of fructose by fructokinase and hexokinase (Fig. 1) it would appear that insulin acts to accelerate the initial phosphorylation of glucose and fructose by hexokinase. It is probable that fructose metabolism in intact animals proceeds almost entirely by way of the fructokinase system since glucose is normally present in tissues *in vivo* in amounts sufficient to inhibit the phosphorylation of fructose by hexokinase. The findings presented in this paper are in agreement with results of experiments *in vivo* by other investigators(7,20-22) suggesting that insulin does not affect the metabolism of fructose in animals and human subjects.

*Summary.* In isolated rat diaphragm, insulin increased the metabolism of fructose and glucose via the hexokinase system but did not affect the utilization of fructose via the fructokinase pathway. The results suggest that insulin affects the metabolism of glucose and fructose by accelerating their initial phosphorylation by hexokinase.

1. Price, W. H., Cori, C. F., and Colowick, S. P., *J. Biol. Chem.*, 1945, v160, 633.

2. Colowick, S. P., Cori, G. T., Slein, M. W., *J. Biol. Chem.*, 1947, v168, 583.

3. Reid, E., Smith, R. H., and Young, F. G., *Biochem. J.*, 1948, v42, XIX.

4. Broh-Kahn, R. H., and Mirsky, I. A., *Science*, 1947, v106, 148.

5. Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, 1949, v177, 311.

6. Stadie, W. C., Haugaard, N., Hills, A. G., and Marsh, J. B., *Am. J. Med. Sc.*, 1949, v218, 275.

7. Chernick, S. S., and Chaikoff, I. L., *J. Biol. Chem.*, 1951, v188, 389.

8. Chernick, S. S., Chaikoff, I. L., and Abraham, S., *J. Biol. Chem.*, 1951, v193, 793.

9. Stadie, W. C., Haugaard, N., and Marsh, J. B., *J. Biol. Chem.*, 1951, v188, 167.

10. Stadie, W. C., Haugaard, N., and Vaughan, M., *J. Biol. Chem.*, 1953, v200, 745.

11. Stadie, W. C., and Zapp, V. A., Jr., *J. Biol. Chem.*, 1947, v170, 55.

12. Roe, J. H., *J. Biol. Chem.*, 1934, v107, 15.

13. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

14. Mackler, B., and Guest, G. M., in press.

15. Cori, G. T., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, 1938, v124, 543.

16. Cori, G. T., and Cori, C. F., PROC. SOC. EXP. BIOL. AND MED., 1938, v39, 337.

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

18. Slein, M. W., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, 1950, v186, 763.

19. Mackler, B., and Guest, G. M., *Am. J. Physiol.*, in press.

20. Levine, R., and Huddlestun, B., *Fed. Proc.*, 1947, v6, 151.

21. Pletcher, A., Fahrlander, H., and Staub, H., *Helvet. Physiol. et Pharmacol. Acta*, 1951, v9, 46.

22. Miller, M., Drucher, W. R., Owens, J. E., Craig, J. W., and Woodward, H., *J. Clin. Invest.*, 1952, v31, 115.

Received May 18, 1953. P.S.E.B.M., 1953, v83.

## Utilization of Sulfur Amino Acids During Healing of Experimental Wounds.* (20350)

MARTIN B. WILLIAMSON AND HERBERT J. FROMM.

*From the Department of Biochemistry, School of Medicine and Graduate School, Loyola University, Chicago.*

The regeneration of tissue during the healing of wounds has been shown to be markedly accelerated by the addition of methionine to the diet(1-7). More recently, the rate of healing of experimental wounds was shown to be increased to the same extent when equivalent amounts of either cystine or methionine were administered(1). Since the conversion of methionine to cystine is irreversible *in vivo* (9,10), this work may be taken to indicate that cystine is the limiting amino acid in the healing process. The possibility still existed that the sulfur amino acids may not be required *per se*, but rather might serve as a source of sulfur for other compounds required

* This work was done under contract with the U. S. Navy, Office of Naval Research.

during healing, as well as their more usual function in protein synthesis.

