956 A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J., 62: 315.
8. Dische, Z. 1955 In: The Nucleic Acids., vol. 1, eds., E. Chargaff and J. N. Davidson. Academic Press, New York.
9. Lowry, O. H., N. J. Rosebrough, H. L. Farr and R. J. Randall 1951 Protein measurement with the folin phenol reagent. J. Biol. Chem., *193:* 265.
10. Warner, R. G. 1962 Nutrient requirements of the laboratory rat. In: Nutrient Requirements of Laboratory Animals, pub. 990. National Academy of Sciences — National Research Council, Washington, D. C., p. 51.
11. Becroft, T., and K. V. Bailey 1965 Supplementary feeding trial in New Guinea highland infants. J. Trop. Pediat. African Child Health, *11*(2): 28.

# Effect of Dietary Iron on Gossypol Toxicity and on Residues of Gossypol in Porcine Liver [1,2]

A. J. CLAWSON AND F. H. SMITH
*Department of Animal Science, North Carolina State University, Raleigh, North Carolina*

*ABSTRACT* A 3 × 3 × 2 factorial design was used to study the effect of gossypol levels (80, 244 and 400 mg/kg of diet), supplemental iron-to-gossypol ratios (0:1, 0.5:1, and 1:1), and sources of supplemental lysine (L-lysine and fish meal) in corn-cottonseed meal rations on performance and tissue residues of gossypol. Six pigs were group-fed each ration. One additional group was fed a corn-soybean meal ration as a control for tissue residues. Free gossypol at the level of 80 mg/kg of diet did not appear to affect pig performance adversely. The iron-to-gossypol ratio of 1:1 was adequate to protect growing pigs against toxicity when up to 400 mg of free gossypol/kg of diet were fed. This ratio of added iron to gossypol prevented accumulation of gossypol in the livers in excess of that observed when 80 mg/kg of diet were fed. The 0.5:1 ratio of iron to gossypol was effective in improving performance but was not as effective in reducing toxicity or in reducing deposition of gossypol in the livers as was the higher level.

The use of cottonseed meal as a dietary source of protein for monogastric animals is limited because of the sensitivity of such animals to gossypol ( a polyphenolic binaphthyl pigment occurring naturally in cottonseed). It has been shown previously that the addition of iron salts to the diet reduces or prevents gossypol toxicity (1–4). The level of dietary iron necessary for the prevention of toxicity, has not been determined for cottonseed meals of varying gossypol content. The levels of iron used have been relatively high and have caused a dark discoloration of the feces of animals to which it is fed. For these or other reasons this method of alleviating the toxicity problem has not been widely practiced.

The present study was conducted to determine the minimal level of iron necessary for the prevention of toxicity in swine and to determine the influence of iron level on the deposition of gossypol in the liver.

## EXPERIMENTAL PROCEDURE

A 3 × 3 × 2 factorial design was used to study 3 ration levels of free gossypol, 3 ratios of added iron to free gossypol and 2 sources of supplemental lysine. Levels of 80, 244 and 400 mg of free gossypol/kg of diet were obtained from 2 cottonseed meals containing 0.07 and 0.32% of free gossypol used singly or as a mixture of equal parts of each at a level of 12.5% of the diet. Iron was added as ferrous sulfate monohydrate at the expense of corn in weight ratios of 0:1, 0.5:1 and 1:1 to free gossypol. Additional lysine was obtained by the substitution of 3.9% fish meal for soybean meal which resulted in a total dietary lysine level of 0.6% or by supplemental L-lysine to provide a dietary level of 1%. The effects of added zinc and copper on gossypol toxicity were also studied. Zinc levels of 100 and 200 mg/kg of diet supplied from zinc oxide or copper at a level of 190 mg/kg of diet supplied from copper sulfate were used. The zinc and copper were added to the corn-cottonseed meal-fish meal diet which contained 244 mg of free gossypol/kg. The corn-soybean meal control ration and the basal rations containing cottonseed meal with added fish meal or L-lysine are presented in table 1.[3]

Received for publication January 26, 1966.

[1] Contribution from the Animal Science Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper no. 2111.

[2] This study was supported partially by the National Cottonseed Products Association, Memphis, Tennessee and by a Public Health Service Research Grant no. AM-07039 from the National Institute of Arthritis and Metabolic Diseases.

[3] The authors wish to acknowledge the assistance of Merck and Company, Inc., Rahway, New Jersey, for supplying the L-lysine, and of Charles Pfizer and Company, Inc., Terre Haute, Indiana, for supplying the vitamin supplement.

Six pigs were group-fed each ration, making a total of 132. The pigs weighed an average of 25 kg initially and the experiment was continued for approximately 90 days or until the pigs died. Pigs that died were autopsied to ascertain the cause of death and their livers were recovered for chemical determination of dry matter, free and bound gossypol. Pigs that survived during the entire study to a weight of approximately 100 kg were slaughtered at a local packing plant. Their livers were also collected and frozen (−18°) for analysis. The methods described previously (5) were used to determine tissue gossypol levels.

## RESULTS

Performance of pigs within the 2 replications was not significantly different; therefore, the data were combined and are shown in table 2. No gross clinical symptoms of toxicity were noted during the first 5 weeks of the test. Feed intake and daily gain were not greatly different between gossypol levels or between iron-to-gossypol-ratios when supplemental iron was present. In the absence of supplemental iron, daily feed intake and rate of gain were reduced in pigs receiving diets containing 400 mg of free gossypol/kg of diet. This trend toward reduced feed intake and daily gains became more pronounced as the feeding period progressed. Poor feed consumption has previously been reported (6) as a symptom of gossypol toxicity. Performance of pigs fed diets containing 244 mg of free gossypol/kg of diet and no iron also became ad-

TABLE 1

*Rations used to obtain graded levels of gossypol*

| | Cottonseed meal + L-lysine | Cottonseed meal + fish meal | Soybean meal control |
|---|---|---|---|
| | % | % | % |
| Ground yellow corn [1] | 79.18 | 81.40 | 83.40 |
| Cottonseed meal (41%) [2] | 12.50 | 12.50 | |
| Fish meal [3] | | 3.90 | |
| Soybean meal [4] | 4.50 | | 14.00 |
| L-Lysine supplement [5] | 1.02 | | |
| Defluorinated phosphate [6] | 0.80 | 0.30 | 1.00 |
| Ground limestone | 1.00 | 0.90 | 0.60 |
| Trace mineral salt [7] | 0.50 | 0.50 | 0.50 |
| Vitamin supplement [8] | 0.50 | 0.50 | 0.50 |

[1] Contained: 0.015% iron, potassium thiocyanate method.
[2] Two cottonseed meals: 1) a prepress solvent meal containing 0.07% free gossypol, 0.028% iron, 1.5% lysine and a nitrogen solubility of 67%; and 2) a direct solvent meal containing 0.32% free gossypol, 0.043% iron, 1.5% lysine and a nitrogen solubility of 74%, were used.
[3] Contained: 66% protein, 0.225% iron, 5.84% lysine and 12% fat.
[4] Contained: 49.5% protein, 0.032% iron and 3.08% lysine.
[5] Contained: 50% L-lysine.
[6] Contained: 34% calcium and 18% phosphorus.
[7] Contained: salt, 94 to 97%; zinc, 0.8%, from zinc oxide; manganese, 0.6%, from manganous oxide; iron, 0.2%, from ferrous carbonate; copper, 0.06%, from copper oxide; iodine, 0.016%, from calcium iodate; and cobalt, 0.015%, from cobalt carbonate.
[8] Supplied: vitamin A, 2200 IU; vitamin D, 220 IU; riboflavin, 1.1 mg; pantothenic acid, 5.5 mg; nicotinic acid, 16.5 mg; choline chloride, 110 mg; vitamin $B_{12}$, 17.6 μg; and butylated hydroxy toluene, 250 mg/kg of diet.

TABLE 2

*Performance of pigs fed rations containing graded levels of gossypol and iron* [1]

| | Gossypol level, mg/kg | | | | | | | | | Corn-soybean meal control |
|---|---|---|---|---|---|---|---|---|---|---|
| | 80 | 244 | 400 | 80 | 244 | 400 | 80 | 244 | 400 | |
| | Iron level, mg/kg | | | | | | | | | |
| | 0 | 0 | 0 | 40 | 122 | 200 | 80 | 244 | 400 | |
| Avg daily feed, kg | 2.73 | 2.12 | 1.47 | 2.61 | 2.68 | 2.52 | 2.67 | 2.83 | 2.74 | 2.76 |
| Avg daily gain, kg | 0.74 | 0.55 | 0.39 | 0.73 | 0.69 | 0.65 | 0.71 | 0.76 | 0.75 | 0.79 |
| Feed/kg gain | 3.67 | 3.86 | 3.78 | 3.60 | 3.90 | 3.86 | 3.70 | 3.74 | 3.64 | 3.51 |
| No. of deaths | 0 | 4 | 9 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |

[1] Combined data for 2 replications of 6 pigs each, except for one replication only for corn-soybean meal control.

versely affected as the feeding period progressed. There was no indication that the added iron per se affected daily feed intake when compared with that of pigs receiving the low level of gossypol and no iron.

Daily gains, over the entire feeding period, were significantly ($P < 0.01$) influenced by both dietary gossypol and by iron level. The interaction term between gossypol and iron was also shown to be significant ($P < 0.01$). The significant differences between gossypol levels evident across all treatments was not significant when supplemental iron was present. There was no significant difference in daily gains between lysine sources.

The first symptoms of toxicity were noted after the experimental diets had been consumed for about 35 days. Eight out of twelve pigs, receiving diets containing 400 mg of gossypol/kg and no added iron died between 37 and 57 days after the experiment was started. The other 4 pigs receiving these diets were showing signs of toxicity (labored jerky breathing and purple coloration of the snout and ears) at the time of the latter deaths. However, they greatly reduced their feed intake for about 4 weeks which resulted in a remission of the symptoms. At that time they started consuming more feed, toxicity symptoms reappeared and one additional pig died. Acute and chronic toxicity from gossypol have been previously reported (7). Four pigs fed diets containing 244 mg of gossypol/kg also died. Their deaths were delayed somewhat, occurring between 48 and 68 days after the trial started. Performance of pigs fed rations containing iron and gossypol at the 1:1 ratio was almost equal to those fed the corn-soybean meal ration which contained no gossypol. There were no deaths or signs of toxicity when the 1:1 ratio of iron to gossypol was used. The 0.5:1 ratio of iron to gossypol also afforded partial protection.

The addition of zinc oxide to provide levels of 100 and 200 mg of zinc/kg of diet or the addition of copper sulfate to provide 190 mg of dietary copper/kg to the corn-cottonseed meal ration containing 244 mg of gossypol/kg was of no observable benefit in preventing toxicity.

Statistical analysis, of the data concerning levels of gossypol and iron in the livers, revealed no difference between the treatments with added lysine or fish meal therefore the data were combined and are shown in table 3.

Levels of both free and bound gossypol in the livers tended to increase significantly ($P < 0.01$) with an increasing dietary gossypol level from 80 to 244 mg/kg. This was true at all levels of added iron. A further increase in dietary gossypol level, to 400 mg/kg, resulted in no additional increase in the liver level of gossypol. This can be explained, at least in part, for those pigs fed no added dietary iron. The pigs fed the higher level of gossypol (400 mg/kg) died before maximal accumulation had occurred, whereas fewer pigs fed the lower (244 mg/kg) level died and they survived for a longer

TABLE 3

*Gossypol and iron content of livers of pigs fed rations containing varying ratios of iron to gossypol*

| | Gossypol level, mg/kg | | | | | | | | | Corn-soybean meal control |
|---|---|---|---|---|---|---|---|---|---|---|
| | 80 | 244 | 400 | 80 | 244 | 400 | 80 | 244 | 400 | |
| | Iron level, mg/kg | | | | | | | | | |
| | 0 | 0 | 0 | 40 | 122 | 200 | 80 | 244 | 400 | |
| Liver gossypol [1,2] | | | | | | | | | | |
| Free | 157 | 226 | 203 | 145 | 248 | 244 | 114 | 168 | 162 | 14 |
| Bound | 162 | 263 | 253 | 153 | 275 | 265 | 121 | 176 | 203 | 8 |
| Liver iron,[3] μg/g | 341 | 646 | 960 | 466 | 482 | 500 | 561 | 553 | 560 | 419 |

[1] Expressed as mean for 12 pigs/treatment in micrograms of gossypol/gram of dry matter.
[2] L.S.D. for comparing means of iron to gossypol ratios are as follows: for free gossypol, 24 and 31; bound gossypol, 26 and 35; and for iron, 110 and 145, respectively, for the 0.05 and 0.01 levels of significance.
[3] L.S.D. for comparing mean gossypol levels within iron to gossypol ratios are as follows: for free gossypol, 41 and 55; for bound gossypol, 45 and 60; and for iron 60 and 79, respectively, for the 0.05 and 0.01 levels of significance.

period of time. It has been shown previously (8) that gossypol does accumulate in the tissues in relation to the length of time that it is fed. For those treatments in which iron was added, it appears that the higher ratio of iron to gossypol was more effective in preventing the accumulation of gossypol in the livers than was the lower ratio. Dietary iron has previously been shown to reduce both free and bound gossypol in organ tissues of pigs (9). The higher level of iron provided by the 1:1 ratio of iron to gossypol significantly reduced the liver level of both free and bound gossypol, whereas the lower level 0.5:1 did not. The level of both free and bound gossypol in the livers was also significantly ($P < 0.01$) different between the 2 ratios of added iron.

The level of iron observed in the livers was variable. The iron level in the livers from pigs receiving no added iron tended to increase with increasing levels of gossypol. This trend was significant ($P < 0.05$). This observation is not in agreement with a previous report (10) in which an inverse relationship between liver gossypol and iron level was shown. In the present study, nine of the twelve pigs fed diets containing 400 mg of free gossypol/kg of diet died. Their livers were gorged with blood. The higher iron content of blood which was present in these livers may account for the high iron levels observed in the livers. Liver dry-matter values for the pigs from treatments receiving diets containing 80, 244 and 400 mg/kg of gossypol with no added iron were 31, 26 and 24% respectively, whereas for all other treatments liver dry matter ranged between 30 and 31%. A similar trend in liver iron level occurred with the 0.5:1 ratio of iron to gossypol. That is, liver iron level tended to increase as dietary gossypol level was increased. Liver iron levels were similar across gossypol treatments at the higher (1:1) ratio of added iron to gossypol.

ACKNOWLEDGMENT

The authors are grateful to Carolina Packers, Inc., Smithfield, North Carolina, for their cooperation in slaugher, and for obtaining tissues for analysis.

LITERATURE CITED

1. Withers, W. A., and F. E. Carruth 1917 Iron as an antidote to cottonseed meal injury. J. Biol. Chem., 32: 245.
2. Gallup, W. D. 1928 The value of iron salts in counteracting the toxic effects of gossypol. J. Biol. Chem., 77: 437.
3. Robison, W. L. 1936 Making cottonseed meal safe and effective when fed as the only source of protein concentrate to pigs in dry-lot. Proceedings of the American Society of Animal Production, p. 87.
4. Barrick, E. R., F. H. Smith, G. Matrone, H. A. Stewart and G. H. Wise 1950 Some factors affecting the nutritive value of cottonseed meal for swine. J. Animal Sci., 9: 646.
5. Smith, F. H. 1965 The determination of free and bound gossypol in swine tissues. J. Am. Oil. Chem. Soc., 42: 145.
6. Clawson, A. J., F. H. Smith, J. C. Osborne and E. R. Barrick 1961 Effect of protein source, autoclaving, and lysine supplementation on gossypol toxicity. J. Animal Sci., 20: 547.
7. Kornegay, E. T., A. J. Clawson, F. H. Smith and E. R. Barrick 1961 Influence of protein source on toxicity of gossypol in swine rations. J. Animal Sci., 20: 597.
8. Sharma, M. P., F. H. Smith and A. J. Clawson 1966 Effects of levels of protein and gossypol, and length of feeding period on the accumulation of gossypol in tissues of swine. J. Nutrition, 88: 434.
9. Smith, F. H., and A. J. Clawson 1965 The effect of diet on the accumulation of gossypol in the organs of swine. J. Nutrition, 87: 317.
10. Clawson, A. J., F. H. Smith and E. R. Barrick 1962 Accumulation of gossypol in the liver and factors influencing the toxicity of injected gossypol. J. Animal Sci., 21: 911.

# Rate of Passage of Calcium-45 and Yttrium-91 Along the Intestine, and Calcium Absorption in the Laying Fowl [1]

S. HURWITZ AND A. BAR
*The Volcani Institute of Agricultural Research, The National and University Institute of Agriculture, Rehovot, Israel*

*ABSTRACT* The passage of $^{45}Ca$ and yttrium-91 ($^{91}Y$) along the gastrointestinal tract of the laying fowl was compared by dosing either the crop or the duodenum with a double tracer dose. Passage time in the crop and along the intestinal tract was similar for both isotopes but $^{45}Ca$ appears to have moved more rapidly than $^{91}Y$ out of the stomach. The passage rate was highest in the duodenum and decreased progressively down the intestine. Outflow of $^{45}Ca$ from the intestine to the blood was rapid during the first 30 minutes after the duodenal administration, by which time the isotope hardly reached beyond the jejunum. The outflow appears to have slowed down considerably after 30 minutes, as also indicated by the specific activity of $^{45}Ca$ in blood plasma. It was concluded that the most effective site of calcium absorption in the laying hen is the jejunum. The possible errors in the determination of absorption with the aid of unabsorbed markers, are discussed.

One of the first steps in investigating intestinal absorption is the identification of the absorption site. Results of a previous trial (1) with laying hens suggested that the absorption of calcium was almost completely restricted to the duodenum and jejunum, with hardly any absorption in the ileum and colon. This conclusion was based on the assumption that the rate of passage of calcium along the intestine was the same as that of yttrium-91 ($^{91}Y$), so that the cumulative absorption could be obtained from the $Ca/^{91}Y$ ratios. Marcus and Lengemann (2, 3) have used the transit time of $^{91}Y$ in order to identify the site of absorption of $^{45}Ca$ and $^{85}Sr$ in the rat. Renner (4) calculated the absorption of fat along the chick-intestine from fat-to-chromic oxide ratios. Undoubtedly, the assumption made above holds for their work as well.

In contrast with results with the hen (1), results with rats (3) and dogs (5) indicated that the ileum was the most effective segment in calcium absorption due to the relatively long transit time in this segment.

The present study was designed to (a) test the previous assumption that the passage time through the intestine is the same for calcium and $^{91}Y$, and (b) evaluate the sites of effective absorption of $^{45}Ca$ by comparing the recovery of the isotope with its progress through the intestine, and by measuring its plasma concentration as a function of time following the delivery of a single dose into the duodenum.

## METHODS

*General procedure.* White Leghorn hens, 14 months old and in good egg production, were used in both trials. At appropriate intervals after dosing they were bled by heart puncture and killed by an intracardiac dose of sodium pentobarbital. Both ends of the intestinal tract were ligated and the intestine was removed. It was divided immediately into segments by ligatures. With this procedure it is felt that no significant food movement could have occurred during the handling of the intestine. In trial 1, the esophagus and stomach were also utilized. The following portions of the intestine were taken separately: the duodenum, three equal-length portions of the jejunum, three equal-length portions of the ileum, the ceca and the colon. Each of

Received for publication February 1, 1966.

[1] Contribution from The National and University Institute of Agriculture, Rehovot, Israel, 1965 series, no. 957-E. This research has been in part financed by a grant made by the United States Department of Agriculture under P. L. 480.

these portions was placed in a crucible, dried overnight at 105° and ashed at 700°. The ash was dissolved in equal volumes of 5 N hydrochloric acid and transferred quantitatively into volumetric flasks. The final acid concentration in each sample was close to 0.5 N. One-milliliter aliquots were placed in scintillation flasks containing Brey scintillation liquid. The samples were then counted in separate channels for $^{45}Ca$ and $^{91}Y$, in a TriCarb liquid scintillation counter. The instrument had been previously calibrated to obtain channels with maximal separation between the isotopes. Furthermore, the proper conditions were established in which variations in counts due to acid-quenching were insignificant over a wide range of acid concentration, so that a small deviation in the acid concentration of the samples from 0.5 N could not affect the count. No corrections were necessary for the $^{91}Y$ counts, but 14.7% of the $^{91}Y$ counts had to be deducted from those of $^{45}Ca$.

Plasma samples were digested in nitric acid and evaporated to dryness. The remaining ash was dissolved in 0.5 N hydrochloric acid, and aliquots taken for radioassays as above. Stable calcium was determined by a direct EDTA titration with calcein as indicator.

The passage time of $^{91}Y$ was estimated graphically from output curves after corrections for recovery, using a similar method to that of Marcus and Lengemann (2). Percentage output from a respective intestinal segment was calculated as (100 − $^{91}Y$ in preceding segments, − $^{91}Y$ in segment). $^{91}Y$ is given in units of percentage of dose.

Obviously, the output from one segment was the input into the next segment. The passage time through any segment was taken as the time interval between 50% output from the preceding segment and 50% output from the segment.

Passage time was also calculated from the results of the previous trial (1) by a method adapted from Persson and Svensson (6). When $^{91}Y$ is fed continuously, the passage time can be calculated by: $^{91}Y$ in segment × 1440/daily $^{91}Y$ intake; the factor of 1440 is the number of minutes per day.

*Trial 1.* This trial was originally designed to compare the passage of $^{45}Ca$ and $^{91}Y$ along the entire gastrointestinal tract.

One hundred and sixty microcuries of $^{45}Ca$ and $^{91}Y$ each, as chlorides, were added to 10 g of laying ration. The mixture was dried, pulverized, mixed well, and equal portions were weighed into gelatin capsules.

Twenty hens were used in this experiment. The feeders were removed the preceding evening in order to eliminate the early morning feed consumption. At 8 AM the feeders were returned for 5 minutes and each hen was given one gelatin capsule per os, into the crop. The hens were then allowed to eat ad libitum. Groups of 4 birds each were killed at 30 minutes, 1, 2, 4, and 7 hours, respectively, after dosing, and their gastrointestinal tract was immediately removed and taken for analysis.

*Trial 2.* The purpose of this trial was to evaluate the passage of $^{45}Ca$ and $^{91}Y$ in the intestine.

Twenty White Leghorn hens were used for this trial. Before and during the experiment feed and water were given ad libitum. The diet was a commercial ration containing 3.5% calcium.

The posterior left abdomen of each bird was cleared of feathers and procaine hydrochloride was injected under the skin. A 3-cm incision was made through the skin and peritoneum, parallel to the long axis of the body, just above the tip of the sternum. After removal of some abdominal fat, the duodenum could be clearly identified. One milliliter of a solution containing 4 μCi of $^{45}Ca$ and 4 μCi of $^{91}Y$ and 1 mg of calcium as chloride was injected with a 25G hypodermic needle into the upper third of the duodenum. The peritoneum was then sutured and the skin clamped. The entire operation took about 3 minutes and the birds were observed to resume their eating a few minutes after their return to the individual cages.

Groups of 4 birds each were bled and killed 15, 30, 60, 120 and 240 minutes, respectively, after dosing. Their intestines were removed immediately and taken for analysis.

## RESULTS

In trial 1 we observed large individual variations in the emptying time of the crop. These did not interfere with the calculation of passage times through the esophagus and stomach since the latter were relatively long. However, no valid calculations could be made for the intestinal segments, where passage times are considerably shorter.

Percentage outputs from the esophagus and stomach (table 1) indicate that both $^{45}Ca$ and $^{91}Y$ were emptied from the esophagus at a similar rate. However, $^{45}Ca$ appeared to have left the stomach at a faster rate than did $^{91}Y$; passage time (50% output) in the esophagus was about 3 hours for both isotopes. Passage time through the stomach was between 1 to 2 hours for $^{91}Y$ and 0.5 to 1 hour for $^{45}Ca$.

From the results of trial 2 it is apparent that almost the entire dose of $^{91}Y$ was recovered in the intestine up to 2 hours after dosing (fig. 1). Since $^{91}Y$ was not found to be absorbed by birds (1), the reduced recovery of $^{91}Y$ at 4 hours after dosing is probably due to fecal excretion. Thirty minutes after dosing the recovery of $^{45}Ca$ in the intestine was as low as 41%, indicating extensive outflow of this isotope from the intestine during this short period. From 30 to 120 minutes after dosing the decline in $^{45}Ca$ recovery was markedly reduced probably due to a reduced outflow. The further reduction in recovery of $^{45}Ca$ 4 hours after dosing may be attributed mainly to fecal excretion similarly to that of $^{91}Y$.

Specific activity of $^{45}Ca$ in blood plasma was relatively high after 15 minutes but

TABLE 1

*Output of $^{45}Ca$ and $^{91}Y$ from the esophagus and stomach of laying fowls* [1]

| Time after dosing | Esophagus | | Stomach [2] | |
|---|---|---|---|---|
| | $^{91}Y$ | $^{45}Ca$ | $^{91}Y$ | $^{45}Ca$ |
| *min* | *% dose* | *% dose* | *% dose* | *% dose* |
| 30 | 1.9 | 3.1 | 0.6 | 1.3 |
| 60 | 21.5 | 21.7 | 10.6 | 12.9 |
| 120 | 19.4 | 23.5 | 13.8 | 19.4 |
| 240 | 71.1 | 76.6 | 48.5 | 58.5 |
| 420 | 77.7 | 80.1 | 75.2 | 78.5 |

[1] A double tracer dose of 8 μCi $^{91}Y$ and 8 μCi of $^{45}Ca$ was forced into the crop in a gelatin capsule.
[2] The stomach includes the proventriculus and the gizzard.

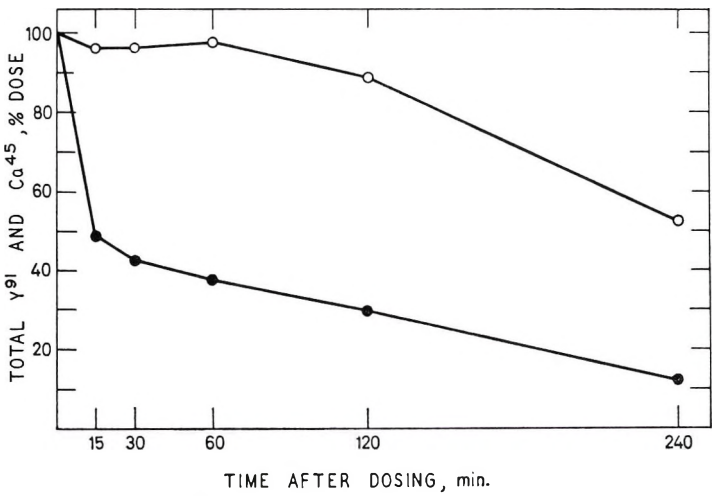


Fig. 1 Total recovery of $^{45}Ca$ (●) and $^{91}Y$ (○) in the entire intestine after the administration of a double tracer dose into the duodenum.

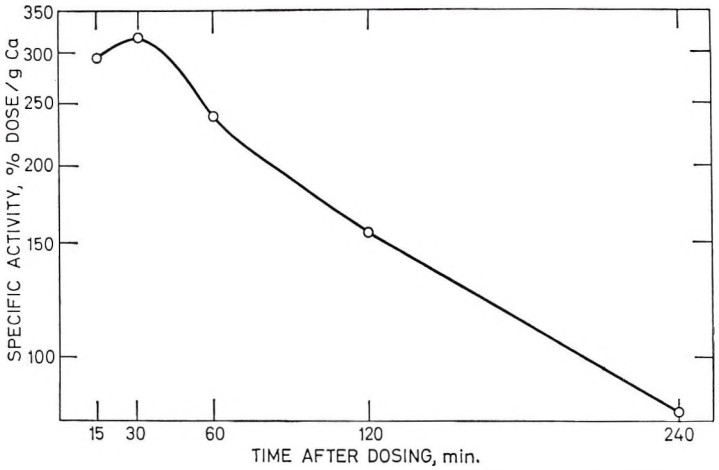


Fig. 2 The specific activity of blood plasma as $^{45}Ca$ following the administration of the isotope into the duodenum.

reached a peak 30 minutes after dosing (fig. 2). It then declined sharply. Radioactivity of blood plasma measured in the $^{91}Y$ channel was small and insignificant, providing additional evidence that the absorption of this isotope in the fowl intestine is poor.

Fifteen minutes after dosing, peak activity of both isotopes was found in the middle jejunum (fig. 3). After longer intervals the peaks were observed in further segments, and by 4 hours most of the activity of both isotopes was concentrated in the colon. In general, the passage rate appeared to be similar for both $^{45}Ca$ and $^{91}Y$, but the distribution of both isotopes among the various intestinal segments was not uniform, probably due to outflow of $^{45}Ca$ from some segments, and inflow into others. It is recalled that the specific activity of blood plasma was high soon after dosing, and inflow could contribute significantly to the $^{45}Ca$ activity in the intestine. Outflow of $^{45}Ca$ from the upper segments can also be responsible for the slight apparent tendency of $^{45}Ca$ to appear in relatively greater concentrations than $^{91}Y$ in the lower segments, after 1 and 2 hours.

Output curves for $^{91}Y$, and the calculated passage times and rates are given in figure 4 and table 2, respectively. The passage time through the duodenum was very short; it was longer in the jejunum and longest in the ileum. Within the jejunum,

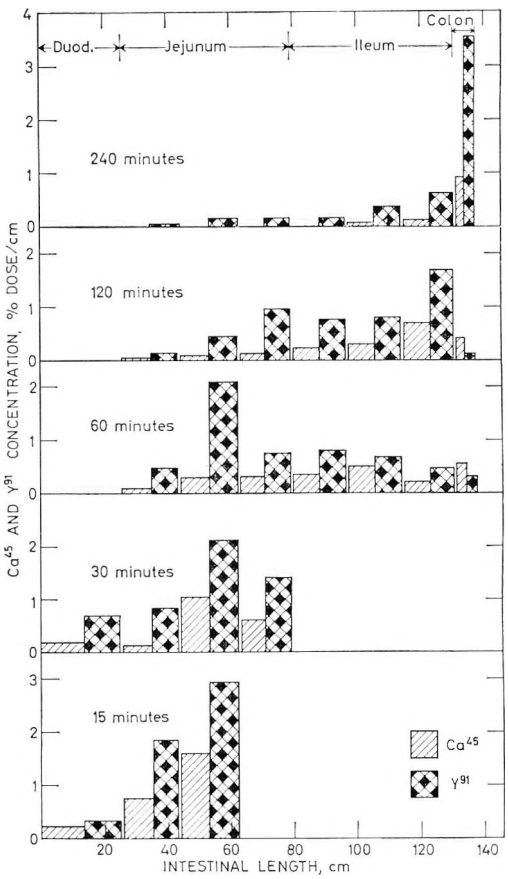


Fig. 3 Concentration of $^{45}Ca$ and $^{91}Y$ along the intestine of laying fowls following a double tracer dose administration into the duodenum.

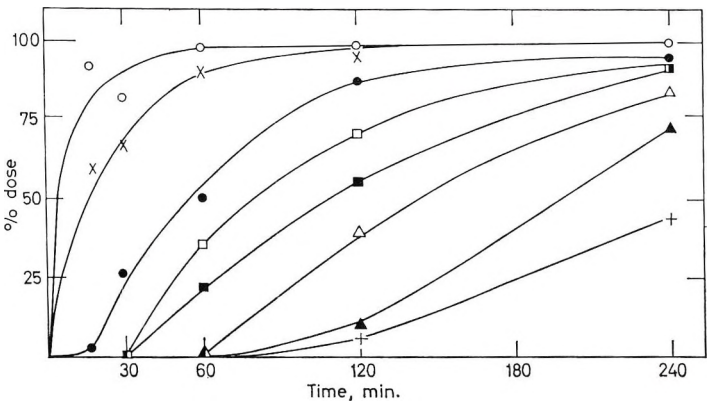


Fig. 4 Cumulative output of $^{91}Y$ from various intestinal segments following the administration of the isotope into the duodenum. Duodenum, (○); upper jejunum, (×); middle jejunum, (●); lower jejunum, (□); upper ileum, (■); middle ileum (△); lower ileum, (▲); and colon, (+).

TABLE 2

*Passage time and rate of $^{91}Y$ in the intestine of laying fowls*

| Intestinal segment | Passage time | | | Passage rate |
|---|---|---|---|---|
| | Present study | | Hurwitz and Bar (1) | |
| | *min* | *min* | *min* | *cm/min* |
| Duodenum | | 4 | 4 | 6.50 |
| Jejunum, upper | 12 | | | 1.50 |
| middle | 42 | | | 0.43 |
| lower | 24 | | | 0.75 |
| whole | | 78 | 75 | 0.70 |
| Ileum, upper | 24 | | | 0.75 |
| middle | 36 | | | 0.50 |
| lower | 60 | | | 0.30 |
| whole | | 120 | 120 | 0.45 |
| Colon | | 48 | 24 | 0.19 |
| Whole intestine | | 250 | 217 | 0.56 |

passage time was longest in the middle and shortest in the upper portion. However, passage time increased progressively down the ileum. Except for the colon, the passage times calculated from the results of the previous study (1) with continuous $^{91}Y$ feeding agree closely with the present calculated values. The differences in the colon may have resulted from the different killing methods used in both studies, namely, neck dislocation in the previous study, and intracardiac sodium pentabarbitol in the present.

## DISCUSSION

Unabsorbed markers have been used commonly to determine the digestibility of various nutrients (7). However, any difference in the rate of passage between the marker and the test nutrient may limit the use of such markers for estimation of absorption along the intestine. As was observed in this study, an effort to compare the passage rate of a marker to the test nutrient may be complicated by outflow of the marker in the upper intestine and possible inflow into the lower intestine. Due to these, the rate of passage through the intestine may appear to be greater for the test nutrient than the marker. The calculation of the passage rate of any absorbed nutrient is also hindered unless a complicated method is devised which can account for the outflow and inflow of the nutrient.

