were made at 7 to 9-day intervals, and fluid on the infected cultures was frequently changed once or twice in the interim. On the 14th day of the 6th passage the original inoculum had been diluted 10^{-11} by the transfers and fluid changes. Fluid harvested at that time was infective for 13-day embryonated eggs as demonstrated by fluorescent antibody staining of lung sections, indicating that the agent had replicated in the entodermal cell cultures. The agent was still present in fluids removed from cultures at 16-18 days of incubation. Specific antigen, however, was not visualized when infected coverslip preparations were tested by the fluorescent antibody technic and no cytopathic effects were observed.

Comments. Chick embryo lungs infected with the FH strain may contain 10^5 or 10^6 embryo infective doses per 0.2 ml(3). Although the original inoculum in these series was not titrated, the finding of the infective agent after 10 and 6 passages, in the 2 series, at estimated dilutions of the original inoculum of 10^{-11} or greater, leaves no doubt that the agent increased in the chick entodermal cell cultures. No titrations of the culture harvests were made and there is no evidence that any degree of adaptation to these cells occurred. The identity of the agent cultured was established by characteristic appearance after fluorescent staining of subcultures in embryo lung with known antisera (convalescent), and the more extensive serologic examination applied to later cell cultures infected by passage from entodermal cells, as described in the accompanying paper(8). Unfortunately, this method of cultivation in entodermal cells does not provide any improvement in current technics. However, this first step in propagation of the Eaton agent in cultured cells suggests that better *in vitro* methods can be found.

Summary. The Eaton agent of primary atypical pneumonia was carried through 2 series of 10 and 6 passages respectively, in chick entodermal cell cultures, indicating growth of the agent in this cell system. Cytopathic effects were not seen and fluorescent antibody staining of the infected cells by the indirect method was negative. The agent was demonstrated in fluids from the infected cultures by subinoculation to chick embryos and fluorescent antibody staining of lung sections.

The authors gratefully acknowledge the technical assistance of George W. Gary, Jr., and Luther B. Townley.

1. Eaton, M. D., Meiklejohn, G., van Herick, W., *J. Exp. Med.*, 1944, v79, 649.

2. Eaton, M. D., Meiklejohn, G., van Herick, W., Corey, M., *ibid.*, 1945, v82, 317.

3. Liu, C., *ibid.*, 1957, v106, 455.

4. Weiss, E., Dressler, H. R., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 691.

5. Gordon, F. B., Quan, A. L., Trimmer, R. W., *Science*, 1960, v131, 733.

6. Chanock, R. M., Cook, M. K., Fox, H. H., Parrott, R. H., Huebner, R. J., *New Eng. J. Med.*, 1960, v262, 648.

7. Donald, H. B., Liu, C., *Virol.*, 1959, v9, 20.

8. Chanock, R. M., Fox, H. H., James, W. D., Bloom, H., Mufson, M. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 116.

Received August 30, 1960. P.S.E.B.M., 1960, v105.

Oxidative Phosphorylation and Amino Acid Incorporation into Protein in Regenerating Rat Liver. (26116)

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It has been shown that incorporation rate of labeled amino acids into protein(1-4) and oxygen consumption(5,6) is higher in the regenerating than in the normal rat liver. Since in this condition, incorporation of amino acids into protein depends upon aerobically formed high energy phosphate(7), it is possible that the increased oxidative proc-

TABLE I. Oxygen Consumption, P/O Ratio and Incorporation of C^{14}-Labeled Amino Acids into Protein of Normal and Regenerating Rat Liver. No. of experiments in parentheses. Mean values ± S.E.