It has been shown also that there is a small but definite retention of sulfur during healing, even when a negative nitrogen balance is in evidence(1,2,8). Further, the retention of sulfur becomes more significant as the rate of healing increases. Thus, there appears to be a correlation between the sulfur retention and the *healing index*. The *healing index* is a measurable numerical evaluation of a function which is directly proportional to the rate of healing(1,2). The metabolic fate of sulfur and sulfur containing amino acids during the healing of experimental wounds should give an insight into the disposition of the retained sulfur. The utilization of the cystine by the regenerating wound tissue and its relation to the function measured by the *healing index* are considered in this report.

*Experimental.* The experiments to be described were carried out on two groups of female albino rats (18 animals per group), weighing 170 ± 20 g. The animals were maintained on a basal 0% protein diet for 5 days prior to wounding. After wounding, one group of rats was continued on the 0% protein diet supplemented with 60 mg of DL-alanine per 100 g of diet, while the second group received the 0% protein diet supplemented with 100 mg of DL-methionine per 100 g of diet. The basal diet consisted of 83 g sucrose, 10 g lard, 2 g corn oil, 5 g salt mixture(15), 1 mg thiamine HCl, 1 mg riboflavin, 1 mg pyridoxine HCl, 4 mg calcium pantothenate, 10 mg nicotinic acid amide, 0.5 mg 2-methyl-naphthoquinone, 1550 IU vit. A,† and 210 IU vit. D.† The animals were provided with 7 g of diet per day, which was completely consumed before the next daily feeding period. Distilled water was permitted *ad libitum*. After removing the hair, the wounds were made on the back of the neck of the rats by tracing the outline of a coin (3 cm diameter), and cutting on the marked tracing. The circle of skin was cut down to the layer of the *fascia* and removed with any adhering connective tissue, according to the method described by Paul *et al.*(16). Two days after wounding each rat was injected subcutaneously with 0.85 mg of $S^{35}$-labeled L-methionine, representing 8.9 x $10^6$ counts per minute. One-third of the animals in each group were sacrificed on the 5th, 8th and 12th day after wounding. Samples of regenerating wound tissue, normal unwounded skin, liver and muscle were taken from each rat and hydrolyzed in 6 N HCl. Great care was taken to insure that no normal tissue was included in the sample with the regenerating wound tissue. Normal unwounded skin tissue was obtained from the area near the site of the wound. This tissue was completely freed of hair before analysis. The above tissue samples were analyzed for total sulfur(11), total nitrogen (microkjeldahl), and cysteine + cystine (designated as *cystine*)(12,13). The relatively small amounts of sample available made it necessary to calculate the methionine present in the tissues by difference. Although the methionine-sulfur fractions probably included sulfur from compounds other than methionine, by far the largest part of these fractions of the sulfur comes from the methionine. Besides determining the total $S^{35}$ in the tissue samples, aliquots were partitioned into cysteine + cystine and non-cysteine + cystine fraction by the method of Zittle and O'Dell(14), and the $S^{35}$ determined in the fractions. The latter fraction was designated as the methionine-$S^{35}$ fraction.

*Results and discussion.* In Table I is shown the $S^{35}$ content of the various tissues which were analyzed in terms of counts per minute per gram of tissue. It should be noted that there is a very marked increase in the $S^{35}$ content of the regenerating wound tissue with time. This increase reflects the accretion of

TABLE I. Effect of Dietary Methionine on Distribution of Methionine-$S^{35}$ in Wounded Rats.

| Days after wounding | Counts/min./g of tissue: Wound tissue | Skin | Liver | Muscle |
|---|---|---|---|---|
| | 0% protein diet | | | |
| 5 | 6065 | 2327 | 7986 | 1660 |
| 8 | 7222 | 1836 | 5154 | 1324 |
| 12 | 9351 | 1690 | 4220 | 924 |
| | 0% protein diet + methionine | | | |
| 5 | 6272 | 2705 | 7475 | 1252 |
| 8 | 9117 | 2265 | 5030 | 1155 |
| 12 | 11565 | 1967 | 3740 | 865 |

† From *oleum percomorphum*.