The difference in passage times between $^{45}Ca$ and $^{91}Y$ in the stomach leads to the conclusion that equality of passage times

of the test marker and the nutrient cannot be always assumed. Such an assumption may lead to erroneous absorption values, especially in the upper gastrointestinal tract. More caution should, therefore, be applied with the use of an unabsorbed marker.

Except for the difference in the stomach, both $^{45}Ca$ and $^{91}Y$ appeared to pass through the intestine at a similar rate. Yttrium-91 was found by Marcus and Lengemann (2) to be strongly adsorbed on solid particles. Due to differences in chemical properties, $^{45}Ca$ should have a lesser tendency of adsorption. The pyloric action in the bird's gizzard results in longer retention of the larger particles. Therefore, the adsorbed $^{91}Y$ may be expected to remain in the stomach for a longer period of time than $^{45}Ca$. In the intestine, however, digesta appear to move as a whole, and the rate of passage would be similar for the various substances. These observations confirm the assumption made in the previous report (1) and offer further proof that calcium is absorbed in the duodenum and jejunum, and absorbed very little in the ileum and colon. The marked outflow of $^{45}Ca$ during the first 30 minutes after the introduction of the isotope into the duodenum, and the appearance of the isotope in blood plasma also supports this conclusion. Although the rate of $^{45}Ca$ outflow was high in the duodenum, the jejunum, due to the slower passage rate, appears to play a more important role in the overall calcium absorption. These conclusions are clearly different from those based on observations with rats (3) and dogs (5) in which the ileum was identified as the most effective site of calcium or strontium absorption.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Mrs. M. Cotter.

LITERATURE CITED

1. Hurwitz, S., and A. Bar 1965 Absorption of calcium and phosphorus along the gastrointestinal tract of the laying hen, as influenced by dietary calcium and egg shell formation. J. Nutrition, *86:* 433.
2. Marcus, C. S., and F. W. Lengemann 1962 Use of radioyttrium to study food movement in the small intestine of the rat. J. Nutrition, *76:* 179.
3. Marcus, C. S., and F. W. Lengemann 1962 Absorption of $Ca^{45}$ and $Sr^{85}$ from solid and liquid food at various levels of the alimentary tract of the rat. J. Nutrition, *77:* 155.
4. Renner, R. 1961 Site of fat absorption in the chick. Poultry Sci., *40:* 1447.
5. Cramer, C. F. 1965 Sites of calcium absorption and the calcium concentration of gut contents in the dog. Canad. J. Physiol. Pharmacol., *43:* 75.
6. Persson, S., and S. A. Svensson 1960 Die Durchgangszeit des Futters durch den Verdauungstraktus von Kueken. Arch. Geflugelkunde, *24:* 407.
7. Berger, E. 1963 Techniques for studying ion transfer across the alimentary tract. In: Transfer of Calcium and Strontium Across Biological Membranes, ed., R. H. Wasserman. Academic Press, New York, p. 57.

# Effect of Exogenous Glucose on Glucose Metabolism in Dairy Cows [1]

J. C. BARTLEY [2] AND A. L. BLACK
*Department of Physiological Sciences, University of California, Davis, California*

*ABSTRACT* Ruminants receive little alimentary glucose because of rumen fermentation, and hence they depend largely on gluconeogenesis for their metabolically essential glucose. Because of these circumstances, it seemed worthwhile to determine how glucose metabolism would be affected if large amounts of exogenous glucose were supplied to cows. Cows were given glucose-U-$^{14}$C intravenously (jugular or ruminal vein) or into their duodenal lumen and the transfer of $^{14}$C from plasma glucose to respired $CO_2$ and milk components was assessed. The effect of various treatments on metabolism of glucose-U-$^{14}$C was evaluated from changes in the specific activity of respired $CO_2$, plasma glucose and milk components. Treatments included infusion of unlabeled glucose into blood or into the duodenum, in short-term studies, and infusion of glucose into the duodenum at the rate of 1.5 kg/day for periods of 21 days. Long-term duodenal infusion of glucose had no apparent effect on rate of glucose absorption from the intestine, but exogenous glucose delivered into blood or the duodenum increased the transfer of $^{14}$C from glucose to respiratory $CO_2$ and to milk components. Under normal conditions lactating cows derive about 10 to 13% of their metabolic $CO_2$ from glucose oxidation but, with an exogenous supply, glucose oxidation increased to a level providing 20 to 23% of the metabolic $CO_2$. When glucose was supplied exogenously to cows, the glucose transfer rate increased from 1.2 g/minute to 1.7 g/minute and there was a decrease in endogenous glucose production. The mammary gland appears to have first-priority on blood glucose and may be responsible for the relatively low oxidation of glucose in lactating cows. The percentage of respiratory $CO_2$ derived from glucose increased in this order: hypoglycemic ketotic cows (5%), lactating cows (10–13%) and non-lactating cow (20%) or lactating cow with exogenous glucose (20–23%).

The mature ruminant has a paucity of alimentary glucose (1, 2) and depends largely on endogenous sources to meet its metabolic requirements, which in the lactating cows can be quite large. Prior to the time of rumen development, the young ruminant is essentially monogastric but after 6 to 8 weeks of age (3, 4) rumen microflora begin to convert dietary carbohydrate into volatile fatty acids (5–7). As the volatile fatty acids increase, the blood glucose concentration decreases (8) to 40 to 60 mg/100 ml, and gluconeogenesis, presumably from propionate (7, 9) becomes of major importance.

It seemed worthwhile to determine what metabolic changes would occur in cows 1) if large amounts of glucose were added to the duodenum, a point in the gastrointestinal tract below the rumen and one that normally receives little, if any, free glucose in the adult ruminant; or 2) if glucose were infused intravenously.

Results reported here, based on utilization of uniformly labeled $^{14}$C-glucose, indicated that prolonged duodenal administration of glucose did not increase the rate of glucose absorption from the alimentary tract of the cow. Exogenous glucose added to the duodenum or injected into hepatic portal blood caused sharp increases in the rate and extent of glucose metabolism and decreased endogenous glucose production.

## MATERIALS AND METHODS

Glucose, uniformly labeled with $^{14}$C (glucose-U-$^{14}$C), was prepared photosynthetically using the leaf of *Canna indicus* and was isolated chromatographically according to methods described elsewhere (10).

Received for publication February 14, 1966.

[1] Supported by Public Health Service Research Grant no. GM-08183 from the Division of General Medical Science.

[2] Supported by Public Health Service Post-Doctoral Fellowship, AF-11,212 from the National Institute of Arthritis and Metabolic Diseases. Present address: Department of Physiology, University of California, Berkeley, California.

Glucose prepared by this method has been shown to be equally labeled in all 6 carbon atoms (11).

Characteristics of the cows and the amount of $^{14}C$ injected are given in table 1. The weight of radioactive glucose infused, less than 250 mg/trial, is negligible compared with the cow's glucose pool (12). Cows received high quality alfalfa hay, ad libitum, 3.6 kg/day of a standard dairy ration.[3] Surgical procedures, including duodenal enterostomy (13) and ruminal vein catheterization (14) were carried out at least one week before the trials. The jugular vein was catheterized the evening before the trial by threading about 25 cm of plastic tubing through a needle into the jugular vein, removing the needle and securing the tubing with a single stitch. The area was anesthetized with a local injection of a solution of procaine hydrochloride prior to catheterization.

Each cow was injected with $^{14}C$-glucose at least twice, except for cow 258, trial 6. Trials were first carried out on the cows after they had recovered from surgery and prior to the time they first received exogenous glucose. These are considered as trials on normal cows (trials 1, 2 and 4). In trials 3 and 5, the cows were given 1.5 kg of glucose/day by intraduodenal infusion of a 50% aqueous solution of glucose for 21 days preceding the trials.

Cow 258, trial 6, acted as its own control. The first 3 hours of the trial were comparable to the trials on normal cows. During the final 4 hours, one gram of glucose/minute was infused into the ruminal vein along with the labeled glucose.

Glucose-U-$^{14}C$ was infused at a constant rate for 7 hours via the jugular vein in trials 2 and 3 and via the ruminal vein in trials 1 and 6. In trials 4 and 5, 2 mCi of glucose-U-$^{14}C$ were given in a single injection into the lumen of the duodenum.

Ninety minutes after the start of trial 3, 1.5 kg of non-labeled glucose was infused into the duodenum as it had been on the previous 20 days. Unlabeled glucose was not infused into the duodenum during the other trials. The duodenal infusion had been stopped 14 hours prior to trial 3 and 7 hours prior to trial 5.

*Chemical methods and counting procedures.* The glucose concentration of plasma, urine, and ingesta was determined by a glucose oxidase method (15). Respired carbon dioxide was collected continuously during the trials and samples pooled for assay every 4 minutes. The methods for the collection of respired air and the determination of its rate of production and specific activity have been described in detail elsewhere (16). Milk was collected periodically during the trials and the milk components separated by methods described previously (17). Blood samples were taken every 15 minutes during the trials and pooled hourly. The phenylosazone derivative of glucose was prepared and the specific activity determined by methods described by Feller et al. (18).

## RESULTS

In earlier studies on glucose metabolism in cows (19), $^{14}C$-glucose was injected into the conveniently accessible jugular blood with the tacit assumption that the results would reflect the metabolic fate of glucose in the cow. This assumption was based on the fact that jugular blood passes directly to the heart and then to peripheral tissues; thus the $^{14}C$-glucose would have the same distribution as glucose released from

[3] Rolled barley 40%, rolled oats 35%, wheat bran 10%, cottonseed meal 14%, salt mixture 1%.

TABLE 1

*Description of cows used in glucose metabolism trials*

| Trial no. | Cow no. | Body wt | Milk production | Primer dose | Infusion dose | Route of infusion |
|---|---|---|---|---|---|---|
| | | *kg* | *kg/day* | *μCi* | *μCi/min* | |
| 1 | 203 | 393 | 8 | 630 | 12.0 | ruminal vein |
| 2 | 203 | 354 | 6 | 500 | 8.5 | jugular vein |
| 3 | 203 | 350 | 5 | 500 | 8.5 | jugular vein |
| 4 | 253 | 437 | dry | 2000 | 0 | duodenum |
| 5 | 253 | 454 | dry | 2000 | 0 | duodenum |
| 6 | 258 | 478 | 7 | 360 | 6.0 | ruminal vein |

liver following gluconeogenesis, the major source of glucose in the cow. When glucose is absorbed from the intestine, it enters portal blood and passes first through the liver before reaching peripheral tissues. Thus its metabolic fate will depend in part on biochemical changes, if any, that occur in the liver.

The first 2 trials were designed to establish whether $^{14}$C-glucose would have a different metabolic fate when it was introduced into portal blood as compared with that introduced into jugular blood. In trial 1, a primer dose of $^{14}$C-glucose was injected into portal blood followed by a constant infusion of $^{14}$C-glucose for a period of 6 hours.

The specific activity of respired $CO_2$, shown in figure 1, increased with time up to 3 hours and then remained constant during the remainder of the $^{14}$C-glucose infusion.

In the second trial the same procedure was repeated except that the $^{14}$C-glucose was infused into the cow's jugular blood. In this case the specific activity of respired $CO_2$ increased at a slower rate and did not level off until several hours had elapsed. The final level of specific activity was nearly the same. In both trials (fig. 1) the specific activity of plasma glucose was constant at $8.9 \times 10^3$ standard specific activity units [4] during the latter part of the trials. Since the plateaus of the specific activity of plasma glucose and respired $CO_2$ were the same in each trial, it appears that the percentage of respired $CO_2$ derived from plasma glucose was unaffected by the route of administration of the labeled glucose.

[4] Specific activity is expressed as microcuries of $^{14}$C/g atom carbon. This value is divided by the microcuries infused per minute (trials 1, 2, 3 and 6) or microcuries injected as a single dose (trials 4 and 5) per kilogram of body weight to give the standardized specific activity.

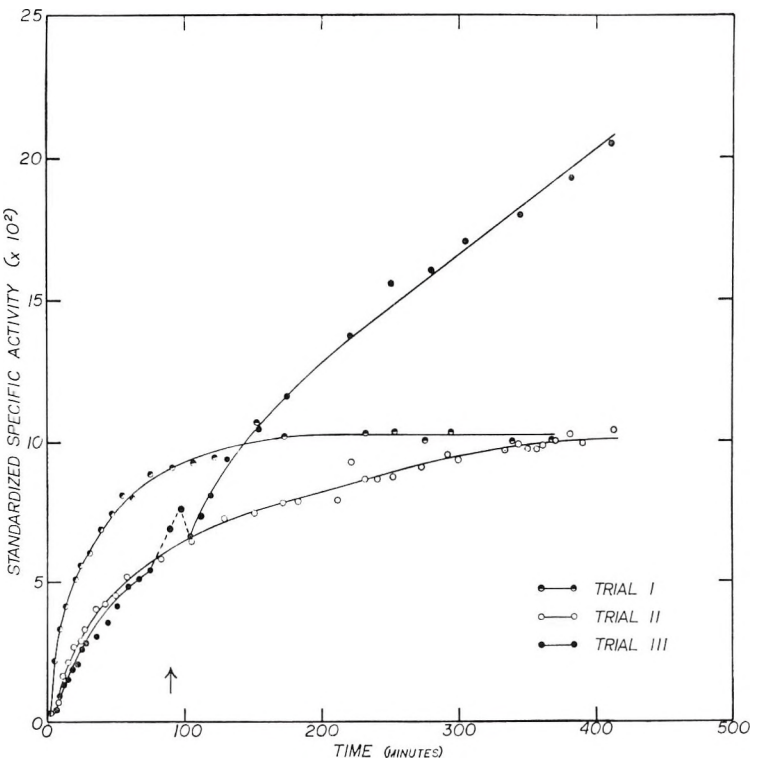


Fig. 1 Standardized specific activity of respired $CO_2$ during constant infusion of glucose-U-$^{14}$C into ruminal vein (trial 1) or jugular vein (trials 2 and 3). In trial 3, a 50% solution of unlabeled glucose was infused into the duodenum (arrow designates starting time) at the rate of 273 g glucose/hour for the last 5.5 hours of the trial.

The more rapid increase in specific activity in trial 1 indicates that the $^{14}$C-glucose was oxidized more rapidly when infused into portal blood. In fact, during the first hour after beginning the infusions approximately twice as much of the $^{14}$C infused appeared in respiratory $CO_2$ after portal infusion (trial 1) compared with jugular infusion (trial 2).

Variation in the rate of oxidation of glucose can occur in the same animal at different times, but the much steeper slope for the $CO_2$ specific activity after portal infusion (see fig. 1) supports the idea that glucose was either more rapidly oxidized by the liver than by peripheral tissues or that the liver converted glucose into other metabolites that were oxidized in peripheral tissues more rapidly than glucose. In either case, the result demonstrates that glucose is readily metabolized in the cow's liver and in this sense, the cow is not different from those animals that normally receive substantial amounts of alimentary glucose.

The specific activities of milk products synthesized by the cow during continuous infusion of $^{14}$C-glucose into jugular or portal blood are listed in table 2. The results for trials 1 and 2 are essentially in agreement and confirm results obtained after a single injection of $^{14}$C-glucose (19). Most of the $^{14}$C from glucose appeared in lactose whether the glucose passed first through the liver or not; smaller amounts appeared in citrate, protein and fat, in that order.

The same cow used for trials 1 and 2 received glucose through a duodenal cannula at the rate of 1.5 kg/day for a period of 3 weeks. This amount of glucose was calculated to be at least one-half of the normal daily caloric intake of the cow. On the day following the last duodenal infusion, $^{14}$C-glucose was infused into jugular blood in the same manner as in trial 2. The specific activity of respired $CO_2$ in this case, increased at the same rate as in trial 2, as shown in figure 1. Thus it appears that the infusion of 31.5 kg glucose (1.5 kg/day for 3 weeks) into the cow's duodenum had not caused metabolic changes that lead to more rapid oxidation of $^{14}$C-glucose.

One and one-half hours after the start of trial 3 and while the rate of $^{14}$C-glucose infusion remained constant, unlabeled glucose was introduced into the duodenum through a cannula at the rate of 4 g/minute for 5.5 hours, the same rate used during the 3-week treatment period. The respired $CO_2$ (fig. 1) which was collected continuously gives the best indication of the effect of the duodenal infusion of glucose on $^{14}$C-glucose metabolism. Within 10 mintues after starting the duodenal infusion, there was an increase in the specific activity of respired $CO_2$ that continued throughout the remainder of the trial, a period of more than 5 hours. The final specific activity of $CO_2$ was at least twice that reached in trials 1 and 2.

The changes in the concentration and specific activities of the plasma glucose during trial 3 are shown in figure 2. Shortly after glucose was introduced into the duodenal lumen, it was entering the plasma more rapidly than it was leaving as shown by the fact that the plasma glucose increased from an initial level of about 50 mg/100 ml to an average of 120 mg/100 ml during the third hour. Subsequently, the concentration of glucose dropped to 80 mg/100 ml, reflecting increased glucose withdrawal from the blood, presumably as a result of an insulin release following the hyperglycemia. After this response and while the duodenal infusion continued, the cow maintained 80 mg/100 ml plasma glucose, a level on the high side of the normal range for ruminants and corresponding more closely to levels found in plasma of non-ruminants.

The specific activity of the plasma glucose showed a reciprocal change, decreasing from a value of about 15 units, initially, to 10 units of specific activity during the last 4.5 hours (fig. 2). This decrease can be attributed to the influx of unlabeled glucose from the duodenum. The fact that the specific activity of plasma glucose remained constant during the time the concentration of plasma glucose was decreasing from 120 mg/100 ml to 80 mg/100 ml indicates that this change in concentration resulted from greater glucose uptake by the tissues without concomitant decrease in rate of glucose addition to the plasma. During the last three hours of the trial the rate of absorption of glucose from the duodenum plus the rate of delivery of endoge-

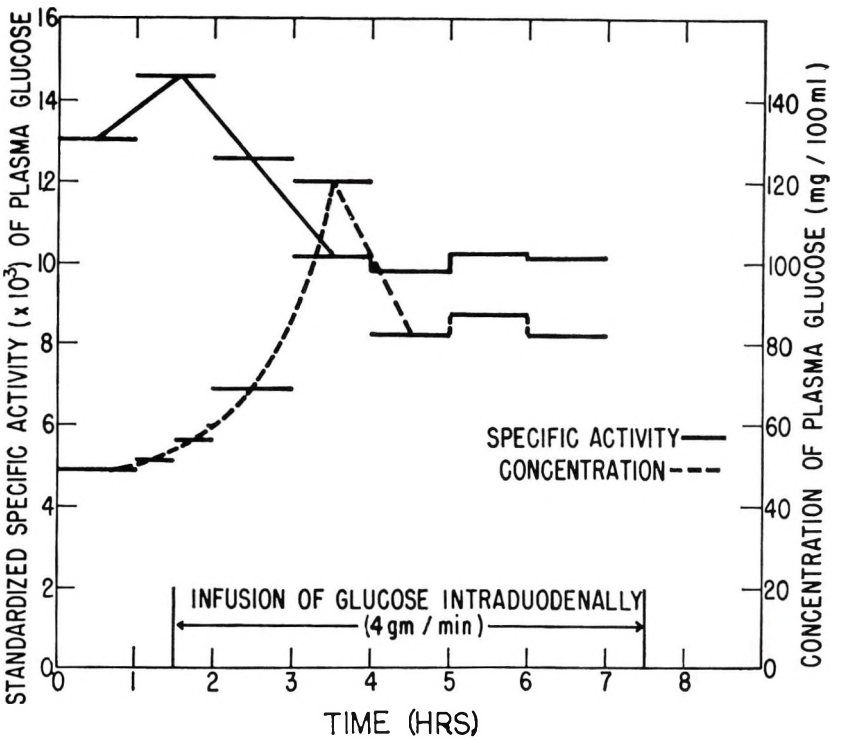


Fig. 2 The specific activity of plasma glucose and the plasma glucose concentration during trial 3 are plotted as horizontal lines showing the mean values for the interval during which the blood sample was collected. The mean values are connected by a line to show the trend with time.

nous glucose to the blood was equal to the rate of uptake by the tissues since the plasma glucose concentration remained constant. The constant specific activity of plasma glucose shows that the rate of delivery of unlabeled glucose to blood (endogenous plus exogenous) must also have remained constant.

Since the specific activity of plasma glucose decreased after unlabeled glucose was added to the duodenum (see fig. 2, from 1.5 to 7.5 hours), it would be expected that there would be a lower specific activity in products derived from blood glucose, in proportion to the isotope dilution. Contrary to this expectation, the specific activity of respired $CO_2$ increased throughout the duodenal glucose infusion (fig. 1) even though the rate of $^{14}$C-glucose infusion remained constant and the specific activity of plasma glucose decreased and then remained constant (see fig. 2). The rate of oxidation of glucose was increased to a greater extent than the dilution of $^{14}$C-glucose by the unlabeled glucose entering the blood. In other words, the response to duodenal glucose infusion reflects more than a simple mass-action effect.

The specific activity of the milk products synthesized by the cow showed a similar change (last column, table 2). The relative distribution of $^{14}$C among the milk components was the same as in trials 1 and 2, but the specific activity of each component was 30 to 200% higher in trial 3. Thus, the influx of unlabeled glucose did not reduce the specific activity of any of the products derived from plasma glucose which indicates that the utilization of glucose for synthesis of milk components increased more than the dilution of the $^{14}$C-glucose due to exogenous glucose absorption.

Trials 4 and 5 were designed to answer the questions whether duodenal administration of glucose would enhance the abil-

ity of the cow to absorb glucose from its duodenum. In trial 4, a tracer amount of $^{14}$C-glucose was added in a single dose through a cannula into the lumen of the duodenum. The specific activity of the cow's respired $CO_2$ reached a maximum of 7 standardized specific activity units about 1.5 hours after the $^{14}$C-glucose was administered (fig. 3). The result can be compared with a maximal level of 8 to 9 standard specific activity units which occurred 45 minutes after cows were injected intravenously with a single tracer dose of $^{14}$C-glucose (19). The longer time-interval before reaching maximal specific activity presumably reflects the delay involved in

TABLE 2

*Standardized specific activity of organic components isolated from milk samples of cow[1] infused at a constant rate for 7 hours with glucose-U-$^{14}$C*

| Sample | Milk component | Standardized specific activity [2] | | |
|---|---|---|---|---|
| | | Trial 1 | Trial 2 | Trial 3 |
| Milk 3 | lactose | 7318 | 6966 | 9385 |
| | citrate | 1770 | 1312 | 2946 |
| | whey-protein | 511 | 469 | 724 |
| | casein | 446 | 571 | 819 |
| | fat | 295 | lost | 370 |
| Milk 4 | lactose | 7495 | 7504 | 8766 |
| | citrate | 1574 | 1812 | 3222 |
| | whey-protein | 488 | 633 | 872 |
| | casein | 446 | 688 | 946 |
| | fat | 393 | lost | 465 |

[1] Glucose-U-$^{14}$C was infused into the ruminal vein in trial 1 and into the jugular vein in trial 2. The cow received daily infusions of glucose into its duodenum for 3 weeks prior to jugular infusion of glucose-U-$^{14}$C in trial 3. The cow was milked at 1, 3, 5 and 7 hours after starting the infusion. Results are given for milk samples 3 and 4 collected at 5 and 7 hours, respectively.

[2] Standardized specific activity is the microcuries $^{14}$C/g atom carbon divided by the microcuries of $^{14}$C infused/minute/kg body weight.

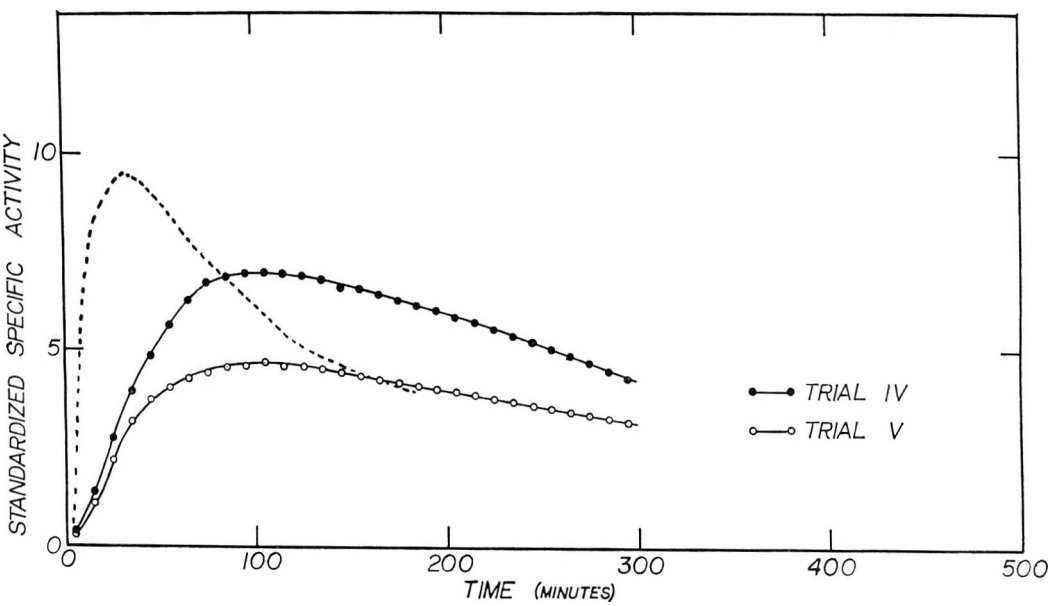


Fig. 3 Standardized specific activity of cow's respired $CO_2$ after a single injection of glucose-U-$^{14}$C into its duodenum. Cow received 1.5 g/day unlabeled glucose infused into its duodenum for 21 days between trial 4 and 5. Dotted line shows the change with time of the specific activity of respired $CO_2$ after single intravenous injection of glucose-U-$^{14}$C into a cow (19).

the absorption of the $^{14}C$-glucose from the duodenum.

Prior to trial 5, a glucose solution was continuously infused into the cow's duodenum at a constant rate (1.5 kg glucose/day) for a period of 3 weeks. The day following the last treatment, the cow again received a tracer amount of $^{14}C$-glucose as a single dose in its duodenum, as it had in trial 4. The specific activity in respired $CO_2$ increased at nearly the same rate in both trials (see fig. 3). From this result it appears that the 3-week infusion of glucose had no enhancing effect on glucose absorption. If anything the initial slope of the respired $CO_2$ curve in trial 5 may have been slightly lower than in trial 4, while the maximal level reached was about 60% as great in the post-treatment trial. This difference might indicate a slower absorption of glucose post-treatment, but a more likely explanation would be that the $^{14}C$-glucose had been diluted by small amounts of unlabeled glucose remaining in the intestine from the earlier treatment with unlabeled glucose. There is reason to expect that some of the glucose solution added to the duodenum (1.5 kg during a 6-hour interval) may have backed up into the cow's abomasum (stomach) and then was slowly released. This material remaining in the lumen or a high intracellular glucose (or glycogen) in cells of the intestinal tract may have diluted the $^{14}C$-glucose in trial 5 causing the lower specific activity in respired $CO_2$ in that trial compared with trial 4. The specific activity of plasma glucose (fig. 4) during the 2 trials shows the same general relationship as the respiratory $CO_2$, namely, a lower specific activity in the plasma glucose after the treatment period (trial 5), although the change of specific activity with time was similar in both trials. In the absence of any positive indication of an increased rate of glucose absorption beyond that expected due to mass effect, it must be tentatively concluded that giving glucose into the duodenum for

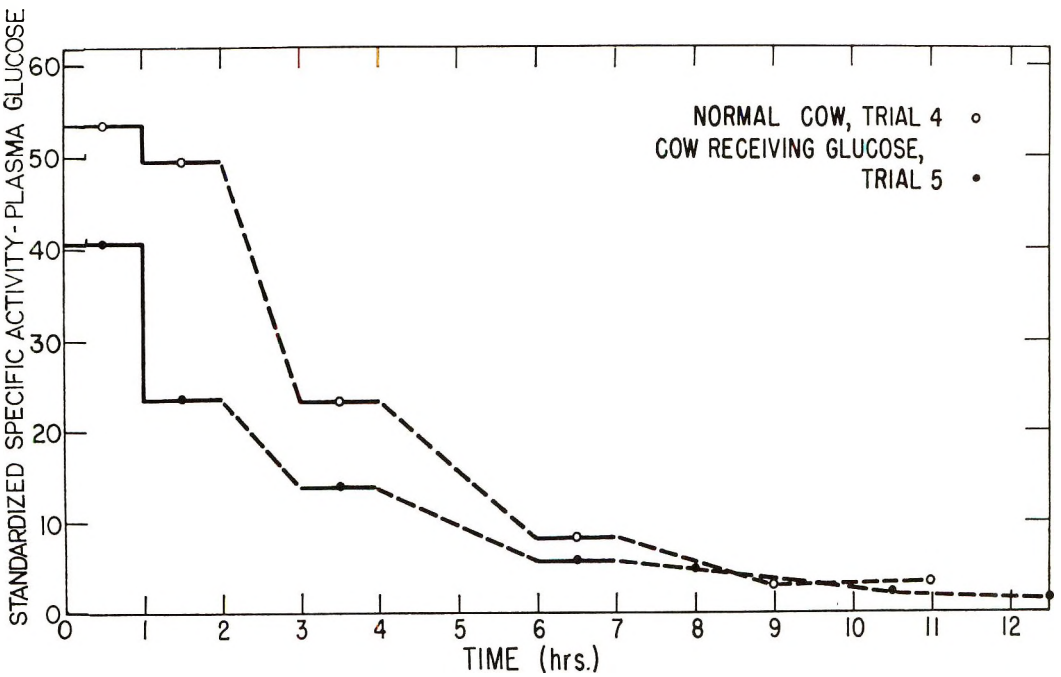


Fig. 4 Specific activity of plasma glucose at various times after an injection of glucose-U-$^{14}C$ into the duodenum of a normal cow (trial 4) and the same cow after receiving 1.5 g glucose/day for 20 days (trial 5). Mean values are shown as horizontal lines for the interval during which blood samples were pooled.

21 days did not affect the capacity for glucose absorption from the intestine.

The effect of a glucose load on the metabolism of $^{14}$C-glucose was studied further in trial 6, in this case by infusing the unlabeled glucose together with the $^{14}$C-glucose into the hepatic-portal venous system. At the beginning of the trial a primer dose of $^{14}$C-glucose (360 μCi) was injected into the ruminal vein followed by a continuous infusion of 6.0 μCi/minute for a period of 7 hours. Three hours after the start of the $^{14}$C-glucose infusion non-radioactive glucose was introduced (together with the $^{14}$C-glucose) at the rate of 1 g/minute, the amount estimated to be entering the plasma of the cow in trial 3. The effect of this unlabeled glucose was the same as in trial 3, when it was introduced via the duodenum, namely, a transient decrease in the specific activity of respired $CO_2$ followed by a sustained increase for a period of at least 3 hours (fig. 5). The rate of increase of specific activity in the respired $CO_2$, however, was only about one-half as great as it was in trial 3 when 4 g/minute of glucose was infused into the cow's duodenum.

The concentration of plasma glucose and its specific activity during trial 6 is shown in figure 6.

The intravenous infusion of glucose (1 g/minute) resulted in an increase in plasma glucose from about 60 mg/100 ml to 100 mg/100 ml and at the same time a decrease from 12 to 8 specific activity units. During the last 3.5 hours of the trial, and while the specific activity of

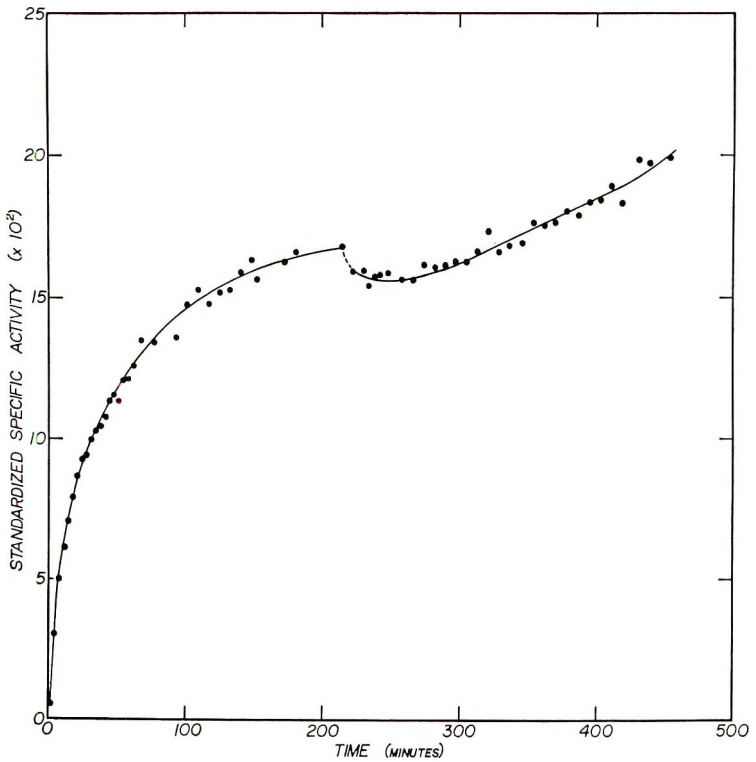


Fig. 5 Specific activity of respired carbon dioxide at various times during trial 6. The cow was injected with a primer dose followed by a continuous infusion of glucose-U-$^{14}$C, at a constant rate, via the ruminal vein. Unlabeled glucose was infused into the ruminal vein at the rate of 1 g/minute beginning at approximately 3 hours and lasting until the end of the trial.