Hr after hepatectomy	Qo_2 (slices)	P/O (homogenate)		Incorporation into protein (slices) (μmoles/g protein/hr)		
		Succinate	α-Keto-glutarate	Glycine-1-C^{14}	L-leucine-C^{14}	L-phenyl-alanine-C^{14}
0‡	5.06 ± .22 (24)	1.87 ± .05 (6)	3.56 ± .12 (19)	3.19 ± .26 (8)	2.63 ± .05 (8)	1.30 ± .11 (8)
84	5.82 ± .28* (24)	1.28 ± .06† (5)	2.39 ± .11† (37)	5.56 ± .40† (8)	4.34 ± .21† (8)	3.38 ± .31† (8)
168	6.20 ± .24† (24)	1.57 ± .19 (6)	3.21 ± .12* (17)	5.84 ± .46† (8)	3.36 ± .15† (8)	2.39 ± .21† (8)
240	5.45 ± .24 (24)	1.91 ± .04 (7)	3.71 ± .09 (18)	4.28 ± .22† (8)	3.23 ± .15† (8)	1.54 ± .07 (8)

* $p < .05$, compared to normal liver. † $p < .01$, compared to normal liver. ‡ Control.

esses may result in a proportional increase in ~ P which, in turn, could support a faster rate of protein synthesis by the multiplying hepatocytes. To investigate this possibility, oxygen consumption, oxidative phosphorylation and incorporation of some amino acids into protein of regenerating rat liver were measured.

Methods. Wistar rats of either sex, weighing about 250 g were partially hepatectomized according to the technic of Higgins and Anderson(8). After a 12-hour fast, normal and hepatectomized rats were decapitated 84, 168 and 240 hours after operation and their livers were removed. Oxidative phosphorylation, amino acid incorporation into protein and oxygen consumption were measured on separate fractions of liver as follows: *1. Oxidative phosphorylation.* The phosphorylation quotient (P/O ratio), with succinate and α-ketoglutarate as substrates, was determined according to the procedure described by Krebs *et al.*(9), based on simultaneous measurement of incorporation rate of labeled inorganic phosphate into ATP and of oxygen consumption by tissue homogenates. Phosphate fractions were separated by paper chromatography(10) and ATP turnover rate was calculated with the formula proposed by Krebs *et al.*(11). Radioactivity was measured with a liquid type Geiger-Müller counter. *2. Incorporation of amino acids into protein and oxygen consumption.* About 50 mg of liver slices were incubated in Warburg flasks for one hour at 38°C in air and in a medium containing NaCl, 1.28 x 10^{-1} M; KCl, 1.35 x 10^{-2} M; $CaCl_2$, 1.94 x 10^{-3} M; $MgSO_4$, 6.8 x 10^{-4} M; Na phosphate buffer, 1 x 10^{-2} M (pH 7.2); and either glycine-1-C^{14}, L-leucine-U-C^{14} or L-phenylalanine-U-C^{14} 1 x 10^{-3} M (the amino acid was added after 10 minutes of equilibration). After incubation the flasks were quickly chilled in an ice bath, the slices were removed and the proteins were purified as described previously (4). Samples were plated and counted using a mica window Geiger-Müller counter. Oxygen consumption of liver slices was measured in an identical system, except without added amino acids, using the standard Warburg technic(12).

Results and discussion. Results are shown in Table I. A moderate and statistically significant increase in oxygen consumption by regenerating liver slices occurs 84, 168 hours after partial hepatectomy and can still be seen 240 hours thereafter, although at this time it is no longer significant. This increase is of the same order of magnitude of that previously reported(5,6). A statistically significant lowering of the P/O ratio occurs 84 hours after partial hepatectomy, using either succinate or α-ketoglutarate as a substrate. When α-ketoglutarate is used, the ratio is still below normal 168 hours after operation, although, at this time, using succinate, the decrease is no longer significant. Two hundred and forty hours after operation, P/O ratio returns to normal with either substrate. In partial agreement with our results, Palladina and Gudina(13) reported a P/O ratio slightly lower than normal, using succinate,

