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# THE INCORPORATION OF C<sup>14</sup> GLYCINE INTO PROTEIN BY HUMAN LIVER SLICES<sup>1</sup>

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Present knowledge of protein synthesis and indeed of intermediary metabolism in general stems from extensive work in many laboratories using a variety of species. Information concerning human intermediary metabolism has not kept pace with the experimental animal work for obvious reasons. Known species differences make it essential to study humans, either to corroborate the findings in experimental animals or to delineate the areas of difference. While recent reports (1, 2) have indicated that tracer doses of C<sup>14</sup> compounds may be administered to humans with apparent safety, nevertheless their usefulness at the present time seems limited because of the unknown factors involved and the long half-life.

The *in vitro* techniques with human tissue offer a means of obviating the radiation hazard and of exploiting the value of the C<sup>14</sup> tool. Biochemical studies of surviving human tissues are few. There are reports on respiration of brain (3, 4) and skin (5), on the cytochrome oxidase system of a variety of normal and malignant tissues (6, 7), on the fate of testosterone in liver (8), and on the incorporation of P<sup>32</sup> into human leucocytes (9). Recently Tagnon, Schulman, Brunshwig and Lieberman reported on the action of human liver slices on alpha-estradiol (10).

It is the purpose of this report to show that biopsy samples of human liver are capable of being handled in the same manner as tissue from experimental animals, and more specifically to present data on the incorporation of carboxyl C<sup>14</sup> labeled glycine into the protein by liver slices.

## MATERIAL AND METHODS

Biopsies of the liver were obtained during laparotomies which were performed upon patients on the Surgical Service of the Multnomah County Hospital. An attempt

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was made to obtain specimens from patients whose livers were presumed to be normal. A wedge of tissue, approximately 1.0 gm., was removed and immediately placed into chilled buffer (0° C.). It was rapidly carried to the laboratory and sliced by hand with a razor blade, maintaining the temperature as close to 0° C. as possible. The slices were blotted with filter paper and weighed rapidly. Carboxyl labeled glycine<sup>2</sup> (0.33 millicurie) and 2 cc. of buffer were added to 75–150 mg. of tissue in a 50 cc. glass-stoppered Erlenmeyer flask. The flasks were shaken for four hours at 37° C. in an atmosphere of 95% oxygen and 5% CO<sub>2</sub>. The incubation was terminated by precipitating the protein with boiling 1 M. acetate (pH 4.5), and the flasks were placed in a boiling water bath for 10 minutes. The contents of the flasks were transferred to 12 cc. heavy-walled centrifuge tubes and the precipitated protein separated by centrifugation at 2,000–2,500 r.p.m. for three to five minutes. The residue was homogenized with 1 M. acetate in a Potter-Elvehjem glass homogenizer, and the precipitate again separated by centrifugation. The protein was washed six times with 8–10 cc. aliquots of cold 1 M. acetate in the centrifuge tube. Non-radioactive glycine was added to one of the washes to help remove unincorporated labeled glycine by dilution of the isotope. The protein residue was then extracted two times with 3:1 alcohol-ether at temperature of 55° to 60° for three to five minutes and once with ether. The dried protein was suspended in 3:1 petroleum ether-acetone with the aid of the glass homogenizer and poured on an aluminum disc which was enclosed between the inverted upper half of a 3.0 cm. diameter one-ounce jar and its screw top (11). The radioactivity was then counted after drying in air. As controls, tissues were precipitated in zero time in the presence of labeled glycine and otherwise treated as were the experimental samples. Such controls were performed in each experiment. Duplicate flasks were used for each set of conditions, although in later experiments a single zero time control was used.

The buffers used were as follows: 1) 0.9% KCl and 0.4% KHCO<sub>3</sub> saturated with 95% O<sub>2</sub>–5% CO<sub>2</sub>, pH 7.5, and 2) Krebs-Henseleit buffer containing NaCl 0.72%, NaHCO<sub>3</sub> 0.25%, KH<sub>2</sub>PO<sub>4</sub> 0.10%, MgSO<sub>4</sub>–7 H<sub>2</sub>O 0.06%, glucose 0.02%, Na citrate 0.03%, and CaCl<sub>2</sub> 0.03%. (The CaCl<sub>2</sub> is made up in a 1% solution and 0.3 cc. added to 10 cc. of the remainder of the mixture just before using. Otherwise a precipitate forms on standing.) The solu-

<sup>2</sup> Glycine, 0.5 millicurie per millimole, purchased from Tracerlab, Inc., Boston, Mass.

tion, prior to adding the calcium, was saturated with a 95% O<sub>2</sub> - 5% CO<sub>2</sub> mixture.

Radioactivity was expressed as specific activities, *i.e.*, the counts per minute per milligram dry weight of protein, after appropriate corrections for background and self-absorption, the latter being done by means of an empirical correction curve obtained under experimental conditions (12). All counts were made with a flow gas counter. Values are expressed as the means of duplicate pairs.