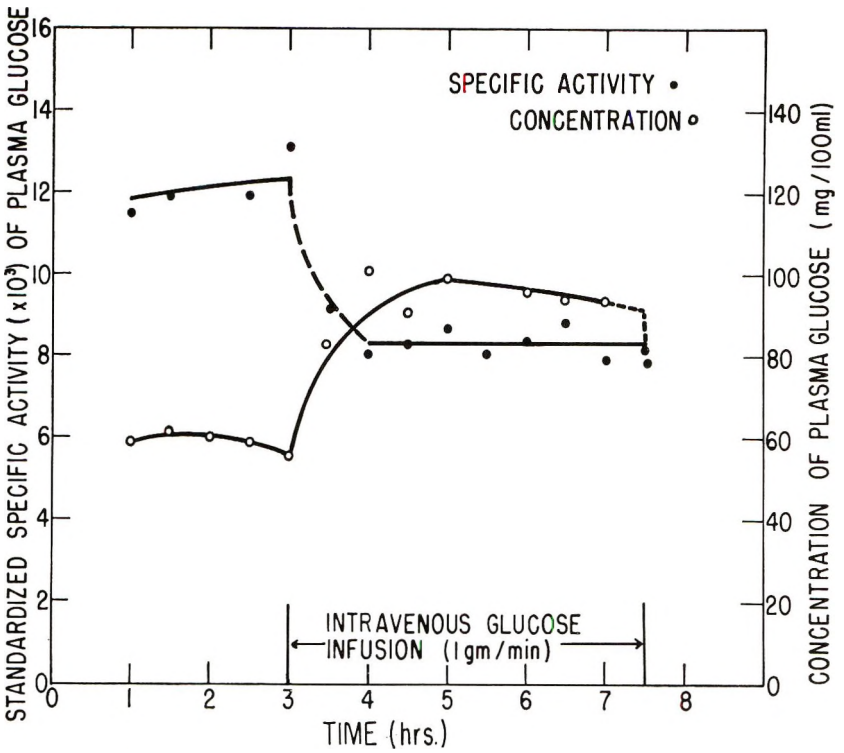


Fig. 6 Specific activity of plasma glucose and plasma glucose concentration during trial 6. A primer dose, followed by a continuous infusion of glucose-U-$^{14}C$ at a constant rate, was given via the ruminal vein. Unlabeled glucose was infused into the ruminal vein at the rate of 1 g/minute starting at 180 minutes after the start of the trial.

plasma glucose was nearly constant, the specific activity of respired $CO_2$ increased at least 30% (see fig. 5). These results show that the glucose infusion resulted in glucose providing a much greater fraction of the metabolic $CO_2$, the same response seen after infusing glucose duodenally, as discussed above.

## DISCUSSION

Determination of the glucose concentration of the duodenal contents of normal cows verified the work of Heald (2) that very little glucose normally reaches the intestine of the ruminant. If a rate of flow of intestinal contents of 160 to 170 liters/day is assumed (20), the average duodenal concentration of glucose, measured in cows with duodenal fistulas, 5 mg/100 ml, would provide only 8 g of glucose/day. Since this amount of glucose would be quantitatively insignificant to the cow, its supply of glucose must arise endogenously, presumably by gluconeogenesis in the liver. Under these conditions, the rate of endogenous glucose production would approximate the glucose transfer rate in the normal cow.

When the specific activity of plasma glucose becomes constant during continuous infusion, an estimate can be made of the rate of transfer of glucose as the rate of dilution of $^{14}C$-glucose by non-labeled glucose entering the plasma pool from the relationship:

$$\frac{(\mu Ci/min)\ ^{14}C\text{-glucose infusion} \times 180/6}{\text{Specific activity of plasma glucose } (\mu Ci/g \text{ atom C})} = \text{g glucose entering/min.}$$

When the plasma glucose concentration is constant, the entry rate will equal the exit rate from the plasma glucose pool and should approximate the rate of glucose utilization by the animal.

The transfer rate of glucose calculated from the trials on normal lactating cows (trials 1, 2, 4 and 6a) was 1.2 g/minute, which is similar to values estimated from single injection trials in normal dairy cows (12, 21). The estimated rate of transfer was the same when glucose was given via the peripheral venous blood (jugular vein infusion) or the hepatic-portal system (ruminal vein infusion). When glucose was supplied exogenously, either duodenally or intravenously, the glucose transfer rate was increased to 1.7 g/minute. A similar increase has been reported for sheep receiving a glucose load (22).

In trial 6, during an intravenous glucose infusion at the rate of 1 g/minute, the calculated transfer rate was 1.7 g/minute. The difference between these values, 0.7 g/minute, reflects the rate of endogenous glucose production. This means that during glucose loading, endogenous glucose production had been depressed to about 60% (0.7 g/minute/1.2 g/minute) of the normal rate. The effect of exogenous glucose on the endogenous production of glucose in non-ruminants has been debated for some years (18, 23–25), but it is now generally agreed that a depression does take place (26). The present results with cows, along with results of Annison and White (22) with sheep, indicate that in this respect the ruminant responds to a glucose load in a manner similar to that of monogastric mammals, namely, by decreasing the endogenous production of glucose.

The fraction of a product derived from a precursor, referred to as the transfer quotient, can be estimated from the results of constant infusion trials by calculating the ratio of the specific activity of the product to that of the precursor, for periods when the specific activity of the product and precursor are constant. In making this calculation for milk components, the average of the last 2 samples collected was used (table 2). The transfer quotient to respired $CO_2$ was calculated from the horizontal part of the curves in trials 1, 2 and 6a, and from the final values measured in trial 3 and 6b. When the specific activity of the product is still increasing, as in the latter 2 trials, then the calculated values are minimal estimates of the transfer quotient. Following single injection of $^{14}C$-glucose (trials 4 and 5) the transfer quotient was calculated by ratio of the area under the specific activity curve of the product to that of the precursor from time zero to infinity (27).

The transfer quotient is more reliable for comparative purposes than the specific activity of the products since it takes into account differences in the specific activity of the precursor. The results of calculations, given in table 3, show that large quantities of glucose given either intraduodenally or intravenously increased the incorporation of glucose into all milk components except fat and doubled, at least, the fraction of respiratory $CO_2$ directly derived from plasma glucose.

The increased utilization of glucose when exogenous glucose is supplied to the cow indicates that it may normally be present in limiting amounts and that it displaces other metabolites as a source of carbon for biosynthesis and as a source of metabolic energy as the supply increases.

The greater oxidation of glucose accounts for most of the observed increase in glucose transfer rate as shown by the following calculation. These cows were producing, on the average, 5.3 mols $CO_2$/hour (4.5 to 6.2 mols/hour) of which about 10% (0.53 mols/hour) was derived from glucose oxidation. With exogenous supply, glucose oxidation doubled which means that an additional 0.53 mols of $CO_2$/hour were being produced from glucose oxidation. This quantity of $CO_2$ would require that an additional 90 millimols/hour of glucose undergo oxidation, or about 0.3 g/minute. The latter value amounts to 60% of the increase in glucose transfer (from 1.2 g/minute to 1.7 g/minute) that occurred when cows received exogenous glucose.

That lactating cows derive a much lower fraction of their metabolic $CO_2$ from glucose than monogastric animals has been shown previously (12). The results of the present study indicate that the utilization of glucose by the mammary gland may

TABLE 3

*Transfer quotients [1] from plasma glucose to respired $CO_2$ and organic components of milk after injecting cows with glucose-U-$^{14}C$*

| Sample | Single injection trial [2] | Control trials | | | | Infusion trials | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 [3] | 2 | 4 | 6a [4] | 3 | 5 | 6b [4] |
| $CO_2$ of respired air | 0.10 | 0.11 | 0.11 | 0.20 | 0.13 | 0.20 | 0.21 | 0.23 |
| Milk components: | | | | | | | | |
| Lactose | 0.80 | 0.83 | 0.81 | dry | | 0.91 | dry | |
| Citrate | 0.19 | 0.18 | 0.18 | dry | | 0.31 | dry | |
| Whey protein | | 0.06 | 0.06 | dry | | 0.08 | dry | |
| Casein | 0.05 | 0.05 | 0.07 | dry | | 0.09 | dry | |
| Fat | 0.05 | 0.04 | 0.03 | dry | | 0.04 | dry | |

[1] In trials 1, 2, 3 and 6 the transfer quotient was calculated by the ratio: $\left(\frac{\text{specific activity of product}}{\text{specific activity of plasma glucose}}\right)$ for the time period when these values were constant, or nearly so. In trials 4 and 5 the transfer quotient was calculated by dividing the area under the curve of the specific activity of respired $CO_2$ by that under the specific activity of plasma glucose from time zero to infinity.
[2] Baxter et al., (12) and Kleiber et al., (19).
[3] Isotope given via ruminal vein.
[4] Isotope infused via ruminal vein. Glucose load given into ruminal vein 3 hours after start of trial. Trial 6a is period prior to load and trial 6b is period during glucose loading of 1 g/min. This cow was lactating, but milk samples were not collected during the trial.
[5] Glucose load into the duodenum 1.5 hours after the start of the trial.

account for part of this difference. In the non-lactating cow (see table 3, trial 4) glucose oxidation provides about 20% of the metabolic $CO_2$, even in the absence of exogenous glucose. When glucose was supplied (trial 5) the transfer quotient increased only slightly (0.21 vs. 0.20) indicating that gluconeogenesis in the non-lactating cow is already sufficient to saturate the enzyme system for glucose oxidation. In contrast, in lactating cows, exogenous glucose doubled the rate of glucose oxidation.

Glucose utilization by the intact bovine mammary gland has not been measured, but it is probably similar to that measured in goats, 8.5 g glucose/100 ml of milk secreted (28). Thus, the glucose withdrawn by the mammary gland can be very large in animals with high levels of lactation.

Results obtained with ketotic cows also support the idea that availability of glucose for oxidation is influenced by the mammary gland. It appears likely that the glucose supply is severely limited during ketosis since the affected animals have marked hypoglycemia (29). Yet Kronfeld et al. (21) have calculated that the transfer rate of glucose was not significantly decreased during ketosis. This presumably occurs because the mammary gland continues milk synthesis at a high rate. In contrast, the transfer quotient of glucose to $CO_2$, as estimated by the method used in trials 4 and 5 from the data of Tombropoulos,[5] was only one-half that of normal cows, 0.05.

It appears that glucose can be oxidized as readily in the cow as in monogastric animals, but other metabolites are ordinarily utilized because the supply of glucose is limited to that produced endogenously and the secretion and synthesis (30) of milk components by the mammary gland take large amounts of the available glucose. When the availability of glucose is decreased even further, as in bovine ketosis, synthesis of milk components continues to pre-empt a large portion of the available glucose so that the oxidation of glucose is decreased further.

The results stress the critical nature of glucose metabolism in lactating dairy cows. The utilization of glucose by the mammary gland for biosynthesis of all organic milk components except fat, and its dependence on gluconeogenesis due to limited availability of glucose from the alimentary tract, places glucostasis in the lactating cow in a delicate balance.

LITERATURE CITED

1. Schambye, P. 1951 Volatile acids and glucose in portal blood of sheep. Nord. Vet. Med., 3: 555.

[5] Data for this calculation were taken from the Doctoral Dissertation: The Metabolism of Specifically Labelled Glucose in Bovine Ketosis, by Dr. E. G. Tombropoulos, University of California, Davis, 1959.

2. Heald, P. J. 1952 The assessment of glucose-containing substances in rumen microorganisms during a digestion cycle in sheep. Brit. J. Nutrition, 5: 84.
3. Conrad, H. R., J. W. Hibbs and N. Frank 1958 High roughage system for raising calves based on early development of rumen function. IX. Effect of rumen inoculations and chlorotetracycline on rumen function of calves fed high roughage pellets. J. Dairy Sci., *41:* 1248.
4. Bryant, M. P., N. Small, C. Bouma and I. Robinson 1958 Studies on the composition of the ruminal flora and fauna of young calves. J. Dairy Sci., *41:* 1747.
5. Elsden, S. R. 1945 The fermentation of carbohydrates in the rumen of the sheep. J. Exp. Biol., 22: 51.
6. Heald, P. J., and A. E. Oxford 1953 Fermentation of soluble sugars by anaerobic Holstrich ciliate protozoa of the genera *Isotrichia* and *Dasytrichia*. Biochem. J., 53: 506.
7. Otagaki, K. K., A. L. Black, J. C. Bartley, M. Kleiber and B. O. Eggum 1963 Metabolism of uniformly labeled glucose-$C^{14}$ introduced into the rumen of a lactating cow. I. Transfer of $C^{14}$ to respired air, volatile fatty acids and major milk constituents. J. Dairy Sci., *46:* 690.
8. McCandless, E. L., and J. A. Dye 1950 Physiological changes in intermediary metabolism of various species of ruminants incidental to functional development of rumen. Am. J. Physiol., *162:* 434.
9. Lindsay, D. B. 1959 The significance of carbohydrate in ruminant metabolism. Vet. Rev. Annot., 5: 103.
10. Putman, E. W., and W. Z. Hassid 1952 Isolation and purification of radioactive sugers by means of paper chromatography. J. Biol. Chem., *196:* 749.
11. Abraham, S., and W. Z. Hassid 1959 The synthesis and degradation of isotopically labeled carbohydrates and carbohydrate intermediates. In: Methods of Enzymology, vol. 4, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 489.
12. Baxter, C. F., M. Kleiber and A. L. Black 1955 Glucose metabolism in the lactating dairy cow. Biochim. Biophys. Acta, *17:* 354.
13. Wangesteen, O. H. 1949 A Textbook of Surgery, ed., F. Christopher. W. B. Saunders Company, Philadelphia, pp. 1045–1047.
14. Parker, H. R., M. Simenson and J. R. Luick 1963 A technique for obtaining portal blood from cattle. Nord. Vet. Med., *15:* 1.
15. Campbell, L. A., and D. S. Kronfeld 1961 Estimation of low concentrations of plasma glucose using glucose oxidase. Am. J. Vet. Res., 22: 587.
16. Kleiber, M. and M. Edick 1952 A respiration apparatus for $C^{14}$ studies with cows. J. Animal Sci., *11:* 61.
17. Kleiber, M., A. H. Smith and A. L. Black 1952 Carbonate as a precursor of milk constituents in the intact dairy cow. J. Biol. Chem., *195:* 707.
18. Feller, D. D., E. H. Strisower and I. L. Chaikoff 1950 Turnover and oxidation of body glucose in normal and alloxan-diabetic rats. J. Biol. Chem., *187:* 571.
19. Kleiber, M., A. L. Black, M. A. Brown, C. F. Baxter, J. R. Luick and F. H. Stadtman 1955 Glucose as a precursor of milk constituents in the intact dairy cow. Biochim. Biophys. Acta, *17:* 252.
20. Hyden, S. 1961 The use of reference substances and measurement of flow in the alimentary tract. In: Digestive Physiology and Nutrition of the Ruminant, ed., Dyfed Lewis. Butterworths, London, pp. 35–47.
21. Kronfeld, D. S., E. G. Tombropoulos and M. Kleiber 1959 Glucose biokinetics in normal and ketotic cows. J. Appl. Physiol., *14:* 1026.
22. Annison, E. F., and R. R. White 1961 Glucose utilization in sheep. Biochem. J., *80:* 162.
23. Combes, B., R. H. Adams, W. Strickland and L. L. Madison 1961 The physiological significance of the secretion of endogenous insulin into the portal circulation. IV. Hepatic uptake of glucose during glucose infusion in non-diabetic dogs. J. Clin. Invest., *40:* 1706.
24. Landau, B. R., J. R. Leonards and F. M. Barry 1961 Regulation of blood glucose concentration: Response of liver to glucose administration. Am. J. Physiol., *201:* 41.
25. Steele, R., J. S. Bishop and R. Levine 1959 Does a glucose load inhibit hepatic sugar output? $C^{14}$ Glucose studies in eviscerated dogs. Am. J. Physiol., *197:* 60.
26. Steele, R., J. S. Bishop, A. Dunn, N. Altszuler, J. Rathgeb and R. G. deBodo 1965 Inhibition by insulin of hepatic glucose production in the normal dog. Am. J. Physiol., 208: 301.
27. Kleiber, M. and A. L. Black 1956 Tracer studies on milk formation in the intact dairy cow. Radioactive Isotopes in Agriculture, AEC document TID 7512. U. S. Government Printing Office, Washington, pp. 395–401.
28. Linzell, J. L. 1960 Mammary gland blood flow and oxygen, glucose and volatile fatty acid uptake in the conscious goat. J. Physiol., *133:* 492 (as corrected in J. Physiol., *163:* 1, 1962).
29. Marshak, R. R. 1958 The pathological physiology of bovine ketosis. J. Am. Vet. Med. A., *132:* 317.
30. Hardwick, D. C., J. L. Linzell and S. M. Price 1961 The effect of glucose and acetate on milk secretion by the perfused goat udder. Biochem. J., 80: 37.

# Effects of Diet, Chronic Inactivity, and Exercise on Growth Performance and Dehydrogenase Activities in Hepatic and Adipose Tissues [1]

FRANK KONISHI
*Department of Food and Nutrition, Southern Illinois University, Carbondale, Illinois*

*ABSTRACT* The effects of diet quality, chronic inactivity, and forced exercise on the growth performance of rats and levels of glucose 6-phosphate dehydrogenase (glucose-6-P dehydrogenase) and 6-phosphogluconate dehydrogenase (6-P-gluconate dehydrogenase) activities in hepatic and adipose tissues were investigated. The mean body weights of the restricted activity rats were significantly lower irrespective of diet quality. Diet quality also significantly influenced the growth as well as the activities of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase. The dehydrogenase activities were significantly higher in the tissues of rats fed a diet high in carbohydrate than those fed diets high in protein or fat. The levels of adipose glucose-6-P dehydrogenase or 6-P-gluconate dehydrogenase were increased by the chronic inactivity in rats fed the high fat diet. Forced exercise however, significantly decreased the adipose and hepatic glucose-6-P dehydrogenase particularly when the exercise preceded the feeding period.

The pathogenesis of obesity is multiple and complex although, basically, it can arise only when the energy intake exceeds physiological needs. This is not to imply that obese individuals always eat excessively since studies have shown that in some cases they consume less than thin people (1, 2). The obese, however, may be less active physically (1, 3, 4). Differences in eating patterns also should be considered. For example, Stunkard et al. (5) described a "night-eating syndrome" in some obese people characterized by nocturnal hyperphagia with subsequent insomnia and anorexia the following morning. Whether this syndrome favors lipogenesis in man has not been demonstrated clearly. However, experiments with animals have shown that manipulation of eating patterns definitely influences the metabolism of tissues (6) and lipogenesis (7). Force-feeding, realimentation after fasting, and rats trained to eat their food in a limited period of time also have led to increased lipogenesis (8–13) as indicated by increase in glucose 6-phosphate (glucose-6-P) dehydrogenase and 6-phosphogluconate (6-P-gluconate) dehydrogenase activities in hepatic and adipose tissues.

The above observations illustrate but a few of the multiple problems associated with the etiology of obesity. Additional studies are necessary to adequately define the interrelationships between patterns of energy intake and expenditure to lipogenesis. The present report gives the results of a study to evaluate the effects of diet quality, chronic inactivity and forced exercise on body and organ growth, and on glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase activities in hepatic and adipose tissues of rats.

## METHODS

Male rats of the Sprague-Dawley strain were used in this study. They were obtained at weanling age and divided into four equal groups according to diet and into 2 groups of 32 and 28 rats each according to restricted and non-restricted activity, respectively. The non-restricted or control animals were housed individually in regular screen-bottom cages (24 cm × 18 cm × 18 cm), and the chronically restricted rats were housed in cages reduced by an adjustable spacer to measure 6 cm

Received for publication February 3, 1966.
[1] This investigation was supported in part by Public Health Service Research Grant no. AM-07754 from the National Institutes of Health.

× 18 cm × 18 cm in depth, height, and width, respectively.

The composition of the 4 diets fed is shown in table 1. All rats were trained and allowed to eat ad libitum quantities of their respective diets for a 2-hour period daily. The 2-hour feeding period was adopted to control any metabolic affects resulting from differences in eating patterns. Water was available at all times. Daily records were kept of the feed consumption and body weights of each rat for 12 weeks. At the end of this period, and immediately after the 2-hour feeding period, the rats were killed by cervical fracture and approximately one-gram samples of epididymal fat and liver were obtained, weighed on a torsion balance, and homogenized in 3 ml of ice-cold 0.25 M sucrose using a Tri-R Teflon tissue homogenizer. The cell-free cytoplasmic suspension for the enzyme measurements was obtained after centrifugation at 100 × g for 10 minutes at 0°. The glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase activities were assayed by the Fitch et al. (14) modification of the Glock and McLean method (15). The enzyme activity units are expressed as millimicromoles of NADPH formed per minute per gram of wet liver tissue and per milligram of adipose nitrogen. The amount of NADPH formed was calculated from the change in optical density by using the extinction coefficient $6.22 \times 10^6$ $cm^2$/mole as determined by Horecker and Kornberg (16). The levels of adipose nitrogen were determined by Nesslerization. Organ weights, body and tail lengths also were obtained. The data were analyzed according to standard statistical methods (17).

## RESULTS

The mean body weights, feed intakes, and efficiencies of weight gain of the animals are summarized in table 2. As expected, the rats fed diets 3 and 4, which contained higher levels of fat, were significantly heavier ($P < 0.01$) than rats fed diets 1 and 2. The mean body weights at the end of the experiment were 334, 352, 382, and 397 g for the rats fed diets 1, 2, 3 and 4, respectively. The restriction of physical activity also affected the final body weights. The chronically restricted animals averaged 354 g in body weight, whereas the control rats averaged 381 g. The differences were statistically significant at the 1% level.

The mean feed intakes were significantly greater ($P < 0.01$) in the rats fed the diets lower in caloric density (1 and 2) than those fed the diets high in fat. The differences in daily intake averaged approximately 10 kcal/rat/day between the rats fed the high fat diets (3 and 4) and diets 1 and 2. The modification of physical activity also significantly ($P < 0.01$) influenced the diet intake. The total

TABLE 1

*Composition of diets*

| | Diet no. | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| | % | % | % | % |
| Casein [1] | 25 | 55 | 25 | 35 |
| Fat [2] | 10 | 10 | 45 | 45 |
| Sucrose | 57 | 27 | 22 | 12 |
| Salt mix [3] | 3 | 3 | 3 | 3 |
| Vitamin mix [4] | 2 | 2 | 2 | 2 |
| Non-nutritive cellulose [5] | 3 | 3 | 3 | 3 |
| Total | 100 | 100 | 100 | 100 |

[1] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland.
[2] Crisco, Procter and Gamble, Cincinnati.
[3] Hawk-Oser Salt mixture no. 3; Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry, ed. 13. McGraw-Hill Book Co., Inc., New York.
[4] Vitamin mixture in dextrose containing the following: (in g/kg) vitamin A, 4.5; vitamin D, 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; and Ca pantothenate, 3.0; and (in mg/kg) biotin, 20; folic acid, 90; and vitamin $B_{12}$, 1.35; Nutritional Biochemicals Corporation, Cleveland.
[5] Alphacel, Nutritional Biochemicals Corporation.

TABLE 2

*Effect of diet and restricted activity on mean body weight gains, feed intakes and efficiencies of body growth in rats*

| Diet and treatment [1] | Body weight | | Total feed intake | Growth efficiency [2] |
|---|---|---|---|---|
| | Initial | Final | | |
| | g | g | g | % |
| 1R (8) | 59 ± 2 [3] | 327 ± 15 | 1055 ± 52 | 25.5 ± 1.8 |
| 1C (7) | 52 ± 2 | 343 ± 9 | 1188 ± 144 | 28.1 ± 11.0 |
| 2R (8) | 60 ± 2 | 334 ± 22 | 1083 ± 93 | 25.3 ± 1.3 |
| 2C (7) | 60 ± 3 | 373 ± 11 | 1157 ± 32 | 27.0 ± 1.3 |
| 3R (8) | 60 ± 3 | 371 ± 12 | 893 ± 28 | 34.9 ± 1.4 |
| 3C (7) | 60 ± 3 | 393 ± 46 | 942 ± 85 | 35.6 ± 4.8 |
| 4R (8) | 60 ± 3 | 382 ± 33 | 903 ± 39 | 35.7 ± 2.9 |
| 4C (7) | 60 ± 3 | 413 ± 47 | 1006 ± 104 | 35.1 ± 2.9 |

[1] R = restricted activity; C = non-restricted activity; number of rats in parentheses.

[2] $\frac{\text{Total body weight gain (g)}}{\text{Total feed intake (g)}} \times 100.$

[3] Values include SD.

quantity consumed by the restricted rats averaged 984 g vs. 1073 g for the control rats, or approximately one gram per day difference during the 12-week period.

The growth efficiencies of the rats were not influenced by the chronic restriction of activity. The efficiencies, however, were significantly higher ($P < 0.01$) in the rats fed diets containing high fat (3 and 4).

Statistical evaluation of the mean organ weights indicated a lack of significant difference between the diet groups of the thymus, heart, spleen, and adrenal glands. The relative weights of the kidneys of the rats fed the hyperprotein diet (diet 2) were approximately 28% larger ($P < 0.01$) than those from rats fed the other diets. The relative weights of the liver also were significantly greater ($P < 0.01$) in the rats fed the hyperprotein diet. The mean differences in weights of the gastrocnemius muscle were not great but were significantly smaller ($P < 0.05$) in the rats fed diets 3 and 4 than those fed diet 1.

When the organ weights were evaluated on the basis of activity groups, significant differences were noted in the weights of the liver and testes. The mean hepatic weights were significantly smaller ($P < 0.01$) in the rats housed in the restricted cages irrespective of diet. The hepatic weights averaged 2660 mg/100 cm$^2$ and 2780 mg/100 cm$^2$ for the restricted and control rats, respectively. The mean weights of the testes, however, were significantly larger ($P < 0.01$) in the restricted (784 mg) than in the control rats (740 mg). The mean weights of the thymus, heart, spleen, adrenals, and gastrocnemius muscle, total body and tail lengths were not significantly influenced by the chronic restriction of activity.

The results of the analyses for dehydrogenase activities in the liver and epididymal fat tissues are graphically illustrated in figures 1 and 2. The glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase activities in the liver were not influenced by the restriction of physical

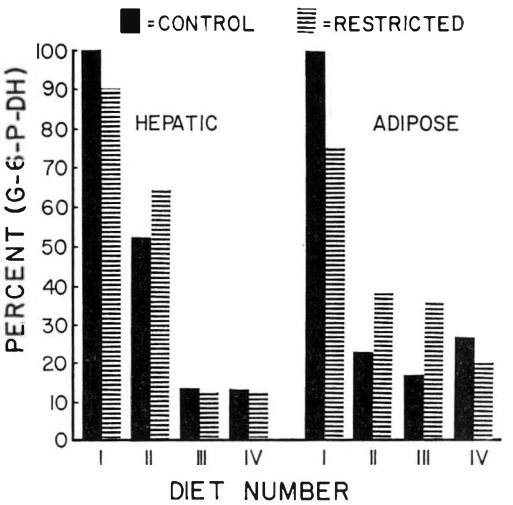


Fig. 1 Relative levels of glucose 6-phosphate dehydrogenase in hepatic and adipose tissues expressed as a percentage of enzymatic activity of animals receiving diet 1 and unrestricted in activity.

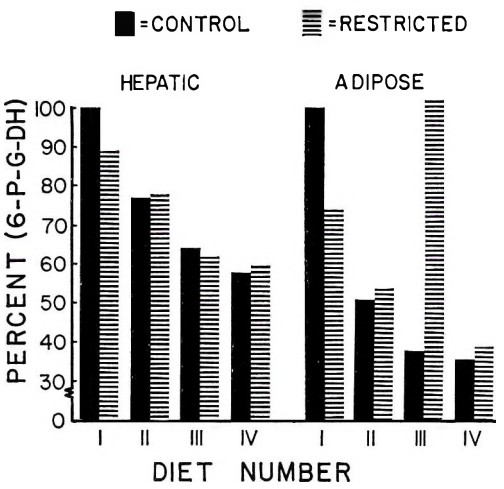


Fig. 2 Relative levels of 6-phosphogluconate dehydrogenase in hepatic and adipose tissues expressed as a percentage of enzymatic activity of animals receiving diet 1 and unrestricted in activity.

activity. However, the quality of the diet definitely influenced the enzyme activities. The hepatic tissues from the rats fed diet 1 were significantly higher ($P < 0.01$) in both glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase activities than those fed diets 2, 3 or 4. The rats fed the hyperprotein diet (diet 2) also showed significantly higher ($P < 0.01$) glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase activities than those fed the diets containing high levels of fat. The interactions between diet and activity were not statistically significant.

The chronic restriction of physical activity significantly increased ($P < 0.01$) the levels of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase in the adipose tissue of rats fed the high fat diet (diet 3) but not in those fed diets 1, 2 or 4. The diet quality also modified the levels of dehydrogenase activity. The glucose-6-P dehydrogenase activity was approximately 3 times higher ($P < 0.01$) in the fat tissues of rats fed diet 1 than those fed the other diets. The 6-P-gluconate dehydrogenase activity of the tissues from rats fed diet 1 also was the highest but, statistically, was only significantly greater ($P < 0.01$) than the tissues from rats fed diet 4.

The lack of a significant enzymatic response to the chronic restriction of physical activity in the rats fed diet 1 prompted a further experiment to assess the metabolic response to forced exercise. Twenty-four male rats were divided into three equal groups and exercised on a motor-driven treadmill at 12.8 meters/minute for one hour daily. They were fed diet 1 for a 2-hour period daily at either one hour before exercise (group A) or one hour (group B) and 3 hours (group C) post-exercise. Twelve additional rats were used as the respective non-exercised controls. The rats were killed immediately after the feeding periods and samples of the liver and epididymal fat tissues were obtained and analyzed as described above.

The results are shown in figure 3 as percentage differences from the respective, non-exercised control values. Statistical analysis of the data indicated that the glucose-6-P dehydrogenase in the liver was significantly lower ($P < 0.05$) in the tissues from the rats that were exercised than from the controls. The time of eating in relation to the exercise period also influenced the level of glucose-6-P dehydrogenase in the liver. The level was significantly lower ($P < 0.01$) in the rats exercised one hour prior to feeding (group B). The liver 6-P-gluconate dehydrogenase levels, however, were not influenced by the exercise or the time of eating.

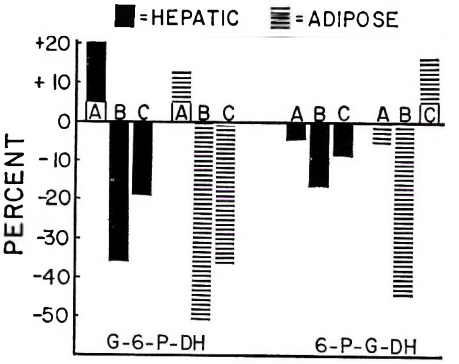


Fig. 3 Relative levels of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in hepatic and adipose tissues of exercised rats expressed as percentage differences from respective control, non-exercised values. A = exercised one hour post-prandially; B = exercised one hour pre-prandially; C = exercised 3 hours pre-prandially.

The exercise also significantly decreased ($P < 0.01$) the adipose glucose-6-P dehydrogenase and the 6-P-gluconate dehydrogenase levels particularly when the exercise period preceded the feeding period by one hour. The interaction between the exercise and time of eating also was statistically significant ($P < 0.01$) suggesting that exercise may affect the availability of intermediary metabolites by modifying the rate of nutrient absorption or metabolic pathway.

## DISCUSSION

The significantly lower body weights of the rats maintained in the restricted cages were unexpected and were in contrast to the earlier observations of Ingle (18). He reported that rats placed in restricted cages became extremely obese with some weighing approximately twice as much as the controls. Studies by Mayer et al. (19) also indicated that non-exercised rats appear to lose their ability to regulate energy intake and consequently become obese. It is possible that such an appetite-regulating mechanism responds differently when the eating patterns are modified as with the 2-hour daily feeding regimen. Whether or not the rats in the present study were adjusting to the apparent decrease in energy output by a compensatory decrease in energy intake was not ascertained. The age and stage of maturity also may be associated with the ability of an animal to control its total energy intake. That is, if the type of appetite-control mechanism is of an instinctive nature, it could be more acute at an early, immature stage as suggested by the studies of Davis (20). Since the rats in the present study were placed in the restricted cages at an earlier age (at about 60 g than the rats used by Ingle (18) (at about 350 g), the latter group may have become less sensitive to the innate regulatory mechanism and reacted accordingly. Additional studies to evaluate the interrelationships between physical activity, age, feeding patterns, and diet quality to the physiological control of appetite and energy intake are indicated.

The compensatory renal hypertrophy in the rats fed the hyperprotein diet is in agreement with that observed in other studies (21, 22). However, the significantly greater hepatic weights in the rats fed the hyperprotein diet were not observed in our previous study (22), although is it realized that the small differences in weight noted in the present study may not be physiologically significant.

The relative activities of the glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase observed in the first study are similar to those reported by others (10, 12, 13, 23, 24) in realimented rats after longer periods of fasting. In all cases, the tissues from rats in unrestricted cages fed the high carbohydrate diet had relatively higher dehydrogenase activity than those fed diets high in either protein or fat. However, the positive enzymatic response to the high fat diet when physical activity was restricted has not been observed previously. The reason for the increase in adipose glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase is unknown but may reflect adaptive changes in patterns of lipogenesis associated with the high fat intake concomitant with the decreased activity. The data suggest increased lipogenesis although this was not supported by corresponding gross body weight changes. Additional studies are necessary to evaluate possible differences in total body composition and in fatty acid composition of adipose tissues.

Forced exercise, however, significantly decreased the enzyme activities. Since glucose-6-P dehydrogenase is associated with the production of NADPH via the hexose monophosphate shunt pathway, which in turn is required for fatty acid and lipid synthesis, decreased levels of glucose-6-P dehydrogenase would indicate a shift away from the shunt pathway and emphasis toward the glycolytic and tricarboxylic acid cycle. This is assuming that the level of an enzyme is directly related to the level of activity of the metabolic pathway in which it participates, as proposed by Fitch and Chaikoff (25). Exercise, then, may modify lipid synthesis by indirectly reducing the supply of available NADPH. From a practical standpoint, the data suggest that the time of exercise in relation to the time of eating as well as the level of exercise are im-

portant factors to consider in the etiology and treatment of obesity.