TABLE II. Sulfur and Nitrogen Content/g of Wounded Rat Tissue.

| Days after wounding | Wound tissue S (mg) | Wound tissue N (mg) | Skin S (mg) | Skin N (mg) | Liver S (mg) | Liver N (mg) | Muscle S (mg) | Muscle N (mg) |
|---|---|---|---|---|---|---|---|---|
| | | | 0% protein diet | | | | | |
| 5 | 1.9 | 20.2 | 2.0 | 39.9 | 3.0 | 34.0 | 2.8 | 34.4 |
| 8 | 2.3 | 22.0 | 1.7 | 40.8 | 2.5 | 31.5 | 2.7 | 33.5 |
| 12 | 2.9 | 27.8 | 1.6 | 42.4 | 2.3 | 28.7 | 2.3 | 35.6 |
| | | | 0% protein diet + methionine | | | | | |
| 5 | 2.0 | 22.4 | 2.1 | 39.2 | 2.9 | 30.5 | 2.7 | 34.2 |
| 8 | 2.8 | 26.3 | 1.6 | 42.1 | 2.5 | 27.1 | 2.5 | 36.1 |
| 12 | 3.6 | 29.8 | 1.6 | 40.2 | 2.3 | 26.7 | 2.4 | 35.6 |

TABLE III. Distribution of $S^{35}$ from Methionine between Cystine and Methionine Fractions of Tissue in Wounded Rats.

| Days after wounding | Counts/min./g tissue: Wound tissue Cystine* | Wound tissue Methionine | Skin tissue Cystine* | Skin tissue Methionine |
|---|---|---|---|---|
| | 0% protein diet | | | |
| 5 | 3536 | 2417 | 1465 | 910 |
| 8 | 4580 | 3205 | 1370 | 730 |
| 12 | 5240 | 4026 | 1171 | 452 |
| | 0% protein diet + methionine | | | |
| 5 | 3907 | 2027 | 1427 | 1377 |
| 8 | 5177 | 3600 | 1085 | 1225 |
| 12 | 6950 | 4072 | 940 | 1003 |

* The "cystine" fraction includes both cysteine and cystine.

new cells and protein in the wound area. Due to metabolic turnover, the $S^{35}$ content of the other tissues decreased with time. Although some small part of the methionine-$S^{35}$ may have been directly utilized, there can be little doubt that the bulk of the wound tissue $S^{35}$ arose from the degradation products of other tissue proteins.

The total sulfur and nitrogen content of these tissues is tabulated in Table II. The general trend of the total sulfur levels in the tissues parallels that observed for the $S^{35}$. However, the changes observed are not as marked as in the case of the radio-sulfur. This may account for the previous difficulty in obtaining significant differences in the sulfur content of wound tissues which had been healing for different lengths of time. Particular note should be made of the fact that the sulfur content of the regenerating wound tissues is very much higher than that found in the other tissues analyzed, based on the nitrogen content of the tissues.

The only significant change in the nitrogen content of the various tissues occurred in the case of the wound tissue, although the small decreases observed in the liver may be reflecting the negative nitrogen balance which existed in these animals. The relatively low nitrogen concentrations appearing in the healing wound tissue samples are consonant with previous work which had shown that this type of tissue has a very high water content(16). From the data in Table II, the water content of the wound tissue appears to diminish gradually with the progression of the healing process.

In Table III, the results of the partition of the $S^{35}$ into *cystine* and methionine-$S^{35}$ fractions is shown. It is of special importance to note that so large a proportion of the injected methionine-$S^{35}$ is converted to cysteine- or cystine-$S^{35}$. In agreement with the data shown in Table I, the sum of the two $S^{35}$ fractions increases with time. However, the concentration of the *cystine*-$S^{35}$ is much greater than is that of the methionine-$S^{35}$. These results would appear to support the previous report(1) showing that although methionine is required during the healing process, there appears to be greater utilization of cystine.

Table IV shows the results of the analysis of the tissues for *cystine* and methionine. The regenerating wound tissues accumulate *cystine* quite rapidly while the concentration of methionine increases at a more leisurely rate. In the liver and muscle, there was a marked decrease in the methionine concentration while the *cystine* remained essentially unchanged throughout the experiment. This might be taken to indicate that the liver and muscle

TABLE IV. Cystine* and Methionine Content/g Tissue in Wounded Rats.