## RESULTS

Table I presents a summary of the clinical data, microscopic evaluation of biopsies, and the experi-

mental observations. Microscopic evaluation of the tissue of all the patients was normal except for J. R. in whom fatty infiltration was present. Liver function tests were not performed for the most part since liver disease was not suspected. Correlations of this type are planned for a future communication. J. R. and H. H. were the only patients who were in poor nutritional condition, having lost approximately 30 and 70 pounds, respectively, at the time of surgery.

It was obviously impossible to control the duration of anaesthesia prior to the biopsy, so that this

TABLE I

Patient	Age	Remarks	Biopsy microscopic evaluation	Protein—counts per minute per milligram							
				A	B	C	D	E	F	G	
				KCl-KHCO <sub>3</sub> *	Complete Krebs-Henseleit	Krebs-Henseleit without calcium	Krebs-Henseleit without magnesium	Krebs-Henseleit without Ca or Mg	Nitrogen	Cyanide .005 M.	
C. A.	77	Cholecystectomy, hydrops	Normal	11.9						13.1 (0)*	
C. S.	61	Ulcer, gastric resection	Normal	7.0						8.1 (0)	
H. J.	54	Ulcer, gastric resection not done	Normal	8.0						7.4 (0)	
J. R.	74	Carcinoma of stomach with metastases	Fatty infiltration	6.4							
A. G.	43	Ulcer, gastric resection	Normal	30.	280						
R. S.	47	Cholecystitis, cholecystectomy	Normal		166	73 (66)				13 (82)	
A. B.	48	Ulcer, gastric resection	Normal		630	250 (60)				23 (96)	
G. N.	67	Cholecystectomy, common duct exploration	Normal		703	480 (32)		203 (71)			
E. H.	66	Cholecystectomy, common duct exploration	Normal		455	236 (48)					50 (89)
R. B.	41	Ulcer, gastric resection	Mild sub-acute cholangitis		613	206 (66)	350 (43)	165 (73)			38† (94)
C. R.	69	Cholecystectomy, stones	Normal		498		138 (72)				
J. B.	62	Common duct exploration	Normal		539		207 (60)			72† (87)	
H. H.	48	Carcinoma of stomach, inoperable	Normal		240		106 (66)				
C. D.	42	Ulcer, gastric resection	Normal		160		49 (69)	65 (59)			
F. P.	42	Cholecystectomy, hydrops	Normal		396					38 (90)	
		Zero time mean ± S.E.		2.1±0.3	8.7±2.3						
		Mean ± S.E.			425±58	249±59	170±52				

\* All figures in parentheses represent % inhibition.

† Single observation. All others are averages of duplicates.

Using as an example the patient C. R., the rate of incorporation of labeled glycine is calculated to be  $7.3 \times 10^{-6}$  mM per gm. of protein per hour starting with a solution containing  $6.65 \times 10^{-4}$  mM glycine and 95 mg. wet weight of liver slices.

TABLE II

Paired experiments	Mean difference*	Number of paired observations	S.E.	t	P
Difference between complete buffer and buffer without calcium	264	5	± 65	4.1	<.025
Difference between complete buffer and buffer without magnesium	235	5	± 53	4.4	<.025
Difference between complete buffer and buffer without either calcium or magnesium	350	3	±122	2.9	>.025

\* This is the mean of the five differences of paired specific activities, e.g., Column B minus Column C (Table I) for each of the five patients.

interval varied from 30 minutes after onset, to five hours (in the case of C. D.). All biopsies, except for C. D., were obtained within three hours from the beginning of anaesthesia.

The data presented show that there is a very significant uptake of the radioactive glycine into the liver protein, and that precipitating the protein at zero time in the presence of the radioactive glycine invariably yielded protein with negligible specific activities. It is apparent that the use of a complete Krebs-Henseleit buffer (Column B) resulted in maximum specific activities (160-703). The use of the KCl-KHCO<sub>3</sub> buffer (Column A), which had no added calcium, magnesium, citrate, or glucose, yielded protein with specific activities ranging from 7 to 30. Five pairs of observations comparing the full Krebs-Henseleit buffer with the same medium, except that calcium had been omitted (Columns B and C), revealed a consistently lower uptake with the latter solution, the difference being significant, as shown in Table II. It should be noted that these pairs of observations compare tissues from a given patient under the two different conditions. A similar, significant difference was found when magnesium sulfate was omitted from the buffer (Columns B and D). When both calcium and magnesium were omitted (Column E), the percentage inhibition seemed to be even greater, except in the case of C. D. This difference is not significant, however, probably because of the fewer observations. A nitrogen atmosphere had no evident depressing effect on the uptake when the KCl-KHCO<sub>3</sub> buffer was used, and a marked inhibiting effect in the presence of the more complete medium (Column F). Cyanide (.005 M.) was an effective inhibitor (Column G).

The figures presented are average values for duplicate pairs of observations for each set of experimental conditions except as noted. The variation of each observation from the mean of its pair of duplicates was less than 15% in 21 out of 30 pairs, and less than 25% in 28 out of 32 pairs.

Data similar to the above have also been obtained using sigmoid colon slices from surgical patients. The specific activities of the protein ranged from 215 to 1,175 under the same experimental conditions. There was apparently the same difference in uptake with buffer variations except that the presence or absence of calcium seemed to have no effect. These observations will be presented in detail at a later date.