ACKNOWLEDGMENT

The author gratefully acknowledges the technical assistance of Ron Harris and Yi-li Chiu for analyses of some of the samples and care of the animals, and the assistance of Richard Chu for statistical analyses of the data.

LITERATURE CITED

1. Johnson, M. L., B. S. Burke and J. Mayer 1956 Relative importance of inactivity and overeating in the energy balance of obese high school girls. Am. J. Clin. Nutrition, *4:* 37.
2. Stefanik, P. A., F. P. Heald, Jr. and J. Mayer 1959 Caloric intake in relation to energy output of obese and non-obese adolescent boys. Am. J. Clin. Nutrition, *7:* 55.
3. Dorris, R. J., and A. J. Stunkard 1957 Physical activity: Performance and attitudes of a group of obese women. Am. J. Med. Sci., *233:* 622.
4. Bullen, B. A., R. B. Reed and J. Mayer 1964 Physical activity of obese and non-obese adolescent girls appraised by motion picture sampling. Am. J. Clin. Nutrition, *14:* 211.
5. Stunkard, A. J., W. J. Grace and H. F. Wolff 1955 The night eating syndrome. A pattern of food intake among certain obese patients. Am. J. Med., *19:* 78.
6. Cohn, C., D. Joseph and M. D. Allweiss 1962 Nutritional effects of feeding frequency. Am. J. Clin. Nutrition, *11:* 356.
7. Cohn, C., and D. Joseph 1960 Role of rate of ingestion of diet on regulation of intermediary metabolism ("meal eating" vs. "nibbling"). Metabolism, *9:* 492.
8. Hollifield, G., and W. Parson 1962 Metabolic adaptations to a "stuff and starve" feeding program. I. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period. J. Clin. Invest., *41:* 245.
9. Hollifield, G., and W. Parson 1962 Metabolic adaptations to a "stuff and starve" feeding program. II. Obesity and the persistence of adaptive changes in adipose tissue and liver occurring in rats limited to a short daily feeding period. J. Clin. Invest., *41:* 250.
10. Tepperman, H. M., and J. Tepperman 1962 Role of hormones in glucose-6-phosphate dehydrogenase adaptation of rat liver. Am. J. Physiol., 202: 401.
11. Lee, M., J. R. Debro and S. P. Lucia 1962 Effects of thyroxine and the pattern of food intake on the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Arch. Biochem. Biophys., *98:* 49.
12. Niemeyer, H., L. Clark-Turri, E. Graces and F. E. Vergara 1962 Selective response of liver enzymes to the administration of different diets after fasting. Arch. Biochem. Biophys., 98: 77.
13. Pande, S. V., R. P. Khan and T. A. Venkitasubramanian 1964 Nicotinamide adenine dinucleotide phosphate-specific dehydrogenases in relation to lipogenesis. Biochim. Biophys. Acta, *84:* 239.
14. Fitch, W. M., R. Hill and I. L. Chaikoff 1959 The effect of fructose feeding on glycolytic enzyme activities of the normal rat liver. J. Biol. Chem., 234: 1048.
15. Glock, G. E., and P. McLean 1953 Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J., 55: 400.
16. Horecker, B. L., and A. Kornberg 1948 The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem., *175:* 385.
17. Snedecor, G. W. 1956 Statistical Methods, ed. 5. The Iowa State College Press, Ames.
18. Ingle, D. J. 1949 A simple means of producing obesity in the rat. Proc. Soc. Exp. Biol. Med., 72: 604.
19. Mayer, J. 1961 Obesity: Physiologic considerations. Am. J. Clin. Nutrition, *9:* 530.
20. Davis, C. M. 1928 Self selection of diet by newly weaned infants. An experimental study. Am. J. Dis. Child., 36: 651.
21. Konishi, F., and R. W. Brauer 1962 Renal hypertrophy and function in irradiated and nephrectomized rats fed a high protein diet. Am. J. Physiol., 202: 88.
22. Konishi, F., V. J. Rosen and R. W. Brauer 1961 The effect of high-protein diets on renal hypertrophy in rats after whole-body irradiation. Radiat. Res., *15:* 822.
23. McDonald, B. E., and B. C. Johnson 1965 Metabolic response to realimentation following chronic starvation in the adult male rat. J. Nutrition, 87: 161.
24. Leveille, G. A., and R. W. Hanson 1965 Influence of periodicity of eating on adipose tissue metabolism in the rat. Canad. J. Physiol. Pharmacol., 43: 857.
25. Fitch, W. M., and I. L. Chaikoff 1960 Extent and patterns of adaptation of enzyme activities in livers of normal rats fed diets high in glucose and fructose. J. Biol. Chem., 235: 554.

# Placental Transfer and Fetal Utilization of Calcium in Developing Swine [1,2]

S. L. HANSARD, H. ITOH,[3] J. C. GLENN AND D. M. THRASHER
*Louisiana Agricultural Experiment Station,*
*Baton Rouge, Louisiana*

*ABSTRACT* Radioisotopic chemical balance and tissue distribution studies were used to investigate the source and fetal-maternal behavior of retained calcium in 38 gilts as a function of gestation age and of time after administration of radioactive calcium. Sows killed during the first, second and third trimester, following oral or intravenous dosing with $^{45}Ca$ showed no significant effects of pregnancy on maternal absorption, plasma $^{45}Ca$ or tissue calcium concentration. At 35, 70, 105 and 114 days' gestation, 0.9, 60, 17.8 and 21.3%, respectively, of the absorbed $^{45}Ca$ was transferred to litters standardized to 8 pigs, whereas less than one per cent was retained in the combined placenta and fluids after 168 hours. Fetal bone calcium values, reflecting degree of mineralization, increased rapidly between trimesters 1 and 2, with less change to parturition. Tissue distribution patterns for retained $^{45}Ca$ paralleled those for the dam, but utilization rate and fetal specific activity values were greater. Fetus-to-dam concentration and fetal specific activity ratios were higher for bone than soft tissue, and decreased generally with gestation age and with time after dosing. Utilization efficiency of absorbed maternal calcium for fetal development increased as pregnancy advanced, reaching 21.3% of the $^{45}Ca$ dose at 114 days' gestation.

The general physiology of placental transfer and of dam and fetal relationships has been treated comprehensively by Barcroft (1). Studies on the transfer of radioactive calcium and strontium to the fetus of mice were reported by Pecher and Pecher (2). Calcium-45 has been demonstrated to cross the placenta of rats at all stages of pregnancy (3), and the rate and quantity increased with advanced fetal age. Plumlee and associates (4) showed $^{45}Ca$ to freely traverse the placenta of cattle at the 3 trimesters of pregnancy, especially during the last stages of gestation. Utilizing 2 isotopes concurrently, Shirley and co-workers (5) have reported free passage of $^{45}Ca$, but no $^{99}Mo$ appeared to cross the sow's placenta. Several of the transport mechanisms for the fetus have been discussed by Widdas (6), and the contribution of radioisotopes to placental calcium transfer studies has been reviewed for cattle by Comar (7) and for swine by Hansard (8).

Previous reports from this laboratory have shown the complex epitheliochorial placenta of the sow to be permeable to iron (9, 10), and to zinc (11), and that membrane permeability was dependent upon the combined effects of several physiological factors (8). The present paper gives data on the placental transfer of calcium in developing swine, the relative uptake and turnover in selected tissues of the dam and the fetus as a function of gestation age, and of time during the last trimester of pregnancy.

## METHODS

Thirty-eight Duroc, Hampshire and Poland China gilts in their first pregnancy were selected at random for killing at 35, 70, 105 and 114 days' gestation following oral or intravenous administration of a single tracer dose of labeled calcium. All sows were maintained with a conventional 16% protein ration (0.7% calcium, 0.5% phosphorus) composed of corn meal 76.5%, soybean oil meal 20%, oyster shell flour 1.0%, dicalcium phosphate 1.0%, iodized

Received for publication January 28, 1966.

[1] Published with the approval of the Director, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana.

[2] We are grateful to International Minerals and Chemical Corporation, Skokie, Illinois and to the National Science Foundation (NSF-1944) for partial support of this study.

[3] Post-doctorate Research Fellow 1964–65, Department of Animal Science, from Tohoku University, Sendai, Japan.

salt 0.5% and vitamin-mineral premix 1.0%.[4] Following a 168-hour blood-balance period (12) each sow was killed, and selected fetal and maternal tissue samples were taken for isotopic chemical analyses. Seven of the gilts, during the third trimester, were similarly dosed and killed 8, 14, 48, 68 and 96 hours after dose administration for comparison with those killed after 168 hours. Fetuses were separated from the placenta, weighed, measured and either ashed for whole body analyses, or selected tissue and organs were separated for tissue calcium distribution studies. Total ash, phosphorus, calcium and labeled calcium measurements were carried out as described in previous reports (3, 4, 14). Sow weights ranged from 122 to 195 kg (mean 156). Therefore, for direct comparison, all maternal data were calculated to the same standard dose and body weight.

## RESULTS AND DISCUSSION

*Calcium and $^{45}Ca$ absorption.* To permit calculations of fetal tissue values on the bases of retained calcium and $^{45}Ca$, and in order to prevent differences due to gastrointestinal absorption from masking actual maternal absorption and placental permeability values, 7-day radioisotopic chemical balance data were collected for each individual sow. Results were not significantly different from those reported for swine earlier (15), and for brevity only the mean results, essential for maternal tissue calculations, are presented. These first-litter gilts excreted 79 ± 5% of the 30 ± 6 g dietary calcium/day, and there was no significant effect of gestation upon absorption. Seventy per cent of the oral dose and 13% of the intravenous $^{45}Ca$ was excreted. Endogenous fecal calcium was calculated (15), and the daily retention values averaged 31 ± 3% of 30 g, or 9.3 g available calcium for deposition in the dam or for placental transfer.

*Maternal tissue calcium and $^{45}Ca$ values.* Average calcium, labeled calcium and specific activity values for selected maternal tissues of the 38 gilts are shown in table 1. Limited animal numbers and the wide individual concentration variations prevented detection of possible significant difference due either to pregnancy or to stage of gestation (3, 4). For convenience of presentation, therefore, all treatments were pooled and labeled calcium was calculated as percentage retained dose $^{45}Ca \times 10^{-4}$ per g fresh weight, corrected to 150 kg body weight for comparative purposes. Maternal bones contained most of the total calcium, and after 168 hours the greater part of the $^{45}Ca$ was in the bone, with concentration highest in trabecular bone where contact with blood vessels occurs, and in areas of higher metabolic activity (3, 16, 17). This was reflected in the high specific activity for soft tissues and sternum,

[4] The vitamin-mineral premix contained: zinc oxide, 30 g; vitamin A, 1,140 IU; vitamin $B_{12}$, 10 mg; riboflavin, 1.0 g; niacin, 2.0 g; and pantothenic acid, 4.5 g, mixed with 4.1 kg ground yellow corn.

TABLE 1

*Calcium and radioactive calcium [1] concentration in maternal tissues of swine*

| Tissue | Calcium | $^{45}Ca$ | Specific activity [2] |
|---|---|---|---|
| | *mg/g* | *% dose/g × $10^{-4}$* | |
| Muscle | 0.061 ± 0.011 [3] | 0.121 ± 0.031 | 198 |
| Liver | 0.071 ± 0.013 | 0.140 ± 0.028 | 197 |
| Kidney | 0.101 ± 0.010 | 0.123 ± 0.031 | 122 |
| Spleen | 0.083 ± 0.010 | 0.109 ± 0.021 | 131 |
| Mammary tissue | 0.091 ± 0.012 | 0.111 ± 0.011 | 122 |
| Femur shaft | 234 ± 36 | 60.2 ± 5.1 | 26 |
| Femur end | 105 ± 17 | 92.1 ± 7.3 | 88 |
| Rib shaft | 145 ± 35 | 54.8 ± 4.8 | 38 |
| Rib end | 74 ± 8 | 157.6 ± 18.1 | 212 |
| Mandible | 151 ± 9 | 96.4 ± 9.4 | 64 |
| Sternum | 85 ± 7 | 174.2 ± 22.1 | 205 |

[1] Tissue $^{45}Ca$ calculated as % absorbed dose/g fresh tissue × $10^{-4}$ 168 hours after dosing, and corrected to 150 kg body weight.

[2] Specific activity or turnover rate calculated as $\frac{\%\ \text{dose/g} \times 10^{-4}}{\text{mg Ca/g}} \times 100$.

[3] Mean ± SD.

rib and femur epiphyses, with usual lower values for areas of cortical bone.

*Physical changes in products of conception during gestation.* The progressive weight increase of the products of conception in sows, including their 362 fetuses, during advancing pregnancy is shown in figure 1. The most critical period for membrane growth appeared to be during the first 70 days, when fetal weight equaled weight of membranes.

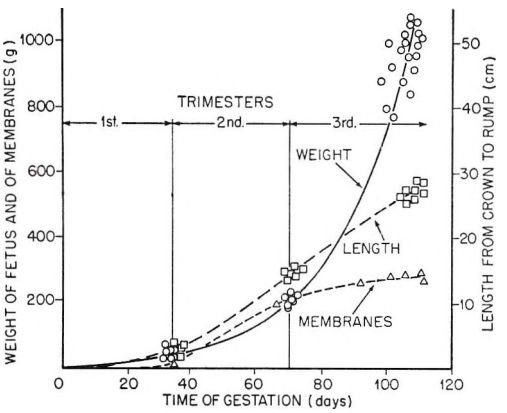


Fig. 1 Fetal and placental membrane growth during gestation in swine.

Greatest relative development occurred during the second trimester, when fetal weight increased 30 times and length, 7 times. However, total mass gain was fastest during the dynamic final trimester, when weight again increased 3 times and length was nearly doubled. Although placental fluid volume and fetal weight increased markedly during the third trimester, there was little weight change in fetal membranes, implying a stretching and expansion of the placenta with increased size of developing fetuses. The morphological relationship thus created over a considerable area of the placenta offers the thinnest laminae of epithelial cytoplasm separating the fetal and maternal vascular plexuses, and the intra-epithelial capillaries acquire significance as an anatomical means for increased mineral exchange between dam and fetus (18).

*Total calcium and $^{45}Ca$ partition in products of conception during gestation.* The effects of fetal age upon calcium and labeled calcium concentration in the placenta, fluids and litter of swine after 168 hours are shown in table 2. Litters, 35 days of age, from sows killed 7 days after receiving a single dose of $^{45}Ca$ contained less than 1% of the absorbed labeled calcium. However, this was 5 times greater than the total $^{45}Ca$ in the combined placenta and fluids. The dam, therefore, retained nearly 99% of the absorbed $^{45}Ca$ dose for body deposition during this first trimester. Fetuses, standardized to a litter of eight, retained approximately 6% at 70 days of age, 17.8% at 105 days, and 114-day-old fetuses contained 21.3% of the absorbed $^{45}Ca$ dose. Total calcium deposition in the fetus increased 4.5 times between 35 and 70 days, and another 9 times between age 70 and 114 days. During the final trimester, therefore, dams retained about 79% of the absorbed $^{45}Ca$, and transferred the remaining 21% to their fetuses. This value was less than the 40% $^{45}Ca$ retained by developing rats (3), but probably reflects differences in placental types (8).

If it is assumed that dietary calcium and $^{45}Ca$ are similarly utilized, it can be calculated that a sow with a litter of 8 pigs, absorbing 9.3 g from the 30 g/day calcium intake, was depositing, at 70 days gesta-

TABLE 2

*Gestation age effects upon absorbed[1] $^{45}Ca$ and total calcium partition in swine products of conception*

| Tissue | Days of gestation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 35 ± 4 | | 70 ± 3 | | 105 ± 4 | | 114 ± 3 | |
| | $^{45}Ca$ | Ca | $^{45}Ca$ | Ca | $^{45}Ca$ | Ca | $^{45}Ca$ | Ca |
| | % | g | % | g | % | g | % | g |
| Placenta | 0.132 | 0.490 | 0.080 | 0.812 | 0.070 | 0.990 | 0.06 | 1.01 |
| Placental fluids | 0.081 | 0.350 | 0.077 | 0.498 | 0.095 | 0.585 | 0.082 | 0.575 |
| Litter (8 fetuses) | 0.904 | 2.0 | 5.9 | 9.5 | 17.8 | 75.0 | 21.3 | 85.0 |

[1] $^{45}Ca$ calculated as percentage of absorbed dose corrected to 150-kg dam, killed 168 hours after dosing.

tion, 5.9% or 0.55 g of the absorbed calcium in its fetuses. Between 70 and 114 days' gestation, deposition progressively increases to 21.3% of 9.3 g or 1.98 g/day. This value approximates the total calcium accretion value of 1.7 g/day calculated from the chemical analyses for this period. The apparent discrepancy between values could be due to the accelerated deposition with increasing age, inherent errors in balance measurements, or possibly to the limited calcium fetal-dam feedback that has been observed in other species (6, 17).

*Labeled calcium distribution and turnover rates in products of conception.* The effect of time upon total transfer of $^{45}Ca$ to the placenta, placental fluid and whole swine fetuses during the third trimester of pregnancy is shown for 7 sows in table 3. These data indicate that the fetus accounted for the greater part of the transferred $^{45}Ca$, and that after 8 hours 13.3% was deposited in a litter of 8 pigs, whereas slightly more than 1% $^{45}Ca$ was transferred to other components of the placental complex. Values decreased for placenta and fluids in a regular manner with time, but $^{45}Ca$ deposition and subsequent specific activity increased for fetuses and after 68 hours 19.7% of the absorbed dose was in the litter, with less than 0.5% remaining in the placenta and surrounding fluids. Labeled calcium approached equilibrium at this time and there was little change to 168 hours, except that values for placenta and placental fluids decreased to less than 0.1% of the retained dose.

These data indicate that (a) fetuses receive and retain most of the maternal $^{45}Ca$ transmitted, (b) contribution of placenta or fluids to fetal $^{45}Ca$ is either exceedingly rapid or insignificant, and (c) maternal blood must be the primary calcium source for developing swine fetuses.

Mathematically the disappearance rate for $^{45}Ca$ from the placenta and fluids, and subsequent deposition in the fetus can be explained as a first-order reaction following the law of mass action, as:

$$\frac{-dA_p}{dt} = k_p[A_p] \qquad (1)$$

This means that the rate $\frac{dA}{dt}$ is proportional to $^{45}Ca$ concentration at time $t$. By integration and rearrangement equation 1 becomes:

$$\ln A = \ln A_o - kt \qquad (2)$$

The regression coefficient $k$ then, calculated from the placental data, was $-0.00346$, indicating a disappearance rate for $^{45}Ca$ to be 0.346% /hour.

In the same manner $^{45}Ca$ disappearance rate from the placental fluids was calculated to be 0.552% /hour.

Labeled calcium uptake by the fetus followed the same law, but the sign was reversed:

$$\frac{dA_f}{dt} = k_f[A_f] \qquad (3)$$

The regression coefficient $k_f = 0.867\%$ / hour for $^{45}Ca$ fetal accretion during the third trimester of pregnancy.

The $^{45}Ca$ disappearance rates were less for the placenta and fluids than the rate for $^{45}Ca$ accretion by the developing fetus. This indicates further the limited contribution of placenta and fluids as a fetal source of calcium.

*Fetal age effects upon tissue $^{45}Ca$ concentration and specific activity.* Concentration of $^{45}Ca$ and specific activity in selected tissues of the developing swine

TABLE 3

*$^{45}Ca$ distribution [1] and turnover rates in the total products of conception of swine during final trimester of pregnancy*

| | Hours after dosing | | | | | | Turnover rate |
|---|---|---|---|---|---|---|---|
| | 8 | 14 | 48 | 68 | 96 | 168 | |
| | | | | | | | % /hr [2] |
| Placenta | 0.97 | 0.78 | 0.43 | 0.33 | 0.22 | 0.07 | 0.346 |
| Placental fluids | 0.49 | 0.42 | 0.10 | 0.10 | 0.09 | 0.07 | 0.552 |
| Litter of 8 | 13.3 | 14.40 | 17.50 | 19.70 | 20.90 | 21.30 | 0.867 |

[1] Calculated as % absorbed $^{45}Ca$ dose in total tissue of fetuses killed during third trimester of pregnancy (standardized to 1-kg fetuses).
[2] See text for calculations.

fetus at the 3 trimesters of pregnancy are shown in table 4. These values reflect the progressive mineralization of fetal bones with increased age, and subsequent high $^{45}Ca$ deposition, 168 hours after dose administration to the dam. Soft tissue concentration values for both calcium and labeled calcium were greater during early gestation, and the high turnover rates (specific activity) for both bone and organs reflect the dynamic state during these developmental periods. Deposition patterns followed closely those observed for maternal tissues (table 1), with highest concentrations in bone and in tissue areas of greater metabolic activity. Concentration and specific activity values were higher for bone than soft tissue and decreased with fetal age probably because of the dilution effects of increased demands for bone growth and the subsequent calcium deposition. For like reason, fetal-to-dam specific activity ratios decreased as gestation advanced, with that of soft tissues approaching unity in the final trimester. Ratios for bone were much greater, reflecting the avidity of fetal bone for the available labeled calcium (3, 4, 8). However, the same general decreasing pattern with increased fetal age was evidenced.

The relationship of selected fetal tissue $^{45}Ca$ concentration to that of the whole fetus during the third trimester of pregnancy is illustrated in figure 2. These values demonstrate the magnitude of the rapid changes occurring in the whole fetus and in representative bone areas during the last 2 weeks of pregnancy, and provide further evidence of relative $^{45}Ca$ concentration in the fetus to that of its component parts. The observed unusually low labeled calcium values for the femur end in these fetuses was undoubtedly due to sampling error and dilution with adjacent cartilage.

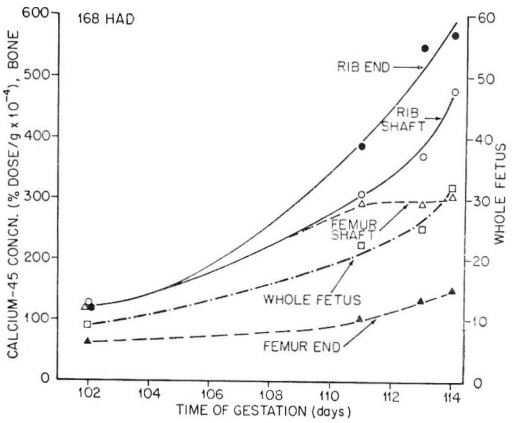


Fig. 2 Gestation age effects upon $^{45}Ca$ concentration in selected bones and in developing swine fetuses killed 168 hours after maternal administration of labeled calcium.

These data substantiate the progressive and rapid rate of calcium deposition during the final trimester in developing swine, and the values parallel laydown patterns observed in other species (2–4, 8). Maternal calcium transferred to the fetal blood then is apparently removed rapidly by the

TABLE 4

*$^{45}Ca$ concentration [1] and specific activity [2] of selected fetal tissues at 3 trimesters of pregnancy*

| | Days of gestation | | | | | |
|---|---|---|---|---|---|---|
| | 35 | | 70 | | 105–114 | |
| | $^{45}Ca$ [1] | Specific activity [2] | $^{45}Ca$ | Specific activity | $^{45}Ca$ | Specific activity |
| | % | | % | | % | |
| Muscle | 0.38 | 290 | 0.39 | 264 | 0.18 | 159 |
| Liver | 0.36 | 296 | 0.20 | 256 | 0.15 | 174 |
| Kidney | 0.35 | 205 | 0.29 | 181 | 0.22 | 139 |
| Spleen | 0.16 | 121 | 0.14 | 116 | 0.15 | 168 |
| Rib shaft | 512 | 853 | 970 | 735 | 285 | 226 |
| Rib ends | 451 | 2255 | 405 | 604 | 513 | 457 |
| Femur shaft | 680 | 829 | 641 | 465 | 291 | 194 |
| Femur ends | 183 | 871 | 581 | 717 | 135 | 233 |
| Mandible | 240 | 800 | 310 | 518 | 189 | 250 |

[1] Tissue $^{45}Ca$ concentration calculated as % absorbed dose/g fresh weight $\times 10^{-4}$, 168 hours after dose administration to dam.

[2] Specific activity calculated from $^{45}Ca$ and tissue calcium values as $\frac{\%\ \text{dose/g} \times 10^{-4}}{\text{mg Ca/g}} \times 100$.

same competing processes discussed for the dam. Absorbed ions are mixed with the body fluids and deposited selectively in the soft tissue and bone areas at rates that increase with the demands for growth and development as gestation advances, suggesting fetal physiological needs as the major incentive for calcium transfer to the products of conception in swine.

ACKNOWLEDGMENTS

Appreciation is expressed to Sharon Nichelson and William Whitfield for assistance with the radioisotopic chemical analyses, and to Karel Vohnout and Carol Bradley for help with the calculations and data tabulation.

LITERATURE CITED

1. Barcroft, J. 1946 Researches on pre-natal life. Blackwell Scientific Publications, Oxford.
2. Pecher, C., and J. Pecher 1941 Radiocalcium and radiostrontium metabolism in pregnant mice. Proc. Soc. Exp. Biol. Med., *46:* 91.
3. Feaster, J. P., S. L. Hansard, J. C. Outler and G. K. Davis 1956 Placental transfer of $Ca^{45}$ in the rat. J. Nutrition, *58:* 399.
4. Plumlee, M. P., S. L. Hansard, C. L. Comar and W. M. Beeson 1952 Placental transfer and deposition of labeled calcium in the developing bovine fetus. Am. J. Physiol., *171:* 678.
5. Shirley, R. L., M. A. Jeter, J. P. Feaster, J. T. McCall, J. C. Outler and G. K. Davis 1954 Placental transfer of $Mo^{99}$ and $Ca^{45}$ in swine. J. Nutrition, *54:* 59.
6. Widdas, W. F. 1961 Transport mechanisms in the fetus. Brit. Med. Bull., *17:* 107.
7. Comar, C. L. 1956 Radiocalcium studies in pregnancy. Ann. New York Acad. Sci., *64:* 281.
8. Hansard, S. L. 1965 Placental transfer and fetal utilization of absorbed minerals by developing swine. Hanford Symposium Proc. Swine in Biomedical Research, Hanford, Washington.
9. Hoskins, F. H., and S. L. Hansard 1964 Placental transfer of iron in swine as a function of gestation age. Proc. Soc. Exp. Biol. Med., *116:* 7.
10. Hansard, S. L. 1964 The physiological role of iron in nutrition of growing pigs. Prague Symposium on Radioisotopes in Animal Nutrition and Physiology, pp. 319–337.
11. Hansard, S. L., H. Itoh, F. H. Hoskins, D. M. Thrasher and J. C. Glenn 1965 Zinc absorption and placental transfer in swine. J. Animal Sci., *24:* 920.
12. Hansard, S. L. 1951 Radioisotope procedures with farm animals. I. Metabolism facilities. Nucleonics, *9:* 13.
13. Hansard, S. L., C. L. Comar and M. P. Plumlee 1951 Radioisotope procedures with farm animals. II. Quantitative administration and sampling techniques. Nucleonics, *9:* 38.
14. Comar, C. L., S. L. Hansard, S. L. Hood, M. P. Plumlee and B. F. Barrentine 1951 Use of $Ca^{45}$ in biological studies. Nucleonics, *8:* 19.
15. Hansard, S. L., W. A. Lyke and H. M. Crowder 1961 Absorption, excretion and utilization of calcium by swine. J. Animal Sci., *20:* 292.
16. Hansard, S. L., C. L. Comar and G. K. Davis 1954 Effects of age upon the physiological behavior of calcium in cattle. Am. J. Physiol., *177:* 383.
17. MacDonald, N. S., D. L. Hutchinson, M. Hepler and E. Flynn 1965 Movement of calcium in both directions across the primate placenta. Proc. Soc. Exp. Biol. Med., *119:* 476.
18. Amoroso, E. C. 1961 Histology of the placenta. Brit. Med. Bull., *17:* 81.

# Evidence for an Unidentified Growth Factor(s) from Alfalfa and Other Plant Sources for Young Guinea Pigs [1,2]

RAJ K. LAKHANPAL, JEAN REICHE DAVIS, JOHN T. TYPPO AND GEORGE M. BRIGGS

*Department of Nutritional Sciences, University of California, Berkeley, California*

*ABSTRACT* An improved purified diet for the guinea pig has been described which is suitable for assay of a proposed unidentified growth factor(s) found in alfalfa and other plant sources (such as soybean meal, dried broccoli, and dried grass clippings). Improvement in growth of up to 50 g over a 3-week assay period was obtained by adding 10% of dehydrated alfalfa to the diet. The factor(s) appears to be distinct from all known vitamins, minerals, amino acids, or other nutrients. It appears to be organic in nature, since it is not found in the ash of alfalfa. The relationship of this "plant factor" to the "grass juice factor" or to other reported unidentified factors for other animals is unknown at this stage. Attempts to identify the factor(s) are in progress.

Although it is possible today to rear and reproduce experimental animals with diets consisting of synthetic or highly purified ingredients, reports are still being published (chiefly of studies utilizing the fowl) of unidentified growth factors present in a variety of natural ingredients such as fish meal, condensed fish solubles, meat meal, distillers solubles, green plants, and alfalfa.

Shortly after the development of an improved semipurified diet for young guinea pigs in 1953 (1), an improved commercial stock diet [3] became available which resulted in 10 to 15% greater early growth than that obtained with a semipurified diet.[4] It soon became evident, in experiments reported here, that this difference in growth could be overcome largely by adding either 20% of the stock diet or a wide variety of plant materials to the semipurified diet. Dried alfalfa was found to be one of the better sources of the growth factor(s).

Alfalfa has been reported to contain an unidentified factor(s) for poultry (2–6), for a microorganism (7), and for the pig (8). In 1938, Kohler et al. (9) studied the growth effect of the "grass juice factor" in guinea pigs, although this factor has since been shown to be largely replaced by a variety of known essential nutrients (10), and its status is uncertain. In 1957, Ershoff (11) reported that both dried alfalfa juice and water-washed alfalfa pulp had significant growth-promoting activity for guinea pigs fed a "mineralized spray-process dried milk" ration (with unstated, and uncertain, amounts of all nutrients now known to be required by the guinea pig). Supplementation of alfalfa meal reduced mortality in guinea pigs exposed to irradiation in studies made by Calloway et al. (12). Reid and Mickelsen (13) confirmed our earlier report [5] that dried alfalfa increased the growth of guinea pigs when added to semipurified diets very similar to those used here.

The purpose of this study is to provide information on various crude sources of the unidentified growth factor(s), on an

Received for publication January 3, 1966.

[1] The authors are indebted to the USDA Western Regional Research Laboratory, Albany, California, for partial financial support of part of this study and to the former Nutrition Section, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, where most of the data were obtained (1954–1960) on the sources of factor(s).

[2] A preliminary report of part of this study was presented at the American Institute of Nutrition meeting in April, 1961 (Federation Proc., 20: 236, 1961). Details of these and related experiments may be found in the M.S. theses of Jean Reiche Davis, 1961, and Raj K. Lakhanpal, 1963, deposited with the Graduate Division of the University of California, Berkeley.

[3] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.

[4] Unpublished data, G. M. Briggs.

[5] See footnote 2.

mproved assay, on some minor attempts of preliminary fractionation, and on differentiation of the factor(s) from known nutrients.

## EXPERIMENTAL

*Animals and their care.* More than the usual details of animal care are given here because of their importance to the success of the experiments and because of the sensitivity of the very young guinea pig to environmental changes. Guinea pigs of mixed strains and mixed sexes, 6 to 9 days old and weighing 95 to 120 g, were obtained commercially.[6] For the first 2 or 3 days, before the start of the experiments, groups of 4 to 6 animals were maintained with the basal diet in double-size cages in order to facilitate learning the use of the drinking apparatus and feed cups. The animals were then divided into uniform groups of five or more according to sex and weight, placed individually in single cages, and fed the various diets. The metal cages (24 × 18 × 18 cm) had 0.6-cm mesh floors the first week, and 1.3-cm mesh floors thereafter. Shallow china food cups (30 mm high and 70 mm in diameter) were used; each cup had a metal top with a large round hole (45-mm diameter) in the center to keep food contamination by the young guinea pigs at a minimum. After the first week, larger cups (60 mm high and 65 mm in diameter) without metal tops were used. Each feed cup was kept in a small wire-mesh frame attached to the cages to prevent tipping. Tap water was supplied from inverted bottles with copper or stainless steel drinking tubes.

The young guinea pigs were handled gently and special attention was given to see that they learned to eat and drink readily. They were checked several times a day for the first few days to see that food and water supplies were clean, available, and being consumed. Cages were cleaned and sterilized 3 times per week or more often, as needed. The animals were weighed daily for the first 3 days and 3 times per week for the rest of the experimental period. Their food consumption was recorded and estimates for food wastage and spillage were made daily.

Most of the earlier feeding trials with young guinea pigs were of 2 weeks' duration. Because the latter phase of the study utilized animals from new sources and these were highly susceptible to infections and environmental changes during the early period, the feeding trials were changed to a 3-week period. Under these conditions, when an occasional animal died during the first week (an average of about 1 in 10 animals, but in some experiments as many as 1 out of 4), it was replaced with an extra animal of the same sex and of similar weight that had been fed the basal diet. Such deaths were obviously due either to an infection or to a refusal to eat or drink and not to the effects of the nutritive value of the diet. Few animals died after the first week, as shown in the tables.