| Days after wounding | Wound tissue Cys | Wound tissue Meth | Skin Cys | Skin Meth | Liver Cys | Liver Meth | Muscle Cys | Muscle Meth |
|---|---|---|---|---|---|---|---|---|
| | mg | | | | | | | |
| | 0% protein diet | | | | | | | |
| 5 | 3.6 | 4.0 | 3.0 | 5.2 | 4.2 | 7.9 | 4.1 | 7.5 |
| 8 | 5.3 | 4.2 | 2.9 | 5.3 | 5.0 | 5.9 | 4.3 | 7.4 |
| 12 | 7.3 | 5.4 | 2.9 | 5.4 | 4.7 | 4.4 | 3.9 | 6.1 |
| | 0% protein diet + methionine | | | | | | | |
| 5 | 4.0 | 4.1 | 3.0 | 4.9 | 4.5 | 8.3 | 4.1 | 8.3 |
| 8 | 6.7 | 4.7 | 2.7 | 4.9 | 4.6 | 6.6 | 4.1 | 6.5 |
| 12 | 9.5 | 5.9 | 2.8 | 4.5 | 5.1 | 4.7 | 4.0 | 6.3 |

* The "cystine" fraction includes both cysteine and cystine.

methionine sulfur are the principal sources for the wound tissue sulfur. It would also follow that a large portion of the metabolized liver and muscle methionine is converted to cysteine or cystine, which may be utilized by the regenerating wound tissue. This is supported by the fact that, in the animals fed the 0% protein diet, the ratio of *cystine* to methionine excreted was found to be approximately 5:1.

Previously reported analyses, as well as those shown here, indicate that there is usually appreciably more methionine found in most tissues, than there is *cystine*. This, however, is not the case in the regenerating wound tissue. In the wound tissue, the concentration of *cystine* becomes increasingly greater than the level of methionine during the course of healing. This fact might be interpreted to mean that there are two types of proteins being synthesized in the wound area. The first type might be considered to be synthesized relatively slowly and to contain a higher proportion of methionine than *cystine*. The characteristics of this type of protein are probably similar to skin proteins in general. The second type, although not synthesized until at least some of the first type of protein has already been laid down in the wound area, is synthesized more rapidly and contains a higher proportion of *cystine* than methionine. Fractionation studies of the proteins in regenerating wound tissue at various stages of development, and in normal skin tissue, are now under way to test this hypothesis.

The rates of healing of wounds in rats fed a 0% protein diet and a 0% protein diet supplemented with methionine were determined as previously described(1,2). The results are shown in Fig. 1 to be essentially straight lines of different slopes (*healing indices*). When the *cystine* per gram of wound tissue is plotted against time, straight lines also result (Fig. 1). These lines may be represented by the following equations:

$$C = k_1 t + a$$
$$T = k_2 t + b$$

where $C$ is the *cystine* concentration in the wound tissues, T, the tensile strength, t, the time in days after wounding, and the other symbols represent constants. It would then follow that: $C = k_3 T + c$. If this equation is valid, a straight line should result from a plot of tensile strength against the *cystine* content of the wound tissue. That this is actually the case is shown in Fig. 2. It may then be concluded that the rate of deposition of *cystine* in the wound tissue is proportional to the rate of increase in tensile strength of the wound (healing index). Since the rate of healing is proportional to the *healing index*, it can now be measured by two separate functions, the tensile strength of the wound, and the cystine content of the wound area.

It might be assumed that the measurement of the tensile strength of wound tissue, requires, among other factors, the rupture of bonds between relatively large molecules which are not in solution, but are arranged in some sort of colloid meshwork. It would then seem probable that the *cystine* in the wound tissue is preponderantly, if not completely, associated with protein molecules.

*Summary.* Wounded rats, injected with L-methionine-$S^{35}$, accumulate the radio-sulfur in the wound tissue, even when the $S^{35}$ content of the other tissues is decreasing. The largest