guanine and xanthine as substrates, 7 days after hepatectomy. The coupling between oxidation and phosphorylation is a labile mechanism strictly dependent upon the integrity of the mitochondria(14). Since the regenerating hepatocytes easily undergo fatty infiltration and cell vacuolation, it is possible that these regressive processes may bring about sufficient mitochondrial damage to cause the observed decrease in P/O ratio. This hypothesis is consistent with the observation that during liver regeneration, the activity of certain mitochondrial enzymes such as glutamic dehydrogenase(15), succinic dehydrogenase and cytochrome oxidase(5) decreases more markedly than would be expected from the decrease in mitochondrial population(16). The rates of glycine-1-C^{14}, L-leucine-C^{14} and L-phenylalanine-C^{14} incorporation into proteins are markedly enhanced in the regenerating liver in comparison to the controls. The difference is statistically significant 84, 168 and 240 hours after partial hepatectomy, except for the increased incorporation of phenylalanine which lasts only 168 hours. This marked increase in protein synthesis occurs simultaneously with a moderate increase in oxygen consumption and a decrease of P/O ratio, a situation in which the formation of high energy phosphates not only does not increase, but may actually decrease. These results suggest that, in the normal liver, the availability of ~ P greatly exceeds that needed for protein synthesis, as shown also by the calculations of Quastel and Bickis(7). In the normal liver, the excess of available energy could be utilized for other hepatic functions, whereas, in the regenerating liver, a greater part of energy would be used to support the increase in protein synthesis and other endergonic processes such as deoxyribonucleic, ribonucleic acid(17) and phospholipid(18,19) synthesis.

It should be noted that cellular multiplication is not the only condition in which an increased amount of energy is channeled toward synthetic processes, since an increased incorporation of amino acid into protein accompanied by normal oxygen consumption and moderate uncoupling of oxidative phosphorylation, has been described also in a regressive process, such as cloudy swelling(14,20). In addition, if we assume that the relative amounts of labeled glycine, leucine and phenylalanine incorporated into liver proteins correspond to the proportions of the 3 amino acids in these proteins, our results suggest that no striking changes in liver protein composition take place during the process of parenchymal regeneration, at least as far as these 3 amino acids are concerned. This is in agreement with previous results demonstrating the similarity between the electrophoretic pattern of soluble proteins(21,22) and the immunologic specificity of mitochondrial and microsomal proteins(23) obtained from regenerating and normal rat liver.

Summary. In slices from regenerating rat liver 84 and 168 hours after hepatectomy, oxygen consumption is moderately increased and glycine-1-C^{14}, L-leucine-C^{14} and L-phenylalanine-C^{14} incorporation into protein is markedly increased as compared with normal liver slices. On the other hand, in liver homogenates, incubated with succinate or α-ketoglutarate as substrates, oxidative phosphorylation is decreased in the early stages (84 hours) of regeneration. The results suggest the following conclusions: 1) in regenerating liver tissue, amino acid incorporation into protein can increase even without a parallel increase in the aerobic formation of high energy phosphates; 2) in both normal and regenerating rat liver, the availability of ~ P greatly exceeds that needed for protein synthesis.

1. Eliasson, N. A., Hammarsten, E., Reichard, P., Åqvist, A., Thorell, B., Ehrensvärd, G., *Acta Chem. Scand.*, 1951, v5, 431.

2. Campbell, P. N., Greengard, O., Jones, H. E. H., *Exp. Cell Research*, 1957, v12, 689.

3. Hultin, T., Von Der Decken, A., *ibid.*, 1957, v13, 83.

4. Guidotti, G., Clerici, A., Bazzano, E., *Minerva Nucleare*, 1958, v2, 14.

5. Perkinson, J. D., Jr., Irving, C. C., *Cancer Research*, 1956, v16, 496.

6. Schwartz, H. S., Barker, S. B., *Am. J. Physiol.*, 1957, v190, 209.

7. Quastel, J. H., Bickis, I. J., *Nature*, 1959, v183, 281.

8. Higgins, G. M., Anderson, R. M., *Arch. Path.*, 1931, v12, 186.

9. Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V., Hems, R., *Biochem. J.*, 1953, v54, 107.