*Experimental diets.* The composition of diets GPD2 and GPD3 is shown in table 1. Diet GPD2 is a modification of diet

TABLE 1

*Composition of basal diets*

| | Diet GPD2 | Diet GPD3 |
|---|---|---|
| | *g/kg* | *g/kg* |
| Casein (vitamin-free) | 300 | 300 |
| Cornstarch | 200 | — |
| Glucose hydrate [1] | 64 | 64 |
| Sucrose | 100 | 462 |
| Cellulose [2] | 150 | — |
| Corn oil | 50 | 50 |
| Mineral mixture (HMW) [3] | 60 | 50 |
| Potassium acetate | 25 | 25 |
| Magnesium oxide | 5 | 5 |
| Zinc carbonate | 0.13 | 0.13 |
| Ascorbic acid | 2 | 2 |
| Choline chloride | 2 | 2 |
| Inositol | 2 | — |
| B-vitamin mix in glucose [4] | 20 | 20 |
| Fat-soluble vitamin mix in corn oil [5] | 20 | 20 |
| Total | 1000.13 | 1000.13 |

[1] Cerelose, Corn Products Company, San Francisco.
[2] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.
[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman. J. Nutrition, *14*: 273, 1937; obtained from Nutritional Biochemicals Corporation, Cleveland.
[4] The following amounts were present per 20 g glucose: (in mg) thiamine·HCl, 16; riboflavin, 16; pyridoxine·HCl, 16; Ca pantothenate, 40; niacin, 200; biotin, 0.5; folic acid, 10; and vitamin $B_{12}$, 0.05.
[5] The following were present per 20 g of corn oil: (in mg) vitamin A acetate, 6; α-tocopheryl acetate, 20; vitamin $D_3$, 0.04; vitamin K (menadione), 2.

[6] Supplied by Dependable Animal Supply Company, Martinez, California. In the early studies, guinea pigs from the N.I.H. colony were used.

GP13 of Reid and Briggs (1). Diet GPD3, a further modification, contains no added source of inositol, cellulose, nor starch. These deletions were made in an attempt to remove possible contaminating sources of sparing factor(s) or the unidentified factor(s) itself. Diet GPD3 also contained 1% less mineral mixture to avoid slightly detrimental effects with the larger amounts in this ration. Supplements were added to the diets at the expense of starch and sucrose, respectively. Fresh diets were mixed [7] every 2 weeks and stored in refrigerators when not in use. Commercial pelleted diets [8] for guinea pigs were used as a control diet (stock diet).

*Fractionation of alfalfa.* Weighed quantities of dehydrated alfalfa (commercial alfalfa meal, 17% protein) were extracted with 95% ethyl alcohol for 48 hours in a large Soxhlet apparatus. The alcohol extract was concentrated under vacuum in glass to a thick syrup which was carefully collected and weighed. The residue left in the thimble was dried at room temperature. The water-soluble "alfalfa juice concentrate" was prepared according to the method of Kohler and Graham (2).[9] The "dry" ash of alfalfa was prepared in a muffle furnace at slowly increasing temperatures to permit slow charring yet to allow removal of organic material within a 24-hour period. These fractions were fed at levels equivalent to 10% of the original dehydrated alfalfa meal.

A variety of substances, including alfalfa, as well as certain pure components, were tested either singularly or in various combinations, at levels indicated later, for their growth-promoting activity.

## RESULTS

The effect of adding different levels of alfalfa to diet GPD2 in the early phases of this work is summarized in table 2, part 1. Optimal growth increases were obtained in 2-week studies with a minimum of 10% of alfalfa, the level chosen for use in later experiments.

In later 3-week studies (table 2, part 2), significant growth responses were again obtained with the addition of 10% dried alfalfa to the diet. The addition of

[7] Details of mixing the diets may be found in the theses mentioned in footnote 2.
[8] See footnote 3.
[9] Kindly supplied by Dr. George Kohler, Western Regional Research Laboratory, Albany, California.

TABLE 2

*Supplementation of diet GPD2 with various levels of alfalfa meal*

| Supplement to diet GPD2 [1] | No. of experiments | No. of guinea pigs | Gain | Gain over basal |
|---|---|---|---|---|
| | | | *g* | *g* |
| Part 1. Experiments conducted 1960–62, 2-week tests [2] | | | | |
| None (basal) | 28 | 140 | 61 | — |
| 5% alfalfa meal (17%) | 2 | 10 | 70 | 9 |
| 10% alfalfa meal | 9 | 45 | 92 | 31 |
| 20% alfalfa meal | 2 | 10 | 80 | 19 |
| 40% alfalfa meal | 1 | 5 | 69 | 8 |
| 10% stock diet [3] | 3 | 14 | 87 | 26 |
| 100% stock diet [3] | 9 | 54 | 98 | 37 |
| Part 2. Experiments conducted 1962–63, 3-week tests [4] | | | | |
| None | 3 | 17(17) | 104 | — |
| 10% alfalfa meal | 5 | 31(30) | 135 | 31 |
| Alcohol extract of alfalfa (equivalent to 10% alfalfa) | 3 | 19(19) | 110 | 6 |
| Alcohol residue of alfalfa (equivalent to 10% alfalfa) | 4 | 25(25) | 112 | 8 |
| 10% dried grass clippings | 1 | 6(6) | 136 | 32 |
| 100% stock diet [3] | 2 | 9(8) | 140 | 36 |

[1] Supplements added at the expense of starch.
[2] Average starting weight, 100 g; mortality was less than 5% of total.
[3] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.
[4] Average starting weights, 106 to 110 g; number of animals at end of experiment given in parentheses.

an equivalent amount of alcohol extract of alfalfa or of the alcohol residue was not effective, but a combination of the 2 fractions was not tested.

In the last half of 1962, only insignificant growth responses were obtained with alfalfa in some experiments, even when they were continued an extra week. The reason for this was unknown but was thought to be due to the presence of impurities or sparing factors in the basal ration, as well as to the loss of a possible labile factor in the alfalfa sample. Therefore, the basal diet was changed to GPD3 to improve the test. Results with this more simplified diet are listed in table 3. Animals fed the basal diet (GPD3) had a higher mortality rate than those fed the former diet. Significant growth responses were obtained with the 2 samples of dried alfalfa (the original sample and a new supply which has been used since, indicating that the factor(s) responsible for the increase in growth was reasonably stable when stored at cold temperatures for long periods). The separate additions of 0.2% inositol and 5 or 15% of cellophane [10] (ingredients in GPD2) were without effect. When an additional 1% of mineral mixture in combination with 3% of cellophane (simulating the mineral and cellulose content of 10% of alfalfa) was fed, there was no stimulation of growth, nor was there any effect of adding alfalfa ash equivalent to 10% of alfalfa. The crude grass juice fraction obtained from alfalfa gave a small but statistically significant response. (Whether the factor(s) is partially destroyed by the small amount of heat necessary to make the concentrate or whether a factor(s) present in the extracted residue must be present to realize the full growth-stimulating effect cannot be answered at this stage.)

A number of different foodstuffs and miscellaneous ingredients were tested for their growth-promoting activities before 1963, using the 2-weeks' test. Good responses, equivalent to alfalfa, were obtained in one or more experiments with a number of feedstuffs from plant sources (level fed given in parentheses): soybean meal (10%), dried broccoli (10%), dried grass clippings (10%), and stock diet (20%). Moderate growth responses were observed with dried kale (10%), corn meal (10%), whey (dried spray) (10%), corn cob flour (10%), distillers solubles (2%), and wheat bran (10%). Insignificant responses were obtained with lactose (10%), liver residue (3%), liver powder (3%), fish meal (3%), pinitol (0.2%) (14), inositol (0.2%), starch (20%), cellulose (15%), oxalic acid (0.2%), levulinic acid (0.1%), furfuraldehyde (0.2%), carotene (0.2%), a commerical

[10] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.

TABLE 3

*Supplementation of diet GPD3*

| | Additions to diet GPD3 [1] | No. of guinea pigs | No. of experiments | Gain in 3 weeks [3] | Gain over basal |
|---|---|---|---|---|---|
| | | | | *g* | *g* |
| 1 | None | 62(51) | 9 | 72 ± 5.2 [4] | — |
| 2 | 10% dried alfalfa, sample 1 | 17(15) | 2 | 112 ± 5.3 | +40 |
| 3 | 10% dried alfalfa, sample 2 | 40(36) | 6 | 122 ± 3.8 | +50 |
| 4 | 0.2% inositol | 7(5) | 1 | 72 ± 11.8 | — |
| 5 | 5% cellulose [5] | 6(6) | 1 | 88 ± 9.1 | +16 |
| 6 | 15% cellulose | 7(6) | 1 | 74 ± 9.0 | + 2 |
| 7 | 1% HMW salts [6] + 3% cellulose | 16(12) | 2 | 70 ± 4.6 | − 2 |
| 8 | 1.8% alfalfa ash | 10(9) | 2 | 65 ± 6.2 | − 7 |
| 9 | Alfalfa juice concentrate (equivalent to 10% alfalfa) | 34(27) | 5 | 95 ± 7.7 | +23 |
| 10 | Stock diet [7] | 38(35) | 5 | 118 ± 4.9 | +46 |

[1] Supplement added at the expense of sucrose.
[2] Number of animals left at end of experiment given in parentheses.
[3] Average starting weights, 102 to 106 g.
[4] Mean ± SE.
[5] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.
[6] See table 1, footnote 3.
[7] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.

source of an unidentified factor used in poultry rations (0.3%), citrus tannins (0.3%), saponin (0.1%), Torula yeast (5%), brewer's yeast (5%), xylose (3%), a variety of flavoring agents (0.3%), additional amounts of all vitamins, and various mixtures of amino acids, including arginine and methionine alone or in combination. Two acetone-soluble fractions of alfalfa juice concentrate [11] were inactive. A combination of 0.1% inositol, 15% cellulose, and 20% starch (as in GPD2) gave a small but definite growth response that none of the components gave when fed alone.

## DISCUSSION

The studies of Reid (10) on vitamin requirements of the guinea pig have resulted in improved knowledge of their nutrient requirements. However, little information is known about possible unidentified factors for guinea pigs. The review of Mannering (15) was published before the above review by Reid, and it is now evident that diets used to test former "unidentified factors" were low in either known vitamins, such as folic acid or thiamine, or in minerals, such as potassium, magnesium, or zinc. In the present studies, the addition of 10% of dehydrated alfalfa to the guinea pig's semipurified diet invariably resulted in better growth than that obtained when guinea pigs were fed alfalfa-free diets containing what is believed to be adequate amounts of all known vitamins, minerals, and amino acids.

These studies indicate that the reduced growth of young guinea pigs is due to the absence of some unidentified growth factor(s). Since the supplementation with alfalfa meal and many other ingredients of plant origin gave a significant growth response, it appears that the unknown growth factor(s) is widely distributed in plant sources. The animal products tested were either inactive or had low growth-promoting activity. The relationship of this "plant factor(s)" to the "grass juice factor(s)" of Kohler and co-workers (2, 9) or to other reported unidentified factors found in alfalfa or similar plant substances cannot be postulated at this stage. Also, we do not know whether the unidentified factor(s) acts by suppressing low-grade infections, but this is a possibility. Various combinations of antibiotics fed at different levels have not given similar results. Studies are in progress, in cooperation with Kohler and his co-workers at the USDA Western Regional Research Laboratory, in attempts to define the properties and nature of this unidentified factor(s).

## ACKNOWLEDGMENTS

The authors are grateful for the help and advice of Dr. George Kohler, Western Regional Research Laboratory, USDA, Albany, California, and Dr. Harold Olcott, Department of Nutritional Sciences, University of California, Berkeley, California. Also, our thanks go to Mrs. Bennie Thomas and Mrs. Esther Hurley for their assistance in animal care.

## LITERATURE CITED

1. Reid, M. E., and G. M. Briggs 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, *51:* 341.
2. Kohler, G. O., and W. R. Graham, Jr. 1951 A chick growth factor found in leafy green vegetation. Poultry Sci., *30:* 484.
3. Fisher, H., H. M. Scott and R. G. Hansen 1954 Further studies on the alfalfa factor and its relation to the liver and whey factors. J. Nutrition, *52:* 13.
4. Jacobs, R. L., J. F. Elam, J. H. Quisenberry and J. R. Couch 1953 Dehydrated alfalfa leaf meal as a source of vitamins and unidentified factors for the mature fowl. Poultry Sci., *32:* 812.
5. Vavich, M. G., A. Wertz and A. R. Kemmerer 1953 Growth-stimulating factors in alfalfa for chicks. Poultry Sci., *32:* 433.
6. Liuzzo, J. A., J. G. Lee, A. B. Watts, E. A. Fieger and A. F. Novak 1960 Stimulation of chick growth with alfalfa concentrates. Poultry Sci., *39:* 823.
7. Lee, J. G., J. A. Liuzzo and A. F. Novak 1961 Alfalfa extracts which stimulate growth of *Neurospora sitophilia*. I. Preparation and activation of crude extracts. J. Pharmacol. Sci., *50:* 737.
8. Gard, D. I., D. E. Becker, S. W. Terrill, H. W. Norton and A. V. Nalbandov 1955 Sources of unidentified factors for the pig. J. Animal Sci., *14:* 532.
9. Kohler, G. O., C. A. Elvehjem and E. B. Hart 1938 The relation of the "grass juice factor" to guinea pig nutrition. J. Nutrition, *15:* 445.
10. National Research Council, Committee on Animal Nutrition 1962 Nutrient Require-

[11] See footnote 9.

ments of Domestic Animals, no. 10. Nutrient Requirements of Laboratory Animals, pub. 990. National Academy of Sciences — National Research Council, Washington, D. C.
11. Ershoff, B. H. 1957 Beneficial effects of alfalfa and other succulent plants on the growth of immature guinea pigs fed a mineralized dried milk ration. J. Nutrition, *62:* 295.
12. Calloway, D. H., G. W. Newell, W. K. Calhoun and A. H. Munson 1963 Further studies of the influence of diet on radiosensitivity of guinea pigs, with special reference to broccoli and alfalfa. J. Nutrition, *79:* 340.
13. Reid, M. E., and O. Mickelsen 1963 Nutritional studies with the guinea pig. VIII. Effect of different proteins, with and without amino acid supplementation, on growth. J. Nutrition, *80:* 25.
14. Rendig, V. V., and E. A. McComb 1962 Pinitol as a constituent of *Medicago sativa*. Arch. Biochem. Biophys., *96:* 455.
15. Mannering, G. J. 1949 Vitamin requirements of the guinea pig. Vitamins and Hormones, *7:* 201.

# A Complex Growth Factor in Duodenal Tissue [1]

C. J. ACKERMAN
*Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia*

*ABSTRACT* A commercial preparation of dry duodenal tissue was hydrolyzed and fractionated to determine whether this material contained thyroid hormones. Five fractions, capable of reinstating growth of growth-arrested, sulfaguanidine-fed rats, were obtained. All were stable to acid hydrolysis and insoluble in 0.1 N HCl. They differed from each other with respect to their solubility in benzene, acetone and methanol, and their stability to alkaline hydrolysis. Neither thyroxine nor triiodothyronine could be detected in four of the fractions. It was concluded that the ability of duodenal powder to reinstate growth of thyroid hormone-deficient rats was not due to thyroxine or triiodothyronine but to unknown factors.

When duodenal powder or fractions of duodenal powder were included in the diet of growth-arrested, sulfaguanidine-fed rats, growth was reinstated (1–4). Such results suggest that the samples tested contained thyroid hormones in physiologically effective concentrations. Numerous attempts to establish the presence of thyroid hormones in duodenal powder have been unsuccessful. However, it was demonstrated that the active principle in one fraction obtained from duodenal powder was neither thyroxine nor triiodothyronine since its stability to HCl hydrolysis, its failure to react with ninhydrin and its $R_F$ on paper chromatograms contrasted with that of the thyroid hormones (4).

Such results warranted further investigation as to the nature of the growth factor but attempts to purify the growth factor consistently produced from 3 to 5 fractions each of which was capable of reinstating growth of growth-arrested rats (4).[2] It is the purpose of the present report to describe the preparation of 5 fractions of duodenal powder, each of which is capable of reinstating growth of growth-arrested rats, and to show that four of these fractions contain neither thyroxine nor triiodothyronine.

## EXPERIMENTAL

*Materials.* 3,5,3′-Triiodo-L-thyronine was recrystallized from hot 2 N HCl, mp 202–204° (5). Duodenal powder [3] was a partially defatted, dry preparation which, when fed to growth-arrested sulfaguanidine-fed rats at a level of 4 g/100 g of diet, produced a gain of 35 ± 3 (SE) g in 2 weeks (2). Pancreatin, papain, and pepsin, were purchased from commercial supply houses.[4] Acidified acetone and acidified methanol were prepared by adding 10 ml of 2 N HCl to 90 ml of the solvent. The Dowex 1–$Cl^-$ anion exchange resin (analytical grade) [5] was 200–400 mesh, 4% cross linked.

*Growth assays.* Growth arrest was achieved by feeding weanling male rats of the Sprague-Dawley strain a diet containing 1% sulfaguanidine (2) for at least 10 weeks. These rats weighed 110 to 145 g and no rat had gained more than 5 g in the 2 weeks prior to its use in an assay. For this reason, it was possible to use one rat for each assay to follow the isolation of the growth factor.

A fraction to be assayed was evaporated to dryness in a stream of air at room temperature. The residue, dissolved in 3 to 7 ml of acidified acetone or methanol, was mixed into 100 g of diet, and fed ad libitum to one rat. The gain in body weight at the end of 14 days was a qual-

Received for publication December 9, 1965.
[1] This work was supported in part by Research Grant no. G13165 from the National Science Foundation.
[2] Abstracts, 5th International Congress on Nutrition, September 1960, Washington, D. C., p. 27.
[3] Duodenal Substance, Cudahy Laboratories, Omaha, Nebraska.
[4] Pancreatin, N. F., Fisher Scientific Company, Silver Spring, Maryland; Papain, N. F., Difco Laboratories, Detroit; Pepsin (1–10,000), Nutritional Biochemicals Corporation, Cleveland.
[5] Bio-Rad Laboratories, Richmond, California.

itative measure of the growth activity of the sample assayed. For reasons described below (Results section), that quantity of each fraction which produced a gain in body weight of 30 g in 14 days, was considered as "one unit" of growth factor.

*Stability to NaOH.* One-unit quantities of the growth factor and one-fourth-unit quantities to which had been added 2.7 μg of triiodothyronine were evaporated to dryness at reduced pressure in test tubes. Three milliliters of 2 N NaOH containing 0.001 M thiouracil as an antioxidant were added. The tubes were evacuated for 10 minutes with a water aspirator during which time the tubes were flushed out 3 times with nitrogen. The tubes were sealed and heated in a boiling water bath (96°) for 16 hours. The hydrolysates were neutralized with HCl and then added directly to 100 g of diet and assayed for growth activity. The tube containing triiodothyronine served as a control to test the stability of the hormone under these conditions of hydrolysis.

*Paper chromatography.* One-unit quantities of the growth factor and one-unit quantities to which had been added 2.7 μg of triiodothyronine were hydrolyzed with 2 N NaOH under nitrogen as described above. The hydrolysates were extracted with 1 volume and 3 times with one-half volumes of *n*-butanol. The extracts were acidified to pH 1 with HCl and air-dried.

The residues were dissolved in 5 to 6 ml of acetone. One-half of the acetone was applied to Whatman 3MM paper in a narrow band 2 to 4 cm long which was then developed (ascending) with *n*-butanol:acetic acid:water (75:10:15). With this solvent system, thyroxine and triiodothyronine move to the same position (6). Thyroid hormones were detected on the chromatograms by the ceric sulfate-arsenious acid procedure of Kono et al. (6).

The approximate $R_F$ values of the growth factor on paper chromatograms were determined by streaking 1.5 units of the growth factor in a narrow band 40 cm long on Whatman 3MM paper. The paper was developed with *n*-butanol: acetic acid:water (75:10:15) (ascending). When dried, the paper was cut into bands at right angles to the solvent flow. Each band was extracted in a Soxhlet extractor for 3 hours with acidified methanol. The methanol extracts were then assayed for growth activity.

*Preparation of growth factors.* Duodenal powder (400 g) was suspended in 800 ml of the following boiling solvents and then filtered: methanol, acetone, benzene, chloroform, and hexane. The insoluble residue, suspended in 800 ml of Krebs-Ringer prosphate buffer, was incubated at 39° for 48 hours with each of the following enzymes: 8 g of pancreatin, pH 8.4; 8 g of papain, pH 4.5; and 4 g of pepsin, pH 2.0. The pH was adjusted with HCl after each 48-hour period.

After the addition of an equal volume of acetone and centrifugation the supernatant was evaporated to a thick syrup at reduced pressure which was then refluxed for 22 hours in 400 ml of 5 N HCl. The following description of the separation of the growth factors from this hydrolysate is schematically depicted in figure 1. The entire hydrolysate was extracted once with an equal volume of *n*-butanol:benzene (1:1) and twice with one-half volumes of the same mixture. The combined extracts were concentrated by boiling to approxi-

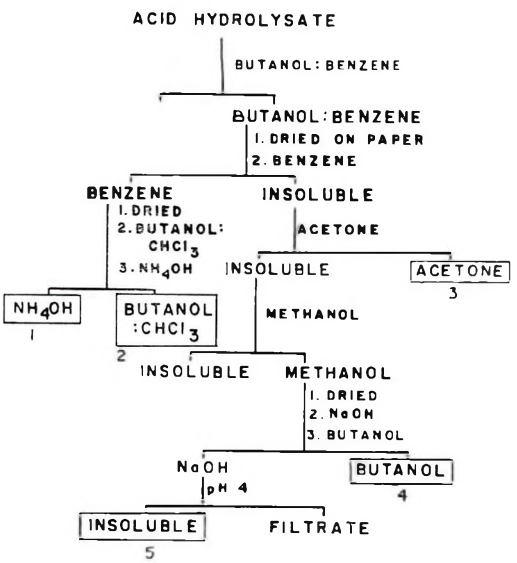


Fig. 1 A schematic representation of the preparation of 5 growth factors from an acid hydrolysate of duodenal powder. The active fractions are those in the rectangles and the numbers correspond to the fractions described in the text.

mately 75 ml. The concentrate was allowed to evaporate onto large sheets of Whatman 3MM filter paper using methanol to quantitatively transfer the concentrate to the paper. When air-dried, the paper was stored for 4 days in large glass jars over NaOH pellets to absorb excess HCl.

The paper was extracted for 6 hours with benzene in Soxhlet extractors and then for 6 hours with acetone, and finally with methanol for 6 hours. Each extract was filtered and assayed at various levels to determine that amount which would produce a gain of 30 g in 2 weeks. These were fractionated further as follows:

A. *Benzene extract.* This was evaporated to dryness at room temperature and the black residue was dissolved in 200 ml of *n*-butanol to which was added 200 ml of chloroform. The solution was extracted once with 400 ml and 3 times with 200 ml of 2 N $NH_4OH$. Thyroid hormones, if present, would be found in the $NH_4OH$ extract (6). The black $NH_4OH$ extract was evaporated to dryness at room temperature and the residue was dissolved is 200 ml of acidified acetone and then assayed for growth activity. This was fraction 1. It was tested for its solubility in 0.1 N HCl, its stability to NaOH hydrolysis, and for the presence of triiodothyronine by paper chromatography. The approximate $R_F$ of the growth factor on paper chromatograms was determined in 2 experiments.

Fraction 2 was the butanol:chloroform phase after it had been extracted with $NH_4OH$. The air-dried residue from one unit of fraction 2 was taken up in 4 ml of 2 N NaOH and then extracted 3 times with an equal volume of benzene. The benzene extract was assayed. Further work on this fraction was not attempted.

B. *Acetone extract.* This solution was evaporated to dryness at room temperature, dissolved in 0.3 N NaOH and filtered. The filtrate was adjusted to pH 8 with acetic acid, cooled overnight and filtered. The precipitate was washed 3 times with 150 ml of hot (70°) water and then redissolved in a minimal amount of 0.2 N NaOH which was then acidified to pH 1 with HCl. A black precipitate was filtered out, washed 4 times with 10 ml of 0.1 N HCl and then dissolved in 300 ml of acidified acetone. This was fraction 3. Aliquots of fraction 3 were assayed for growth activity before and after NaOH hydrolysis, and it was tested for the presence of triiodothyronine by paper chromatography.

Eight units of fraction 3 in aqueous solution at pH 9 were added to a Dowex 1-$Cl^-$ column (15 × 3.1 $cm^2$) which was then eluted with 100 ml of each of the following: water, 0.4 N HCl, 0.8 N HCl, water, propanol:0.12 N HCl (20:80), propanol:0.1 N HCl (50:50). One-eighth and one-fourth of each eluate was assayed.

C. *Methanol extract.* No pH 8 precipitate appeared when this solution was treated as described for the acetone extract. Two active fractions were obtained from this extract by the following procedure. The extract was evaporated to dryness at room temperature and the residue was suspended in 100 ml of 2 N NaOH and filtered. The NaOH solution was extracted with 100 ml and 3 times with 50 ml of *n*-butanol, and then 3 times with 50 ml of *n*-hexane to remove butanol from the NaOH phase.

The combined butanol and hexane extract was evaporated to dryness at reduced pressure. The residue was extracted 4 times with 100 ml of 0.1 N HCl and then dissolved in acidified methanol. This was fraction 4. It was assayed before and after NaOH hydrolysis and it was also tested for the presence of triiodothyronine by paper chromatography.

The NaOH phase was adjusted to pH 4 with acetic acid, cooled overnight (5°) and centrifuged. The resultant black precipitate was dissolved in acidified methanol. This was fraction 5. This fraction was assayed before and after NaOH hydrolysis and it was tested for triiodothyronine by paper chromatography. An attempt was made to determine the $R_F$ of the growth factor in this fraction by developing 1.5 units on paper and assaying the extracts of 4 sections of the chromatogram. Also, 6 units in aqueous solution at pH 9 were added to a Dowex 1-$Cl^-$ column (10 × 3.1 $cm^2$). The column was eluted successively with 250 ml each of water, 0.3 N HCl, 2 N HCl, water, *n*-propanol: $H_2O$ (1:1), water, *n*-propanol:

TABLE 1

*Growth response of growth-arrested, sulfaguanidine-fed rats to dietary triiodothyronine*

| Triiodothyronine added to diet | Avg initial body wt [1] | Avg body wt gain |
|---|---|---|
| *μg/100 g diet* | *g* | *g/2 weeks* |
| None | 145 | 1.6 ± 0.03 [2] |
| 1 | 133 | 10.1 ± 4.4 |
| 2 | 139 | 16.0 ± 3.1 |
| 2.7 | 141 | 29.6 ± 1.4 |

[1] Six male rats/group. The maximum gain in body weight of any rat was 2 g during the 2 weeks prior to this experiment.
[2] Average ± SD.

0.12 N HCl (20:80), and *n*-propanol: 0.2 N HCl (50:50). Aliquots of all eluates were assayed.

## RESULTS

Table 1 illustrates again (2) the characteristic growth arrest of sulfaguanidine-fed rats, and, that 1 μg of triiodothyronine in 100 g of diet can be detected by a growth response in such rats. The growth response to 2.7 μg of triiodothyronine/100 g of diet was approximately 30 g in 14 days. Therefore, if the growth responses to the various fractions of duodenal powder were due to thyroid hormones, it may be assumed that a gain of 30 g in 14 days was a response to approximately 2.7 μg of triiodothyronine (or its equivalent of thyroxine, approximately 5 μg).[6] This is an amount easily detected on paper chromatograms (6) and for this reason various aliquots of the fractions were assayed to determine that amount which would produce a gain of 30 g in 14 days. That quantity of the growth factors which produced a gain of 30 g in 2 weeks was considered "one unit."

The growth responses of growth-arrested rats to the various extracts isolated from duodenal powder are summarized in table 2. Only the growth responses to those quantities which produced a gain of approximately 30 g in 14 days are shown. Based on these results, an approximation of the available growth units in each extract is given in parentheses together with the total volume of each extract. "One-unit" quantities were subjected to further treatment and the growth responses of the rats to the treated samples provided a qualitative measure of the recovery of the growth factor.

Two growth factors were present in the benzene extract. Both the $NH_4OH$ extract (fraction 1) and the butanol:$CHCl_3$ phase (fraction 2) were active when the residue from the benzene extract, dissolved in butanol:$CHCl_3$, was extracted with $NH_4OH$ (table 2 and fig. 1). If present, thyroid hormones would be expected in the $NH_4OH$ extract (6) but neither thyroxine nor triiodothyronine could be detected in this fraction by paper chromatography (fig. 2, fraction 1), and it was concluded that the growth response to this fraction was not due to thyroid hormones. The growth factor in fraction 1 was insoluble in 0.1 N HCl and it was stable to NaOH hydrolysis. On paper chromatograms, it was recovered from the $R_F$ section 0.7 to 1.0 (table 2). The paper chromatography was repeated but also, 3 μg of triiodothyronine were co-chromatographed with approximately one-tenth of one unit of fraction 1. When developed, the paper strips were divided into 4 $R_F$ sections, and assayed. As indicated by the growth response (table 2), triiodothyronine was recovered in the area $R_F$ 0.7 to 0.85, but the growth factor appeared to be equally divided between $R_F$ 0.70 to 0.85 and 0.85 to 1.0.

The growth factor in fraction 2 (the butanol:$CHCl_3$ phase after extraction with $NH_4OH$) was stable to NaOH hydrolysis and it could be extracted from NaOH with benzene (table 2). This fraction could not be chromatographed on paper.

Fraction 3 was insoluble in benzene, insoluble at pH 8, insoluble in hot water and in 0.1 N HCl. It was stable to alkaline hydrolysis and it could be extracted from 2 N NaOH with *n*-butanol. No activity was recovered in the NaOH phase. Neither thyroxine nor triiodothyronine could be detected on paper chromatograms of this fraction (fig. 2, fraction 3). Approximately one-half of this growth factor added to a Dowex 1-$Cl^-$ column was lost. Only the *n*-propanol: 0.2 N HCl (1:1) effluent of the column contained any growth activity (table 2).

Two growth factors (fractions 4 and 5) were present in the methanol extract.

[6] Unpublished experiments.

TABLE 2

*Growth response of growth-arrested [1] rats to the fractions prepared from duodenal powder and to these fractions after various treatments*

| Fraction, total volume, total units,[2] and treatment | Wt gain [3] |
|---|---|
| | *g/2 weeks* |
| A. Benzene extract (1440 ml, 27 units) [4] | 31, 33, 34 |
| Fraction 1 ($NH_4OH$ extract, 200 ml, 20 units) | 28, 32, 35 |
| a. Residue washed with 0.1 N HCl | 31 |
| b. Hydrolyzed in 2 N NaOH, 16 hours | 33, 34 |
| c. 1/4 unit plus 2.7 μg of $T_3$[5] hydrolyzed in 2 N NaOH, 16 hours | 28 |
| d. Paper chromatography [6] | |
| $R_F$ 0–0.2; 0.2–0.5; 0.5–0.7; 0.7–1.0 | 2, 6, 3, 32 |
| Co-chromatographed with and without 3 μg of $T_3$ | |
| plus $T_3$: $R_F$ 0–0.3; 0.3–0.7; 0.7–0.85; 0.85–1.0 | 0, 1, 31, 4 |
| minus $T_3$: $R_F$ 0–0.3; 0.3–0.7; 0.7–0.85; 0.85–1.0 | 1, 4, 18, 18 |
| Fraction 2 (butanol:$CHCl_3$ phase, 200 ml, 17 units) | 34, 35, 38 |
| a. Hydrolyzed in 2 N NaOH, 16 hours | 29, 32 |
| b. 1/4 unit + 2.7 μg of $T_3$, hydrolyzed 2 N NaOH, 16 hours | 29 |
| c. Benzene extract of 2 N NaOH solution | 31 |
| B. Acetone extract (1700 ml, 26 units) | 27, 34, 35 |
| Fraction 3 (HCl-insoluble, pH 8-insoluble, 100 ml, 23 units) | 32, 32, 34 |
| a. Hydrolyzed in 2 N NaOH, 16 hours | 30, 34 |
| b. 1/4 unit + 2.7 μg of $T_3$ hydrolyzed 2 N NaOH, 16 hours | 29 |
| c. Butanol extract of NaOH solution | 30, 36 |
| NaOH phase after butanol extraction | 6, 8 |
| d. Eight units on Dowex 1-$Cl^-$ column. Propanol:0.2 N HCl (1:1) effluent | |
| 1/8 of effluent | 14, 16 |
| 2/8 of effluent | 26, 28 |
| C. Methanol extract (1230 ml, 18 units) | 28, 29 |
| Fraction 4 (butanol extract of NaOH solutions, 17 units) | 33, 35, 36 |
| a. Residue washed with 0.1 N HCl | 27, 29 |
| b. Hydrolyzed 2 N NaOH, 16 hours | 24, 26 |
| c. 1/4 unit plus 2.7 μg of $T_3$ hydrolyzed 2 N NaOH, 16 hours | 32 |
| Fraction 5 (pH 4-insoluble after butanol extraction, 13 units) | 27, 29 |
| a. Residue washed with 0.1 N HCl | 26, 27 |
| b. Hydrolyzed 2 N NaOH, 16 hours | 6, 8 |
| c. 1/4 unit plus 2.7 μg of $T_3$, hydrolyzed 2 N NaOH, 16 hours | 27, 30 |
| d. Paper chromatography [6] $R_F$ 0–0.2; 0.3–0.5; 0.5–0.7; 0.7–1.0 | 2, 2, −1, 4 |
| e. Six units on Dowex 1-$Cl^-$ column. Propanol:0.2 N HCl (1:1) effluent | |
| 1/6 of effluent | 6 |
| 1/2 of effluent | 34 |

[1] No rat had gained more than 4 g during the 2 weeks prior to its use in an assay.

[2] One unit is that amount of a fraction which will produce a gain of 30 g in 2 weeks when fed in 100 g of diet to one rat, and this is equivalent to the growth response of one rat to 2.7 μg of 3,5,3′-triiodo-L-thyronine fed in 100 g of diet.