#### DISCUSSION

There have been numerous reports on studies of this type with tissues from experimental animals (11, 13-16). Since the classic work of Schoenheimer (17) the lability of tissue protein and its existence in dynamic equilibrium with its component parts has become an accepted concept and the incorporation of labeled amino acids into protein of adult tissues is an accepted fact. With respect to the relationship between incorporation and protein synthesis there has been considerable discussion among biochemists (18). When an *in vivo* technique is used, there is little doubt that the level of incorporation is a measure of synthesis. When tissue slices and homogenates have been used, however, some doubt has been expressed as to the meaning of "incorporation." However, the weight of existing evidence presented by Greenberg, Borsook, Tarver, Zamecnik, and others indicates that incorporation by tissues in the flask probably reflects protein synthesis. Indeed Peters and Anfinsen have obtained from chicken liver slices a radioactive protein which behaved immunologically and electrophoretically like serum albumin (19).

Comparison of these data with other results in animals is difficult because of the different experimental conditions. In our laboratory rat liver slices yielded protein with a specific activity of 250, using the complete Krebs-Henseleit buffer. This is certainly the same order of magnitude as the results with human liver, which was somewhat surprising in view of the report by Sprinson and Rittenberg that the turnover of tissue protein (as

measured by feeding N<sup>15</sup> labeled glycine) in the rat is five times that of man (20). Whereas under the conditions of the present experiments one specific activity is easily compared with another it must be pointed out that such a comparison does not apply to the specific activities reported by other investigators. In any given experiment the specific activity obtained depends upon the following variables: 1) the specific activity of the amino acid added at the beginning of the experiment; 2) the number of hours incubated; 3) the weight of tissue and its protein concentration; 4) the efficiency of the counting equipment (the flow-gas counter being three to four times more efficient than the Geiger end-window tube); 5) the concentration of various ions in the media; and 6) the concentration of O<sub>2</sub> available. It might nevertheless be of interest to mention several comparative results. Winnick, Friedberg and Greenberg found a comparable depression in uptake by insoluble particles of rat liver homogenate when calcium was omitted from the buffer, but when magnesium was omitted, the degree of inhibition was considerably less than in the current experiments (21). Borsook and his associates reported comparable data (22). Depression of the uptake by a nitrogen atmosphere is the universal finding with all animal tissues with the exception of the uptake of labeled lysine by whole homogenate of guinea pig liver (22). It is likely that nitrogen produced no depression in the experiments using the KCl-KHCO<sub>3</sub> buffer because the system was already depressed by operating under sub-optimal conditions.

With the complete buffer the specific activities cluster symmetrically around the mean with a normal type distribution. It is conceivable that the specific activity obtained in C. D. might have been higher had the biopsy been taken earlier in the course of his surgery. The effect of the anaesthesia and the duration thereof are unknown. The marked sensitivity to small amounts of added calcium and magnesium emphasizes the need for carefully defined conditions as a corollary of a "normal" figure.

The incorporation of the labeled glycine into the protein has all the characteristics of an enzymatic reaction which may well be the process whereby the whole organism synthesizes protein. It is obviously an oxygen requiring reaction since it is al-

most entirely abolished by a nitrogen environment or cyanide. The requirements for added calcium and magnesium ions are striking, and one might infer that phosphorylation is a fundamental part of this mechanism of synthesis. Such data have been reported with experimental animal tissue, and the reports support the conclusion that the requirements include oxygen, adenosinetriphosphate (ATP), magnesium and calcium ions. ATP has been added to animal liver homogenates without significantly increasing the uptake of labeled amino acid. However, Winnick has demonstrated that particles of rat liver will not incorporate labeled glycine after dialysis unless ATP is added (23). Since some glycine is incorporated into protein with the KCl-KHCO<sub>3</sub> buffer, it is inferred that the concentration in the human liver slices of some of the components is too low for optimal incorporation, and that this deficiency is easily overcome by adding the desirable components.

Experimental evidence to date is quite convincing that the liver synthesizes serum albumin from the constituent amino acids. This well accepted concept has been reinforced by the recent evidence of Tarver and Reinhardt that the hepatectomized dog incorporates practically no radioactive methionine into serum albumin (24). Low serum albumin concentration is well correlated with advanced hepatic damage and a logical inference is that the liver is incapable of synthesizing albumin at a normal rate. The ability of the liver slice to incorporate radioactive glycine into its protein is quite probably a reflection of the rate of protein synthesis. With this in mind one might expect to find reduced incorporation by pathological liver slices especially where serum albumin concentration is below normal. Such experiments are currently in progress.

#### SUMMARY

The incorporation of carboxyl C<sup>14</sup> labeled glycine into the protein of human liver slices has been studied. The specific activities of the protein were higher when the tissue was incubated with Krebs-Henseleit buffer as compared to a KCl-KHCO<sub>3</sub> buffer.

The reaction was inhibited by nitrogen, cyanide, and failure to add calcium or magnesium.

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