10. Krebs, H. A., Hems, R., *Biochim. Biophys. Acta*, 1953, v12, 172.

11. Krebs, H. A., Eggleston, L. V., Terner, C., *Biochem. J.*, 1951, v48, 530.

12. Umbreit, W. W., Burris, R. H., Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, Minneapolis, 1949, Burgess Publishing Co.

13. Palladina, L. I., Gudina, A. M., *Ukrain. Biokhim. Zhur.*, 1958, v30, 1958.

14. Fonnesu, A., Severi, C., *J. Biophys. Biochem. Cytol.*, 1956, v2, 293.

15. Greenbaum, A. L., Greenwood, F. C., Harkness, R. D., *J. Physiol.*, 1954, v125, 251.

16. Allard, C., DeLamirande, G., Cantero, A., *Cancer Research*, 1952, v12, 580.

17. Hecht, L. I., Potter, Van R., *ibid.*, 1958, v18, 186.

18. Levin, E., Albert, S., Johnson, R. M., *Arch. Biochem. Biophys.*, 1956, v64, 272.

19. Clerici, E., Guidotti, G., Sambo, G., *Sperimentale, Arch. Biol.*, 1958, v108, 283.

20. Ciaranfi, E., Fonnesu, A., Guidotti, G., *Atti Soc. It. Patol.*, 1957, v5, 521.

21. Sorof, S., Claus, B., Cohen, P., *Cancer Research*, 1951, v11, 873.

22. Guidotti, G., Clerici, E., *Experientia*, 1958, v14, 341.

23. Clerici, E., Guidotti, G., Bazzano, E., *Tumori*, 1958, v44, 285.

Received August 30, 1960. P.S.E.B.M., 1960, v105.

Effect of Dietary Proline and Hydroxyproline on Tensile Strength of Healing Wounds.* (26117)

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The deleterious effects of ascorbic acid deficiency and protein depletion on collagen synthesis in healing wounds have been well established. Analysis of tissue from the wounds of scorbutic animals reveals abnormally high levels of the amino acid-proline and abnormally low levels of hydroxyproline (2). The reversal of the normal hydroxyproline-proline ratio in scurvy and the finding of low hydroxyproline levels in protein depletion states have suggested that an inability to hydroxylate proline may be an important factor in the retarded synthesis of collagen in both conditions(11). Abnormalities in the ground substance, such as a reduction in the acid mucopolysaccharides, may also be responsible for the failure to develop normal tensile strength which requires, in addition to collagen synthesis, that newly created tropocollagen particles be organized into purposeful patterns. However, if there were not serious fundamental defects in the intracellular synthesis of the collagen molecule, one would expect to find normal proline-hydroxyproline ratios in the wound tissues because the amino acid composition of tropocollagen is essentially the same as that of polymerized or mature collagen(6).

We have postulated that, if there is a defect in synthesis of collagen by fibroblasts in scorbutic or protein depleted animals (presumably at the hydroxylation of proline stage), tensile strength of their wounds might be favorably influenced by supplementing their diets with pure synthetic hydroxyproline. Previous investigations of the metabolism of hydroxyproline by Stetten(3), Green and Lowther(9), and Wolf and Berger(10) strongly suggest that free hydroxyproline probably would not be utilized in production of collagen during healing. In normal animals there is very little incorporation of dietary hydroxyproline in the general protein pool; nearly all of N^{15} labeled hydroxyproline can be recovered in the urine and stools (Stetten(9)). However, there is an extremely low turnover of the amino acids in a normal animal's collagen (Neuberger and Slack(8)), therefore Stetten's experiments do not necessarily mean that an animal which was ac-

* Supported by grant from Nat. Inst. of Arthritis and Metab. Dis., U.S.P.H.S., Bethesda, Md.