[3] Each figure is the growth response of one rat to one unit of the indicated preparation, or to one unit of the preparation after it was treated as indicated. Each sample assayed was mixed with 100 g of diet and fed ad libitum until it was consumed. When the assay was duplicated or triplicated, the weight gain of each rat is shown. To compare the stability of triiodothyronine to NaOH hydrolysis with the stability of the growth factors, the hormone was added to a small amount of the growth factors before hydrolysis.

[4] The figures in parentheses are the total volumes of the active fractions obtained from 400 g of duodenal powder and the total number of growth units that this volume contained.

[5] $T_3$: 3,5,3′-triiodo-L-thyronine.

[6] Solvent system: *n*-butanol:acetic acid:water (75:10:15). Extracts of the indicated $R_F$ sections were assayed and the sequence of the weight gains shown in the right hand column correspond to the sequence of the indicated $R_F$ sections.

The growth factor in fraction 4 was insoluble in 0.1 N HCl, stable to alkaline hydrolysis (table 2) and contained no iodine (fig. 2, fraction 4).

Fraction 5 was the precipitate obtained when the butanol-extracted NaOH solution was adjusted to pH 4. This was also insoluble in 0.1 N HCl but it was destroyed by alkaline hydrolysis (table 2). Triiodothyronine added to this fraction was not destroyed by alkaline hydrolysis, demonstrating that the growth factor in fraction

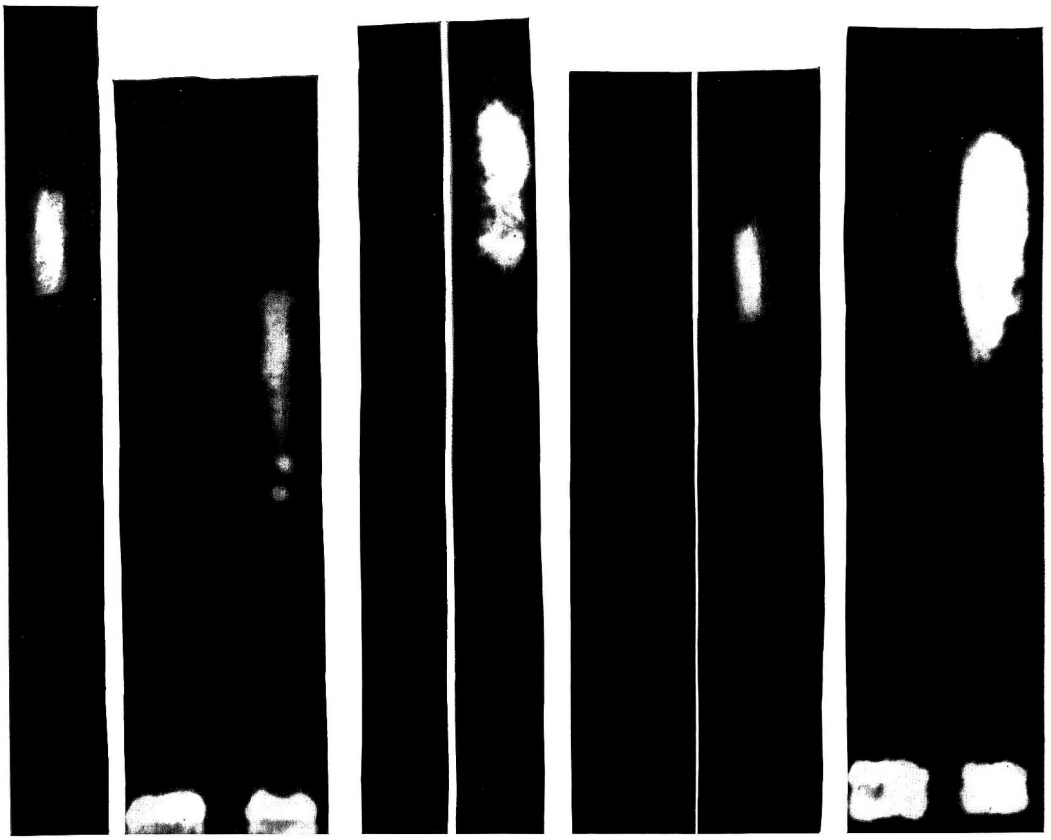


Fig. 2 Paper chromatography of fractions 1, 3, 4, and 5 with and without added triiodothyronine. Each fraction with and without 2.7 $\mu$g of added triiodothyronine ($T_3$) was hydrolyzed under $N_2$ with 2 N NaOH for 16 hours. The hydrolysates were extracted with *n*-butanol and one-half of this extract was applied in bands on Whatman 3MM paper. Developed with *n*-butanol: acetic acid: water (75:10:15). The papers were treated with ceric sulfate-arsenious acid to detect iodine. A faint band near the origin (not visible on the photograph), was visible on the chromatogram of fraction 3 (without added $T_3$). No iodine was detected on the chromatogram of fraction 4.

5 was not triiodothyronine. This is supported by the failure to detect any thyroid hormones on paper chromatograms (fig. 2, fraction 5). Fraction 5 appeared to be less stable than any of the other fractions. No activity could be recovered from paper chromatograms and when 6 units of activity were added to a Dowex 1-$Cl^-$ column, only 2 units were recovered. Only the *n*-propanol: 0.2 N HCl (1:1) effluent of the column contained any activity. At the time of this writing, 6 months after fraction 5 was first prepared, all activity had disappeared. No loss in activity was apparent in fractions 1 to 4.

## DISCUSSION

Further purification of the growth factors was accompanied by extensive losses in activity. They were strongly adsorbed or destroyed on charcoal, ion exchange resins, and paper. This property and the tedious assay handicapped attempts to clarify the relationship of the growth factors to each other. However, all fractions and precipitates that were obtained during this fractionation procedure were assayed and the 5 active fractions described appear to represent all of the growth activity of the original duodenal powder. Since neither thyroxine nor triiodothyro-

nine could be detected on paper chromatograms of four of these fractions, and the behavior of the fifth fraction (fraction 2) contrasts with the behavior of thyroid hormones, it was concluded that the reported growth response of thyroid hormone-deficient rats to dietary duodenal powder (2, 3) was not due to thyroid hormones.

That the growth factors may be related to each other arises from the observation in numerous experiments that the properties of a given active fraction may change with time. For example, it was previously reported (4) that a fraction, soluble in NaOH, became insoluble in alkaline solutions after standing for one week and the growth factor in this preparation could be extracted with benzene. In addition, the water-soluble preparation isolated from enzymatically hydrolyzed duodenal powder (3) became insoluble in water after storage at freezing temperatures for one year, but the growth factor could be extracted from this preparation with benzene. The benzene-soluble growth factor has been the most difficult to prepare consistently.

It has also been noted frequently, that an active fraction initially isolated as a precipitate at pH 8, became soluble at pH 8 after long standing or after passage through an anion exchange column. The growth factor could be recovered, in part, as a precipitate at pH 6. Such behavior suggests structural changes in the growth factor that are not necessarily accompanied by a loss of activity. These changes could be esterification, lactone or lactam formation, formation of complexes, etc.

Possibly related to structural changes of the growth factor is the observation that the division of an active fraction into 2 subfractions yields products more active than the original fraction. This was noted in a previous report (4) but it also occurred in these experiments. As indicated in table 2, the benzene extract contained 27 units, but when this was divided into fractions 1 and 2, a total of 36 units was recovered. Also, the methanol extract contained 18 units which yielded a total of 30 units when this extract was divided into fractions 4 and 5. This could not occur if the division of one fraction into 2 active subfractions was the result of the division of a single substance into 2 parts, unless the process of fractionation induced structural changes such that the subfractions were physiologically more effective than the original.

The significance of the growth factors remains obscure. A variety of tissues have been shown to be capable of reinstating growth of growth-arrested rats but these have not been consistently active when different lots from different sources were assayed (1). In addition, thymus gland, duodenum and pancreas from calves, steers, and pigs slaughtered at this Institute were also inactive. It is, therefore, possible that the presence of growth factors in certain tissues occurs as a result of a particular biological event. That this may be pregnancy is suggested by the activity of ovarian powder (1) and a recent observation that commercial corpus luteum powder [7] is 4 times more effective than the duodenal powder used in this report.

By a process similar to that described in this report, 3 active fractions have been separated from a commercial preparation of corpus luteum powder. Since the corpora lutea exist in mature females for only a short period of time, this would explain the lack of response to some of the numerous tissues that have been assayed. Inactive tissues may have been obtained from immature animals, males, or from females before or after the appearance of corpora lutea. The corpora lutea secrete progesterone, but it may be stated here that up to 8 mg of progesterone /100 g of diet failed to produce a growth response in growth-arrested, sulfaguanidine-fed rats.

ACKNOWLEDGMENT

The author wishes to thank William Bradley for the photograph of figure 2.

LITERATURE CITED

1. Ackerman, C. J., and V. Tsou 1961 Effect of various tissues on the growth of sulfaguanidine-fed rats. J. Nutrition, 74: 84.
2. Ackerman, C. J. 1963 Effect of potassium iodide and duodenal powder on the growth and organ weights of goitrogen-fed rats. J. Nutrition, 74: 140.

[7] Nutritional Biochemicals Corporation, Cleveland.

3. Ackerman, C. J., and V. Mah 1963 Response of thyroid hormone-deficient rats to a growth factor in duodenal tissue. J. Nutrition, 80: 263.
4. Ackerman, C. J. 1964 The absence of thyroid hormones in a growth factor of duodenal powder. J. Nutrition, 85: 150.
5. Gross, J., and R. Pitt-Rivers 1953 3, 5, 3'-Triiodothyronine. I. Isolation from thyroid gland and synthesis. Biochem. J., 53: 645.
6. Kono, T., L. Van Middlesworth and E. B. Astwood 1960 Chemical identification of iodine containing compounds in human serum. Endocrinology, 66: 845.

# Effect of Severe Protein-Calorie Malnutrition in the Baby Pig upon Relative Utilization of Different Dietary Proteins [1]

R. H. BARNES, W. G. POND, E. KWONG AND I. REID
*Graduate School of Nutrition and Department of Animal Husbandry, New York State College of Agriculture, Cornell University, Ithaca, New York*

*ABSTRACT* A comparison was made of the nutritive value of several protein sources in normal rats using the conventional protein efficiency ratio, and in baby pigs that were malnourished by subjecting them to a diet low in protein and high in fat. The dietary proteins included casein, a heat-treated soybean meal, 3 different cottonseed preparations and a fish flour that had been used by others in infant feeding studies. In the protein efficiency measurements most of the proteins were essentially equivalent, although two of the cottonseed preparations were somewhat lower than the other proteins. In the malnourished baby pig casein was definitely superior to all other proteins on the basis of the rate of return of serum protein values to normal and the rate of weight gain. These observations have been related to recent reports showing the superiority of milk over fish protein concentrate in stimulating serum protein and growth rate responses in infants recovering from protein-calorie malnutrition. It is proposed that the nutritional quality of protein for feeding malnourished infants should be evaluated in experimental animals simulating protein-calorie malnutrition in addition to conventional procedures utilizing the normal animal. Furthermore, the hypothesis is presented that the superiority of milk protein is due to its better proteolytic digestion under conditions in which digestive enzymes are seriously deficient.

Many different protein sources are being examined for utility in providing dietary protein to combat protein-calorie malnutrition in the developing countries of the world. One extremely important population group where such dietary supplements might find usefulness is the protein-calorie malnourished pre-school child. The evaluation of nutritional quality of protein sources that are being examined for these purposes is characteristically conducted in normal animals by such procedures as the protein efficiency determination (1) or one of its modifications, such as the NPU measurement (2). Sometimes a protein-depleted adult rat is fed the test protein in the well-established depletion-repletion test of protein quality or by some modification as described by Frost (3). Seldom, if ever, is any attempt made to evaluate the nutritive quality of the protein source in an animal in which, at an early age, severe protein-calorie malnutrition has been induced — in other words, in an experimental situation resembling to some degree the malnourished pre-school child in which the protein source may have a most important nutritional role. One difficulty in using such a test system is that with the most extensively used experimental animal, the rat, signs of protein-calorie malnutrition such as low serum proteins and fatty livers develop only with extremely time-consuming procedures such as force-feeding of all food. On the other hand, these signs of malnutrition can be induced readily with ad libitum feeding in the baby pig (4, 5). The present study provides a comparison of nutritive values as determined by the well-known protein efficiency ratio procedure in weanling rats and by growth and serum protein concentration rates in the protein-calorie malnourished baby pig.

## EXPERIMENTAL

*First series.* Twenty Yorkshire pigs, 21 days of age and averaging approximately 5 kg body weight, were fed the depletion

Received for publication February 23, 1966.

[1] This research was supported in part by funds provided through the State University of New York, a Public Health Service Research Grant no. A-3620 from the National Institutes of Health and a research grant from the National Science Foundation.

diet (table 1) that has been reported to result in the extreme signs of protein-calorie malnutrition. After receiving this diet for 8 weeks, the pigs were distributed into 5 groups of 4 pigs each and were fed ad libitum the 18% protein level diets (repletion) described in table 1. The animals were weighed each week and blood samples were drawn from the superior vena cava into heparinized syringes. These procedures were continued for 4 weeks, at which time certain of the test protein supplies had become exhausted. Blood samples were centrifuged immediately and total serum protein was determined by the biuret method of Gomall et al. (6). Another sample of serum was used for paper electrophoretic separation and densitometer quantitation of classes of serum proteins. Four positive control pigs were fed the basic rehabilitation diet, but with 25% casein (control diet, table 1), from the beginning of the study (pigs 3 weeks of age) to the end (pigs 15 weeks of age).

Samples of each of the experimental diets were fed ad libitum to male, weanling rats for 2 weeks. The usual conditions of individual caging and temperature control at approximately 22° were followed.

The protein sources were identified as follows: 1) casein (as a control) — a commercial grade of feed casein; 2) fish flour — a composite of 5 samples taken from the UNICEF plant established in Quintero, Chile; 3) soybean meal — a commerical, feed grade, extracted and toasted soybean meal; 4) a commerical cottonseed flour manufactured by Traders Oil Mill Company,[2] CSF–21 — an extracted cottonseed experimentally produced by the Southern Regional Research Laboratories of the Department of Agriculture, and given the batch designation of CSF–21 by UNICEF.

*Second series.* In a second series of studies, 3-week-old pigs were fed the low protein-high fat diet (depletion diet, table 1) for 8 weeks and then rehabilitated with different test proteins as before. Five groups of 4 pigs each were used and, as in the previous study, an additional group was fed a normal control diet from the

[2] Proflo, Traders Oil Mill Company, Fort Worth, Texas.

TABLE 1

*Composition of diets*

| | First series | | | Second series | | |
|---|---|---|---|---|---|---|
| | Depletion | Repletion | Control | Depletion | Repletion | Control |
| | % | % | % | % | % | % |
| Glucose [1] | 40.6 | 58.6 [2] | 33.6 | 40.6 | 58.6 [2] | 37.4 |
| Dextrin [3] | 25.0 | 25.0 | 25.0 | 25.0 | 25.0 | 25.0 |
| Corn oil [4] | 25.0 | 10.0 | 10.0 | 25.0 | 10.0 | 10.0 |
| Isolated soy protein [5] | 3.0 | — | — | 3.0 | — | 21.2 |
| Casein [6] | — | — | 25.0 | — | — | — |
| Minerals [7] | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 |
| Vitamins 1 [8] | 1.0 | 1.0 | 1.0 | — | — | — |
| Vitamins 2 [9] | — | — | — | 1.0 | 1.0 | 1.0 |
| Protein level | 2.55 | 18.0 | 21.25 | 2.55 | 15.0 [10] | 18.0 |
| Fat level | 25.0 | 10.0 | 10.0 | 25.0 | 10.0 | 10.0 |

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Protein sources (see text) used for repletion were incorporated into diets by replacing an equivalent quantity of glucose.
[3] White technical, Nutritional Biochemicals Corporation, Cleveland.
[4] Mazola, Corn Products Company, Argo, Illinois.
[5] Promine, Central Soya, Chicago.
[6] Crude, 30-mesh, National Casein Company, New York.
[7] Composition, g/kg diet: $CaHPO_4 \cdot 2H_2O$, 15.4; $CaCO_3$, 12.30; $KH_2PO_4$, 17.20; NaCl, 6.1; $CuSO_4$, 0.57; $FeSO_4$, 0.66; $MnSO_4$, 0.185; $ZnCO_3$, 0.66; MgO, 0.82; $CoCl_2$, 0.011; and KI, 0.0004.
[8] Composition, units/kg diet: vitamin A, 2400 IU; vitamin D, 600 IU; and (in milligrams) thiamine·HCl, 4.0; riboflavin, 10.0; niacin, 60.0; Ca D-pantothenate, 40.0; pyridoxine·HCl, 3.3; choline dihydrogen citrate, 4000; inositol, 200.0; folic acid, 3.0; α-tocopheryl acetate, 5.0; menadione, 5.0; and vitamin $B_{12}$, 70.0 μg.
[9] Composition, units/kg diet: vitamin A, 2400 IU; vitamin D, 600 IU; and (in milligrams) thiamine·HCl, 4.0; riboflavin, 10.0; niacin, 60.0; Ca D-pantothenate, 40.0; pyridoxine·HCl, 3.3; choline dihydrogen citrate, 6458.0; inositol, 200; folic acid, 3.0; α-tocopheryl acetate, 5.0; biotin, 0.001; menadione, 5.0; and $Na_2SeO_3$, 0.01 g; and vitamin $B_{12}$, 70.0 μg.
[10] In the rat experiment of the second series, protein level was adjusted to 10.0% by changing the amount of glucose and the protein source.

first of the experiment. In this series the isolated soybean protein Promine [3] was used to supply 3% protein in the depletion period and to supply 18% protein in the so-called normal control diet. For rehabilitation the test proteins were included in the diet in amounts necessary to provide 15% protein and the fat content was adjusted to 10% of the diet, as shown in table 1. Test proteins were fed for a total of 8 weeks and weekly body weights were measured and blood samples for serum protein determinations taken.

The test protein sources were then incorporated into diet formulations for feeding to rats at levels to provide 10% protein. Groups of 10 male, weanling rats, caged individually, were fed the diets ad libitum. Growth rate and food consumption were recorded weekly for 4 weeks and protein efficiency ratios calculated on the basis of grams gain in weight per gram of protein consumed.

The test protein sources were defined as follows: 1) casein — the same as in the first series; 2) cottonseed flour — USDA solvent-extracted product from the Southern Regional Research Laboratories, identified by UNICEF as CF-32; 3) cottonseed flour, Peru — special cottonseed flour from the Agrarian University, La Molina, Peru, UNICEF identification CF-33; 4) commercial cottonseed flour — a commercial product purchased from Traders Oil Mill Company; and 5) fish flour — prepared in the UNICEF plant in Quintero, Chile, and identified as FF-Q-65G. Amino acid analyses were run on acid hydrolysates of these materials using the Technicon autoanalyzer. Results are given in table 2.

## RESULTS

*First series.* Four-week rehabilitation growth curves for pigs are given in figure 1. The commercial cottonseed was inferior to the other products, with casein and the commercial soybean meal providing most rapid growth during the relatively short rehabilitation period. Total serum proteins and serum albumin regen-

[3] Promine, Central Soya, Chicago.

TABLE 2

*Amino acid composition of protein sources* [1]

| Amino acid | Sample no.[2] | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| | *g amino acid/16 g nitrogen* | | | | |
| Lysine | 7.94 | 7.51 | 4.10 | 4.12 | 3.88 |
| Histidine | 2.86 | 1.96 | 2.68 | 2.65 | 2.59 |
| Ammonia | 2.04 | 1.14 | 1.54 | 1.92 | 1.82 |
| Arginine | 3.87 | 6.66 | 10.23 | 11.78 | 11.34 |
| Cysteic acid | 0.12 | 0.24 | tr | tr | tr |
| Aspartic acid | 6.82 | 10.10 | 9.95 | 8.67 | 9.48 |
| Threonine | 4.16 | 4.91 | 3.76 | 3.26 | 3.40 |
| Serine | 5.42 | 4.35 | 5.17 | 4.55 | 4.67 |
| Glutamic acid | 23.70 | 16.48 | 22.05 | 20.63 | 21.36 |
| Proline | 10.40 | 4.66 | 4.11 | 3.87 | 4.03 |
| Glycine | 1.78 | 7.15 | 4.51 | 4.00 | 4.27 |
| Alanine | 2.97 | 6.51 | 4.32 | 3.78 | 4.02 |
| Cystine (Schram hydrol.) | 0.43 | 0.80 | 1.98 | 1.79 | 1.72 |
| Valine | 6.72 | 5.06 | 3.45 | 4.84 | 3.54 |
| Methionine | 2.83 | 3.09 | 1.47 | 1.41 | 1.39 |
| Isoleucine | 5.29 | 4.36 | 3.44 | 3.14 | 3.69 |
| Leucine | 9.07 | 7.60 | 6.11 | 5.55 | 5.91 |
| Tyrosine | 5.61 | 3.42 | 3.21 | 2.96 | 3.22 |
| Phenylalanine | 5.04 | 4.04 | 5.53 | 5.11 | 5.38 |
| Alloisoleucine | tr | tr | tr | tr | tr |
| Methionine sulfoxides | tr | tr | tr | tr | tr |
| % Nitrogen by Kjeldahl (as is) | 14.9 | 13.2 | 8.1 | 9.7 | 9.0 |
| % Moisture | 5.1 | 4.5 | 7.4 | 5.7 | 7.1 |

[1] These samples were analyzed by Mr. Ray Herness of the research laboratories at the Central Soya Chemurgy Division, Chicago.
[2] Sample 1, Casein; Sample 2, Fish flour, FF-Q-65G; Sample 3, Cottonseed flour, Peru — CF-33; Sample 4, Cottonseed flour, USDA — CF-32; Sample 5, Cottonseed flour, commercial product.

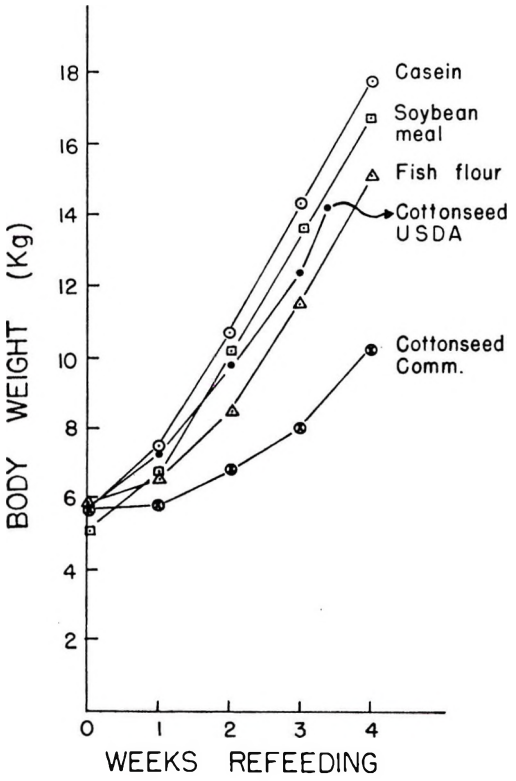


Fig. 1 Weight gain during refeeding (first series).

eration results are given in figures 2 and 3. The casein-fed animals were somewhat superior to the other groups in serum albumin recovery, but the time of rehabilitation feeding was too short to permit values to return to normal and the groups appeared to overlap to an extent that prohibited final conclusions regarding relative regeneration rates.

Only small samples of the swine diets were available for feeding to weanling rats and therefore 2-week weight gains were the extent of observations possible in the normal animal for purposes of comparison with the swine results. These data are given in table 3. From these figures there is no indication that casein had any superiority in growth promotion over the other protein sources except for the commercial cottonseed flour which gave the lowest weight gain obtained in the study. These results were suggestive of a difference in the nutritive quality of casein as measured in the normal, weanling rat and the severely protein-calorie malnourished baby pig. A second study was carried out with changes in experimental plan introduced so as to overcome some of the weaknesses that were apparent in the original experiment.

*Second series.* Twenty pigs at 3 weeks of age were fed the depletion diet in the same manner as was described for the first series. A control group was fed a diet containing 18% protein in the form of isolated soybean protein. After receiving the deficient diet for 8 weeks, the pigs were divided into 5 groups of 4 pigs each and fed ad libitum the rehabilitation diet shown in table 1, which provided 15% protein from the 5 sources as follows: casein, fish flour, cottonseed flour, USDA-CF-32; cottonseed flour, Peru; and the commercial cottonseed flour. All of

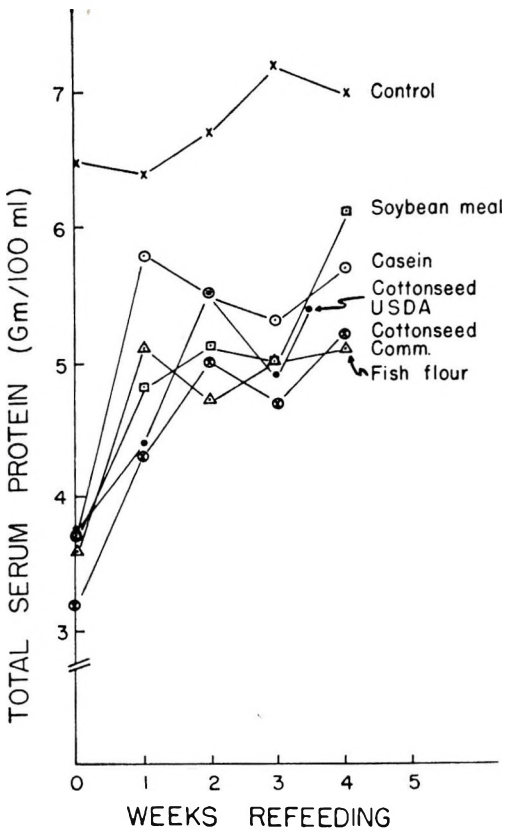


Fig. 2 Total serum protein during refeeding (first series).

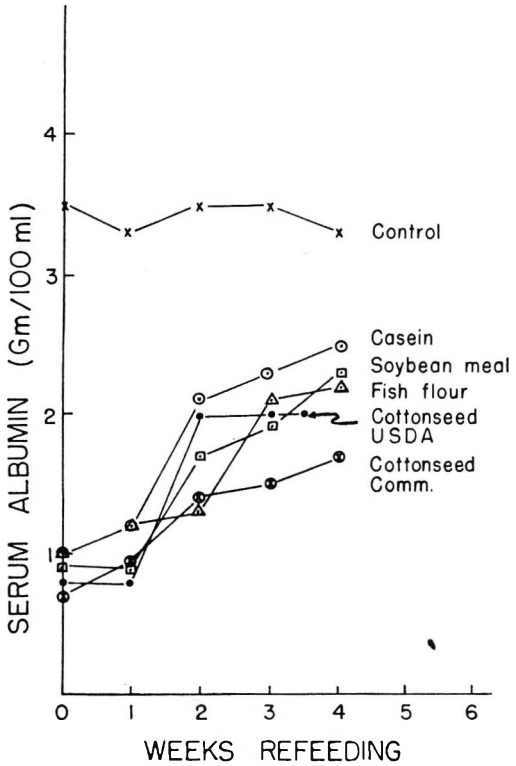


Fig. 3 Serum albumin during refeeding (first series).

these materials were described in the Methods section.

Growth responses are represented graphically in figure 4. With the slightly lower protein content of the diet than in the first series and an experimental period of 8 weeks, a marked superiority of casein can be seen, with two of the cottonseed flours, the commercial and the Peru preparations giving the poorest growth. The USDA cottonseed and fish flour diets were intermediate, but definitely inferior to the casein diet.

Serum protein concentration, shown in figures 5 and 6, clearly illustrates the superior performance of casein, but with these measurements all other protein sources grouped together so that there was no evidence of differences among them.

These 5 protein sources were incorporated into semipurified rat diets so as to provide 10% protein. Groups of 10 male, weanling rats were fed these diets for 4 weeks. Growth rates and food consumption figures are given in table 4. Casein did not provide for better growth than the other protein sources under the experimental conditions of a PER determination. In confirmation of the first series, the USDA cottonseed preparation gave the greatest growth and the commercial cottonseed flour, the poorest. Casein and fish flours were intermediate in growth promotion. PER calculations show that casein, fish flour and the USDA cottonseed flour were essentially equal, and superior to the Peru and the commercial cottonseed flours.

## DISCUSSION

The nutritional quality of the several protein sources studied differs in the 2 types of test procedures used. The second series, which is the more definitive study for reasons of the longer rehabilitation period and the use of a lower protein level in the rehabilitation diets, gives no clear-cut differentiation between the 3 cottonseed flours and the fish flour. This is particularly true in the rate of serum protein recovery. In growth of the depleted pigs, these 4 protein sources do group themselves in the general order observed in the rat PER studies, namely, the USDA cottonseed and fish flours being essentially equal, and superior to the com-

TABLE 3

*Growth of male weanling rats fed 18% protein diets*

| | 2-Week wt gain | Protein consumed | g gain / g protein |
|---|---|---|---|
| | *g* | *g* | |
| Casein | 88 | 18.4 | 4.8 |
| Fish flour | 88 | 16.5 | 5.3 |
| Cottonseed, USDA | 97 | 21.1 | 4.6 |
| Cottonseed, commercial [1] | 75 | 18.6 | 4.0 |
| Soybean meal | 88 | 22.1 | 4.0 |

[1] Proflo, Traders Oil Mill Company, Fort Worth, Texas.

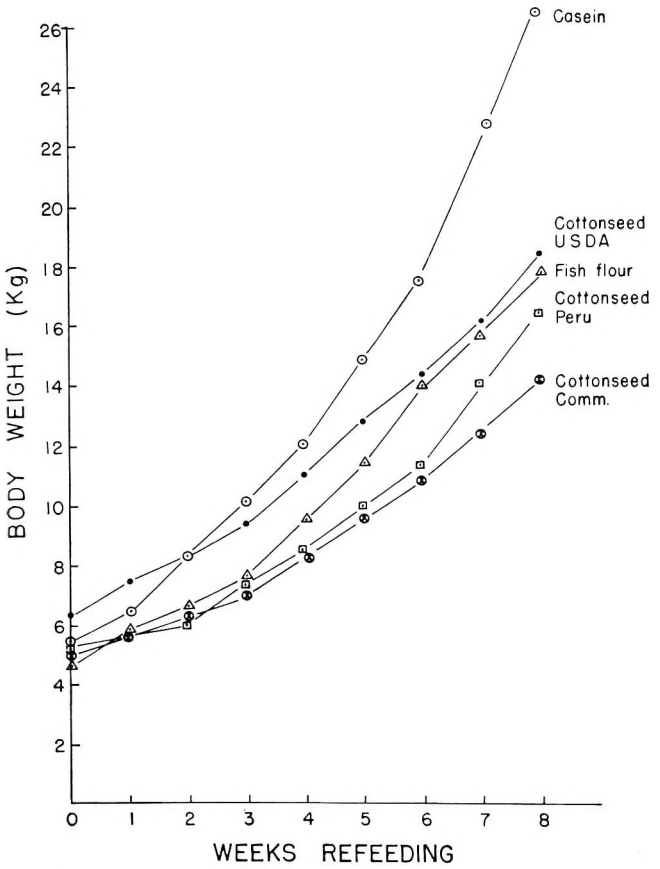


Fig. 4 Weight gain during refeeding (second series).

mercial and Peru cottonseed flour preparations. The percentage differences in growth rates between the USDA cottonseed flour and fish flour, on the one hand, and the two other cottonseed flours on the other, are similar in both depleted pigs and the normal weanling rats. The obvious exception in the 2 types of study is casein, which equals fish flour and the USDA cottonseed flour in the rat PER test, but is far superior in growth stimulation, and both total protein and serum albumin regeneration in the depleted pigs.

This major difference in nutritional quality in the 2 types of test might be due to a species difference or to an effect related to the severely depleted state of the baby pigs. Many studies of nutritional quality of protein have been made in both rats and swine and there is no information known to the authors which would support the view that major differences in amino acid requirements or in response to dietary differences in protein quality exist in the 2 species. It appears much more logical to conclude that the differences noted in the present study are related to the nutritional status of the animals. Long periods of protein depletion in the young or old rat lead to minimal reductions in serum proteins and other signs of protein-calorie malnutrition. The usual depletion-repletion study of protein quality does not involve the use of an animal in the severe stages of protein-calorie malnutrition that were evident in the baby pigs used in the present study (3). In the repletion test no major differences with other types of nutritional quality measurements were noted (3). The pigs used in the present studies represent not only a more severe degree of protein

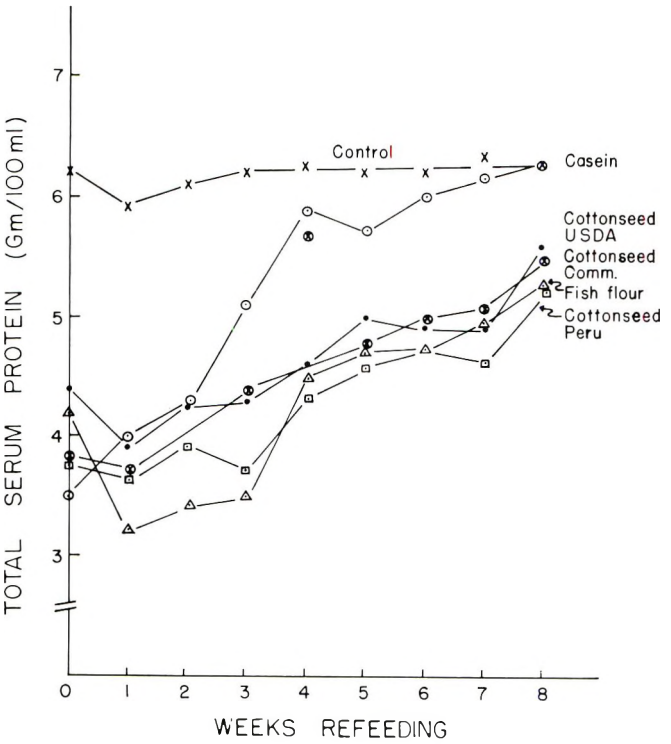


Fig. 5 Total serum protein during refeeding (second series).