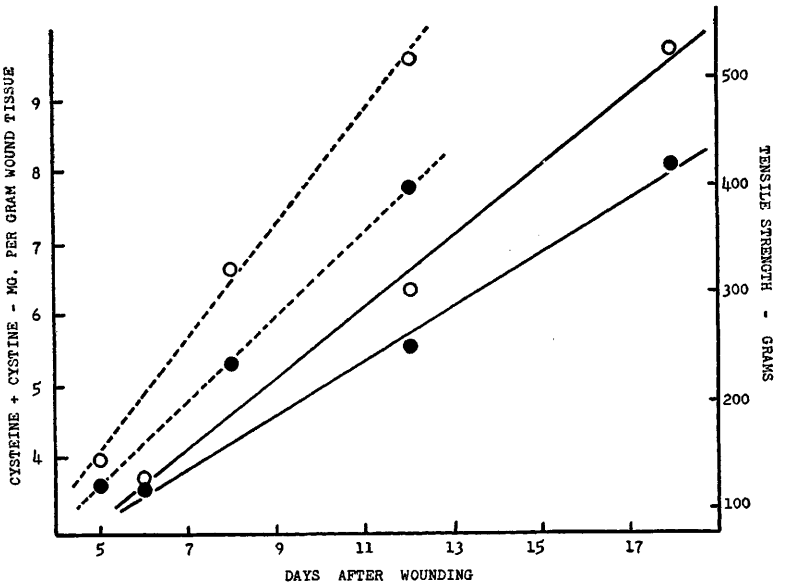


FIG. 1. Tensile strength and cysteine + cystine concentration of healing wounds in rats on a non-protein diet plotted against time. Solid circles, diet supplemented with alanine; open circles, diet supplemented with methionine. Solid lines, tensile strength data; broken lines, cysteine + cystine data. Significance between the mean values of tensile strength is "p" $<0.02$. Significance between the mean values of the cysteine + cystine is "p" $<0.05$.

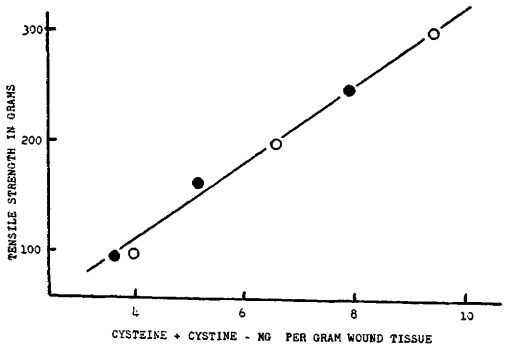


FIG. 2. Tensile strength of healing wound in rats on a non-protein diet plotted against the cysteine + cystine concentration of the regenerating wound tissue. Solid circles, diet supplemented with alanine; open circles, diet supplemented with methionine.

part of the $S^{35}$ in the wound tissue appears as cystine-$S^{35}$. The rate of deposition of cystine in the regenerating wound tissue is greater than that of methionine. The rate of healing (as measured by the *healing index*) is a function of the cystine content of the wound tissue.

1. Williamson, M. B., and Fromm, H. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 623.

2. Williamson, M. B., McCarthy, T. H., and Fromm, H. J., PROC. SOC. EXP. BIOL. AND MED, 1951, v77, 302.

3. ———, *Fed. Proc.*, 1951, v10, 270.

4. Cuthbertson, D. P., *Proc. Nutr. Soc.*, 1946, v4, 185.

5. Localio, S. A., Morgan, M. E., and Hinton, J. W., *Surg. Gyn. Obst.*, 1948, v86, 582.

6. Peters, R. A., *Brit. Med. Bull.*, 1945, v3, 81.

7. Croft, P. B., and Peters, R. A., *Nature*, 1945, v155, 175.

8. Williamson, M. B., and Fromm, H. J., *Fed. Proc.*, 1953, v12, 291.

9. Binkley, F., *J. Biol. Chem.*, 1944, v155, 39.

10. DuVigneaud, V., Kilmer, G. W., Rachele, J. R., and Cohn, M., *J. Biol. Chem.*, 1944, v155, 645.

11. Treon, J. B., and Crutchfield, W. E., *Ind. Eng. Chem.*, 1942, v14, 119.

12. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, 1929, v83, 109.

13. Block, R. J., and Bolling, D., *The determination of amino acids*, 1948, Burgess Pub. Co., Minneapolis.

14. Zittle, C. A., and O'Dell, R. A., *J. Biol. Chem.*, 1942, v139, 753.

15. Hubbel, R. B., Mendel, L. B., and Wakeman, J., *J. Nutr.*, 1937, v14, 237.

16. Paul, H. E., Paul, M. B., Taylor, J. D., and Marsters, R. W., *Arch. Biochem.*, 1945, v7, 231.

Received May 18, 1953. P.S.E.B.M., 1953, v83.