TABLE 4

*Nutritive quality of protein sources in the standard 4-week rat growth study (10% protein) (second series)* [1]

| Protein source | Starting wt | Final wt | Wt gain | Protein consumed | PER |
|---|---|---|---|---|---|
| | *g* | *g* | *g* | *g* | |
| Casein | 65 | 168 | 103 | 37.9 | 2.7 |
| Fish flour | 65 | 174 | 109 | 38.4 | 2.8 |
| Cottonseed, Peru | 65 | 136 | 71 | 35.2 | 2.0 |
| Cottonseed, USDA | 65 | 181 | 116 | 38.4 | 3.0 |
| Cottonseed, commercial [2] | 65 | 132 | 67 | 35.0 | 1.9 |

[1] Ten male weanling rats/group.
[2] Proflo, Traders Oil Mill Company, Fort Worth, Texas.

depletion than others have used in measuring protein quality, but also the animals are very young in contrast with those of older ages — dogs and other species — used in plasma protein regeneration studies. In both rats and pigs, the males grew more rapidly than the females. In the rat studies and in the first pig study, only males were used. In the second pig study random sex distribution was used. Therefore, a preponderance of one sex in any study did not take place and sex distribution could not have influenced the results. It is our conclusion that the alterations in relative nutritive quality that have been noted in the present study are not due to species differences, but are reflections of the severe condition of protein depletion and the very young age of the pigs.

There is always a hazard in attempting to relate experimental observations in an animal to a disease condition in the human being. Nevertheless, there are similarities in the effects noted in the present studies and the responses to various die-

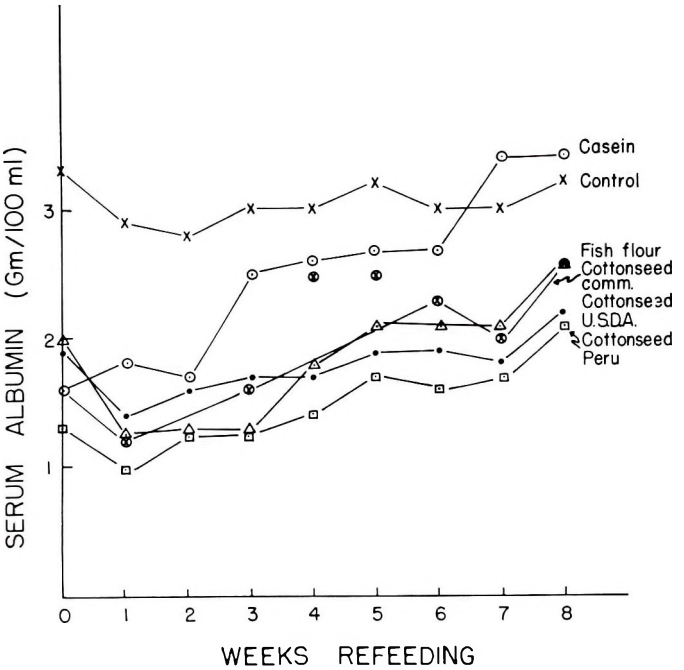


Fig. 6 Serum albumin during refeeding (second series).

tary proteins that have been noted by several clinical investigators studying the recovery of children from severe protein-calorie malnutrition. Pretorius and Wehmeyer (7) compared 2 fish protein preparations with dried skim milk in infants recovering from protein-calorie malnutrition. One of the fish protein products was definitely inferior. Very small differences in favor of milk over the second fish preparation were found for weight gain and serum albumin recovery, but these differences were not statistically significant. Graham and his associates (8) compared 2 fish protein concentrates alone or in combination with wheat with a modified cows' milk in malnourished infants. They reported similar growth responses and nitrogen retention with the different protein sources, but milk was superior as a dietary protein in returning serum albumin levels to normal. These authors confirmed the inferior ability of fish protein alone or in combination with wheat flour to correct hypoalbuminemia or to maintain serum albumin levels in severely malnourished infants (9). Milk protein was superior when the responses were compared, but growth and nitrogen retention were similar with all of these protein sources. On the other hand, Srikantia and Gopalan (10) have reported that many children hospitalized with kwashiorkor did not accept diets in which the major source of protein was from fish protein concentrate. In those children that did accept these diets, both clinical and biochemical responses compared favorably with those of malnourished children receiving diets containing milk protein. The major difference between the 2 dietary treatments was that growth response after disappearance of edema was inferior with the fish protein group.

In the present studies with malnourished baby pigs, both serum albumin responses and growth rate were poorer with fish protein and certain plant proteins than with the milk protein, casein. It might be that the same metabolic failure in handling non-milk protein was observed in the malnourished pigs as in the malnourished human infants. Obviously, in the pig studies better control of such factors as age, state of protein depletion and nutrient intake could be achieved. This

may explain the fact that both growth rate and serum protein responses were inferior with non-milk-protein-containing diets in the pig studies.

A question of importance is to determine whether the protein-depleted baby pig has an increased capacity to utilize casein or whether the other 4 protein sources cannot be properly handled by the deficient animal. A study of the amino acid composition of the different protein sources shown in table 2 provides no clue as to why the cottonseed and fish protein sources should be so inferior to casein in the depleted pig. The inferior utilization of two of the cottonseed preparations in the rat PER test is probably related to the gossypol content of these materials and the relative lack of availability of lysine. Amino acid analysis does not help in defining the cause of low utilization.

As a working hypothesis it is concluded that the cottonseed and fish protein sources are poorly utilized in the pigs because of lack of availability of amino acids which we interpret to mean lack of intestinal absorbability resulting from the experimentally induced condition of the animals. Supposedly, this could happen if proteolytic enzyme formation and secretion into the intestinal tract were severely depressed by the combination of the very young age of the animals and the extent of protein depletion that was imposed. This combination of effects is known to have a marked effect upon the morphology and exocrine function of the pancreas (11). In experimental animals such as the pig, which has a large microbial population in the small intestine (12), it is possible that unabsorbed protein may be degraded by bacterial enzymes, and nitrogenous products such as amines could be formed and absorbed. As a result, poor proteolytic hydrolysis of protein need not be reflected in a marked increase in fecal nitrogen. Casein might remain in the stomach for a longer time than the other proteins that were examined. This would give casein some advantage in being digested by intestinal enzymes. There is some evidence that milk protein is superior to soybean protein in the baby pig and that this is due largely to the smaller dependency upon pancreatic exocrine function for digestion of milk protein. This concept has been substantiated in a study of the relative digestibility of milk protein and soybean protein in pigs as influenced by ligation of the pancreatic ducts (13). This study, which utilized baby pigs 2 to 6 weeks of age, gave evidence that in the absence of pancreatic secretion, milk protein was approximately twice as digestible as soybean protein, indicating that milk protein can be more completely degraded by intestinal and gastric secretions than soybean protein. These were normal animals with pancreatic ducts ligated, but the net effect might have been similar to the results of the present study in which severely protein malnourished pigs were used, even though they were 11 weeks old at the time of the growth and serum protein recovery studies. Furthermore, Maner et al. (14) have shown that in the baby pig the rate of passage through the intestinal tract is much more rapid for soybean protein than for casein. These observations are believed to support the hypothesis that casein is superior in nutritional quality to other proteins under the experimental conditions used, because casein is more readily digested and absorbed than the other proteins studied. This may also be the explanation for the superiority of milk protein in malnourished human infants. The results obtained and the proposed explanation for these results support the conclusion that studies of nutritive quality of proteins intended for infant and preschool feeding programs where malnutrition is endemic should include evaluation in animals simulating the human situation. The protein-calorie malnourished baby pig may be useful for this purpose.

## LITERATURE CITED

1. Osborne, T. B., L. B. Mendel and E. L. Ferry 1919 A method of expressing numerically the growth-promoting value of proteins. J. Biol. Chem., 37: 223.
2. Miller, D. S., and A. E. Bender 1955 The determination of the net utilization of proteins by a shortened method. Brit. J. Nutrition, 9: 382.
3. Frost, D. V. 1959 Methods of measuring the nutritive value of proteins, protein hydrolysates, and amino acid mixtures. The repletion method. In: Protein and Amino Acid Nutrition, ed., A. A. Albanese. Academic Press, New York, p. 225.

4. Lowrey, R. S., W. G. Pond, R. H. Barnes, L. Krook and J. K. Loosli 1962 Influence of caloric level and protein quality on the manifestations of protein deficiency in the young pig. J. Nutrition, *78*: 245.
5. Pond, W. G., R. H. Barnes, R. B. Bradfield, E. Kwong and L. Krook 1965 Effect of dietary energy intake on protein deficiency symptoms and body composition of baby pigs fed equalized but suboptimal amounts of protein. J. Nutrition, *85*: 57.
6. Gornall, A. G., C. J. Bardawill and M. M. David 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., *177*: 751.
7. Pretorius, P. J., and A. S. Wehmeyer 1964 An assessment of nutritive value of fish flour in the treatment of convalescent kwashiorkor patients. Am. J. Clin. Nutrition, *14*: 147.
8. Graham, G. G., J. M. Baertl and A. Cordano 1965 Dietary protein quality in infants and children. Am. J. Dis. Child., *110*: 248.
9. Graham, G. G., J. M. Baertl and A. Cordano 1966 Studies in infantile malnutrition. V. The effect of dietary protein source on serum proteins. Am. J. Clin. Nutrition, *18*: 16.
10. Srekantia, S. G., and C. Gopalan 1966 Fish protein concentrates in the treatment of kwashiorkor. Am. J. Clin. Nutrition, *18*: 34.
11. Véghelyi, P. V., and T. T. Kemény 1961 Protein metabolism and pancreatic function. In: The Exocrine Pancreas, Ciba Foundation Symposium. Little Brown and Company, Boston.
12. Wilbur, R. D., D. V. Catron, L. Y. Quinn, V. C. Speer and V. W. Hays 1960 Intestinal flora of the pig as influenced by diet and age. J. Nutrition, *71*: 168.
13. Pekas, J. C., V. W. Hays and A. M. Thompson 1964 Exclusion of the exocrine pancreatic secretion: effect on digestibility of soybean and milk protein by baby pigs at various ages. J. Nutrition, *82*: 277.
14. Maner, J. H., W. G. Pond, J. K. Loosli and R. S. Lowrey 1962 Effect of isolated soybean protein and casein on the gastric pH and rate of passage of food residues in baby pigs. J. Animal Sci., *21*: 49.

# Copper, Molybdenum and Zinc Interrelationships: The influence of inorganic sulfate upon distribution and excretion of $^{65}Zn$ and $^{99}Mo$ in pregnant rats

KENNETH E. KINNAMON

*United States Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado*

*ABSTRACT* The effects of dietary sulfate, alone and in combination with dietary copper, molybdenum, and zinc, were studied in the pregnant rat with respect to distribution and excretion of administered $^{65}Zn$ and $^{99}Mo$. Increased dietary sulfate alone or in combination with any of these 3 elements was found not to significantly affect: $^{99}Mo$ or $^{65}Zn$ retention in fetal tissues or placental membranes (amnion, chorion, yolk sac, allantois, and decidua); $^{99}Mo$ digestive tract absorption or excretion via urine or feces; $^{99}Mo$ blood levels; $^{65}Zn$ excretion via urine or feces; or volume of urine excreted. Diets high in sulfate with copper and molybdenum added did affect urinary excretion of $^{99}Mo$. In rats of the latter group, urinary excretion of labeled molybdenum was significantly lower than in animals fed any other ration containing elevated levels of molybdenum. Animals fed high molybdenum rations showed lower blood, fetal, and placental structure retention and higher urine excretion of labeled molybdenum after 48 hours.

The dietary requirements for a particular nutrient may be significantly influenced by the presence and quantities of other nutrients. For example, symptoms attributed to magnesium deficiency in adult rats can be overcome by altering the calcium-to-phosphorus ratio in the diet (1). It is reasonable to assume that the growing mammalian fetus may be subjected to unfavorable conditions due to similar mineral nutrient interrelationships.

There is ample evidence to support the hypothesis that interrelationships exist between copper, molybdenum, and zinc (2–6) and that inorganic sulfate is probably a part of this interrelationship (7–10). The role of dietary sulfate in molybdenum toxicity is particularly confusing at this time. For example, the ability of inorganic sulfate to alleviate growth suppression in rats, caused by excess dietary molybdenum, has been established (9–10), but how this is brought about is not known. Reduction of intestinal absorption (11), increased urinary excretion (11–14), and the simple replacement value of sulfate (15) are among the explanations offered. Too, the extent to which copper alleviates molybdenum toxicity in many cases is difficult to assess because the selected therapeutic copper compound was in the form of sulfate (4, 16, 19). It also appears possible that a similar difficulty exists in evaluating the influence of zinc upon distribution of $^{65}Zn$ since $ZnSO_4$ is often used in experiments (20–22).

The importance of copper, molybdenum and zinc in many significant aspects of fetal metabolism, the evidence of various interactions existing among these nutrients, and the evidence of sulfate participation in this interrelationship prompted the present study of the role of dietary sulfate in relation to each of these elemental nutrients.

## MATERIALS AND METHODS

This experimental work was divided into two parts — the first, a study of sulfate interrelationships in pregnant rats, and the resultant influence upon the injection of $^{65}Zn$, and the second, a similar experiment to determine the influence upon administered radioactive molybdenum. In both experiments, rats of the Sprague-Dawley strain,[1] 6 weeks of age, were used. Before administration of tracer

Received for publication November 6, 1965.

[1] Obtained from Sprague-Dawley, Inc., Madison, Wisconsin.

radioactive material the experimental animals were housed individually in stainless steel suspension-type cages having solid walls but wire-mesh floors and front. Dimensions were 25.4 cm × 20.3 cm × 17.7 cm. Food containers were glass cylinders equipped with white lacquered screw-cap metal lids having a 3.2-cm hole stamped through the center. After radioisotope administration and until the animals were killed,[2] they were maintained in cages constructed essentially as described above except that the dimensions were 15.2 cm × 11.4 cm × 11.1 cm and were equipped with special cone dividers in the excreta funnel so that urine and feces were collected separately. While the rats were kept in cages of the latter type urine and feces were collected daily. Throughout both experiments food and water were offered ad libitum; food consumption was recorded daily, except during the 5-day breeding period. Weight gain was recorded 3 times per week.

*Experiment 1. $^{65}Zn$ administration.* Sixty-four female animals were placed at random in 8 groups of 8 rats each and fed a basal diet of finely ground commercial laboratory ration[3] containing 18 ppm copper, 70 ppm zinc, and less than 1 ppm molybdenum (23). One or more of the following ingredients was added to the basal diet: 0.01% copper as $Cu(CH_3COO)_2 \cdot H_2O$; 0.08% molybdenum as $Na_2MoO_4 \cdot 2H_2O$; 0.50% zinc as $ZnCO_3$; and 0.15% sulfate as $Na_2SO_4$ to obtain the following 8 treatments: 1) basal ration (control); 2) Cu; 3) Mo; 4) Zn; 5) $SO_4$; 6) Cu + $SO_4$; 7) Mo + $SO_4$; 8)Zn + $SO_4$. After these groups were maintained with their respective rations for a period of 5 weeks a healthy Sprague-Dawley male rat was housed with each experimental animal. Daily vaginal smears were made during this 5-day period to ascertain the day of conception. Conception was assumed to have occurred when sperm were observed in a smear. At the end of 7 weeks all of the animals were given a 10-μCi intraperitoneal injection of aqueous $^{65}ZnCl_2$ solution[4] and subjected to ether euthanasia 96 hours after receiving this tracer injection. Fetuses and placental structures (amnion, chorion, yolk sac, allantois, and decidua) were removed, counted, and weighed. Representative samples of fetuses and placental structures, along with a well-mixed portion of the daily urine collections and all the daily fecal collections were measured for radioactivity by counting in a Packard Auto-Gamma[5] sodium iodide scintillation detector.

All data were subjected to an analysis of variance. Significant differences between any two treatment means were determined by using the multiple range test of Duncan as described by Li (24) with some modifications derived from Linquist (26). Data analyzed as percentages were first submitted to an inverse sine transformation (26).

*Experiment 2. $^{99}Mo$ administration.* Ninety-six rats were assigned at random to 8 groups of 12 animals each. The groups were designated as follows: 1) basal ration (control); 2) Cu; 3) Mo; 4) $SO_4$; 5) Cu + $SO_4$; 6) Mo + $SO_4$; 7) Cu + Mo; 8) Cu + Mo + $SO_4$. One or more of the following at the levels indicated were added to the basal ration to obtain the 8 treatments: 0.02% copper as acetate; 0.02% molybdenum as molybdate; and 0.20% sulfate as the sodium salt. The basal ration had the following percentage composition by weight: starch, 44.4; vitamin-free casein, 18.0; sucrose, 22.4; fiber, 4.0; salt mixture W,[6] 4.0; vitamins[7] in dextrose, 2.2; and corn oil, 5.0. Mineral supplements were added to the basal ration at the expense of starch.

The groups were maintained with their respective rations for a period of 4 weeks

[2] The Principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed.

[3] Purina Laboratory Chow, Ralston Purina Company, Davenport, Iowa.

[4] Obtained from Abbott Laboratories, Oak Ridge, Tennessee.

[5] Packard Instrument Company, Inc., LaGrange, Illinois.

[6] Ingredients and amounts in grams to give one kilogram of salt mixture are as follows: NaCl, 105; KCl, 120; $H_2PO_4$, 310; $Ca_3(PO_4)_2$, 149; $CaCO_3$, 210; $MgSO_4$(anhydr.), 90; $FePO_4 \cdot H_2O$, 14.7; $MnSO_4$(anhydr.), 0.20; $K_2Al_2(SO_4)_4 \cdot 24H_2O$, 0.09; $CuSO_4 \cdot 5H_2O$, 0.39; NaF, 0.57; and KI, 0.05. Formulated according to Wesson, L. G., Science, 75: 339, 1932; obtained from Nutritional Biochemicals Corporation, Cleveland.

[7] The vitamin mixture contained the following per kilogram of ration: vitamin A, 19,800 IU; vitamin D, 2200 IU; and (in milligrams) α-tocopherol, 110; inositol, 110; ascorbic acid, 990; choline chloride, 1650; menadione, 49.5; p-aminobenzoic acid, 110; niacin, 99; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; folic acid, 1.98; and (in micrograms) biotin, 440; vitamin $B_{12}$, 29.7 (Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation).

before normal male Sprague-Dawley rats were placed with the females for a period of 5 days. Vaginal smears were made as described in experiment 1 above. At the end of 6 weeks and 2 days, each animal was given a 50-μCi dose of $^{99}Mo$ [8] administered by stomach tube as a 3-ml aqueous solution of molybdate. Forty-eight hours after $^{99}Mo$ administration, each animal was anesthetized with ether to allow 4 ml of blood to be drawn from the inferior vena cava then killed with an ether "overdose." A weighed portion of each heparinized 4-ml whole blood sample was placed in a counting tube; a second well-mixed portion was used to determine microhematocrits in duplicate; and a third aliquot was centrifuged and a weighed portion of the plasma placed in a counting tube. Fetuses and placental structures were handled as described in experiment 1.

## RESULTS

### *Growth, feed consumption, and litter size*

Significant differences in weight gain were noted only in experiment 1 (table 1, column 1). When compared with controls, significant differences ($P < 0.05$) in weight gain were noted only in those rats supplemented with 0.08% molybdenum. Weight gain in this Mo group was also significantly lower ($P < 0.01$) than in the Mo + $SO_4$ rats. Weight gain in the Cu-supplemented rats was higher than those in the Mo group ($P < 0.01$), $SO_4$ group ($P < 0.05$), and in the Zn + $SO_4$ group ($P < 0.05$). No significant differences were observed in feed consumption, litter size, or average fetus weights in either experiment.

### *Distribution of radioactivity in the fetus and placental structures*

Between-group comparisons were made on the basis of percentage activity retained per the following: 1) fetus or placental structures, 2) gram of wet weight of fetus or placental structures, 3) total wet weight of all fetuses per rat; or 4) total wet weight of all placental structures per rat.

In an earlier study (23) it was found that although there is a possible 120-hour variation in fetal age, the $^{65}Zn$ activity/g of fetus or placental structures within any treatment group, 96 hours after injections, is directly proportional to its wet weight. That is, the variation in percentage of injected dose observed within a given treatment group, whether evaluated in terms of activity per fetus, per placental structures, or per litter can be attributed solely to the variation in the wet weight of that tissue which is being compared. Therefore, when using the activity per gram of tissue method of evaluation, not only can significant differences between treatments be shown in fetuses or placental structures of comparable weight

[8] Obtained from ISO/Serve Inc., Cambridge, Massachusetts.

TABLE 1

*Average weight gain of animals in experiment 1 and the percentage of retained $^{65}Zn$ present in their fetuses and placental structure 96 hours after administration*

| | Treatment group | Wt gain | $^{65}Zn$ retained in | |
|---|---|---|---|---|
| | | | Fetus | Placental structure |
| | | *g* | *% of dose/g (wet wt)* | *% of dose/g (wet wt)* |
| 1 | Basal ration (controls) | 125 | 0.470 | 0.475 |
| 2 | Cu | 134 | 0.318** | 0.305** |
| 3 | Mo | 109* | 0.392 | 0.428 |
| 4 | Zn | 121 | 0.176** | 0.194** |
| 5 | $SO_4$ | 119 | 0.386 | 0.467 |
| 6 | Cu + $SO_4$ | 123 | 0.322** | 0.307** |
| 7 | Mo + $SO_4$ | 128 | 0.402 | 0.392 |
| 8 | Zn + $SO_4$ | 121 | 0.156** | 0.156** |

* Indicates a significant difference at the 5% level when compared with controls.
** Indicates a significant difference at the 1% level when compared with controls.

and development, but the same significant differences are noted when all fetuses or placental structures of all weights within each treatment are considered as a single group and compared. The results in the present study confirm this earlier observation and in addition, show clearly that a similar relationship exists between the fetus and placental structures and $^{99}$Mo retention 48 hours after administration of this isotope.

Thus, only percentage activity retained per gram of wet weight of fetus or placental structures are presented for either $^{65}$Zn or $^{99}$Mo retention.

*Experiment 1. $^{65}$Zn administration.* The radioactivity per gram of fetuses and placental structures are shown in table 1, columns 2 and 3. Over all treatment groups, no significant difference was noted when the percentage per gram of fetus was compared with the percentage per gram of placental structures. When compared with controls, $^{65}$Zn uptake was significantly lower ($P < 0.01$) not only in those rats maintained with rations increased in zinc content but also in rats fed copper-containing rations. Furthermore, uptake with the zinc-containing rations was significantly lower ($P < 0.01$) than in animals fed copper rations. The presence of increased sulfate appeared to have no effect on the uptake of labeled zinc by the fetuses or placental structures, i.e., no significant differences were noted between the following groups: Mo vs. Mo + $SO_4$; Cu vs. Cu +$SO_4$; Zn vs. Zn + $SO_4$.

*Experiment 2. Molybdenum administration.* Results are shown in table 2, columns 1 and 2. Over all treatment groups the activity per gram of fetus was much lower ($P < 0.01$) than activity per gram of placenta. Between-group comparisons showed that in both type tissues the retention of $^{99}$Mo 48 hours after administration was much lower ($P < 0.01$) in those animals fed a molybdenum-containing diet. Elevated dietary sulfate had no apparent effect upon retention of labeled molybdenum. No significant differences were found in either type of tissues when rats in the following groups were compared: controls vs. $SO_4$; Mo vs. Mo + $SO_4$; Cu vs. Cu + $SO_4$; neither was the Mo group significantly different from the Cu + Mo group.

### *Blood*

The percentages per gram of retained $^{99}$Mo, 48 hours after administration in whole blood plasma and red blood cells are shown in table 2, columns 3 and 4. Values for whole blood and plasma were determined by direct measurement, whereas the value for red blood cells was computed.[9] Over all groups the percentage per gram in the red blood cells was significantly lower than the percentage per gram in the plasma. Between-group differences in levels of retained $^{99}$Mo in plasma and red blood cells are almost identical in relative magnitude to the differences shown for fetuses and placental structures of the same experiment. Hem-

[9] $$\%/g \text{ in rbc's} = \%/g \text{ in whole blood} - \frac{(\%/g \text{ in plasma} \times (1 - \text{hematocrit}))}{\text{Hematocrit}}$$

TABLE 2

*Percentage per gram of retained $^{99}$Mo present in fetuses, placental structures and blood 48 hours after administration of $^{99}$Mo (exp. 2)*

| | Treatments | $^{99}$Mo retained in | | | |
|---|---|---|---|---|---|
| | | Fetus | Placental structure | Plasma | Red blood cells |
| | | *% of dose/100 g wet wt* | | | |
| 1 | Basal ration (controls) | 2.37 | 6.43 | 1.94 | 0.65 |
| 2 | Cu | 2.07 | 4.97 | 1.64 | 0.55 |
| 3 | Mo | 0.08** | 0.44** | 0.24** | 0.14** |
| 4 | $SO_4$ | 1.97 | 5.67 | 2.05 | 0.45 |
| 5 | Cu + Mo | 0.17** | 0.34** | 0.32** | 0.03** |
| 6 | Cu + $SO_4$ | 1.85 | 4.42 | 1.36 | 0.48 |
| 7 | Mo + $SO_4$ | 0.22** | 0.48** | 0.28** | 0.04** |
| 8 | Cu + Mo + $SO_4$ | 0.09** | 0.29** | 0.39** | 0.00+** |

** Indicates a significant difference at the 1% level when compared with control animals.

atocrits between groups were not significantly different.

### *Excretion*

*Experiment 1. $^{65}Zn$ administration.* Labeled zinc in both urine and feces during the 96 hours following injection was significantly higher ($P < 0.01$) in animals maintained with high zinc rations when compared with any group of non-zinc rations (table 3). No other statistically significant differences were observed. A comparison of columns 1 and 2 of table 3 shows that the feces are the principal route of excretion of labeled zinc. Daily urine volume appeared to be the same in all groups.

*Experiment 2. Molybdenum administration.* Data for excretion of labeled molybdenum for experiment 2 are summarized in table 4. Molybdenum-99 elimination via the urine on day 1 was significantly higher ($P < 0.01$) than normal in all of the animals fed high molybdenum diets. The reverse was true of radioactivity eliminated on day 2, i.e., $^{99}Mo$ excretion on the second day after tracer administration was lower than in the control animals. Presence of high dietary sulfate in combination with dietary molybdenum had no apparent effect on $^{99}Mo$ excretion; sulfate, however, in rations containing both added copper and molybdenum did effect tracer molybdenum elimination. In the rats of the latter group, urinary excretion of labeled molybdenum was significantly lower ($P < 0.01$) than in animals fed any other ration containing elevated levels of molybdenum. No significant differences were noted in urine volume or feces excretion of Mo.

## DISCUSSION

At the levels used in this study, dietary $Na_2SO_4$, alone or in combination with rations high in copper, molybdenum, or

TABLE 3

*Percentage of $^{65}Zn$ excreted 96 hours after administration (exp. 1)*

| | Treatment group | $^{65}Zn$ excreted | | |
|---|---|---|---|---|
| | | Urine | Feces | Total |
| | | % | % | % |
| 1 | Basal ration (control) | 0.81 | 52.20 | 53.01 |
| 2 | Cu | 0.84 | 58.13 | 58.97 |
| 3 | Mo | 0.95 | 58.41 | 59.36 |
| 4 | Zn | 5.11** | 86.26** | 91.37** |
| 5 | $SO_4$ | 0.74 | 65.01 | 65.75 |
| 6 | $Cu + SO_4$ | 1.18 | 56.17 | 57.35 |
| 7 | $Mo + SO_4$ | 1.04 | 59.04 | 60.08 |
| 8 | $Zn + SO_4$ | 4.17** | 82.69** | 86.86** |

** Indicates a significant difference at the 1% level when compared with controls.

TABLE 4

*Percentage of $^{99}Mo$ excreted by rats of experiment 2*

| | Treatment group | $^{99}Mo$ excreted | | | | |
|---|---|---|---|---|---|---|
| | | Urine | | | Feces | Urine and feces |
| | | 1st 24 hr | 2nd 24 hr | Total | Total | Total |
| | | % | % | % | % | % |
| 1 | Basal ration (control) | 24.41 | 8.29 | 32.70 | 8.17 | 40.87 |
| 2 | Cu | 18.66 | 12.15 | 30.81 | 6.66 | 37.47 |
| 3 | Mo | 78.70** | 1.68* | 80.38 | 2.94 | 83.32 |
| 4 | $SO_4$ | 30.30 | 8.86 | 39.16 | 5.94 | 45.10 |
| 5 | Cu + Mo | 67.10** | 2.14* | 69.24 | 12.59 | 81.83 |
| 6 | $Cu + SO_4$ | 18.44 | 9.02 | 27.46 | 13.49 | 40.95 |
| 7 | $Mo + SO_4$ | 77.34** | 2.52* | 79.86 | 3.19 | 83.05 |
| 8 | $Cu + Mo + SO_4$ | 39.03** | 1.70* | 40.73 | 7.44 | 48.17 |

* Indicates a significant difference at the 5% level when compared with controls.
** Indicates a significant difference at the 1% level when compared with controls.

zinc has been shown not to affect significantly: 1) $^{99}$Mo or $^{65}$Zn retention in fetal tissues or placental membranes, 2) $^{99}$Mo gastrointestinal absorption or excretion via urine or feces, 3) $^{99}$Mo blood levels, 4) $^{65}$Zn excretion via urine or feces, or 5) volume of urine excreted. Increased dietary sulfate did alleviate a growth depression caused by diets containing 0.08% molybdenum.

It has been suggested that the tissue retention of molybdenum in sheep was due in part to an effect of sulfate in reducing intestinal absorption and the prevention of renal tubule reabsorption (11). On the basis of the present study, it appears that such a mechanism is not operating in the pregnant rat. Sulfate in dietary combinations with both copper and molybdenum did significantly depress the increased urine $^{99}$Mo elimination caused by other diets high in molybdenum (table 4, columns 1, 2 and 3). The significance of this is unexplained at this time; however, the fact that the biological activity of each of these is significantly influenced by the ratio of these three present in the body has been emphasized (11). Underwood (27) referring to the work of Dick (12, 28–31) states that "molybdenum exerts its limiting effect on copper retention in the sheep only in the presence of inorganic sulfate; that neither molybdenum nor sulfate *alone* interferes with copper retention; and that the effectiveness of either is increased to a maximum as the intake of the other is increased." Recently, Gray and Daniel (32) have shown that the effect of dietary molybdenum or sulfate or of both, upon rats is profoundly influenced by the status of the animal's stored copper. They reported that when rats' stored copper was low and when a copper-deficient diet was fed, small amounts of molybdenum produced toxic symptoms which were intensified by sulfate. However, when copper stores and copper intake were adequate, sulfate prevented harmful effects caused by molybdenum.

Retention of radioactive molybdenum in fetuses, placental structures and blood (table 2) 48 hours after tracer administration was shown to be much lower in animals fed high molybdenum diets. Molybdenum-99 absorption, however, was apparently not a factor in this retention since feces levels (table 4, column 4) of $^{99}$Mo were not significantly altered, whereas there was considerable radioactivity in the urine of these groups (table 4, column 3). In rats maintained with nonmolybdenum-containing rations, fetal retention of $^{99}$Mo was approximately 0.02% /g, whereas retention in rats of the Mo groups was approximately 15 times less. Within each treatment group, radioactivity in the placental structures was at least 2 times higher than that of the fetal tissue. This within-group difference in retention of labeled molybdenum at 48 hours between fetuses and placental structures differs from retention of labeled zinc in these 2 tissues at 96 hours. As previously reported (23) and repeated in this study, retention of labeled zinc in these 2 tissues is essentially the same.

Blood retention of $^{99}$Mo has been shown to be significantly depressed in rats fed high dietary molybdenum. Retention was 5 to 8 times lower than in the nonmolybdenum groups. However, in all groups concentration of labeled molybdenum was considerably lower in red blood cells than in plasma (table 2, lines 3 and 4). The percentage of labeled molybdenum observed in the red blood cells of the rats fed the nonmolybdenum-containing diets was similar to that reported for naturally occurring red-cell molybdenum of sheep blood (13). The chemical form or combination in which molybdenum exists in blood has not been studied extensively. Scaife (13) reports red blood cell and plasma molybdenum to be readily dialyzable and wholly present as an anion, probably molybdate.

High molybdenum rations greatly accelerated the urinary excretion of tracer molybdenum (table 4, column 3). The rapid elimination of $^{99}$Mo in rats fed high molybdenum diets could be due to a more rapid turnover or perhaps to a homeostatic control mechanism similar to the one reported for zinc (20, 33).

This study has confirmed an earlier report (23) that rations high in copper as well as those high in zinc cause a significant decrease in the fetal or placental membrane's retention of injected labeled

zinc. Too, high zinc rations greatly accelerate tracer zinc elimination. Cotzias et al. (33) have reported similar observations for excretion, using a whole body counting technique. Accordingly, rats in the high zinc groups had eliminated via urine and feces approximately 70% more than controls by 96 hours after administration.

The increased weight gain observed in the Cu group is not unlike that resulting from the growth-promoting effect of copper reported for growing pigs (34). Since food consumption in this group was not significantly higher the weight gain is most likely due to an improvement in efficiency of food conversion.

ACKNOWLEDGMENTS

The author wishes to acknowledge the technical assistance of Joseph E. Abernathy and Wallace J. Murray. He is also grateful to Gerhard J. Isaac for help and advice in conducting the statistical analyses and to Dr. Wayne O. Evans for his help, interest and criticism.

LITERATURE CITED

1. Forbes, R. M. 1963 Mineral utilization in the rat. I. Effects of varying dietary ratios of calcium, magnesium and phosphorus. J. Nutrition, *80:* 321.
2. Breslow, E., and F. R. N. Gurd 1963 Interaction of cupric and zinc ions with sperm whale metmyoglobin. J. Biol. Chem., *238:* 1332.
3. Brinkman, G. L., and R. L. Miller 1961 Influence of cage type and dietary zinc oxide upon molybdenum toxicity. Science, *134:* 1531.
4. Comar, C. L., L. Singer and G. K. Davis 1949 Molybdenum metabolism and interrelationships with copper and phosphorus. J. Biol. Chem., *180:* 913.
5. Magee, A. C., and G. Matrone 1960 Studies on growth, copper metabolism and iron metabolism of rats fed high levels of zinc. J. Nutrition, *72:* 233.
6. Ritchie, H. D., R. W. Luecke, B. V. Baltzer, E. R. Miller, D. E. Ullrey and J. A. Hoefer 1963 Copper and zinc interrelationships in the pig. J. Nutrition, *79:* 117.
7. Dick, A. T. 1953 Influence of inorganic sulfate in the copper-molybdenum interrelationship in sheep. Nature, *172:* 637.
8. Johnson, H. L., and R. F. Miller 1961 The interrelationships between dietary molybdenum, copper, sulfate, femur alkaline phosphatase activity and growth of the rat. J. Nutrition, *75:* 459.
9. Miller, R. L., W. O. Price and R. W. Engel 1956 Added dietary inorganic sulfate and its effect upon rats fed molybdenum. J. Nutrition, *60:* 539.
10. Van Reen, R., and M. A. Williams 1956 Studies on the influence of sulfur compounds on molybdenum toxicity in rats. Arch. Biochem., Biophys., *63:* 1.
11. Dick, A. T. 1956 Molybdenum and copper relationships in animal nutrition. In: A Symposium on Inorganic Nitrogen Metabolism, eds., W. D. McElroy and B. Glass. John Hopkins Press, Baltimore, Maryland, p. 445.
12. Dick, A. T. 1953 The effect of inorganic sulfate on the excretion of molybdenum in the sheep. Australian Vet. J., *29:* 18.
13. Scaife, J. F. 1956 Molybdenum excretion and retention in the sheep. New Zealand J. Sci. Technol., *38A:* 293.
14. Barker, S. 1960 Effect of inorganic sulfates in molybdenum excretion by a marsupial. Nature, *185:* 41.
15. Feaster, J. P., and G. K. Davis 1959 Sulfate metabolism in rabbits on high molybdenum intake. J. Nutrition, *67:* 319.
16. Arrington, L. R., and G. K. Davis 1953 Molybdenum toxicity in the rabbit. J. Nutrition, *51:* 295.
17. Ferguson, W. S., A. H. Lewis and S. J. Watson 1943 The tert pastures of Somerset. I. The cause and cure of tertness. J. Agr. Sci., *33:* 44.
18. Gray, L. F., and G. H. Ellis 1950 Some interrelationships of copper, molybdenum, zinc and lead in the nutrition of the rat. J. Nutrition, *40:* 441.
19. Neilands, J. B., F. M. Strong and C. A. Elvehjem 1948 Molybdenum in the nutrition of the rat. J. Biol. Chem., *172:* 431.
20. Cotzias, G. C., and P. S. Papavasiliou 1964 Specificity of zinc pathway through the body: homeostatic considerations. Am. J. Physiol., *206:* 787.
21. Martin, W. G., and H. Patrick 1961 Radionuclide studies. 3. The effect of breed and dietary zinc, calcium and vitamin $D_3$ on the retention of $zinc^{65}$ in chicks. Poultry Sci., *40:* 1004.
22. Sastry, K. S., P. R. Adiga, V. Venkatasubramanyam and P. S. Sarma 1962 Interrelationships in trace-element metabolism in metal toxicities in *Neurospora crassa*. Biochem. J., *85:* 486.
23. Kinnamon, K. E. 1963 Some independent and combined effects of copper, molybdenum and zinc on the placental transfer of zinc-65 in the rat. J. Nutrition, *81:* 312.
24. Li, J. C. R. 1957 Introduction to Statistical Inference. Edward Brothers, Ann Arbor, Michigan, p. 221.
25. Linquist, E. F. 1956 Design and Analysis of Experiments in Psychology and Education. Riverside Press, Cambridge, p. 266.
26. Johnson, P. O. 1949 Statistical Methods in Research. Prentice Hall, New York, p. 149.

27. Underwood, E. J. 1962 Trace elements in human and animal nutrition. Academic Press, New York, p. 100.
28. Dick, A. T. 1952 The effect of diet and of molybdenum on copper metabolism in sheep. Australian Vet. J., 28: 30.
29. Dick, A. T. 1953 The control of copper storage in the liver of sheep by inorganic sulphate and molybdenum. Australian Vet. J., 29: 233.
30. Dick, A. T. 1954 Preliminary observations on the effect of high intakes of molybdenum and of inorganic sulphate on blood, copper and on fleece character in crossbred sheep. Australian Vet. J., 30: 196.
31. Dick, A. T. 1954 Studies on the assimilation and storage of copper in crossbred sheep. Australian J. Agr. Res., 5: 511.
32. Gray, L. F., and L. J. Daniel 1964 Effect of the copper status of the rat on the copper-molybdenum-sulfate interaction. J. Nutrition, *84:* 31.
33. Cotzias, G. C., D. C. Borg and B. Selleck 1962 Specificity of zinc pathway through the body: Turnover of $^{65}Zn$ in the mouse. Am. J. Physiol., 202: 359.
34. Allen, M. M., R. S. Barber, R. Braude and K. G. Mitchell 1961 Further studies on various aspects of the use of high-copper supplements for growing pigs. Brit. J. Nutrition, *15:* 507.

# Metabolism of Lipids in Rats Exposed to Heat under Conditions of a Normal and a High Fat-High Cholesterol Diet

PAVEL BOBEK AND EMIL GINTER
*Laboratory Department of the Institute of Human Nutrition Research, Bratislava, Czechoslovakia*

*ABSTRACT* Prolonged intermittent exposure to heat "34°" exerts strong influence on lipid metabolism in rats maintained with a nutritionally well-balanced diet; a general tendency to decreased cholesterol concentration was observed in the tissues. At the same time a decrease of the biosynthesis of cholesterol from acetate-1-$^{14}$C occurred in the liver and a decrease of the $\beta$-fraction of serum lipoproteins was observed. Exposure to elevated temperature caused a decrease of esterified fatty acid levels in the blood serum and liver; in epididymal fat tissue an increased concentration of these substances took place. The biosynthesis of fatty acids from acetate-1-$^{14}$C exhibited a marked decrease in the liver. Heat exposure caused a decrease of the iodine number of depot lipids. The decrease of hepatic lipogenesis indicates a general slowing of lipid metabolism under the influence of exposure to elevated temperature. With the high fat-high cholesterol diet no marked differences were noted between the groups investigated, since the markedly unbalanced diet had a more profound metabolic influence than the effect of heat exposure. The morphological picture of the myocardium and aorta was likewise not influenced by heat exposure.

Although the effect of low environmental temperatures on fat metabolism has been studied rather extensively, relatively few data are available on the influence of exposure to heat. Young and Cook (1) reported that mice kept at 35°, although consuming less food, showed an increase in total body lipids in comparison with that of mice kept at 24°. Similar results were obtained by Mefferd et al. (2) in rats acclimated to a temperature of 35°. In the latter, a decrease in specific activity of total body cholesterol following the administration of 1-$^{14}$C-acetate and in the activity of succinic dehydrogenase in the liver was observed. The results indicate that in rats exposed to higher temperatures, the turnover of body lipids is reduced.

The aim of the present study was to investigate the influence of intermittent exposure of rats to heat on lipid metabolism in various organs of rats fed a well-balanced diet and in those fed a high fat-high cholesterol diet.

## METHODS

In 3 series of experiments, white, male rats of the Wistar strain, weighing 180 to 200 g, were used. Sixty animals of each series were divided into 4 groups, each containing 15 animals. Groups 1 and 2 received the Larsen diet (3),[1] whereas groups 3 and 4 received a high fat-high cholesterol diet (table 1).

Groups 2, and 4 were maintained at a temperature of 22 to 23° and served as controls. Animals of groups 1 and 3 were exposed twice daily, in the morning and in the afternoon, to a temperature of 34 ± 1°, each time for a period of 2 hours. The heat exposure was carried out by means of 300-w radiators equipped with parabolic covers and a thermoregulative device. After a 14-day adaptation of the animals, the final dose of exposure was set to a gradually increasing environmental temperature. Food and water were given ad libitum. Experimental conditions were equal in all experimental series; a difference between the series consisted only in the length of the experiment (first and third series, 13 weeks; second series 7 weeks), and in the observations made.

At the end of the experimental period of series 1 the animals were decapitated.

Received for publication September 7, 1965.

[1] The Larsen diet contained the following: (in grams) coarse wheat flour, 1860; dried milk, 570; casein, 510; alfalfa, 90; $CaCO_3$, 48; hydrogenated fat, 150; cod liver oil, 21; and NaCl, 6.

TABLE 1

*Composition of high fat — high cholesterol diet*

| | g |
|---|---|
| Hydrogenated vegetable fat [1] | 400 |
| Sucrose | 203 |
| Casein | 150 |
| NaCl | 100 |
| Cellulose | 40 |
| Salt mixture [2] | 40 |
| Cholesterol | 40 |
| Dried yeast | 20 |
| Cholic acid | 5 |
| Choline chloride | 2 |

[1] The hydrogenated vegetable fat was fortified with the following/kg of ration: 10 mg vitamin K (menadiol diacetate); 25,000 IU of vitamin $A_1$; 0.125 mg vitamin $D_2$; 125 mg α-tocopheryl acetate; and 2 ml cod liver oil.

[2] Osborne, T. B., and L. B. Mendel. J. Biol. Chem., *15*: 317, 1913.

Subsequently, concentrations of cholesterol (4), esterified fatty acids (5), lipid phosphorus (6) and the iodine number [2] of fats (7, 8) were determined in the blood serum. Lipoprotein fractions were determined by paper electrophoresis (9). In a chloroform-methanol extract obtained according to Folch et al. (10) from liver and lung homogenates the concentrations of cholesterol, esterified fatty acids and lipid phosphorus were determined by the methods described above. Iodine number was determined for the epididymal fat. The heart and the thoracic section of the aorta were subjected to histological examination (hematoxylin-eosin, Sudan IV, Mallory, PAS, Hale, Hotchkiss and alcian blue).

In series 2 values for the lipid fractions in the adrenals, glycogen concentration in the liver (11), and the concentration of cholesterol and esterified fatty acids in the epididymal fat were determined by the methods described above.

In the third experimental series, the animals, 3 hours before killing, received intraperitoneally 40 μc of Na acetate-1-$^{14}$C for 100 g of body weight. Cholesterol was isolated from the liver and the adrenals according to the method of Sperry and Webb (12), and total fatty acids from the liver (13). At the same time concentrations of cholesterol (12) and total fatty acids (14) were determined in these tissues. The activity of the samples, which were applied in thin layers on small aluminum plates, was measured by means of an end-window thin-wall GM tube.

The results were analyzed statistically by the *t* test.

## RESULTS

The mean weight curves of animals of all groups from the first experimental series are shown in figure 1. There was an increase in the weight of animals fed the Larsen diet, and the curve was similar for the group exposed to heat. Weight increase in rats fed the high fat diet and kept at room temperature was minimal, and the weight of animals of this group as compared with that of rats fed the Larsen diet was significantly lower ($P < 0.001$) in the sixth and thirteenth experimental week. Heat exposure under conditions of feeding the high fat diet caused a decrease of body weight which was statistically significant in weeks 4, 8 and 12 of the experiment ($P < 0.001$ in all 3 cases). In this group the mortality rate was high, about 40%.

In rats fed the Larsen diet, heat exposure caused a decrease of cholesterol levels in most of the tissues examined. With feeding the high fat diet there was a marked increase in cholesterol concentration in all the tissues examined except those of the lungs. On the other hand, exposure to elevated temperature did not markedly affect the cholesterol levels; a marked decrease occurred only in the epididymal fat (table 2).

In the liver and blood serum of rats fed the Larsen diet and exposed to heat, a statistically significant decrease in the level of esterified fatty acids was observed. On the other hand, the quantity of these substances increased markedly in the epididymal fat. With the high fat diet, heat exposure resulted in a decrease of esterified fatty acids in the adrenals (table 3).

With heat exposure, there was a significant decrease in phospholipid concentration in the blood serum and the adrenals of rats fed the Larsen diet; in rats fed the high fat diet and exposed to heat the decrease was statistically significant only in the adrenals (table 4).

The iodine number of blood lipids under conditions of heat exposure increased sig-

[2] Iodine number has been defined by the authors as the milligrams of iodine bound by the lipids extracted from 100 ml of serum or 100 g of adipose tissue. This does not conform with the definition commonly used in the United States.

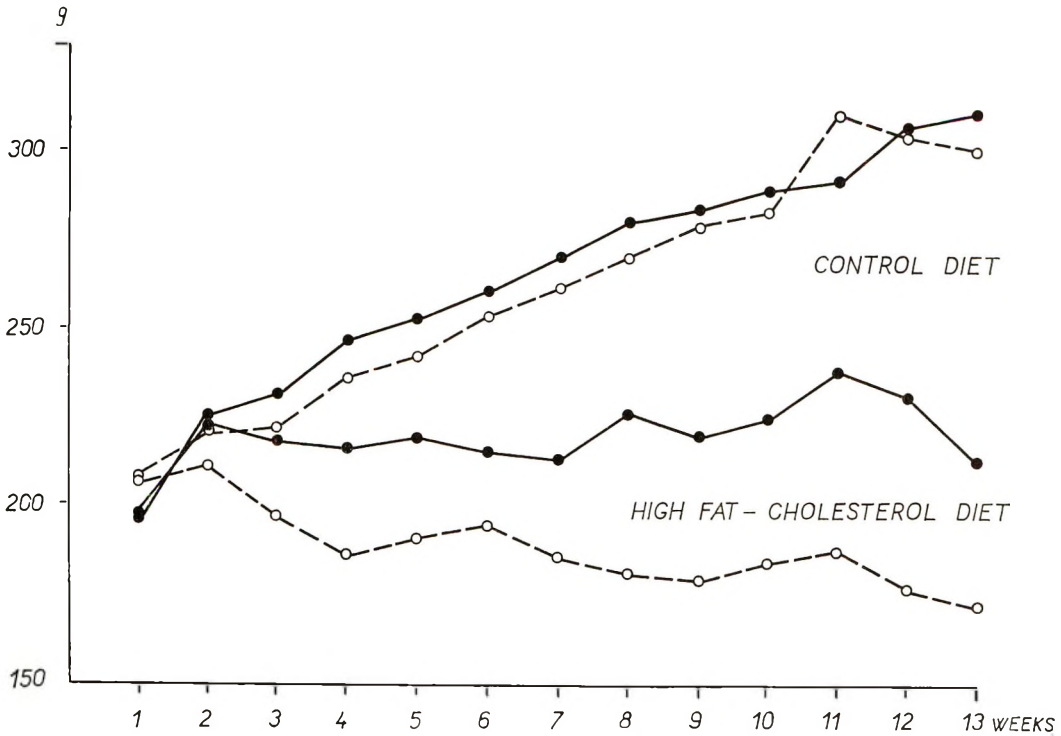


Fig. 1 Effect of exposure to elevated environmental temperature on mean weight curves of rats maintained with a well-balanced diet, and with a high fat-high cholesterol diet (series 1). Solid lines, groups maintained at 22° to 23°; dotted lines, groups maintained at 34 ± 1°.

TABLE 2

*Cholesterol levels of serum and tissues of rats exposed to heat while maintained with a normal or a high fat — high cholesterol diet*

| | Normal diet | | High fat — high cholesterol diet | |
|---|---|---|---|---|
| | 22–23° | 34 ± 1° | 22–23° | 34 ± 1° |
| Blood serum | 58 ± 3 [1] | 52 ± 2 | 271 ± 34 | 317 ± 48 |
| Liver | 403 ± 15 | 352 ± 13* | 10.048 ± 783 | 8.642 ± 1.228 |
| Lungs | 583 ± 17 | 512 ± 10** | 620 ± 19 | 718 ± 23** |
| Adrenals | 2.962 ± 190 | 3.429 ± 247 | 8.829 ± 794 | 7.776 ± 1.348 |
| Epididymal fat | 136 ± 8 | 98 ± 10** | 234 ± 16 | 130 ± 13** |

[1] Mean values expressed in mg/100 ml or 100 g wet tissue ± SE.
* Differs from the values indicated in the left column at $P < 0.05$.
** Differs from the values indicated in the left column at $P < 0.01$.

TABLE 3

*Levels of esterified fatty acids in serum and tissues of rats exposed to heat while maintained with a normal or a high fat — high cholesterol diet*

| | Normal diet | | High fat — high cholesterol diet | |
|---|---|---|---|---|
| | 22–23° | 34 ± 1° | 22–23° | 34 ± 1° |
| Blood serum | 0.76 ± 0.05 [1] | 0.56 ± 0.02* | 0.83 ± 0.05 | 0.97 ± 0.08 |
| Liver | 17.1 ± 1.0 | 14.1 ± 0.6* | 21.9 ± 1.1 | 19.0 ± 0.9 |
| Lungs | 6.7 ± 0.3 | 6.3 ± 0.3 | 9.1 ± 0.4 | 9.6 ± 0.4 |
| Adrenals | 41.6 ± 3.4 | 41.4 ± 3.6 | 38.0 ± 5.3 | 22.1 ± 3.9* |
| Epididymal fat | 215.9 ± 15.6 | 279.6 ± 14.8* | 119.4 ± 13.9 | 95.8 ± 8.1 |

[1] Mean values expressed in milliequivalents/100 ml or 100 g wet tissue ± SE.
* Significantly different from the values indicated in the left column at $P < 0.05$.

nificantly both in rats fed the Larsen diet and in those fed the high fat diet. The iodine number for the epididymal fat of rats fed the Larsen diet and exposed to heat decreased markedly. Feeding the high fat diet with a high content of saturated fatty acids caused a sharp decrease of the iodine number of the depot lipids; under these conditions heat exposure resulted in a significant increase in the iodine number. With heat exposure there was a significant decrease in the relative concentration of blood serum β-lipoproteins in rats fed the Larsen diet. Feeding the high fat diet resulted in an increase of serum β-lipoproteins, not influenced by the higher environmental temperature.

In animals maintained with the well-balanced diet and exposed to heat there was a sharp decrease in liver glycogen concentration; in animals maintained at room temperature, feeding of the high fat diet caused a sharp decrease in hepatic glycogen. Under these conditions, heat exposure had a tendency to normalize glycogen concentration in the liver. Table 5 shows the summarized data concerning values of the iodine number, serum β-lipoproteins and hepatic glycogen.

In rats fed the well-balanced diet and exposed to heat, there was a decrease in the specific activity of liver cholesterol which indicates a decrease in the biosynthesis of cholesterol from acetate-1-$^{14}$C. Feeding of the high fat diet depressed the endogenous synthesis of cholesterol in the liver and in the adrenals to values so low that the activity of isolated cholesterol was too low to measure, and thus it was not possible to compare the rate of this process in rats maintained at room temperature and at the elevated temperature. The specific activity of total fatty acids in the liver of rats exposed to heat decreased significantly only when the Larsen diet was fed (table 6).

In rats fed the Larsen diet, no lesions were observed in the myocardium or the aorta. In some animals maintained at room temperature, feeding of the high fat

TABLE 4

*Levels of phospholipids in serum and tissues of rats exposed to heat while maintained with a normal or a high fat — high cholesterol diet*

| | Normal diet | | High fat — high cholesterol diet | |
|---|---|---|---|---|
| | 22–23° | 34 ± 1° | 22–23° | 34 ± 1° |
| Blood serum | 120 ± 5 [1] | 90 ± 2* | 127 ± 7 | 157 ± 15 |
| Liver | 4.225 ± 125 | 4.075 ± 125 | 3.400 ± 125 | 3.500 ± 75 |
| Lungs | 2.575 ± 75 | 2.400 ± 75 | 2.825 ± 100 | 2.875 ± 100 |
| Adrenals | 4.925 ± 175 | 3.375 ± 200* | 4.075 ± 200 | 1.450 ± 125* |

[1] Mean values expressed in mg/100 ml or 100 g wet tissue ± SE.
* Significantly different from the values indicated in the left column at $P < 0.001$.

TABLE 5

*Iodine bound by serum and epididymal tissue lipids, serum β-lipoproteins; and hepatic glycogen in rats exposed to heat while maintained with a normal or a high fat — high cholesterol diet*

| | Normal diet | | High fat — high cholesterol diet | |
|---|---|---|---|---|
| | 22–23° | 34 ± 1° | 22–23° | 34 ± 1° |
| | | Iodine | | |
| Blood serum, mg/100 ml | 96 ± 11 [1] | 211 ± 14** | 161 ± 9 | 275 ± 11** |
| Epididymal fat, mg/100 g | 9265 ± 264 | 5106 ± 375** | 1201 ± 109 | 1927 ± 226* |
| | | β-lipoproteins | | |
| Blood serum, % [2] | 49.1 ± 1.2 | 40.3 ± 1.6** | 56.7 ± 2.8 | 55.2 ± 1.8 |
| | | Glycogen | | |
| Liver, g/100 g wet tissue | 2.7 ± 0.2 | 0.7 ± 0.2** | 0.6 ± 0.1 | 1.9 ± 0.3** |

[1] Mean ± SE.
[2] Relative per cent of sum of 0 + α- + β-lipoprotein fractions.
* Differs from the values indicated in the left column at $P < 0.01$.
** Differs from the values indicated in the left column at $P < 0.001$.

TABLE 6

*Specific activities of total cholesterol and fatty acids in the liver and adrenals of rats exposed to heat while maintained with a normal or a high fat — high cholesterol diet*

| | Normal diet | | High fat — high cholesterol diet | |
|---|---|---|---|---|
| | 22–23° | 34 ± 1° | 22–23° | 34 ± 1° |
| Cholesterol | | | | |
| Liver | 626 ± 149 [1] | 106 ± 25* | — | — |
| Adrenals | 282 ± 106 | 66 ± 45 | — | — |
| Total fatty acids of liver | 12.039 ± 2.468 [2] | 2.899 ± 958* | 5.442 ± 2.349 | 1.245 ± 187 |

[1] Mean values expressed in counts/min/mg ± SE.
[2] Mean values expressed in counts/min/milliequivalents ± SE.
* Significantly different from the values indicated in the left column at $P < 0.01$.

diet produced small subepicardial foci of necrosis surrounded by vacuolized muscle fibers. Small rami of the coronary arteries were edematous and vacuolized, they showed a slightly positive reaction to PAS, and also a weak reaction to alcian blue. Except in one instance of moderate vacuolization of the arterial wall, the findings in the aortas were negative. Staining for fats yielded negative results. In the group exposed to heat, defects of small groups of muscle cells, replaced by a small focus of granular tissue, were observed in the myocardium. In the aorta only moderate edema was noted. In general, the group exposed to heat did not exhibit substantial differences from the group kept at room temperature.

## DISCUSSION

The mean weight curves demonstrate that exposure to heat had no effect on the weight increase in rats fed the Larsen diet. On the other hand, feeding of the high fat diet caused no increase in the mean weight of the experimental animals; and under these conditions, heat exposure caused a decrease in body weight. The high mortality in this group of animals also demonstrates that exposure to heat represented an extreme stress to the animals fed the high fat diet.

In rats maintained with the Larsen diet and exposed to heat, cholesterol concentration in different tissues tended to decrease. At the same time a moderate decrease in cholesterol biosynthesis from acetate-1-$^{14}$C was observed in the liver. The mechanism whereby cholesterologenesis is decreased in the livers of heat-exposed rats is not known; it may be connected with the decrease in liver glycogen level demonstrated previously in this group. Bucher and McGarahan (15) have observed that under certain conditions, cholesterol biosynthesis in the liver depends upon glycogen. The decrease in cholesterologenesis is also correlated with the decrease in the β-lipoprotein level in the blood serum of rats exposed to heat. In animals fed the high fat diet, both cholesterol concentrations in the serum and the liver and serum β-lipoproteins were increased, but not further affected by elevated environmental temperatures. In these animals, heat exposure resulted in diverse changes of cholesterol concentration in the various organs. The decisive factor here was by no means the rate of cholesterol biosynthesis since, in agreement with others (16, 17),[3] a decrease was observed in cholesterologenesis from acetate-1-$^{14}$C as a consequence of extraordinarily high amounts of cholesterol ingested in food. Hence, the cholesterol level in the different organs is likely the product of the rate and direction of transport processes conveying cholesterol from the gastrointestinal tract into different tissue, and of cholesterol catabolism.

When the well balanced diet was fed, heat exposure caused a decrease in esterified fatty acid concentration in the blood serum and in the liver, and a pronounced increase in epididymal fat. This might point to a possible decrease in the mobilization of fatty acids from depot tissues. At the same time, a significant decrease was noted in acetate-1-$^{14}$C utilization for the synthesis of fatty acids in the liver of heat-exposed rats. The decrease in esterified

[3] Gould, R. G., and C. B. Taylor 1950 Effect of dietary cholesterol on hepatic cholesterol synthesis. Federation Proc., 9: 179 (abstract).

fatty acid levels in the livers of these animals is presumably due to a decreased supply of fatty acids from the depots and to reduced lipogenesis in the liver. This conclusion is in fair agreement with the observations of other authors (1, 2), who described lowered lipid turnover in animals acclimated to elevated environmental temperature.

In rats fed the high fat diet, exposure to heat failed to affect the levels of esterified fatty acids in most of the organs.

Exposure of animals to elevated environmental temperatures appeared to interfere also with phospholipid turnover; this may be inferred from the sharp decline in phospholipid concentration in the adrenals of heat-exposed rats fed either the normal diet or high fat diet. The mechanism of this phenomenon remains obscure; it is possibly connected with the decreased fatty acid biosynthesis in the tissues of rats acclimated to heat.

Young and Cook (1) have observed the melting point of the total body fat to be increased in rats acclimated to heat. Likewise, in rats acclimated to heat, the rate of labeled acetate conversion into fatty acids was more markedly reduced for the fraction of liquid fatty acids (2); this may indicate that in an organism acclimated to heat, the proportion of fatty acids with a higher melting point rises. In line with the above observation, we also noted a marked decrease in the iodine number of the epididymal fat of rats fed the Larsen diet; this appears to indicate that under the influence of elevated environmental temperatures, increased amounts of saturated fatty acids with a higher melting point were present in the epididymal fat. In animals fed the high fat diet, there was, as expected, a decrease in the iodine number of depot fat, and which increased with exposure to heat. The mechanism of this phenomenon and also the increase in the iodine number of blood lipids in rats exposed to heat remain unexplained.

Morphological examination of the myocardium and the aorta did not reveal any substantial differences between groups kept at temperatures of 22 and 34°. It appears that in contrast with the atherogenous action of exposure to cold (18–21), exposure to elevated temperatures does not entail increased deposition of lipids into the arterial system of rats.

An evaluation of our results leads to the conclusion that in animals fed the well-balanced diet, exposure to elevated temperature lowers the rate of lipid metabolism (turnover); this is evidenced by decreased mobilization of fatty acids from depot tissues and by decreased lipid biosynthesis in the liver tissue. In animals fed the high fat-high cholesterol diet, the dietary regimen had a more profound metabolic influence than the effect of the elevated environmental temperature.

## LITERATURE CITED


1. Young, D. R., and S. F. Cook 1955 Body lipids in small mammals following prolonged exposure to high and low temperature. Am. J. Physiol., *181:* 72.
2. Mefferd, R. B., M. H. Nyman and W. W. Webster 1958 Whole body lipid metabolism of rats after chronic exposure to adverse environments. Am. J. Physiol., *195:* 744.
3. Fábry, P. 1955 The influence of diet composition upon the daily rhythmical fluctuation of glycide reserves. Čsl. Fysiol., *4:* 1.
4. Hořejší, J. 1964 Chemical Examinations in Medicine. State Medical Publishing House, Praha, p. 399.
5. Stern, J., and B. Shapiro 1953 A rapid and simple method for the determination of esterified fatty acids. J. Clin. Pathol., *6:* 158.
6. Stewart, C. P., and E. B. Hendry 1935 The phospholipids of blood. Biochem. J., *29:* 1683.
7. Trappe, W. 1938 Über die Ergebnisse mit der Jodzahlbestimmungsmethode nach H. P. Kaufman. Biochem. Ztschr., *7:* 296.
8. Šulc, M., J. Měštan and Č. Michalec 1961 The lipids in pathology. I. Iodine number and clinical atherosclerosis. Sborn. Lék., *63:* 33.
9. Swahn, B. 1953 A micromethod for the determination of total lipids in serum. Scand. J. Clin. Invest., 9 (*suppl. 5*): 1.
10. Folch, J., M. Lees and G. H. Sloane-Stanley 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., *226:* 497.
11. Good, C. A., H. Kramer and M. Somogyi 1933 The determination of glycogen. J. Biol. Chem., *100:* 485.
12. Sperry, W. M., and M. Webb 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97.
13. Tretjakova, K. A., and D. E. Grozdenskij 1959 The influence of the ionizing irradiation upon the biosynthesis of cholesterol and fatty acids in the rat liver and adrenals. Vopr. Med. Chim., *5:* 362.
14. Van de Kamer, J. H., H. T. Huinink and H. A. Weyers 1949 Rapid method for the de-

termination of fat in feces. J. Biol. Chem., *177:* 347.
15. Bucher, N. L. R., and K. McGarrahan 1956 The biosynthesis of cholesterol from acetate-1-$C^{14}$ by cellular fractions of rat liver. J. Biol. Chem., 222: 1.
16. Biggs, M. W., and D. Kritchevsky 1951 Observations with radioactive hydrogen ($H^3$) in experimental atherosclerosis. Circulation, *4:* 34.
17. Tomkins, G. M., H. Sheppard and I. L. Chaikoff 1953 Cholesterol synthesis by liver. III. Its regulation by ingested cholesterol. J. Biol. Chem., *201:* 137.
18. Sellers, E. A., and R. W. You 1956 Deposition of fat in coronary arteries after exposure to cold. Brit. Med. J., *1:* 815.
19. Wilgram, C. F. 1959 Experimental atherosclerosis and cardiac infarcts in rats. J. Exp. Med., *109:* 293.
20. Sellers, E. A., and D. C. Baker 1960 Coronary atherosclerosis in rats exposed to cold. Canad. Med. Assn. J., *83:* 6.
21. Sodeman, W. A., and J. T. Logue 1960 Effect of environmental temperature upon cholesterol-induced aortic atherosclerosis in the rabbit. Proc. Soc. Exp. Biol. Med., *103:* 255.

# Invitation for Nominations for 1967 American Institute of Nutrition Awards

Nominations are requested for the 1967 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. *Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1966*, to be considered for the 1967 awards.

*General regulations for A.I.N. awards.* Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made at the annual dinner at the annual meeting.

### *1967 Borden Award in Nutrition*

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper
1966 – R. T. Holman

NOMINATING COMMITTEE:

P. H. WESWIG, *Chairman*
E. L. HOVE
K. E. HARSHBARGER

Send nominations to:

DR. P. H. WESWIG
*Department of Agricultural Chemistry*
*Oregon State University*
*Corvallis, Oregon 97331*

### *1967 Osborne and Mendel Award*

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.
1965 – D. M. Hegsted
1966 – H. H. Mitchell

NOMINATING COMMITTEE:

M. O. SCHULTZE, *Chairman*
ALEX BLACK
L. E. HOLT, JR.

Send nominations to:

DR. M. O. SCHULTZE
*Department of Biochemistry*
*University of Minnesota*
*St. Paul, Minnesota 55101*

### *1967 Mead Johnson Award for Research in Nutrition*

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri
1966 – M. Daniel Lane

NOMINATING COMMITTEE:

P. L. DAY, *Chairman*
A. R. KEMMERER
L. M. HENDERSON

Send nominations to:

DR. P. L. DAY
*National Heart Institute*
*National Institutes of Health*
*Bethesda, Maryland 20014*

### *1967 Conrad A. Elvehjem Award for Public Service in Nutrition*

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

The first recipient of this award was:

1966 – C. Glen King

NOMINATING COMMITTEE:

E. E. HOWE, *Chairman*
F. W. QUACKENBUSH
R. E. SHANK

Send nominations to:

DR. E. E. HOWE
*Merck Institute for Therapeutic Research*
*Rahway, New Jersey 07065*

# Invitation for Nominations for 1967 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

*Fellows Committee:*

S. L. HANSARD, *Chairman*
PAUL E. JOHNSON
W. H. GRIFFITH
AGNES F. MORGAN
RICHARD M. FORBES

*Send nominations to:*

DR. S. L. HANSARD
*Department of Animal Science*
*Louisiana State University*
*Baton Rouge, Louisiana 70803*

The following persons have been elected previously as Fellows of the Society:

J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Max Kleiber (1966)
Samuel Lepkovsky (1966)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

# Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

*Committee on Honorary Memberships:*

PAUL GYÖRGY, *Chairman*
GRACE GOLDSMITH
R. W. ENGEL

*Send nominations to:*

DR. PAUL GYÖRGY
*University of Pennsylvania*
*Pennsylvania General Hospital*
*Philadelphia, Pennsylvania 19104*

The following persons have been elected previously as Honorary Members of the Society:

Kunitaro Arimoto
W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Harriette Chick
F. W. A. Clements
David P. Cuthbertson
Herbert M. Evans
Joachim Kühnau
Toshio Oiso
H. A. P. C. Oomen
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
B. S. Platt
Emile F. Terroine
Jean Tremolieres
Eric John Underwood
Artturi I. Virtanen